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Biochemistry and Cell Biology of Ageing: Part I Biomedical Science

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Foreword: Biochemistry and Cell Biology of Ageing – Parts I and II

Never has it been more important or timely for new volumes on the science of ageing to be produced. Around the world, continuing gains in life expectancy coupled with declining fertility rates in many countries are producing profound shifts in demographic profiles. A growing fraction of the population is living to advanced old age, bringing with it increased prevalence of a wide range of age-related chronic diseases. Whereas it was once thought that ageing was something that just happened and that was relatively low on the priority list for research, recent decades have seen exciting advances in probing the complex mechanisms through which the ageing process develops.

We have come a long way from the days when it was simply assumed there was some internal biological clock that would allow us an allotted span of "threescore years and ten" and then kill us. Few had questioned why ageing should impose this fate upon us. It was loosely supposed that it was nature's way of creating living space for the next generation and securing evolutionary succession. We now know that these old-fashioned concepts have little credence. During our evolution, our genomes evolved impressive systems to try and preserve functional homeostasis in the biochemistry and cell biology of our bodies. The trouble is that there was never the evolutionary pressure to make these systems good enough completely to prevent damage from accumulating. Gradually, and at first unobtrusively, things begin to go wrong, starting from the earliest stages of life. And, it is not one thing above all others – many systems experience deterioration at the same time. Herein lies the intriguing challenge of trying to unpick the contributions of the individual mechanisms that are being found to play their part in ageing and then of putting it all together.

Understanding the biochemistry of ageing is among the most complex of problems in the life sciences. On the one hand, we need to be intensively reductionist. We need to identify the fine detail of each one of the many biochemical mechanisms that contribute to functional decline. On the other hand, we need to appreciate that knowing everything there is to know about one particular mechanism may tell us rather little about the ageing process itself. To get the bigger picture, we must acknowledge that it makes little sense to argue the case for this mechanism versus

that mechanism and so on. It is not a matter of simple alternatives. Instead of rooting for mechanism A *or* B *or* … *or* Z, we must learn to appreciate that it is A *and* B *and* … *and* Z. Whether we call this integrative biology, or systems biology, or some other term of a similar nature, the bottom line is that we need to join forces and learn as much as we can about the different biochemical mechanisms and their often synergistic interactions. In some ways, the science of ageing is the science of life itself. In the traditional school of biochemistry, we learn about how life has evolved the remarkable processes of DNA replication, transcription, translation, turnover, signal transduction, cell division, and all the rest. These systems are so beautifully coordinated that we might marvel at first at how well they work. But the underlying molecular interactions are noisy and subject to perturbations of all kinds and at all times. It is this reverse side of the orderliness of biochemical processes that we need to appreciate to understand ageing.

In clinical terms, ageing is equally complex and challenging. Age is much the largest risk factor for a whole spectrum of different diseases, dwarfing the contributions from genetic, lifestyle, and environmental risk factors. Furthermore, the fact that so many conditions share ageing as their dominant risk factor means it is no surprise that very old people commonly exhibit extensive multi-morbidity. But is ageing normal, or is it a disease? The answer is that ageing is a normal biological process but it has the distinctive property that it makes us more vulnerable to diseases of many kinds. So, it is a bit of a hybrid – both normal and also the source of pathologies. The old arguments about whether ageing is normal or disease are not particularly helpful. Ageing is driven by the accumulation of damage in our cells and organs, and the same is true of age-related, chronic diseases. Thus, there is a huge overlap. Once we understand the basic mechanisms of ageing itself, we will gain valuable knowledge about the many diseases which may affect us in later life. Thus, the study of the biochemistry and cell biology of ageing should seek to combine biomedical and clinical science. It is to be warmly welcomed, therefore, that J. Robin Harris and Viktor Korolchuk have produced these twin volumes, bringing into intimate juxtaposition a collection of state-of-the-art reviews of the biochemistry of ageing from both perspectives.

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Preface

This book, *Biochemistry and Cell Biology of Ageing: Part I, Biomedical Science* (along with Part II, Clinical Science), was conceived following the reading (by JRH) of Lewis Wolpert's controversial yet thoroughly enjoyable 2011 book *You're Looking Very Well: The Surprising Nature of Getting Old*. As a broad discipline, *Ageing* has been deemed to fit in well with the diverse content of the Springer *Subcellular Biochemistry* series, and the two books covering Biomedical Science and Clinical Science were duly commissioned.

We have attempted to compile a list of chapters written by authoritative authors to cover the field as thoroughly as possible. Along the way to production, a few chapters failed to appear! Nevertheless, the remaining 17 chapters provide a good coverage of the subject. To place the available chapters in a logical sequence has defeated us; we have simply presented them here as they appear in our initial list of agreed chapters, at the time of compilation. Each Biological Science chapter stands firmly on its own merit, with correlation to the Clinical Science book chapters in some cases. Over recent decades, ageing research has expanded enormously worldwide, responding to the increased importance to the general population, where there is an obvious desire to retain "quality of life," health, and self-sufficiency into the later years.

The Contents list page, immediately following this Preface, shows the range of topics that are included. Without singling out any individual topic and author(s), it is clear that most of the important aspects of ageing research are included. Together they provide an in-depth survey of fundamental biomedical science within the field of ageing research. We hope that the book will be of value to undergraduate biomedical science students, medical students, postgraduate researchers, and academics involved and interested in aspects of ageing research.

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July, 2018

Contents

Chapter 1
Antioxidant Vitamins and Ageing

Irina Milisav, Samo Ribarič, and Borut Poljsak

Abstract The free radical theory of ageing (FRTA), presented by Denham Harman in 1950s, proposed that aerobic organisms age due to reactive oxygen species (ROS)/free radical induced damage that accumulates in cells over time. Since antioxidants can neutralize free radicals by electron donation, the most logical approach was to use them as supplements in order to prevent ageing. In this chapter, we will discuss the inability of antioxidant supplementation to improve health and longevity.

Although many antioxidants are efficient free radical quenchers *in vitro*, their *in vivo* effects are less clear. Recent evidence from human trials implies that antioxidant supplements do not increase lifespan and can even increase the incidence of diseases. Synthetic antioxidants were unable to consistently prevent ROS-induced damage *in vivo*, possibly as dietary antioxidants may not act only as ROS scavengers. Antioxidants can have dichotomous roles on ROS production. They are easily oxidized and can act as oxidants to induce damage when present in large concentrations. In appropriate amounts, they can modulate cellular metabolism by induction of cell stress responses and/or activate cell damage repair and maintenance systems. Therefore, the antioxidants' beneficial role may be reversed/

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prevented by excessive amounts of antioxidant supplements. On the other hand, ROS are also involved in many important physiological processes in humans, such as induction of stress responses, pathogen defence, and systemic signalling. Thus, both "anti-oxidative or reductive stress" (the excess of antioxidants) as well as oxidative stress (the excess of ROS) can be damaging and contribute to the ageing processes.

Keywords Antioxidants · Ageing · ROS · Dietary supplements · Longevity

Introduction

Despite decades of intensive research, the two key questions in ageing research still remain unanswered:

- *Is the age-related increase in oxidative stress, leading to accumulation of oxidative damage to macromolecules, the cause or the consequence of human ageing?*

and

- *Are there any other mechanisms underlying the ageing process (e.g. lack of intracellular nicotinamide adenine dinucleotide)?*

Oxidative stress, a state when there are high levels of reactive oxygen species (ROS) compared to antioxidant defenses or the pro- anti- oxidant disturbance (Sies 1991), is a hallmark of many chronic age-related disorders (Ames et al. 1993), including Alzheimer's disease (Markesbery 1997), Parkinson's disease (Jenner 2003), atherosclerosis (Singh and Jialal 2006; Tsutsui et al. 2011), chronic inflammation (Federico et al. 2007), kidney disease (Ozbek 2012), many types of metabolic disorders (Roberts and Sindhu 2009; Furukawa et al. 2004), myocardial infarction (Di Filippo et al. 2006), stroke (Allen and Bayraktutan 2009), some cancers (Sosa et al. 2013; Fiaschi and Chiarugi 2012) and also ageing (Halliwel and Gutteridge 2005). ROS include free radicals - the reactive chemicals with an unpaired electron in an outer orbit, like superoxide ($O_2^{\bullet -}$), singlet oxygen ($^{1/2}O_2$), and hydroxyl radical ($HO^{\bullet}$) and non-free radical oxygen containing molecules, such as hydrogen peroxide (H_2O_2). There are also reactive nitrogen, iron, copper, and sulfur species (Deguillaume et al. 2005; Riley 1994) that could contribute to increased ROS formation, oxidative stress and impaired redox balance. Endogenous free radicals are produced in metabolic reactions when cells convert the energy from food in the presence of oxygen. The mitochondrial electron transport chain, as the main site of intracellular oxygen consumption, is the main source of ROS formation (Ames 2004; Perez Campo et al. 1998; Barja 1998); the nitric oxide synthase reaction is another endogenous source of ROS. Increased levels of ROS are produced when organisms are exposed to microbial infections, inflammation, perform extensive exercise, or are exposed to exoge-

nous ROS sources, like environmental pollutants and toxins such as alcohol, cigarette smoke, ionizing/UV radiations and excessive amounts of redox-active metals. Most of the free radicals are generated inside the cells (Richter et al. 1988; Sastre et al. 2000). To prevent oxidative damage, it is important that excessive ROS are neutralized by antioxidants and antioxidative responses. Increased levels of oxidative damage of DNA, proteins and lipids were found in aged human tissues and animal models (Bokov et al. 2004). Lower oxidative damage and increased resistance to oxidative stress is observed in long-lived compared to the short-lived animals (Bokov et al. 2004; Sohal and Weindruch 1996; Barja 2002).

Some encouraging experimental results to prevent senescence were obtained by mitochondrial-targeted antioxidants, such as triphenylphosphonium-conjugated antioxidants plastoquinone (SkQ1) and MitoQ (Skulachev et al. 2011; Anisimov et al. 2008; Rocha et al. 2010). An increased production of ROS is the main reason for oxidative stress associated with many before mentioned pathologies.

Free Radical Theory of Ageing

The Free radical theory of ageing or the Mitochondrial free radical theory of ageing (MFRTA) postulated by Harman in the 1950s, proposed that ageing results from the accumulation of oxidative damage, closely linked with the release of ROS from mitochondria (Liu et al. 2014; Piotrowska and Bartnik 2014). According to MFRTA, the accumulation of oxidative damage is the main driving force of the ageing process. Supporting evidence are the inverse correlations between species' lifespan and oxidative damage, however with exceptions. For example, the longest-living rodent, the naked mole rat (*Heterocephalus glaber*), has high levels of oxidative modifications - protein carbonylation, lipid peroxidation and oxidative damage of DNA (Sanz and Stefanatos 2008; Perez et al. 2009a, b). Strenuous physical activity/exercise is another example that contradicts MFRTA, since the increased oxygen consumption and ROS formation is without the concomitant decrease in lifespan.

Antioxidants

Natural Antioxidants

Antioxidants are substances that delay, prevent or remove oxidative damage to/from a target molecule (Halliwell 2007; Halliwell and Gutteridge 2015). An alternative definition is that they are substances that directly scavenge ROS, up-regulate antioxidants defenses or inhibit ROS production (Khlebnikov et al. 2007). Free radical-induced oxidative stress is attenuated by preventive and repair mechanisms, or physical and antioxidant defenses (Valko et al. 2007). Endogenous antioxidative cell defenses include enzymatic and non-enzymatic molecules that are distributed within the cytoplasm and cell organelles. Endogenous defenses against ROS include

the enzymes superoxide dismutase (intracellular Cu/Zn-SOD, Mn-SOD and extracellular SOD), glutathione peroxidase, catalase, peroxiredoxins and the nonenzymatic antioxidants, glutathione, thioredoxin, and uric acid (Poljsak 2011). Enzymatic antioxidants are divided into primary and secondary. The **primary antioxidant enzymes**, for example SOD, several peroxidases and catalase, catalyze a cascade of reactions that convert ROS to more stable molecules, such as H_2O and O_2. SOD converts superoxide anions into hydrogen peroxide as a substrate for catalase (Rahman 2007). It has been suggested that, *in vivo*, the activation of enzymatic antioxidant defenses by exogenous antioxidants ingested from food is more important than the ROS scavenging (Forman et al. 2014). To conserve the cell's energy, endogenous antioxidant defenses and repair systems are only triggered by elevated ROS formation (Poljsak et al. 2011). Besides primary enzymes, a large number of **secondary antioxidant enzymes** (glutathione reductase and glucose-6-phosphate dehydrogenase) neutralize ROS indirectly, in association with other endogenous antioxidants. Glutathione reductase reduces glutathione and recycles it to enable more ROS neutralization. Glucose-6-phosphate dehydrogenase regenerates nicotinamide adenine dinucleotide phosphate (NADPH) into a reduced state (Turunen et al. 2004). There are many **nonenzymatic endogenous antioxidants**. Cofactor coenzyme Q is present in cells and membranes and plays an important role in cellular metabolism and in the respiratory chain; its free radical-scavenging antioxidant properties are due to iron–sulfur clusters that accept electrons. Vitamin A combines with peroxyl radicals thus preventing lipid peroxidation (Jee et al. 2006). Uric acid prevents lysis of erythrocytes and is also an important scavenger of singlet oxygen (Kand'ar et al. 2006). Other small molecular-weight nonenzymatic antioxidants include vitamins E and C, and many minerals, like selenium and zinc. Selenium is the integral part of the antioxidant enzyme glutathione peroxidase (Tabassum et al. 2010). Flavonoids (i.e. flavonols, flavanols, anthocyanins, isoflavonoids, flavonones and flavones and phenolic acids) act as chelators of transition metal ions involved in Fenton's chemistry and ROS scavengers.

ROS formation, and related oxidative damage, is a continuous process in the human body. The human cells have a second category of antioxidant defense molecules that remove and repair damaged biomolecules thus limiting their accumulation and detrimental effects (Cheeseman and Slater 1993). These include enzymes for reparation of oxidatively damaged nucleic acids, oxidized proteins and oxidized lipids (Cheeseman and Slater 1993). Many essential maintenance repair systems are deficient in senescent cells, which is consistent with the theory of cell damage accumulation over time (Terman and Brunk 2006; Brunk et al. 1992).

Do Exogenous Antioxidants Promote Longevity by Attenuating ROS Induced Cell Damage?

Does the intake of natural extracts from fruit and vegetables have any advantages over ingestion of synthetic antioxidants? In principle, yes. Due to their regenerative potential, antioxidants should be ingested as an integral part of a daily diet and not

as a supplement. People who regularly eat fresh fruits and vegetables, a good source of antioxidants and other phytochemicals, have a lower risk for heart disease and for some neurological diseases (Stanner et al. 2004). There is also evidence for some types of vegetables and fruits to reduce the risk for a number of cancers (World Cancer Research Fund 2017). Beneficial health effects have been demonstrated for several chemicals isolated from plants (Raskin et al. 2002; Reddy et al. 2003). Initially it was assumed that the antioxidant activity of phytochemicals is responsible for their health benefits (Bravo 1998). Later it was shown that other substances in fruits and vegetables, or a complex mix of substances (e.g., inhibitors of cell proliferation like green tea polyphenols, resveratrol, curcumin; inhibitors of P450 from grapefruit and garlic; antagonists of estrogen, like some flavonoids; inhibitors of angiogenesis, like genistein, *epigallocatechin gallate*) contribute to a better health and a decreased incidence of age-related diseases, observed in the individuals with a high intake of fruit and vegetables (Cherubini et al. 2005; Lotito and Frei 2006) compared to the general population with a low intake. Diet modulates gene expression (Alam et al. 2016). For example, phytochemicals modulate stress induced pathways via activation of the transcription factor Nrf-2; sulforaphane and curcumin activate the antioxidant response element (ARE) (Lee and Surh 2005); resveratrol activates histone deacetylases and their target FOXO transcription factors (Frescas et al. 2005); and capsaicin and allicin activate the transient receptor potential calcium channels (Bautista et al. 2005). These beneficial dietary effects are summarized in the "xenohormesis hypothesis" (Lamming et al. 2004) that attributes the health benefits from dietary phytochemicals, particularly from secondary metabolites produced by plants under stress, to the activation of stress signaling pathways in animals and fungi (Sinclair and Howitz 2006).

Increased fruit and vegetable consumption lowers overall mortality in humans (Bellavia et al. 2013; Zhan et al. 2017), while intake of synthetic antioxidants increases overall mortality (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994). Can it be concluded that the antioxidants in fruit and vegetable have different effects on human lifespan than the synthetic ones? Lifespan increasing agents for rodents are, for example, extracts from pomegranate, blueberry, black and green tea, curcumin, cinnamon, morin, sesame, pycnogenol, quercetin, and taxifolin (Spindler et al. 2013). However, the authors found no significant effect of these extracts on the lifespan of male F1 hybrid mice. Therefore, isolated phytonutrient anti-oxidants and anti-inflammatories cannot be considered as potential longevity therapeutics, even though consumption of whole fruits and vegetables is associated with extended healthspan and lifespan. The relevance of rodent studies for humans is not clear. For example, one-hundred-six studies, evaluating potential longevity compounds or combinations of longevity compounds were reviewed by Spindler (2012) and only six studies reported both lifespan extension and food consumption data, thereby excluding the potential effects of caloric restriction which is the only scientifically proven method to extend lifespan in many species tested.

Why Is Ingestion of Synthetic Antioxidants Without Health Benefits?

Studies in invertebrates (e.g., the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila*) and rodents report a positive correlation between increased lifespan and resistance to oxidative stress. Only a few studies with transgenic *Drosophila,* which overexpress antioxidant enzymes, imply that alterations in oxidative damage/stress has a role in ageing (Perez et al. 2009a, b). Surprisingly, the maximum lifespan correlates with the rate of DNA repair only; it correlates inversely with antioxidant enzyme levels and free-radical production (Perez Campo et al. 1998). Additionally, mice deficient in both Mn-SOD and glutathione peroxidase-1 have an increased oxidative damage and a greater incidence of pathology but no reduction in lifespan (Zhang et al. 2009). SOD knockout mice have a higher oxidative damage to DNA yet show a slightly longer lifespan than controls (Van Remmen et al. 2003). Deletions of various SOD isoforms in *C. elegans* did not shorten lifespan despite the attenuated protection against oxidative stress (Gems and Doonan 2009; Doonan et al. 2008; Yang et al. 2007). However, deletion of the antioxidant defense in *C. elegans* elicited a tenfold extension of both median and maximum adult lifespan (Ayyadevara et al. 2008). Similarly, the longest living rodent, the naked mole rat (maximum lifespan 28.3 years vs. mice 3.5 years), exhibits increased levels of lipid peroxidation, protein carbonylation, and DNA oxidative damage even at a young age (Perez et al. 2009a, b). Bats have a higher metabolic rate and a greater production of mitochondrial ROS, but nevertheless they live much longer than mice, with the lifespan of about 20 years (Finch 1990). Genetically modified mice that possess extra mitochondria live almost 2 years longer than the wild-type mice, despite more ROS being formed during the oxidative phosphorylation (Hanson and Hakimi 2008). Two conclusions can be made on the basis of the presented animal experiments. Firstly, overexpressing or knocking down antioxidant levels modulates oxidative damage and can even increase cancer incidence, but does not modulate lifespan (Lewis et al. 2013). Secondly, human studies, although long and costly, are the only way to evaluate hypothetical benefits of synthetic antioxidants on human lifespan.

Antioxidant Epidemiological Studies

Many *in vitro* human studies imply that synthetic antioxidant or plant extracts supplementation decreases oxidative stress and oxidative damage (reviewed in Poljsak and Fink 2014). According to the free radical theory of ageing, the antioxidant use should reduce the incidence of cancer risk and other chronical degenerative diseases and prolong lifespan by neutralizing damage-causing free radicals. However, this assumption is not consistently supported by epidemiological studies/human trials that assessed the antioxidants' effect on the incidence of specific diseases and/or lifespan (Bjelakovic et al. 2004; Miller et al. 2005a, b; Vivekananthan et al. 2003;

Caraballoso et al. 2003). Bjelakovic et al. (2007) published a systematic review of the literature on antioxidant studies from 1977 to 2006. They selected and analyzed 47 most rigorously designed studies of supplement effectiveness. The antioxidants assessed were beta-carotene, vitamin A, vitamin C, vitamin E, and/or selenium and the result showed increased mortality among supplement users. This result is consistent with the study where large doses of vitamin E increased the risk of prostate cancer by 17% in 35,500 men over 50 and ingestion of the antioxidant beta-carotene and retinol significantly increased the risk of lung cancer in male smokers (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994). The authors concluded that antioxidants might increase ROS formation by the Fenton reaction. However, they considered it more likely that antioxidants did not trigger cancer but rather accelerated the progression of existing undiagnosed cancers by increasing cellular proliferation. There are some data in animal models and humans on the extended lifespan, by lowering all-cause mortality, by ibuprofen and aspirin in low doses (He et al. 2014, Kaiser 2012) and vitamin D in large doses (Kupferschmidt 2012).

Why Were Clinical Trials with One or Multiple Synthetic Antioxidants Unsuccessful?

First, homeostatic mechanisms regulate the amount of antioxidant activity in cells. There may be substances other than antioxidants in fruits and vegetables that improve cardiovascular health and decrease cancer incidence (Cherubini et al. 2005; Lotito and Frei 2006).

Second, the endogenous antioxidative cell defense or activation of cell death pathways may be changed by the intake of only one antioxidant. The overall antioxidant capacity remains unaffected, as changes in the level of only one antioxidant may trigger a compensatory adjustment in the others. Cell exposure to exogenous antioxidants could decrease the rate of synthesis or the uptake of endogenous antioxidants, thus the total "cell antioxidant potential" remains unaltered (reviewed in Poljsak 2011). Ristow and colleagues reported that nutritive antioxidants annuled lifespan extension by mitohormesis inhibition (Schulz et al. 2007). Supplementation with antioxidants or radical-scavenging supplements could neutralize the ROS which triggers the release of Nrf2 that produces hormesis during low cell stress. Antioxidant supplementation, when the cell's stress is within the hormesis range, buffers ROS and thus "turns off" the stress activated keap1-Nrf2 pathway; therefore, turning off hormesis. For example, taking antioxidants during exercise abolishes the exercise's hormetic health benefits (Ristow et al. 2009). Supplementation with some antioxidants (e.g. vitamin E and α-lipoic acid) suppresses skeletal muscle mitochondrial biogenesis, regardless of training status (Strobel et al. 2011). Antioxidant therapy, with vitamins A, C, E, and resveratrol, can suppress the synthesis of endogenous antioxidants, most probably due to the reduced mitochondrial biogenesis, which is stimulated by excessive ROS formation (Strobel et al. 2011), thus prevent-

ing the beneficial effects obtained with regular exercise (Gomez Cabrera et al. 2008; Gliemann et al. 2013; Donato et al. 2010). Schulz et al., demonstrated that nutritive antioxidants completely prevented lifespan extension in *C. elegans* by inhibiting mitohormesis (Schulz et al. 2007). Additionally, the imbalance caused by reducing ROS, and/or increasing the antioxidant capacity, could affect cellular signaling and attenuate the mitohormesis mediated training benefits (Richardson et al. 2007).

Third, antioxidants that are reducing agents can act as prooxidants in higher doses or in the presence of redox cycling metal ions, promoting an increased free radical formation. The superoxide anion that is mainly produced during mitochondrial metabolism, is neutralized by superoxide dismutase, which generates hydrogen peroxide (H_2O_2). H_2O_2 is formed during autoxidation of synthetic antioxidants vitamin C and E (Poljsak and Raspor 2008; Poljsak et al. 2006). This ROS is not very reactive *per se*; nevertheless, the presence of transition metal ions may trigger the formation of highly reactive free radicals by a Fenton-like reaction. Vitamins C and E reduce metal ions, for example iron to the form of Fe^{2+}, which generate free radicals through the Fenton reaction:

(i) $2Fe^{3+} + \text{Ascorbate} \rightarrow 2Fe^{2+} + \text{Dehydroascorbate}$

(ii) $2Fe^{2+} + 2H_2O_2 \rightarrow 2Fe^{3+} + 2OH\cdot + 2OH^-$

Redox cycling metal ions (such as iron, copper, chromium, cobalt and vanadium) and antioxidant vitamins increase free radical formation. Increased levels of superoxide anion, hydrogen peroxide, antioxidant vitamins, or the redox-active metal ions increase formation of hydroxyl radical. For example, the Iowa Women's Health Study data imply that dietary vitamin and mineral supplements are associated with increased mortality, especially in the presence of iron supplements (Mursu et al. 2011). Metal-chelating antioxidants, (e.g. albumin, ceruloplasmin, ferritin and transferrin) attenuate ROS production by inhibiting the Fenton reaction catalyzed by copper and iron (Imlay 2003, Terman and Brunk 2006). Flavonoids also act as chelators of transition metal ions involved in Fenton's chemistry preventing ROS formation (Mira et al. 2002; Bhuiyan et al. 2017; Zhang et al. 2016a, b).

Fourth, ROS are also signaling molecules. Cells generate hydrogen peroxide to regulate glucose metabolism, cellular growth and proliferation, and as a cell defense against pathogens (Rhee 1999). The main synthesized ROS are superoxide radical and nitric oxide, which are produced by NADPH oxidases and NO synthases (Bedard and Krause 2007). ROS are also physiological mediators that modulate transcription factors (Nordberg and Arner 2001), such as the redox-sensitive transcription factor nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). ROS in moderate concentrations also act as essential mediators for the body to dispose of unwanted cells by apoptosis. If administration of antioxidant supplements decreases ROS, it may also interfere with apoptosis and attenuate elimination of damaged cells, including those that are precancerous and cancerous (Salganik 2001).

Fifth, antioxidants cannot distinguish among the beneficial radicals and those causing oxidative damage to biomolecules. Thus, both the "anti-oxidative or reduc-

tive stress" (antioxidative imbalance) as well as the oxidative stresses can be damaging for organisms and may promote cancerogenesis and ageing, respectively (reviewed in Poljsak and Milisav 2012).

Last, some ROS are extremely reactive; the estimated half-life of the $OH^{\cdot}$ is 10^{-9} seconds. $OH^{\cdot}$ can diffuse the distance between 3 to 10 nm within this time (Halliwell and Gutteridge 1984) and reacts with the surrounding molecules within three molecular diameters (Pryor 1994). Therefore, $OH^{\cdot}$ is not scavenged by any synthetic antioxidant (e.g. vitamin C, E), unless the antioxidant is present in excessive amounts to the biomolecules (Cheeseman and Slater 1993). As this is not feasible, even supplementation of dietary antioxidants cannot efficiently scavenge all of the ROS formed within the cells. Enzymatic antioxidants accelerate chemical reactions and have a higher capacity to eliminate ROS compared to the dietary antioxidants. The human body produces 10^{23} free radicals per hour (Lodish et al. 2004); therefore, the equimolar ratio of antioxidant enzymes (catalase, superoxide dismutase) quenches much more free radicals then the non-enzymatic antioxidants.

In summary, an increase in cellular antioxidants may trigger either increased ROS formation or neutralize free radicals, causing redox and signal transduction dysregulation. Nonselective elimination of free radicals by synthetic antioxidants is therefore more likely to disrupt, rather than extend, the normal cell function.

What Can Be Learned from Exceptionally Long-Living Species?

Both, mitochondrial energy production and their membrane phospholipid content influence lifespan of animals. Stabilization of mitochondrial structure and energy efficiency reduces oxidative stress and promotes longevity in various species. A highly peroxidation-resistant membrane composition was observed in the exceptionally long-living mammals and birds compared to the shorter-living animals of similar size (Hulbert 2008; Hulbert et al. 2007). Saturated and monounsaturated fatty acids are more resistant to peroxidative damage than the polyunsaturated ones. The mitochondrial respiratory chain complexes of long lived birds generate fewer free radicals than the mitochondrial respiratory chain complexes of mammals with similar metabolic rate and size (Hulbert et al. 2007). Efficient DNA repair contributes to longevity in Blanding's (*Emydoidea blandingii*) and painted turtles (*Chrysemys picta*), which have not shown any signs of ageing in studies lasting decades (Congdon et al. 2001; Congdon et al. 2003); this is attributed to their efficient telomere maintenance (Girondot and Garcia 1999; Lutz et al. 2003). Could approaches to increase the cell's repair systems be more important than boosting the cell's antioxidative defense?

Increasing Repair Systems for Increased Lifespan

Moderate oscillations in antioxidants and ROS formation contribute less to ageing and age-related diseases than repair system failures (Gems and Doonan 2009; Perez et al. 2009a, b; Yang and Remmen 2009). As the clearance efficiency of malfunctioning mitochondria decreases with age, the more superoxide is produced (Wallace 2005; Sohal and Weindruch 1996). Also, cytosolic defense mechanisms and processes involved in cellular repair and maintenance decline with age. Therefore, the age-related increase in DNA damage may result from (a) the increased ROS generation and (b) the decline of DNA repair mechanisms and clearance.

The DNA's excision repair capacity positively correlates with the maximum lifespan of diverse mammalian species (Hart and Setlow 1974, Perez Campo et al. 1998). Experimentally enhanced oxidative stress in laboratory animals has neither shortened lifespan nor induced age-related signs, perhaps due to a high rate of DNA repair and a low rate of free radical production near the DNA. DNA repair capacity correlates with species-specific lifespan. The importance of equilibrium between cell damage and repair was observed in centenarians. On the one hand, there was an age-associated decrease in the levels of proteins that participate in nucleotide excision repair, like proliferating cell nuclear antigen (PCNA), replication protein A (RPA), excision repair 3 (ERCC3), DNA damage recognition and repair factor XPA, and p53 (Goukassian et al. 2000). On the other hand, there was an 1.6-fold increased poly(ADP-ribose) polymerase (PARP) activity, an immediate response of eukaryotic cells to oxidative and other types of DNA damage (Muiras et al. 1998). PARP activation is an immediate cellular response to chemical, metabolic or radiation-induced DNA single-strand DNA breaks (Bürkle et al. 2005). Longevity is associated with a high poly-ADP-ribosylation capacity (Muiras et al. 1998); poly(ADP-ribose) polymerase activity of 13 mammalian species correlates with a species-specific lifespan (Grube and Bürkle 1992). Stimulation of DNA repair seems a better alternative to antioxidant supplementation for increasing lifespan; however, the clinical effectiveness and modes of administration and utilization of inducers were not validated and require further study (Emanuel and Scheinfeld 2007; Poljsak 2011).

Hormesis: The Induction of Adaptive Responses to Stress Conditions

Hormesis is an adaptive response to a low-to-moderate-intensity stressor exposure that causes an initial disruption in homeostasis. Toxicologists use the term hormesis to refer to a biphasic dose response to an environmental agent that has a beneficial effect at low doses and a toxic one at high doses (Mattson 2008a, b).

ROS-induced stress/damage may be attenuated by triggering an adaptive stress response that increases endogenous antioxidant activity and damage repair pro-

cesses. Moderate stress induced by physical activity, caloric restriction (CR) or mimetic compounds may induce hormesis. These processes do not seem to interfere with ROS-dependent cellular signaling and may increase cellular resistance to subsequent more severe stress.

The mechanisms of stress responses include cell cycle arrest needed for DNA damage repair, activation of cellular signaling pathways and appropriate transcription factors. The key regulators of cellular stress response pathways include mammalian target of rapamycin (mTOR), proteins involved in insulin/insulin-like growth factor (IGF) signaling, sirtuins and AMP-activated protein kinase (AMPK) pathways (Kenyon 2005).

Different phytochemicals, for example sulforaphane and curcumin, activate the Nrf-2-ARE hormetic pathway (Mattson 2008a, b; Fulgencio et al. 2001; Spindler et al. 2003). Many of the chemicals that trigger an adaptive stress response and induce hormesis may be mistakenly believed to act as antioxidants. An initial mitochondrial or cytosolic ROS formation induced by phytochemicals may trigger mild cellular stress responses leading to increased production of cytoprotective and restorative proteins (Birringer 2011). Some compounds with antioxidant activities may delay ageing because of their role in damage repair stimulation that was triggered paradoxically because of their pro-oxidant activity.

Hormesis is induced by many "stressors", including aldehydes, prooxidants, irradiation and UV radiation, caloric restriction, heat, heat shock, cold and hypergravity. There is a cross-resistance to various stressors, including to some phytochemicals, heat shock, ischemia, exercise, dietary energy restriction oxidants and UV-radiation, in organisms from bacteria to humans (reviewed in Milisav et al. 2012). Finkel and Holbrook (Finkel and Holbrook 2000) suggested that oxidative stress induced hormesis is the best strategy to enhance endogenous antioxidant levels. The cell's defense and repair efficiency may be enhanced after exposure to "moderate" levels of ROS, since expression of many DNA repair enzymes is upregulated during the mild oxidative or other kind of stresses (Schulz et al. 2007; Wani et al. 1998; Bases et al. 1992). Therefore, low and intermittent doses of ROS could promote health and increase lifespan through activation of adaptive response pathways by increasing the synthesis of antioxidant and other defense and repair systems (Halliwell 1999). Many DNA repair enzymes are upregulated after oxidative stress (Schulz et al. 2007; Wani et al. 1998; Bases et al. 1992). Components of the heart-healthy lifestyle, like polyunsaturated fats, physical activity and moderate alcohol consumption, are pro-oxidant (Williams and Fisher 2005). The effects of ROS, whether damaging, protective or signaling, depend on the equilibrium between the ROS production, scavenging and time of exposure (Gratão et al. 2005). Halliwell has proposed that stimulation of increased levels of endogenous antioxidants, by some pro-oxidants, was more effective than consuming additional dietary antioxidants (Halliwell 2011).

An alternative to activating repair systems in a physiological manner with mild damage (Fig. 1.1), is the use of certain compounds that could "mimic" damage formation and thus activate stress or damage signaling pathways to upregulate repair. Ideally, hormetic compounds would be used to activate the repair processes in the

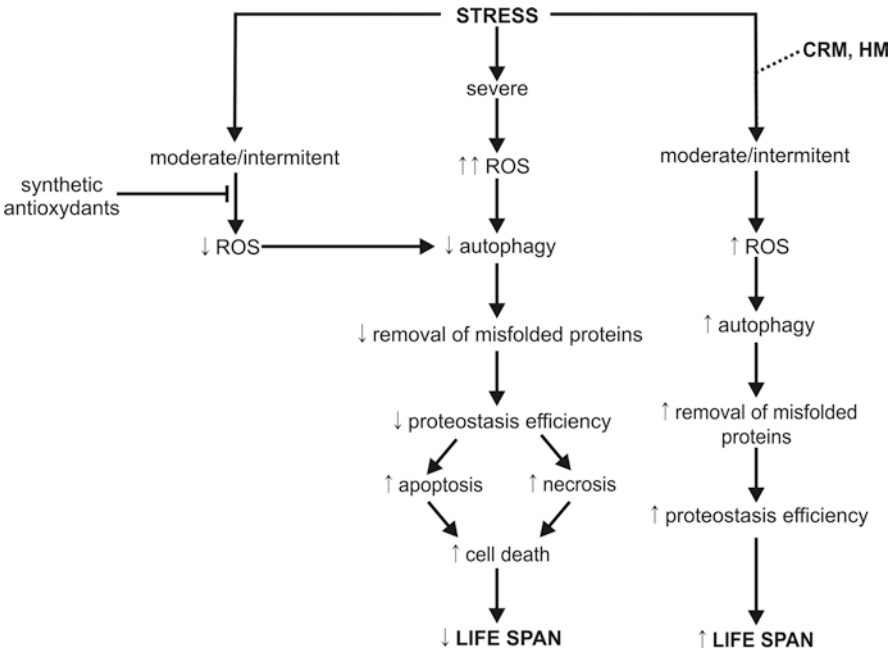

Fig. 1.1 Hypothetical effects of synthetic antioxidants and severe, moderate or intermittent stress on lifespan. Abbreviations: *CRM* caloric restriction mimetics, *HM* hormesis mimetics, *ROS* reactive oxygen species

absence of mild tissue damage. Some CR mimetic (CRM) compounds have physiological and anti-ageing effects similar to CR. The ideal CRM modulates the pathways regulating energy metabolism to induce the health-promoting and anti-ageing effects of CR without calory restriction (Lane et al. 2007). Candidate CRM compounds inhibit glycolysis (2-deoxyglucose), enhance insulin metabolic actions (metformin), or affect stress signaling pathways (resveratrol) (Ingram et al. 2006). Resveratrol and related polyphenol compounds are CRM that extend life-span of yeast, *C. elegans, Drosophila* (Howitz et al. 2003; Wood et al. 2004) and mice on high fat diet (Baur et al. 2006); their effect on human lifespan is not known. At present, the use of hormesis, to prevent ageing and ageing-related diseases in humans, is in the initial stages of research.

Is NAD+ the Missing Link Between Oxidative Stress, CR, Exercise, DNA Repair and Increased Healthspan?

The nicotinamide adenine dinucleotide (NAD+) coenzyme participates in reduction-oxidation reactions and is an essential co-substrate for other enzymes such as sirtuins and poly(adenosine diphosphate–ribose) polymerases which regulate apoptosis,

DNA repair, stress resistance, metabolism, and endocrine signaling (Sauve 2008). NAD(+) depletion promotes the ageing process by limiting (1) energy production, (2) DNA repair and (3) genomic signaling. NAD(+) regulates lifespan as a cofactor in redox reactions and as a coenzyme in metabolic processes. NAD(+) activity declines with ageing and NAD(+) restoration can reverse cell ageing by inducing cellular repair and stress resistance (reviewed in Poljsak and Milisav 2016). Many studies imply that ageing and age-associated diseases could be ameliorated with increased NAD+ availability (Houtkooper et al. 2010; Khan et al. 2007; Ying 2007; Bieganowski and Brenner 2004; Imai 2010, Trammel et al. 2016). NAD(+) levels can be increased by different means offering several approaches to develop prevention and treatment interventions for ageing and age-associated diseases. For example, aerobic exercise, CR and fasting increase NAD(+) levels, as well as mitochondrial and sirtuin activity (Hipkiss 2008; Morris et al. 2011; Morselli et al. 2010). Ingestion of NAD+ intermediates, like nicotinamide riboside and nicotinamide mononucleotide, increases NAD+ levels (Bogan and Brenner 2008; Canto et al. 2012; Mills et al. 2016; Zhang et al. 2016a, b).

NAD+ enables optimal functioning of poly ADP ribose polymerase (PARP). Longevity is associated with a high poly-ADP-ribosylation capacity: PARP is increased by 1.6-fold in centenarians (Muiras et al. 1998) and PARP activity of 13 mammalian species correlates with a species-specific lifespan (Grube and Bürkle 1992). Nicotinamide phosphoribosyltransferase (NAMPT)-mediated nicotinamide adenine dinucleotide (NAD+) levels might be the missing link between ageing, cell cycle control, DNA damage repair and cellular metabolism on the one hand and the ageing clock, on the other (reviewed in Poljsak 2017). Since the research in NAD+s role in lifespan regulation is in the early stages, the answer to the question on whether NAD+ is the real "fountain of youth" lies in the future.

Conclusion

Antioxidant Vitamin Supplements

Epidemiological studies on synthetic antioxidants failed to justify addition of these compounds to our diet on grounds of health improvement and lifespan prolongation. However, this does not negate the necessity to search for alternative interventions to ameliorate the progressive, ageing related increase in oxidative stress and ROS-induced cell damage. There is no official recommendation for the number or the amount of antioxidants we need (Argüelles et al. 2007); therefore, the best source to obtain antioxidants is from a varied diet. Daily intake of at least 400 g of fruits and vegetables has been recommended by the World Health Organisation (World Health Organization, 2017) to obtain enough protective compounds from the regular diet to neutralize ROS. However, 80% of American children and adolescents and 68% of adults do not eat enough fruit and vegetables to meet the WHO

five portions a day recommendation (Ames 2001). Additionally, the effect and quantity of antioxidants in fruits and vegetables is reduced by (a) an excessive consumption of energy-rich, micronutrient-poor, and refined food (Ames 2006); (b) an impoverishment of the soil that is reflected in the low antioxidant and micronutrient food content (from excessive exploitation of the soil itself, acid rain, increasing desertification, pollution, etc.), (c) an uncontrolled use of pesticides and (d) a post-harvest and industrial processing of food to improve storage, transport and shelf life that reduces the antioxidant and micronutrient content of fruits and vegetables (Tijskens 2004). Degradation in nutrition value of fruits and vegetables might reach a degree to justify diet supplementation with synthetic antioxidant vitamins, although in principle additional vitamin and mineral supplements are not needed for a healthy adult person, if he/she eats varied and diverse foods with a sufficient energy intake.

Stimulation of the Repair Systems

Prevention of excessive mitochondrial ROS generation seems to be a more efficient approach for decreasing oxidative stress than quenching free radicals with antioxidants. The putative enhanced protection from ROS, by overexpression of antioxidant enzymes, failed to lead to an extended life-span (Seto et al. 1990; Orr and Sohal 1993; Mockett et al. 1999). Numerous studies on model organisms were unable to find any evidence to support the hypothesis that lowering ROS promotes longevity, nor that increasing antioxidant capacity extends the lifespan (Ristow and Schmeisser 2011). Numerous clinical trials, in which individuals received one or more synthetic antioxidants, failed to demonstrate any benefits of antioxidant supplementation. There are several possible explanations for these results that could occur individually or in combination: (a) increased antioxidative stress is harmful due to disrupted ROS signaling; (b) homeostatic mechanisms in cells prevent beneficial effects of the exogenously added antioxidants; (c) the rate of synthesis or the uptake of endogenous antioxidants may decrease when adding the exogenous antioxidants, so that the total "cell antioxidant potential" remains unaltered; (d) there are additional substances in fruits and vegetables that improve cardiovascular health and decrease the cancer risk (Cherubini et al. 2005; Lotito and Frei 2006); (e) many antioxidants do not reach the sites of free-radical generation or the antioxidants targeted to mitochondria, like MitoQ or plastoquinone, and substances or approaches that promote new mitochondria or enhance their efficiency (e.g. ketogenic diet, caloric restriction, exercise, NAD+ precursors) have only a marginal effect, (f) low bioavailability of some antioxidants because of: poor absorbance (low membrane transport), non penetration of the blood-brain barrier, conversion of antioxidants to prooxidants under certain physiological conditions, e.g. in the presence of free unbound iron ions and (g) oxidative stress and oxidative damage do not significantly influence healthspan or lifespan.

Increased oxidative damage had no impact on age-related mortality profiles of studied animals (Speakman and Selman 2011). Thus, modulating the concentration of antioxidants by gene manipulation or dietary intake of synthetic antioxidants does not seem to influence longevity or prevent oxidative stress/damage since paradoxically the synthetic antioxidant supplementation may even increase mortality in humans (Bjelakovic et al. 2004, 2008; Miller et al. 2005a, b; Kim et al. 2002; Vivekananthan et al. 2003; Caraballoso et al. 2003). The inverese correlation between the levels of antioxidants and longevity in mammals was observed for many different enzymatic and non-enzymatic antioxidants in different types of tissues (Barja 2012). Long-lived animals have lower levels of mitochondrial ROS formation, no matter if their metabolic rate is low (e.g. larger mammals) or high (e.g. birds) (Gredilla and Barja 2003). There are at least two main characteristics of long-lived species: a high rate of DNA repair and a low rate of free radical production near the DNA. Also, long-lived animals have fewer double bonds in their unsaturated lipids, so they are less vulnerable to ROS mediated changes (Perez Campo et al. 1998).

Although antioxidants do not promote longevity, oxidative stress still remains the most important contributor to ageing. Thus, alternative approaches to prevent or attenuate oxidative stress tissue damage should be considered, for example stimulation of cellular damage repair. Although the oxidative damage increases with age (Fraga et al. 1990; Oliver et al. 1987; Hamilton et al. 2001), some data imply that the rate of oxidative DNA repair and other cell repair mechanisms decrease with age (Little 1976; Ralser and Benjamin 2008). The duration of life-span and health-span may thus be improved by activating signaling pathways that boost cellular repair and maintenance processes. ROS are involved in intracellular signaling and redox regulation. As some ROS seem to act as signaling molecules in cellular signaling pathways, any generalized lowering of the levels of oxidative stress by antioxidant supplements cannot be beneficial. Thus, life-span-extending approaches should use hormetic compounds or mild to moderate stress inducing physical activity or dietary interventions to selectively activate stress response pathways and promote defense and repair mechanisms (Poljsak and Milisav 2014). Modulation of mitochondrial function is key to the success of life-span-extending approaches. Interventions that increase mitochondrial activity extend lifespan. Such examples are CR, pharmacological treatments and moderate physical exercise (Ames 2005; Warburton et al. 2006; Lanza et al. 2008; Schulz et al. 2010). Reduced mitochondrial activity decreases lifespan in *S. cerevisiae*, *C. elegans* and in rodents (Bonawitz et al. 2006; Zarse et al. 2007; Thierbach et al. 2005). However, it still remains to be answered whether continuous activation of repair processes provides sustained and only beneficial results, since chronic repair processes (e.g. inflammation) also promote collateral, pathologic tissue remodeling, for example, fibrosis.

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Chapter 2
Autophagy: 'Self-Eating' Your Way to Longevity

Charlotte J. Pattison and Viktor I. Korolchuk

Abstract Ageing is the gradual decline in biological function both at the cellular and organismal level. One of the key characteristics of cellular ageing is the accumulation of damaged proteins and organelles which, in turn, can cause cellular toxicity and death. Autophagy is an evolutionarily conserved process that is responsible for the sequestration of damaged or surplus cytoplasmic components which are then delivered to the lysosome for degradation. This house-keeping mechanism is essential to maintain cellular homeostasis and survival, particularly during stress. A decline or loss of sensitivity/responsiveness of autophagy is intimately linked with an accelerated rate of ageing as well as many age-related diseases including neurodegeneration, cancer and metabolic disease where damage accumulation exceeds damage removal. This chapter summarises current knowledge regarding the relationship between autophagy and ageing and outlines some strategies that can be implemented to promote the anti-ageing effects of autophagy to improve human health and lifespan.

Keywords Autophagy · Damaged proteins · Lysosome · Cellular homeostasis · Age-related disease · Anti-ageing

Introduction

The maintenance of cellular homeostasis requires the careful, constant sensing of and responding to the intra- and extra-cellular environment. The availability of growth promoting mitogenic cues such as growth factors, amino acids, oxygen and reactive oxygen species are integrated by a vast array of cell signalling pathways to dictate the level of cell metabolism, DNA replication, cell growth and ultimately

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proliferation. The process of autophagy is an important catabolic process that further functions to support energy liberation and anabolism. Derived from the Greek words *auto* and *phagin,* autophagy literally means 'self-eating' and is a process via which old, damaged, dysfunctional and foreign cytoplasmic contents are delivered to the lysosome. The lysosome is a degradative organelle that houses a vast array of digestive enzymes including proteases and glycosidases. Here, the cargo delivered via autophagy is degraded and recycled to provide fuel for biosynthetic processes (Bejarano and Cuervo 2010). Three types of autophagy have been described – microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy, each differing in the way material is taken up and delivered to the lysosome.

Microautophagy refers to the direct lysosomal uptake of cytoplasmic content as a result of invagination of the lysosomal membrane (Sattler and Mayer 2000). In contrast, proteins targeted for degradation by CMA contain a specific peptide motif biochemically related to KFERQ which is recognised by the chaperone protein Hsp70. The cargo-chaperone complex is then recruited to the lysosomal membrane for it to bind to the receptor protein LAMP2A, inducing its multimerisation which is required for the translocation of the cargo into the lysosomal lumen (Dice 1990; Bandyopadhyay et al. 2008). The third type of autophagy, macroautophagy, is the best studied type and will be referred to simply as autophagy from here on. Autophagy was classically considered to be a non-selective and bulk degradation process which involves the sequestration of cytoplasmic contents into a *de novo*, double-membrane structure called a phagophore (Fig. 2.1). The phagophore grows around the cargo that is to be degraded to form an autophagosome which is trafficked to and fuses with the lysosome (Kopitz 1990; Levine and Kroemer 2008a). Increasingly however, mechanisms of more selective forms of autophagy are being

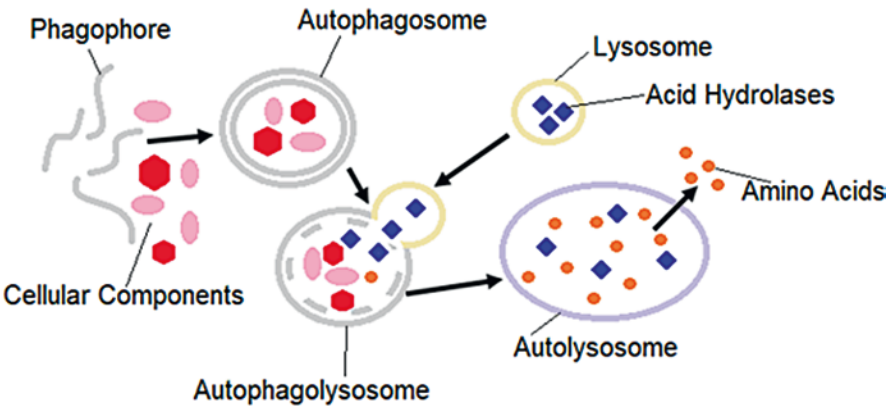

Fig. 2.1 Diagram depicting the process of macroautophagy. Autophagy is induced in response to stress such as oxygen and nutrient starvation. A phagophore forms arounds cellular components that have been targeted for degradation, engulfing them into an autophagosome. Autophagosomes fuse with lysosomes where the contents are degraded by lysosomal acidic hydrolyses into the constituent amino, nucleic and fatty acids. These basic structures can then be recycled and reused as a source of energy and for the biosynthesis of new cellular organelles and components

described that, similar to classic macroautophagy, are dependent on autophagosome cargo delivery to the lysosome and share much of the same molecular machinery. Most notably, these include the selective degradation of mitochondria ('mitophagy'), endoplasmic reticulum ('ERphagy'), protein aggregates ('aggrephagy'), intracellular bacteria ('xenophagy') or peroxisomes ('pexophagy') (Kirkin et al. 2009). As illustrated in Fig. 2.1, autophagy involves the sequestration of cellular components and their digestion into simple biosynthetic building blocks which are then liberated and reused within the cell (Mizushima 2007; Van Zutphen et al. 2013).

Autophagy and Its Molecular Components

Autophagy is highly regulated and continuously occurs at a basal level within all cells, performing a housekeeping role to clear misfolded, old and damaged proteins and organelles that would otherwise accumulate and cause toxicity. Autophagy is strongly upregulated via both direct and indirect mechanisms in times of cellular stress, for example during nutrient or energy deprivation, oxidative stress or pathogen invasion. This cytoprotective role of autophagy allows for the liberation of amino acids or energy to support cell survival, as well as clearing potentially toxic oxidised proteins or pathogens (He and Klionsky 2009; Cuervo and Macian 2012).

Nutrient starvation is one of the most potent activators of autophagy and works via the mammalian (also referred to as mechanistic) target of rapamycin complex 1 (mTORC1). In nutrient replete conditions, mTORC1, the master regulator of cell growth, drives biosynthesis, specifically protein translation, lipid and nucleotide synthesis and inhibits autophagy. This inhibition of autophagy however is relieved in nutrient deprivation (Laplante and Sabatini 2012). mTORC1 is a multiprotein complex that consists of mTOR, a 298 kDa serine/threonine protein kinase that functions as the catalytic subunit of the complex, and the mTOR-interacting proteins Raptor, mLST8, Deptor and PAS40 (Peterson et al. 2009). There are many excellent reviews on this topic (Jung et al. 2010; Jewell and Guan 2013; Russell et al. 2013; Rabanal-Ruiz et al. 2017) so here we will only provide an outline of the upstream regulation of mTORC1. In brief, the activation of Rag GTPases, primarily by amino acids, supports the recruitment of mTORC1 to the cytoplasmic surface of the lysosomal membrane where the small GTPase Rheb, the master activator of mTORC1, resides (Sancak et al. 2010; Kim et al. 2008). In its active form, mTORC1 inhibits autophagy via the phosphorylation of ULK1 (also known as Autophagy-related (Atg) 1) at Ser-757 and Atg13 at Ser-258. Both of these proteins are found in a stable complex with 200 kDa FAK family kinase-interacting proteins (FIP200) (Puente et al. 2016). In contrast, during nutrient stress, mTORC1 is inactivated leading to ULK1 autophosphorylation and ULK1-dependent phosphorylation of FIP200 and Atg13. This leads to the translocation of the complex to the site of phagophore initiation and supports the recruitment of a second protein kinase complex, the VPS34 complex, consisting of the class III PI3K (PI3KC3) VPS34, AMBRA1, Beclin-1, p150/VPS15 and Atg14. Evidence suggests that ULK1-dependent

phosphorylation of AMBRA1 within this complex may promote this localisation. ULK1 phosphorylation also activates Beclin-1, a transient component of the VPS34 complex usually found in association with the anti-apoptotic protein, Bcl-2. Upon its phosphorylation at the Ser-14 site by ULK1, Beclin-1 dissociates from Bcl-2, and instead associates with PI3KC3. Based on this observation, Bcl-2 can be described as an anti-autophagic protein, as well as anti-apoptotic (Jung et al. 2010; Jewell and Guan 2013; Russell et al. 2013; Rabanal-Ruiz et al. 2017).

Together, these interactions downstream of mTORC1 inactivation promote the VPS34-dependent conversion of phosphoinositol to phosphoinositol-3-phosphate (PI3P). The local enrichment of PI3P promotes the recruitment of PI3P-binding proteins such as WD repeat domain phosphoinositide-interacting protein (WIPI) 2B and double FYVE-containing protein (DFCP) 1 to help support formation and elongation of the phagophore membrane. A third complex, made up of Atg16L, Atg5 and Atg12 is also involved in phagophore elongation. It supports the conjugation of the cytosolic microtubule-associate protein 1A/1B light chain (LC3-I), formed by the cleavage of pro-LC3, to phosphatidylethanolamine (PE) thus producing LC3-II which is incorporated into the growing membrane. A number of sources of membrane have been shown to contribute to phagophore formation and elongation including the ER-mitochondrial contact sites, ER exit sites (ERES), ER-Golgi intermediate compartment (ERGIC), Golgi, plasma membrane and recycling endosomes (Ravikumar et al. 2010; Van der Vaart and Reggiori 2010; Hailey et al. 2010; Tooze et al. 2014). LC3-II is an essential marker of autophagy which is important for cargo recognition, autophagosome formation and autophagosome-lysosome fusion (Axe et al. 2008; Ganley et al. 2009; Bartolomeo et al. 2010; Decuypere et al. 2012; Marquez and Xu 2012; Lindqvist et al. 2014; Nguyen et al. 2016; Tsuboyama et al. 2016; Pengo et al. 2017).

The fusion of the fully formed autophagosomes with late endosomes or directly with lysosomes to form autolysosomes is the final step of the autophagy process. Key molecular players required for the successful fusion include the endosomal sorting complex required for transport (ESCRT), Rab7, soluble NSF attachment receptor (SNARE) proteins such as syntaxin17 and lysosomal-associated membrane proteins (LAMP) 1 and 2 (Huynh et al. 2007; Itakura et al. 2012; Zhou et al. 2013). Lysosomes contain digestive enzymes necessary to degrade the autophagosome cargo into simple components such as amino acids, which are transported back into the cytoplasm and reused. Lysosomes have an internal pH of 4.5-5, which is optimal for acidic hydrolases to function (Appelqvist et al. 2013; Yapici et al. 2015). This low pH is tightly regulated and maintained primarily by the active pumping of protons from the cytosol by the vacuolar-type H^+-ATPase (V-ATPase). As the cytosol has a neutral pH of ~7.2, a leak of these enzymes out of lysosomes is not destructive to healthy components of the cell (Mindell 2012). Furthermore the lysosome and integral membrane proteins, including the V-ATPase, amino acid transporter SLC38A9 and cholesterol transporter NPC1, all participate in controlling mTORC1 activity and therefore indirectly, autophagy (Castellano et al. 2017; Wang et al. 2017; Yao et al. 2017). As mTORC1 activity is regulated by its localisation to the lysosome, the aforementioned membrane-spanning proteins mediate

signals from intra-lysosomal amino acids and cholesterol, and furthermore the release of free amino acids into the cytoplasm has also been implicated in activating mTORC1 (Rebsamen et al. 2015; Wang et al. 2015a; Castellano et al. 2017).

Autophagy, Ageing and Disease

A number of studies have intimately lined ageing and changes in autophagy potential. Moreover, age-related changes in autophagic activity have been linked to many diseases including neurodegeneration, cancer, cardiovascular disease, metabolic disease and macular degeneration (Nassif and Hetz 2012; Carroll et al. 2013; Li et al. 2015). Ageing can be defined as a progressive decline in function at a cellular, tissue and organ level (Holliday 1995). This loss of function is associated with a decline in the ability to sense and respond appropriately to environmental stresses and is associated with increased vulnerability to illness and disease (Fedarko 2011). Studies performed in yeast and *Drosophila* models found that mutations in *Atg* genes were associated with significantly shortened organismal lifespan (Juhasz et al. 2007; Fabrizio et al. 2010; Matecic et al. 2010). For example, the study conducted by Juhasz et al. (2007) demonstrated that loss of *Atg7* in *Drosophila* resulted in the inability to appropriately respond to nutrient stress, accumulation of protein aggregates in brain cells and significantly reduced lifespan. In aged *Drosophila* and rat models, the expression of autophagy genes including *Atg1, Atg8a, Atg10* and *Atg18*, at either the mRNA or protein levels, has been shown to be reduced (Omata et al. 2014; Nooraei et al. 2018). Furthermore, hypermethylation of autophagy genes such as *Atg5* and *LC3B* has also been correlated with failed autophagy, tissue degeneration and premature death (Mizushima and Levine 2010; Khalil et al. 2016). Together with the observations in animal models with genetic and pharmacological disruption of autophagy, these results strongly support the notion that age-related impairment of autophagy contributes to the age-related organismal decline.

While many studies indicate that reduced autophagy contributes to age-related deterioration as a result of the accumulation of cellular damage (Cuervo 2008; Carroll et al. 2013; Martinez-Lopez et al. 2015), other studies suggest that at least some aspects of autophagy may be upregulated during ageing, specifically the co-chaperone protein Bcl-2 associated athanogene (BAG) 3. BAG proteins regulate the activity of cellular chaperones by interacting with the Heat shock proteins (Hsp) 70. The upregulation of BAG1 correlates with the increased degradation activity through the ubiquitin-proteasome system (UPS), a process by which damaged and misfolded proteins are ubiquitinated, allowing their recognition by the multi-subunit 26S proteasome, and to be subsequently degraded. However, oxidised and cross-linked proteins have inhibitory effects on the proteasome and thus cannot be degraded and instead accumulate. The accumulation of oxidative damage is a classic hallmark of ageing. It therefore has been hypothesised that, due to the age-dependent reduction of UPS activity, aged cells can compensate by increasing autophagy through upregulation of BAG3 (Gamerdinger et al. 2009; Crippa et al.

2013; Höhn and Grune 2013). BAG3 forms a stable complex with HspB8 and Hsp70 and can indirectly phosphorylate the eukaryotic translation initiation factor 2-alpha (eIF2α) through an unknown mechanism, which ultimately promotes the inhibition of protein synthesis and activation of the autophagic process. HspB8-BAG3-Hsp70 also complexes with the ubiquitin-binding scaffold protein p62 and LC3. These interactions thereby deliver targeted proteins to autophagosomes for their degradation via autophagy (Carra 2009; Gamerdinger et al. 2009, 2011).

Ageing is also associated with the state of cellular senescence. First described by Hayflick and Moorhead (1961), senescent cells are defined by a permanent cell cycle arrest. In this state they are incapable of proliferating, as proteins involved in regulating cell cycle progression such as p16 are upregulated. Senescent cells are characteristically large in size, accumulate mitochondrial dysfunction and secrete proteases and various pro-inflammatory cytokines and chemokines, including IL-6 and IL-8 (Coppé et al. 2008; Korolchuk et al. 2017; Carroll and Korolchuk 2018). Cellular senescence is a normal process and considered to be an important tumour suppressor mechanism in order to prevent the uncontrolled proliferation of cells and, essentially, protect against the initiation and development of cancer (Campisi 2001). At the same time accumulation of senescent cells has been shown to positively correlate with ageing and contribute to age-related organismal decline. One of the factors which contribute to this pro-ageing role of senescent cells is the release of pro-inflammatory cytokines and chemokines by senescent cells. These can induce inflammation, a hallmark of ageing strongly linked to the development of many age-related conditions, most notably cancer (Van Deursen 2014). Studies in progeroid and normally aged mice demonstrated that clearance of senescent cells could delay, alleviate and even reverse signs of age-related tissue degeneration (Baker et al. 2011, 2016). These findings have prompted research looking into senolytic agents as a therapeutic strategy against age-related diseases (Baker et al. 2016). Although the relationship of senescence and autophagy is complex, it is possible that inducing autophagy in senescent cells may act as a senolytic treatment, which, may ultimately improve health and prevent the onset of many age-related conditions (Korolchuk et al. 2017).

Age-Related Neurodegenerative Diseases and Autophagy Dysfunction

Alzheimer's Disease

Alzheimer's disease is the most common neurodegenerative disease, affecting approximately one in ten of all adults over 65 (Karlawish et al. 2017). The main hallmarks of Alzheimer's disease include the accumulation of amyloid-beta (Aβ) peptides and the aggregation of misfolded tau proteins, both of which are toxic and causative in the death of neurons, loss of brain network connections to various

regions of the brain and ultimately cognitive decline (Sanz-Arigita et al. 2010; Serrano-Pozo et al. 2011; Williams 2013). Aβ peptides are formed from proteolytic cleavage of an amyloid precursor protein (APP) by the β- and γ-secretase complexes which interestingly can reside on the inner membrane of autophagosomes (Jutras et al. 2005; Lichtenthaler 2012). These Aβ peptides form soluble oligomeric structures that can be eliminated via autophagy (Jaeger et al. 2010). In some cases however this oligomerisation becomes uncontrolled and incorporation of more peptides into these misfolded oligomers gradually accumulate into an insoluble fibrous plaque. Plaque formation can contribute to cell death and the release of these intracellular Aβ plaques into the extracellular environment. The two main products of APP cleavage are Aβ of 40 and 42 amino acids in length. It is $A\beta_{1\text{-}42}$ that predisposes the protein towards amyloid aggregation and ultimately plaques (Citron et al. 1996; Chow et al. 2009; Friedrich et al. 2010).

Tau is involved in microtubule assembly and stability (Kadavath et al. 2015). While tau is normally modified by phosphorylation in Alzheimer's patients, tau is noted to be hyper-phosphorylated which causes the protein to aggregate with other tau proteins and form neurofibrillary tangles within neurons in the brain. These tangles contribute to synapse loss, cell death and consequently Alzheimer's (Liu et al. 2015). Hyper-phosphorylation of tau affects microtubule stability which has wide implications for all membrane trafficking events in the cell, including autophagosome-lysosome fusion. This is particularly acute in neurons due to their biology and the long distances between the axon and cell body. The subsequent accumulation of autophagosomes and damaged proteins contributes further to neuronal cell death (Funderburk et al. 2010).

Autophagy is widely noted to be reduced or defective in Alzheimer's patients. Research strongly suggests that disruption of the final stages of the autophagy pathway (fusion between autophagosomes and lysosomes and proteolysis of autophagy cargo) underlie autophagy failure, the accumulation of Aβ and tau proteins in the brain and, consequently, the onset of Alzheimer's disease (Zare-Shahabadi et al. 2015). A mutation in the *PSEN1* gene, encoding presenelin-1 (PS1), is the most common cause of familial Alzheimer's disease (Kelleher and Shen 2017). In addition to PS1 functioning as the catalytic subunit of the γ-secretase complex, the protein plays an important role in lysosomal acidification and the activation of lysosomal hydrolases (Lee et al. 2010). Disease-associated mutations in the PSEN1 gene, therefore, affect the ability of lysosomes to degrade cargo as acidification within the lysosomal compartment does not occur and the lysosomal enzymes do not function, as a result leading to accumulation of toxic proteins in neurons (Lee et al. 2010). Autophagosomes containing engulfed APP tend to accumulate in Alzheimer's patients; Aβ fragments continue to be produced as a result of secretase activity within autophagosomes, resulting in elevated levels of Aβ fragments given that they are not successively broken down (Funderburk et al. 2010; Barnett and Brewer 2011). In some cases, these autophagosomes rupture and release Aβ into the cytosol of the cell. One particular consequence of this release is mitochondrial dysfunction. Several *in vitro* studies have demonstrated that these Aβ peptides localise to the mitochondria and block the movement of the nuclear-encoded proteins mtHsp70

and Tom20 into the mitochondria. This results in a decrease to the mitochondrial membrane potential, an increase in ROS production as well as mitochondrial fragmentation via Aβ activation of Drp1 and Fis1, proteins involved in mitochondrial fission (Anandatheerthavarada et al. 2003; Barsoum et al. 2006; Sirk et al. 2007). ROS release, in turn, promotes the formation of more autophagosomes – a vicious cycle accelerating neurodegeneration (Manczak et al. 2006; Dumont et al. 2009; Barnett and Brewer 2011).

Age-Related Macular Degeneration

Dysregulation of autophagy has been implicated in the pathophysiology of age-related macular degeneration (AMD), the leading cause of vision loss in the developed world (Pascolini et al. 2004; Michalska-Małecka et al. 2015).

The macula consists of specialised retinal pigment epithelia (RPE) which functions to absorb light, provide a retinal-blood barrier and is important in ion transport. The RPE is constantly exposed to photo-oxidative stress and exhibits robust levels of basal autophagy which is required to protect the cells from the accumulation of oxidative damage. Indeed AMD is generally associated with a decrease in autophagy and an increase in susceptibility to oxidative stress as well as an accumulation of damaged mitochondria, dysfunctional lysosomes and lipofuscin deposits. These specific hallmarks of AMD can directly and indirectly impact on autophagy potential (although a direct causative association has not been necessarily identified). For example, lipofuscin deposits are yellow-brown electron-dense granules that accumulate in the lysosomes of many tissues with age, including RPE cells. This accumulation causes lysosomal dysfunction, altering lysosomal v-ATPase activity and preventing efficient autophagosome-lysosome fusion which ultimately undermines the quality control role of the autophagosome-lysosome pathway (Terman and Brunk 1998; Holz et al. 1999; Bergmann et al. 2001, 2004; Krohne et al. 2010; Blasiak et al. 2014). Indeed, RPE cells from AMD patients show decreased autophagic flux upon nutrient starvation which appears to be due to defects in autophagosome-lysosome fusion, as well as decreased tolerance to oxidative stress (Golestaneh et al. 2017). Moreover, lipofuscins can generate ROS which can further contribute to autophagy impairment (Jarrett and Boulton 2012). At the same time, a direct reduction in expression of Atg proteins including Beclin-1, Atg9 and Atg7 has been shown in the retinae of AMD patients, especially those with more advanced stages (Jarrett and Boulton 2012; Karlsson et al. 2013; Whitmore et al. 2013; Mitter et al. 2014; Hallam et al. 2017). Furthermore, consistent with the importance of functional autophagy for eye homeostasis, *Beclin-1*$^{-/-}$ and *Atg7*$^{-/-}$ knockout mouse models rapidly develop retinal degeneration when exposed to light-induced oxidative stress (Chen et al. 2013a).

A reduction in autophagy potential has been implicated in reduced mitochondrial fitness in AMD. RPE cells contain high numbers of mitochondria and have a high oxygen consumption rate which supports their function and AMD is associated with

an accumulation of damaged mitochondrial DNA and functionally impaired mitochondria (Karunadharma et al. 2010; Golestaneh et al. 2017). This reduction in mitochondrial fitness contributes significant intracellular ROS which can causes extensive damage, including to autophagy proteins and *in vitro,* AMD patient-derived RPE cells show increased reliance on glycolysis to produce ATP (Mitter et al. 2014; Golestaneh et al. 2017). Furthermore, mitochondria DNA damage has been associated with increased autophagy and exosome release which may directly contribute to the age-related deposition of drusen underneath the RPE which is a risk factor for AMD (Wang et al. 2009). Although it has yet to be formally studied, it would be interesting to investigate the rate of mitochondria-specific autophagy (termed 'mitophagy') in AMD patients and whether a reduction in this specific pathway contributes to cell death and macular degeneration.

Although there are currently no existing treatments available to patients suffering AMD, there are efforts in progress to combat the disease. A small number of drugs are currently in clinical trials, and not surprisingly, several of them have been designed to increase the activity of autophagic pathways within the retina (Taskintuna et al. 2016; Li et al. 2017).

Cancer

The role autophagy plays in cancer is complex and often contradictory; it acts as an important tumour suppressor mechanism in most cases, however, it can also function as a tumour promoter. Evidence that autophagy suppresses tumorigenesis is linked to the discovery of *Beclin-1* mutations in human cancers. Heterozygous loss of the *Beclin-1* allele, located on chromosome 17q21, has been found across breast, ovary, prostate and some brain cancers (Liang et al. 1999; Miracco et al. 2007). In mouse models, heterozygous loss of *Beclin-1* was also shown to increase tumour occurrence, knockout of both *Beclin-1* alleles resulted in death whilst overexpression of *Beclin-1* inhibited tumour development (Liang et al. 1999; Yue et al. 2003). Studies have shown that heterozygous loss of *Beclin-1* resulted in increased intracellular ROS levels, DNA damage and inflammation, suggested that failed autophagy, due to its reduced induction, allows accumulation of damage in cellular components such as mitochondria, giving rise to the deregulation of intracellular ROS levels (Mathew et al. 2009; Kongara et al. 2010). Elevated ROS levels, DNA damage and inflammation are all well-known drivers of tumour initiation and progression (Mathew et al. 2009). Aside from *Beclin-1*, the activation of many oncogenes leads to the upregulation of mTORC1 and the consequent suppression of autophagy to further promote tumorigenesis (Ilagan and Manning 2016).

In certain contexts however, autophagy has also been shown to have a pro-tumour role. Cancer cells have high metabolic demands to support increased growth and proliferation. The induction of autophagy in these cells is essential to supply the necessary energy and nutrients and overcome the stresses such as oxygen and nutrient deprivation (Guo et al. 2016). Thus, some cancer cells, such as those harbouring

oncogenic Ras, are often described as 'autophagy addicted' (Guo et al. 2011; Strohecker et al. 2013). Pharmacological inhibition of autophagy in these aggressive cancers has been successful in reducing tumour growth, increasing sensitivity to chemotherapies and increasing patient survival rates (Yang et al. 2011; Guo et al. 2012).

Another example of the pro-tumour role autophagy can play is with regard to high mobility group box 1 (HMGB1) proteins, regulators of DNA transcription. Usually, the secretion of HMGB1 into the extracellular environment by cells signals tissue damage and initiates a pro-inflammatory response and the degradation of the damaged cells. Some cancer cells, when in an hypoxic environment, can secrete HMGB1, activating the inflammatory response, the secretion of pro-inflammatory cytokines. This constitutes a positive feedback loop further accentuating the inflammatory response which supports the formation, growth and spread of the tumour, while HMGB1-induced autophagy supports the high energy demands of metastatic cancers (Palumbo et al. 2004; Jube et al. 2012; Han et al. 2015).

Myocardial Ischemia-Reperfusion Injury

Ischemia-reperfusion injury of the heart refers to cardiac damage that is caused by the return of oxygen and blood flow after a period of oxygen and glucose deprivation (ischemia) (Zarbock et al. 2014). The decreased function of the myocardium is associated with increased ageing leading to an increase of myocardial apoptosis and the incidence of myocardial ischemia-reperfusion injury (Liu et al. 2011). During ischemia, oxygen and nutrient deprivation results in mitochondrial damage and myocardial dysfunction (Levine and Kroemer 2008b). Autophagy during the ischemic stages is often described as having a beneficial and pro-survival role. However during reperfusion, autophagy often has detrimental effects to cardiomyocytes (Thapalia et al. 2014). This is summarised in Fig. 2.2.

Autophagy is upregulated during ischemia through a 5′-adenosine monophosphate-activated protein kinase (AMPK)-mTOR-ULK1 dependent mechanism (Matsui et al. 2007; Egan et al. 2011). Furthermore, oxygen deprivation promotes the activation of hypoxia inducible factor-α (HIF-1α) which signals for the upregulation of Beclin-1 and autophagy (Zhang et al. 2008). In rat ischemia models, significant autophagosome and lysosome presence was observed compared to the control group (Hariharan et al. 2011). Autophagy supports survival of cardiomyocytes in part through the liberation of amino acids and ATP but it also functions to compensate for ischemia-induced dysfunction of the ubiquitin-proteasome system (Mizushima 2003; Yan et al. 2005; Calise and Powell 2013). Drug-induced inhibition of autophagy in ischemia-subjected mice resulted in a significant reduction of ATP production and increased death of cardiomyocytes (Matsui et al. 2007).

Upon reperfusion, rather than autophagy levels returning to low, basal levels, they increase further in a Beclin-1-dependent manner, and is widely reported as detrimental to cardiac health. The restoration of oxygen to the heart often results in ROS-induced ROS release as the activity down the mitochondrial electron trans-

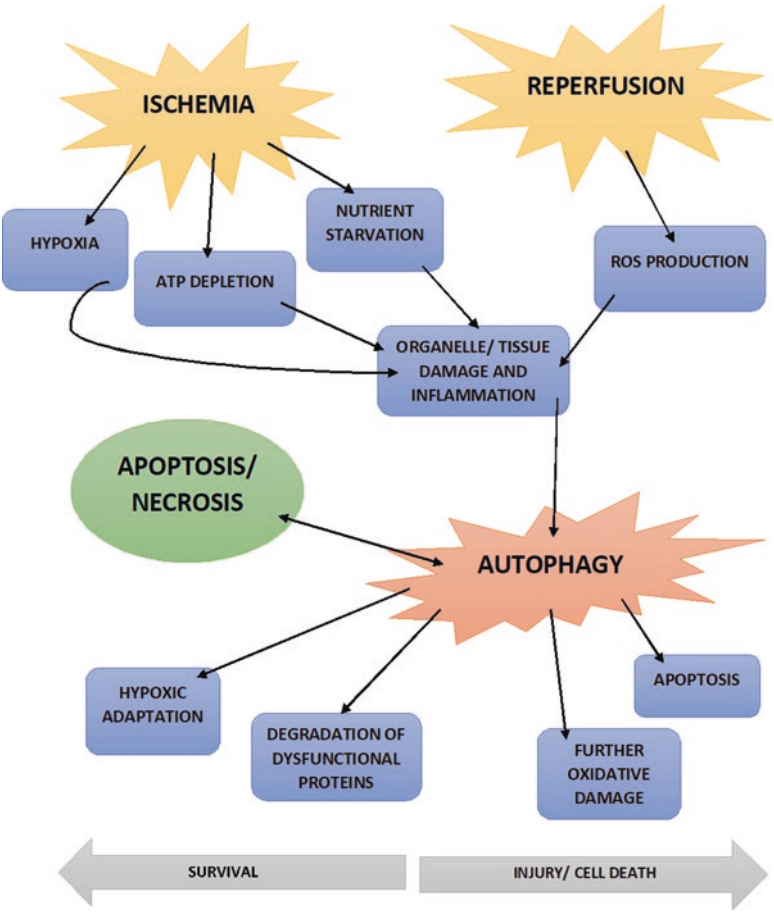

Fig. 2.2 The role of autophagy in Ischemia-Reperfusion Injury. During ischemia, nutrient starvation, oxidative stress and organelle damage strongly activate autophagy. Its induction is initially protective however, autophagic activity further increases upon the reintroduction of oxygen as this reperfusion causes a mass release of ROS. Excessive levels of autophagy may induce cell death and have detrimental effects to the myocardium as more damage is caused to the surrounding tissue affecting its ability to function

port chain restarts. The presence of ROS can cause damage to intracellular proteins. Importantly for autophagy, this has been shown to cause an inhibition of the cysteine protease activity of Atg4 which is involved in the pre-processing and delipidation of LC3 (Scherz-Shouval et al. 2007; Kongara and Karantza 2012; Kalogeris et al. 2014). Consequently, cardiomyocytes suffer severe damage and over-activation of autophagy ultimately leading to cell death (Hamacher-Brady et al. 2006; Sadoshima 2008). Consistent with this conclusion, inhibition of Beclin-1 in a rat model of reperfusion has been demonstrated to suppress cell death (Valentim et al. 2006).

Strategies to Induce Autophagy and Slow Ageing

The world population is ageing which subsequently increases the incidence of age-related diseases and puts a much pressure on communities and healthcare systems. While in some cases the inhibition of autophagy would be beneficial to prevent disease, generally the induction of autophagy is considered to be a strategy to slow the aging process and increase longevity. A number of approaches exist that promote the induction of autophagy including calorie restriction, sunlight exposure, caffeine and green tea consumption, as well as the use of mTORC1 inhibiting drugs like rapamycin and metformin.

Calorie Restriction

Calorie restriction (CR) has been extensively studied as a practical anti-ageing strategy as it is the most potent inducer of autophagy through the activation of AMPK and Sirtuin 1 as well as the inhibition of the insulin/insulin-like growth factor 1 (IGF-1) and mTORC1 pathways (Barzilai and Ferrucci 2012; Chen et al. 2013b; Wang et al. 2015b). Arguably, the most beneficial anti-ageing effect of CR is its ability to minimise oxidative damage given that ageing and age-related diseases are most often characterised by the accumulation of oxidative damage leading to DNA damage and protein accumulation (Harman 1956; Gladyshev 2014). It has also been shown that CR increases the activation of forkhead box O (FoxO) transcription factors as well as decreasing the activation of nuclear factor kappa B (NFκB) (Kim et al. 2007; Jung et al. 2009; Yamaza et al. 2010). FoxO transcription factors are involved in regulating many genes controlling apoptosis, ROS detoxification and DNA repair. Increased activation of FoxO proteins supports longevity as reactive species are removed quickly and cellular damage can be repaired, restoring function and preventing cell death (Lu and Huang 2011; Ju et al. 2014). Similarly, NFκB functions as a transcription factor, however, its activation results in pro-inflammatory gene expression and increases cell proliferation. NFκB therefore can promote the induction of cancer and further oxidative damage to cells (Pahl 1999; Yamamoto and Gaynor 2001).

Although a vast amount of scientific literature indicates that CR has beneficial effects on organismal physiology and slows down ageing, a study conducted by Morley et al. (2010) demonstrated that CR may, in some cases, have more negative health effects than positive. The study, which also used mice on calorie-restricted diets, found that CR restriction was not effective in older mice. Loss of muscle mass, muscle strength and bone density were instead consequences of CR. The study therefore speculated that CR in older people may not the best anti-ageing strategy as it has many health risks and lead to age-related diseases such as osteoporosis and increased susceptibility to various infections (Meisenberg and Simmons 2017).

An intervention similar to CR is intermittent feeding (IF), a dietary pattern that involves fasting on alternate days. IF has been suggested to have a greater anti-aging potential than CR, particularly with regards to brain health and delaying the onset of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease (Anson et al. 2003). While CR upregulates autophagy in various organs in the body, autophagy may not be upregulated in the brain during mild nutrient deprivation due to it being a 'metabolically-privileged' site (Alirezaei et al. 2010). Short term fasting periods, however, induce a massive upregulation of autophagy in the brain, particularly in the cortical and Purkinje neurons as the number of autophagosomes in these areas is observed to significantly increase (Alirezaei et al. 2010). Additionally, research using intermittently fed mice found that these animals had an increased resistance to excitotoxic stimuli and exhibited improved neuronal function and fewer clinical symptoms of neurodegenerative diseases compared to mice on calorie-restricted diet or fed *ad libitum* (Anson et al. 2003). IF also increases the amount of circulating ghrelin, a hunger hormone that stimulates autophagy in cortical neurons. In addition to the health and anti-aging benefits autophagy brings about, ghrelin also regulates cardiac function, increases osteoblast cell differentiation and bone mineralisation as well as suppresses pro-inflammatory cytokines such as IL-1α and IL-6. While CR also causes a release of ghrelin, IF does so to a much greater extent. This means that the associated effects on healthspan and lifespan extension are also much greater. Many current clinical trials are investigating ghrelin as a potential therapeutic strategy for many age-related conditions including chronic heart failure, cancer, frailty and depression (Ferreira-Marques et al. 2016; Bayliss et al. 2016).

Sunlight Exposure – Vitamin D

Another powerful inducer of autophagy is the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (Wu and Sun 2011). Increasing sunlight exposure is an easy life change people can make to improve health and slow ageing as short wave ultraviolet B (UVB) energy is converted to vitamin D in the liver (Bikle 2014). Vitamin D3 induces autophagy via several pathways. For example, vitamin D3 increases PI3K and Beclin-1 expression levels thereby increases phagophore formation. Vitamin D3 also increases Atg16 recruitment, necessary for membrane elongation and initiation of lysosomal degradation (Sly et al. 2001; Wang et al. 2008; Travassos et al. 2009; Wu and Sun 2011). Vitamin D3 signalling also results in an increase to the size of lysosomal lumens and enhances the activity of the lysosomal enzymes, and therefore supports the autophagosomal maturation and degradation stages (Høyer-Hansen et al. 2005). Additionally, vitamin D3 increases intracellular calcium levels and increases the activation of Ca^{2+}/calmodulin-dependent kinases and phosphatases which initiate the recruitment of cathelicidin to autophagosomes, a small protein necessary for autophagosome formation and fusion with lysosomes (Yuk et al. 2009). Another key finding that further supports the role of vitamin D3 in

autophagy initiation is that it activates the transcription and translation of the DNA damage-inducible transcript 4 protein (DDIT4). DDIT4 is a DNA damage response protein that stimulates the formation of the TSC1/2 complex and suppression of mTORC1 activity (Wang et al. 2016). A final example is that vitamin D has been noted to activate FoxO signalling, which again functions to inhibit mTORC1 via direct and indirect mechanisms, to allow autophagy to occur (Chen et al. 2010; Yildizgören and Togral 2014). A deficiency of vitamin D, or the body's defective ability to generate it, has proven to contribute to calcium signalling dysregulation resulting in mitochondrial dysfunction and increased ROS production, and consequently accelerating the ageing process (Holick and Schlogl 2014; Berridge 2015).

Exercise

Autophagy is essential during exercise due to the high energy demands of skeletal muscle cells and is activated by AMPK and through the disruption of the Bcl-2/Beclin-1 complex upon stress stimuli, typically oxygen or ATP depletion. With exercise, stress stimuli induce the phosphorylation of Bcl-2 resulting in the dissociation of Bcl-2 from Beclin-1 and consequently the induction of autophagy via Beclin-1 (He et al. 2012; Dagon et al. 2015). By subjecting mice to periods of running, it was found that after 30 min of exercise, autophagy levels in skeletal and cardiac cells had increased by 40%, and reached a maximum at 80 min. In addition to the induction of autophagy, exercise also stimulates skeletal muscle to increase its glucose uptake and consumption efficiency by increasing insulin sensitivity and upregulating GLUT4 receptors on the plasma membrane, to further accommodate the high metabolic demands of skeletal muscle cells (Richter and Hargreaves 2013). A study showed that mutating a phosphorylation site of Bcl-2 impaired autophagy induction (He et al. 2012). Due to this mutation, phosphorylation of the protein in response to stress stimuli could not occur, preventing Bcl-2 dissociation from Beclin-1 and the induction of autophagy. Interestingly, this mutation also resulted in a reduction of GLUT4 upregulation at the plasma membrane and reduced insulin sensitivity. To confirm that this altered glucose metabolism observation was due to the failed induction of autophagy, the same study was conducted on *Becn1*$^{+/-}$ mice, in which a similar outcome was observed, further suggesting increased glucose metabolism is dependent on the activation of autophagy (He et al. 2012). Failed autophagy therefore may be causative of the deleterious effects of diabetes mellitus type 2 (T2DM) and obesity, two common conditions also associated with ageing. These findings therefore indicate that exercise-induced autophagy plays a protective role against the development of T2DM and obesity (Liu et al. 2009; Van Dalfsen and Moore 2013). Physical exercise is arguably the easiest lifestyle change a person can make to slow ageing and improve longevity.

Concluding Remarks

A plethora of evidence demonstrates that autophagy is an important process for protecting cells from stress, delaying the process of ageing and development of disease, as well as enhancing lifespan. The relationship between autophagy and ageing was identified using genetic screens of yeast and *Drosophila* in which altered expression or knockout of autophagy-related genes resulted in reduced, or even failed, autophagy, an increased rate of ageing and the development of age-related disease. For this reason, many therapeutic strategies for such diseases upregulate and activate autophagy to aid clearance of toxic protein accumulations and prevent further tissue degeneration; the prevention of neurodegenerative diseases such as Alzheimer's disease being a strong example. At the same time, in some cancers and cardiovascular diseases, autophagy can have detrimental effects on health. In these circumstances, the induction of autophagy would not be appropriate. However, for the majority of cases, the activation of autophagy through simple life changes such as calorie restriction, increased exercise participation and sunlight exposure are strategies to slow ageing and improve human health.

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Chapter 3
Nutrient Sensing, Signaling and Ageing: The Role of IGF-1 and mTOR in Ageing and Age-Related Disease

Simon C. Johnson

Abstract Nutrient signaling through insulin/IGF-1 was the first pathway demonstrated to regulate ageing and age-related disease in model organisms. Pharmacological or dietary interventions targeting nutrient signaling pathways have been shown to robustly attenuate ageing in many organisms. Caloric restriction, the most widely studied longevity promoting intervention, works through multiple nutrient signaling pathways, while inhibition of mTOR through treatment with rapamycin reproducibly delays ageing and disease through specific inhibition of the mTOR complexes. Although the benefits of reduced insulin/IGF-1 in lifespan and health are well documented in model organisms, defining the precise role of the IGF-1 in human ageing and age-related disease has proven more difficult. Association studies provide some insight but also reveal paradoxes. Low serum IGF-1 predicts longevity, but IGF-1 decreases with age and IGF-1 therapy benefits some of age-related pathologies. Circulating IGF-1 has been associated both positively and negatively with risk of age-related diseases in humans, and in some cases both activation and inhibition of IGF-1 signaling have provided benefit in animal models of the same diseases. Interventions designed modulate the nutrient sensing signaling pathways positively or negatively are already available for clinical use, highlighting the need for a clear understanding of the role of nutrient signaling in ageing and age-related disease. This chapter examines data from model organisms and human genetic association studies, with a special emphasis on IGF-1 and mTOR, and discusses potential models for resolving the paradoxes surrounding IGF-1 data.

Keywords mTOR · Nutrient signaling · Ageing · IGF-1 · PI3K

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Introduction

Modern molecular gerontology is in many senses a chaotic and eclectic field of research. Model system approaches to elucidating the mechanisms of ageing range from measuring replicative lifespan studies in budding yeast *Saccharomyces cerevisiae*, consisting of physically counting the number of 'daughter' cells individual 'mother' yeast cells can produce, to large scale genetic studies in human centenarians, which involve massive long-term clinical follow-up and large-scale next-generation sequencing endeavors. The spectrum of organisms used in modern ageing research includes nematode (*Caenorhabditis elegans* and others) and fly (*Drosophila melanogaster*) models, mice and rats, non-human primates, comparative studies utilizing everything from North Atlantic *Arctica islandica* clams to naked mole rats (*Heterocephalus glaber*), and a whole catalog of exotic 'emerging' models aimed at providing fresh perspectives to the ageing field. While this complex mixture of complementary approaches has powered many theories of ageing, the identification of nutrient sensing and signaling pathways as regulators of longevity is arguably the most important discovery in ageing research to date. Nutrient sensing and signaling has been shown to regulate ageing in eukaryotic organisms from yeast to humans through dietary, genetic, and pharmacological manipulation, mutagenesis and RNAi screening, comparative biology, genome-wide association studies (GWAS), and rare genetic variant analysis. Lifespan extending genetic manipulations in nutrient signaling pathways helped legitimize the study of ageing, and more recently have led to the extraordinary – small molecule interventions that modify the underlying process of ageing, improving lifespan and preventing or delaying age-related disease.

The benefits of reduced nutrient signaling on longevity are well-established and broadly conserved across model systems, but a variety of questions remain regarding the impact of these pathways on normal human ageing and age-related disease. What are the downstream effectors of greatest importance? What therapeutic strategies will provide the greatest benefit with the lowest off-target effects? How can we bridge the gap between pre-clinical studies and human treatments? What are the limitations of targeting nutrient sensing in human health? Here, we discuss the role of the major nutrient sensing and signaling pathways in ageing and provide an up to date discussion of these questions, with an emphasis on how NSS impacts human ageing.

Nutrient Sensing Signaling

Insulin/IGF-1 Signaling – the First Pathway of Ageing

The insulin/insulin-like growth factor 1 (IGF-1) signaling pathway was the first defined genetic pathway regulating ageing and age-related disease in model organisms (Kenyon 2011), detailed in landmark studies that provided the first evidence

that genetic manipulation of nutrient sensing signaling (NSS) can modify lifespan (Kenyon et al. 1993, Dorman et al. 1995). Subsequent early studies suggested a linear membrane bound receptor to transcription factor pathway, comprised of the cell surface receptor DAF-2 (homolog of the mammalian IGF-1 Receptor), the PI3 kinase AGE-1, the intracellular kinase AKT/PKB, and the fork head transcription factor DAF-16 (homolog of human Foxo3a) (Paradis and Ruvkun 1998). Numerous additional players have since been identified, with dozens of modifying factors surrounding a central IGF-1/IGF1R/PI3K/AKT/mTOR pathway (see Figs. 3.1 and 3.2). The core intracellular components of NSS (such as TOR, AMPK, and AKT) are widely conserved across the Eukarya domain; for example, the yeast homologs of mTOR, AMPK, and AKT are Tor, Snf1, and Sch9, respectively (see Fig. 3.1).

As the complexity of NSS has been revealed, the linear pathway model for the physical signaling events has become obsolete, making way a more nuanced understanding of NSS. The linear model still provides a reasonable representation of the overall role of NSS in ageing and the benefits of general NSS targeting interventions such as caloric restriction (CR) (see Fig. 3.2), but it is now clear that complex, multi-layered networks of sensors and effectors including feed-back loops, tissue and cell type specific factors, and species specific pathways, modify the core NSS paradigm. Tangled in these networks are multiple points of intracellular and systemic surveillance of nutrient levels and growth favorable conditions, an array of downstream effector pathways and molecules, and a relatively small number of key, highly conserved, intracellular signal hubs which coordinate the many inputs and outputs. Early hopes that a single transcription factor could underlie the majority of the benefits of NSS pathway modulation have proven premature, but intracellular signaling hubs have taken their place as the lead candidates in pharmacological attenuation of ageing. The premier examples are the mechanistic Target of Rapamycin complexes (mTORC1 and mTORC2), central mediators of NSS and established pharmacological targets (see Fig. 3.1; discussed in detail below). Circulating systemic factors, in particular IGF-1 in mammals, are also viewed as potential therapeutic targets in ageing and age-related disease.

Regulation of Nutrient Signaling

Systemic Signaling, Circulating Factors

The canonical PI3K/AKT pathway of ageing can be activated by any of a broad range of hormones, growth factors, and cytokines, acting through either receptor tyrosine kinases (such as IGF-1R/Daf-2 or INSR) or G protein-coupled receptors. In theory, any or all of these may contribute to ageing, but causal evidence is mainly associated with growth hormone (GH) and IGF-1. Growth hormone (GH) is itself the primary driver of circulating IGF-1 levels, driving production through activation of hepatic IGF-1 synthesis. As for GH, it is secreted by the pituitary gland in response to hypothalamic GH-releasing hormone (GHRH), insulin-induced

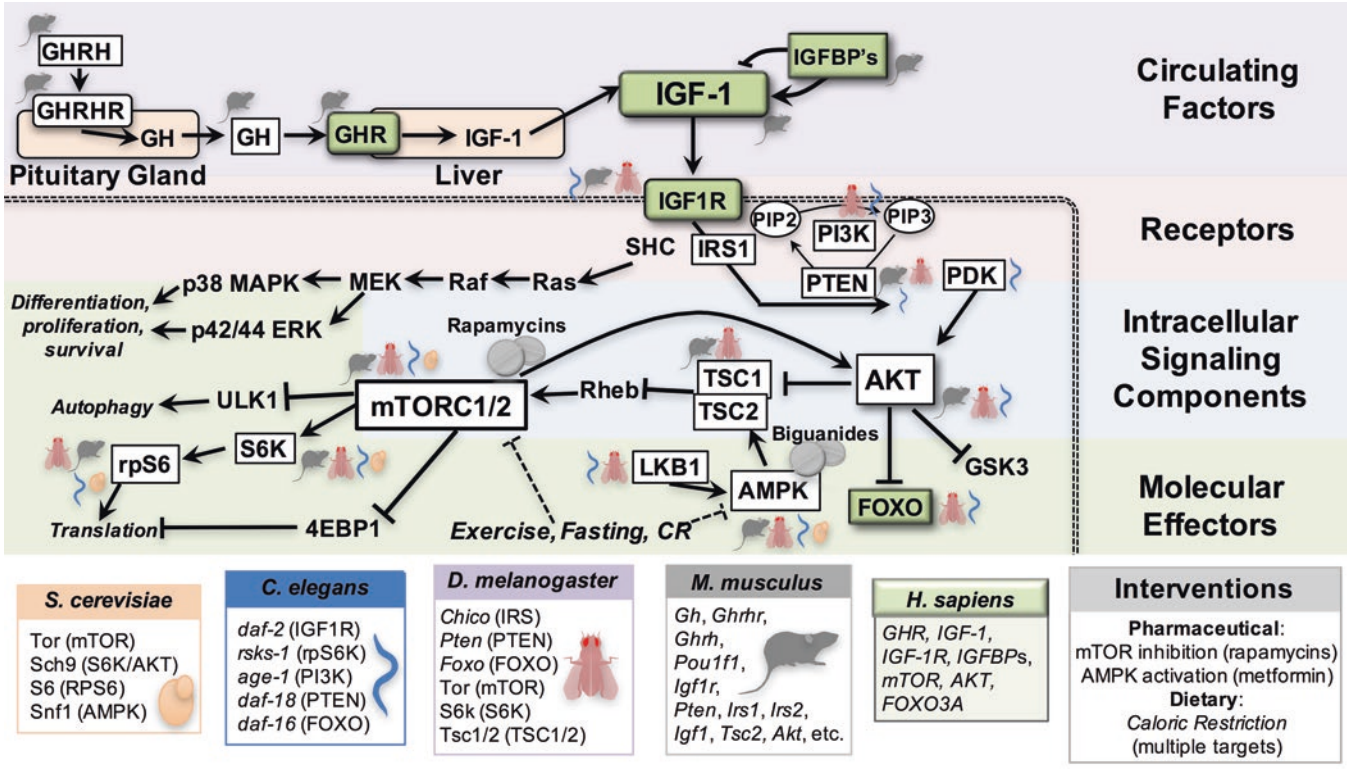

Fig. 3.1 Key Components of Nutrient Sensing Signaling that Regulate Ageing in Eukaryotes. A simplified schematic of core nutrient sensing signaling pathways that regulate ageing, including insulin/IGF-1, mTOR, AMPK, and AKT. Nutrient sensing signaling pathways include circulating factors, cell surface receptors, intracellular signaling components, and molecular effectors. Putative regulators of ageing have been found in each of these categories of macromolecules. Model organisms indicate species in which genetic studies have implicated given factors, with the homolog names of key components listed below. Genes linked to human ageing through genome-wide association or candidate gene genetic association studies are indicated in green. Pharmacological and dietary interventions modulate ageing through their actions on nutrient sensing signaling pathways

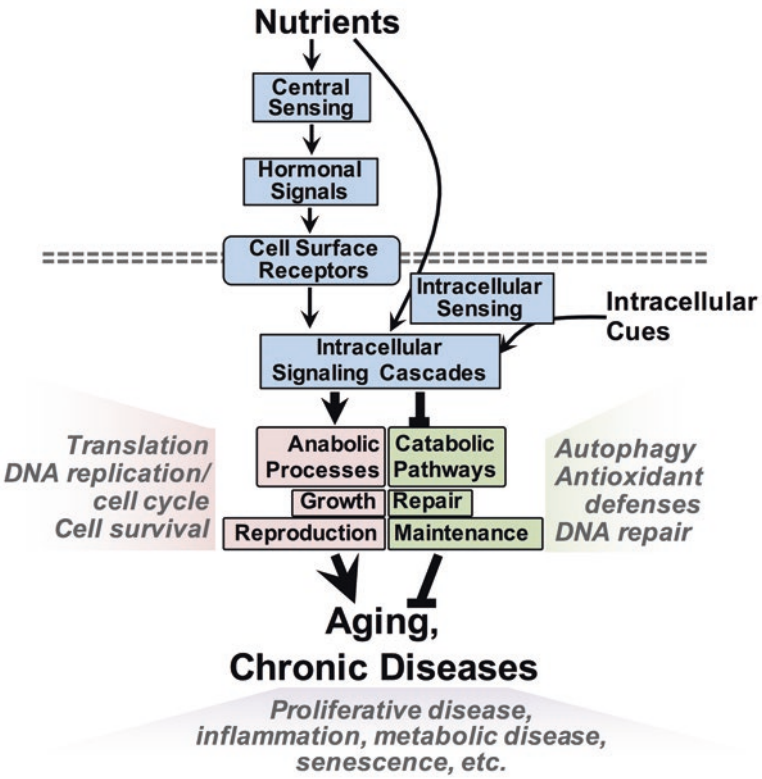

Fig. 3.2 A Linear Model for Nutrient Sensing Signaling in Ageing and Age-related Disease. A linear model provides a simplified representation of the role of nutrient sensing signaling in ageing and age-related disease. In this model, overall nutritional status in multicellular organisms is sensed through central mechanisms as well as through intracellular nutrient sensing cues. Central sensing regulates the production of circulating hormonal signals, including GH, IGF-1, and insulin, which activate cell surface receptors and stimulate intracellular nutrient signaling pathways. Intracellular cues, such as ATP/ADP, NADH/NAD+, amino acid levels, and ribosome assembly, regulate intracellular pathways directly and modifying cellular response to circulating factors. Tissue and cell type specific responses in multicellular organisms are coordinated by differential expression of intracellular factors and cell surface receptors, specificity of systemic factors, and by bioactivity-modifying tissue bound and circulating factors. Together, these signaling cascades promote growth and fecundity at the expense of repair and maintenance. Chronic activation of nutrient sensing signaling drives ageing and many chronic diseases associated with ageing, while targeting nutrient signaling pathways attenuates ageing and age-related disease, as discussed

hypoglycemia, and vigorous exercise; GH production is inhibited by hyperglycemia, glucocorticoids, and negative feedback induced by IGF-1 (Buckler 1971; Barbetti et al. 1990) (see Fig. 3.1). IGF-1 feedback on GH is mediated by inhibition of the cyclic AMP response element binding protein (CREB) binding protein (CBP), a transcriptional co-factor necessary for GH production (Romero et al. 2012).

Low circulating IGF-1 in under-nutrition or fasting results from both decreased GH production and enhanced turnover of serum proteins. IGF-1 bioavailability is modulated by a binding proteins including the IGF-1 binding proteins (IGFBP's) and IGF-1 acid labile subunit (IGFALS) and the ratios of IGFBP's to IGF-1 have been associated with some human diseases (Arafat et al. 2009; Gokulakrishnan et al. 2012). IGF-1 is also produced locally in a tissue-specific manner in response to stimuli such as mechanical stress and injury (Pelosi et al. 2007). IGFBP's, IGFALS, locally produced IGF-1, and factors that modulate signal transduction at or downstream of the IGF-1 receptor (IGF1R) all complicate the interpretation of circulating IGF-1 levels.

IGF-1 has been a major focus of biogerontology, but evidence suggests it may not be the most important circulating factor in mammalian ageing. GH and GHR knockout mice are viable and exceptionally long lived, while knockout of either IGF-1R or IGF-1 are lethal and partial loss only modestly alters lifespan. Intriguingly, liver specific knockout of IGF-1 results in an ~75% reduction in circulating IGF-1, but these mice had normal body growth and produced IGF-1 in several non-hepatic tissues in response to GH (Yakar et al. 1999). This uncoupling of GH and IGF-1/IGF-1R will be discussed further below.

Cellular Interface and Intracellular Regulation

At the cell surface, circulating nutrient sensing signaling factors activate membrane bound receptors and stimulate intracellular signaling cascades. GH acts through the homodimeric receptor tyrosine kinase GHR. IGF-1 activates homodimeric IGF-1R as well as insulin-receptor (INSR) /IGF1R hybrid receptor heterodimers, and, with only weak affinity, homodimer INSR. Insulin similarly activates both INSR and IGF1R. Receptor/ligand binding of IGF-1, Insulin, or GH results in activation of PI3K/AKT and MAPK/ERK signaling through intracellular insulin receptor substrates (IRS1-4) and SHC, respectively (Fig. 3.1). AKT activation leads to inhibition of glycogen synthase kinase 3 (GSK-3) and forkhead box O transcription factors (FOXO's), including FoxO3a, as well as activation of the mechanistic target of rapamycin (mTOR).

mTOR promotes mRNA translation through rpS6K/rpS6 and the eukaryotic initiation factor binding protein 4E-BP1 and decreases autophagy through inhibition of ULK1. The ERK/MAPK pathway activates mitogenic factors such as the proto-oncogene c-MYC, and promotes translation through activation of rpS6 via phosphorylation at rpS6K independent sites (Pende et al. 2004). Together, these processes enact intracellular growth signaling, mRNA translation, catabolic pathways, and metabolism, which together modulate eukaryotic healthspan and longevity (Fig. 3.1).

Intracellular Sensing

Nutrient sensing occurs intracellularly through a number of distinct nutrient, energy, and growth permissive condition monitoring systems. These include ATP level surveillance by the AMP activated protein kinase AMPK; NADH/NAD+ monitoring by NAD+ dependent protein post-translational modification regulators Sirtuins (not discussed in this chapter); oxygen sensing by REDD1/REDD2, the Hif-1 pathway; small molecule sensing at the lysosome; and various ligand nutrient/hormone ligand dependent transcription factors such as Peroxisome proliferator-activated receptor gamma (PPAR-γ). While many of these individual sensors has been tied to ageing and disease in one or more model systems, one intracellular sensing hub has stood out as a major, and modifiable, target in ageing: mTOR.

mTOR is a key intracellular mediator of nutrient sensing signaling and, as a result, of ageing and disease (Johnson et al. 2013). The mTOR complexes, mTORC1 and mTORC2, are uniquely important for two reasons: first, these complexes synthesize input nutrient and growth information from a broad array of unique molecular signals and coordinate extensive cellular responses by tuning anabolic and catabolic pathways (Fig. 3.3). Second, key mTORC components show sufficient structural conservation from yeast to man that antifungal macrolides targeting Tor in yeast also robustly and specifically inhibit mTOR in mammals (discussed below).

In addition to activation downstream of cell surface receptors, mTOR is involved in cellular response to many of the nutrient sensing factors detailed above. Well-established points of regulation include activation by amino acid sensing at the lysosome mediated by the Ragulator complex (Kim and Kim 2016); inhibition resulting from a low cellular ATP/ADP ratio mediated by AMPK; inhibition by low oxygen levels mediated by the intracellular sensors REDD1 and REDD2 (Vadysirisack and Ellisen 2012); and ribosome capacity sensing by mTORC2, which directly couples nutrient sensing signaling at mTOR to cellular ability to enact mRNA translation (Zinzalla et al. 2011). A variety of additional signals have been shown to attenuate mTOR activity, such as glucose concentration and the NADH/NAD+ ratio (through the action of Sirtuins and other factors), but the precise mechanisms mediating these points of control remain to be fully defined. In sum, ATP status, oxygen level, ribosome capacity, amino acid concentration, systemic growth signaling, and other yet to be defined sensors all converge at mTORC1 and/or mTORC2, which together coordinate cellular proliferative and maintenance programs in response to these inputs.

Downstream of mTOR

mTORC1 drives proliferation and growth through inhibition of autophagy, activation of mRNA translation/protein synthesis, and regulation of metabolism. mTORC1 promotes protein synthesis by activating ribosomal protein subunit S6, downstream of S6 kinase, and by releasing the eukaryotic translation initiation binding factor eIF4E-BP1 (or 4E-BP1) and allowing for cap-dependent mRNA translation.

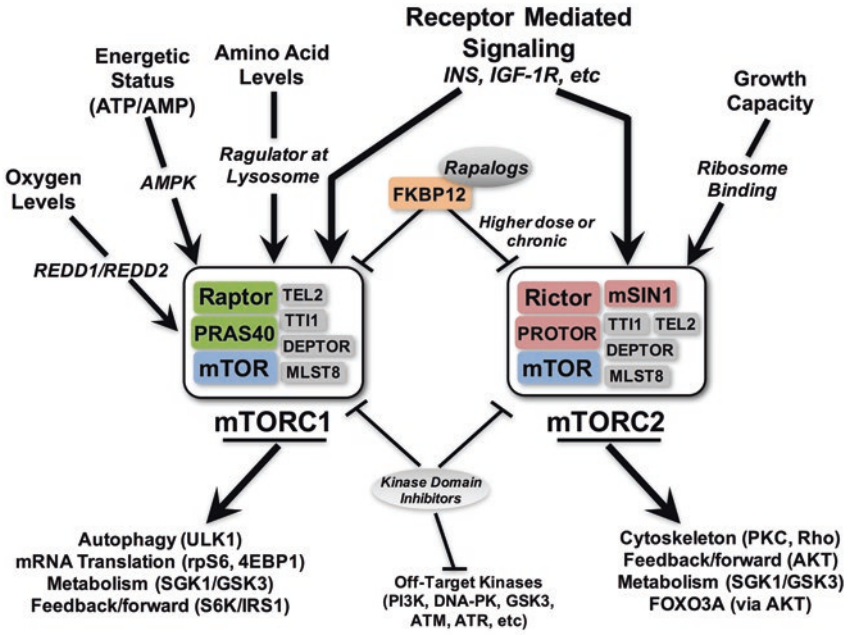

Fig. 3.3 Intracellular Integration of Nutrient Sensing Signals through mTOR Signaling. A simplified representation of mTOR complexes 1 and 2 as intracellular hubs for nutrient sensing signaling. mTORC1 and 2 are activated or inhibited by a variety of intracellular cues, including amino acid sensing, energetic status, and oxygen levels, as well as downstream of cell-surface receptors for extracellular signals (directionality of individual pathways not shown). mTORC1 and 2 integrate these inputs and enact various downstream cellular processes through their protein kinase activities. Key outputs of mTORC1 include regulation of mRNA translation through rpS6 and 4EBP1, autophagy through ULK1, metabolism through SGK1 and GSK3, and feedback regulation of receptor mediated signaling through modulation of IRS1 phosphorylation. mTORC2 regulates cytoskeletal organization through PKC and Rho Kinase, metabolism through SGK1 and GSK3, and Foxo3a activity and positive and negative feedback loops through actions on AKT. mTOR inhibitors target both complexes with differential action on the two dependent on dose, duration, cell type, and precise pharmacological target. Rapamycins or rapalogs inhibit mTORC1 and 2 in complex with the protein FKBP12. Kinase domain targeting inhibitors provide more complete inhibition, but with off-target effects on other kinases

Autophagy, the intracellular catabolic process of recycling through lysosome-mediated degradation, is inhibited by active mTOR. Thus, active mTOR promotes synthesis over recycling, while mTOR inhibition permits increased catabolism while dampening the biosynthetic process of protein synthesis. This shift exemplifies the role of mTOR in cellular adaptations to conditions permissive of or unsuitable for proliferation.

Both mRNA translation and autophagy appear to be key players in ageing and age-related pathologies. Decreasing mRNA translation increases longevity in multiple models; knockdown or deletion of ribosomal proteins increases lifespan in yeast, flies and nematodes, and S6K deletion extends lifespan and decreases body

size in mice. Pharmacological inhibition of translation has also been shown to increase lifespan in yeast. Some studies suggest that differential translation of certain mRNA's that rely on cap-dependent translation, rather than simply overall decreased translation, may play a role in the benefits of mTOR inhibition. Such a model has been established in budding yeast – translation of the low-nutrient response transcription factor Gcn4 is preferentially increased when global translation is reduced, and Gcn4 is necessary for the benefits of caloric restriction in this model. How differential mRNA translation impacts nutrient responses in mammals remains to be clarified.

Induction of autophagy is necessary for the benefits of reduced nutrient sensing signaling, but unlike translation has not been shown to be sufficient for lifespan extension. Damaged macromolecules, including aggregated proteins, oxidized lipids, and dysfunctional organelles, are known to accumulate during ageing and thought to contribute to cellular and tissue dysfunction.

Finally, mTORC2 contributes to nutrient responses by regulating the cytoskeleton, modifying metabolism, and providing a feed-forward activation of AKT through phosphorylation at serine 473. The feed-forward to AKT provides a clear role for mTORC2 in linking ribosome capacity to growth signaling through mTORC1.

Pharmacological Targeting of mTOR

The importance of mTOR in ageing has been demonstrated through genetic studies, as described, as well as through the NIH intervention testing program (ITP) experiments which utilized large-scale, multi-center, blinded mouse trials to test the efficacy of mTOR inhibition in mouse ageing using the compound rapamycin (Warner 2015). These trials demonstrated that mTOR inhibition reproducibly and significantly increases mouse lifespan in mammals, even when treatment begins late in life, and that lifespan increases are both dose-dependent and occur in both males and females with similar efficacy when equivalent blood levels are achieved (Harrison et al. 2009; Wilkinson et al. 2012). These findings have proven highly reproducible, and recent work has shown that even transient treatment at higher doses can dramatically increase survival (Bitto et al. 2016).

Rapamycin, or sirolimus, was the first identified pharmacological inhibitor of mTOR, for which yeast target of rapamycin, aka Tor, was named. Various modified forms with improved solubility or stability are now available, including temsirolimus, everolimus, and deforolimus (Nasr et al. 2015). These agents, collectively 'rapamycins', are special among small molecule inhibitors in that they do not bind directly to mTOR but, rather, bind the adapter protein FKBP12 (RBP1 in yeast) and only the FKBP12-rapamycin complex inhibits mTOR (Koltin et al. 1991). This two-tier mechanism provides incredible target specificity which has not yet been demonstrated with any of the newer mTOR active-site inhibitors, and the maximum biological effect of rapamycins is limited by cellular levels of FKBP12. Active site inhibitors, including Torin 1 and 2, can provide greater levels of mTOR inhibition

by binding directly to mTOR, but these compounds also show substantial off-target inhibition of other, structurally related, protein kinases such as DNA-PK, GSK3, ATM, and ATR (see Fig. 3.3), particularly at high concentrations (Liu et al. 2013). Rapamycins, conversely, remain specific to mTOR even at high doses.

Specific targeting of mTORC1 versus mTORC2 has been a major focus of recent work in mTOR. The rationale for this goal is that mTORC1 directed processes have been robustly associated with disease and ageing, while mTORC2 is considered by many to be involved only in off-target effects of mTOR inhibition such as altered glucose handling. Rapamycins are often described as mTORC1 specific inhibitors, but this oft-repeated statement has been proven a historic fallacy. mTORC1 and mTORC2 are both inhibited by rapamycin when treatment is chronic or in 'high dose' paradigms (Sarbassov et al. 2006). Various factors influence the relative sensitivity of mTORC1 and mTORC2 to rapamycin, as well the differential sensitivities of individual downstream targets (Mukhopadhyay et al. 2016). While very acute treatment with low concentration rapamycin may be mTORC1 specific, these conditions represent the exception rather than the rule.

mTORC1 and mTORC2 specific inhibitors are beginning to become available, and the next few years should shed new insight into the relative importance of these two complexes. The focus in ageing have been mTORC1, but the rationale for this focus has largely been a result of the convenience of following up on a better characterized complex coupled with regular misinterpretations of the primary literature. As discussed above, rapamycin is often called mTORC1 specific, but the statement in this form is not supported by evidence. In addition, the glucose-handling effects have been largely attributed to mTORC2, while a very modest and gender specific lifespan extension in mice appears to be possible with specific inhibition of mTORC1 (Lamming et al. 2012). Though intriguing, this partial genetic uncoupling neither precludes a benefit from mTORC2 inhibition, nor definitively demonstrates that mTORC1 inhibition alone can recapitulate the benefits of rapamycin. And, critically, the 'off-target' effects on glucose handling have not truly been demonstrated to be off-target at all – rather, the full benefits of mTOR inhibition may rely on mechanisms that modify glucose tolerance in vivo. Common non-physiologically relevant methods for measuring glucose handling likely misrepresent biology in vivo, and differences in glucose handling following bolus delivery may be misleading. This is exemplified by so-called 'hunger diabetes' in caloric restriction, and the fact that both caloric restriction and rapamycin treatment extend lifespan and prevent diabetes related diseases while also resulting in 'abnormal' glucose handling and insulin sensitivity in common bolus response paradigms (Blagosklonny 2011; Piguet et al. 2012).

There is also direct evidence suggesting mTORC2 specific pathways are important to at least some age-related diseases. For example, the mTORC2 driven regulation of lipid synthesis and cytoskeletal functions appear to be key factors in cancer (Benavides-Serrato et al. 2017; Bian et al. 2017; Guri et al. 2017) and mTORC2 has been shown to mediate inflammation related dermal ageing (Choi et al. 2016), while a number of the cytoskeletal and metabolic pathways directly regulated by mTORC2 have been linked to ageing and age-related diseases. In

particular, cytoskeletal regulation by Rho kinase has now been shown to delay cellular senescence and has been linked to disease progression in multile age-related neurodegenerative diseases (Feng et al. 2016; Henderson et al. 2016; Kumper et al. 2016). Determining the relative importance of mTORC1 and 2 in ageing and age-related disease is a major focus of current research in ageing.

Nutrient Signaling in Ageing

Several components of NSS regulate lifespan and healthspan, the period of an organism's life spent free from significant morbidities, in model organisms or have been associated with heathy ageing in humans (Fig. 3.1). Dwarf mice defective in GH are long-lived and, as in humans, disease resistant, as are GHRH deficient and *Irs1* null mice (Selman et al. 2008; Sun et al. 2013). Interventions reducing IGF-1 signaling are associated with improved outcome in some murine models of AD and proteotoxicity models in invertebrates (Cohen et al. 2009; Cohen 2011; Parrella et al. 2013). Long-lived *Gh*, *S6K*, and *Igf1r* mutant mice have a markedly attenuated onset and severity of age-related pathologies including age-associated cardiac dysfunction, cancers, and age-related proteotoxicities (Cohen et al. 2009; Selman et al. 2009). PTEN, an antagonist of PI3K/IGF-1 signaling through de-phosphorylation of PIP3, promotes longevity in worms, flies, and mice, and is necessary for lifespan extension in IGF1R mutants (Ortega-Molina et al. 2012). PDK mediates signaling to AKT, limiting lifespan in worms (Paradis et al. 1999). Depletion of rpS6 or S6K increases lifespan in yeast, worms, and mice (Selman et al. 2009). FoxO3a, a transcription factor which is inhibited by IGF-1 signaling, is required for lifespan extension by reduced NSS in worms and flies and variants in the *FOXO3A* locus have been reproducibly associated with human longevity (Flachsbart et al. 2009). Both common and rare variants in *AKT* and *IGF1R* have also been associated with human lifespan (Pawlikowska et al. 2009).

Multiple evolutionarily conserved nutrient/growth signaling pathways involved in nutrient sensing influence healthspan and lifespan (Laplante and Sabatini 2012; Johnson et al. 2013). AMP activated protein kinase (AMPK) is activated by low energy levels, inhibited by insulin/IGF-1 signaling, and is a positive regulator of lifespan in eukaryotes from yeast to mice (Martin-Montalvo et al. 2013). Metformin, an AMPK activator, has been shown to increase lifespan in *C. elegans* (Chen et al. 2017) and extend healthspan in mouse studies (although lifespan was not increased) and is the first compound headed to human trials tracking multiple age-related diseases (ARD's) (Check Hayden 2015; Barzilai et al. 2016). As discussed, mTOR is a key mediator of NSS and a well-established regulator of lifespan in eukaryotes (discussed above) (Johnson et al. 2013). Inhibition of mTOR by rapamycin, genetic disruption of the mTOR complexes, and hypomorphic mTOR alleles all extend murine lifespan and slow mouse ageing (Lamming et al. 2012; Wu et al. 2013). Finally, caloric restriction (CR), the oldest and most widely reported longevity-enhancing and ARD delaying intervention, acts through attenuation of all these pathways. CR is

the only longevity intervention reported in primates; two recent studies reported healthspan benefits, one also reporting increase lifespan (Mattison et al. 2012; Colman et al. 2014). Moreover, clinical trials examining the safety of CR in healthy individuals have very recently been published, with some early signs of benefits (Fontana et al. 2016; Martin et al. 2016; Romashkan et al. 2016). Interestingly, these studied report that in humans circulating inhibitory IGFBP-1 is significantly increased by CR but circulating IGF-1 is unaltered, a deviation from CR results in rodents.

In addition to these reported benefits of reduced NSS, elevated IGF-1 is associated with some age-related human diseases. High circulating IGF-1 is associated with the progression of prostate, breast, pancreatic, bladder, and small-cell lung cancer (Schernhammer et al. 2005; Fidler et al. 2012; Price et al. 2012; Belardi et al. 2013; Kubasiak et al. 2015). These observations led to the development of IGF-1R neutralizing antibodies as a therapeutic for cancer, although they have not consistently proven effective as monotherapies (You et al. 2013). High circulating IGF-1 has also been associated with chronic heart failure and increased all-cause mortality and is positively associated with risk of metabolic syndrome in longitudinal studies (Andreassen et al. 2009; Chisalita et al. 2011; Friedrich et al. 2013). Taken together, this evidence establishes IGF-1 and the growth and nutrient/growth signaling network surrounding it as central regulators of eukaryotic healthspan and lifespan and suggests that interventions designed to decrease IGF-1 signaling might promote health and longevity in humans.

IGF-1 in Human Health

In humans, strong defects in IGF-1 signaling cause dwarfism but protect against some age-related diseases, while some evidence suggests that subtler deficiencies confer resistance to diseases of ageing without marked effects on growth. Although the benefits of reduced insulin/IGF-1 in lifespan and health are well documented, defining the precise role of the IGF-1 in age-related disease, particularly human age-related diseases, has remained a complex problem, with many apparent paradoxes involving IGF-1. Low serum IGF-1 predicts longevity, but IGF-1 decreases with age and IGF-1 therapy benefits some of age-related pathologies. Circulating IGF-1 has been associated both positively and negatively with risk of age-related diseases in humans, and in some cases both activation and inhibition of IGF-1 signaling have provided benefit in animal models of the same diseases. Interventions designed modulate the insulin/IGF-1 pathway positively or negatively are already available for clinical use, highlighting the need for a clear understanding of the role of IGF-1 in ageing and age-related disease.

Insulin-like growth factor 1, IGF-1, is a small hormone protein with endocrine, paracrine, and autocrine functions. IGF-1 was first described as the serum factor responsible for stimulating protein synthesis following growth hormone (GH) treatment, having insulin-like properties not repressible by insulin neutralizing antibodies (Froesch et al. 1963). IGF-1 stimulates growth in most mammalian cell types

and is critical for normal development. GH receptor (GHR) defects or production of GHR neutralizing antibodies leads to impaired IGF-1 production and Laron syndrome dwarfism. Both primary IGF-1 deficiency and Laron syndrome are treated using recombinant IGF-1.

While the clinical significance of IGF-1 in dwarfism is well established, the role of IGF-1 in chronic and age-related diseases remains controversial. Genetic manipulation in model organisms and comparative genetics using human centenarians have demonstrated that insulin/IGF-1 signaling drives age-related pathologies but, conversely, IGF-1 therapy has been shown to benefit certain models of age-related disease and low serum IGF-1 is a predictor of disease risk in many human association studies. These paradoxical results have led to both pro- and anti- NSS strategies for overlapping pathologies, most notably in neurodegenerative diseases. Given the availability of both activating and inhibitory interventions targeting the IGF-1 pathway there is an urgency to clarify the seemingly paradoxical roles of IGF-1 in human disease (further discussed below in *Resolving the Paradoxes – Competing Models*).

Nutrient Signaling in Age-related Disease – a Focus on IGF-1

Neurodegenerative Disease

Clinical studies and rodent models give a mixed view of NSS in AD, Huntington's disease (HD), and dementia. IGF-1 resistance has been reported in mouse models of neurodegenerative disease and in human AD and HD patients, and intranasal insulin and IGF-1 are under consideration as a therapeutic strategy in AD, HD, and stroke (Hanson and Frey 2008; Lopes et al. 2014; Lioutas et al. 2015). A number of animal studies have reported beneficial effects of intranasal insulin or IGF-1 in models of stroke, AD, and HD, and injury-induced neurological damage, and preliminary human data suggests intranasal administration of the long-acting insulin analogue Detemir improves cognition in adults with mild cognitive impairment or early stage AD dementia (Cai et al. 2011; Chen et al. 2014; Lopes et al. 2014; Claxton et al. 2015; Lioutas et al. 2015; Mao et al. 2016).

On the other hand, decreased NSS is associated with reduced risk of age-related neurological decline in model organisms. In humans high serum IGF-1 has been associated with increased risk of AD, independent of ApoE status (van Exel et al. 2014), and two recent longitudinal reports describe a human IGF-1 allele enriched in AD patients and associated with increased circulating IGF-1 (Vargas et al. 2011; Wang et al. 2012a, b). Additionally, serum IGF-1 is significantly increased in the offspring of Alzheimer's patients compared to individuals with no family history of Alzheimer's, independently of ApoE status, suggesting a heritable mechanistic link (van Exel et al. 2014), and IGF-1 receptor activating activity of serum, a bioactivity measure that is thought to reflect IGF-1 function better than serum levels alone, was also recently shown to associate with a higher prevalence and incidence of dementia and Alzheimer's (de Bruijn et al. 2014).

Ischemic Stroke and Cardiovascular Disease

Single nucleotide polymorphisms, SNPs, in the *IGF1* gene associate with ischemic stroke and cardiovascular disease (CVD) risk in candidate-based studies, suggesting some role for IGF-1 in these diseases (Aoi et al. 2012). In mice, treatment with IGF-1 following ischemic injury is beneficial, but increasing IGF-1 *prior* to an ischemic event results in a greater infarct size and worsened pathology; in agreement, decreasing IGF-1 prior to the ischemic injury through preconditioning attenuates disease (see Fig. 3.4) (Endres et al. 2007; Zhu et al. 2008). In humans, a recent study identified an *IGF1* SNP associated with increased serum IGF-1 levels and improved post-stroke outcome but found no *IGF1* variant associated with the risk of having a stroke (Aberg et al. 2013). The uncoupling of stroke risk and post-stroke outcome suggests that IGF-1 may have unique roles in each setting.

The relationship between ischemic CVD risk and serum IGF-1 is similarly complex. Some studies have suggest a u-shaped relationship between serum IGF-1 and CVD, both high and low IGF-1 predicting CVD mortality, but these reports are limited by their use of prospective design using already aged participants (van Bunderen et al. 2013). A recent study examining nearly 4,000 elderly men followed over a 4–6 year period found no association between serum IGF-1 and CVD-related mortality or overall mortality but did observe levels of both IGFBP1 and IGFBP3 to be predictive, IGFBP1 positively and IGFBP3 negatively, of survival (Yeap et al.

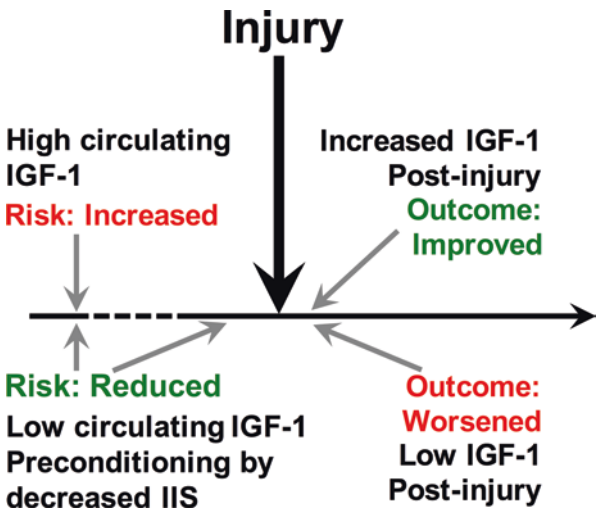

Fig. 3.4 Temporal Specificity of IGF-1 in Ischemic Injury. Temporal complexities of IGF-1 in response to injury. Available evidence indicates that IGF-1 is necessary for repair responses to ischemic injury events, while high-IGF-1/IIS signaling prior to an ischemic event is associated with poor outcome. This setting highlights the complexities of modeling and analyzing age-related pathologies. To clarify the role of IGF-1 in acute disease events pre- and post- injury levels are needed, and event risk versus response must be carefully distinguished

2011). Long-term longitudinal studies starting with healthy cohorts and measuring IGF-1, IGFBPs, and IGF-1 signaling in affected tissues will be necessary to uncover the true relationship between serum IGF-1 and ischemic stroke or CVD. Identifying gene variants that impact IGF-1 levels or signaling throughout life will also allow for better assessment of the role of IGF-1 in human ischemic disease in humans.

Sarcopenia

Much of the data supporting IGF-1 treatment as an intervention in age-related muscle disease are based on rodent models using acute injury. Among these are skeletal muscle injury models of sarcopenia using denervation, hind-limb unloading, and cardiotoxin injection. The beneficial effects of IGF-1 in these settings have been well reported, but their ability to accurately model age-related disease, versus acute injury, is not clear so they will not be discussed here.

A recent study examining the role of IGF-1 in normative human and rodent ageing found that although serum IGF-1 levels decrease during ageing, skeletal muscle NSS did not decrease in human or mouse; in fact, mTOR/S6 kinase activity actually increased with age (Sandri et al. 2013). Genetically increasing AKT activity in old mice resulted in exacerbated muscle decline and reduced lifespan, supporting a pro-ageing role for NSS in skeletal muscle. An independent study found that while chronic exercise prevents age-related sarcopenia in mouse quadriceps muscles, overexpression of IGF-1 in skeletal muscle had no benefit (McMahon et al. 2014).

Age-related Bone Loss

The relationship between the GH/NSS axis and bone health is complex. Circulating IGF-1 positively associates with bone mineral density (BMD) in post-menopausal women, is reduced in osteoporosis patients, and GH therapy in adults with GH deficiency improves BMD (Appelman-Dijkstra et al. 2014; Mo et al. 2015). In contrast, while GHR deficiency in Laron dwarfism is associated with dramatic reduction in circulating IGF-1 and overall body size, it does not appear to result in decreased BMD (Benbassat et al. 2003). GH drives circulating IGF-1, and consequently serum IGF-1 levels reflect GH status, so reported associations between IGF-1 and BMD may simply reflect the GH/BMD relationship. Further complicating the subject, it has been argued that GH impacts BMD predominately through changes in skeletal muscle mass, rather than direct effects on bone, and that there is a limited direct role for either serum GH or IGF-1 on bone (Klefter and Feldt-Rasmussen 2009).

Recent studies have begun to address some of these questions using tissue specific modulation of IGF-1. Osteocyte specific *Igf1* deletion suggest that local, but not circulating, IGF-1 is important for bone mineral metabolism (Sheng et al. 2014). Hepatic IGF-1 null mice with increased GH and GH overexpressing mice both show impaired bone architecture, while loss of hepatic IGF-1 in the context of normal GH has no impact on bone (Nordstrom et al. 2011; Lim et al. 2015). In agreement, it was

recently demonstrated that deletion of IGF-1 during early post-natal development results in a 67% increase in bone volume and increased density and trabecular number (Ashpole et al. 2015); consequently, BMD appears to be acutely sensitive to GH levels, with circulating IGF-1 impacting bone primarily via its role in feedback inhibition of GH, while locally produced IGF-1 plays a direct role in bone maintenance. The extent to which skeletal muscle mass plays a role in each of these settings remains to be defined.

Metabolic Syndrome and Obesity

Metabolic syndrome (MS) and obesity have repeatedly been associated with circulating IGF-1 levels in humans but a clear role for the factor has been elusive. Recent data suggests that study design may account for some of the discrepancies. A recent report comparing cross-sectional and longitudinal data from the same cohort found that while a cross-sectional analysis suggests a relationship between low serum IGF-1 and the prevalence of MS, a longitudinal assessment of the same population revealed that high serum IGF-1 is a predictive risk factor for the development of MS and serum IGF-1 levels decrease as MS progresses (Friedrich et al. 2013). Similarly, while cross sectional data suggests that low serum IGF-1 is associated with obesity, early life IGF-1 levels positively associate with risk of later life obesity (Madsen et al. 2011).

Evidence from Genome-wide Association Studies

A number of genes encoding factors involved in the insulin and IGF-1 signaling have been linked to human disease through genome-wide association studies (GWAS) (Table 3.1 and Fig. 3.1). These studies provide strong evidence that genetic variation in NSS influences a wide range of human diseases from cancer to autism and include many classic age-related pathologies such as Alzheimer's disease, age-related hearing loss, and cardiovascular disease. Meta-analyses of the NHGRI GWAS catalog, which acts as a repository for all reported significant GWAS findings, indicate that genes in NSS are enriched among age-related diseases, consistent with the notion that this pathway is a key regulator of ageing and age-related disease in humans (Cluett and Melzer 2009, Johnson et al. 2015). While GWAS provide robust evidence that identified genetic loci influence traits, they lack information regarding the directional impact of identified genetic variation or the mechanistic role of trait associated factors. Candidate gene studies in humans and model organisms have been critical in providing functional evidence linking NSS to disease and ageing, though these studies, particularly human clinical association studies, have provided a mixed view of NSS in disease.

Table 3.1 Genes in Insulin/IGF-1 signaling linked to human disease through genome wide association studies

Gene	Disease	References
AKT3	Schizophrenia, diabetic retinopathy	Grassi et al. (2011), Ripke et al. (2013) and Schizophrenia Working Group of the Psychiatric Genomics (2014)
EIF4E2	Non-small cell lung cancer (survival)	Sato et al. (2011)
EIF4ENIF1	Age-related hearing impairment	Fransen et al. (2015)
GHR	Systemic lupus erythematosus	Hom et al. (2008)
MRAS	Coronary heart and artery disease, amyotrophic lateral sclerosis (sporadic)	Erdmann et al. (2009), Schunkert et al. (2011), Dichgans et al. (2014) and Xie et al. (2014)
RRAS	Schizophrenia	Schizophrenia Working Group of the Psychiatric Genomics (2014)
RRAS2	Alzheimer's disease	Sherva et al. (2014)
NRAS	Autism	Xia et al. (2014)
IGFBP7	Age-related macular degeneration	Arakawa et al. (2011)
IGF1R	Arthritis (juvenile idiopathic)	Thompson et al. (2012)
IGF2, INS	Prostate cancer, type 1 diabetes autoantibodies	Eeles et al. (2009) and Plagnol et al. (2011)
IGF2BP2	Type 2 diabetes, gestational diabetes	Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research et al. (2007), Scott et al. (2007), Zeggini et al. (2007), Unoki et al. (2008), Zeggini et al. (2008), Takeuchi et al. (2009), Voight et al. (2010), Parra et al. (2011), Saxena et al. (2013), Hara et al. (2014), Replication et al. (2014) and Anderson et al. (2015)
IGF2R	Brain lesion load, periodontitis	Baranzini et al. (2009) and Teumer et al. (2013)
IGFB2	Esophageal cancer	Wu et al. (2012)
IGFBP1	Rheumatoid arthritis	Padyukov et al. (2011)
IGFBP1, IGFBP3	Major depressive disorder	Investigators et al. (2013)
IGFBP5, IGFBP2	Mitral valve prolapse	Dina et al. (2015)
IRS1	Type 2 diabetes	Rung et al. (2009), Voight et al. (2010) and Replication et al. (2014)
STK11	Psychosis in Alzheimer's disease	Hollingworth et al. (2012)

(continued)

Table 3.1 (continued)

Gene	Disease	References
MAP2K5	Obesity	Speliotes et al. (2010), Wen et al. (2012), Berndt et al. (2013), Wen et al. (2014) and Locke et al. (2015)
MAP3K1	Breast cancer, type 2 diabetes	Easton et al. (2007), Thomas et al. (2009), Turnbull et al. (2010), Michailidou et al. (2013), Tabassum et al. (2013) and Ahsan et al. (2014)
MAP3K11	Gout	Matsuo et al. (2016)
MAP3K13	Airflow obstruction	Wilk et al. (2012)
MAP3K14	Multiple sclerosis	International Multiple Sclerosis Genetics et al. (2011)
MAP3K4	Ageing	Edwards et al. (2013)
MAP3K7	Amyotrophic lateral sclerosis, Graves' disease, celiac disease	Dubois et al. (2010), Chu et al. (2011) and Xie et al. (2014)
MAP3K7IP1	Primary biliary cirrhosis, Crohn's disease	Franke et al. (2010) and Mells et al. (2011)
MAP3K7IP2	Crohn's disease, inflammatory bowel disease, diabetic retinopathy	Grassi et al. (2011) and Liu et al. (2015)
MAP3K8	Inflammatory bowel disease	Jostins et al. (2012)
MAP4K4	Psychiatric disorders (combined)	Cross-Disorder Group of the Psychiatric Genomics (2013)
MAP4K5	Lupus nephritis in systemic lupus erythematosus	Chung et al. (2014)
MAPK1	Multiple sclerosis	International Multiple Sclerosis Genetics et al. (2011)
MAPK10	Peripheral artery disease	Kullo et al. (2014)
PDK1	Erectile dysfunction in type 1 diabetes	Hotaling et al. (2012)
PIK3C2A	Schizophrenia or bipolar disorder	Ruderfer et al. (2014)
PIK3C3	Periodontitis	Teumer et al. (2013)
PIK3R1	Alzheimer's disease	Ramanan et al. (2014)
PTEN	Type 2 diabetes, periodontitis	Teumer et al. (2013) and Replication et al. (2014)
PRKAA1	Gastric cancer	Shi et al. (2011) and Hu et al. (2016)
PRKAB1	Ulcerative colitis, inflammatory bowel disease	Liu et al. (2015)
PRKACB	Breast cancer (male)	Orr et al. (2012)

(continued)

Table 3.1 (continued)

Gene	Disease	References
PRKAG2	Chronic kidney disease, bipolar disorder	Kottgen et al. (2010) and Belmonte Mahon et al. (2011)
RAF1	Cardiac hypertrophy	Parsa et al. (2011)
RPS6KA1	Amyotrophic lateral sclerosis (sporadic)	Xie et al. (2014)
RPS6KA2	Inflammatory bowel disease, dental caries	Jostins et al. (2012), Wang et al. (2012a, b) and Zeng et al. (2014)
RPS6KA4	Primary biliary cirrhosis, leprosy	Mells et al. (2011) and Zhang et al. (2011)
RPS6KB1	Multiple sclerosis, inflammatory bowel disease	International Multiple Sclerosis Genetics et al. (2011), Jostins et al. (2012)
SHC1	Prostate cancer	Eeles et al. (2013)
NR	Schizophrenia	Goes et al. (2015)
SHC4	Major depressive disorder, eating disorders	Aragam et al. (2011)
TSC1	Psoriasis, migraine without aura	Nair et al. (2009), Anttila et al. (2013)

A summary of gene-disease associations identified through genome-wide association studies involving key human genes in NSS, as shown in Fig. 3.1. Associations here achieved a genome-wide significance association threshold of $p<10\text{-}5$. Endophenotype associations, including associations with disease markers, not shown. GWAS data publicly available through the National Human Genome Research Institute GWAS Catalog

Paradoxes of IGF-1

The longevity and healthspan promoting benefits of reduced NSS are generally undisputed, but the impact of individual factors tends to be much more controversial, as in the case of IGF-1. IGF-1 has been a major focus of biogerontology, likely owing both to its historic context (the discovery of the nematode insulin/IGF-1 like receptor Daf-2) and the relative ease of measuring circulating IGF-1 in human cohorts for correlative studies. Although widely studied, the precise role of IGF-1 has remained stubbornly obscure. Mixed reports of IGF-1 in age-related diseases have led to various non-mutually exclusive models describing IGF-1 in human health. Both pro- and anti- IGF-1 therapies are in various stages of clinical trials, giving urgency to the paradoxes of IGF-1. Given the potential impact of these

therapeutic approaches to human health and the substantial attention given to this molecule in biogerontology, a detailed inspection of the data surrounding IGF-1 itself in ageing and age-related disease is warranted.

Evidence for Benefits of Reducing IGF-1 Signaling in Ageing

As discussed, the insulin/IGF-1-like signaling pathway was the first identified and is arguably the best characterized genetic pathway regulating lifespan in evolutionarily diverse organisms including nematodes, flies, and mice, with intracellular components also regulating lifespan in single cell eukaryotes (Fig. 3.1). Low serum IGF-1 is a positive predictor of lifespan in genetically heterogeneous mice, and humans with low IGF-1 resulting from GHR defects have a reduced incidence of age-associated cancers and metabolic disease (Harper et al. 2004; Guevara-Aguirre et al. 2011). Mx-cre driven deletion of IGF-1 in the liver dramatically reduces circulating IGF-1 and increases lifespan in mice (Svensson et al. 2011). A recent human genome-wide association study found that variants associated with low serum IGF-1 are also associated with increased likelihood of survival beyond 90 years (Teumer et al. 2016). Likewise, low IGF-1 is positively predictive of survival in already long-lived humans, and the offspring of centenarians tend to have low serum levels and bioactivity of IGF-1 (Guevara-Aguirre et al. 2011; Vitale et al. 2012; Milman et al. 2014). Human centenarians are enriched for rare variants in the *IGF1R* that reduce receptor function and impair IGF-1 stimulation of signaling in cultured cells (Tazearslan et al. 2011). Notably, *IGF1R* reduction of function allele carriers have increased serum IGF-1, presumably due to altered feedback inhibition of IGF-1 production. Together, this data strongly suggests that IGF-1 driven signaling promotes ageing and age-related disease.

Evidence for a Beneficial Role of IGF-1 in Disease

The evidence that IGF-1 signaling promotes ageing and age-related pathologies is substantial, but the precise role of IGF-1 in human age-related diseases in many instances remains controversial (excluding cancer, which will not be discussed here, as the disease-promoting role of NSS is well established). Serum IGF-1 was found in a 1985 study to decline during human ageing and it was suggested that this partly explains age-related bone and muscle loss. Supporting this view, low serum IGF-1 has been associated with metabolic syndrome (MS), cardiovascular disease (CVD) mortality, and hepatic steatosis (Oh et al. 2012), and low IGF-1 has been positively associated with mortality risk in some clinical studies (Tang et al. 2014). At least one recent study suggests there is a healthspan tradeoff of reduced NSS in invertebrates (Bansal et al. 2015), but the majority of paradoxical NSS data has arisen from studies of disease in rodents and humans.

Resolving the Paradoxes of IGF-1 – Competing Models

Central Versus Peripheral IGF-1

Given that IGF-1 has regulatory functions in the central nervous system, including regulation of GH at the pituitary gland, distinct from those of circulating IGF-1 it has been proposed that benefits and detriments of IGF-1 can be separated by uncoupling central (brain) and peripheral levels (Fig. 3.5) (Huffman et al. 2016; Milman et al. 2016). In this model systemic (hepatic) IGF-1 drives diseases such as cancer while central IGF-1 is necessary for proper regulatory function. Consistent with this model it has recently been demonstrated that intracerebroventricular infusion of IGF-1 in old rats rescues age-related declines in whole-body insulin sensitivity and glucose metabolism (Huffman et al. 2016). A related idea linked to the mixed neurodegenerative disease data is that neuronal health itself depends on central IGF-1 and neurodegenerative disease simply has a different relationship with IGF-1 than other age-related pathologies. While it remains to be seen if specific perturbations of brain versus peripheral IGF-1 will result in greater benefits than reducing NSS systemically in normal ageing or disease, and the mechanistic relationship further probed, it is an intriguing model which, given the existing intranasal delivery route, could lead to novel approaches to treating human age-related disease.

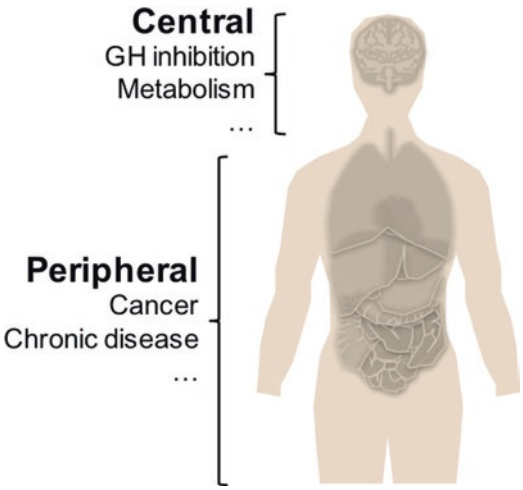

Fig. 3.5 Central Versus Peripheral IGF-1. The central versus peripheral model for the complex role of IGF-1 in ageing and age-related disease. In this model, circulating IGF-1, predominately produced in the liver, drives tissue ageing and age-related diseases, particularly cancer, in peripheral tissues, while brain-localized IGF-1 drives centrally regulated processes and neuron survival that combat age-related neurodegenerative diseases. While highly controversial, this model highlights the tissue-specificity of IGF-1 actions and may provide a partial explanation for complexity of IGF-1 in ageing

Temporal Specificity

One explanation for the discordant data in ischemic disease and injury models is that the role of IGF-1 in acute injury is highly dependent on timing. Lifespan studies generally show protective effects of reduced IGF-1 on chronic, including ischemic vascular, diseases, whereas injury models often show that IGF-1 treatment improves outcome. These observations support a model where chronic IGF-1 signaling drives risk of ischemic injury whereas NSS is necessary for a proper response to injury. In agreement, IGF-1 expression is induced during injury, and IGF-1 has been shown to play pleotropic roles in ischemic stroke, cardiovascular disease, and sarcopenia models (including acute injury models), as discussed (see Fig. 3.4) (Wagner et al. 2003). A precedent for this model has been established by recent studies of the senescence associated secretory phenotype (SASP). SASP is a well-documented driver of chronic and ARD's and reduced SASP signaling delays ARD's, while activation of SASP is beneficial in promoting wound repair (Demaria et al. 2014; Baker et al. 2016).

IGF-1 Resistance

The mixed role of IGF-1 in Alzheimer's disease may also be partly explained by observations that Alzheimer's brains show IGF-1 resistance and IGF-1 therapy provides a benefit in this setting, whereas genetic models suggest that chronic IGF-1 stimulation promotes the pathogenesis of IGF-1 resistance itself (de la Monte 2012; Zemva and Schubert 2014). Neuronal insulin/IGF-1 resistance precipitates a variety of defects, including altered glucose metabolism and neuronal viability, which are attenuated by IGF-1 treatment (Chen and Zhong 2013; Zemva and Schubert 2014). This model suggests IGF-1 benefits in AD are mechanistic similar to insulin injection in type 2 diabetes (T2D) – insulin prevents morbidities by normalizing blood glucose but does not improve the underlying defect of insulin insensitivity. Early routine use of insulin is associated with a variety of side-effects; behavioral modification and insulin-sensitizing agents, such as metformin, are preferred therapies (Lebovitz 2011). It seems prudent that caution be exercised in considering IGF-1 as a therapy in neurodegenerative disorders, but the lack of available treatment options and late-onset of the diseases should be weighed against potential side-effects.

While perhaps best-supported by experiments in AD models, age-related IGF-1 resistance may explain other observed benefits of IGF-1 therapy in aged animals and warrants further direct study.

Optimal Dose, Context Specificity

Perhaps the simplest model for IGF-1 in ARD and ageing is the notion that there is an ideal dosage which balances the beneficial and detrimental effects of IGF-1 and maximizes lifespan (Fig. 3.6). This model, supported by human clinical data (Burgers

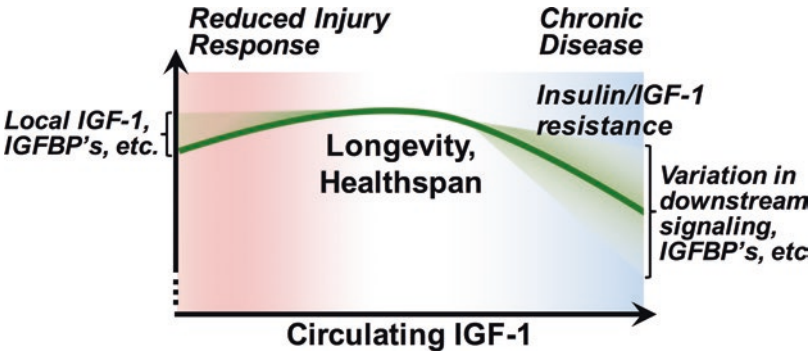

Fig. 3.6 Optimal Dose and IGF-1 Resistance. A dose-dependent model for the overall role of IGF-1 follows a u-shaped curve. IGF-1 levels may include IGF-1 produced in the liver and tissue localized production, depending on the context. At sub-optimal IGF-1 concentrations responses to acute injury are negatively affected. At high concentrations diseases are promoted by chronic IGF-1 stimulation. Chronic stimulation is associated with insulin/IGF-1 resistance, explaining the apparent benefits of local IGF-1 treatment in some neurodegenerative disease models. Circulating and intracellular factors modify signaling at both ends of the u-curve. Sufficient local IGF-1 production may be sufficient to negate the detrimental effects of reduced circulating IGF-1 in the context of acute injury

et al. 2011), likely accounts for the overall pleiotropy of IGF-1 but alone provides limited framework to consider interventions targeting this factor. Some intermediate level of circulating IGF-1 may in fact limit the development of chronic diseases without leading to detrimental effects, such as reduced wound healing, but tissue targeted and context specific interventions would undoubtedly provide greater benefit.

As stated, these models are non-mutually exclusive, and it is likely that each are at least partially true, or true in specific context. Which, if any, provide efficacious new approaches to age-related disease is the question at hand.

Circulating Factors – More Than Just IGF-1

IGF-1, acting through the IGF-1 receptor, is largely treated as the only, or at least the primary, mediator of systemic NSS in mammals. This has largely been historically driven; IGF-1 and IGF-1R represented attractive targets after the 1997 report that the longevity regulating DAF-2 receptor in *C. elegans* is a homolog of the human insulin/IGF-1 receptor (Kimura et al. 1997). While available data does support the notion that IGF-1 and IGF-1R do act as regulators of longevity in mammals, it is important to note the distinctions between nematode and mammalian insulin/IGF-1 signaling and, critically, the data suggesting that alternative systemic nutrient signals play equal or greater role in ageing. GH and GHRH are not only upstream of IGF-1 but activate intracellular NSS pathways themselves. GH, through GHR, stimulates PI3K/AKT/mTOR and MAPK/ERK pathways independent of

IGF-1. In addition, insulin and IGF-2 both have overlapping roles with IGF-1. Even in *C. elegans*, where the IGF-1R homolog DAF-2 has strongly influences lifespan, there are over 30 insulin/IGF-1 like signaling molecules and the relative contribution of each is unclear (Gahoi and Gautam 2016). Thus, hindsight would suggest that IGF-1 may not be as important in isolate as initially assumed.

The relatively overemphasized role of IGF-1/IGF-1R *per se* is highlighted by genetic models of longevity in mice: pituitary loss of function Ames and Snell dwarf mice, and GH, GHR, or GHRH knockout animals all show substantially increased lifespan compared to normal animals, with median survival improved by 50–70% (Sun et al. 2013). IGF-1R heterozygous mice are reportedly long-lived, but this phenotype is milder and appears to be gender and strain specific. Conditional knockout of IGF-1 in liver, which produces ~80% of circulating levels, has been found to extend median lifespan of mice but only by ~10% (Svensson et al. 2011). This extends beyond the upstream regulators of IGF-1; overexpression of FGF21, a fasting hormone secreted by the liver, was shown to increase median lifespan in mice by 36%, reportedly through modulation of mTOR, AKT, and the GH-IGF-1 axis in liver (Zhang et al. 2012). Direct relative effect comparisons between these studies are impossible given the complexity of the experiments and the complications associated with deleting IGF-1 and IGF-1R which, when homozygous deleted, are neonatal lethal (Epaud et al. 2012; Pais et al. 2013). Nevertheless, the general trend would suggest that circulating factors other than IGF-1 may prove better candidates for intervention in ageing and warrant further attention.

Experimental Considerations and Future Directions

While association studies are valuable for linking phenotypes to genetic variation or biomarkers, results should be interpreted with extreme caution. In particular, cross-sectional association data involving a dynamic parameter like IGF-1, which is strongly influenced by health status, should be approached with great caution. Chronic renal failure, hepatic dysfunction, and malnutrition all cause a reduction in levels or serum bioactivity of IGF-1, among broader changes to circulating factors (Moller and Becker 1990; Moller and Becker 1992; Tonshoff et al. 2005; Sirbu et al. 2013). Since IGF-1 is itself altered by the presence of underlying pathology causality should not be inferred from cross sectional data alone even when robust associations are observed.

Similarly, genetic modeling provides an immense amount of information regarding the role of individual factors in ageing and disease, but results from genetic models must always be interpreted with care. The more complex the genetic modulation, the greater the room for unintended consequences. Complex heterozygotes or conditional mutants may provide useful complimentary data, but off-target effects,

temporal or spacial gene functions (including developmental functions), leaky promoters, and gene dosing effects are all complicating factors that are too often ignored when model data is published. Pharmacological approaches have also been limited by over-interpretation or lack of proper controls. The ageing literature is littered with unrepeated studies identifying intervention strategies of unclear validity. Greater effort should be focused on multi-center collaborations, as typified by the intervention testing program, where published findings are least likely to be influenced by bias. The sum of available data on nutrient sensing signaling paints a clear landscape where reduced signaling delays pathologies of ageing, but individual studies must be taken in context.

Study design is critical for interpreting association data, but pleotropic effects can dramatically complicate interpretation even in the most rigorously designed study. As discussed, experimental evidence suggests that nutrient signaling plays different roles in altering the risk of an ischemic events versus promoting recovery following such events. For this reason, abstract or overly simplified models for age-related disease may not accurately reflect the intended pathology. The most notable examples are acute injury in muscle models using toxins or physical injury (as discussed), but examples of age-related disease models with no clear link to actual ageing are abundant. There are also important considerations that must be accounted for in clinical studies; retrospective studies will likely be enriched for patients harboring factors that promote survival and may be misleading if they are used to predict the effects of genetic variability on risk, while prospective studies that track only incidence but lack survival data may obscure important associations.

Remaining unanswered questions regarding the role of nutrient sensing signaling in disease will require tissue and context specific animal models and cautious interpretation of experimental findings. In human studies, functional genetics defining the impact of disease or longevity associated genetic variation on the expression of IGF-1 or related factors will provide further insight into the relationship between IGF-1 and disease. Creative synthesis of modern high-throughput datasets to identify and describe genetic variants with IGF-1 eQTL's, including those with tissue specific impact, will add new depth to our current understanding of the regulation and role of IGF-1 in human disease. Circulating NSS factors, including IGF-1 modifiers such as the IGFBP's, deserve greater attention in studies focused on systemic signaling; they are both major confounding factors in NSS studies and promising targets for future therapies. Addressing these issues and identifying the downstream targets of importance will lead to novel therapeutic strategies and pharmacological targets.

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Chapter 4
The Interplay Between Cholesterol Metabolism and Intrinsic Ageing

Mark Tomás Mc Auley

Abstract The last few decades have witnessed remarkable progress in our understanding of ageing. From an evolutionary standpoint it is generally accepted that ageing is a non-adaptive process which is underscored by a decrease in the force of natural selection with time. From a mechanistic perspective ageing is characterized by a wide variety of cellular mechanisms, including processes such as cellular senescence, telomere attrition, oxidative damage, molecular chaperone activity, and the regulation of biochemical pathways by sirtuins. These biological findings have been accompanied by an unrelenting rise in both life expectancy and the number of older people globally. However, despite age being recognized demographically as a risk factor for healthspan, the processes associated with ageing are routinely overlooked in disease mechanisms. Thus, a central goal of biogerontology is to understand how diseases such as cardiovascular disease (CVD) are shaped by ageing. This challenge cannot be ignored because CVD is the main cause of morbidity in older people. A worthwhile way to examine how ageing intersects with CVD is to consider the effects ageing has on cholesterol metabolism, because dysregualted cholesterol metabolism is the key factor which underpins the pathology of CVD. The aim of this chapter is to outline a hypothesis which accounts for how ageing intersects with intracellular cholesterol metabolism. Moreover, we discuss the implications of this relationship for the onset of disease in the 'oldest old' (individuals ≥85 years of age). We conclude the chapter by discussing the important role mathematical modelling has to play in improving our understanding of cholesterol metabolism and ageing.

Keywords Cholesterol metabolism · Reactive oxygen species · Cardiovascular disease · 'Oldest old'

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Introduction

Ageing is a phenomenon which has troubled scholars, philosophers, writers and scientists for centuries. However, humanity had to wait until the end of the nineteenth century for the first meaningful biological explanation as to why we age. This was proposed by the German biologist August Weisman. Weisman adopted an evolutionary framework and posited an adaptive theory to account for why we age (Kirkwood and Cremer 1982). Weisman proposed that senescence is a direct consequence of evolved limitations to the division potential of somatic cells and suggested this deficiency evolved because it is beneficial to eliminate old individuals from a population, thus extricating resources for younger individuals. Weismann mused senescence or "programmed death" as it has been coined was an evolved trait and its function was to remove decrepit individuals from a population. As an explanation for why we age this theory remains contentious for several reasons; first, if a biological characteristic was to be selected for by evolution, for example, "programmed ageing/death" it would have been essential for it to manifest itself in a manner which has a bearing on the survival of the organism; and because ageing is seldom observed in the wild. Weisman did go on to refine his ideas and proposed a theory that considered old individuals as neutral and the evolution of ageing as a "panmixia," where neutral characters decline during evolution. Thus, Weismann's original idea of "programmed ageing" has been superseded by several non-adaptive theories which were introduced throughout the nineteenth century. The cornerstone of all the main non-adaptive evolutionary theories of ageing is as follows, the force of natural selection declines with age. The idea that ageing can be described by a decline in the force of natural selection was initially recognized by JBS Haldane and RA Fisher in the 1920s (Rose 1991), however it was Peter Medawar who formalized this idea to propose what has become known as the mutation accumulation theory (Medawar 1952). Medawar suggested ageing could be accounted for by the accumulation of deleterious alleles (mutations) accumulating in the later stages of life. The reason for their accumulation is due to the diminishing force of natural selection. An extension of this theory was proposed by Williams in 1957, it is known as the antagonistic pleiotropy theory and it suggests certain genes have a positive effect in early life but have a deleterious effect in later years (Williams 1957). The next significant evolutionary theory was proposed in the late 1970s (Kirkwood 1977). This theory was put forward by Thomas Kirkwood and centers on the life history of an organism. It is referred to as the disposable soma theory (DST). According to the DST organisms have a finite amount of energy which is divided between reproduction or investment in somatic maintenance. Because extrinsic mortality is high in the wild, the ideal strategy for the allocation of this energy is based on the life expectancy of the organism. If maintenance is set too high, energy will be wasted when the organism dies. If maintenance is set too low, this will result in premature intrinsic mortality. Therefore, based on the DST organisms have evolved so the quantity of energy directed towards somatic maintenance is sufficient to reach reproductive years but less than needed for indefinite survival.

What makes the DST theory unique as an evolutionary account of ageing is that it seamlessly interconnects with the cellular maintenance and repair systems which have been identified as key components of cell ageing. In turn, maintenance and repair systems integrate with a suite of cellular processes and metabolic networks which have been implicated with ageing. The first of these processes to be truly associated with cellular ageing was identified by the paradigm shifting work of Hayflick and Moorehead in the 1960s, who demonstrated cellular senescence occurs when differentiating cells stop dividing (Hayflick and Moorhead 1961). Up until this point it had been widely regarded that cells could divide indefinitely and are essentially immortal, provided they have an adequate environment and supply of nutrients. What fueled this misconception was the misguided work of an American based French physician called Alexis Carrel at the turn of the twentieth century (Carrel 1912). Carrell claimed to have grown a single line of chicken embryo fibroblasts for over 30 years. However, in light of the careful work of Hayflick and others it has been suggested some of the materials used by Carrel to feed the chicken fibroblast cultures were contaminated with fresh chicken embryo cells. Thus, Carrel's experiments gave the false impression that cells were immortal when this was clearly not the case. It can be argued the illuminating work of Hayflick and Moorhead opened the door for scientists to actively commence seeking other processes associated with cell ageing. To this end the genesis for a more integrated view of ageing commenced in the 1970s, when the theoretical biologist Alexei Olovnikov reconciled the idea of cellular senescence with chromosomal biology, by deducing that chromosomes are incapable of complete end-replication (Olovnikov 1973). Olovnikov postulated this was due to DNA attrition each time a somatic cell divides. Moreover, it was thought that when chromosomal erosion reached a critical point cell division would cease. The theoretical ideas of Olovnikov were confirmed experimentally in the seventies and eighties when it was found that chromosomal ends are protected by telomeres, and that the repetitive TTAGGG sequence of the telomere is maintained by the enzyme telomerase, which is a key regulator of the rate of telomere attrition (Greider and Blackburn 1985; Blackburn and Gall 1978). The significance of telomere loss to ageing in humans was emphasized in the 1990s when it was found both the quantity and size of telomeric DNA in human fibroblasts diminish with age *in vitro* (Harley et al. 1990).

The integrated nature of ageing is further emphasized when the "free-radical theory" of ageing is considered (Harman 1956). The essence of Harman's theory is, that oxygen free radicals damage the cell. The build-up of this damage eventually constitutes ageing. It is logical to see why this hypothesis is attractive, when one considers ageing has traditionally been defined as the gradual decline in the integrity of an organism over time leaving it vulnerable to mortality (Comfort 1964). Due to the appealing nature of this theory, it has built a significant reputation within biogerontology. However, it is imperative to stress, that although the damage caused by reactive oxidative species (ROS) most likely influences the trajectory of ageing, it is naïve to suggest it is the single factor which underpins ageing. Rather, as our group suggested recently it is a convenient framework for integrating many aspects of ageing (Mooney et al. 2016). Moreover, it is necessary to appreciate that ROS

affect cellular processes both negatively and positively. An example of the former is oxidative stress can augment the rate of telomere attrition. This was revealed in the 2000s when it was demonstrated that continued exposure to oxidative stress affects telomere integrity and augments the onset of senescence (Kurz et al. 2004). Moreover, it has been found that the advantageous effects of caloric restriction (CR) could be precipitated *via* a decline in the generation of ROS (Qiu et al. 2010). This study found the deleterious impact of ROS declined in mice lacking SIRT3. In addition, it was found CR was associated with a decrease in the accumulation of oxidative damage in rhesus monkeys (Mattison et al. 2017). On the positive side, mild exposure to ROS has been suggested to have a beneficial effect on the ageing process. This idea fits within the concept of hormesis, whereby low doses of an otherwise harmful substance improve the functional ability of an organism (Gems and Partridge 2008).

Additional mechanisms have been associated with ageing (Mc Auley et al. 2017a). For example, the accumulation of unrepaired DNA damage has been suggested to be a significant component of ageing (Freitas and de Magalhaes 2011). DNA is susceptible to damage from extrinsic factors, including pollutants and ultra violet light, or intrinsic factors such as ROS (Cadet and Wagner 2013). The majority of DNA damage is rectified as part of a DNA-damage response (DDR) maintenance system, of which the p53 signaling pathway is the central player (Williams and Schumacher 2016). Interestingly, it would appear the DDR system is closely coupled with telomere maintenance, because proteins associated with DDR also play a role in telomere maintenance (Arnoult and Karlseder 2015). In addition protein levels are regulated by quality control systems, which overlap with many other processes associated with ageing (Hartl 2016). For instance, ROS are known to compromise the integrity of proteins (Hartl 2016), some of which can be rectified by molecular chaperones (Klaips et al. 2018). However, over time the efficiency of the chaperone system diminishes and its dysregulation leads to a build-up of protein damage (Koga et al. 2011). In addition to chaperones an array of other intracellular maintenance and repair systems are part of a broad suite of biological entities which have been identified as key to how ageing unfolds. In the last few decades biogerontology has made remarkable progress in understanding these processes. However, despite our growing knowledge of these biological mechanisms, to date we only have a rudimentary understanding of how they interact to shape the trajectory of ageing and the onset of disease. This is particularly frustrating because ageing as a risk factor is often recognized demographically, however ageing processes are routinely overlooked in disease mechanisms. Thus, a central goal of ageing research is to understand how diseases such as cardiovascular disease (CVD) are shaped by the biological mechanisms which are synonymous with ageing. This is a significant challenge because CVD is the main cause of morbidity in older people (Mc Auley and Mooney 2014). A worthwhile place to begin disentangling the relationship between CVD and these processes is to firstly consider how ageing impacts lipid metabolism, and more specifically how it effects cholesterol metabolism (Morgan et al. 2016a; Mc Auley and Mooney 2014; Mooney and Mc Auley 2016). There is a long-established relationship between plasma cholesterol levels and CVD risk

(Nelson 2013). The biological underpinnings of this relationship are in part due to the strong association which exists between elevated total cholesterol/low-density lipoprotein cholesterol (LDL-C) and atherosclerosis (Roy 2014). Atherosclerosis is considered the underlying vascular processes which represents the pathological explanation for coronary heart disease (CHD) and stroke; the principal clinical manifestations of CVD (Libby et al. 2013). In contrast to total cholesterol and LDL-C, high-density lipoprotein cholesterol (HDL-C) has an anti-atherogenic role to play in reducing CVD risk (Murphy et al. 2012). In this chapter we will pay particular attention to how ageing intersects with intracellular cholesterol metabolism and the implications of this for the onset of disease.

Whole Body Cholesterol Metabolism and the Impact of Ageing

To appreciate the impact ageing has on cholesterol metabolism and the significance of this for older people, it is necessary to understand the key mechanisms which control whole-body cholesterol balance (Fig. 4.1). The body maintains cholesterol balance by responding to changes in ingestion, absorption, synthesis and excretion (Mc Auley and Mooney 2015). For example, a drop in cholesterol production results in an increase in cholesterol absorption and *vice versa* (Cohen 2008). Humans, ingest a negligible quantity of dietary cholesterol, which mixes intestinally with cholesterol originating intrinsically. Intestinal absorption is regulated by cholesterol ester hydrolase which releases long chains of cholesterol esters, facilitating their incorporation into bile acid micelles (Hernell et al. 1990). The intestinal protein Niemann-Pick C1-Like 1 (NPC1L1) regulates cholesterol absorption into the enterocyte by clathrin-mediated endocytosis (Park 2013). The ATP-binding cassette (ABC) transporters G5 and G8 (ABCG5/G8) control the efflux of cholesterol in the enterocytes from the lumen (Repa et al. 2002). Embedded in the enterocyte, acetyl CoA acetyltransferase 2 (ACAT2) takes part in the re-esterification of cholesterol (Joyce et al. 1999). Esterified cholesterol links with apo B-48, triacylglycerols (TGs) and phospholipids, which together comprise a chylomicron. Chylomicrons join the lymph, where the enzyme lipoprotein lipase (LPL) works on their TGs releasing free fatty acids (FFAs) (Santamarina-Fojo and Dugi 1994). Chylomicron remnants are eliminated from the blood by the liver *via* hepatic remnant receptors (Rensen et al. 1997). The liver is the major site for the manufacture of endogenous cholesterol (Dietschy et al. 1993). As a result the liver contains a substantial pool of cholesterol. This reservoir provides the cholesterol which is incorporated as part of TG rich very low density lipoproteins (VLDLs) (Tiwari and Siddiqi 2012). VLDLs are synthesised primarily to enable the transport of endogenously synthesised TG to the tissues (Tiwari and Siddiqi 2012). On joining the plasma VLDL is in part hydrolysed by LPL to form IDL. Most of the cholesterol within VLDL is separated from the circulation *via* hepatic receptors and re-joins the liver. IDL is in turn metabolised

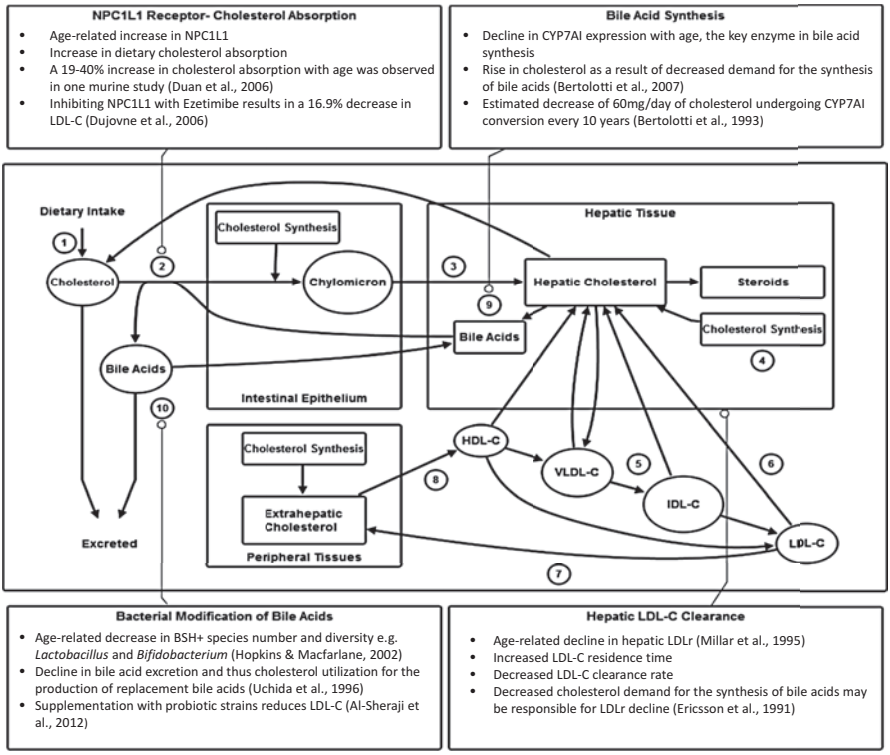

Fig. 4.1 The impact of ageing on whole body cholesterol metabolism. (Figure taken from Morgan et al. 2016a, b, c)

to LDL (Ramasamy 2014). Removal of circulating cholesterol occurs *via* the uptake of IDL, VLDL and LDL. LDL-C is removed by receptor mediated endocytosis via the LDL receptor (LDLR) (Goldstein and Brown 2015). The uptake of cholesterol is modulated by intracellular cholesterol levels. A change to cellular cholesterol concentration elicits two separate negative-feedback loops, one regulates the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR), the rate limiting enzyme in cholesterol biosynthesis (Goldstein and Brown 2015). The second feedback loop down regulates LDLR synthesis. In addition to activating these feedback loops, cholesterol entering the cell as part of LDL triggers ACAT2. Esterification of cholesterol by ACAT2 converts free cholesterol (FC) to cholesterol esters, which in a hepatic cell are incorporated into VLDL and returned to the plasma (Goldstein and Brown 2015). Reverse cholesterol transport (RCT) is a necessity for removing excess cholesterol from peripheral tissue (Rader et al. 2009). HDL is key to this pathway. HDLs, work by removing excess cholesterol to produce HDL-C (Tuteja and Rader 2014). HDLs ferry peripheral cholesterol to the liver where it joins the hepatic pool of FC. Alternatively the excess cholesterol can be converted to bile salts (Groen et al. 2004). This means the excess peripheral

cholesterol can be excreted from the body as bile salts during enterohepatic circulation. Vital to RCT is the ferrying of FC and phospholipids to lipid-free apo A-I in a process regulated by ATP-binding cassette transporter 1 (ABCA1). ABCA1 mediates the efflux of cholesterol lipid-poor apolipoproteins (apo-A1 and apoE), which then form nascent high-density lipoproteins (HDL), a crucial early stage in RCT (Huang et al. 2015). Nascent HDL particles progress to mature HDLs as a result of the esterification of cholesterol by lecithin-cholesterol acyltransferase (LCAT) (Saeedi et al. 2015). Cholesterol within HDLs can follow one of two routes to the liver. Firstly, HDL particles can go straight to the liver and release their cholesterol by engaging with scavenger receptor class B, type I (SR-BI) receptors (Shen et al. 2014). Secondly, cholesterol can be transferred to the liver as a result of cholesteryl ester transfer protein (CETP), redistributing cholesterol to other lipoproteins, such as LDL and VLDL (Wang et al. 2018). Consequently RCT is ubiquitously suggested as an anti-atherogenic pathway and HDLs are key to it. Evidence of this is provided by a multitude of epidemiological and clinical studies (Vergeer et al. 2010). These studies have shown that an inverse relationship exists between the concentration of HDL-C and the development of premature CVD (Wilson et al. 1988). Collectively, these processes help to maintain whole-body cholesterol balance. However, the ageing process has a significant impact on cholesterol metabolism (Morgan et al. 2016b). Figure 4.1 illustrates the impact ageing has on these processes and the challenge which is faced when we consider studying cholesterol metabolism and its interaction with ageing. In the next section we will focus on how ageing effects cholesterol metabolism in older people and the implications this has for healthspan.

Cholesterol Metabolism Is Different in the Oldest Old

As outlined in the previous sections both LDL-C and HDL-C levels have long established relationships with CVD risk. Specifically the significance of LDL-C as a risk factor for CVD is underscored by epidemiological evidence which illustrates LDL-C increases up until the midpoint of life in males and females and then decreases in the latter decades of life (Felix-Redondo et al. 2013). It is not certain why LDL-C levels gradually increase in so many people up until middle age, and then diminish in later years. This phenomenon becomes increasingly intriguing when one considers the findings of a seminal study which examined cholesterol metabolism in individuals ≥85 years of age (Weverling-Rijnsburger et al. 2003). In this study it was found that both high and low levels of LDL-C impacted mortality risk in these individuals to the same extent (Lv et al. 2015). This counter intuitive finding has also been witnessed in similar studies (Lv et al. 2015), while much has been made of the utility of LDL-C as a risk factor for CVD (Ravnskov et al. 2016). Recently, we proposed a hypothesis to account for these intriguing findings (Mc Auley and Mooney 2017). Our hypothesis is grounded in the mechanisms which underpin intracellular hepatic cholesterol homeostasis. In order to appreciate our

hypothesis it is necessary to reaffirm the processes which govern intracellular cholesterol homeostasis in hepatic cells and how these impact physiological levels of LDL-C. Firstly, as outlined above it is important to understand that circulating levels of LDL-C are the result of a series of biochemical processes which begin with the entry of VLDL into the circulation from the liver. As this metabolic cascade unfolds, a significant proportion of cholesterol re-enters the liver as a result of the uptake of VLDL and LDL. The kingpin of these events is the LDLR. This receptor is fine-tuned by evolution to 'hoover up' circulating LDL-C. The activity of the LDLR receptor is governed by an equally wonderful set of intracellular regulatory processes. Firstly, when the concentration of FC changes, this invokes two negative feedback loops (1) HMGR synthesis is suppressed (2) there is a reduction in the rate of LDLR synthesis. Finally, as cholesterol is trafficked into the cell it elicits ACAT2. Esterification of cholesterol by ACAT2 converts FC to cholesterol esters (CE), which in a hepatic cell are incorporated into VLDL and returned to the plasma. The deployment these fundamental processes are central to how cholesterol levels are homeostatically maintained within the cell. In the next section we will critically examine how ageing could impact intracellular cholesterol homeostasis.

How Could Ageing Impact Intracellular Cholesterol Homeostasis?

There is ground for believing ageing has an impact on intracellular cholesterol homeostasis. Moreover, this could account for LDL-C diminishing as a risk factor in some older people. Based on our recent hypothesis we suggest cholesterol homeostasis in certain older people is defined by two distinct metabolic scenarios. These scenarios are outlined in Fig. 4.2. In the first metabolic state hepatic endogenous antioxidant capacity is insufficient to deal with increased ROS levels which accompany ageing. The metabolic consequences of this deficiency are that increasing ROS levels result in the total activation of HMGR, which increases the synthesis of FC. This elicits a corresponding rise in plasma LDL-C due to the increased secretion of VLDL-C. Such individuals are at risk of developing CVD as a result of atherosclerosis. In the second scenario which accounts for cholesterol metabolism in very old people, endogenous antioxidant capacity is also overwhelmed by rising intracellular ROS levels. This results in the total activation of HMGR; however, the defining feature of this situation is the low activity of ACAT2. The low activity of ACAT2 causes a reduction in the secretion of VLDL-C, consequently there is a drop in LDL-C levels. It is our conjecture that this is a particularly detrimental situation, because despite a drop in LDL-C, FC increases. Moreover, we are of the opinion that the accumulation of hepatic FC could contribute to the onset of nonalcoholic fatty liver disease (NAFLD). NAFLD has been correlated with an increased risk of

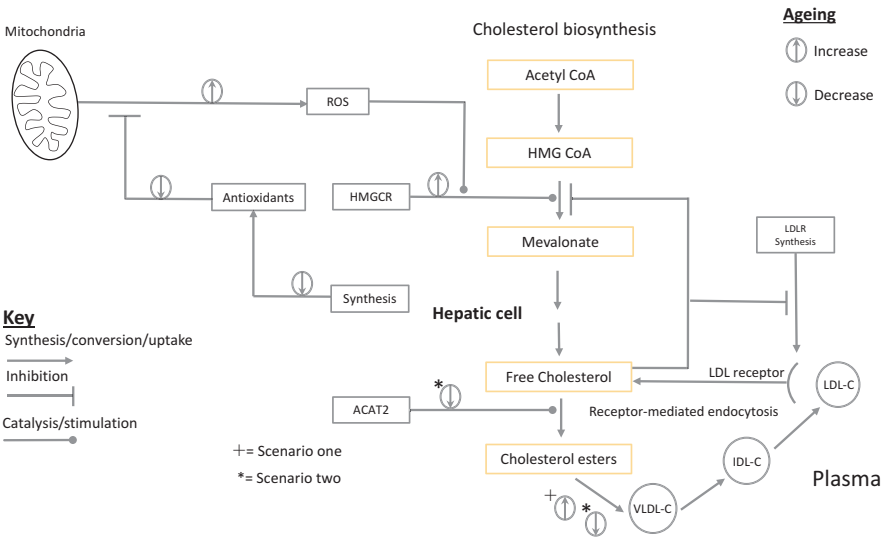

Fig. 4.2 The putative effects of ageing on intracellular cholesterol homeostasis. (Figure taken from Mc Auley et al. 2017a, b). According to our hypothesis a decrease in hepatic antioxidant capacity and a rise in ROS elicits increased cholesterol biosynthesis, which raises LDL-C. On the other hand when an increase in ROS is accompanied by a decline in the activity of ACAT2 this results in decreased LDL-C with an associated rise in hepatic FC. These changes ultimately could mask underlying pathology in the 'oldest old'

CVD (Patil and Sood 2017). Thus, based on our reasoning we suggested the inverse correlation between risk of CVD mortality and LDL-C is a result of these changes to intracellular cholesterol homeostasis. Our hypothesis is based on a number of key experimental findings. Firstly, several studies have suggested the hepatic antioxidant capacity diminishes with age. For instance, it has been found that the activities of superoxide dismutase (SOD) and catalase decreased substantially with age in rat liver (Semsei et al. 1989; Ji 1993).

Several lines of evidence support the idea that ROS induce the total activation of HMGR. For example, it has been found ROS have a role to play in the age-associated dysregulation of HMGR related to hypercholesterolemia in rats (Pallottini et al. 2005). Furthermore, it has been found that ROS has the capacity to impinge on the activity of HMGR as part of thioacetamide-induced hepatic injury (Pallottini et al. 2006). Finally, it has been found that ROS levels effect HMGR dephosphorylation (Pallottini et al. 2007). Taken together, these findings add weight to the idea that ROS have an impact on intracellular cholesterol homeostasis. The next aspect of our recent hypothesis which required inspection is the notion that intrinsic ageing causes a drop in the activity of ACAT2. Experimental evidence for this argument is lacking somewhat, however support was found in work which examined homozygous Watanabe heritable hyperlipidemic rabbits; an animal model lacking in

LDLRs (Shiomi et al. 2000). Despite this limitation, these animals manifest a significant drop in their plasma concentrations of LDL-C with age. Intriguingly, in these animals it has been found there is a decrease in ACAT activity in conjunction with a drop in the hepatic release of VLDL-C. This finding dovetails neatly with our hypothesis, as it is likely a reduction in ACAT activity would correlate with increased hepatic FC. This change could have health implications, as the accumulation of FC hepatically is likely a precursor to non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (Arguello et al. 2015). In summary, an important inference from this hypothesis is intrinsic age-related changes to hepatic cholesterol metabolism could result in a decrease in LDL-C, which has been observed in very old people. Moreover, these changes could account for the inverse association which has been observed between LDL-C levels and risk of cardiovascular mortality in the oldest old.

The Impact of Other Ageing Associated Processes on Intracellular Cholesterol Metabolism

Other processes associated with cellular ageing have been shown to interact with cholesterol homeostasis. One such entity are silent information regulator proteins, otherwise known as sirtuins. Sirtuins, are NAD+–dependent deacetylases (Imai and Guarente 2016). To date seven known mammalian sirtuins have been identified, which are localized to different regions of the cell. They engage in a variety of tasks, from nutrient sensing to DNA damage/repair (Imai and Guarente 2016). There are 7 known mammalian sirtuins, that function as NAD+–dependent deacetylases, which are involved in a wide range of cellular activities including nutrient sensing and DNA repair (Guarente 2011). The most well studied of the sirtuins, SIRT1, plays a role in various metabolic activities that permit the cell to adapt to perturbations in nutrient levels (Chang and Guarente 2014). For example, SIRT1 plays a part in modulating liver gluconeogenesis, insulin secretion and fat mobilization (Aditya et al. 2017). Sirtuins also impact cholesterol biosynthesis. For instance, SIRT1 deacetylates the nuclear receptor liver X receptor α (LXRα) to provoke the manufacture of ABCA1. The importance of SIRT1 to cholesterol metabolism is further emphasized when this gene is knocked out in mice. Mice suffer a reduction in plasma HDL-C levels and cholesterol accumulates in their liver (Li et al. 2007). It has also been found SIRT2 can reduce sterol biosynthesis by decreased trafficking of SREBP-2 (Luthi-Carter et al. 2010). Moreover, it has been suggested Sirt6 is vital for Srebp2 gene regulation (Tao et al. 2013). Specifically this study found hepatic deficiency of Sirt6 in mice resulted in raised serum and hepatic cholesterol levels. Thus, sirtuins appear to have a key part to play in the regulation of intracellular cholesterol homeostasis.

The Role of Mathematical Modelling in Understanding Cholesterol Metabolism and Ageing

In the last few decades mathematical modeling has come to the fore as a key tool for understanding complex biological processes (Mc Auley et al. 2013, 2015a; Salcedo-Sora and Mc Auley 2016; Choi et al. 2015; Kilner et al. 2016). Moreover, the role mathematical modeling has to play in ageing research is increasingly growing (McAuley et al. 2009, 2015b, 2016, 2017b; Mc Auley and Mooney 2018). The reason mathematical modelling is so essential to help improve our understanding of ageing is that it offers an ideal framework for dealing with the complexities associated with ageing. Over the years many different aspects of ageing have been modelled mathematically and we have gained an additional insight into the ageing process as a result. Until recently, however there has not been a specific focus on how ageing impacts cholesterol metabolism. The goal of our group is to utilize mechanistic kinetic mathematical models to infer how ageing impacts the overall dynamics of cholesterol metabolism (Mc Auley et al. 2005; Mc Auley and Mooney 2015). A kinetic model utilizes rate expressions to represent the velocity at which biochemical reactions occur. The rate equations are then used to inform the assembly of a series of differential equations which are used to simulate the temporal behavior of the biochemical entities.

As mentioned previously, although intrinsic ageing has not been explicitly included in models of cholesterol metabolism until our recent work, it is however worthwhile outlining the impact modelling has had on our understanding of cholesterol metabolism. Moreover, it is important to stress the significance of these findings for understanding ageing. Over the years an array of different kinetic models have been used to encapsulate the dynamics of many aspects of cholesterol metabolism. Such models have included mathematical representations of LDLR dynamics, intracellular cholesterol homeostasis/cholesterol biosynthesis, and whole-body mathematical conceptualizations of cholesterol metabolism. As early as 1985 Chun and colleagues assembled a kinetic model of the recycling of LDLRs in human skin fibroblasts (Chun et al. 1985). This model had significant merit and the general features of the model were able to corroborate experimental work which showed oxycholesterol/hydroxycholesterol down-regulates the synthesis of cell surface receptors, and in this environment, the binding of normal LDL is independent of receptor regulation. An adjunct to the model was it predicted that the degradation of LDL and the simultaneous rise of cholesterol had no clear inhibitory effect on the down-regulation of receptor synthesis. Over a decade later another model which focused on LDLR mediated endocytosis was assembled. This mathematical model was informed by previous kinetic experiments and its utility was validated by the use of labelled [14C] sucrose-LDL in Hep-G2 cells. Using this model as a template the underlying kinetic processes which govern receptor-mediated endocytosis of [14C] sucrose-LDL in Hep-G2 cells were delineated (Harwood Jr and Pellarin 1997).

A further model which centered on cholesterol metabolism was that of Cobbold and colleagues (Cobbold et al. 2002). This model is of significance as it incorporated a key age related process, namely atherosclerosis. This model accounted for the build-up of cholesterol within sub-endothelial cells to form a fatty streak; a process suggested to be the prelude to atherosclerosis. Furthermore, one of the most characteristic features of this model was the inclusion of the underlying rational which is generally thought to account for the accumulation of cholesterol in the artery wall, namely that it is a consequence of the oxidation of LDL-C. A useful feature of this model was its ability to encapsulate the dynamics of LDL oxidation. The utility of the model from an ageing and health-span perspective was underscored by its predictions. For instance, the model was able to predict that a negligible rate of oxidant production could be dramatically increased by a harmful extrinsic event, such as an alteration to diet. Intriguingly this model was used to show that supplementation with ascorbate had a negligible effect on lipid peroxidation. Moreover, the model was able to demonstrate that increasing the flux of HDL particles into the intima resulted in a decrease in oxidant levels. This was an intriguing finding of the model as it offered the possibility of HDL having anti-atherosclerotic properties or perhaps even antioxidant capabilities. Following on from this work August and colleagues in 2007 created a mathematical model which integrated a number of key processes involved in cholesterol metabolism (August et al. 2007). Specifically the model was able to capture the metabolic cascade associated with the removal of cholesterol from the liver. The main findings to emerge from this model centered on the lipoprotein cascade and intracellular cholesterol homeostasis. Firstly, it was discovered that intracellular levels of cholesterol are robust to changes in the parameters which govern the synthesis and regulation of intracellular cholesterol synthesis. Secondly, changes to the parameter values which govern plasma cholesterol levels can result in plasma cholesterol levels varying considerably. Clearly there are important inferences to be drawn from these findings for ageing and healthspan. Firstly, it is possible that plasma cholesterol is not a tightly regulated component of cholesterol metabolism. In addition even though inter-individual LDL-C plasma concentrations are heterogeneous; such population variability is still reconcilable with the tight regulation of cholesterol biosynthesis which takes place in the cell. Thus, from a healthy ageing perspective these are factors which require careful consideration when studying the impact ageing has on cholesterol metabolism. Another model which focused on lipoprotein dynamics was constructed in the 2000s (Hübner et al. 2008). The focus of this model was a population of healthy subjects. This model uncovered heterogeneous lipoprotein distributions within the lipoprotein sub-fractions and predicted changes in lipoprotein distribution as a consequence of disease.

Other models have focused on receptor–ligand binding and trafficking (Shankaran et al. 2007). A key discovery of this research was that avidly is the central controller of the activity of the LDLR. From an ageing perspective this is an important finding because experimental work has shown the dysregulation of the LDLR is age dependent (Segatto et al. 2011). Additional models have targeted the LDLR. For instance, a receptor mediated endocytosis model compared different responses of the receptor

to a bolus of extracellular LDL versus a continuous supply of LDL (Wattis et al. 2008). The model revealed competition between LDL and VLDL particles for binding to cellular surface pits affects the cellular cholesterol levels. Specifically, it was uncovered that when there is a continuous flux of low levels of lipoproteins to the cell surface, more VLDL than LDL occupies the pit, since VLDL are better competitors for receptor binding. Another model which focused on this area was that of Tindall and colleagues who created a mathematical model which represented the uptake of LDL and VLDL in a hepatocyte (Tindall et al. 2009). The mathematical model reviled that the binding, unbinding and internalisation rates, combined with the fraction of receptors recycled and the rate at which the cholesterol dependent free receptors are synthesized are key to the uptake of both LDL and VLDL lipoproteins. The next model of cholesterol metabolism was assembled by Eussen and colleagues (Eussen et al. 2011). This mathematical model was significantly different from the models which proceeded it because it outlined cholesterol metabolism in a more integrated manner. However, its main deficiency was that it excluded many of the key regulatory processes which help to maintain whole body cholesterol balance. Essentially the model was made up of compartments, these included, the liver, blood and extrahepatic tissue. From a healthy ageing perspective the main utility of this model was its ability to predict that the daily consumption of 29 g of plant sterols was able to reduce LDL-C concentration by 8–9%. In a similar fashion van de Pas et al. (2012) created a whole body of cholesterol metabolism, which was developed by modifying a previous mathematical model which was designed based on mice studies. However, up to this point no group had created a whole-body mathematical model of cholesterol metabolism which included the role ageing has to play in the dysregulation of this system.

In 2012 a shift in the trajectory of research in this field occurred when the first whole body mathematical model of cholesterol metabolism was developed which focused specifically on ageing (Mc Auley et al. 2012). This model placed aged-related changes to cholesterol metabolism at its center. The model was validated using the findings from a meta-analysis and experimental literature. It was used to explore how changes to cholesterol metabolism with age impact LDL-C levels. The mathematical model was able to predict that each 10% increase in the rate of cholesterol absorption resulted in a 12.5 mg/dl increase in LDL-C levels. Furthermore, the model predicted a 30% increase in cholesterol absorption between the ages of 20 and 60 years would result in a 34 mg/dl increase in plasma LDL-C. However, the key finding of the model centered on how the age-associated changes to the number of hepatic LDLR impact plasma LDL-C. It was revealed that a drop in hepatic LDLR by 50% by 65 years resulted in an increase in LDL-C. This was a seminal discovery, as it has been observed experimentally that the activity of LDLR decreases with age. Furthermore, this finding resonates with the LDLR models discussed previously in this chapter. Our model was heavily adopted in 2014 to identify a flip response in plasma cholesterol levels due to varying the sensitivity of cholesterol absorption and intestinal bile salt levels (Mishra et al. 2014). Our model was also used in 2015 to demonstrate that 6 weeks of simvastatin therapy reduced LDL-C by 14%, and after 1 year LDL-C reduced by 33% (Paalvast et al. 2015).

According to this group, this result was comparable with experimental findings which showed this drug reduces LDL-C levels by 30–40%.

In 2013 a model of the flux regulation in the cholesterol biosynthesis pathway was created (Watterson et al. 2013). This mathematical model lucidly uncovered that the down-regulation of the enzymes which are the central mediators of this pathway resulted in a tapered drop in the flux along the pathway. Moreover, when the mathematical model was used to replicate pharmacological interventions, this resulted in a concurrent degree of down-regulation in cholesterol synthesis, in a step change manner. It was postulated by the authors that the coordinate regulation of cholesterol biosynthesis displayed a long-term evolutionary advantage over single enzyme regulation. In light of this work we updated our 2012 model to incorporate cholesterol biosynthesis (Morgan et al. 2016c, 2017). Moreover, we included several other regulatory processes which were not part of the first mathematical model. The new version of the model was utilized to show that the parameters associated with the less characterized aspects of cholesterol metabolism are most sensitive to parameter variation. The areas were RCT, lipoprotein dynamics and enterohepatic bile acid circulation. These are aspects of cholesterol metabolism which remain poorly understood mechanistically. The cholesterol biosynthesis component of our new mathematical model behaved in a robust manner. Clearly, this finding resonates with the models discussed previously in this section. In particular, this finding resonates with one of the key findings of the August et al. (2007) model, thus emphasizing the importance of these aspects of cholesterol metabolism for the dysregulation of this system with age.

Closing Remarks

No single gene, molecular mechanism or intracellular biochemical process governs human ageing. Rather, ageing can be thought of as the manifestation of a plethora of biological changes which interact with one another to gradually render us closer to mortality. Invariably our mortality is precipitated by age-related disease. Of the diseases of ageing, CVD places the most significant burden on older people. The sharp rise in CVD during middle age is an example of the declining force of natural selection, thus this phenomenon can be conveniently accounted for when ageing is viewed through the lens of a non-adaptive evolutionary framework. Therefore, many aspects of the mainstream non-adaptive evolutionary theories of ageing can meaningfully account for an increase in the prevalence in CVD with age. This increase has been well recognized demographically, however, what is not fully appreciated and is routinely overlooked is the contribution mechanisms associated with ageing make to the onset of diseases such as CVD. In particular when considering CVD, it is necessary to focus on how ageing impacts cholesterol metabolism. In this chapter we have highlighted the importance of considering the ageing process in a holistic fashion. Moreover, we have emphasized how ageing has the potential to disrupt hepatic cholesterol metabolism. In particular we outlined our hypothesis

which attempts to account for the unusual findings in the oldest old which have shown that in some older people there is an inverse association between LDL-C levels and CVD mortality. As part of our hypothesis we highlighted that ROS have the potential to increase the rate of cholesterol biosynthesis. When this ageing effect is combined with a decrease in ACAT2 activity this could have the potential to lead to increased levels of hepatic free cholesterol and to low levels of plasma LDL-C. Thus, in our opinion it is imperative that future investigations which examine cholesterol metabolism in the oldest old pay careful attention to the association between LDL-C levels and disorders including NAFLD. According to our idea it is conceivable low levels of LDL-C in the oldest old could be used as a proxy indicator of underlying hepatic pathology. Additional work also needs to be carried out into ACAT2 in humans and its possible drop in activity during ageing. Specifically, it would be interesting to find out if the activity of ACAT2 is vulnerable to ROS. This would suggest a mechanism which disrupts it and help to explain why certain middle aged people have normal/high levels of LDL-C which progressively decline with age. In this chapter we also stressed the important role mathematical modelling has to play in representing the interactions between the processes which define ageing and how these interactions ultimately contribute to the onset of disease. We presented a number of mathematical models which have been developed to represent various aspects of cholesterol metabolism. Models of this type have provided a valuable insight into how ageing impacts key biological systems such as cholesterol metabolism. Moreover, they will provide a useful means for investigating ageing and the onset of disease in the future.

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Chapter 5
Key Age-Imposed Signaling Changes That Are Responsible for the Decline of Stem Cell Function

Melod Mehdipour, Yutong Liu, Chao Liu, Binod Kumar, Daehwan Kim, Ranveer Gathwala, and Irina M. Conboy

Abstract This chapter analyzes recent developments in the field of signal transduction of ageing with the focus on the age-imposed changes in TGF-beta/pSmad, Notch, Wnt/beta-catenin, and Jak/Stat networks. Specifically, this chapter delineates how the above-mentioned evolutionary-conserved morphogenic signaling pathways operate in young versus aged mammalian tissues, with insights into how the age-specific broad decline of stem cell function is precipitated by the deregulation of these key cell signaling networks. This chapter also provides perspectives onto the development of defined therapeutic approaches that aim to calibrate intensity of the determinant signal transduction to health-youth, thereby rejuvenating multiple tissues in older people.

Keywords Ageing · Signaling pathways · Altered signaling cascades · Stem cells · Stem cell nice · TGF-β · Smad · Delta/Notch · Wnt · Beta-catenin · Jak/STAT · MAPK · Cell growth · Self-renewal · Differentiation · Inflammation · Tissue injury · Tissue regeneration · Tissue health

The Transforming Growth Factor Beta (TGF-β)

The Transforming Growth Factor Beta (TGF-β) superfamily regulates many key events in normal tissue growth and development, maintenance, and homeostasis (Kingsley 1994). These homodimeric secreted proteins are capable of signaling almost every cell type, are produced by many cells and are broadly responsible for cell proliferation, differentiation, apoptosis, and inflammation in various tissues in adults, and during development, in such evolutionary distinct species as mammals,

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flies and worms (Massagué 1998; Massagué and Chen 2000; Derynck and Zhang 2003). A TGF-β ligand initiates signaling by binding to a Type II receptor serine/threonine kinase, which then dimerizes with a Type I receptor present on the cell surface. Dimerization initiates phosphorylation of the Receptor I kinase domain, which then propagates the signal by phosphorylating pathway-restricted SMAD (a combination of Caenorhabditis elegans SMA and Drosophila MAD) proteins (Heldin et al. 1997; Shi and Massagué 2003). These pSMADs then oligmerize with the common mediator SMAD4 to translocate to the nucleus, where they mediate transcriptional regulation of numerous target genes in response to TGF-β (Heldin et al. 1997). The name of "transforming growth factor" is partially misleading, because TGF-β has pleiotropic effects, and more typically inhibits the proliferation and differentiation of many different cell lines. Additionally, TGF-β family members are morphogenic: they activate and inhibit separate transcriptional targets at different levels (Derynck and Zhang 2003). Here we provide a brief overview of recent studies on the role of TGF-β in modulating cell proliferation and differentiation focusing on its role in controlling the regenerative capacity of adult stem cells and the effects of TGF-β on mammalian tissue ageing.

TGF-β is abundant in the bone where it regulates osteoblasts, and has been reported to exhibit both positive and negative effects on bone (likely depending on the concentration and timing when this morphogen is experimentally altered). Specifically, TGF-β has been suggested to modulate the proliferation rate and functional activity of the bone-forming osteoblasts; and bone loss related to ageing is characterized by diminished osteoblast proliferation, that has been correlated with reduced concentrations of Insulin like Growth Factors (IGFs) and TGF-β (Marie 1997). It is reported that skeletal content of TGF-beta also decreases with age in human bone samples obtained from both men and women, with a net loss of ~25% in TGF-β between the ages of 20–60 years (Nicolas et al. 1994). Additionally, mRNA expression of inhibitory SMADs, SMAD6 and SMAD7, is decreased in mesenchymal stem cells that are derived from old mice, suggesting either a decreased activity of TGF-β/BMP signaling pathway, which positively regulates SMAD6 and SMAD7, or more likely enhanced TGF-beta signaling when negative regulators of this pathway are attenuated (Moerman et al. 2004). Administration of exogenous TGF-beta has been reported to increase osteoblast recruitment and differentiation, arguing against a proliferative effects of TGF-beta1 on these bone forming cells, and leading to enhanced bone formation and decreased bone loss in rodent models of ageing-induced osteopenia (Noda and Camilliere 1989; Joyce et al. 1990; Marie 1997). However, osteoblast-specific overexpression of TGF-β2 in transgenic mice resulted in a progressive bone loss with an osteoporosis-like phenotype, defective bone mineralization, and severe hypoplasia of the clavicles (Erlebacher and Derynck 1996). Furthermore, in some experimental models, TGF-β inhibits osteoblast differentiation by modulating the transcriptional activity of Runx2 (Alliston et al. 2001). Summarily, TGF-beta signaling exhibits multiple positive and negative effects on the bone, which are still being deciphered with respect to the ageing of this tissue.

TGF-β is well known to control inflammation and it is a prototypical immunomodulator that inhibits major histocompatibility complex gene expression (MHCI and MHCII) (Kaminska et al. 2005; Katsimpardi et al. 2014). However, with ageing, the role of TGF-beta1 changes from anti-inflammatory to pro-inflammatory in multiple tissues such as muscle and brain (Yousef et al. 2015; Rebo et al. 2016). Similarly, in the thyroid, TNF-alpha induced strong TGF-β production in aged thyroid cells, but not young, indicating that old thyroid cells become more sensitive to inflammation and produce more TGF-β (Pekary et al. 1995). TGF-β signaling is shown to increase with ageing systemically and in multiple tissues (Carlson et al. 2009b; Yousef et al. 2015). Such an increase seen in brain likely reflects activated microglia, the local myeloid sub-set of cells (Lodge and Sriram 1996; Doyle et al. 2010; Butovsky et al. 2014) for which TGF-β is a major differentiation factor. And, as expected, TGF-beta signaling is also needed for differentiation and function of neurons; accordingly, a lack of TGF-β1 causes late onset motor dysfunction, and defects in glutamate recycling and synaptic plasticity (Lodge and Sriram 1996; Butovsky et al. 2014). TGF-β's role in controlling the diseases of the central nervous system generally depend on the levels of this morphogenic cytokine (similarly to the situation in muscle, bone, etc.). Additionally, the activation of microglia by TGF-beta 1 has been suggested to combat Alzheimer's disease (AD), by inducing phagocytosis of Aβ. For example, an experimental increase in astroglial TGF-β1 production in aged mice reduced the number of parenchymal amyloid plaques and the overall Aβ load in the hippocampus and neocortex, and decreased the number of dystrophic neurites, in association with a strong activation of microglia (Wyss-Coray et al. 2001).

TGF-β is well known to inhibit proliferation of many different cell types in favor of differentiation. Being faithful to its name, however, fibroblast subsets (including the mesenchymal cells) proliferate in response to TGF-beta 1; this causes the known phenotype of tissue fibrosis either with ageing or pathology, or when ectopic TGF-beta1 is administered (Goumans and Mummery 2000; Carlson et al. 2009b; Yousef et al. 2015). Furthermore, subcutaneous administration of TGF-β induces rapid fibrosis and angiogenesis in tissue, and TGF-β expression is correlated with muscle fibrosis in dystrophic patients (Bernasconi et al. 1995). As a result, TGF-β antagonists are used as anti-fibrotic agents, and have been shown to improve the health of various organs and tissues including kidney, skin, lung, brain, joint, and arterial wall (Border and Noble 1994). Accordingly, a topical application of low dose TGF-β accelerates cutaneous wound healing and cartilage repair in old mice, but repeated injection of higher doses induces generalized tissue fibrosis at injection sites in kidney and liver (Beck et al. 1993; Border and Noble 1994; O'Kane and Ferguson 1997; Blaney Davidson et al. 2005).

TGF-β inhibits the proliferation of primary and secondary cultures of epithelial cells, including endothelium, bronchial epithelial cells, mammary cells, and hepatocytes, and in some cases, this inhibition is associated with terminal differentiation (Holley et al. 1983; Masui et al. 1986; Knabbe et al. 1987). The loss of this negative regulation is thought to contribute to cell over-proliferation and tumor development (Markowitz et al. 1995), suggesting that TGF-beta1 is anti-oncogenic. However, an

increase in TGF-beta1 causes epithelial to mesenchymal cell fate transition, promoting cancer metastasis (Cunha and Pietras 2011; Sheen et al. 2013). Hence, once again, specific levels of this multi-functional cytokine reflect health versus pathology.

In skeletal muscle an age-specific increase in TGF-beta1 causes decline in satellite cell proliferation and muscle regeneration (Carlson et al. 2008, 2009b; Conboy et al. 2015; Yousef et al. 2015; Rebo et al. 2016). Failure of muscle regeneration due to excessive TGF-β has been also observed in a mouse model of Duchenne muscular dystrophy (Cohn et al. 2007). However, TGF-β had been also suggested to inhibit differentiation of cultured myoblasts through SMAD3-mediated repression of MyoD, of IGF-II, and reduced IGF-I receptor activation (Liu et al. 2001; Rao and Kadesch 2003; Janzen et al. 2006; Carlson et al. 2009c; Gardner et al. 2011). TGF-beta has been also reported by several groups to antagonize the activation of Notch (an age-specific determinant of satellite cell activation and muscle repair (Beck et al. 1993; Border and Noble 1994; Bernasconi et al. 1995; O'Kane and Ferguson 1997; Conboy and Rando 2002; Conboy et al. 2003; Blaney Davidson et al. 2005). In addition to the direct inhibitory effects of TGF-beta/pSmad on proliferation of satellite cells (via induction of CDK inhibitors) (Carlson et al. 2009a; Yousef et al. 2015), an increase in local and circulating TGF-β1 contribute to the excessive connective tissues and thickened extracellular matrix, making a non-permissive satellite cell niche that inhibits activation or proliferation of these muscle stem cells (Allen and Boxhorn 1989).

Another known activity of TGF-β is the control of cell-cycle regulators, such as induction of CDK inhibitors and Smad3-mediated transcriptional repression of growth-promoting genes: c-Myc and *Id* 1 (Chen et al. 2002; Derynck and Zhang 2003; Kang et al. 2003). In this regard, an age specific evolutionary conserved (in mice and humans) increase in systemic TGF-β1 (and resulting excessive pSmad2, 3 signaling) suppresses regenerative potential in satellite cells and Mv1Lu epithelial cells (Rao and Kadesch 2003; Janzen et al. 2006; Carlson et al. 2009c). Interestingly, delivery of a TGF-β neutralizing antibody *in vivo* fails to reduce the systemic level of TGF-β and rescue myogenesis in old mice, but systemic delivery of a TGF-β receptor inhibitor (Alk5 inhibitor of TGF-beta receptor kinase) improves muscle regeneration (Carlson et al. 2009b). One of the CDK inhibitors that is positively regulated by TGF-bets, $p16^{INK4a}$, accumulates in multiple mammalian tissues with age, and in the bone marrow, age-elevated p16 is responsible for the decline of HSC function (Janzen et al. 2006).

An interesting and controversial TGF-beta superfamily member with respect to effects on ageing tissue health, is GDF11, a circulating factor that is highly homologous to GDF8 (myostatin) (Sinha et al. 2014; Brun and Rudnicki 2015; Egerman et al. 2015). As many other TGF-beta family members, GDF11 is angiogenic (Arthur et al. 2009), and as expected, when added ectopically, it improves the cerebral vasculature with indication to enhance olfactory bulb neurogenesis in old mice (Katsimpardi et al. 2014). On the flip side of this coin, GDF11 has high association with human colon cancer, which could be due to promoting tumor vascularization

(Yokoe et al. 2007; Jakobsson and van Meeteren 2013). GDF11 has been also described as a key rejuvenative systemic factor that is responsible for positive effects of heterochronic parabiosis on old skeletal muscle (Sinha et al. 2014). However, depletion of GDF11 during parabiosis or joining GDF11 knock-out (KO) animals were not studied, and many of the key findings on diminished systemic levels of GDF11 with age or the positive effects of this protein on multiple tissues have been reported difficult to reproduce (Loffredo et al. 2013; Katsimpardi et al. 2014; Sinha et al. 2014; Brun and Rudnicki 2015; Egerman et al. 2015; Zhang et al. 2016; Hammers et al. 2017).

TGFβ and its numerous, yet related ligands initiate the signaling pathway by first binding onto their Type 2 receptors. Type 2 receptors then heterodimerize with Type 1 receptors. Next, the intracellular domain of Type 2 transphosphorylates the Type 1 intracellular domain, thus activating the signaling cascade. Inactive Smads 2 and 3 are phosphorylated by activated receptor, in turn activating Smad2 and Smad 3. Phosphorylated Smad2 and 3 are bound together by the mediating Smad 4, forming a heterotrimer. This complex of Smad proteins is then translocated into the nucleus to induce a great variety of genes that are responsible for cell proliferation, cell growth, inflammation, differentiation, apoptosis, and tissue development. Shown are a few downstream representative phenotypes that change with age and injury in adult tissues, as controlled by the TGFβ pathway (Fig. 5.1). Stem cells in adult tissues exhibit a decline in cell proliferation and tend to express factors that favor differentiation. In the context of injury, each of the presented activities are induced by TGFβ signaling in an effort to patch damaged tissue. TGFβ, on the contrary, can be responsible for increased and chronic tissue inflammation and the decreased

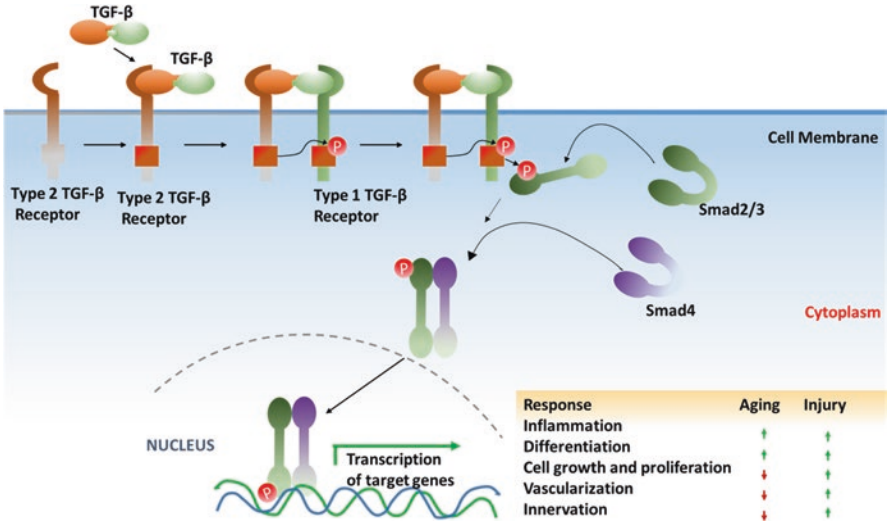

Fig. 5.1 Schematic depiction of TGFβ signaling in a cell and the respective changes on downstream phenotypes during ageing and tissue injury

capability of tissue vascularization (Egerman et al. 2015) and innervation (Macpherson et al. 2015). These functions indicate the vital and dynamic roles of the TGFβ pathway on tissue health and regeneration.

The Notch Signaling Pathway

The Notch signaling pathway is highly conserved in evolution and between prenatal and postnatal organogenesis systems that mediates short-range cell-cell communication. In mammals, in addition to four Notch receptors (Notch 1–4) and five ligands belonging to the Delta-like (Delta-like ligand Dll1, 3, 4) and Jagged (Jagged1,2) family, this network also comprises transcriptional complex components and target genes. In the canonical Notch signaling pathway, Notch receptors are activated by ligands that are located on the surface of adjacent cells, or the same cell can express both Notch receptors and ligands. Upon ligand binding, the Notch receptor is sequentially cleaved by several proteases, resulting in the release of the active form of Notch (the Notch intra-cellular domain, NICD), which translocates to the nucleus and forms a complex with the DNA-binding transcription factor RBP-Jk. This interaction turns transcriptional repression into activation, thereby regulating diverse target genes (Hori et al. 2013).

The canonical Notch pathway signaling has been shown to play a key role in regenerative potential; for example, decreased basal Notch signaling activity has been found in mesenchymal stem cells from geriatric mice, which was characterized by reduced expression of the Notch target genes Hey 1, Hey L, and Hey 2 (Mutyaba et al. 2014). In recent years, a number of studies have shed light on the role of Notch signaling pathway in age-associated disorders, which include but are not limited to the ageing of skeletal muscle, the ageing of the heart and the vascular system, and the ageing of the neural system.

With respect to skeletal muscle ageing, an insufficient expression of Delta, and subsequent lack of Notch activation in satellite cells was identified as the key age-specific determinant of diminished muscle regeneration in the mouse (Conboy et al. 2003). Substantiating the evolutionary conservation of this phenomenon, significantly reduced gene expressions of Notch1, Jagged1, and Delta-like 1 has been documented in skeletal muscle biopsies from an older group (60–75 years old) compared to muscle from a younger group (18–25 years old) (Carey et al. 2007). Interestingly, exposure of satellite cells from old mice to young serum boosted the expression of the Notch ligand Delta, enhanced Notch activation, and increased proliferation *in vitro*, suggesting that the age-specific intensity of cell-cell interactions through Delta/Notch is somehow regulated systemically (Conboy et al. 2005).

In the field of arterial and cardiac ageing, it has been suggested that ischemic stress generates a significantly higher degree of contractile impairment and cellular damage in aged versus young hearts; and this was, in part, correlated with a substantially

modified transcriptome of Notch-target genes in aged versus young hearts (Ashton et al. 2006). Implying that Notch activity is also important in cardiovascular transplantation, during vein graft adaptation to the arterial environment, both Dll-4 and Notch-4 expression were down-regulated in an aged, but not a young, background (Kondo et al. 2010).

With respect to neurogenesis, it is well known that formation of new neurons by neural stem cells continues in the ventricular-subventricular zone (V-SVZ) of the adult forebrain; and a recent study showed that Notch2 deficiency resulted in accelerated exhaustion of neural stem cells in the V-SVZ, promoting an ageing-like phenotype (Engler et al. 2018). Moreover, linking Notch deficiency with a key age-associated brain pathology, an *in vitro* model of AD revealed that ageing was related to a decrease in Notch-1 signaling in the rat hippocampus and cortex. Therefore, dysregulation of Notch signaling might conduce to reduced neurogenesis and damaged hippocampal function during ageing, and this might play a role in AD development and progression (Tanveer et al. 2012).

When it comes to the relationship between Notch and tissue damage, activation of Notch by its ligands is induced in space and time at the site of injury or degeneration, for instance in experimentally injured skeletal muscle, thereby promoting the activation of Notch-expressing adult stem cells for proliferation and tissue repair (Conboy and Rando 2002; Conboy et al. 2003). In a recent study that explored whether Notch1 signaling was in connection with angiogenesis after focal cerebral ischemia in the human brain, it was found that the levels of NICD were elevated in endothelial cells of the peri-infarct region. And similar to the situation in muscle, the Notch1 ligand Jagged1 was also more abundant in the peri-infarct regions of human brain (Ren et al. 2018). Similar results have been seen with a rat spinal cord injury model and in a lung injury model where the expression of Notch1 *in vivo* was enhanced in response to injury (Yamamoto et al. 2001; Yao et al. 2017).

Summarily, Notch signaling pathway plays an important positive role in activating adult tissue stem cells for maintenance and repair of multiple organ systems. Local, injury-triggered induction of Notch ligands and Notch levels give cells instruction on the timing and space of tissue regeneration. An age-specific decline in Notch signaling contributes to degenerative diseases of multiple tissues in mice and people (Fig. 5.2).

Notch receptors are activated by ligands through cell-cell interactions. In mammals, Delta/Jagged ligand binding leads to proteolytic cleavage of the Notch receptor, resulting in the release of the active form of Notch (Notch intra-cellular domain, NICD), which translocates to the nucleus and forms a complex with the DNA-binding transcription factor RBP-Jk, thereby regulating transcription of diverse target genes. Expression of Notch ligands is induced in the damaged tissues, which localizes Notch activation to the stem cells in the vicinity of the injury, where repair is needed. In the ageing state, the expression of Notch ligands is reduced, thus causing a decrease of NICD, and diminished expression of the Notch target genes.

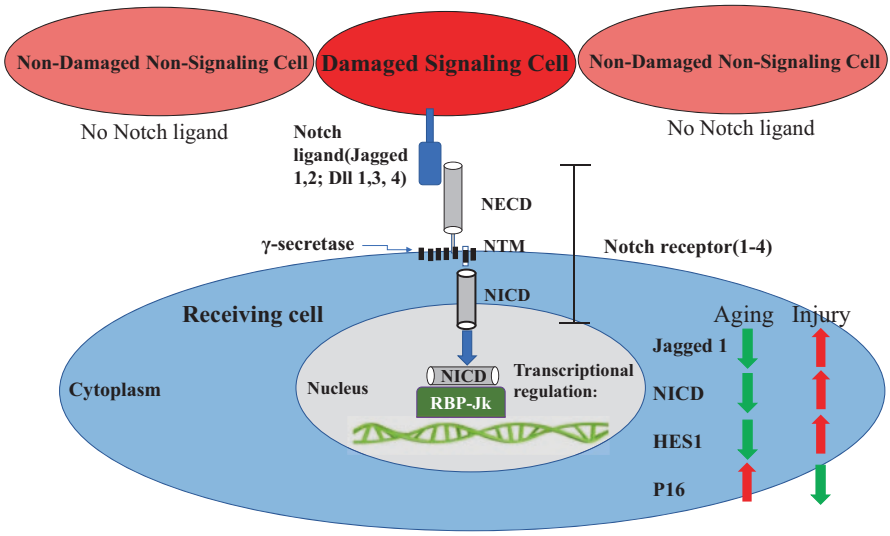

Fig. 5.2 The Notch signaling cascade and its changes during ageing

The Wnt/Beta-Catenin Pathway

Wnt proteins are a family of soluble cysteine-rich glycosylated proteins with conserved signaling pathways. These proteins are pleiotropic, evolutionary and developmentally conserved determinants of a broad range cell functions: proliferation, differentiation, survival, and death (Wodarz and Nusse 1998; Wright et al. 1999; Li et al. 2006). In adult mammalian tissues, the Wnt pathway is involved in stem cell renewal, differentiation and homeostatic maintenance of a number of organs, including skeletal muscle and bone (Chen et al. 2007; Ito et al. 2007; Brack et al. 2008; Carlson et al. 2009b), brain – hippocampal neurogenesis (Zhou 2004; Lie et al. 2005), hair follicle homeostasis and cellular senescence (Ross 2000; Reya et al. 2003; Willert et al. 2003; He et al. 2004; Canalis et al. 2007).

There is some evidence that age-specific elevation in Wnt signaling is involved with the deregulation of muscle and skin homeostasis and repair; at the same time, several lines of research have suggested that Wnt signaling positively regulates stem cell and progenitor cell renewal and that cellular senescence accompanies a decrease in Wnt signaling (Brack et al. 2007; Brack and Rando 2007).

During past years, several studies have suggested that canonical Wnt signaling positively regulates adult stem cell renewal and thereby promotes healthy tissue maintenance (Hoffman et al. 2004; Kuhnert et al. 2004). In agreement with this notion, inhibition of canonical Wnt signaling through adenoviral expression of the Wnt antagonist Dickkopf-1 (DKK1) caused a significant decline of cell proliferation in the small intestine and colon, a loss of regenerating crypts, and eventual architectural degeneration (Hoffman et al. 2004; Kuhnert et al. 2004). In concert

with these observations, experimentally diminished DKK1 expression at later time points rescues crypt and villus regeneration (Hoffman et al. 2004; Kuhnert et al. 2004), demonstrating a reliance on Wnt signaling for productive maintenance of adult intestine and colon.

The dependence on Wnt pathway for homeostatic tissue maintenance is also clear in bone, where DKK1 interferes with fracture repair (Chen et al. 2007); and in the brain, where Wnt/β-catenin is active in the hippocampal neurogenic niche and where an over-expression of Wnt3 increases neurogenesis while blocked of Wnt signaling abolishes neurogenesis, and a mutation in the Wnt signaling co-receptor, LRP6 leads to malformation of the dentate gyrus (Zhou 2004; Lie et al. 2005). Survivin, a known mitotic regulator, is demonstrated to regulate the cell cycle of neural progenitor cells (NPCs) through Wnt signaling. Activation of the canonical Wnt signaling pathway can lead to an increase in survivin protein level and rescue proliferation-deficit NPCs (Miranda et al. 2012).

In addition to the neurodegenerative diseases, a dysfunctional Wnt pathway also promotes heart failure (Soriano et al. 2001; Marambaud et al. 2002; Barandon et al. 2003, 2005; Morin et al. 2004; Li et al. 2006; Wiedau-Pazos et al. 2009). In skeletal muscle, Wnt has pro-myogenic activity and its signaling is indispensable for cellular homeostasis and specifically, for the appropriate differentiation of intermediate progenitors into fusion-competent myoblasts and myotubes (Brack et al. 2008; Carlson et al. 2009b).

With respect to the phenomenon of cellular senescence, in cultured human fibroblasts development of senescence accompanies a decrease in canonical Wnt signaling (decreased Wnt2) and increased GSK-3β, a negative regulator of this pathway, and senescence can be partially reversed by the addition of Wnt3A (Ye et al. 2007). In further support for the senomorphic properties of Wnt, downregulation of its signaling creates an onset of senescence in primary human cells (Ye et al. 2007). Furthermore, sustained over-expression of GSK-3β induces the senescence of human and mouse cells, suggesting that a repressed Wnt signaling cascade may contribute to the permanent cell cycle arrest (Zmijewski and Jope 2004; Kortlever et al. 2006). Overall, these studies support the hypothesis that Wnt signaling antagonizes key attributes of ageing: the reduction of regenerative proliferative responses and increased cell senescence.

While a growing number of studies support the positive role of Wnt signaling in attenuating the ageing process, excessive signaling by this pathway was shown to promote fibrosis of skeletal muscle with a link to systemic nature of age-elevated Wnt (Brack et al. 2007). Similar negative consequences of excessive Wnt have been reported in other organs; for example, Kirstetter et al. have shown that constitutive activation of Wnt through β-catenin over-expression leads to loss of hematopoietic stem cell multi-lineage differentiation and repopulation (Kirstetter et al. 2006). Similarly, studies by Scheller et al. suggest that activation of Wnt forced cell cycle re-entry of hematopoietic stem cells, and thus led to HSC exhaustion in the long term (Scheller et al. 2006). Augmented Wnt signaling was also detected in mouse models of premature ageing, *e.g.* Klotho knockouts, which was suggested to reduce a secreted Wnt antagonist (Liu et al. 2007). Physiologic elevation of Wnt signaling

is implicated in arterial ageing in humans, where the expression of Frizzled 4 (Fzd4), a Wnt receptor, and several targets of the Wnt/β-catenin/TCF pathway become excessive (Marchand et al. 2011).

So does Wnt attenuate or accelerate ageing? The answer is likely in the details of this canonical signaling and the specific studies that assay a specific facet of this pathway. The timing of the experimental perturbations in Wnt and the dosage of agonists/antagonists is likely to produce different results, since Wnt is a morphogen and also has different effects on stem versus progenitor and differentiated cells (*e.g.* during lineage progression). For example, activation of Wnt signaling was shown to promote the expansion and renewal of HSCs, but prolonged over-signaling results in rapid exhaustion of hematopoietic stem cell pool in the long term, resulting in hematologic abnormalities (Reya et al. 2003; Willert et al. 2003; Kirstetter et al. 2006; Scheller et al. 2006). In a similar vein, persistent expression of Wnt1 caused rapid growth of hair follicles initially, but then resulted in epithelial cell senescence, disappearance of the epidermal stem cell compartment, and hair loss (Castilho et al. 2009). And similarly, exogenous Wnt3A inhibits satellite cell activation (Brack et al. 2007), because it inhibits Delta/Notch signaling that is needed for satellite cell proliferation (Conboy and Rando 2002; Brack et al. 2008). However, physiologic Wnt promotes muscle regeneration at later time points after tissue damage, by ensuring the correct differentiation of precursor cells into *de-novo* myofibers (Conboy and Rando 2002; Brack et al. 2008).

To add more complexity, Wnt is highly interactive and has canonical and non-canonical branches that cross-talk with the Notch and TGF-beta pathways in multi-functional networks. The large number and diversity of components that participate in Wnt signal transduction is astounding, which partially explains the diverse view-points on its role in ageing in different studies.

Given the extensive involvement of Wnt signaling with multi-tissue homeostasis and the potential to interfere with ageing (Fig. 5.3), additional studies are clearly warranted to understand the details that make the difference between promoting youth-health versus ageing-disease.

The Jak/STAT Signaling Pathway

The Janus Kinase/Signal Transducers and Activators of Transcription pathway, colloquially known as Jak/STAT, is one of the many pleiotropic signaling pathways that can transduce a great variety of signals in evolutionary distinct animals. Post-natally, Jak/STAT is responsible for induction of the expression and production of a plethora of growth factors and cytokines (Rawlings et al. 2004). During embryonic development and tissue repair, many interactive signaling networks that orchestrate organ formation and homeostasis are maintained by Jak/STAT. As such, Jak/STAT is pleiotropic in its effects on organ systems, including the regulation of immune responses, cell division, apoptosis, and tumor formation. Accordingly, mutations that deregulate the Jak/STAT pathway (inhibit or hyper-activate it) affect multiple

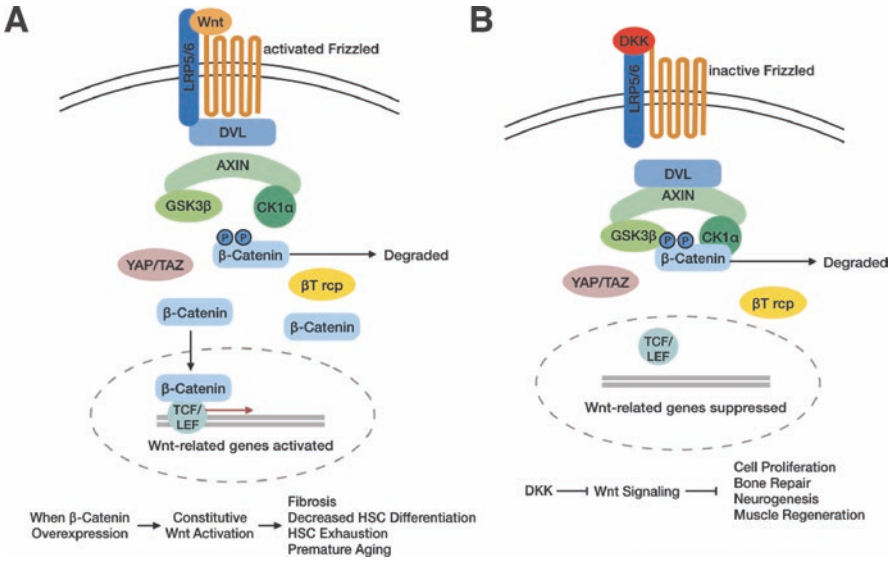

Fig. 5.3 Wnt signaling and its age-imposed changes. (**a**) When Wnt binds and activates Frizzled, the complex made of Axin, APC, GSK3β, CK1α, etc. gets disrupted. This allows unphosphorylated β-catenin to accumulate and localize to the nucleus and induce gene transcription alongside with TCF/LEF. However, despite its regenerative role, excessive or constitutive Wnt signaling leads to negative consequences such as fibrosis, HSC exhaustion, and premature ageing phenotypes. (**b**) In the presence of DKK or age-related decrease of Wnt signaling, canonical Wnt signaling is suppressed, beta-catenin is phosphorylated and gets degraded by proteosomes; therefore, Wnt-related genes in the nucleus are not activated, resulting in a decreased spectrum of regenerative responses

organismal processes (Igaz et al. 2001; O'Shea et al. 2002; Rawlings et al. 2004); with chronic inflammatory disease, flares of leukemia, erythrocytosis, and gigantism, as specific examples of overly activated Jak/STAT.

Jak-STAT signaling cascade plays a major role in cytokine signaling. Some examples of these include IFN-gama, IL-2, IL-4, and IL-10 (Rodig et al. 1998; Smith et al. 2016). The signaling mechanism is ultimately initiated by extracellular soluble cytokines that bind onto specific transmembrane receptors of cells in target tissues (Aaronson and Horvath 2002). Upon ligand activation, the receptors multimerize and subsequently recruit Jak tyrosine kinases to their intracellular SH2 domains (Jatiani et al. 2010). This multimerization brings the Jak proteins into close proximity with one another, thus allowing them to trans-phosphorylate each other. In mammals, there are four members in the Jak family: Jak1, Jak2, Jak3, and Tyk2 (Kisseleva et al. 2002; O'Shea et al. 2002; Heinrich et al. 2003; Rawlings et al. 2004). The phosphorylated, or activated, Jaks in turn bind and phosphorylate additional STAT substrates. Mammals have seven STAT genes: *STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B,* and *STAT6* (Aaronson and Horvath 2002). The diverse STAT proteins allow for tissue-specific responses that vary in intensity and duration. When the pathway is off, STAT proteins are inactive transcription factors

that reside in the cytosol. Activated Jaks bind and phosphorylate these STATs, which when activated by Jaks, homo- or heterodimerize and translocate into the nucleus to induce the expression of target genes. The negative feed-back in this pathway is comprised of JAK and STAT phosphatases called suppressor of cytokine signaling (SOCS) (Croker et al. 2008; Tamiya et al. 2011; Price et al. 2014).

The Jak/STAT pathway is interactive with other signaling cascades, including but not limited to the PI3K/Akt and MAPK/Erk pathways (Jain et al. 1998; Rawlings et al. 2004). For example, PI3K proteins can bind to SH2 domains of activated cytokine receptors; and Jak/STAT can cross over into the MAPK/Erk pathway through a mediator Grb2, thereby activating SOS and the MAPK/ERK pathway (Rawlings et al. 2004). Interestingly, MAPK can phosphorylate STAT proteins, providing the positive feed-back to the Jak/STAT signaling (Rawlings et al. 2004). Other notable examples of Jak/STAT cross talk include the integration of signaling following the activation of MAPK and AKT by inflammatory cytokines such as TNF-alpha, Toll-like receptors, and ITAM associated receptors (Ivashkiv 2012).

The Jak/STAT is the signaling determinant in various tissues in fruit flies and mammals, particularly during organogenesis and regeneration, including the neural stem cell and myogenic stem cell niches (Miller and Gauthier 2007; Jang and Baik 2013; Stine and Matunis 2013; Doles and Olwin 2014).

In the brain's stem cell niche, the Jak/STAT pathway is highly active during development, but the expression of JAK1, JAK2, STAT1, STAT3, and STAT6 decrease in adulthood (Claudio et al. 1998; Nicolas et al. 2013). In the young adult brain, Interleukin-15 is highly expressed in the subventricular zone (SVZ). This activates STAT's 1, 3, & 5, and increases the proliferation of these neural stem cells (NSC) (Sylvian 2009; Gómez-Nicola et al. 2011; Nicolas et al. 2013). Leptin, which is known to decline with age, (Garza et al. 2008) can activate proliferation in the dentate gyrus (DG) of adult mice through the activation of STAT3 and Akt (Garza et al. 2008). Different branches of the pathway control different cell-fates: JAK1 appears to be chiefly involved with astrocyte differentiation (Nicolas et al. 2013), JAK2 modulates NSC proliferation (Garza et al. 2008; Hee et al. 2010) and JAK3 signaling appears to be involved with differentiating NSC's into oligodendrocytes and neurons (Hee et al. 2010). Additionally, the Jak/STAT pathway plays a crucial role in synaptic plasticity and the various AMPA and NMDA receptors associated with it (Mahmoud and Grover 2006; Nicolas et al. 2013).

Jak/STAT signaling plays a crucial role in skeletal muscle stem cell self-renewal, which becomes altered with age (Dumont et al. 2015; Rebo et al. 2016). JAK1 and STAT's 1&3 promote proliferation of myoblasts, while preventing premature differentiation (Sun et al. 2007), as demonstrated by the upregulation of JAK1-STAT1-STAT3 *in vivo* after experimental muscle injury (Jang and Baik 2013). And, a block of myogenic differentiation is seen when the JAK1 and STAT 1&3 pathways are activated by oncostatin M (Xiao et al. 2010). The timing of Jak/STAT singling is important: LIF induction of JAK2 and STAT3 contributes to satellite cell proliferation (Spangenburg and Booth 2002), while later on, the activated JAK2 and STAT's 2&3 promote skeletal muscle differentiation (Wang et al. 2008). It was observed that the pro-differentiation activity of this pathway is partially mediated by MyoD

and MEF2 (Wang et al. 2008; Jang and Baik 2013). The Akt and ERK pathways can also contribute to muscle stem cell differentiation by inhibiting JAK3, which induces the expression of pro-differentiation markers including MGN (early marker), and MHC (late marker) (Jang et al. 2012).

Jak/STAT signaling changes with age, which profoundly influences the decline of stem cell function in muscle and brain. For example, there is an increase of Jak/STAT signaling in aged muscles, which contributes to diminished regeneration (Price et al. 2014). The Jak/STAT signaling intensity elevated with age, that negatively impacts myogenesis and neurogenesis, results from the chronic tissue inflammation that increases with age (Chazaud and Mouchiroud 2018). In this regard, STAT3 has been closely associated with mediating the pro-inflammatory signals, shifting the balance of muscle repair from stem cell proliferation towards differentiation under chronic inflammatory conditions (Price et al. 2014; Tierney et al. 2014; Chazaud and Mouchiroud 2018). In the brain, the negative effects of long term potentiation are brought about by an imbalance in pro-inflammatory cytokine expression, and the likely consequential deregulation of Jak/STAT (Nicolas et al. 2013). Not surprisingly, Jak/STAT modifiers are emerging as novel therapeutics in an effort to quell many age-related or inflammatory-related pathologies in muscle and brain (Price et al. 2014; Qin et al. 2016).

Jak/STAT extensively integrates into inflammatory signaling (Ivashkiv 2012; Banerjee et al. 2017) and is triggered by the secretome of senescent cells; both of these events, chronic inflammation and accumulation of senescence, become elevated with age (Xu et al. 2016). Senescent cells secrete a plethora of pro-inflammatory cytokines and growth factors known as the senescence-associated secretory phenotype (SASP) (Kuilman et al. 2008; Coppé et al. 2008). SASP and the chronic inflammatory state that is typical for aged mammals, continuously simulate the Jak/STAT cascade, causing such deleterious organism-wide phenotypes as adipose tissue dysfunction, frailty, and poor metabolic dysfunction (Yu et al. 2009; Meyer and Levine 2014) (Fig. 5.4).

The MAPK/ERK Signaling Pathway

The mitogen activated protein kinases (MAPK) signal transduction pathway is found in all eukaryotes and senses external stimuli such as growth hormones, cytokines and stress. The function of MAPK is attributed to the presence of distinct kinase modules that relay signal via phosphorylation and dephosphorylation (Chang and Karin 2001). The modules involved in the MAPK signaling cascade are MAPKs, MAPKKs (MAPK kinase) and MAPKKKs (MAPKK kinase) which function with the help of several other intermediary proteins. MAPK is phosphorylated by MAPK kinase which in turn is phosphorylated by MAPKKK. The activation of the MAPKKKs is achieved by phosphorylation by the MAPK kinase or by the protein kinase C (with the G-Protein Coupled Receptor, GPCR). MAPKs belong to the

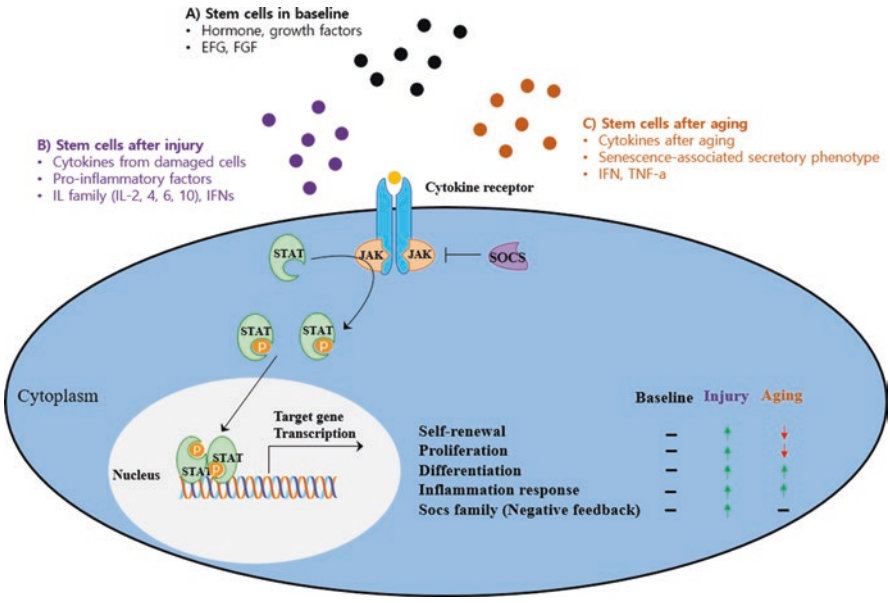

Fig. 5.4 Schematic representation of the Jak/STAT signaling pathway and its relative change of activity under baseline, injury, and ageing conditions. (**a**) Adult stem cells (ASC's) under baseline conditions generally maintain their quiescence by balancing proliferation and differentiation signals. These signals are mainly brought about by hormones and growth factors (Jiang et al. 2009). Single, upward-pointing green arrows under "Baseline" qualitatively denote baseline downstream activities of Jak/STAT signaling, as illustrated. (**b**) Upon tissue injury, ASC's receive various cytokine signals from damaged cells or infiltrating leukocytes (Mohri et al. 2012; Doles and Olwin 2014). These signals relay through the Jak/STAT pathway to activate quiescent ASC's prompting them to rapidly proliferate. Over time, Jak/STAT plays a role in attenuating proliferation and induces differentiation & self-renewal. The ASC derived differentiated cells replace damaged tissue and Jak/STAT signaling reverts back to its baseline state in quiescent resident stem cells. These are reflected by the increased numbers of green arrows under "Injury". (**c**) During the ageing process, stem cells could also be affected by inflammatory cytokines, including those secreted from senescent cells. The activation the Jak/STAT pathway in this manner seems to bolster differentiation and inhibit proliferation of stem cells to an extent that is far greater than baseline (indicated by green and red arrows respectively). The self-renewal capability of Jak/STAT is also hampered with advanced ageing and this consequently results in a decline of regenerative capacity

serine/threonine protein kinases and consist of three major groups, the extracellular signal-regulated kinase (ERK), p38 and the c-Jun NH_2-terminal kinases(Schaeffer and Weber 1999).

Different groups of MAPKs function in stem cell maintenance, proliferation, asymmetric division and differentiation.

The age-specific changes in the MAPK pathway have been well explored in skeletal muscle, which the conclusion that the regenerative capacity of the old muscle cell diminishes with ageing as a consequence of decreased mitogen-activated protein kinase (MAPK) pathway signaling (Conboy and Rando 2005, 2012; Carlson et al.

2009c; Yousef et al. 2013). The p38 represents the MAPK branch that is best known for transducing cellular stress signals, and it can be also activated by cytokines, growth factors and cell to cell contact (Segalés et al. 2016). p38 has four members: MAPK14 (p38α), MAPK11 (p38β), MAPK12 (p38γ), and MAPK13 (p38δ). p38 MAPK has been shown to have pleiotropic roles in myogenesis: breaking stem cell quiescence, asymmetric division and differentiation (Jones et al. 2001; Chakkalakal et al. 2012; Troy et al. 2012). p38 is generally considered to promote cell differentiation by down-regulating cyclin D through inhibitory effects of p38 on JNK. Namely, p38α down-regulates JNK pathway through induction of MKP-1 – a JNK phosphatase, thus inhibiting cell proliferation in favor of differentiation (Perdiguero et al. 2007). In concert with its morphogenic nature, p38 isoforms can also inhibit differentiation: treatment with recombinant p38αβ antagonizes myoblast fusion into differentiated myotubes (Lluís et al. 2006); and p38γ has been shown to prevent myogenic differentiation through phosphorylation of the MyoD transcriptional factor, so that it represses myogenin expression (Gillespie et al. 2009; Segalés et al. 2016).

Interestingly, several studies point towards not only positive but also negative roles of MAPK in myogenesis, which is likely due to the pleiotropic nature of this pathway and different roles that it plays at different stages of tissue maintenance and repair (Milasincic et al. 1996; Weyman et al. 1997; Gredinger et al. 1998; Kastner et al. 2000; Jones et al. 2001).

Abnormal activation of the p38 MAPK pathway interferes with asymmetric muscle stem cell division, resulting in reduced self-renewal and regenerative potential. Neutralizing the over-expression of p38α/β MAPK with specific inhibitors in a hydrogel setting provided for improved stem cell engraftment and muscle regeneration in the old (Williamson et al. 2003; Segalés et al. 2016).

In addition to its pivotal role in maintenance and regeneration of skeletal muscle, MAPK signaling regulates homeostasis of many other tissues, including brain, liver and bone. For instance, this pathway affects neuronal properties, including structural characteristics such as spine density, synaptic plasticity, and neuroinflammation (Zhen et al. 1999; Jeanneteau and Deinhardt 2011; Maphis et al. 2016). The p38 mitogen-activated protein kinase is responsible for transducing inflammatory signals in the brain and for initiating cell apoptosis. In AD, increased levels of the phosphorylated p38 were found to be associated with neuritic plaques, neuropil threads, and neurofibrillary tangle-bearing neurons (Hensley et al. 1999). Additionally, hyper-phosphorylation and aggregation of tau protein, tied with AD, was ameliorated upon oral administration of an isoform-selective, brain-permeable small molecule inhibitor of p38α MAPK (Hensley et al. 1999; Hamanoue et al. 2016). Expression of the *mkp-1* (the phosphatase that antagonizes MAPK) directly correlates with the levels of BDNF and with the increase in the post-BDNF excitatory neuronal activity (Thouverey and Caverzasio 2015).

In the liver, mitogen-activated protein kinases, such as the c-Jun N-terminal kinase (JNK) and p38, play significant roles in regulating metabolism. The activity of pERK is up-regulated in livers of obese mice, as compared to lean animals. Activation of pERK in the livers of lean mice leads to decreased metabolic rate, lower expression of genes involved in fatty acid oxidation, and an increase in fasting

hyperglycemia that accompanies systemic insulin resistance (Jiao et al. 2013; Xiao et al. 2017). Hepatic p38 MAPK antagonizes the c-Jun N-terminal kinase (JNK) to down-regulate fatty liver disease (hepatic steatosis) by maintaining hepatic bile acid synthesis and fatty acid oxidation (Jiao et al. 2013).

The p38 MAPK signaling pathway is crucial for skeletal development and bone homeostasis. Mutations in genes affecting this pathway result in developmental bone disorders, such as chondrodysplasia, cleidocranail dysplasia, or faciogenetical dysaplsia (Greenblatt et al. 2010; Thouverey and Caverzasio 2015). In concert with its stress-transducing role, p38α is abundant in the bone afflicted by osteoporosis and inflammatory osteolysis (Greenblatt et al. 2010). The mechanisms of MAPK function in the bone are many and include phosphate inhibition, scaffold protein production, and induction of autophagy.

MAPK is highly interactive with other key cell-signaling pathways (as described with respect to Jak/STAT above). And, MAPK is known to induce Notch activation in evolutionary distinct species through the up-regulation of Notch ligands. In fact, one reason for diminished muscle regeneration with age is the lack of MAPK/ERK induction of Delta in the aged skeletal muscle (Conboy and Rando 2002; Conboy et al. 2003; Elabd et al. 2014). While there are likely many physiological ligands of MAPK in mammals, one age-specific factor is oxytocin which declines in older mice and humans and signals via MAPK to maintain health of muscle and bone, as well as healthy metabolism and mental well-being (Elabd et al. 2014). In bone, the MAPK pathway synergizes with TGF-beta-activated kinase 1 (TAK1; MAP 3 K7) as one of the primary activators upstream of p38 in osteoblasts. Deletion of the TAK1 gene results in clavicular hypoplasia and delayed fontanelle fusion, leading to under-developed bones and teeth. This phenotype, cleidocreanial dystosis (CCD), is observed in humans with haploinsufficiency for the transcription factor runt-related transcription factor 2 (Ruxn2) (Greenblatt et al. 2010).

In summary, MAP kinase is an important pathway that senses external stimuli such as stress, growth factors and cytokines and allows cells of multiple tissues to adapt to the changes in their complex multi-scale environments. MAPK is pleiotropic and it controls the stem cell quiescence, expansion of intermediate precursor cells, asymmetric cell-cycling, and terminal differentiation. With age, MAPK generally declines, which perturbs tissue homeostasis and repair by dedicated stem cells (both directly and through skewing of other signaling networks, which interact with MAPK, such as Notch and Jak/STAT, for instance). At the same time, in the chronic inflammatory states (typical of aged tissues), the p38 signaling branch of MAPK becomes excessive, which also diminishes tissue homeostasis and repair.

Figure 5.5 shows the four groups of the MAPK subfamily: (ERK), p38 MAPK, Jun N-terminal kinases (JNK) and ERK5. An external signal in the form of growth factors, cytokines or stress is sensed by the receptors such as GPCR, RTK, cytokine receptors and transmitted to the upstream elements (*e.g.* PKC and RAS). Further signaling occurs *via* a series of phosphorylation and dephosphorylation events that are carried out by MKKK, MKK and MAPK modules. The activated ERK, p38 MAPK and Jun N-terminal kinases (JNK) activate transcription factors locate inside the nucleus and bring about the desired response.

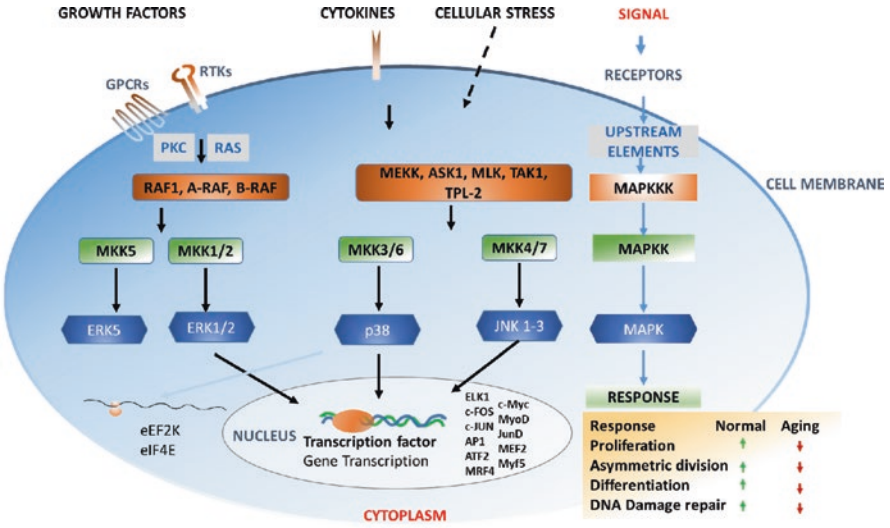

Fig. 5.5 Figure showing an overview of the Mitogen activated protein kinase (MAPK) signaling pathway

Authors' Contributions Melod Mehdipour edited and referenced the chapter and contributed to the Jak/Stat sub-chapter, Yutong Liu contributed to the TGF-beta and Wnt sub-chapters, Chao Liu contributed to the Notch sub-chapter, Daehwan Kim contributed the Jak/Stat sub-chapter, Binod Kumar and Ranveer Gathwala contributed to the MAPK sub-chapter, Irina Conboy planed and directed the chapter composition.

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Chapter 6
Creatine, Creatine Kinase, and Aging

Nathalie Sumien, Ritu A. Shetty, and Eric B. Gonzales

Abstract With an ever aging population, identifying interventions that can alleviate age-related functional declines has become increasingly important. Dietary supplements have taken center stage based on various health claims and have become a multi-million dollar business. One such supplement is creatine, a major contributor to normal cellular physiology. Creatine, an energy source that can be endogenously synthesized or obtained through diet and supplement, is involved primarily in cellular metabolism via ATP replenishment. The goal of this chapter is to summarize how creatine and its associated enzyme, creatine kinase, act under normal physiological conditions, and how altered levels of either may lead to detrimental functional outcomes. Furthermore, we will focus on the effect of aging on the creatine system and how supplementation may affect the aging process and perhaps reverse it.

Keywords Creatine · Creatine kinase · Aging · Supplementation · Muscle function · Motor and cognitive function · Anti-aging intervention

Creatine

Aging is associated with declines in motor and cognitive functions. Humans have always been searching for a "fountain of youth", and dietary supplements have been at the center of this search. One supplement that has garnered some support and is the focus of this chapter is creatine. Creatine, a derivative of the guanidinium cation

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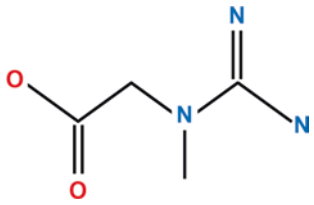

Fig. 6.1 Creatine molecule. Oxygen atoms of the carboxylic acid group are red and the nitrogen atoms from the guanidine group are blue. Hydrogens have been omitted for clarity

involved in a variety of cellular functions, specifically those involved in cellular energy, was discovered by the French organic chemist Michel Eugene Chevreul (Fig. 6.1) (Chevreul 1835). Chevreul found creatine as an isolate from meat and derived the name from *kreas*, the Greek word for "meat." In 1847, German chemist Justus von Liebig replicated Chevreul's work and isolated crystalline creatine (Liebig 1847). Both scientists identified muscle as being the major reservoir for creatine.

Creatine is considered a non-essential nutrient that can be synthesized or obtained from a normal diet. The mammalian body produces the molecule in the liver, kidney, and pancreas with the amino acids arginine, glycine, and methionine, as the foundation for its biosynthesis (Brosnan et al. 2011). Arginine can be classified as a semi-essential or essential amino acid, where the distinction is dependent on the developmental stage of the subject. Pre-term infants are unable to synthesize arginine sufficiently due to paucity of arginine synthesis enzymes in the intestine, which can ultimately affect creatine concentrations. (Wu et al. 2004). Glycine is an amino acid that is considered essential in the human diet as the levels may vary in pre-term infants. This simple amino acid serves as a neurotransmitter for inhibitory chloride ion channels in the spinal cord (Graham et al. 1967), a co-agonist neurotransmitter that enhances the activity of excitatory glutamate channels in the brain (Henneberger et al. 2013), and as a precursor to creatine (Bloch and Schoenheimer 1941). Glycine is produced when serine hydroxymethyl transferase removes a methyl group from serine for placement on a tetrahydrofolate molecule. This reaction can occur in a variety of tissues and organisms as discussed in Hatefi et al. (1957). Furthermore, arginine and glycine come together and through the action of L-arginine:glycine amidinotransferase (AGAT), guanidinoacetic acid, an immediate precursor to creatine is formed and transported to the liver for further enzymatic reaction. In the liver, guanidinoacetic acid reacts with S-adenosylmethionine (product of methionine via a reaction by the methionine adenosyl transferase enzyme) to form creatine (Fig. 6.2) (Bera et al. 2008).

From the liver, creatine is transported into the bloodstream and is distributed throughout the body. Up to 95% of creatine is stored in skeletal muscle, while the remaining 5% can be found in other tissues, including the brain, testes, kidneys, and liver (Walker 1979). Under normal conditions, creatine is synthesized constantly to meet the energy demands of cells, as it is rapidly used in ATP production. According to a study by Hoberman et al., the human body loses 2 g of creatine per day due to normal degradation (e.g., molecular and cellular breakdown) (Hoberman et al. 1948). To recover this loss each day, creatine must either be synthesized de novo or obtained from exogenous sources, such as diet or supplementation. Following creatine intake and absorption, it enters the cells via a cell specific 'symporter', the

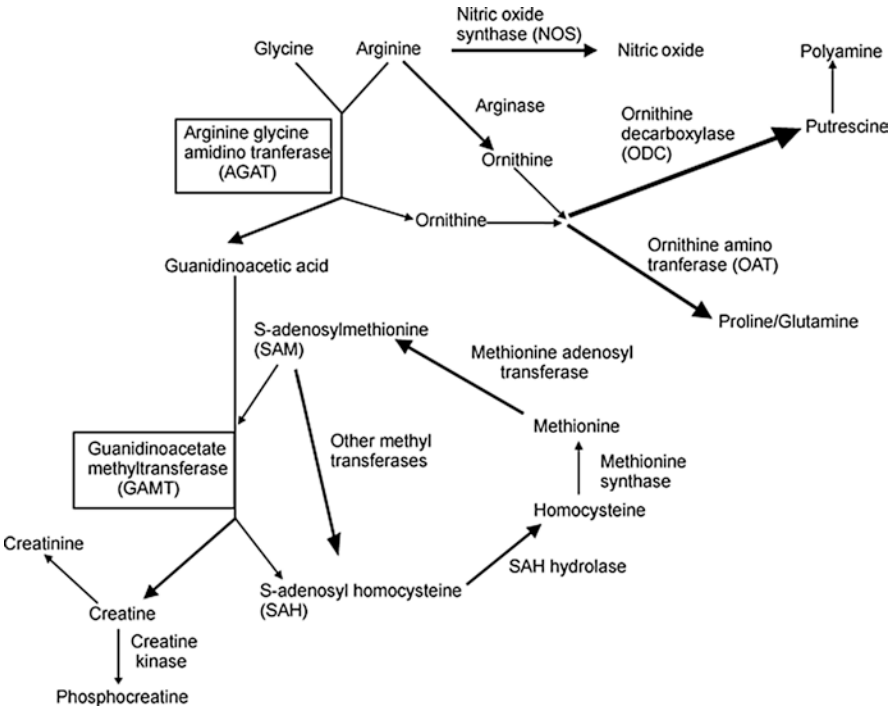

Fig. 6.2 Diagram of creatine and creatine interaction in other biosynthetic pathways. Schematic used with permission from Bera et al. (2008)

sodium- and chloride-dependent creatine transporter 1, a 635 amino acid protein encoded by the SLC6A8 gene (Nash et al. 1994). This protein is comprised of 12 transmembrane domains, with domains 1, 3, 6, and 8 contributing to the transport pathway. Creatine is transported into the cell along with chloride utilizing the sodium gradient. The transporter is part of the neurotransmitter sodium symporter family, which include the ɣ-aminobutyric acid, serotonin, norepinephrine, and dopamine transporters (Gozzo et al. 1993). The first image of the 3-dimensional protein structure was determined when a bacterial homologue to these proteins was used to form X-ray diffracting crystals to yield a high resolution data set. The *Aquifex aeolicus* leucine transporter, or LeuTAa, provided the crystallographic data to show the arrangement of the transmembrane domains as well as the active transport center of the protein (Yamashita et al. 2005).

Although creatine is endogenously synthesized, it can be obtained through exogenous means, such as a normal diet or supplementation. Beef and fish are abundant sources of creatine. One question that arises from the supplementation of the body's own mechanism for obtaining creatine is how is exogenous creatine absorbed? Creatine may be absorbed within the gastrointestinal tract. While the exact mechanism of creatine absorption in the gut remains unclear, positive indication of the creatine transporter's presence has been observed in the intestine, suggesting that dietary creatine supplementation may be absorbed here (Nash et al. 1994).

Peral et al. (2002) narrowed down the location of these transporters to the small intestine, where active creatine transporters were found.

Movement of creatine into cells depends significantly on the expression of the creatine transporter. The creatine transporter was first cloned from rabbit brain and shown to be expressed in all tissues but the liver and intestine (Guimbal and Kilimann 1993). In this first study, the highest levels of creatine transporter mRNA were found in the heart, muscle, and kidney. Researchers cloned the human creatine transporter with similar organ expression (Gonzalez and Uhl 1994; Sora et al. 1994). It stands to reason that, to have successful absorption of creatine, there must be adequate levels of creatine transporter present. However, a study by Guerrero-Ontiveros and Wallimann suggested that the creatine transporter expression can be reduced by chronic intake of creatine (Guerrero-Ontiveros and Wallimann 1998). The investigators in the aforementioned study, administered dietary creatine (diet containing 4% creatine and 50 mM creatine in the water supply) for 3–6 months to rats and observed a reduction in creatine transporter expression in rat quadriceps muscles.

As a source of energy for cellular activities, creatine plays a vital role in early development and good health in humans. However, there are genetic disorders that can disrupt creatine synthesis, absorption, and distribution in the body. These are termed "creatine deficiency syndromes" and individuals afflicted by these syndromes have low cellular levels of creatine. Each of the deficiencies discussed below have a common outcome, but vary in the path to creatine deficiency. These deficiencies involved enzymes that lead to the synthesis of creatine at critical reactions or the protein transporting synthesized creatine into cells.

Creatine Deficiencies

Creatine Transporter Defect

As described above, the creatine transporter moves creatine into a cell against the creatine concentration gradient. This is achieved through co-transport with sodium and chloride ions. When working normally, the creatine transporter maintains creatine levels in cells, including brain and muscle tissue. The creatine transporter gene, SLC6A8 gene, is located on chromosome Xq28, at the tip of the X chromosome (Salomons et al. 2001). To understand the role of this gene, the investigators studied a young boy who was experiencing diminished maturity in speech and language abilities. Upon examination with magnetic resonance spectroscopy, it was determined that the levels of creatine in the child's brain was undetectable. Furthermore, the patient's creatine transporter gene had a single nucleotide substitution, a cytosine to thymine (Cecil et al. 2001). Ultimately, the expressed creatine transporter protein had 122 fewer amino acids than the wild type transporter that has 625 amino acids. In an effort to slow the progression of the disease and increase the creatine levels in the brain, the patient was placed on an oral creatine

supplementation (creatine monohydrate in solution) regimen of 2.5 g creatine, administered three times a day. Nonetheless, the supplementation failed to increase the levels of creatine in brain and was therefore discontinued.

Arginine:Glycine Amidinotransferase Defect

There are several critical enzymatic steps in the pathway to creatine synthesis. One of these steps in this creatine pathway is the arginine: glycine amidinotransferase (AGAT). The AGAT enzyme reacts with arginine and glycine to form the products ornithine and guanidinoacetate, the immediate precursor to creatine. Patients with a deficiency in the AGAT enzyme presented with significantly low concentrations of creatine or phosphocreatine, as seen in the brain of female siblings (Item et al. 2001). These patients were observed to have reduced mental capacity (mental retardation) and low levels of guanidinoacetate in their urine. The AGAT enzyme in these patients had a single point mutation in the gene (mapped to chromosome 15q11.2), a substitution of guanine to adenosine, which introduced a stop codon in place of a tryptophan codon. This mutation resulted in expression of a gene that encodes a truncated (shortened) and non-functioning enzyme. The AGAT deficiency is recessive, requiring two copies of the mutant gene to exhibit the phenotype. The truncated AGAT protein was incapable of catalyzing arginine and glycine substrates to guanidinoacetate due to the absence of the enzyme's active-site. The younger brother of the aforementioned sisters had the same mutation but had normal development by 18 months of age due to early intervention (Battini et al. 2006). In this case the newborn patient received creatine supplementation via two methods: the mother's breast milk followed by direct supplementation. The authors point out that creatine supplementation of the mother's milk was used due to a lack of toxicology data for direct creatine supplementation to a newborn patient. The mother's diet was supplemented with creatine to achieve this goal, and this supplementation led to an intake range from 3 to 9 g of creatine per day. At 4 months of age, the patient received a direct supplementation of 100 mg/kg/day of creatine for the remainder of the time observed (age 18 months). During this time, plasma and urine creatine levels increased and remained elevated. Of note, the urine creatine levels of the patient were significantly higher than the normal control range (Battini et al. 2006). Following supplementation it was reported that, remarkably, the patient's cognitive development remained normal throughout periodic observation until age 18 months.

Guanidinoacetate Methyltransferase (GAMT) Deficiency

Guanidinoacetate methytransferase (GAMT), also known as *S*-adenosyl-L-methionine:N-guanidinoacetate methyltransferase, takes a methyl group from *S*-adenosyl-L-methionine and adds it to guanidinoacetate. From this reaction, guanidinoacetate becomes the key reactant in the synthesis of creatine. Should the

GAMT enzyme not function properly, no creatine is synthesized and guanidinoacetate accumulates within the body. This enzymatic dysfunction was reported in a patient who had low creatine levels and an extrapyramidal movement disorder (Stockler et al. 1994). Despite a normal birth, the 5 year old patient could not perform normal basic functions like roll over, sit, and swallow. Upon study with phosphorous magnetic resonance spectroscopy and proton spectroscopy, the patient had no detectable levels of creatine or phosphocreatine. Arginine supplementation failed to yield improvement in function, but creatine supplementation at 400 mg/kg/d in the absence of arginine resulted in an improvement in the male patient's condition. The patient was "more alert, followed with his eyes, grasped and moved small toys…and began to turn over and crawl" (Stockler et al. 1994).

GAMT enzyme malfunction has been associated with mutations within the gene. The gene was first cloned in 1995 (Isbrandt and von Figura 1995) and later found to map with human chromosome 19p 13.3 (Chae et al. 1998). The patient exhibited seizures, low muscle tone, and developmental delay. Similarly to the male patient described in the previous paragraph, the GAMT deficiency was also observed in an unrelated female patient exhibiting seizures, low muscle tone, and developmental delay (Stockler et al. 1996). Liver samples were taken from the previously described male and female patients with GAMT deficiency and subjected to DNA sequencing. The male patient's GAMT gene DNA sequence showed mutations. There were four identified transcripts of the GAMT enzyme in the male patient: an insertion of 13 nucleotides following position 309 in the GAMT gene, a guanine to adenosine mutation at position 327 followed by an insertion of 44 nucleotides, a transcript that combined these two mutations/insertions, and deletion of 146 nucleotides following position 181. These combinations of transcripts reflect the results of splice variants of the same mutant gene. The father was heterozygous with copies of the wild type and position 327 guanine to adenosine and 44 nucleotide insertion alleles while the mother was heterozygous for the wild type and the insertion of 13 nucleotides following position 309 alleles. The patient's brother was heterozygous with the wild type and the position 309 insertion of 13 nucleotides mutant alleles. The unrelated female patient was homozygous with the position 327 G to A mutation with insertion of 44 nucleotides in the GAMT gene along with the insertion of the 146 nucleotide deletion. As compared to the unrelated male patient, the female patient was homozygous this mutant gene. Further analysis showed that the 327 G to A mutation leads to the deletion of 146 nucleotides following position 181.

In 2014, Stockler-Ipsiroglu et al. reported on the collected data for 48 patients with GAMT deficiency (Stockler-Ipsiroglu et al. 2014). Of the 48 patients, 30 patients had increased cerebral creatine levels following creatine supplementation (ranging from 300–800 mg/kg). The effects of creatine supplementation appeared to reduce symptoms associated with epilepsy, movement disorders, and increased brain creatine levels. While creatine supplementation alone failed to reverse intellectual disabilities, combinatorial therapy, such as including L-ornithine with creatine supplementation, showed reductions of guanidinoacetate and improvements in one patient, in the areas of social skills and attention. Interestingly, those patients that had mutant GAMT gene who began treatment at prenatal, 1 week, 3 weeks, and 9 months of age were observed to be either normal (prenatal, 1 week, and 3 week

old subjects) or borderline (9 month old) in measures of developmental delay and intellectual disability. Each of these patients were treated with creatine supplementation, a high-dose of ornithine, and a low protein diet. After months of treatment, three of these patients remained normal while the 9 month old was near normal. One could characterize these as prophylactic creatine supplementation, as the more severe deficits had not been observed in these patients at the time of the study. This may indicate that early and consistent creatine intervention is needed to avoid developmental delay. However, patients who began treatment at age 10 and 11 months showed mild and moderate symptoms of the deficiency. Based on the available data, an early intervention has potential in maintaining near normal behavior in patients with GAMT deficiency.

Creatine Supplementation in Healthy Young Individuals

Dietary supplementation was projected to be a $14 billion industry in 2000 (Zeisel 1999). Consumer surveys on dietary supplementation revealed that most individuals take supplements to either improve or maintain their health (Bailey et al. 2013). Dietary supplements consist of vitamins, minerals, and other over the counter products that may not face scrutiny for efficacy and safety from the Food and Drug Administration. In spite of a lack of regulation, patients continue to complement their diets with these supplements, and they are often consumed without the guidance of their primary care physician.

Creatine supplementation has shown therapeutic efficacy in cases of creatine deficiency, as described above in the case of patients with low to no detectable creatine levels. While replacing creatine reversed some of the effects of movement disorders and slowed the decline in cognitive abilities, it did not affect established pathologies. Creatine supplementation maintained the current condition of the patient at the time of treatment onset and served as a prophylactic, or preventative, intervention before pathology began. As is the case of many prophylactic interventions, the efficacy of the treatment depends on when the intervention is administered and how long it is maintained.

The aforementioned case studies focused on genetic deficiencies that benefit from creatine supplementation and may suggest to lay individuals that creatine supplementation may be of benefit to them as well. The nutritional supplement industry touts the benefits of creatine supplementation and based on these claims, one would consider that supplementation of a nutrient like creatine could have added benefit in healthy individuals. Creatine is a popular supplement for athletes, but has also generated some interest amongst non-athletes.

Typically, the human body will produce 2 g of creatine per day, either from ingestion of food or synthesis in the body. This is added to the normal amount of creatine found in the body (approximately 120 g) (Walker 1979). Creatine supplementation is common in the realm of athletics, as they use it as a source of energy to replenish what is lost in working muscles. In a review that focused on the use of creatine in sports, the authors found that there were a variety of dosing regimens for creatine (Butts et al. 2018). In athletes, one regimen suggested the use of loading

doses and maintenance doses. For example, an athlete may take 20 g per day in the loading phase then switch to a lower dose during the maintenance phase, in order to keep creatine levels elevated (Mesa et al. 2002). The length of the loading phase varies between studies. Creatine loading in men, 20 g per day of creatine for 6 days followed by 2 g per day of creatine in the maintenance phase was reported as the rapid way to load creatine in muscle. However, a daily dose of 3 g of creatine was sufficient to achieve the same elevated levels of creatine as the 20 g loading dose/2 g maintenance dose protocol (Hultman et al. 1996). An optimized proposed protocol for oral creatine supplementation suggested that the loading phase consist of 2 days, where 5 g of creatine is ingested four time each day (a total of 20 g creatine per day) and be followed by maintenance doses of 3–5 g creatine during the maintenance phase (Mesa et al. 2002). Related to athletic performance, Havenetidis reviewed research on the use of creatine in the military (Havenetidis 2016). Within these studies, approximately 27% of supplement users consumed creatine. Surprisingly, creatine failed to provide significant enhancement of performance in the majority of the studies investigated. However, creatine did show effectiveness as a supplement in anaerobic tasks, such as muscle strength and power lifting (Law et al. 2009). As pointed out by Havenetidis, anaerobic exercise depends on creatine phosphate stores in the skeletal muscle for energy, not on typical aerobic energy sources. Thus, a study that focuses on an aerobic-dependent energy source may not be the appropriate measure of efficacy of creatine. Furthermore, creatine has been used as a supplement for a variety of conditions, mostly based on anecdotal evidence of efficacy. For some disorders, there are published research studies that suggest creatine is helpful in treating symptoms of the disease. Creatine has shown positive effects in studies of osteoarthritis (Neves et al. 2011), muscular dystrophy (Nabuurs et al. 2013), and fibromyalgia (Leader et al. 2009; Alves et al. 2013). A recent report evaluated creatine as an anti-nociceptive compound in an animal model of thermal and inflammatory pain (Izurieta-Munoz et al. 2017). In this study, creatine supplementation decreased nociceptive behaviors in response to inflammation and supports an anti-nociception activity with the use of creatine.

Creatine Kinase

History

In 1927, Eggleton and Eggleton described the existence of an extremely labile phosphate compound in the muscle fiber, which was later isolated and characterized as creatine phosphoric acid (Fiske and Subbarow 1925). In 1936, further experimentation by Karl Lohmann established that hydrolysis of creatine phosphoric acid in an adenylic system was reversible; this reaction, as discussed in the next section, is now known as ‘high energy transfer reaction’ and plays a major role in ATP production and energy consumption both in mitochondria and cytosol (Rapoport 1978). During the investigation of the role of creatine kinase (CK), Banga and Askonas

described the role of CK in two enzyme systems; one system catalyzed the hydrolysis of ATP and the other transferred phosphate from creatine phosphate (Cr~P) to adenosine-diphosphate (ADP) (Banga et al. 1939; Askonas 1951). However, it was only in 1954 that Kuby and colleagues were able to isolate and partially crystalize the enzyme CK from rabbit muscle (Kuby et al. 1954). Over the years the use of serum CK, as an indicator of muscle degradation, started gaining popularity, and advances led to reports of the existence of different isoenzymes. It was initially suggested by Dance (1962) that this enzyme exists as a dimer and that the isoenzymes can be separated upon electrophoresis (Dawson et al. 1965; Dawson and Fine 1967). As described in detail below, CK exists in three different types of isoenzymes; namely brain and muscle (cardiac and skeletal) which have homologous dimers and other tissues that have heterologous dimers consisting of both muscle and brain (Eppenberger 1994). In 1986, Perrymen and colleagues isolated a full length cDNA for human muscle (M) creatine kinase (MCK) and further, following the expansion of molecular genetics, significant advances were made in understanding the structure and location of specific genes for the enzyme. As a result, the location of MCK was narrowed down to human chromosome 19 and mapped to 19q13.2-q13.3 (Nigro et al. 1987; Qin et al. 1998; Perryman et al. 1986).

Structure, Function, and Role in Biology

Evolutionarily, creatine kinase is a 40 kDa polypeptide structure, which consists of some highly conserved and some variable parts. The highly conserved sites of the framework of CK are responsible for basic functions like substrate binding while the variable sites are responsible for isoenzymes or species specific functions. The highly conserved part of this enzyme is retained across all species and all isoforms (Muhlebach et al. 1994). Creatine kinase is a member of the phosphagen kinase family of guanidine kinases, and the primary role of these enzymes is to assist in ATP hydrolysis (Muhlebach et al. 1994). It was originally considered predominantly to be a vertebrate phosphagen, but it was subsequently discovered that this enzyme is also present in invertebrates like sponges, mollusca, and arthropoda (Ellington 2000; Suzuki and Furukohri 1994). To be functionally active, CK needs to form dimers to create a protein structure of ~ 84 KDa units. Creatine kinase is typically present in tissues that demand a high level of energy like brain and muscle. Most vertebrate animals also have a tissue and compartment specific isoenzyme of CK (Schlattner et al. 2006; Schlegel et al. 1988). The type of CK found in the cytosol is composed of two polypeptide subunits of 42 kDa units each. These subunits can either be B (brain type) or M (muscle type) and these subunits come together to form dimers that are present in three different isoenzymes: CK- BB (brain), CK-MM (skeletal muscle) and CK-MB (cardiac muscle). Another form of CK is localized in between the cristae and intermembrane space of mitochondria (Mt-CK). The two different isoenzymes of Mt-CK are ubiquitous and sarcomeric. The ubiquitous form is expressed in brain, smooth muscle and sperm while the sarcomeric form is

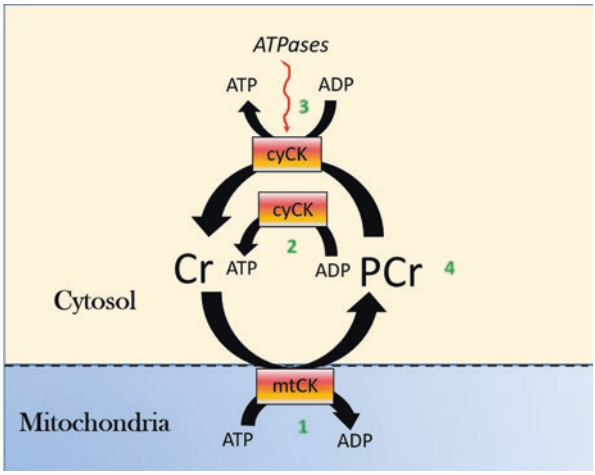

Fig. 6.3 Creatine/Phosphocreatine shuttle system to maintain cellular energy homeostasis; based on (Schlattner et al. 2006). The schematic depicts compartment specific CK (mitochondrial CK-mtCK and cytosol CK-cyCK). (1)-Decrease ATP/ADP in the mitochondria (2)-Increase ATP/ADP ratio in the cytosol (3)-Increase in ATPases activity to consume ATP in the cytosol (4)-Cr/PCr shuttle system to maintain ATP/ADP ratios globally both in mitochondria and cytosol

expressed in striated muscle found in cardiac and skeletal muscle. To form a functional entity, the Mt-CK complex is expressed as an octameric structure (Schlegel et al. 1988; Schlattner et al. 2006; Liu et al. 2010).

To fulfill demands of cellular bioenergetics, the interplay between the cytosolic and mitochondrial isoenzymes of CK is essential. Cytosolic Creatine (Cr) is reversibly converted to a phosphorylated form phosphocreatine (PCr) which regulates the concentration of ATP in the cell; both cytosolic and mitochondrial. These two isoenzymes are connected by a shuttle or circuit PCr/Cr (Fig. 6.3). The compartmentalization of these isoenzymes and shuttling of PCr into the cytosol helps phosphocreatine (PCr) generated in the mitochondria to be shuttled to the cytoplasm, and be further utilized to regenerate ATP in the cytoplasm. This regeneration of ATP takes place in the presence of ATPases present in the cytoplasm during muscle contraction or in kidneys during sodium retention. Models depicting the function of CK system explain that Cr/PCr act as a 'temporal' and 'spatial' energy buffering system. According to these models, Cr/PCr shuttle system has five main functions: (1) acts as a temporal energy buffer; (2) acts as a spatial energy buffer; (3) prevents rise in intracellular free ADP by seizing the free ADP present in the cytoplasm and converting it to ATP, thus regulating the net pool of adenine nucleotide in the cell (Iyengar et al. 1982; Iyengar 1984); (4) acts as a proton buffering system by preventing acidification of the tissues both at cellular or global levels during ATP hydrolysis, and; (5) maintains a low ATP/ADP ratio in the mitochondria while maintaining a high ATP/ADP ratio in the cytosol, which is important to stimulate oxidative phosphorylation in the mitochondria and ATP consumption in the cytosol (Wallimann et al. 1989; Eppenberger 1994).

Distribution in Tissues

The ratio of the subunits of CK found in the cytosol varies according to the tissue. Skeletal muscle typically contains 98% MM and 2% MB and cardiac muscle contains 70–80% MM and 20–30% MB, while the brain predominantly has only the BB type of the isoenzyme (Schlattner et al. 2006; Wallimann and Hemmer 1994). High amounts of energy is utilized in the brain and with only small amount of energy stores like glucose, glycogen from aerobic respiration, the organ has to rely on the Cr/PCr shuttle system for its energy source (Norwood et al. 1983). CK from brain mitochondria is very specific and different from the isoenzyme found in the heart mitochondria (Schlegel et al. 1988; Wyss et al. 2000). Adult human brain also contains MM-CK and is restricted to specific regions of the brain (Hamburg et al. 1990). There are huge regional differences in the activity of CK within the different brain regions. In the cerebellar cortex, higher levels of CK and PCr were measured in the molecular layer than in the white matter (Maker et al. 1973). Further, the activity of CK was higher in the cerebellum, striatum and pyramidal cells when compared to whole brain (Manos et al. 1991). Creatine kinase is not limited to neuronal cells but is also present in glial cells, astrocytes and oligodendrocytes (Manos et al. 1991; Yoshimine et al. 1983). In these cells, CK function is coupled with myelin synthesis, transport and assembly and therefore is postulated to play a significant role in certain neurodegenerative diseases (David et al. 1998).

The MM-CK muscle specific CK is able to interact with M-band (myomesin) of sarcomere and functionally is coupled with a variety of ATPase pumps present at the sarcoplasmic reticulum and regulates various ATP dependent functions (Saks et al. 1978). The myofibrils in the heart also contain specific MM-CK and Mt-CK isoenzymes which are localized in the sarcoplasmic reticulum, plasma membrane, myofilaments, mitochondria and glycolytic complexes of cardiac muscles (Ventura-Clapier et al. 1987). In smooth muscles all types of CK isoenzymes were detected (BB, MB and MM), however, BB-CK and Mt-CK are the main isoforms that were identified in smooth muscle fibers and may perform specific functions associated with that cell (Clark 1994). CK is also present in cells like spermatozoa, retinal cells, pancreas, placenta, thyroid, thymus, brush-border of the intestine, cartilage and bone cells (Wallimann and Hemmer 1994). The tissue specific distribution of the CK enzyme in different cellular organelles further suggest that the presence of Cr/PCr shuttle system is able to fulfill the intermittent and/or high energy demands of a cell.

Factors Affecting Level of Creatine Kinase

According to the standard established by the International Federation of Clinical Chemistry (IFCC) the upper reference level for CK for males is 171 U/I and for females is 145 U/I (Schumann and Klauke 2003). The sex difference in CK levels

could be attributed to higher proportion of muscle mass in males (Neal et al. 2009). Similarly, creatine kinase levels in the plasma can be altered by a myriad of factors that include race, age, physical activity, medications, minor injuries and diseased states. Medications like statins are known to cause muscle damage, and therefore as an indicator of muscle damage, it is common practice by physicians to monitor CK levels in patients on statins. Recent reports from large number of clinical studies conducted to study the effects of statins on different age groups and ethnicity has led to a better understanding of differences in creatine kinase levels (Neal et al. 2009). Creatine kinase levels in African Americans in both sexes were significantly higher in comparison to other races such as Caucasians, Hispanics and South Asians (Neal et al. 2009). However, CK levels among non-African Americans were similar. A post-mortem study in black and white males, comparing CK levels in tissues from different organs such as cerebrum, cerebellum, heart, renal artery and skeletal muscle reported a 70% increase in CK in African American males (Brewster et al. 2012). The results from recent studies highlighting the racial differences in CK levels are in concurrence with the some studies conducted 3–4 decades ago (Meltzer and Holy 1974; Wong et al. 1983; Black et al. 1986). Therefore, the National Muscle Expert Association has recommended 'race-ethnicity-and-sex'- specific values for upper limits of normal (ULN) for diagnoses of abnormal CK levels (Thompson et al. 2006).

Age is another factor that has an impact on CK levels. In newborns, levels of CK are reported to be 10 times higher than in adults (Gilboa and Swanson 1976). Gilboa and Swanson reported a high increase in CK activity in the first 4 days following delivery and pointed that the increase in activity could be explained by increased physical stress endured by the fetus during birth. Further, these levels returned to normal levels by 6–10 weeks post birth (Gilboa and Swanson 1976; Zellweger and Antonik 1975; Rudolph and Gross 1966). A study in females revealed changes in CK levels throughout their life; the CK levels increased dramatically during pre-puberty, pregnancy, and postmenopause (Bundey et al. 1979; Fukutake and Hattori 2001).

Exercise is also a major factor affecting CK levels. In males who trained for distance running, CK levels were elevated to 168 ± 15 U/L during training and these levels were significantly lower when the training was reduced (Houmard et al. 1990). Therefore, it can be concluded that low to moderate exercise causes an increase in level of serum CK; however, these levels are back to baseline levels within 7–9 days once training is discontinued. During mild to moderate exercise the body is able to facilitate the repair of the damaged muscle tissue, to regulate the metabolic disruption of cellular components in muscle and ultimately restore normal serum CK levels (Totsuka et al. 2002). However, excessive physical exertion like running marathons, can cause skeletal muscle damage and in some extreme cases, in untrained individuals could lead to a condition called as rhabdomyolysis (Morandi et al. 2006). This disease is characterized by symptoms such as muscle pain, soreness, increased weakness, and darkened urine. During this state, lysis of skeletal muscle cells releases intracellular toxins into the systemic circulation and if left untreated can result in kidney damage (Morandi et al. 2006). Creatine kinase levels are highly sensitive to muscle injury and can therefore be used as a tool to diagnose muscle damage. Serum CK levels during rhadomyolysis can raise up to 300×10^6 U/L (Efstratiadis et al. 2007). However, the exact mechanism resulting in

such an increase in CK levels is poorly understood. Direct muscle injuries include injuries occurring from natural disasters like, earthquakes or car and industrial accidents. Such individuals undergo severe pathophysiological changes due to extreme trauma and require dialysis to prevent rhabdomyolysis-induced renal failure (Criddle 2003; Vanholder et al. 2000). In patients with metabolic-endocrine disorders like hypokalemia, hyponatremia, hypophosphatemia, and hypothyroidism, the permeability of the sarcolemma can be altered distorting the function of sarcomere resulting in rhabdomyolysis (Vanholder et al. 2000; Shiber and Mattu 2002). Similarly, drugs like statins, fenofibrates, antiretrovirals, angiotensin-II receptor antagonist, immunosuppressants, and hydroxychloroquine result in significant muscle damage and may also contribute to rhabdomyolysis (Jamal et al. 2004; Warren et al. 2002). Patients who suffer from genetic disorder like Duchenne and Becker muscular dystrophies suffer from rapid progressive degeneration of the muscle fibers beginning at 3–5 years of age due to faulty dystrophin gene. These individuals are susceptible to complications like malignant hyperthermia as a result of anesthesia used during surgery. Acute rhabdomyolysis is one of the key contributing factors for malignant hyperthermia and eventual death of these patients (Morris 1997; Hayes et al. 2008). Creatine kinase is also increased in patients suffering from certain autoimmune disease like polymyositis and dermatomyositis (Thakur et al. 1996; Galarraga et al. 2003). Therefore, it can be concluded that early and continuous monitoring of serum CK can be used as a clinical tool to detect onset and severity of muscle disorders.

Aging and Creatine

Oxidative Capacity and Aging: Levels of Creatine

In a normal 70 kg human, the total creatine reserve is 120 g, with 2 g/day being produced from endogenous sources (Walker 1979). The levels of creatine and the activity of creatine kinase seem to decrease as a function of age in both animals and humans.

Skeletal muscle changes with age are associated with diminished capacity to accomplish daily tasks. Reports are inconsistent regarding whether these functional changes are also associated with decreases in oxidative capacity of the muscles. Mitochondrial enzymes and respiratory rates of muscles seem to be decreased with age in humans (Short et al. 2005), and oxidative capacity, measured by *in vivo* phosphorus magnetic resonance spectroscopy (MRS), has been shown to be lower in gastronecmius (McCully et al. 1993) and vastus lateralis (Conley et al. 2000) in older individuals than in young ones. However, other laboratories reported conserved levels of oxidative metabolism with age in humans when matching for physical activity (Rasmussen et al. 2003). This was supported by studies using phosphocreatine (PCr) rate of recovery post-contraction as a MRS measure of oxidative capacity showing no differences between young and old subjects (Lanza et al. 2005). The inconsistency of outcomes may be due to a variety of factors, including the level of activity of an individual, and muscle differences. A study by

Larsen et al. tackled this potential issue and determined that the differences in oxidative capacity, measured by PCr recovery from contraction using magnetic resonance spectroscopy, were sex- and muscle- dependent and that physical activity influenced the outcome (Larsen et al. 2012).

Using volumetric proton magnetic resonance spectroscopic imaging to map brain metabolites, it was determined that creatine was higher in gray matter than in white matter, and that its levels were higher in old individuals than in young ones (Pfefferbaum et al. 1999). Increased levels of creatine signify a decrease in PCr levels, and thereby a decrease in overall oxidative metabolism. Furthermore, in post-mortem samples of the frontal and occipital regions, creatine kinase activity was decreased in old controls vs. young controls (Smith et al. 1991). Interestingly, in neuronal cultures from hippocampus, Aksenova et al. reported a gradual increase with "age" in creatine kinase levels associated with a decreased enzymatic activity, thereby suggesting an accumulation of inactive creatine kinase molecules (Aksenova et al. 1999). In gerbils, the activity of CK was found to be decreased with aging in the brain, along with other markers of oxidative stress (Carney et al. 1994).

The creatine/phosphocreatine (Cr/PCr) system is important for metabolism and therefore plays a vital role in the normal cellular function, in particular high energy demand cells such as muscle and brain cells. Depletion of creatine has been associated with brain disorders including cognitive and motor impairments, and language/speech issues (van de Kamp et al. 2014; Joncquel-Chevalier Curt et al. 2015). A study of the cerebral creatine deficiency syndrome-2 mice, which have a mutation in the creatine transporter, reported impairments of short- and long-term memory along with other markers of brain dysfunction (Baroncelli et al. 2016). Another model of creatine deficiency, creatine transporter knockout mice, have shown severe deficits in cognitive function in males (Skelton et al. 2011), and relatively mild deficits in the females (Hautman et al. 2014).

Overall, cell, rodent and human studies indicated a derangement of the creatine, phosphocreatine and creatine kinase system at least in muscle and brain tissues associated with aging. Furthermore, creatine depletion has been shown to lead to phenotypes of motor and cognitive impairments, and motor and cognitive dysfunctions are hallmarks of the aging process. Overall, these studies suggest that supplementation with creatine has the potential to reverse functional declines associated with aging (and to some extent age-associated diseases).

Creatine Supplementation, Diseases and Aging

Creatine and Diseases

Creatine is an ergogenic compound that is attractive to athletes due to its propensity to increase ATP formation (Hall and Trojian 2013) and the very few unwanted side effects associated with its intake (Juhn and Tarnopolsky 1998). Its supplementation leads to increase PCr which is a critical form of energy storage needed for high intensity exercise. Creatine supplementation has been extensively studied in young

individuals and in association with exercise (Bemben and Lamont 2005). With the emphasis on the effect of creatine on metabolism and its other properties, the focus of creatine supplementation shifted from athletes to a plethora of diseases; diseases involving neuromuscular disorders and/or mitochondrial dysfunction such as Parkinson's and Huntington's diseases and others (Smith et al. 2014). Furthermore, creatine is found to be relatively safe, can penetrate the blood-brain barrier, and has shown some efficacy in animal models, thus making it a good candidate as a neuroprotective agent. A randomized double-blind study determined that creatine could not be rejected as futile for therapy of Parkinsonism, and could be considered for Phase III trials for Parkinson's Disease (Investigators 2006). A thorough review by Persky and Brazeau provided mechanisms of actions and a summary of the effects of creatine supplementation in animal and human studies for pathological conditions such as heart disease, musculoskeletal disorders, depression associated with stroke, gyrate atrophy, and nephrotoxicity (Persky and Brazeau 2001). Other studies have proposed that creatine could benefit patients with fibromyalgia (Leader et al. 2009), and could reduce morbidity and mortality associated with high-risk pregnancies (Dickinson et al. 2014). Another nice review by Klopstock et al. reported the outcomes of creatine supplementation in rodent and human studies of neurodegenerative diseases such as Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, and reflected that while creatine seemed promising in mice studies, it did not translate well in human clinical trials. However, it is noteworthy that there has not been many published reports of creatine supplementation in humans, and that perhaps the mice models used are to be questioned as to how well they reflect human conditions (Klopstock et al. 2011).

Creatine Supplementation and Aging

Aging studies of creatine supplementation are sparse; however some parallel to successful intervention such as caloric restriction can be drawn making it a worthy compound to alleviate the negative effects of aging. It has been shown to have anti-apoptotic (O'Gorman et al. 1997) and antioxidative properties (Lawler et al. 2002), two capacities that should help reverse the effects of age.

Sarcopenia, an age-related loss of muscle mass and function, and its associated bone loss results in frailty and increased risk of fall, leading to decreased independence, increased social isolation and depression, and possibly death. Finding ways to reduce or reverse age-related declines in function are of the utmost importance to improve survival and quality of life as we age. Creatine is a particularly attractive supplement especially in the elderly due to its safety potential with few unwanted side effects. However, caution must be advised, as long-term studies of creatine supplementation are lacking, and there is a potential risk of side effects that is increased especially in individuals with kidney diseases/issues (which is a likely scenario in older individuals). After only 5 days of creatine intake, middle-aged individuals had a higher PCr availability and PCr resynthesis than younger subjects, and increased time to exhaustion to the same degree in both age groups (Smith et al. 1998). A meta-analysis revealed that creatine supplementation associated with

resistance training increased muscle mass, and improved upper body strength to a further extent that training alone (Candow et al. 2014). Several studies have determined that intake of creatine have anabolic (muscle mass, strength, volume training) and anti-catabolic (protein catabolism, oxidation) effects (Dalbo et al. 2009). On the other hand, other studies have reported a lack of effect on sarcopenia, in this case in a mouse model of accelerated aging (Derave et al. 2005). Older, healthy and normally active men and women responded positively to creatine intake for 7 days and exhibited improvements in muscle strength and performance on physical tasks (Gotshalk et al. 2002, 2008). In 70 year old males, creatine supplementation along with strength training improved leg strength, endurance and power as well as lean tissue mass (Chrusch et al. 2001). In a group of 75 ± 6 year old men and women, creatine supplementation for 14 days resulted in increased upper body strength and increased fatigue threshold (Stout et al. 2007). More recently, a review and meta-analysis by Chilibeck et al. revealed that creatine supplementation had beneficial effect in old participants during resistance training, with increased lean tissue mass and, muscular strength (Chilibeck et al. 2017). Creatine may also have beneficial effect on bone health via direct or indirect (byproduct of improved muscle health) actions, associated with improved bone health in older males (Chilibeck et al. 2005). In *in-vivo* work on osteoblast-like cells, creatine's presence increased mineralization and metabolic activity supporting potential beneficial effect of creatine on bone repair (Gerber et al. 2005).

Studies of creatine supplementation have been well documented in neuromuscular and neurodegenerative diseases (Wallimann et al. 2011), however studies in healthy individuals are less common. The neuroprotective effects of creatine have been hypothesized to be due to its antioxidative, antiapopototic and bioenergetics properties (Wallimann et al. 2011). Aging is associated with redox and bienergetic dysregulation, therefore creatine could be a useful supplement to reverse effects of aging on overall health and more specifically brain health. In healthy mice, creatine supplementation increased their median life span, and improved healthspan, along with measures of oxidative stress and neuroprotection (Bender et al. 2008). In a human study, creatine intake for 2 weeks was reported to improve memory on most tasks, however the study had low power (McMorris et al. 2007). In the aging rodent a study of creatine supplementation by Bender et al. (2008) found that some biomarkers such as DNA oxidation, age pigment lipofuscin, and BDNF (Brain-derived neurotrophic factor) followed the same trends as observed with caloric restriction (Sohal and Weindruch 1996; Duan et al. 2001). While these effects were relatively small, they amounted to an overall antiaging effect of creatine which warrants more studies. However, it is noteworthy that clinical trials of antiaging interventions are seldomly done and funding is extremely difficult to gain.

Is Creatine a Viable Option to Alleviate Age–Related Dysfunction?

Creatine is a safe and inexpensive supplement that has shown to have numerous benefits in the athletic population. However, studies of the effects of creatine in the elderly population remain sparse, and the outcomes of these studies are equivocal

and are relatively small (see review from Dalbo et al. for details of studies). As for any antiaging interventions, there are many factors to consider including early vs. late life implementation, the duration and the age and sex of the subjects, all of which may influence the outcome. Prolonged exposure to high creatine concentration may also lead to down-regulation of the creatine transporter, but would need to be studied to determine whether on/off supplementation can lead to further benefits compared to continuous supplementation. While some age-related adaptations may decrease the response of older individuals to creatine supplementation, there remains strong support for creatine enhancing muscular performance in short intervention times (5–7 days). The US Society for Sarcopenia, Cachexia and Wasting Disease has reviewed the literature and made recommendations on supplements (Morley 2015). They suggest that short-term creatine may be of benefit along with exercise for sarcopenia, however long-term studies for this and other conditions are needed. Effects on neurodegenerative diseases remain to be seen in clinical trials, and consistent effects on age-associated declines need to be further studied.

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Chapter 7
Extracellular Matrix and Ageing

Helen L. Birch

Abstract The extracellular matrix (ECM) provides the environment for many cells types within the body and, in addition to the well recognised role as a structural support, influences many important cell process within the body. As a result, age-related changes to the proteins of the ECM have far reaching consequences with the potential to disrupt many different aspects of homeostasis and healthy function. The proteins collagen and elastin are the most abundant in the ECM and their ability to function as a structural support and provide mechanical stability results from the formation of supra-molecular structures. Collagen and elastin have a long half-life, as required by their structural role, which leaves them vulnerable to a range of post-translational modifications. In this chapter the role of the ECM is discussed and the component proteins introduced. Major age-related modifications including glycation, carbamylation and fragmentation and the impact these have on ECM function are reviewed.

Keywords Collagen · Elastin · Ageing · Advanced glycation end-products · Fragmentation

Extracellular Matrix Distribution

The extracellular matrix (ECM) refers to the many proteins and glycoproteins that provide the milieu for the cells within the body. Cell behavior is profoundly effected by the environment in which they residue, thus ageing of the ECM is central to understanding age-related changes to cell metabolism and function. In addition, age-related changes to the ECM directly impact on the ability of the ECM to provide physical function. Most of the attention on the ECM has come from the study

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of connective tissues, where the ECM is profuse and the cell population relatively sparse. Connective tissues form a diverse group and are the most abundant and widely spread tissue types within the body. Some connective tissues are well known such as bone and cartilage and the dense regular connective tissues, tendon and ligament and the dense irregular connective tissues, dermis and heart values. Others, such as areolar and reticular loose connective tissues, are less often appreciated but perform vital functions within the body providing support around blood vessels, nerves and other body organs, and forming the stroma of organs such as liver, spleen and lymph nodes and part of the basement membrane. The ubiquitous and diverse nature of connective tissues and their ECM characteristics results in broad and varied roles, influencing almost every process within the body.

Role of the Extracellular Matrix

The ECM is best recognized for its role as a scaffold, supporting the delicate cells of the body and providing the appropriate mechanical properties for physiological function (Fig. 7.1). The mechanical properties vary from tissue to tissue; for example in bone high compressive strength is required to bear load without buckling while in tendon, strength under tensile loads is necessary while maintaining the ability to flex around joints and other structures. The areolar tissue surrounding blood vessels and nerves and in the subcutaneous layer of skin requires a greater degree of stretch and flexibility and a more porous structure to allow passage of interstitial fluid. Even within a tissue type, a range of mechanical properties is required and these are achieved by subtle differences in the ECM composition. In addition to providing structure and tissue boundaries, the ECM is a key component in matrix to cell and cell-to-cell signaling. Matrix to cell signaling allows tissues to respond to mechanical cues in a process known as mechano-transduction. Integrin receptors on the cell surface interact with ECM proteins through specific protein sequences allowing sensing of the mechanical environment (Fig. 7.1). In addition to the extracellular attachment to the matrix, integrin receptors have an intracellular connection to the cell cytoskeleton, enabling the translation of mechanical signals into an intracellular chemical response. Through this process the mechanical environment can regulate the composition and turnover of the ECM. The ECM also provides an adhesive substrate for cell migration through a range of membrane-bound cell adhesion molecules and can direct cell migration in development, regeneration and the repair process (Fig. 7.1). The ECM is an important source of growth factors providing attachment and controlled release of many growth factors and signaling molecules (Fig. 7.1), thereby aiding cell-to-cell signalling. Furthermore the ECM can itself be a source of bioactive fragments through the controlled degradation of components of the ECM (Fig. 7.1).

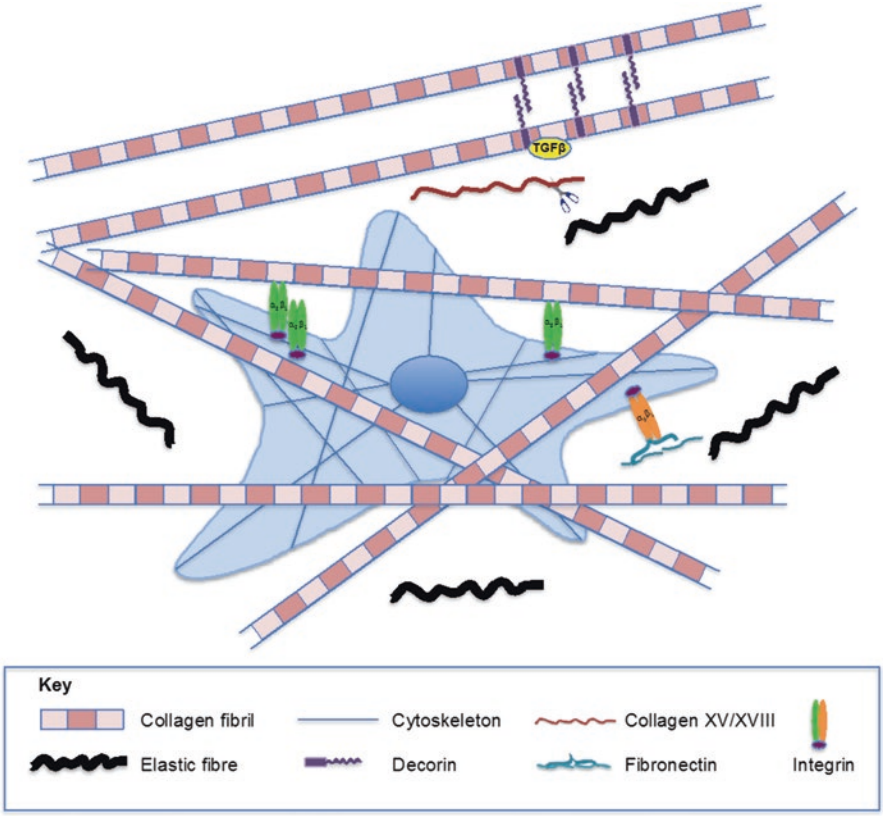

Fig. 7.1 Schematic representation of the ECM showing the diversity of functions. Matrix proteins provide a structural support (e.g. collagen type I, elastic fibres); interact with cell surface receptors to provide mechanical cues (e.g. collagen and $\alpha_1\beta_2$ integrin); direct cell migration (e.g. fibronectin and $\alpha_5\beta_1$ integrin), sequester growth factors (e.g. decorin and TGFβ) and, through degradation, provide bioactive signaling molecules (e.g. collagens XV and XVIII release endostatin)

Extracellular Matrix Composition

Using a bioinformatics approach, over 1000 proteins have been identified as comprising the ECM and the set of genes encoding these proteins has been termed the 'matrisome' (Naba et al. 2017). The current version of the matrisome (http://matrisomeproject.mit.edu) (Naba et al. 2016) shows some variation between species and includes 1027 genes from the human genome and 1110 genes from the mouse genome. A further division of the matrisome has been added to identify core matrisome proteins, based on structural and functional features, and matrisome-associated proteins, which includes regulators of the ECM and secreted factors that may interact with core ECM proteins (Fig. 7.2). The core matrisome proteins include collagens, elastin, proteoglycans and glycoproteins and although less numerous than the

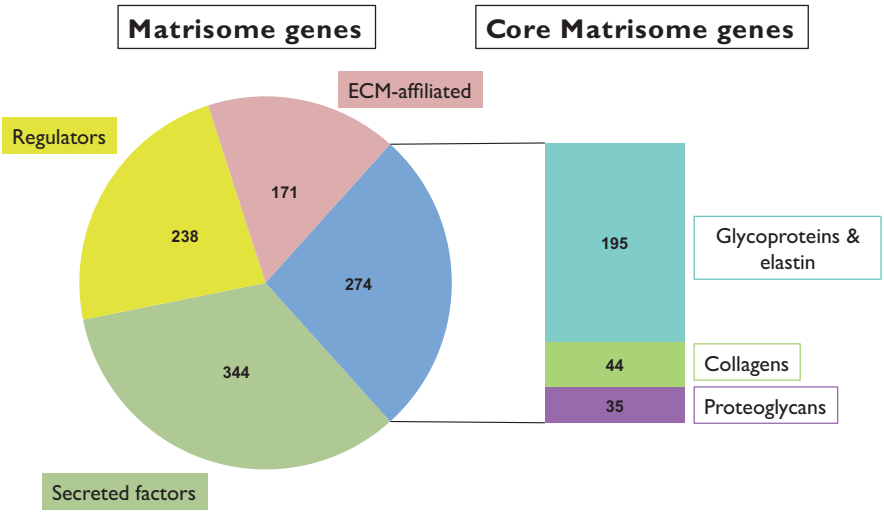

Fig. 7.2 Categories of the matrisome showing the number of genes in each category for the human genome. (Adapted from Naba et al. 2016)

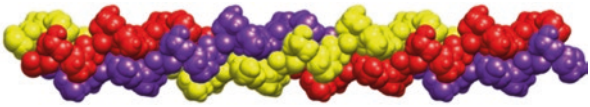

Fig. 7.3 Space-filling model of collagen triple helix showing three polypeptide chains (yellow, red and purple) in right-handed supercoil. (Modified from Bella 2016)

matrisome-associated proteins in terms of different genes, these proteins form the majority of the mass of the ECM and provide the structural scaffold.

Collagens are recognized as the main structural components of the ECM and form a family of 28 different types of collagen coded for by 44 different genes (for a review see Kadler et al. 2007). The characteristics of the collagen family members are a repeating triplet sequence of amino acids with glycine every third residue followed frequently by proline and hydroxyproline. This repeating sequence allows three polypeptide chains to form the unique triple helical structure of the collagen molecule (Fig. 7.3). The polypeptide chains and collagen molecules undergo extensive post-translational modifications, including covalent crosslinking between collagen molecules through hydroxylysine and lysine residues. A further requirement for inclusion within the collagen family is that the protein must be extracellular and have a structural role in maintaining tissue integrity, which excludes a small number of proteins possessing regions with a collagen triple helical-like structure. Within the collagen family, proteins are further classified into subgroups based on related structural features and their supra-molecular organization. The fibril-forming collagens, which include types I, II, III, V, XI, XXIV and XXVII are by far the most abundant collagen types within the body.

The protein elastin has particular relevance to the ageing of the ECM. Unlike collagen, elastin is a single gene product and variation in the synthesised protein is due to alternative splicing of the mRNA resulting in isoforms of elastin. Like collagen, elastin forms fibres, in this case through deposition onto a microfilament scaffold. Elastic fibres provide elasticity and extensibility to tissues such as the dermis and elastic blood vessels.

Proteoglycans form the next most significant group of molecules by mass in the ECM and are characterized by a large carbohydrate component and a relatively small protein core. The carbohydrate component is present as long unbranched chains of repeating disaccharide units known as glycosaminoglycans (GAGs) and these are attached to the protein core through an O-glycosidic linkage. One of the disaccharide units of the GAG chain is an amino sugar and the other sugar, and sometimes both sugars, is modified with negatively charged sulphate or carboxyl groups. The variation in disaccharide units gives rise to a number of different GAG chains including chondroitin sulphate, decorin sulphate and keratan sulphate. The most recent and comprehension classification of proteoglycans is based on their location as intracellular, cell-surface, pericellular or extracellular proteoglycans and these classes are further sub-grouped by common structural features of their protein cores (Iozzo and Schaefer 2015). Those most relevant to the ECM are the hyalectans such as aggrecan and vesican and the small leucine rich proteoglycans (SLRPs) such as decorin, biglycan, fibromdulin and lumican.

Glycoproteins, in contrast to proteoglycans, are composed predominately of protein with a smaller carbohydrate component and although in terms of different species are greater in number, form a smaller proportion of the ECM by mass. Examples of important glycoproteins in the ECM include fibronectin, collagen oligomeric matrix protein (COMP) and lubricin.

Extracellular Matrix Susceptibility to Ageing Changes

A key feature of the ECM is that it provides a stable structural support for the body, for organs within the body and for resident cells within a tissue. Structural stability is achieved through the formation of supra-molecular protein structures, which by their nature are metabolised relatively slowly, if at all. These supra-molecular structures are well suited for their structural role but as a consequence of their slow turnover they are susceptible to age-related chemical and enzymatic modifications that can accumulate within the tissue. The majority of the protein in the ECM is collagen, specifically the fibril-forming collagens types I, II and III, named by their ability to form fibril-based supra-molecular organisations. The fibril-forming collagens are long rod-like molecules with a molecular length of approximately 300 nm and a diameter of 1.5 nm. Once synthesised and secreted from the cell these molecules spontaneously align in a quarter-staggered arrangement to form fibrils (Petruska and Hodge 1964). Within the fibril, collagen molecules are staggered relative to their neighbour by 67 nm, a distance referred to as the D period. A gap of

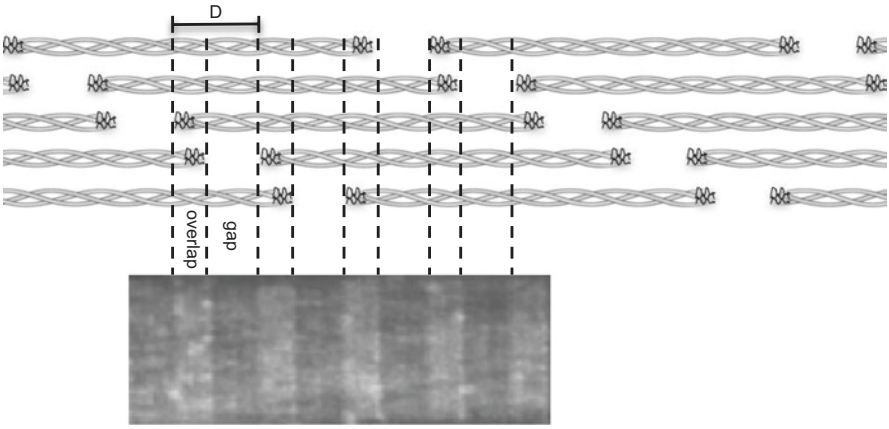

Fig. 7.4 Schematic representation of the quarter-staggered arrangement of collagen molecules within a fibril giving rise to a gap and overlap region and a banded appearance under the electron microscope

0.54D (36 nm) is left between the ends of sequential collagen molecules resulting in a 0.46D (31 nm) overlap with the adjacent collagen molecule (Fig. 7.4). This characteristic packing results in a banded appearance when viewed under an electron microscope or by atomic force microscopy. Once fibril formation has occurred, the extracellular enzyme lysyl oxidase is able to bind to the fibrillar collagen and catalyse the oxidative deamination of lysyl or hydroxylysyl residues in the telopeptide (non-helical end region) of an adjacent collagen molecule resulting in an aldehyde. In type I collagen three aldehydes can form at the N terminal telopeptide and two at the C terminal telopeptide. A series of spontaneous reactions then occurs where the aldehyde reacts with the hydroxylysyl residue of the neighbouring molecule to form a bivalent crosslink. Over time the lysyl or hydroxylysyl aldehyde in a third collagen molecule reacts with a bivalent crosslink forming a series of trivalent pyridinoline and pyrrole mature crosslinks (for a review of collagen crosslinking see Bailey et al. 1998). The particular species of crosslink formed depends on the extent of hydroxylation of lysine resides and the balance between hydroxylation in the helical and telopeptide regions of the collagen molecule. Due to the positioning of the binding site for lysyl oxidase, these crosslinks are restricted to the telopeptide regions of the collagen molecules with one crosslink forming per molecule in the N-telopeptide and two crosslinks at the C-telepeptide. These covalent crosslinks between molecules within a collagen fibril provide a high degree of mechanical and chemical stability to the ECM but also predispose the collagen to accumulation of age-related modifications by virtue of the long half-life.

Elastic fibres result from the deposition of elastin on to a microfibril scaffold composed of fibrillin and a number of other glycoproteins and proteins. These highly organized supra-molecular structures form despite the relative lack of an organised structure to the elastin molecules themselves (Baldock et al. 2011).

Elastin molecules aggregate through the interaction of hydrophobic domains to form amorphous clumps. Following the concentration and alignment of elastin molecules, lysine residues undergo oxidative deamination by the enzyme lysyl oxidase to form aldehydes, which then undergo further reactions with other lysine aldehydes and lysine residues to form the elastin crosslinks desmosine and isodesmosine. Unlike collagen, these crosslinks form along the entire length of the elastin molecule and involve four separate lysine residues linking two elastin molecules together. The formation of crosslinks is extensive and renders elastin extremely insoluble and resistant to enzymatic digestion.

Extracellular Matrix Turnover

Information in the literature with regard to turnover of the ECM is scant and this is likely due to the difficulty in measuring turnover in long-lived proteins. A method that has been most widely applied is measuring the ratio of L- and D- enantiomers of amino acids, specifically aspartic acid (Ritz-Timme and Collins 2002). During ribosomal protein synthesis amino acids are incorporated into proteins in the L form however over time, spontaneous, post-translational racemization slowly converts L-form amino acids into a racemic mixture of L- and D- enantiomers. By comparison of the racemization rate of aspartic acid in the protein of interest with that of a tissue where the protein is essentially inert, a protein half-life can be calculated. Using this technique the half-life of collagen in human femoral condyle articular cartilage has been calculated to be 117 years (Verzijl et al. 2000) and in hip articular cartilage more than 200 years (Maroudas et al. 1992) compared to a half-life of 15 years for collagen in skin (Verzijl et al. 2000). In healthy human intervertebral disc the D:L ratio of aspartic acid predicted a half-life for collagen of 78–216 years depending on the donor age, with longer half-life at older ages (Sivan et al. 2008). The same technique suggests that the proteoglycan aggrecan in intervertebral disc turns over more readily with a half-life of 4.5–5.7 years (Sivan et al. 2006). In equine digital flexor tendon tissue the D:L ratio of aspartic acid predicted a the half-life for collagen of 198 years compared to half-life of 2.18 years for non-collagenous proteins (Thorpe et al. 2010). Interestingly, collagen half-life was significantly lower for a tendon with different mechanical requirements; the equine digital extensor tendon, at 34 years (Thorpe et al. 2010). In human tendon, the persistence of ^{14}C metabolic labeling of proteins as a result of nuclear weapons testing, suggested that collagen in human tendon is essentially inert (Heinemeier et al. 2013). In healthy and degenerate human intervertebral disc elastin was found to be stable and long-lived (Sivan et al. 2012). The age of elastin in lung tissue calculated using D:L aspartic acid levels closely correlated with donor age suggesting essentially no turnover while ^{14}C residence time in elastin gave a mean of 74 years (Campbell et al. 1991). The half-life of these ECM proteins is considerably longer than the majority of other proteins. For example, turn over studies in mice found the average half-lives of proteins in the brain, liver, and blood to be between 3 and 9 days (Price et al. 2010).

Post-translational Modifications to the Extracellular Matrix

All proteins are susceptible to spontaneous non-enzymatic chemical modifications and these can be reversible or irreversible. There is now an appreciation that reversible modifications play an important role in a range of cell processes, adding an additional layer of complexity to control of cell behavior under normal physiological conditions (Harmel and Fiedler 2018). Irreversible modifications however are likely to be detrimental and in proteins with a long half-life such as collagen and elastin these modifications accumulate within the tissue with increasing age. The simplest of modifications results from a rearrangement of functional groups to form stereoisomers, such as amino acid racemisation, as discussed above. Racemisation can occur to all amino acids except glycine but the rate differs between amino acids and is most rapid in aspartic acid, hence its use as a marker for protein age. Other chemical modifications involve the removal or addition of chemical groups following attack on the protein by reactive species. These modifications include oxidation, deamidation, carbonylation, glycation, succination and carbamylation. Modifications most relevant to the ECM are those that affect amino acids with a high representation in collagen, such as lysine. The most widely studied, although still relatively little understood, is glycation due to its role in diabetes.

Collagen Glycation and Formation of Advanced Glycation End-Products

Advanced glycation end-products (AGE) form through a series of reactions initiated by a reactive carbonyl group on a reducing sugar attacking the nucleophilic amino group on lysine and arginine amino acid side-chains in proteins in a process known as the Maillard reaction. The initial product is a Schiff base, which then undergoes a rearrangement to form a more stable Amadori product. Over time a series of rearrangements occurs which may culminate in a further reaction with an amino group on a side-chain of a second amino acid resulting in a crosslink. This series of reactions can in theory result in numerous different types of AGEs, some of which affect individual peptides/proteins and some which crosslink polypeptides within and between proteins together (Fig. 7.5). AGEs are often fluorescent and it has been well documented that collagen fluorescence increases with increasing age due to accumulation of these compounds.

The type of AGE formed *in vivo* depends on many factors including the source of reactive carbonyl group, amino acid residues involved and the metabolic status of the individual. Hexose sugars, for example glucose, galactose and fructose or pentose sugars such as ribose and arabinose can take part in the initial reaction. In healthy individuals hexose sugars are the most abundant with glucose being present at levels between 4.4 to 6.1 mM while pentose sugars such as fructose, although more reactive in the glycation reaction, are only present at micro-molar levels

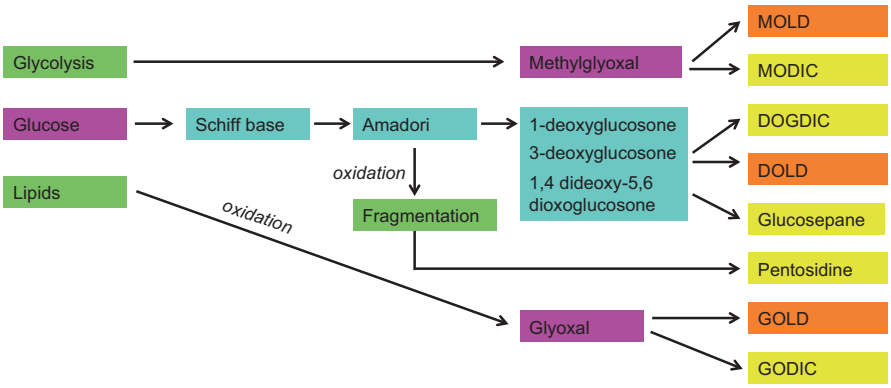

Fig. 7.5 Pathways leading to the formation of AGE crosslinks following the Maillard reaction. Lysine-arginine crosslinks are shown in yellow and lysine-lysine crosslinks shown in orange. (Adapted from Monnier et al. 2005)

(Schalkwijk et al. 2004). Other dicarbonyl species, namely glyoxal, methylglyoxal, 1-deoxyglucosone and 3-deoxyglucosone can result from glycation under oxidative conditions and may take part in the formation of AGE crosslinks. Methylglyoxal can also be formed from intermediates in glycolysis (Phillips and Thornalley 1993) and glyoxal from lipid oxidation. The levels of these two metabolites are however usually low in healthy individuals as they are effectively detoxified by mammalian enzyme systems. The imidazolium crosslinks, GOLD, MOLD and DOLD are formed by glyoxal, methylglyoxal or 3-deoxyglucosone respectively and two lysine residues on neighbouring molecules. A series of lysine-arginine crosslinks are formed by reaction of glyoxal, methylglyoxal or 3-deoxyglucosone with lysine and arginine to form MODIC, GODIC and DOGDIC. An AGE crosslink known as glucosepane is formed from glucose through 1,4-dideoxy-5,6-dioxoglucosone and has starting reactants of glucose, lysine and arginine. The most widely measured AGE crosslink due to its acid stability and inherent fluorescence is pentosidine, although the physiologically relevant crosslink appears to be pentosinane, which becomes oxidised during analytical processing to produce pentosidine (Biemel et al. 2001). Although Amadori breakdown products can result in formation of pentosinane, experimental data suggest that pentose sugars are the predominant pathway (Biemel et al. 2001) resulting in a lysine-arginine crosslink.

Pentosidine, the most widely reported AGE crosslink in the literature, shows a strong correlation with increasing age in tendon (Thorpe et al. 2010; Couppe et al. 2009), however absolute levels in tissues are relatively low. In skin samples, pentosidine levels of between 6–10 mmoles/mole collagen for healthy and diabetic individuals respectively have been measured (Monnier et al. 1999). In tendon tissue measured levels range between 5 mmoles/mole collagen for human posterior tibialis and flexor digitorum longus tendons (Corps et al. 2012), 14 mmoles/mole collagen for equine tendons (Thorpe et al. 2010) and 11–73 mmoles/mole collagen for old and young human patellar tendon (Couppe et al. 2009). DOGDIC, MODIC and

GODIC crosslinks have all been detected in collagen from human skin and glomerular basement membrane samples although levels are low and similar to those for pentosidine (Sell et al. 2005). Glucosepane however has been measured at considerably higher levels in skin samples from diabetic (5000 pmoles/mg collagen) and non-diabetic people (2000 pmoles/mg collagen) (Sell et al. 2005; Monnier et al. 2013). These measured levels approach those for the lysyl oxidase-mediated collagen crosslinks suggesting multiple crosslinks per collagen molecule may form. Furthermore, glucosepane levels show a significant positive correlation with increasing age in skin (Sell et al. 2005) and tendon (unpublished data) suggesting that glucosepane may play a part in age-related decline in collagen function. There is little or no compelling evidence to suggest that AGE crosslinks form in the protein elastin. As elastin is extensively cross-linked through lysine residues with physiological enzyme-mediated crosslinks it may be protected from this particular post-translational modification.

Protein Carbamylation

Carbamylation is a non-enzymatic post-translational modification of proteins that has received less attention than that of glycation until fairly recently. Carbamylation occurs when the amino group a of lysine amino acid residue binds to isocyanic acid forming homocitrilline (Fig. 7.6). Isocyanate is present as a breakdown product of urea thus conditions which cause an increase in blood urea levels such as chronic kidney disease are associated with higher levels of homocitrilline. More recently homocitrilline has also been shown to accumulate in dermis, bone and tendon with increasing age in murine species and dermis in humans and bovine species (Gorisse et al. 2016). Homocitrilline reached higher levels in longer-lived species and was found to be mostly associated with collagen and elastin. In the study by Gorisse et al. (2016), a comparison was made between the absolute levels of homocitrilline and the glycoxidation product carboxymethyl-lysine (CML) to determine the relative importance of these two different pathways in the ageing of ECM proteins.

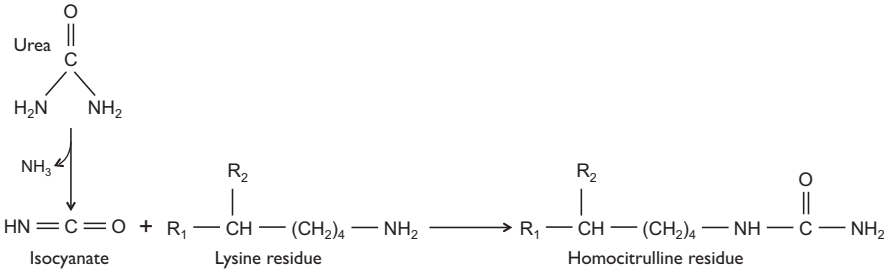

Fig. 7.6 Formation of homocitrulline from isocyanate following urea breakdown in the carbamylation reaction

In human skin collagen samples over 70 years of age, homocitrilline levels were measured at 5.15 mmol/mole lysine compared to 1.96 mmoles/mole of collagen for CML although it should be noted that CML is not the major AGE in human collagen samples, being some four times lower than glucosepane levels (Monnier et al. 2016). In theory any of the lysine residues may be susceptible to carbamylation; studies to determine the site have shown four preferential sites in the alpha-1 chain of type I collagen and one in the alpha-2 chain and some of these sites overlap with CML formation sites (Gorisse et al. 2016). Additionally it has been shown that hydroxylysine can also undergo carbamylation to form hydroxyhomocitrulline (Taga et al. 2017).

Protein Degradation and Fragmentation

Chemical modifications to proteins also include the cleavage of peptide bonds, most often through enzymatic processes. Peptide bond cleavage and degradation of proteins represents a part of normal protein homeostasis, however in ageing tissue the balance may be lost and degradation rate may exceed synthesis. In addition, partially cleaved proteins may be retained within the ECM due to physiological and AGE-related crosslinking leading to an accumulation of dysfunctional protein.

Fragmentation of elastic fibres is often cited in relation to ageing of skin and the vasculature. In the human aorta, histological sections from young donors show parallel, thick elastic fibres while in older donors, elastic fibres appear thinner, fragmented and disorganised as they become separated by increased amount of other ECM components (Zarkovic et al. 2015). Similarly in the dermis, elongated elastic fibres present in young tissue become shorter and fragmented in old specimens (Bonta et al. 2013). It has been suggested that elastic fibre damage is the direct result of mechanical fatigue (O'Rourke 1976) and although mechanical fatigue of porcine aorta elastic fibres following repetitive cyclical loading has been demonstrated *in vitro* (Greenwald 2007), this does not prove that this is the only factor. Indeed other studies have shown an increase in the activity of matrix metallo-proteinases (MMPs) with elastase activity in the aorta wall of rodents and humans suggesting increased elastin degradation (Wang et al. 2015).

In tendon, there is evidence to suggest that partially degraded collagen accumulates in the ECM with increasing age. In equine flexor tendon, the neo-epitope formed from the collagenase (MMP-1 and 13) cleavage of the collagen triple helix was found in significantly higher levels in older horses (Thorpe et al. 2010). In the same tendon samples, the expression of MMP-10 at the messenger RNA level and MMP-3 at the protein level, were increased in older tissue (Thorpe et al. 2016). Proteomic analysis of equine tendon has identified a number of neo-peptides originating from collagens and glycoproteins in old tissue that were not present in young tissue suggesting a change in the degradative profile of the ECM with ageing (Peffers et al. 2014). The challenge now is to identify the enzymes responsible for

the generation of these age-related neo-peptides to provide targets to inhibit age-related fragmentation of the ECM.

Age-related change to the ECM in articular cartilage has received particular attention due to the high prevalence of osteoarthritis; a degenerative disease associated with advancing age. It has been recognised for many years that the proteoglycans in cartilage become depleted and are subjected to proteolytic cleavage. The large aggregating proteoglycan aggrecan is cleaved between the G1 and G2 globular domains by both MMP13 and aggrecanase (ADAMTS5) activity and both fragments have been found to accumulate with increasing age (Lark et al. 1997). The G3 domain at the C-terminal end of aggrecan also becomes depleted with increasing age (Dudhia et al. 1996). In addition, type II collagen cleavage and denaturation has been identified in ageing cartilage (Wu et al. 2002).

Impact of Post-translational Modifications to the ECM on Function

While it is important to determine the changes that occur to the ECM molecules with ageing and to understand the mechanisms responsible, it is imperative that these findings are taken further with an understanding of how they modify the mechanical, chemical and biological functions of ECM.

Changes to Mechanical Properties of Connective Tissue with Increasing Age

Much of the research into the impact of ageing on dense connective tissues is based on the acceptance that the matrix stiffens with increasing age. This however is easily misinterpreted due to the complex mechanical behaviour of soft collagen–rich tissues. The elongation response of these tissues to an applied force is non-linear. Initially, a relatively large amount of elongation is achieved for a relatively low force before the tissue responds in an essentially linear manner obeying Hooke's Law. At high forces, tissue damage occurs and the force/elongation curve flattens out before complete failure occurs (Fig. 7.7). The term 'stiffness' refers to the amount of force required to stretch the tissue by a given amount and when expressed per unit area of tissue provides the material modulus. In most studies, the modulus in the linear portion of the loading curve is most often used to describe the mechanical behaviour of the tissue. Interestingly, a number of *in vitro* studies have found that the linear modulus in human tendon showed no change with increasing age (Flahiff et al. 1995; Hubbard and Soutas-Little 1984; Johnson et al. 1994) or in fact, a decline in the Achilles tendon (Thermann et al. 1995) and patella tendon (Blevins et al. 1994). Findings are similar for *in vivo* studies with a number of studies

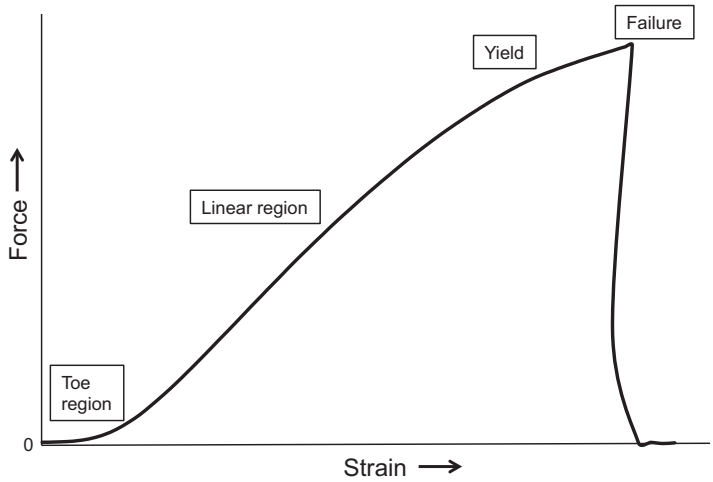

Fig. 7.7 The typical curve shape obtained when strain (elongation) is plotted against applied force for soft collagen-rich tissues such as tendon, ligament and dermis

reporting no change or a decrease in tendon stiffness with increasing age (Csapo et al. 2014; Karamanidis and Arampatzis 2006; Stenroth et al. 2012). Similarly in equine tendon, the linear modulus of the whole tendon was found not to differ significantly in older age horses compared to young horses (Thorpe et al. 2013b).

Studies investigating the sub-components of tendon; fascicles, fibres and the inter-fascicular matrix, suggest that the main changes with ageing are observed at the micro-scale level. These studies have also revealed differences in the mechanism for elongation between spring-like tendons, where a relatively large degree of elongation is required, and positional tendons where efficient function depends on very limited stretch under physiological loads. In the equine superficial digital flexor tendon (SDFT); one of the most highly adapted energy storing tendons, the inter-fascicular matrix is less stiff compared to the 'positional' common digital extensor tendon (CDET) allowing sliding between fascicles (Thorpe et al. 2012). Although the linear modulus of the fascicles does not differ between these two tendon types, elongation is achieved through different mechanisms. In the SDFT fascicle elongation appears to be achieved by the uncoiling of a helical structure of the fascicle whereas in the CDET the fibres within the fascicles slide relative to each other (Thorpe et al. 2013a). Increasing age results in a stiffening of the inter-fascicular matrix in the SDFT, although no change to the modulus of the fascicle (Thorpe et al. 2013b). The stiffening of the inter-fascicular matrix suggests that the fascicles are likely to be loaded earlier during tendon extension in older tendons (Thorpe et al. 2013b). There is an enrichment of elastin in the inter-fascicular matrix of the SDFT and, with ageing, this decreases in quantity and becomes more disorganized (Godinho et al. 2017), which may explain the stiffening as load is transferred to the collagen component of the inter-fascicular matrix. With ageing, the fascicles and the inter-fascicular matrix in the SDFT have a reduced ability to

withstand repeated loading and unloading cycles (Thorpe et al. 2017), and this decrease in fatigue resistance is associated with a reduced helical structure of the fascicle (Thorpe et al. 2014).

In main arteries, such as the aorta and carotid arteries, the evidence for an increase in stiffness is more compelling (Greenwald 2007). The elastin content of these arteries is much higher than in tendon; values of 40% elastin have been measured in human aorta in young individuals (Hosoda et al. 1984) and this provides the artery with a compliant and elastic response to applied force. Elastin content however decreases significantly during ageing and although the absolute amount of collagen may also decrease, the relative proportion of collagen increases (Hosoda et al. 1984). Collagen is 100–1000 times stiffer than elastin (Greenwald 2007) thus it seems likely that mechanical load is transferred to collagen fibres and this is responsible for an overall increase in resistance to stretch and reduced ability to recoil.

In skin, reported values for modulus vary widely (Kalra and Lowe 2016) and this is probably due in part due to the variation in experimental technique for measuring mechanical properties in skin. There is however evidence for an increase in stiffness with increasing age (Diridollou et al. 2001) and the scenario may be similar to blood vessels where a decrease in elastin content results in a more dominant role of collagen and the apparent increase in stiffness.

Effect of AGE Crosslinking on Mechanical Properties of the ECM

A number of studies have attempted to quantify changes to mechanical properties of collagen-rich tissues following AGE modifications using *in vitro* models. Incubation of rabbit Achilles tendon with ribose resulted in increased load to failure, increased maximum stress, increased Young's modulus and increased toughness (energy to failure) and a reduced solubility of tendon (Reddy et al. 2002). Incubation of rabbit Achilles tendon with glucose showed similar changes to those seen with ribose, although glucose incubation had no effect on the mechanical properties of femur and tibia bones from rats (Reddy 2003). Later studies have investigated the effect of incubation of rat-tail tendon fascicles in methylglyoxal at the fascicle, collagen fibre and collagen fibril level. One study showed that methylglyoxal incubation did not change the tendon fascicle stiffness but was associated with an abrupt failure and loss of the yield phase resulting in a higher ultimate stress (Li et al. 2013) while another showed an increase in fascicle failure stress and strain, peak modulus and energy to failure (Svensson et al. 2018). In the study by Li et al. (2013), rat-tail tendon fascicles that had not been incubated in methylglyoxal extended predominantly by fibre sliding however following methylglyoxal incubation, fibre sliding reduced and fibre stretching dominated the extension response; similar results were seen with ribose incubation (Gautieri et al. 2017). At the collagen fibril level, methylglyoxal incubation did not affect the fibril stiffness but resulted in a sudden failure

and higher fibril strain at failure although reduced collagen molecule sliding within the fibril (Fessel et al. 2014) and again similar results were seen following ribose incubation (Gautieri et al. 2017). Another study found that fibril modulus increased following methylglyoxal treatment alongside an increase in failure stress, failure strain and energy to failure (Svensson et al. 2018). There are however limitations to these *in vitro* studies as the chemical nature of the AGE products present was not determined, and the glycation agents used are unlikely to produce physiologically relevant AGE crosslinks.

Glucose is by far the most abundant sugar in healthy individuals and therefore most likely to be involved in the Maillard reaction. The involvement of glucose is supported by the relatively high levels of glucosepane in collagen from aged-healthy and diabetic dermis and glomerular basement membrane compared to AGE crosslinks formed from methylglyoxal and ribose (Sell et al. 2005). The impact that glucosepane has on tissue mechanical properties has not been measured directly but is implied through the association of glucosepane with the complications of diabetes. For example diabetic patients have a higher risk of Achilles tendon injuries (Ranger et al. 2015) and have been shown to have a higher material stiffness in the Achilles tendon compared to age-matched controls (Couppe et al. 2016). Evidence to support an effect of glucosepane on collagen mechanics has also come from computational studies. Using a fully atomistic molecular dynamics simulation of type I collagen in a fibrillar environment, six potential sites were identified where glucosepane formation would be feasible and energetically favourable (Collier et al. 2015). Using a steered molecular dynamics approach, the introduction of glucosepane at each of these sites showed an increase in the tensile Young's modulus by 3.0–8.5% in the initial phase of elongation (0–15%) of the collagen molecule (Collier et al. 2018).

Effect of AGE Crosslinking on Biochemical Properties and Interaction with Other Molecules of the ECM

The addition of chemical groups to collagen molecules changes the charge profile and attraction for other ions and molecules within the matrix as well as resulting in steric hindrance for binding of bioactive molecules and cell-matrix interactions. Collagen molecules have a close association with water molecules, which reside within and around the collagen triple helix in different forms and have an impact on the physical properties of the collagen (Miles et al. 2005; Miles and Ghelashvili 1999). Water molecules form part of the structure of the collagen molecule and include hydrogen-bonded bridges between the polypeptide chains within the collagen molecules (Ramachandran and Chandrasekharan 1968), hydrogen bonded chains of water lying in the cleft of the collagen triple helix and water attracted to the hydrophilic surfaces of the molecule. In addition 'free' water resides around the hydrophobic surfaces of the collagen molecules within the collagen fibril and

between collagen fibrils (Fullerton and Amurao 2006). The AGE crosslink glucosepane has been shown to be very hydrophilic (Nash et al. 2016) and this suggests that there maybe an increase in hydration of collagen fibrils in ageing ECM and associated changes in physical properties.

The locations of PTMs within the collagen molecule, and other matrix proteins, determine the potential for interference with the interaction with other matrix and cell proteins. The six sites identified in the study by Collier et al. (2015) with potential for glucosepane formation in collagen overlap with a number of other important interaction sites (Sweeney et al. 2008) for example decorin, heparin, heat shock protein 47 and interleukin-2 and MMP-1/13 cleavage site (Collier et al. 2015). The close proximity of the collagenase cleavage site to that of AGE crosslink formation suggests that glucosepane may interfere with collagen degradation. Interestingly, experimental studies have shown that collagen fascicles incubated in ribose are five times more resistant to collagenase degradation than control fascicles; the fascicles however were highly susceptible to cleavage when mechanical load was applied (Bourne et al. 2014). Six potential sites for DOGDIC formation in type I collagen have also been identified and only one of these is common to both glucosepane and DOGDIC (Collier et al. 2016) suggesting an AGE crosslink specific impact on collagen function.

Effect of AGE Crosslinking on Matrix-Cell Interactions

The modification of collagen at key cell interaction sites has the potential to disrupt sensing of mechanical environment and interpretation of mechanical signals into an appropriate cell response. For example, the potential sites identified for the formation of glucosepane in collagen (Collier et al. 2015) overlap with integrin binding sites suggesting a possible impact on mechano-transduction and cell phenotype.

In addition to a direct effect on the ECM, AGEs and AGE crosslinks have been found to modulate a range of cell functions suggesting cell surface binding sites for AGE and an indirect effect on the ECM. The receptor found to be responsible for mediating these intracellular events is a 35 kDa cell surface receptor for AGE (RAGE) and a member of the immunoglobulin superfamily (Neeper et al. 1992). The receptor is present on the cell surface of many different cell types including endothelial cells, mononuclear phagocytes, smooth muscle cells, mesangial cells, neurons (Schmidt et al. 1994) and lung epithelial cells and fibroblasts (Queisser et al. 2008). RAGE has been implicated in numerous cell behavior changes and cell signaling processes. The activation of RAGE results in the generation of reactive oxygen species (ROS) through stimulation of NADPH oxidase (Wautier et al. 2001) resulting in oxidative stress (Tan et al. 2007; Wautier et al. 2001). Levels of the transcription factor NFκB are up-regulated in response to ROS resulting in increased expression of the cytokine TNFα (Gao et al. 2008; Kay et al. 2016) and a cascade of inflammatory cytokine release. Other pro-inflammatory cytokines are also up regulated such as IL-β1 and IL-6 providing AGEs and RAGE a clear role in inflammation.

RAGE has been implicated in a broad range of disease processes including diabetes, chronic kidney disease, cardiovascular disease, cancer, alzheimer's disease (Perrone et al. 2012) and Parkinson's disease (Jiang et al. 2018). RAGE may also play a role in ageing in healthy individuals by contributing to 'inflammageing' (Franceschi et al. 2000); the chronic low grade inflammation associated with ageing.

Conclusions

The ECM is composed of a variety of proteins and glycoproteins but is dominated by collagen, and in some more compliant tissues, elastin. Collagen and elastin are long-lived proteins and as a result are particularly susceptible to accumulation of non-enzymatic PTMs and fragmentation resulting from enzymatic cleavage. The most important PTM products appear to be AGEs especially AGE crosslinks and possibly the more recently identified homocitrulline following carbamyalation. These modifications are likely to influence the mechanical and structural role of the ECM directly. In addition, PTMs are likely to impact on function of the ECM indirectly through altered interactions with other bioactive molecules and through cell receptors such as RAGE by activating cell-signalling cascades leading to changed cell behavior, phenotype and inflammation. There is much to be learnt in the ageing of the ECM and protein PTM field of research where a better understanding of the pathways involved, the species formed and the preferential protein sites will allow impact on function to be determined. Strategies to reduce adventitious PTMs over the life course hold great promise for increasing health span in the ageing population.

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Chapter 8
Vitamin D and Ageing

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Abstract One hundred years has passed since the discovery of vitamin D as the active component of cod-liver oil which cured the bone disease rickets. Since then our knowledge of vitamin D has expanded tremendously and has included recognition of the importance of UV radiation as a source of the vitamin as well as the discovery of the vitamin as a nutrient, a pro-hormone and a potent steroid hormone with a major role in calcium and bone metabolism. In the last 25 years or so, the discovery of the vitamin D receptor in over 30 different body tissues together with the existence of the alpha-1-hydroxylase enzyme in these tissues provided evidence of a pleiotropic role of vitamin D outside its classical role in the skeleton. These important discoveries have provided the basis for the increasing interest in vitamin D in the context of nutritional requirements for health including the prevention of chronic diseases of ageing. The recent publication of the Dietary Reference Intake

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report on vitamin D and calcium by the North American Institute of Medicine (IOM) is the most comprehensive report to date on the basis for setting nutritional requirements for vitamin D. This chapter will summarize the nutritional aspects of vitamin D and discuss the changes in vitamin D metabolism and requirements with ageing. It will summarize key evidence on the relationship between vitamin D status and some of the main ageing related health outcomes including bone, muscle and cognitive health as well as survival focusing on the published literature in very-old adults (those >= 85 years of age).

Keywords Vitamin D · Very-old adults · Metabolism and function · Nutritional requirements · Epidemiology · Musculoskeletal health · Cognitive health · Mortality

Vitamin D Metabolism and Function

The term 'vitamin D' was given during the early 1920's to a group of closely-related secosteroids with antirachitic properties. Two of the most important nutritional forms of vitamin D are cholecalciferol (vitamin D_3, derived from animal origin) and ergocalciferol (vitamin D_2, derived from plant origin). However, natural dietary sources of vitamin D are limited with oily fish, egg yolk and meat contributing up to 90% of vitamin D intake from non-fortified food sources (Hill et al. 2004). Vitamin D_3 and D_2 can also be derived by photoirradiation from their precursors 7-dehydrocholesterol and ergosterol, respectively. In vertebrates, the cholesterol-like precursor, 7-dehydrocholesterol, present in the skin epidermis, undergoes photolysis when exposed to UV-B-light of wavelengths 290–315 nm to yield a variety of photoirradiation products including tachysterol, lumisterol and previtamin D_3. Previtamin D_3 then undergoes spontaneous thermal rearrangement to vitamin D_3. Because of the skin's ability to synthesise the vitamin upon exposure to appropriate sunlight, vitamin D is only an essential nutrient when sunlight is limited.

Vitamin D_3 (obtained from dermal synthesis or from dietary sources), which is biologically inactive, is transported via vitamin D binding protein (DBP) to the liver where it is hydroxylated at the C-25 position by the 25-hydroxylase enzyme [CYP2R1] to yield 25-hydroxyvitamin D_3 [25(OH)D or calcidiol] which is the most commonly used index of vitamin D status (Ross et al. 2010). The CYP2R1 enzyme regulates 25-hydroxylation of vitamin D_3 to produce $25(OH)D_3$, which is dependent on the concentrations of vitamin D_3 in circulating/plasma. From the liver, $25(OH)D_3$ is returned to the circulation, bound to DBP, and transported to the kidney where the enzyme 1-α-hydroxylase [CYP27B1] converts it to 1,25-dihydroxycholecalciferol ($1,25(OH)2D3_3$ or calcitriol), which is the major active metabolite of vitamin D. When $1,25(OH)2D3_3$ is in excess, the enzyme 24-hydroxylase (CYP24) in the kidney converts $25(OH)D_3$ to 24,25-dihydroxycholecalciferol, which is believed to be biologically inactive. Furthermore, $25(OH)D_3$ can be converted to other inactive metabolites such as 23,25-dihydroxycholecalciferol, 25,26-dihydroxycholecalciferol and 1,24,25-trihydroxycholecalciferol and

excreted mainly in faeces, but the biological roles of these metabolites are not well understood [for reviews, *see* Horst and Reinhardt 1997; Holick 2003].

The major biological role of $1,25(OH)2D3_3$ is to promote intestinal calcium absorption. In addition, $1,25(OH)2D3_3$ increases the absorption of other essential minerals across the intestine, such as phosphorus, magnesium, zinc and manganese (Biehl et al. 1995; Krejs et al. 1983), and enhances the net renal reabsorption of calcium and phosphorus (Singh and Dash 1997). Thus, $1,25(OH)_2D3_3$ is a major regulator of calcium homeostasis. The classical target organs for $1,25(OH)_2D_3$ are the intestine, bone, the kidneys and the parathyroid glands however, $1,25(OH)_2D_3$ also acts at several sites in the body in an intracrine or paracrine manner (White 2012). Normal physiological concentrations of calcium are required for proper neuromuscular and cellular functions. Low circulating calcium (hypocalcaemia) stimulates the secretion of parathyroid hormone (PTH) from the parathyroid gland, which, in turn, enhances the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ acts on the intestine, kidneys and bone to restore normal circulating calcium concentrations. In addition to PTH, it is also well recognised that other hormones, such as calcitonin, glucocorticoids, growth hormones and sex steroids regulate the production of $1,25(OH)_2D_3$ (Lal et al. 1999). In addition to its classical role in the skeleton, a number of key hydroxylase enzymes together with Vitamin D Receptors (VDR) have been identified in over 30 different extra-skeletal tissues suggesting an important regulatory role of vitamin D in these target tissues (Lal et al. 1999). Furthermore, data from epidemiological and (some) intervention studies have provided fascinating and really exciting hypotheses about relationships between vitamin D status and risk of several chronic conditions [including multiple sclerosis, tuberculosis, rheumatoid arthritis, cardiovascular disease, hypertension, cognitive decline, lung conditions and certain cancers; [for reviews *see* Ross et al. 2010; Wang 2009]. The biological actions of 1,25(OH)2D3 in target tissues are mediated either through:

(i) a nuclear vitamin D receptor (VDR), which, once complexed with $1,25\ (OH)_2\ D_3$ and retinoic acid receptors (RXR), can regulate gene expression (genomic effects),
(ii) intra-cellular signalling pathways activated through putative plasma membrane receptors (non-genomic effects) (Lal et al. 1999).

It is well established that $1,25(OH)_2D_3$ is essential for the normal growth and development of bone. In bone cells, $1,25(OH)_2D_3$ acts on osteoblasts to increase osteoclastogenesis and bone resorption which contribute to mineral homeostasis (Turner et al. 2012). The discovery of the molecular triad of receptor activator of nuclear factor kappa (RANK), RANK–ligand (RANKL) and osteoprotegerin (OPG) [RANK/RANKL/OPG] in the 1990's represented a significant breakthrough in the understanding of the pathophysiology of bone remodelling [for review *see* Theoleyre et al. 2004]. RANK, on the surface of osteoclasts binds to its ligand (RANKL) present on surface of osteoblasts following their stimulation by $1,25(OH)_2D_3$. Binding of RANK to RANKL initiates the maturation of osteoclasts and is enhanced by the antagonistic effect of $1,25(OH)_2D_3$ on the protein OPG. As OPG normally binds RANKL, it prevents binding to RANK therefore inhibiting osteoclast maturation. It should be noted that $1,25(OH)_2D_3$ also regulates the transcription of a number of

key osteoblastic genes such as those coding for the bone proteins osteocalcin, osteopontin, osteonectin and proteoglycan (Martin and Seeman 2008).

Changes in Vitamin D Metabolism with Ageing

Calcium Absorption

Calcium is absorbed from the bowel by an active vitamin D dependent transport mechanism and by passive diffusion. The active transport mechanism plays an important role in calcium homeostasis, as the amount absorbed is inversely related to dietary calcium intake (Ireland and Fordtran 1973). Fractional calcium absorption therefore increases when dietary calcium intake is reduced (Dawson-Hughes et al. 1993). Calcium absorption decreases with advancing age (Bullamore et al. 1970), which has been attributed to a number of mechanisms, including the reduction in circulating 25(OH)D with age (Baker et al. 1980), impaired hydroxylation of 25(OH)D to $1,25(OH)_2D_3$ with declining renal function (Francis et al. 1984), resistance to the action of vitamin D metabolites on the bowel mucosa (Eastell et al. 1991) and low circulating oestrogen concentrations in women after the menopause (Heaney et al. 1989). Increasing circulating 25(OH)D concentrations by oral vitamin D supplementation improves calcium absorption in older women, but this is attenuated by renal impairment (Francis et al. 1983), suggesting that lower levels of substrate circulating 25(OH)D) and impaired hydroxylation of 25(OH)D to $1,25(OH)_2D_3$ both contribute to the decrease in calcium absorption with age. Despite the inverse relationship between dietary calcium intake and calcium absorption, the increase in calcium absorption when dietary calcium is reduced is less marked in older people than younger adults (Ireland and Fordtran 1973). This may be due to reduced production of $1,25(OH)_2D_3$, but it may also reflect resistance to the actions of vitamin D metabolites on the bowel, as some studies have shown an attenuated response in calcium absorption to increases in $1,25(OH)_2D_3$ in older women (Eastell et al. 1991). Although the decline in calcium absorption with advancing age is multifactorial in origin, the improvement in absorption with vitamin D supplementation suggests that vitamin D deficiency is the major cause of malabsorption of calcium in older people (Francis et al. 1983). The positive relationship between circulating 25(OH)D and fractional absorption extends to 25(OH)D concentrations above 100 nmol/L (Francis et al. 1983; Gallagher et al. 2012), leading some experts to advocate that these concentrations are necessary for optimal bone health. Nevertheless, although a recent randomised controlled trial comparing the effect of different doses of vitamin D showed higher calcium absorption in subjects with a circulating 25(OH)D of 75 nmol/L than those with 50 nmol/L, the magnitude of the difference was small (Gallagher et al. 2012).

Renal 1 α Hydroxylase

Renal function declines with advancing age and this is accompanied by a decrease in circulating $1,25(OH)_2D_3$ concentration (Epstein et al. 1986). As mentioned above, the effect of vitamin D supplementation on calcium absorption is attenuated by renal impairment (Francis et al. 1983). An early study showed that as glomerular filtration rate (GFR) falls below 50 ml/min, there is a reduction in circulating $1,25(OH)_2D_3$ and lower fractional absorption of calcium (Francis et al. 1984), together with an increased circulating parathyroid hormone (PTH). Other studies show an inverse relationship between circulating 25(OH)D and PTH across all adult age groups, but that PTH is higher in older people than young adults for any given circulating 25(OH)D concentration (Vieth et al. 2003) possibly due to reduced renal 1 α hydroxylation.

Dermal Vitamin D Production

The dermal capacity to produce vitamin D in persons aged 65 years has been estimated to be about 25% of that in persons aged 20–30 years exposed to the same amount of sunlight (Holick et al. 1989; MacLaughlin and Holick 1985). This reduction cannot be explained by the decrease in mass of the epidermis with ageing, but rather seems to be related to the reduction in the concentration of skin 7-dehydrocholesterol. Other indirect factors which affect exposure to sunlight in older adults include the wearing of more concealing clothing (Matsuoka et al. 1992), an increased use of sunscreen (Holick 1994), and reduced sun exposure, arising from less physical activity and time outdoors compared with younger age groups (Health Survey for England 2008).

Changes in VDR Numbers

Vitamin D deficiency is associated with muscle weakness which potentially increases the risk of falls and fractures, possibly mediated through effects on $1,25(OH)_2D_3$ receptors which have been discovered in muscle (Simpson et al. 1985; Bischoff et al. 2001). Bischoff-Ferrari et al. demonstrated a strong negative correlation between age and VDR expression in muscle as measured by the number of VDR-positive nuclei per 500 counted nuclei (Bischoff-Ferrari et al. 2004). This association was independent of biopsy location and circulating 25(OH)D concentrations. This finding may have significant clinical ramifications in older age owing to the importance of $1,25(OH)_2D_3$ in regulating transcription of muscle related genes. The role of vitamin D in muscle atrophy in older adults has been reviewed by Dawson- Hughes (2012) and will be discussed later in this chapter.

Nutritional Aspects of Vitamin D

Assessment of Vitamin D Status

Circulating 1,25(OH)2D3$_3$ concentrations are under homeostatic control, which limits its value as a nutritional marker of vitamin D status (Ross et al. 2010). However, circulating or plasma total 25(OH)D [i.e. that derived from adding 25(OH)D_2 and 25(OH)D_3] concentration is widely accepted as a good biomarker of vitamin D status, since the concentration of this metabolite closely reflects the amount of vitamin D synthesized in the skin and ingested in the diet (Ross et al. 2010). During winter, in countries of latitudes greater than 40° North or South the skin is incapable of synthesizing vitamin D for 4–5 months of the year as sunlight must pass a much longer distance through the atmosphere and most UV-B-light is absorbed by the atmosphere, preventing any effective UV irradiation of the skin (Webb et al. 1988). Therefore, it is assumed that during winter the circulating 25(OH)D concentration is directly related to late-summer concentrations, oral intake and body stores of its precursor vitamin D_3. While circulating 25(OH)D is generally regarded as a good biomarker of exposure [i.e. that derived from sun and diet], its use as a biomarker of function and outcome is less clear owing to the multitude of factors influencing this prohormone (Prentice et al. 2008). Notwithstanding such difficulties, the concentration of 25(OH)D is widely used to diagnose vitamin D deficiency in both the clinical and non-clinical settings.

Dietary Vitamin D Requirements and Vitamin D Intakes

Using the risk-assessment framework commonly used to set Upper Levels for nutrients, the Institute of Medicine (IOM) in their recent Dietary Reference Intake (DRI) report (Ross et al. 2010) set a 25(OH)D concentration of 30 nmol/L as indicative of vitamin D deficiency based on integrating a number of key bone health outcomes, including rickets, osteomalacia, impaired calcium absorption and lower BMD. The nature of the relationship between 25(OH)D concentration and bone health outcomes will be discussed in detail later in this review. It is noteworthy that the IOM committee concluded that there was insufficient evidence to define vitamin D deficiency based on non-skeletal outcomes. Based on the relationship between 25(OH)D status and those aforementioned bone health outcomes, and using both data from epidemiological and intervention studies, the IOM established a population 25(OH)D concentration of 40 nmol/L and 50 nmol/L as the basis for setting an Estimated Average Requirement (EAR) of 10 μg/day and a Recommended Daily Allowance (RDA) of 15 μg/day, respectively in people aged 1–70 years. The IOM set an RDA of 20 μg/day for individuals aged >70 years, while it could only establish an Adequate Intake (AI) of 5 μg/day for infants <1 year (Ross et al. 2010). The EAR is the amount of a nutrient which meets the needs of half (50%) the population while

the RDA is the amount of a nutrient which will meet the needs of practically all (97.5%) healthy persons in a population. The AI is an estimation of the observed dietary intake of an asymptomatic population. The approach and conclusions of the recent IOM report (Ross et al. 2010) was a significant deviation from those of the previous IOM DRI report of 1997 (Institute of Medicine 1997) in that for the first time an EAR and RDA was established for children and adults. In the past only an AI of 5 μg/day could be derived for persons up to 70 years (Institute of Medicine 1997). Two of the caveats of the IOM report are that the vitamin D recommendations (1) assume an adequate dietary calcium intake and (2) assume a negligible contribution from sunlight to 25(OH)D concentrations. It is also noteworthy that in terms of adverse effects, the Tolerable Upper Intake Level (UL) for vitamin D which is the highest level of daily consumption that current data have shown to cause no side effects is 100 μg/day (Ross et al. 2010) while in the older DRI report (Institute of Medicine 1997) it was set at 50 μg/day. In 1998, the UK Committee on Medical Aspects of Food and Nutrition Policy (COMA) concluded that a prudent public health approach to safeguard against vitamin D deficiency and its adverse effect on bone health would be to retain the Reference Nutrient Intake set in 1991 (10 μg/d for those aged >65 year).

The Scientific Advisory Committee for Nutrition (SACN) in the UK re-evaluated nutritional requirements for vitamin D for the British population in 2016 (SACN 2016). The findings of the report suggests a Reference Nutrient Intake (RNI) of 10 μg/day (400 IU/day), throughout the year, for everyone in the general UK population aged 4 year and above. The approach used in deriving the new RNIs involved determining the dietary input of vitamin D required to keep the circulating 25(OH)D above 25 nmol/L (the population protective cut-off to protect musculoskeletal health) (SACN 2016). The RNI of 10 μg/day (400 IU/day) for the general UK population includes pregnant and lactating women and population groups at increased risk of vitamin D deficiency. Since, there were insufficient data to set RNIs for children aged under 4y, Safe Intakes were recommended for this age group (8.5–10 μg/340–400 IU per day for all infants aged under 1year and 10 μg/400 IU per day for ages 1 up to 4 year). SACN were unable to quantify and take account of sunlight exposure in setting the DRVs because of the number of factors that affect endogenous vitamin D synthesis.

There can be no doubt (and ample evidence exists) that dietary vitamin D intakes are a concern in large proportions of the European population [for review see Kiely and Black 2012]. For example, mean dietary vitamin D intakes (including that from supplements) are between 4 and 5 μg/day among adults from National Diet and Nutrition Surveys in the UK, mostly from meat, fish and eggs, fortified foods and supplements. Therefore, current vitamin D intakes are considerably lower than recommendations and urgent dietary-based strategies are needed to bridge the gap.

Circulating 25(OH)D Concentrations in Older Age

An extensive array of studies including a mix of both representative and convenience sampling frames have reported 25(OH)D concentrations among older adults all over the globe (Wahl et al. 2012; Mithal et al. 2009; Ovesen et al. 2003). Without doubt, the region with the most available data on 25(OH)D concentrations is Europe, followed by North America and Asia. Limited data exist for South America and Africa with very few studies in children and adolescents in these regions (Wahl et al. 2012). Cross sectional data predominate and year round 25(OH)D concentrations are only available in some studies. In addition, comparisons of the prevalence of hypovitaminosis D between studies is compounded by the heterogeneity with regard to circulating 25(OH)D concentrations used to define vitamin D status. Furthermore, the very low calcium intakes seen in some communities complicate the interpretation and subsequent treatment of vitamin D deficiency in these population groups. Data from three multi-centred, standardized studies show that between 17 and 58% of older Europeans are vitamin D deficient (defined as circulating 25(OH)D) less than 30 nmol/L (Van der Wielen et al. 1995; Andersen et al. 2005; Lips et al. 2006). National representative data on 25(OH)D concentrations from the National Diet and Nutrition Surveys in UK adults aged over 64 years show that up to 10% of free-living and 40% of institutionalized adults have plasma 25(OH)D concentrations less than 25 nmol/L throughout the year [reviewed by Lanham-New et al. 2011]. Moreover, if the higher IOM cut point of 40 nmol/L is applied (defining an EAR) the proportion of adults with inadequate 25(OH)D concentrations rises considerably. While older adults are well-established as a 'at risk' group for vitamin D deficiency, it should be noted that ethnic populations residing in less sunnier climates are also particularly at risk of vitamin D deficiency. For example, in a large study of vitamin D status among South Asian (*n* 1105) and Black African and Caribbean adults (*n* 748) >45 years living in the West-Midlands region of the UK (Patel et al. 2013) plasma 25(OH)D concentrations <30 nmol/L were found in 76% of South Asians and 55% of Black African and Caribbean adults throughout the year. Another study involving 35 South Asians living in Surrey (Darling et al. 2013) found that 81% and 79% of the participants had circulating 25(OH)D concentrations <25 nmol/L during winter and autumn, respectively. These studies suggest an extremely high prevalence of vitamin D deficiency in these population groups which require urgent attention. Despite recent concerns about the high prevalence of vitamin D deficiency in much of the British adult and paediatric population [Scientific Advisory Committee for Nutrition 2016] there is a dearth of data on vitamin D status, and its predictors, in very old adults.

Recent data from a large broadly representative cohort of 85 year olds from the Newcastle 85+ study, UK showed that vitamin D deficiency [as defined by a circulating 25(OH)D concentration <30 nmol/L] is alarmingly high at all times of the year but particularly during winter and spring (Hill et al. 2016). Season of the year and use of vitamin D containing preparations (both supplements and medications) were strong predictors of 25(OH)D concentrations in these very old adults. In a cross-sectional investigation of 25(OH)D concentrations among 367 Belgian 80+

year olds, 20% and 66% had circulating 25(OH)D concentrations <25 and 50 nmol/L, respectively (Matheï et al. 2013). In a recent osteoporosis screening trial investigating the anti-fracture efficacy of a new anti-osteoporotic drug, 25(OH)D concentrations were measured at baseline in 1894 individuals aged 80+ years from 9 different European countries (Bruyère et al. 2014). Mean (SD) 25(OH)D concentrations were 53.3 (26.7) nmol/L in the entire cohort while circulating 25(OH)D concentrations showed wide geographical variation with the lowest mean 25(OH)D concentration (45.7 nmol/L) in Belgian participants and the highest mean concentration (81.7 nmol/L) in Spanish participants (Bruyère et al. 2014). The British participants in the Bruyère et al. study (region not specified) had a mean 25(OH)D concentration of 61.8 nmol/L with 22% of the participants having 25(OH)D concentrations <50 nmol/L. These 25(OH)D concentrations are considerably higher than those observed in Newcastle 85+ participants despite the fact that both studies used the same analytical assay for 25(OH)D (DiaSorin RIA).

While no information was available on season from the European study (Bruyère et al. 2014) the use of non-prescribed vitamin D containing supplements was high at >30% among the British participants (Bruyère et al. 2014) and higher than in Newcastle 85+ participants (19%), which agrees with the evidence that vitamin D supplements have a significant effect on circulating 25(OH)D in older adults (Cashman et al. 2009). An extensive array of studies, including both representative and convenience sampling frames have reported 25(OH)D concentrations among the younger old (generally >65 years, but <85 years) all over the globe (Wahl et al. 2012; Mithal et al. 2009). Comparisons of the prevalence of suboptimal D status between studies is compounded by the heterogeneity with regard to circulating 25(OH)D concentrations used to define vitamin D status. For example it has been estimated that between 17 and 58% of older Europeans are vitamin D deficient (circulating 25(OH)D) <25–50 nmol/L (Van Der Wielen et al. 1995; Andersen et al. 2005). Furthermore, the well recognized analytical variability in assays for 25(OH)D compounds further the comparison of vitamin D status measurements between studies (Carter, 2011). In the Newcastle 85+ study, the observation that institutionalized participants had significantly higher circulating 25(OH)D concentrations than their community dwelling counterparts is noteworthy (Hill et al. 2016). These differences are explained primarily by a greater use of prescribed vitamin D containing preparations in institutionalized participants which tend to contain higher amount of vitamin D than over the counter supplements (*see* below). For example, 45% and 14% of institutionalized and community-dwelling participants respectively took prescribed medicines containing vitamin D. Indeed, use of prescribed and non-prescribed vitamin D preparations were strong independent predictors of vitamin D status in the entire cohort. These findings agree with the commonly held view that vitamin D medication and supplement use are strong predictors of 25(OH)D in older adults (Van der Wielen et al. 1995). For example in a large European multicentre study of vitamin D status of older adults >65 years (Van der Wielen et al. 1995) mean circulating 25(OH)D concentrations were significantly higher in Norwegian participants (51 nmol/L) than Spanish participants (34 nmol/L) which was explained in part by high consumption of cod liver oil in Norwegian participants.

Indeed some studies show that 25(OH)D concentrations are inversely associated with risks of death due to cardiovascular disease, cancer, and other causes (reviewed later in the chapter). On the other hand, limited evidence suggests that there may be a U-Shaped association between 25(OH)D and various health outcomes including cognition and all-cause mortality (reviewed later in the chapter). Such findings may lend support to the need to define 'normal' and 'healthy' reference range for 25(OH)D concentrations in very old adults. This is vitally important as a 25(OH)D threshold >70–80 nmol/L across the lifecycle has been regarded by some as 'optimal' for health (Heaney and Holick 2011). Furthermore, there is a need to determine the vitamin D requirements in very old adults specifically since recommendations are generally set for those aged 65+ and the needs of those aged 85+ may not be the same as for those aged >65 but <85 years.

Vitamin D and Bone Health in Older Age

The latest report from the Scientific Advisory Committee on Nutrition (SACN, 2016) defined the threshold of 25 nmol/L as 'population protective level' for musculoskeletal health in the UK population, including older adults, whilst the IOM (Ross et al. 2010) did not support 25(OH)D concentrations >50 nmol/L (i.e. above deficiency threshold) as beneficial for non-skeletal health outcomes, warranting more research. Severe vitamin D deficiency is defined by a circulating 25(OH)D less than 25 nmol/L, which corresponds to the upper end of the range at which vitamin D deficiency osteomalacia and rickets has been observed (Prentice et al. 2008). However, higher levels of circulating 25(OH)D have been associated with secondary hyperparathyroidism, increased bone resorption, bone loss, impaired muscle function and an increased risk of falls and fragility fracture, and there remains contention about the thresholds applied. These outcomes will be reviewed in the next two sections of this chapter.

Osteomalacia

Recommended circulating levels of 25(OH)D in adult life are commonly set against the clinical risk of developing osteomalacia, although falls and fracture risk are important considerations. The gold standard diagnostic test for mineralisation disorder associational with vitamin D deficiency (vitamin D deficiency osteomalacia) is the identification of mineralisation defect with increased osteoid thickness and reduced calcification fronts, which are identified by bone histomorphometry after tetracycline labelling. However, population based studies, using this invasive technique, are impractical. One recent study used bone histomorphometry in post-mortem specimens in Germany, apparently finding that abnormal bone mineralisation was only seen in a proportion of subjects whose circulating 25(OH)D was less than

75 nmol/L (Priemel et al. 2010). The study has been criticised because it uses post mortem bone histomorphometry without tetracycline labelling, so both generalisability is compromised and causes other than vitamin D deficiency may explain histomorphometric changes seen, while the use of such post-mortem data to make dietary recommendations seems bizarre (Aspray and Francis 2013). This theme has been addressed comprehensively in the IOM report (Ross et al. 2010) where, even ignoring the technical limitations in Priemel's study, osteomalacia is sometimes reported at circulating 25(OH)D levels less than 30 nmol/L but rarely observed at 25(OH)D levels greater than 50 nmol/L.

Secondary Hyperparthyroidism

The circulating concentration of 25(OH)D below which parathyroid hormone (PTH) increases outside the normal range may be used to establish a threshold value for vitamin D insufficiency and this is of particular importance for bone metabolism, because an elevated PTH is associated with increased bone loss (Bischoff-Ferrari et al. 2006, 2008; Sahota et al. 2001, 2004; Rejnmark et al. 2011). The relationship of circulating blood levels of 25(OH)D to PTH is contentious. Some studies suggest that PTH reaches a plateau with increasing circulating 25(OH)D concentration (Chapuy et al. 1997; Lappe et al. 2006), while others demonstrate an inverse relationship throughout the physiological range of 25(OH)D concentrations (Vieth et al. 2003; Arabi et al. 2010; Bates et al. 2003; Durazo-Arvizu et al. 2010; Sahota et al. 2006). It is important to consider that the relationship between 25(OH)D and PTH may be influenced by the effects of many other factor including comorbidities. Advancing age, dietary calcium and phosphate intake, renal function, plasma vitamin D binding protein (DBP), magnesium concentration, IGF-1, testosterone, smoking and physical inactivity may all have important roles in the development of secondary hyperparathyroidism (Vieth et al. 2003; Arabi et al. 2010; Durazo-Arvizu et al. 2010; Sahota et al. 2006; Gunnarsson et al. 2009). Moreover, comparisons between studies may be hampered by the use of different assays for 25(OH)D and PTH (Lai et al. 2012; Lips et al. 1999).

Bone Mineral Density

The National Health and Nutrition Examination Survey III (NHANES III) examined the relationship between circulating 25(OH)D and bone mineral density (BMD) at the hip in 4958 women and 5003 men aged 20 years and above (Bischoff-Ferrari et al. 2009a, b). This showed a positive association between circulating 25(OH)D and BMD in both sexes, with the highest BMD found in subjects with a circulating 25(OH)D above 75 nmol/L. Although these results were adjusted for potential confounding variables, the authors acknowledged that one cannot infer a

causal relationship between circulating 25(OH)D and BMD from a cross-sectional study. The evidence based reviews performed for the IOM Report also examined the relationship between vitamin D and BMD (Ross et al. 2010). Among the observational studies reviewed, there was fair evidence to support an association between circulating 25(OH)D levels and BMD or changes in BMD at the femoral neck.

The largest randomised controlled trial (RCT) of the effects of vitamin D supplementation on bone health was the Women's Health Initiative Study (WHI), where 36,282 postmenopausal women aged 50–79 years were randomised to receive calcium (1000 mg) and vitamin D (10 μg) or placebo daily (Jackson et al. 2006). In a sub-set of 2431 women who underwent bone density measurements, there was greater preservation of BMD at the hip with supplementation than with placebo, which comprised 0.59%, 0.86% and 1.06% after 3, 6 and 9 years respectively. The IOM Report highlighted that the combined results of RCTs comparing calcium and vitamin D supplementation with placebo were consistent with a small effect on lumbar spine, femoral neck and total body BMD (Ross et al. 2010). In contrast, in trials comparing combined calcium and vitamin D supplementation with calcium alone, no significant difference in change in BMD was seen, suggesting that vitamin D supplementation may be less beneficial in calcium replete subjects.

Fracture Risk

The IOM Report also examined the relationship between circulating 25(OH)D and fracture risk (Ross et al. 2010). Only one of the three prospective cohort studies reviewed found an inverse relationship between circulating 25(OH)D and fractures, but in contrast nine of the 12 case-control studies observed lower 25(OH)D levels in patients with fractures than in the control subjects. The apparent inconsistency between the results of prospective cohort and case-control studies may reflect a failure to fully adjust for confounding variables in the latter, not least the effect of the fracture, any hospital admission, surgical procedure and associated inflammation on vitamin D production and metabolism (Reid et al. 2011). One of the earliest RCTs investigating the anti-fracture efficacy of vitamin D supplementation compared the effect of combined calcium (1200 mg daily) and vitamin D (20 μg daily) and placebo in 3270 women with an average age of 84 years living in French nursing homes or apartment blocks for the elderly (Chapuy et al. 1992). In a small subset of subject undergoing venepuncture and BMD measurement, there was correction of vitamin D deficiency and secondary hyperparathyroidism with supplementation, together with a small increase in BMD. Intervention also reduced the risk of hip and other non-vertebral fractures. It was unclear from this study if both calcium and vitamin D was required for the beneficial effect of supplementation or if this would be effective in community dwelling older people. The RECORD study sought to address this question, by comparing the effect of placebo or calcium (1000 mg daily) and vitamin D (20 μg daily), either alone or in combination, in 5292 community-dwelling older women or men with a low trauma fracture (Grant et al.

2005). Over the 24–62 month follow-up period there was no difference in the incidence of all clinical fractures or hip fractures. Compliance with supplementation in the RECORD study was relatively poor, especially when this included calcium. Nevertheless, pre-planned analysis showed no difference in outcome in subjects with good compliance with supplementation compared with participants who were less compliant. Although the WHI study showed a small improvement in BMD with calcium (1000 mg) and vitamin D (10 μg) supplementation, there was no overall effect on fracture incidence (Jackson et al. 2006). Among the subjects who remained compliant with supplementation there was a significant reduction in the risk of hip fractures. The results of other RCTs of vitamin D supplementation, with or without additional calcium, on the risk of fracture have yielded inconsistent results.

Meta-analyses indicate that combined calcium and vitamin D supplementation reduces the incidence of hip fractures in older people, but vitamin D alone is ineffective (Boonen et al. 2007; DIPART Group 2010; Chung et al. 2011; Avenell et al. 2009). Nevertheless, much of the beneficial effect of combined supplementation in these meta-analyses is driven by the results of the study in institutionalised French women, where vitamin D deficiency is common. A meta-analysis by Bischoff-Ferrari, which adjusted the dose of vitamin D for compliance, suggested that vitamin D decreased the incidence of non-vertebral fractures independent of additional calcium supplementation (Bischoff-Ferrari et al. 2009a, b). The reduction in fracture risk was more marked in studies where the received vitamin D dose exceeded 10 μg daily, whereas there was no decrease in fractures in studies where the subjects received 10 μg daily or less. An individual patient data meta-analysis by Bischoff-Ferrari, which also adjusted the dose of vitamin D for compliance, showed a trend for reduction in the risk of hip fractures but a small reduction in non-vertebral fractures (Bischoff-Ferrari et al. 2012).

The inconsistency of the results of the anti-fracture trials of vitamin D is likely to reflect heterogeneity in the populations studied, their baseline vitamin D status, dose of vitamin D, frequency and route of administration, compliance with supplementation and the use of additional calcium supplementation Nevertheless, it would appear that vitamin D supplementation is most likely to be beneficial in older people with vitamin D deficiency, such as those who are housebound or living in residential or nursing homes. Although the study in institutionalised French women (Chapuy et al. 1992) and several meta-analyses (Boonen et al. 2007; DIPART Group 2010; Chung et al. 2011; Avenell et al. 2009) suggest that additional calcium supplementation is required, it is unclear if a high dietary calcium intake is sufficient to obtain the benefit of vitamin D supplementation. Although the concept of the annual administration of high dose vitamin D is potentially attractive, either by the intramuscular or oral route, this may be associated with an increase in fracture risk (Sanders et al. 2010; Smith et al. 2007). For example, a study of high dose vitamin D supplementation (12,500 μg once yearly) reported an increased rate of falls and fractures, particularly in the first 3 months (Sanders et al. 2010). Similar findings have been reported in another study which, gave 7500 μg to older people, with a relative risk of hip fracture of 1.49 (1.02–2.18) in older people treated in their own homes for 3 years (Smith et al. 2007) and a non-significant 1% increase in

non-vertebal fractures over 10 months in care home residents (Law et al. 2006). These studies offer a concern with regard to what could be perceived as toxicological doses of vitamin D (i.e. 125 times the IOM UL) and its potential risks. Unfortunately, 25(OH)D and PTH were only measured in a small minority of participants in all of these interventional studies (Francis 2007), limiting the ability to explore the relationship between the circulating 25(OH)D achieved and fracture prevention.

Vitamin D and Muscle Health in Older Age

Finding the likely factors such as circulating 25(OH)D which may help to maintain or improve muscle strength, function, and physical performance well into advanced age to preserve independence bears a great public health importance (Holick 2007). Several lines of evidence have been suggested to support the involvement of 25(OH)D in skeletal muscle strength and function (Bischoff-Ferrari 2012; Ceglia and Harris 2013; Girgis et al. 2013). Firstly, clinical signs of severe 25(OH)D deficiency (<25 nmol/L) (Holick 2007) have been linked to myopathy, muscle pain and impaired gait, and their amelioration by vitamin D supplementation (reviewed by Girgis et al. 2013). Secondly, localisation of vitamin D receptor (VDR) in human muscle cell lines, myoblasts (Olsson et al. 2016), and adult skeletal muscle (Bischoff et al. 2001) although recently challenged (Wang and DeLuca 2011), and functional *in vitro* studies have provided insights into direct biological role of active form of vitamin D, the $1,25(OH)_2D_3$ in regulation of genes and signalling pathways affecting calcium homeostasis, proliferation and differentiation of muscle cells (Ceglia and Harris 2013; Girgis et al. 2013). Thirdly, despite conflicting findings across individual intervention studies, the results of the latest meta-analyses of randomized controlled trials (RCT) of vitamin D supplementation have showed a small but significant improvement in muscle strength and function in older adults who had 25(OH)D concentrations below 30 nmol/L (Beaudart et al. 2014) or 50 nmol/L (Rejnmark 2011), and reduced risk of falls in those with 25(OH)D <25 nmol/L at baseline after vitamin D and calcium co-administration (Murad et al. 2011). Lastly, observational studies (reviewed in; Houston 2015; McCarthy and Kiely 2015), although inconsistent, have suggested that 25(OH)D concentration of <50 nmol/L exerts negative effect on various measures of muscle strength and function and physical performance in older adults aged 60 and over.

Over a dozen prospective studies have examined the role of circulating 25(OH)D in muscle strength and physical performance in older adults (Granic et al. 2017; Houston 2015; Sohl et al. 2013a; Dam et al. 2009; Wicherts et al. 2007; Houston et al. 2011a, b, 2012.

Most have hypothesised a protective effect of higher 25(OH)D concentrations (≥50 or ≥ 75 nmol/L) for muscular health and function. The studies differed, among others, in respect to participants' characteristic, baseline 25(OH)D concentration, muscle strength and functioning measures, and their baseline levels. Only a few

have included the very old (aged ≥85) (Granic et al. 2017; Sohl et al. 2013a; Wicherts et al. 2007; Houston et al. 2011a, b)–the age group at an increased risk of muscle mass and strength loss (Dodds et al. 2017), functional decline (Kempen et al. 2006), and low 25(OH)D (Hill et al. 2016). The Newcastle 85+ study was the first cohort study to test non-linear relationships between 25(OH)D (defined by season-specific quartiles) and decline in grip strength (GS) and timed up and go (TUG) test in the very old (aged ≥85) living in the UK (Granic et al. 2017). The results observed a U-shaped association between 25(OH)D and GS decline in both men and women remained significant only for men in the lowest compared with combined middle quartile. Men in SQ1 [25(OH)D <30 nmol/L] experienced a loss of 1.41 kg/year and accelerated decline of −0.43 kg throughout the 5-year follow-up. Women (but not men) in the lowest and highest 25(OH)D quartile (>47–75 nmol/L) had slower overall TUG times at baseline but not over time. Greater benefits for muscle strength were not observed in participants with 25(OH)D ≥75 nmol/L (Granic et al. 2017). There is only one other study of adults aged ≥80, from Belgium, which has found no association between 25(OH)D concentration and several measures of muscle performance in cross-sectional analysis, although severe vitamin D deficiency (<25 nmol/L) was high in this cohort, especially in winter (Matheï et al. 2013). Therefore, in very old adults it appears that keeping 25(OH)D above 25–30 nmol/L minimum may reduce muscle strength decline, whereas values >50 nmol/L may not confer additional benefits for muscle heath and functioning in the very old.

Several other studies have reported an increased risk of muscle function decline in younger-old participants with low vitamin D status (defined as either <30 or <50 nmol/L or lowest data-driven quartile) (Sohl et al. 2013a, b; Dam et al. 2009; Wicherts et al. 2007; Houston et al. 2011a), others have found no risk (Verreault et al. 2002; Sohl et al. 2013b), and no association with the faster rate of decline in functioning measures (Houston et al. 2011b, 2012). Recently recommended 25(OH)D cut-offs for overall and musculoskeletal health in the UK are much lower (SACN 2016) than the required levels proposed by the IOM (Ross et al. 2010) and The Endocrine Society guidelines (25 vs 50 vs 75 nmol/L, respectively). Despite the differences in hypothesis, definition of exposure (25(OH)D cut-offs), and outcome measures for muscle strength and function, certain parallels between the results in our and the studies that included the very old (Wicherts et al. 2007; Houston et al. 2011a, b, 2012) should be noted. In a sub-group of 979 older adults (aged 65–88 years) from the Longitudinal Aging Study Amsterdam, those with 25(OH)D <25 nmol/L had higher risk of decline in physical performance over 3 years, whilst those in the intermediate group (50–75 nmol/L) did not experience greater rates of decline compared with participants with 25(OH)D >75 nmol/L (Wicherts et al. 2007). In the Cardiovascular Health Study All Stars, participants aged 77–100 with 25(OH)D deficiency (<50 nmol/L) had weaker GS at baseline compared with those in sufficient group (≥75 nmol/L), but did not have an increased risk of GS decline over 3 years (Houston et al. 2011a). The Health, Aging, and Body Composition Study of over 2600 older adults aged 71–80 have determined 25(OH)D threshold

and best performance for physical function and strength at 70–80 nmol/L and 55–70 nmol/L, respectively (Houston et al. 2012). Although participants with 25(OH)D < 50 nmol/L had worse physical performance at baseline and 2- and 4-year follow-up compared with those in sufficient group (≥75 nmol/L), no association was found for GS, and no association with a faster rate of decline in either measures. Increasing 25(OH)D to ≥50 nmol/L was associated with clinically significant improvement in the short physical performance battery over 12 months in older adults aged 70–89 (Houston et al. 2011b). Taken together, the results suggest detrimental effects of low circulating 25(OH)D (<25 nmol/L) and no change (decline) or favourable outcomes for muscle strength and physical performance at both intermediate (>50 nmol/L) and higher (>75 nmol/L) concentrations.

Previous data from the Newcastle 85+ study has identified sex-specific trajectories and baseline determinants of GS decline over 5 years in the very old (Granic et al. 2016). Steeper slopes of GS decline in men compared with women could be partially explained by multi-morbidity (Collerton et al. 2009; Kingston et al. 2014) (a significant predictor of weaker GS in women), body composition (Siervo et al. 2015) (fat mass higher in women despite lower body weight), and survival. Shorter survival time in women in both low and high 25(OH)D groups has also been observed in participants from the Newcastle 85+ study (Granic et al. 2015a). In addition, women's longer life expectancy spend with more diseases and disabilities (Kingston et al. 2014), and selective mortality in men (survival of heathier men), may result in a biased sample, and a lack of power to detect associations in women.

Although recent meta-analyses of RCT have reported a small improvements in muscle strength and function in deficient older adults (25(OH)D <30 or 50 nmol/L), larger scale studies are needed to determine sources and thresholds of 25(OH)D for physical functioning in advanced adulthood. The results of the above studies have several sources of bias related to vitamin D. Most do not report 25(OH)D status prior to baseline or adjust for long-standing vitamin D deficiency which may be corrected by supplementation prior to study commencement (especially in women). Single measures of 25(OH)D may also miss-classify individuals' vitamin D status throughout the year. Other sources of miss-classification in studies may be due to assay variability in measuring 25(OH)D with immunoassays often over estimating 25(OH)D deficiency (<30 nmol/L) (Snellman et al. 2010), particularly in older women (Perna et al. 2012). Loss to follow-up may be another source of bias. As with any cohort of older adults, in particular very old cohorts, mortality is high, thus the results may be influenced by the presence of more robust survivors. There still remains the possibility of residual confounding affecting 25(OH)D status, muscle strength and physical performance relationship.

Vitamin D and Cognitive Health in Older Age

Recent evidence from life sciences and epidemiology points to the role of 25(OH)D in brain function, including cognition, across the life span (Balion et al. 2012; Kesby et al. 2011). Detection of hydroxylases for vitamin D activation and vitamin D receptors in neurons and glia in brain regions essential for cognition and memory implicates their relevance for brain health. Moreover, *in vitro* and *in vivo* studies propose neuroprotective properties of 25(OH)D (Kesby et al. 2011).

Although recent evidence indicates that supplementation improves vitamin D status in older adults without adversely affecting heath and survival (Bjelakovic et al. 2014) there is no consensus on the definition of hypovitaminosis D and upper 25(OH)D thresholds for optimum physical and mental health in old age to prevent problems with under- or over-treatment (Ross et al. 2010; Holick 2008; Sanders et al. 2013; Cranney et al. 2007). Non-optimal concentration of 25(OH)D, variously defined as <25 nmol/L (10 ng/ml) or <50 nmol/L (20 ng/ml), has been implicated as a risk factor for global cognitive impairment (Annweiler et al. 2010; Llewellyn et al. 2011) and weaker performance on domain-specific cognitive tasks (Buell et al. 2009; Hansen et al. 2011; Lee et al. 2009; Seamans et al. 2010) in several, but not all, cross-sectional studies (McGrath et al. 2007) involving adults aged 60+. Only four prospective studies reported an increased risk of cognitive decline in association with lower concentrations of circulating 25(OH)D (≤50 nmol/L) in adults aged 65+ (Slinin et al. 2010, 2012; Llewellyn et al. 2010; Wilson et al. 2014). Studies on 25(OH)D and cognitive decline in those aged 85+ are scarce (Granic et al. 2015b; Formiga et al. 2011; Menant et al. 2012).

The Newcastle 85+ Study tested for the presence of either an inverse or a non-linear association between 25(OH)D concentrations and cognition at baseline and cognitive decline over 3 years, utilizing measures of global and attention-specific cognitive function (Granic et al. 2015b). The investigators found that both low and high season-specific quartiles of 25(OH)D were associated with higher odds of prevalent cognitive impairment (assessed by SMMSE), poorer attention reaction times/processing speed and focused attention/concentration, and greater attention fluctuation (assessed by Cognitive Drug Research battery). Differences remained significant after adjustment for sex, education, lifestyle factors and the presence of several chronic diseases, although effects were small (Granic et al. 2015b). However, the rate of change of all attention measures over 5 years did not vary across 25(OH)D groups, and no association between 25(OH)D and odds of global incident cognitive impairment or decline was found (Granic et al. 2015b). To our knowledge, this is the first prospective study to find evidence for a U-shaped relationship between 25(OH)D and global cognitive function and attention in the very old. Taken together, it could be hypothesized that the neuroprotective effects of vitamin D mediated via expression of proteins that, for example, attenuate the toxicity of reactive oxygen species (Ibi et al. 2001) in very old neurons are attained only at moderate but not at low or high 25(OH)D concentrations.

Thus far, only four prospective studies of older adults aged 65+ have examined the association between 25(OH)D and prevalent and incident global cognitive impairment, and decline in attention and executive function, with inconsistent results. A study of community-dwelling older men (Slinin et al. 2010) found limited evidence of an independent association between lower 25(OH)D concentration (≤19.9 ng/ml) and incident cognitive impairment or decline in global and executive function. A similar study involving community-dwelling older women (Slinin et al. 2012) reported that very low (<25 nmol/L) and low levels (<50 nmol/L) of 25(OH)D were associated with an increased risk of impaired global cognitive function and decline [defined by modified MMSE (3MS)], but not with impaired executive function or decline. Two further studies have also investigated these 25(OH)D cut-points. The InCHIANTI study found that compared with participants with sufficient 25(OH)D (≥75 nmol/L) the deficient group (<25 nmol/L) experienced a substantial global (assessed by MMSE) and executive cognitive decline (assessed by Trails A and B) over 6 years (Llewellyn et al. 2010), whilst the Health, Aging and Body Composition Study confirmed that lower 25(OH)D (<50 nmol/L) was associated with a greater cognitive decline on the 3MS compared with sufficient 25(OH)D (≥75 nmol/L) over 4-year follow-up (Wilson et al. 2014). A similar global cognitive measure as in previous studies was utilized, although different circulating 25(OH)D cut-offs were derived *a posteriori* (Wang et al. 2009), but no association between 25(OH)D and global incident impairment or decline after adjustment for confounders was detected. This lack of association may be due to very old age of study participants, reduced power to detect the association, specific definition of cognitive change at an individual level, and/or changed circulating 25(OH)D status over the 3 years of the study. Increased mortality amongst older women belonging to the lowest and highest season-specific 25(OH)D quartiles as observed in this cohort (Granic et al. 2015a) could be one of the reasons for the loss of analytical power. Nonetheless these findings are in agreement with reports from the NHANES III, a cross-sectional study of the non-institutionalized US population, aged 60–90 years, where the worst performance on learning and memory tasks was associated with the highest quintile of 25(OH)D (McGrath et al. 2007).

There is the possibility of reverse causation (i.e. non-optimal 25(OH)D concentrations being a consequence of prevalent cognitive impairment) (Johansson et al. 2013). To assess change in attention/information processing speed, attention fluctuation and accuracy in relation to 25(OH)D, the CDR system, previously used in dementia studies and clinical trials to discriminate between various types of dementias and to detect change in attention-specific cognitive domains pre and post treatment with millisecond precision, have been employed in previous studies (Wesnes, 2008; Wesnes et al. 2000; Rowan et al. 2007). Future studies should determine whether these attention deficits relate to decline in global cognition and interfere with daily functioning (Bronnick et al. 2006). A small clinical trial of patients aged 65 and over with a history of falls and 25(OH)D insufficiency (≤30 nmol/L) showed an improvement of 0.4 s in CRT, compared with the control group, 6 months after a single intramuscular injection of vitamin D, which increased the average circulating 25(OH)D from 10.4 to 17.5 ng/ml – the latter within the middle quartiles reported

here to be associated with better attention scores (Dhesi et al. 2004). Future prospective studies should test the proposed U-shaped relationship between circulating 25(OH)D and cognition in this age group and determine whether other cognitive domains are affected similarly by 25 (OH)D status.

Vitamin D and Mortality

In the past two decades, accumulated evidence from cellular, animal and population-based studies has indicated the involvement of vitamin D metabolites in immunomodulation, cancer inhibition and cardiovascular, respiratory, brain and muscle function (Christakos et al. 2013; Pludowski et al. 2013; Welsh 2012; IARC 2008; Kesby et al. 2011). These extra-skeletal effects of vitamin D suggest its potential role in overall health and survival (Hossein-Nezhad and Holick 2013). Recent observational studies in the general and older populations (≥65) have shown a non-linear relationship between circulating hydroxyvitamin D [25(OH)D], the major circulatory and storage form of vitamin D, and both disease-specific and all-cause mortality (Michaælsson et al. 2010; Signorello et al. 2013; Amer and Qayyum 2013; Durup et al. 2012; Dror et al. 2013; Melamed et al. 2008). This indicates that moderate rather than low or high concentrations of 25(OH)D may result in more favourable health outcomes and increased survival. Using an evidence-based approach for bone health, the US Institute of Medicine (IOM) has produced its latest report stating that: (i) concentrations of 50 nmol/L 25(OH)D meet the requirements of 97.5% of the North American population; (ii) concentrations of ≥75 nmol/L are not consistently associated with increased health benefits; and (iii) not all persons have inadequate 25(OH)D if concentrations are below 50 nmol/L (Ross et al. 2010). Amongst at-risk groups, older adults are more likely to have lower 25(OH)D levels (Hirani and Primatesta 2005; Ovesen et al. 2003; Shoben et al. 2011) because of reduced skin 7-dehydrocholesterol concentrations (the cutaneous precursor of vitamin D), inefficient renal activation of 25(OH)D and a reduction in outdoor activities with advancing age (Holick 2008). These factors also contribute to greater variability in both circulating 25(OH)D concentrations and in the average requirement for vitamin D supplementation in older adults (Ross et al. 2010; Rosen et al. 2012). The findings of observational studies, randomized control trials (RCTs) and benefit–risk assessments all suggest that vitamin D supplementation in the general and older populations can ameliorate suboptimal 25(OH)D concentrations without adverse effects on disease-specific or all-cause mortality (Neuhouser et al. 2009; Bischoff-Ferrari et al. 2010; Bischoff-Ferrari et al. 2009a, b; LaCroix et al. 2009; Autier and Gandi 2007; Bjelakovic et al. 2014). However, there is no agreement amongst researchers and healthcare professionals about the optimal, beneficial and age-specific 25(OH)D concentrations in relation to extra-skeletal outcomes and mortality [Ross et al. 2010; Holick 2008; Rosen et al. 2012; Brannon 2012; Sanders et al. 2013), especially in older adults. Current evidence supports an inverse or non-linear association between 25(OH) D levels and mortality amongst adults aged 65 years

and older. For example, a meta-analysis including 24,000 participants from nine prospective observational studies demonstrated a 25% increased pooled hazard ratio for all-cause mortality in the lowest compared with the highest 25(OH)D category in those aged ≥65 years (Rush et al. 2013). A similar meta-analysis which included 12 studies (30,000 participants) confirmed an inverse association between 25(OH)D and mortality and a decrease in mortality risk of 8% for an increase in 25(OH)D of 20 nmol/L (SchÖttker et al. 2013). Two recent population-based studies from Denmark (Durup et al. 2012) and Israel (Dror et al. 2013) which both included over 40% of older adults (aged ≥65 years), showed a reversed J- and U-shaped relationship between 25(OH)D concentration and total mortality, respectively, and the best survival for individuals with 25(OH)D levels between 50 and 90 nmol/L. Similarly, an examination of the National Health and Nutrition Examination Survey (NHANES) data (2001–2004) revealed no significant reduction in mortality above a circulating 25(OH)D >52.6 nmol/L (Amer and Qayyum 2013).

To our knowledge, only one prospective cohort study has investigated the relationship between 25(OH)D and mortality in the very old (aged ≥85 years), despite this being the fastest growing segment of many populations worldwide. Furthermore, except for the study conducted amongst members of the Clalit Health Services in Israel (Dror et al. 2013), the numbers of very old adults included in the above-mentioned studies were small. In a prospective cohort study of older adults in the Newcastle 85+ study, we found a dose–response relationship between circulating 25(OH)D and all-cause mortality, with both the lowest and highest season specific 25(OH)D quartiles being associated with higher mortality over 6 years (Granic et al. 2015a). The higher risk of mortality amongst participants with the highest concentrations [a threshold range of ≥47 nmol/L (spring) to ≥69 nmol/L (summer) for the highest season-specific quartile] appeared to be driven largely by women taking vitamin D-containing supplements and/or prescribed medication (Granic et al. 2015a). Furthermore, the greater risk of mortality amongst women with the highest 25(OH)D concentrations (SQ4 or 'sufficient' categories) was independent of their frailty status (Ensrud et al. 2010). To our knowledge, this is the first observational study to suggest a U-shaped relationship between circulating 25(OH)D and all-cause mortality in very old adults. Several recent systematic reviews and meta-analyses of prospective cohort studies investigating the association between 25(OH)D status and risk of mortality [Rush et al. 2013; SchÖttker et al. 2013; Zitterman et al. 2012; Scragg 2011) have demonstrated a shorter survival amongst adults with the lowest (<25 or <50 nmol/L) compared with highest 25(OH)D concentrations, especially in those aged 65 years and older (Rush et al. 2013; Scragg 2011). In other studies, a non-linear relationship was noted with favourable survival outcomes at concentrations between 50 and 90 nmol/L (Amer and Qayyum 2013; Durup et al. 2012; Dror et al. 2013). However, except for the study conducted in Israel (Dror et al. 2013) relatively few participants aged over 85 years were included in these studies.

In a large retrospective study from general practices in Copenhagen (CopD Study) (Durup et al. 2012) an inverse J-shaped relationship between 25(OH)D and mortality was observed, with the longest survival at concentrations of 50–60 nmol/L

during 3 years of follow-up. Similarly, a historical prospective study of more than 420,000 members of the Clalit Health Services in Israel (Dror et al. 2013), which included >20,000 participants aged ≥85 years, found that the lowest risk of mortality and acute coronary syndrome was associated with 25(OH)D in the range of 20–36 ng mL1 (50–90 nmol/L) during 4.5 years of follow-up. A meta-analysis of 14 prospective cohort studies involving the general population (age range 45–80 years and 1.3–27.0 years of follow-up) also suggested a non-linear relationship between 25 (OH)D and mortality, but 25(OH)D levels of ~75–87.5 nmol/L were considered optimal (Zittermann et al. 2012).

The higher mortality rates observed amongst very old women, with higher 25(OH)D concentrations [SQ4 or 'sufficient' (≥75 nmol/L) categories] whether users or nonusers of vitamin D supplements/ medication, respectively, have not been reported previously [Bischoff-Ferrari et al. 2010; Bischoff-Ferrari et al. 2009a, b; LaCroix et al. 2009; Autier and Gandi 2007; Bjelakovic et al. 2014]. The Women's Health Initiative calcium/vitamin D RCT, a 7-year combined therapy intervention (1 g calcium and 400 IU vitamin D daily), reported a trend towards mortality reduction amongst postmenopausal women aged <70 years, but neither a beneficial nor an adverse effect in women aged >70 years (LaCroix et al. 2009). A meta-analysis of 56 RCTs of vitamin D supplementation and survival (Bjelakovic et al. 2014) demonstrated a decrease in all-cause mortality amongst predominantly older adults including women aged ≥70 years, but also found adverse renal outcomes associated with vitamin D3 and calcium combination therapy.

Lower (<37.5 nmol/L) and higher (≥75 nmol/L) concentrations of 25(OH)D have been moderately associated with frailty amongst older women (aged ≥69 years) in the Study of Osteoporotic Fractures (Ensrud et al. 2010), and the risk of death was significantly increased amongst frail NHANES III participants (aged ≥60 years) in the lowest (<49.5 nmol/L) compared with not frail participants in the highest (>84.1 nmol/L) 25(OH)D quartiles (Smit et al. 2012). The results from observational cohort studies exploring sex differences in mortality in relation to 25(OH)D are inconclusive and have not included the very old. The NHANES III showed a U-shaped relationship between 25(OH)D levels and mortality in the general population of women (aged ≥20 years) but not in men at concentrations of <50 and > 125 nmol/L (Melamed et al. 2008). In a study of older men (a birth cohort from 1920 to 1924, aged 71 at baseline) from the Uppsala region, an increased risk of total and cancer mortality was observed at both low (<46 nmol/L) and high (>98 nmol/L) 25(OH)D concentrations over 12.7 years of follow-up (Michaæ lsson et al. 2010). In both these studies, the longest survival was associated with the middle 25(OH)D categories, but the thresholds were much higher than in the present study (NHANES III: 75–100 nmol/L; Uppsala Study of Older Men: 46–98 nmol/L); this difference may be related to the age of participants, habitual diet, supplementation, length of follow-up or other factors/covariates.

Several limitations of the aforementioned studies should be noted. There remains the possibility of residual confounding by additional factors that affect the relationship between circulating 25(OH)D and mortality. Although many of these studies controlled for the number of chronic diseases and for frailty status, increased mor-

tality amongst older women may be mediated by other mechanisms associated with non-optimal 25(OH)D levels such as polypharmacy (Sohl et al. 2012) or an acute inflammatory response (Reid et al. 2011; Waldron et al. 2013). Recent studies have demonstrated a rapid decline in circulating 25 (OH)D after elective hip or knee surgery or after an acute inflammatory insult, thus 25(OH)D may be an unreliable biomarker of vitamin D status up to 3 months after the event (Reid et al. 2011; Waldron et al. 2013). Whilst recognizing that there is seasonal variations in 25(OH)D status, the results of some of the studies above were based on a single measurement, which may misclassify 25(OH)D levels throughout the year. It is also difficult to explore disease-specific mortality in relation to 25(OH)D in very old adults because of high rates of multimorbidity in this age group (Collerton et al. 2009).

Conclusion and Future Direction

The last two decades have seen major advances in our understanding of the metabolism, nutritional requirements and molecular aspects of vitamin D. The upward shift in the target 25(OH)D threshold set by authoritative bodies to define better bone health has been a significant step in recent years and much of the world's elderly population have a vitamin D status below what is considered optimal for bone health. However, the debate surrounding the optimal circulating 25(OH)D concentration for both skeletal and non-skeletal health will continue until significant progress has been made in a number of important areas:

1. The first centres around assay variability for 25(OH)D measurements, which has been addressed somewhat by the recent introduction of the Standard Reference Material (SRM) for vitamin D by the National Institute of Standards and Technology (NIST) in the USA.
2. The second area centres around gaining a better understanding of the production, storage and utilization of 25(OH)D (and it's free form) as biomarkers of effect.

Future intervention studies investigating vitamin D on health outcomes need to carefully choose the dose and form of vitamin D supplementation as well as the appropriate study duration so as to ensure the desired target 25(OH)D concentration is achieved. To date, only limited information is available in studies investigating the relationship between 25(OH)D concentrations and health outcomes in population subgroups, such as infants, adolescents, pregnant and lactating women and in dark-skinned individuals. To address this large research gap, vitamin D researchers need to decide on appropriate endpoints for vitamin D adequacy and insufficiency. The urgent need to undertake more high quality vitamin D intervention studies which quantify the impact on an array of health outcomes (including non-skeletal health) in a broader range of populations needs to be a priority. It is imperative that such studies report all relevant outcomes including adverse events as some recent research suggests (albeit a tentative) a U-shaped relationship between 25(OH)D status and at least some health outcomes. Studies should also give due consideration

to VDR genotype and should control for sun exposure, season, calcium intake, baseline circulating 25(OH)D concentrations and measure potential adverse effects. Studies should take advantage of emerging technology which makes genome-wide analysis possible. Appreciably, genotyping studies will need to be large in study design or analysis, because of the very large sample sizes required to adequately account for genotype effects. The dearth of information in many population subgroups including pregnant women and ethnic minorities should be prioritized in future studies on vitamin D status and health. Finally, in light of the widespread prevalence of dietary and biochemical vitamin D inadequacy in many populations and its negative consequences for bone health, strategies to increase oral vitamin D intake should be a priority.

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Chapter 9
Telomeres, Telomerase and Ageing

Gabriele Saretzki

Abstract Telomeres are specialised structures at the end of linear chromosomes. They consist of tandem repeats of the hexanucleotide sequence TTAGGG, as well as a protein complex called shelterin. Together, they form a protective loop structure against chromosome fusion and degradation. Shortening or damage to telomeres and opening of the loop induce an uncapped state that triggers a DNA damage response resulting in senescence or apoptosis.

Average telomere length, usually measured in human blood lymphocytes, was thought to be a biomarker for ageing, survival and mortality. However, it becomes obvious that regulation of telomere length is very complex and involves multiple processes. For example, the "end replication problem" during DNA replication as well as oxidative stress are responsible for the shortening of telomeres. In contrast, telomerase activity can potentially counteract telomere shortening when it is able to access and interact with telomeres. However, while highly active during development and in cancer cells, the enzyme is down-regulated in most human somatic cells with a few exceptions such as human lymphocytes. In addition, telomeres can be transcribed, and the transcription products called TERRA are involved in telomere length regulation.

Thus, telomere length and their integrity are regulated at many different levels, and we only start to understand this process under conditions of increased oxidative stress, inflammation and during diseases as well as the ageing process.

This chapter aims to describe our current state of knowledge on telomeres and telomerase and their regulation in order to better understand their role for the ageing process.

Keywords Telomerase · Telomere · Senescence · DNA damage · Ageing

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Introduction

Telomerase activity and telomere length have a crucial role in cellular senescence and human ageing as well as in the pathobiology of several human diseases. Telomeres protect chromosome ends and thereby maintain genome stability and have an important role in ageing and tumour development. Telomere length in normal human somatic cells is tightly controlled. In cells without any telomere-lengthening mechanism, telomere length shortens due to an inherent molecular mechanism of DNA replication during cell division, as well as due to increased oxidative stress. However, there is much more to telomeres than just the length of the DNA sequence. Integrity such as telomere capping that prevents the signalling of a DNA damage is important for telomere function. It recently also became obvious that despite long or short telomere, DNA damage can accumulate anywhere within the telomere and can induce a DNA damage response, senescence, ageing or cell death, depending on cellular context. Although intensely investigated over the last decades, telomere homeostasis and its role for human ageing are still not yet well understood. More and more mechanisms are being uncovered that are able to contribute to telomere shortening and homeostasis such as telomere position effect (TPE), telomeric transcription products (TERRA) or rapid telomere trimming. However, not much is known about the role of those newly discovered mechanisms in specific cell types such as human lymphocytes or what significance they might have for the ageing process in general.

In some cell types telomere shortening can be counteracted by a specific enzyme: telomerase reverse transcriptase. However, most human somatic cells do not actively express this enzyme. But some cell types commonly used to analyse telomere lengths in human studies are blood lymphocytes together with other monocytic blood cells, usually referred to as peripheral blood monocytes (PBMCs). These cells are able to up-regulate telomerase activity when activated. Both factors, telomere length and telomerase activity, are regulated independently and at various molecular levels, and their intricate interplay is not yet entirely understood with mechanisms such as oxidative stress, TPE and TERRA interfering with both. Consequently, the evaluation of an average telomere length as a biomarker of ageing is not straightforward. While short telomeres can activate telomerase activity via TERRA regulation or the telomere position effect (TPE), the newly discovered protein TZAP as well as telomerase has been shown to trim down long telomeres as well.

Intense research over the last decades has substantially improved our understanding about telomeres, telomere-associated proteins, and the biogenesis and regulation of the telomerase complex, as well as of mechanisms of telomerase activation and telomere-independent functions of telomerase.

This chapter aims to describe the underlying molecular mechanisms for the regulation of telomere length and its complex relationship with telomerase activity and other telomere-associated processes and mechanisms. In addition, it will briefly outline what is known about the changes of telomeres and telomerase activity during cellular senescence and the ageing process in general. A better understand-

ing of the mechanisms regulating telomere structure and function is essential for the potential prevention and treatment of ageing and age-related diseases. However, for more detailed information on age-related diseases and its correlation with telomeres and telomerase, the reader is referred to other sources, while this chapter is dedicated predominantly to cellular senescence and the ageing process.

Telomeres

Telomeres are special nucleoprotein structures at the end of linear chromosomes (see Fig. 9.1). One of their most important roles is to maintain genomic integrity. While most bacteria and organelles such as mitochondria have circular genomes, eukaryotic organisms have telomere caps at the end of their chromosomes (Blackburn 1994). Telomeres consist of tandemly repeated short DNA sequences that can be different in various organisms. In mammals, the hexanucleotide sequence is TTAGGG, but also lower organisms such as sponges have this same sequence.

Telomere length and structure can vary among species ranging from an irregular repeat array of only 1.3 kilobases (kb) in yeast (D'Mello and Jazwinski 1991) to several kilobases in humans and even longer in some mouse species, which can be as long as 40–80 kB (Blasco et al. 1997). In lower eukaryotic organisms such as yeast (e.g. *Saccharomyces cerevisiae*), telomeres consist of various parts with different sequences and are much shorter (Zakian 1996). However, in all species, telomere length is tightly regulated. The main function of telomeres is to protect chromosomes from damage, degradation and end-to-end fusion and thus to ensure physical integrity of linear eukaryotic chromosomes. Telomeric structures prevent these linear chromosomes from being recognised as double-strand DNA breaks, which could otherwise result in signalling of a DNA damage response (DDR), senescence, apoptosis or chromosomal rearrangements (de Lange 2002).

Telomere DNA is guanosine-rich and can form secondary structures such as G-quadruplexes (Mullins et al. 2016). Telomeres consist mainly of heterochromatin,

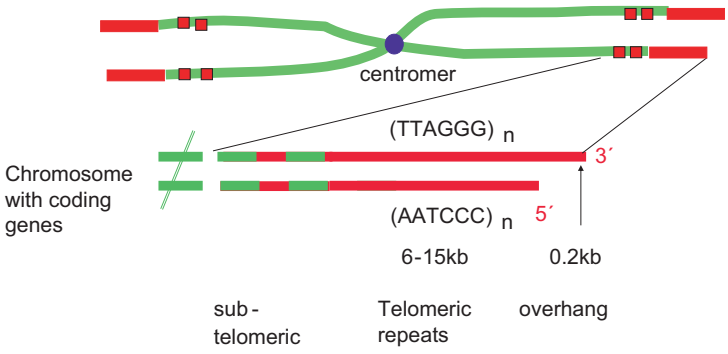

Fig. 9.1 Simplified telomeric DNA structure

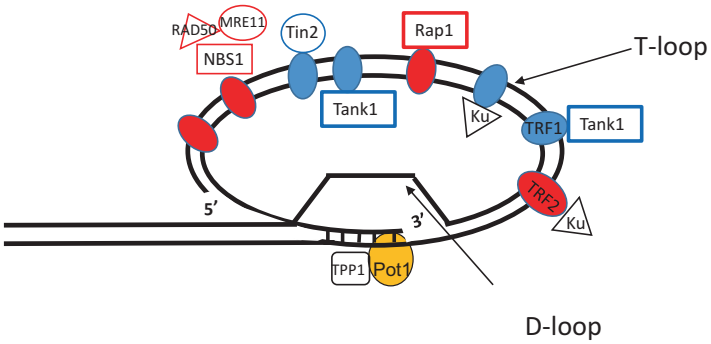

Fig. 9.2 Telomere DNA is associated with shelterin proteins TRF1, TRF2, RAP1, POT1, TPP1 and TIN2 and forms a telomeric T-loop as well as a displacement D-loop. In addition, other proteins such as Ku86, Tankyrase 1 and DNA damage and repair proteins such as NBS1, MRE11 and Rad50 are associated with the telomere

but can be transcribed, as described below. In addition to the telomeric DNA sequences, a specific set of proteins is involved in forming the telomere structure which is collectively referred to as shelterin complex (de Lange 2005; *see also* Fig. 9.2). The most prominent proteins binding the double-stranded DNA are telomeric repeat binding factors 1 and 2 (TRF1 and TRF2). Other proteins such as POT1 bind to the single-stranded overhang at the 3′ end of the telomere and are formed during telomere replication, as described later. All shelterin proteins have diverse structural and functional properties. Due to interactions of DNA and proteins as well as proteins with each other, telomeres form characteristic loop structures: the telomere loop (T-loop) and a displacement loop (D-loop). The latter protects the single-stranded DNA overhang (Henderson and Blackburn 1989; *see also* Fig. 9.1) from degradation by tucking it in with the help of POT1 and TPP1 (Fig. 9.2) (de Lange 2009). Changes in telomere and protein composition are known to accompany changes in telomere length as well as to occur during different cell cycle stages, development and ageing. This will be described in more detail below.

Telomere Structure and the Role of Telomere-Binding Proteins

Telomeres associate with specialised proteins called shelterins. The shelterin complex contains six protein components in humans: telomere repeat factors 1 and 2 (TRF1/TRF2), TRF1-interacting nuclear protein 2 (TIN2), POT1 and TIN2 organising protein 1 (TPP1), protection of telomeres 1 (POT1) and repressor/activator protein 1 (RAP1) (Liu et al. 2004; de Lange 2005; Palm and de Lange 2008; Takai et al. 2010). Three of these proteins bind telomeric DNA directly: TRF1 and TRF2 bind double-stranded DNA, while POT1 associates with the single-stranded 3′ overhang. POT1 is essential for chromosome end protection via D-loop formation (Lei et al.

2004; see Fig. 9.2). Together with its binding partner TPP1, POT1 masks the 3′ telomeric overhang from telomerase and thereby regulates accessibility of telomerase to telomeres (Kelleher et al. 2005; Wang and Lei 2011). Thus, shelterin proteins directly contribute to the association of telomerase with telomeres in late S phase of the cell cycle when most of the genome has already been replicated. The interaction of telomeres and telomerase will be described in more detail below.

The shelterin complex protects the telomere and is essential for the formation of loop structures (Chen et al. 2007, see Fig. 9.2). It has been suggested that a minimum telomere length is required for the binding of shelterin proteins to the telomere (Martínez and Blasco 2011).

The complete telomere proteome consists of around 200 proteins. They are associated with different aspects of telomere biology, including telomere protection, telomere maintenance, DNA repair or prevention of DNA damage signalling. Other proteins interact with those directly binding to telomere DNA: TIN2 binds to TRF1, and RAP1 is recruited through its interaction with TRF2 (Kim et al. 1999; de Lange 2005). In that way, non-DNA-binding proteins are able to influence the properties of DNA-binding shelterin proteins via mutual conformational adjustments (Gaullier et al. 2016), and all these components together form a telomeric interactome (Songyang and Liu 2006). TIN2 is important for the assembly and structural integrity of the shelterin complex and interacts with TRF1, TRF2 and TPP1 (Kim et al. 2004; Ye et al. 2004). TIN2 also binds to TPP1 that connects the TPP1/POT1 heterodimer to TRF1 and TRF2 on duplex telomeric repeats, allowing POT1 to associate with single-stranded telomeric DNA (Hu et al. 2017; Takai et al. 2011). Depletion of TIN2 destabilises the shelterin complex and thus telomeres, activating telomeric DNA damage signalling (Karlseder et al. 1999).

TPP1 also regulates telomerase recruitment to telomeres through the interaction with TERT for telomere maintenance (Nandakumar et al. 2012; Zhong et al. 2012). Inhibition of TIN2 results in less TPP1-mediated telomerase recruitment to telomeres, thus influencing telomere homeostasis (Abreu et al. 2010; Sexton et al. 2014). POT1 and TPP1 bind as a heterodimer to single-stranded telomere DNA to prevent DNA damage responses from capped (functionally and structurally preserved) telomeres. Intriguingly, many DNA damage response proteins are associated with telomeres and contribute to certain aspects of telomere function (for review, *see* de Boeck et al. 2009). The DNA damage repair complex MRE11/Rad50/NBS1 is a constitutive telomere component and interacts with shelterin proteins TRF1 and TRF2 (Karlseder et al. 1999). This complex is also involved in the generation of 3′ G-overhangs and exonucleolytic processing of telomeric DNA (Chai et al. 2006). These findings strongly implicate that telomere integrity and DNA repair pathways interact at the molecular level. The functional status and integrity of telomeres depend on the stability of the telomeric nucleoprotein structure and its length. Telomere uncapping due to loop opening, telomere shortening beyond a critical length as well as withdrawal of shelterin components such as TRF2 result in a DNA damage response (DDR) and result in either cellular senescence, apoptosis or malignant transformation (*see also* Fig. 9.3d). TRF2 specifically prevents

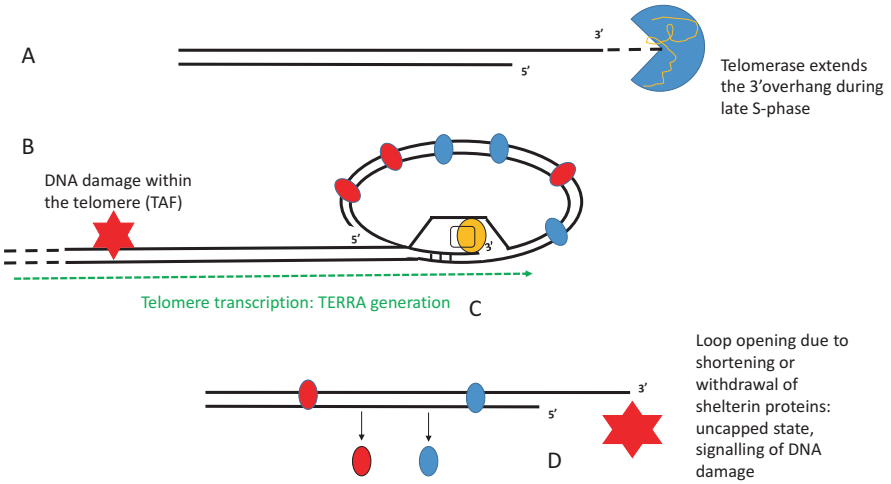

Fig. 9.3 Various processes occur at telomeres: (**a**) elongation of telomeres by telomerase. (**b**) DNA damage within the telomere without shortening or loop opening. (**c**) Telomere transcription (TERRA generation). (**d**) Loop opening due to shortening or withdrawal of shelterin proteins: uncapped state, signalling of DNA damage

telomeres from checkpoint activation such as the ATM kinase signalling pathway and protects telomeres from end fusions caused by the non-homologous end-joining DNA repair pathway (Denchi and de Lange 2007). Overexpression of dominantly negative mutated TRF2 results in T-loop uncapping eventually causing senescence or apoptosis (Karlseder et al. 1999). In contrast, depletion of telomere-binding proteins such as TRF2 causes chromosome end-to-end fusions, genomic instability and cell cycle arrest. Experimental TRF2 removal results in a senescence-like uncapped telomeric state that induces ATM and DDR (Denchi and de Lange 2007; Sfeir and de Lange 2012). TRF1 has been as well shown to participate in telomere replication in mammals. Lack of TRF1 at telomeres activates another checkpoint: the ATR kinase in S phase of the cell cycle. The de Lange group demonstrated that TRF1 promotes the efficient replication of TTAGGG repeats and prevents fork stalling (Sfeir et al. 2009). Two helicases, BLM and RTEL1, were implicated in the removal of G4 quadruplex DNA structures at telomeres for efficient replication (Vannier et al. 2012).

Additional proteins involved in double-stranded break repair (DSB) such as Ku86 and DNA-PKc are also associated with telomeres. These proteins might be involved in post-replicative strand processing (Bailey et al. 2001). DNA-PKc is the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) in the non-homologous end joining of double-strand breaks. It has a role in telomere length maintenance and telomere capping since its lack resulted in chromosome fusions involving telomeres produced by leading-strand synthesis (Espejel et al. 2002a). The subunit Ku86 of the NHEJ factor Ku interacts with TRF1 and TRF2 (Hsu et al. 2000). DNA-PKc is involved in the signalling process of short and uncapped telomeres (Espejel et al. 2002a). However, both DNA-PKc and Ku86 are required for proper telomere capping,

while their absence results in telomere fusion which is independent of telomere lengths (Goytisolo et al. 2001; Samper et al. 2000; Goytisolo and Blasco 2002). Tankyrase1, a poly(ADP-ribose) polymerase, is recruited to the telomere by TRF1 (Smith et al. 1998; Cook et al. 2002). There it is involved in telomere length regulation and sister chromatid separation (Hsiao and Smith 2008).

Recently, a new telomere-associated protein was identified: the telomeric zinc finger-associated protein (TZAP), which binds to long telomeres with a low concentration of shelterin complex competing with TRF1 and TRF2 (Li et al. 2017). TZAP then triggers a trimming process of these long telomeres thereby contributing to telomere length regulation and homeostasis.

Telomeres belong mainly to heterochromatin and contain HP1 (heterochromatin protein 1), and together with subtelomeric regions, they have heterochromatin-like histone marks including H3K9 and H4K20 trimethylation (Benetti et al. 2007). Subtelomeric DNA is heavily methylated by DNA methyltransferases (DNMTs) in human cells (Yehezkel et al. 2008). Thus, genes placed experimentally near telomeres are transcriptionally silenced, known as the "telomere position effect" (TPE) (Kim and Shay 2018; Baur et al. 2001).

Several telomere-binding factors have also telomere-independent functions. For example, RAP1 was identified as a transcription factor exerting a negative effect on metabolism and influencing genes involved in insulin secretion, PPAR (peroxisome proliferator-activated receptor) signalling and growth hormone pathways, ATP-binding cassette transporters and genes involved in type 2 diabetes (Martinez et al. 2010). In addition, RAP1 is a critical modulator of the NF-κB signalling (Teo et al. 2010). It forms a complex with IκB kinases, and RAP1 levels are in turn regulated by NF-κB signalling, thereby establishing a feedback regulatory mechanism. Poly (ADP-ribose) polymerase 1 (PARP1) also has a dual role as a genomic caretaker and mediator of inflammation. PARP1 interacts with telomeric proteins TRF2 and the Werner helicase (WRN), as well as DNA damage signalling proteins such as DNA-PKs, ATM and MRE11 (Mangerich and Burkle 2012). PARP1 also promotes NF-κB-dependent transcription and microglial activation, suggesting a potential role of PARP1 in neuro-inflammation (Chiarugi and Moskowitz 2003).

In addition to its role in shelterin, TIN2 can also localise to mitochondria, where it is post-translationally modified and can influence oxidative phosphorylation (Chen et al. 2012a, b). Depletion of TIN2 increases mitochondrial ATP generation and oxygen consumption while decreasing ROS levels (Chen et al. 2012a, b). These results suggest that TIN2 participates in the regulation of mitochondrial function independent of its role in telomere maintenance (Chen et al. 2012a, b; Sullivan et al. 2012).

T-Loop and D-Loop Structures

Telomeres form both T- and D-loops (Fig. 9.2). The T-loop (telomere loop) is formed from double-stranded telomere DNA and double-strand binding shelterin proteins TRF1 and TRF2 (Smogorzewska and de Lange 2004; Yoshimura et al. 2004; Doksani et al. 2013). The T-loop has been proposed to facilitate a higher-order

structure which seems instrumental in telomeric capping to avoid a DNA damage response. It is also important for telomerase extension of telomeres. The D-loop (displacement loop) involves the invasion of the 100–200 bases of the 3′ single-stranded G-rich overhang pairing with the CCCTAA strand of the telomeric double-stranded telomere structure forming a triple-stranded DNA configuration (Griffith et al. 1999). Both loops are important for telomere capping (Blackburn 2000 Mt Sinai). The 3′ overhang binds POT1 which is instrumental for D-loop formation (Griffith et al. 1999).

T-loop formation with telomeric DNA can be increased *in vitro* when TRF2 binds to tail-loop junctions. The role of TRF2 in T-loop formation is also emphasised by the finding that its inhibition *in vivo* causes opening of the T-loop, resulting in uncapping of telomeres and emanation of a DNA damage response (van Steensel et al. 1998; de Lange 2005). On the other hand, regulated loop opening occurs during telomere replication and during access of telomerase to the 3′ telomere overhang in late S phase of the cell cycle. Thus, telomeres can change composition and function during the cell cycle. The single-stranded telomeric TTAGGG containing 3′overhang is bound by POT1/TPP1 during most of the cell cycle. However, in S phase, ssDNA may also bind to RPA, a protein involved in DNA replication. In addition, the G-rich strand also binds to the CST (CTC1, STN1 and TEN1) complex (Price et al. 2010), which increases at telomeres in late S phase for lagging-strand synthesis during telomere replication and for this to occur has to terminate telomerase-mediated telomere extension (Chen et al. 2012a, b). Many more proteins have been demonstrated to be involved in the interaction between telomeres and telomerase and thus telomere extension and elongation. hnRNPA1 associates with telomere ends and stimulates telomerase activity (Zhang et al. 2006). A G-quadruplex structure at telomeres can form. The authors found that hnRNPA1 binds to single-stranded and G-quadruplex structured human telomeric repeats which could block telomere extension by telomerase. They also found that depletion of hnRNPA1 significantly compromised telomerase activity. The authors suggest that hnRNPA1 stimulates telomere elongation through unwinding of a higher-order telomere structure formed at the translocation step of telomere elongation by telomerase (Zhang et al. 2006).

One important factor for the distinction of scheduled or damage-induced loop opening is the binding of POT1 and RPA to the G-rich overhang which has been shown to be orchestrated by TERRA (Flynn et al. 2011). TERRA is described in detail in the following section.

Telomeric Repeat-Containing RNA (TERRA)

It has recently been discovered that telomeres are not exclusively heterochromatin and transcriptionally silent as originally thought, but can be transcribed from the C-strand into repetitive RNA: long non-coding RNA called TERRA (Azzalin et al. 2007). TERRA in mammals is non-polyadenylated. TERRA is transcribed from

specific promoters within subtelomeric regions of several chromosomes that are not heterochromatinised (Nergadze et al. 2009; Pfeiffer and Lingner 2012). TERRA contains telomeric repeats and can vary in length from around 100 bases to 9 kilobases (Azzalin and Lingner 2008). Human TERRA promoters containing CpG-rich tandem repeats of 39 and 37 base pairs exist in around half of all human subtelomeres (Nergadze et al. 2009). The RNA polymerase that synthesises TERRA molecules is RNA polymerase II (RNAPII) (Schoeftner and Blasco 2008).

Importantly, only a few telomeres are transcribed within a cell, and it is not yet well understood how these are selected or differ between various cells. It has been shown that TERRA overexpression on a single telomere is able to induce premature senescence (Maicher et al. 2012). In contrast, TERRA can also suppress senescence by stimulating telomere recombination (Yu et al. 2014).

Interestingly, TERRA seems to be preferentially expressed from short telomeres by attracting telomerase and binding to it (Graf et al. 2017). The maintenance of a minimal telomere length is essential in order to prevent cellular senescence. When critically short telomeres occur in the absence of telomerase, they can be repaired by homology-directed repair (HDR) to prevent the onset of premature senescence. Intriguingly, preferentially the shortest telomeres are targeted for HDR via increased TERRA generation and formation of HDR-promoting RNA-DNA hybrids (R-loops). The increased level of TERRA and R-loops at short telomeres is the result of a local defect in RNA degradation mechanism. The persistence of R-loops at short telomeres promotes the activation of a DNA damage response (DDR) and recruitment of the Rad51 recombinase. In this way TERRA and R-loops seem to influence the occurrence of replicative senescence (Graf et al. 2017).

TERRA can interact with telomeric DNA to form hybrid DNA-RNA G-quadruplexes that can interact with components of the shelterin complex and also bind to telomerase (Hirashima and Seimiya 2015). TERRA also interacts with TRF1 and TRF2 (Deng et al. 2009).

Intriguingly, these RNAs seem to be involved in telomere maintenance and to contribute to the regulation of the telomere capping state as well as in the DDR response to telomere dysfunction (Montero et al. 2016). Thus, it can be speculated that TERRA transcription could be induced in response to stress or DNA damage to telomeres and subtelomeres (Azzalin and Lingner 2015). TERRA inhibits telomerase activity in *cis in vitro*, while telomere elongation represses TERRA expression (Arnoult et al. 2012). Thus, TERRA promotes telomere shortening by inhibition of telomerase activity (Luke et al. 2008) but at the same time protects telomeric ends.

Furthermore, TERRA can regulate telomere length through modulation of exonuclease 1, resulting in shortening but only of the respective telomeres TERRA derives from, thus in *cis*. Since TERRA contains specific mutations within its sequence, TERRA can specifically bind in *cis* to the telomere of origin, but it can at the same time affect any chromosome in *trans* (Chu et al. 2017). The authors showed that depleting TERRA increases telomerase activity and induces telomeric dysfunction, including formation of telomere-induced DNA damage foci and loss or duplication of telomeric sequences. They conclude that TERRA functions as an epigenomic modulator in *trans* and as an essential regulator of telomeres in *cis*.

Others have demonstrated that TERRA physically and genetically interacts with Ku, and since Ku inhibits Exonuclease 1, TERRA also influences exonuclease function. Thus, TERRA expression is able to control and regulate telomere length through the processing of telomeres independently of telomerase (Pfeiffer and Lingner 2012).

TERRA levels are tightly regulated through the cell cycle. In human cells, TERRA levels are highest in G1, strongly decrease in S phase and start increasing again in G2 of the cell cycle (Porro et al. 2010). TERRA also affects the replication of leading-strand telomeres (Le et al. 2013). TERRA has been shown to influence the binding of POT1 and RPA to the telomere ssDNA and ensures that POT1 displaces RPA immediately after completion of replication in order to avoid RPA activating DDR (Flynn et al. 2011).

Telomere transcription can be induced by telomere damage, and TERRA levels increase in response to telomere uncapping, DNA damage and TRF2 loss even with long telomeres (Porro et al. 2014). These authors showed that TERRA is also involved in the initiation steps of the DDR pathways, thereby promoting changes in the chromatin architecture of damaged telomeres and ensuring an effective DDR and DNA repair. TERRA promotes the recruitment of chromatin modifiers to damaged telomeres and also regulates chromosome-end mobility within the nucleus (Feuerhahn et al. 2010). TERRA has been implicated in modulating the structure and processing of de-protected telomeres. The NHEJ factor Ku physically interacts with TERRA and exonuclease 1 which can shorten/resect telomeres upon their damage or uncapping. TERRA has been shown to associate with histone methyltransferase, which promotes accumulation of H3K9me3 at damaged telomeres and in that way is involved in telomeric DDR. These results suggest that TERRA might be involved in telomere-remodelling events (Porro et al. 2014).

Importantly, uncapped telomeres generate more TERRA. The physical association of TERRA with its telomeres they are generated from, and its known involvement in telomere length regulation and telomere damage repair, suggests that a tight regulation of this specific RNA is essential in order to ensure telomere stability. The nonsense-mediated RNA decay (NMD) mechanism degrades TERRA thereby avoiding excessive accumulation of TERRA at telomeres and telomere instability in human cells. However, the detailed regulation of these tightly intertwined processes remains largely unknown currently (Azzalin and Lingner 2015).

In addition, TERRA also participates in telomere length regulation via alternative telomere maintenance (ALT) mechanisms (Arora et al. 2014; Arora and Azzalin 2015). However, this ALT mechanism of telomere recombination and maintenance is preferentially described in immortalised and cancer cells, while it is not clearly known whether it has also a role in normal somatic cells. However, TERRA has been shown to be active and important during gametogenesis (Reig-Viader et al. 2014) as well as during senescence in normal somatic cells (Rippe and Luke 2015).

Tutton and colleagues recently demonstrated that TERRA transcription can be induced by p53 activation, and noncanonical binding sites for p53 have been identified in human subtelomeres. The p53 binding sites in subtelomeres provide a transcriptional regulation of TERRA in response to various DNA damage and stress

signalling pathways. Cells lacking p53 or the CRISPR-engineered deletion of p53 binding sites in subtelomeres resulted in a loss of TERRA expression and an increase in the accumulation of persistent γH2A.X DNA damage signals at telomeres (Tutton et al. 2016).

In conclusion, TERRA plays multiple roles at the telomere, and it has been proposed that TERRA might coordinate different activities at the telomere that depends on its state (Rippe and Luke 2015).

Mechanisms of Telomere Shortening in the Absence of Telomerase

Over the recent decades, telomeres have been intensively studied and have been suggested as important biomarkers of ageing. The main reason for this is that in many human tissues, telomeres continuously shorten due to the so-called end replication problem (Olovnikov 1971; Watson 1972), oxidative stress (von Zglinicki et al. 1995; von Zglinicki 2000, 2002) as well as end processing mechanisms (Levy et al. 1992; Makarov et al. 1997). In cells without telomerase activity (see below), about 15–50 bp of telomeric DNA is lost during each cell division in fibroblasts *in vitro* (von Zglinicki et al. 1995; Lorenz et al. 2001) and around 15 bp/year *in vivo* (Allsopp et al. 1992).

The "end replication problem" (ERP) describes the inability of eukaryotic DNA polymerases to completely synthesise the lagging strand during DNA replication (Levy et al. 1992). While the DNA polymerase synthesises the complementary daughter strand on the leading strand continuously in 5′-3′ direction, the only direction possible for DNA polymerases, on the lagging strand short DNA fragments called "Okazaki fragments", are synthesised with the help of an 8–12-base-long RNA primer. The latter is later degraded and the fragments stitched together by DNA ligase. However, at the very last distal Okazaki fragment, the RNA primer is degraded and cannot be replaced by DNA. This leaves a part of the newly synthesised DNA strand shorter than the original parental DNA strand which thereby forms a 3′ overhang. This single-stranded structure is protected within the above described D-loop, and its function as a substrate for telomerase binding will be described in conjunction with telomerase. Due to the loss of a small telomeric sequence during each round of cell division and DNA synthesis, telomeres shorten in cells without telomerase which is able to counteract the loss of telomere synthesis by filling in telomeric hexanucleotides onto the 3′ strand, which is then filled up at the 5′ strand during the next replication cycle.

This regular telomere shortening during each round of DNA replication is the basis for the finite number of cell divisions characteristic for most human somatic cells in culture, known as the "Hayflick limit" (Hayflick and Moorhead 1961; Allsopp et al. 1992; von Zglinicki et al. 1995; Blasco et al. 1997). Consecutive telomere shortening eventually results in a critical telomere length which leads to telomere uncapping (Fig. 9.4a) and the activation of a p53-dependent DNA damage

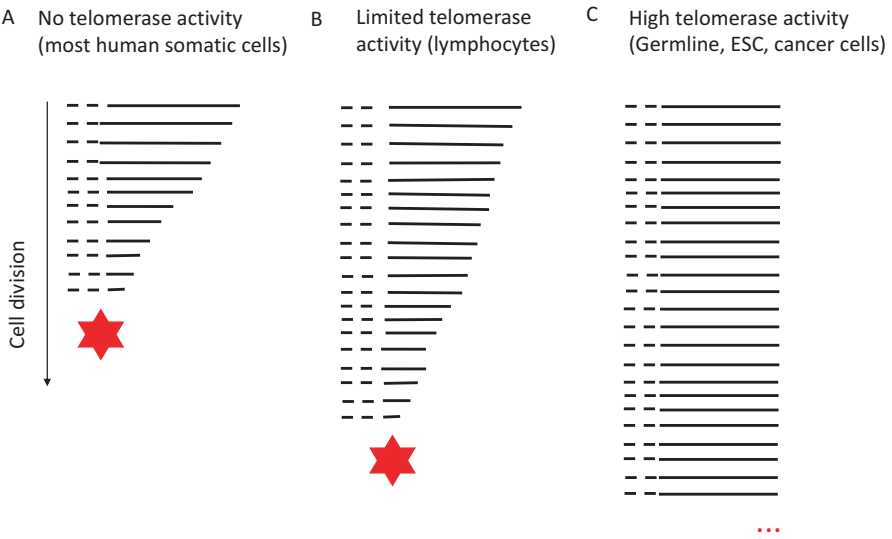

Fig. 9.4 Correlation between telomerase activity and telomere maintenance. (**a**) Telomere shortening induces senescence in human somatic cells. (**b**) Extended cellular lifespan due to some telomerase activity. (**c**) Constitutively high telomerase activity results in telomere maintenance and unlimited proliferation potential

response (DDR) leading to replicative senescence or apoptosis. This will be described in more detail later. Short telomeres are particularly vulnerable against external DNA damage (Goytisolo et al. 2000; González-Suárez et al. 2003; Sedelnikova et al. 2004).

In addition, nucleolytic end processing contributes to telomere shortening. DNA replication generates blunt ends on the leading strands and non-blunt ends on the lagging strands (Makarov et al. 1997; Pfeiffer and Lingner 2012). TRF2 recruits Apollo that resects the 5′ strand from the leading end to generate a 3′ overhang (Wu et al. 2012). Afterwards, Exo1 excessively resects both the leading and lagging strands to generate longer overhangs. Finally, POT1 directs the fill-in of the 5′-strand by the recruiting Pol-α through the CST complex (Bernal and Tusell 2018).

Telomere shortening also depends on environmental conditions and can increase up to tenfold under increased oxidative stress from around 20–50 bp per division in human fibroblasts under normoxic conditions up to 500 bp/cell division under hyperoxic culture conditions (von Zglinicki et al. 1995; von Zglinicki 2000, 2002; Richter and von Zglinicki 2007). Correspondingly, the lifespan of cultured cells varies according to their telomere shortening rates (see Fig. 9.5).

Oxidative stress is mainly derived from reactive oxygen species (ROS) produced at different cellular sites and by different pathways (NADPH oxidases, mitochondria). ROS directly target DNA and can modify it in different ways. It can induce direct breaks such as single-strand breaks into the DNA or modify nucleotides when the DNA consists of 8-oxodG which is then either not repaired, stalls repair mecha-

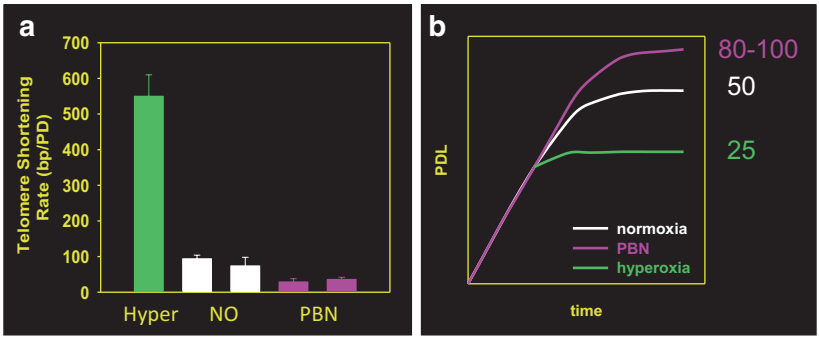

Fig. 9.5 Oxidative stress shortens telomere length (**a**) and reduces replicative lifespan (**b**). Data from von Zglinicki et al. (1995). *Hyper* hyperoxia, *NO* normoxia, *PBN* ROS scavenger that decreases oxidative stress

nisms or is translated into DNA breaks during DNA repair. Breaks which have accumulated when a cell is not dividing will be translated into telomere shortening when the cell divides next time (Sitte et al. 1998). Exposure to inflammation resulting in oxidative stress can also accelerate telomere loss and damage (von Zglinicki 2002; Jurk et al. 2014).

While oxidative stress can accelerate telomere shortening, the shortening rate also depends on the antioxidant capacity of the cell and might therefore be genetically determined (von Zglinicki et al. 2000; Lorenz et al. 2001). It has been demonstrated that different cell types of the same individual (e.g. PBMCs and skin fibroblasts) have corresponding antioxidant capacities (Zglinicki et al. 2000). In other words, cells with a high antioxidant capacity are less sensitive against oxidative stress than cells with a low antioxidant capacity that are very susceptible to oxidative stress. All these properties were thought to make telomeres good biomarkers of ageing. However, there is a downfall to this: telomerase which is active in some cell types such as lymphocytes, is able to counteract telomere shortening.

In addition, various postmitotic cells do not divide, and thus damage to their telomeres can accumulate without being translated into telomere shortening (Hewitt et al. 2012) although telomere uncapping can still occur and result in DNA damage signalling and senescence induction (Blackburn 2000, 2001, 2005). This is described in more detail in the next section (1.4). This situation can, for example, occur in neurons (Jurk et al. 2012).

Due to its high guanine content on one strand which contains the sequence TTAGGG, telomeric DNA seems to be rather sensitive against oxidative damage (Petersen et al. 1998; von Zglinicki et al. 2000b). Experiments on fibroblasts cultivated under different levels of oxidative stress show a greatly increased rate of telomere shortening under hyperoxic culture conditions compared to cells under normoxia (Fig. 9.5) (von Zglinicki et al. 1995). Likewise, decrease of oxidative stress using either physiologically low oxygen (around 3–5%) culture conditions or the addition of antioxidants decreases telomere shortening (von Zglinicki et al.

2000b; Richter and von Zglinicki 2007). This property of telomeres to react to their environment (oxidative stress in particular) together with a lower repair rate due to the high GGG content of telomeres contributes to the sentinel function of telomeres for genomic damage.

There are many more processes and numerous telomere-related factors involved in the fine-tuning of telomere lengths. For example, the role of telomere transcription and TERRA generation was already described above and is involved in the regulation of telomere length via various mechanisms. Among others that are described above, TERRA transcription facilitates the 5′-3′ nuclease activity of Exo1 at telomeres, thereby regulating the telomere shortening rate (Pfeiffer and Lingner 2012).

Moreover, there is a process called "telomere trimming" or "rapid loss of telomeres" involving overly long telomeres by "T-loop resolution" (Pickett et al. 2009, 2011). This process involves the XRCC3 (protein involved in homologous recombination)-mediated removal of telomere loops in the form of t-circles and can occur in the male germline as well as in activated normal lymphocytes (Pickett et al. 2011). A newly discovered mechanism of telomere length control is also known as telomere trimming and requires the protein TZAP. It directly competes with shelterin for telomere binding and thereby facilitates the trimming of too long telomeres by rapid deletion of telomeric repeats (Li et al. 2017). The main mechanisms of regulation of telomere length are summarised in Fig. 9.3.

How Do Dysfunctional (Short or Damaged) Telomeres Induce Senescence?

Cellular senescence is defined as an irreversible loss of replicative capacity occurring in primary somatic cells (Hayflick and Moorhead 1961). Cellular senescence has numerous causes, but one very likely mechanism is telomere shortening (Harley et al. 1990a, b; Herbig et al. 2004; von Zglinicki et al. 2005) or damage to telomeres (Hewitt et al. 2012; Fumagalli et al. 2012; Nakamura et al. 2008). Functional telomeres protect chromosome ends so that these are not recognised as double-strand breaks. Shelterin components at functional telomeres repress the DNA damage response at telomeres, while distinct shelterin proteins suppress specific damage signalling and repair pathways. For examples, defects in TRF2 activate the ATM pathway (van Steensel et al. 1998), while removal of TRF1 and POT1 activates ATR (Carneiro et al. 2010). In contrast, overexpression of TRF2 diminishes the repair of telomeric single-strand breaks and accelerates telomere shortening in human fibroblasts resulting eventually in senescence (Richter et al. 2007).

Telomere shortening in the absence of a compensatory mechanism such as telomerase activity (described below) results in uncapped telomeres. In the presence of intact checkpoints, a DNA damage response is activated that results either in senescence or apoptosis; both are tumour suppressor mechanisms that prevent fur-

ther cell divisions and suppress cancer development efficiently. However, in the event of dysfunctional cell cycle checkpoints, telomeres can shorten further, leading to a telomere crisis (Ramírez et al. 2003), which can again lead to apoptosis, but also to genomic instability and telomere fusions and greatly increase the risk of tumour development. Thus, telomere shortening and the induction of a DDR are also potent tumour suppressor mechanisms.

The causal relationship between telomere shortening and senescence induction was demonstrated when the telomerase protein TERT was overexpressed in various human primary cell types, preventing senescence induction (Bodnar et al. 1998). Human fibroblasts reach replicative senescence with an average telomere length of around 6–8 kb (Allsopp et al. 1992). Critically short telomeres are thought to result in opening of the T-loop and, consequently, an "uncapping" of telomeres exposing the DNA ends to emanate a DNA damage response (Aubert and Lansdorp 2008).

Telomere dysfunction can be caused by (a) shortening of telomeres (von Zglinicki et al. 1995), (b) uncapping of telomeres due to the opening of the protective T- and D-loops, for example, by inhibition of TRF2 (Karlseder et al. 1999) and (c) by DNA damage that occurs anywhere in the telomere (Hewitt et al. 2012; Fumagalli et al. 2012). While telomere shortening requires cell division (Sitte et al. 1998), DNA damage can also accumulate in telomeres of non-dividing cells and result in telomere dysfunction (Hewitt et al. 2012; Jurk et al. 2012). Stabilisation of senescence may occur through the positive feedback loop between pro-inflammatory signalling, telomere dysfunction, senescence-associated ROS production and SASP (Passos et al. 2010; Jurk et al. 2014).

In addition to DNA damage at telomeres, the de Lange lab has demonstrated that the disruption of some shelterin components such as TRF2 and POT1 also resulted in the occurrence of telomere-dysfunction-induced DNA damage-related foci (TIF) (Takai et al. 2003; Denchi and de Lange 2007).

Induction of senescence by the DDR involves the tumour suppressor p53, which in turn activates the cyclin-dependent kinase inhibitor p21 resulting in a halt of cell cycle progression (Di Leonardo et al. 1994; Herbig et al. 2004; d'Adda di Fagagna et al. 2003).

Critically short or damaged telomeres induce p53 activation and a p21 signal, which is then signalled to the sensor kinases ATM/ATR kinases that are recruited to the DNA damage site, then activating downstream mediator kinases, checkpoint kinases I and II (CHK1 and CHK2) (Rouse and Jackson 2002; von Zglinicki et al. 2005). Eventually, DDR proteins such as the histone H2A.X are phosphorylated at Ser-139 (D'Adda di Fagagna et al. 2003), while others such as BP53 are also recruited to so-called DNA damage foci. These can be detected as telomere dysfunction-induced foci (TIF) that appear at the uncapped telomeres (Takai et al. 2003; D'Adda di Fagagna et al. 2003; Kaul et al. 2012) or telomere-associated foci (TAFs) that can occur anywhere along the telomere (Hewitt et al. 2012; Fumagalli et al. 2012), see Fig. 9.3b. As few as five dysfunctional telomeres are sufficient to induce an irreversible cell cycle arrest, replicative senescence in primary human fibroblasts (Kaul et al. 2012).

Several protein kinases from diverse eukaryotes known to perform important roles in DNA repair have been demonstrated to play critical roles in telomere maintenance. The human telomere-associated protein TRF2 is rapidly but transiently (around 2 h) phosphorylated by ataxia-telangiectasia-mutated (ATM) protein kinase in response to DNA damage (Tanaka et al. 2005). These authors showed that the phosphorylated form of TRF2 does not bind to telomeres and rapidly localises to the site of DNA damage. This provides an association between TRF2 phosphorylation to the DNA damage response.

Telomere damage is normally thought to be irreparable (Petersen et al. 1998; Hewitt et al. 2012; Fumagalli et al. 2012), while new data suggest that DSB repair at internal sites of telomeres can occur (Doksani and de Lange 2016). Several groups have recently shown that telomeres can acquire DNA damage independently of length, the presence of telomerase activity or telomere fusion (Hewitt et al. 2012; Fumagalli et al. 2012; Cesare et al. 2009). These results can also explain why mouse telomeres, although very long, can still be damaged and induce a cell cycle arrest (Hewitt et al. 2012). This finding also demonstrates that it is not just short telomere length or telomere uncapping that induces senescence. It is not entirely clear why there is no DNA repair at telomeres, but several studies have suggested that telomere binding proteins such as TRF2 and RAP1 might actively inhibit DNA repair mechanisms such as non-homologous end joining (NHEJ) (Bae and Baumann 2007). Cesare and co-workers suggest that telomeres with a DNA damage response (measured as TIFs) might represent an intermediate configuration between a fully capped and an uncapped state (Cesare et al. 2009). Interestingly, the authors found that in cells with telomerase activity, DDR was associated with lower activity and shorter telomeres, while in cells using the ALT mechanism, TIF number was dependent on telomere length.

Recent data from the de Lange lab show that internal telomere DSB sites are repaired by homologous recombination (HR) and PARP1-dependent end joining utilising recombination events between sister telomeres is not repressed throughout the telomeric DNA. This is suggested to induce a more ALT-like phenotype (Doksani and de Lange 2016). However, the study used an experimental DSB inducing system in immortalised telomerase positive mouse embryonic fibroblasts (MEFs). Thus, it is possible that this process is more relevant in cancer cells.

Inheritance of Telomere Length (TL)

In addition to a gradual telomere erosion over cellular lifetime in dividing tissues due to the ERP and oxidative stress, there is a remarkable interindividual variability in telomere length across the general population that seems to be highly heritable. This corresponds to the results of many cross-sectional studies in the past that showed a high spread of TL over large age ranges (von Zglinicki et al. 2000; Deelen et al. 2014). Analysis of PBMCs of young dizygotic and monozygotic twins showed 78% heritability of TL (Slagboom et al. 1994; Kimura et al. 2008a). Some studies

reported stronger father-offspring than mother-offspring correlations for TL, suggesting that inheritance of this trait might be mainly paternally determined (Nordfjäll et al. 2005, 2010; Njajou et al. 2007). A strong correlation between offspring TL and paternal age at birth has been described (Unryn et al. 2005; De Meyer et al. 2007; Njajou et al. 2007; Kimura et al. 2008a; Arbeev et al. 2011).

Although it is well established that TL shortens with age in most proliferating tissues, sperm TL is an exception: older men have sperm with longer telomeres (Allsopp et al. 1992; Baird et al. 2006; Kimura et al. 2008b). The association of paternal age with longer TL is thought to be due to direct inheritance from longer TL in sperm (Kimura et al. 2008b), while the study could not find evidence that telomere shortening (TS) was delayed in sperm from older fathers. This may be explained by the fact that the activity of telomerase is high in testes (Wright et al. 1996; Zalenskaya and Zalensky 2002). In humans, a comparative study of TL in lymphocytes and sperm in 135 men aged 18–68 found a yearly decline in the former of 19 bp, while the latter gained around 57 bp per year (Aston et al. 2012). Consistent with the fact that offspring inherit half their chromosomes from sperm, offspring of older fathers tend to have longer telomeres (Kimura et al. 2008b; De Meyer et al. 2007; Unryn et al. 2005). Eisenberg et al. (2012a, b) even analysed the grandfather generation. They found evidence for cumulative, multigenerational lengthening of telomeres suggesting that age-related changes in sperm TL can be transmitted across at least two generations and is thus cumulative via paternal inheritance: grandchildren of older paternal grandfathers at the birth of fathers had longer telomeres (Eisenberg et al. 2012a, b).

This finding has serious implications for human TL in the future due to the fact that in many developed countries, average reproductive age of fathers cumulatively influences TL of their offspring. At least in theory, this can in the long run improve longevity, health and life expectancy of the population provided a large enough penetrance of the trait.

Others have also suggested a correlation between maternal TL and offspring TL (Nawrot et al. 2004; Akkad et al. 2006; Broer et al. 2013), but these findings could not be confirmed widely. While male gametes divide continuously throughout life, female oocytes are generated prenatally. This could explain why TL did not vary in relation to maternal age at the time of birth, and consequently, maternal and grand maternal ages were not significantly associated with the child's or the grandchild's TL (Eisenberg et al. 2012a, b).

Importantly, while in early life, there is a strong impact of inheritance on PBMC TL (Slagboom et al. 1994; Graakjaer et al. 2006; Nordfjäll et al. 2005, 2010; Njajou et al. 2007), the strength of the hereditary factor seems to diminish with age corresponding to a lower correlation in TL for older twin pairs (Svenson et al. 2011).

Benetos et al. (2011) suggested that to account for inherited TL, which is much better preserved in non-dividing tissues such as muscle and fat, should be subtracted from the TL of highly proliferative cells such as lymphocytes, which are the common cell type usually analysed in human studies on TL. This way they found a much stronger correlation between age and TL in PBMC which better accounts for any age-related changes. Telomere shortening is largest during the first years of life,

while interestingly, the pattern of chromosome-specific variations in telomere length is maintained throughout life (Graakjaer et al. 2003).

Genetic association studies have also revealed associations between TL and single-nucleotide polymorphisms (SNPs) in genes coding for the telomerase components TERC and TERT (Lee et al. 2014a), and see below. Other studies demonstrated that genes regulating TL may influence human longevity by showing associations between SNPs in TERC and POT1 with human longevity (Deelen et al. 2013a, b).

Consequently, to better understand telomere length biology and its regulation during ageing and disease, longitudinal studies seem the better way forward.

Telomerase

Structure and Telomere-Related Telomerase Function

Telomerase is an RNA-dependent DNA polymerase and reverse transcriptase which in its canonical function extends telomere ends (Greider and Blackburn 1989). It consists of the reverse transcriptase (TERT) catalytic subunit and the RNA part (TERC) containing the template for telomere synthesis. TERT and TERC are both necessary and sufficient for the catalytic activity of telomere maintenance and elongation to take place (Lingner et al. 1997; Weinrich et al. 1997). In addition, both telomerase components interact with specific factors: TERT with Hsp90 and p23 and TERC with L22 and hStau, dyskerin, NHP2, GAR1 and NOP10 (Wang and Meier 2004). Telomerase is related to other reverse transcriptases such as human immuno-deficiency virus (HIV), long terminal repeats (LTRs), group II introns , etc. (Lingner et al. 1997; Nakamura et al. 1997).

The human TERT gene is located on chromosome 5p15.33 (Greenberg et al. 1998), while TERC is located at 3p26.3 (Soder et al. 1997). TERT mRNA in mammals comprises 16 exons and 15 introns across 35kb (Cong et al. 1999). In mammals, TERC comprises 451 nucleotides, while its template part complements the telomeric sequence, which comprises 11 nucleotides (5′-CUAACCCUAAC-3′) (Feng et al. 1995; Chen and Greider 2003; Egan and Collins 2012).

This RNA (hTR or hTERC) has a complex secondary and tertiary structure (Zhang et al. 2011). The 3′ G-rich telomeric overhang, described above under the "end replication problem", is recognised by the telomerase complex and elongated with hexameric TTAGGG telomeric repeats that are complementary to the template region on the TERC RNA component (Blackburn 2005). In this way telomerase via its enzymatic function is able to add DNA sequences to telomeres, thereby compensating for the loss sustained during DNA replication and the ERP.

Canonical (telomere elongation) as well as noncanonical functions have been described for telomerase and its hTERT component. When functioning canonically, telomerase enzymatic activity protects against chromosomal end fusion, chromo-

somal instability and telomere erosion. Noncanonical (non-telomeric) functions of the telomerase subunit hTERT are implicated in various cellular functions such as cell survival and proliferation, mitochondrial function, regulation of gene expression, stem cell biology, chromatin remodelling and cellular transformation as well as a protective function against apoptosis and oxidative stress related to mitochondrial localisation (Cong and Shay 2008; Saretzki 2014; Ahmed et al. 2008; Haendeler et al. 2009; Singhapol et al. 2013). This will be described in more detail below.

Telomerase Activity in Human Tissues

The highly conserved and tightly regulated catalytic subunit TERT (telomerase reverse transcriptase) of the telomerase enzyme complex maintains and restores the length of telomeres by reverse transcribing telomeric hexanucleotides from an RNA template (Smogorzewska and de Lange 2004). However, in humans, only germline stem cells and cancer cells express sufficient levels of telomerase to maintain telomere lengths completely (Kim et al. 1994; Wright et al. 1996; Shay and Bacchetti 1997).

Telomerase is expressed in a tissue-specific manner and decreases in telomerase positive human cell types during ageing (Lin et al. 2015; Young et al. 2003). While telomerase activity is persistent in most adult mouse tissues (Martín-Rivera et al. 1998), telomerase is active in most human embryonic tissues until about week 20 of gestation, at which point its levels gradually decline except, for example, in the liver (Ulaner and Giudice 1997, Ulaner et al. 1998). Activity is still detectable in certain adult human cells, often after activation in lymphocytes, endothelial cells and adult stem cells (Lin et al. 2015; Kurz et al. 2003; Valentijn et al. 2015). Telomerase activity is also high in germline cells, ensuring that offspring starts with sufficiently long telomeres. Embryonic stem cells that are derived from the inner cell mass of blastocysts are characterised by high telomerase activity levels. This is the basis for them being able to grow continuously *in vitro* under cell culture conditions (Saretzki et al. 2008). Finally, telomerase activity is highly up-regulated during tumour development and thus high in most cancer entities (Shay and Bacchetti 1997). One possible reason for the up-regulation of TA is mutations in the hTERT promoter during cancerogenesis (Shimoi et al. 2017; Jung et al. 2017). Another important mechanism of up-regulation of telomerase during tumorigenesis is the interaction with human papillomavirus (HPV) type I6 and 18 E6 protein that can induce telomerase activity in both mammary epithelial cells and primary human keratinocytes (Klingelhutz et al. 1996), mediated by hTERT transcriptional up-regulation (Gewin and Galloway 2001; Veldman et al. 2001). The maintenance of telomeres, even if it occurs at a very short telomere length in cancer cells, allows cells to grow indefinitely and is thus an important prerequisite for cellular immortality.

In addition, there is one alternative mechanism of telomere extension which is based on recombination of telomeric sequences and called “alternative lengthening of telomeres” (ALT) (Dunham et al. 2000). However, it occurs not very frequently

in vivo but mainly during *in vitro* immortalisation of cells of mesenchymal origin and in cancers such as sarcomas that are derived from this cell type (Reddel 2003; Henson and Reddel 2010). Another important difference between telomerase activity in mouse and human cells is its different processivity which is much lower in mouse tissues (Chen and Greider 2012). The mechanisms for these differences are not yet well understood.

Regulation of Telomerase

Telomerase is regulated at multiple molecular levels (for review, *see* Liu 1999; Stewart 2002; Gladych et al. 2011). Firstly, transcription of the hTERT as well as the RNA (called hTR or hTERC) is influenced by various transcription factors such as myc, Sp1 and others which can either activate or inhibit transcription (Cong et al. 1999). The promoter activity of the hTERT gene can also be influenced by different hormones such as oestrogen and progesterone although their mechanism of action is not entirely well understood. Other regulatory mechanisms include hTERT splicing, post-translational modification, binding to and accessing telomeres as well as subcellular localisation.

Regulation of *hTERT* Transcription

Transcription of *hTERT* has been suggested as a preferential step in the regulation of telomerase activity (Shay and Bacchetti 1997). The promoter region of *hTERT* is rather extensive in length (several kilobases), and parts have been cloned (Cong et al. 1999; Takakura et al. 1999). Previous studies on the *hTERT* promoter have defined a core region encompassing 330 bp upstream of the translation start site to 228 bp downstream, extending right into the second exon of the gene (Cong et al. 1999; Horikawa et al. 1999; Takakura et al. 1999). A number of transcription factor binding sites have been identified in this core promoter. The core promoter of the *hTERT* gene contains several known regulatory elements including GC-motifs and E-boxes. Several transcription factors, including c-Myc, Sp1, activating enhancer-binding protein-2 (AP-2), hypoxia-inducible factor (HIF-1), ETS, oestrogen receptor (ER), E2F, activator protein 1 (AP-1), vitamin D receptor (VDR), Wilms' tumour 1 (WT1) and many more, have been found to modulate the transcriptional activity of the *hTERT* promoter (Ramlee et al. 2016). These factors can regulate hTERT transcription either positively or negatively, some form complexes such as myc/mad/max, and others like SP1 can regulate in both directions, depending on cellular context (Oh et al. 2000; Wu et al. 1999; Zhang et al. 2017). For more detail please see recent reviews (Ramlee et al. 2016; Avin et al. 2016; Zhang et al. 2016a, b, c).

Hormonal Regulation of *hTERT* Transcription

In addition to multiple specific transcription factors, hormones seem to play an important role on hTERT transcription in hormone-sensitive tissues (for reviews, *see* Bayne and Liu 2005; Hapangama et al. 2017). However, their binding often occurs far away from the core promoter and is sometimes rather indirect such as for progesterone that utilises MAP kinase signalling (Wang et al. 2000). The oestrogen receptor is a nuclear hormone receptor which binds to oestrogen response elements (EREs) upon stimulation by its ligand. hTERT transcription and telomerase activity are activated by estradiol (E2) in ER-positive cells (Boggess et al. 2006). *In vitro* binding assays showed that ERα specifically binds to two EREs in the *hTERT* promoter (Misiti et al. 2000). Telomerase activation by oestrogen occurs in hormone-responsive tissues (Kyo et al. 1999; Misiti et al. 2000). Telomerase activity, for example, in the human endometrium, correlates to endometrial cell proliferation and is differentially expressed during the menstrual cycle (Kyo et al. 1997; Tanaka et al. 1998; Hapangama et al. 2017). This indicates a role for steroid sex hormones in telomerase activity regulation.

Effect of Viruses on *hTERT* Transcription

Some virus infections are known to induce oncogenic pathways. For example, several high-risk human papillomaviruses (HPVs) are causally involved in the majority of cervical cancers by transactivating the *hTERT* promoter of (Klingelhutz and Roman 2012). The transforming genes of the high-risk HPVs are E6 and E7. Kiyono et al. (1998) demonstrated that E6 and E7 collaborated to immortalise cultured keratinocytes (Kiyono et al. 1998). E6 is able to degrade p53 and also to induce *hTERT* transcription in genital keratinocytes by interacting with the transcription factor myc (Liu et al. 2009). This correlates to an increased cellular telomerase activity and immortalisation. However, the HPVs were not able to transactivate *hTERT* promoters in fibroblasts (Liu et al. 2009).

Various studies over the last 20 years have identified the transcriptional, epigenetic and post-transcriptional roles that high-risk E6 and E7 proteins have in telomerase regulation (Zhang et al. 2017). For review, *see* Katzenellenbogen (2017).

In contrast, the human CMV is able to induce a constitutive hTERT expression and telomerase activation in human fibroblasts depending on Sp1-binding sites in the hTERT promoter and acetylation of histone H3 as well as a reduction in HDAC binding at the core hTERT promoter (Stråat et al. 2009).

Regulation of *hTR* Transcription

hTR is comprised of four domains: the core domain, the conserved regions 4 and 5 (CR4/CR5), the H/ACA-specific RNA (scaRNA) and CR7 (for more details, *see* Zhang et al. 2011). The highly conserved core domain and CR4/CR5 regions are

central for association of hTR with hTERT. The core domain itself contains an 11-nucleotide CAAUCCCAAUC repeat that acts as a template for telomere repeat binding. The H/ACA domain interacts with evolutionarily conserved H/ACA proteins dyskerin, Gar1, Nop10 and Nhp2, forming an H/ACA ribonucleoprotein. Mutations in this domain or interacting proteins commonly cause dyskeratosis congenita. Along with CR7, the H/ACA box recruits two downstream proteins to allow targeting of TR to Cajal bodies. The Cajal bodies act as sites of modification and assembly of various RNPs, including spliceosomal factors (Morris 2008). hTR is thought to be constitutively and ubiquitously expressed in most human tissue but has been shown to be the limiting factor for telomerase activity in human brain during early development (Ishaq et al. 2016). Sp1 and HIF-1 activate *hTR* transcription, while other proteins (Sp3) repress it by silencing the hTR promoter using MAPK signalling cues (reviewed in Cairney and Keith, 2008). *hTR* expression is thought to be rather ubiquitous in most human tissues (Feng et al. 1995). Bilsland et al. (2006) showed that *hTR* promoter activity is repressed through collaboration between Sp3 and MEKK1.

Although it has been suggested for a long time that the regulation of hTERT is the predominant step in the regulation of telomerase activity, new data suggest that there are exceptions to this. Ishaq et al. (2016) analysed the regulation of *hTERT* and *hTR* expression as well as telomerase activity during early human brain development. They found that TA is reduced to undetectable levels after postconception week 14, strongly correlating to a down-regulation of hTR expression, while there were no significant changes in the levels of wild-type or alpha-spliced hTERT (Ishaq et al. 2016). This seems to be the reason for a maintenance of the TERT protein in human and mouse neurons (Spilsbury et al. 2015; Iannilli et al. 2013) which forms the basis for a noncanonical function of the TERT protein in neurons where it is located outside the nucleus (Spilsbury et al. 2015; Iannilli et al. 2013), without the presence of telomerase activity.

Regulation of hTERT by Alternative Splicing

In humans, the hTERT gene spans approximately 42 kb and consists of 16 exons. The hTERT mRNA is frequently alternatively spliced (Ulaner et al. 1998; Colgin et al. 2000; Listerman et al. 2013). Around 22 hTERT splice versions have been identified so far (Hrdlickova et al. 2012). None of these isoforms however – with the exception of the full-length transcript, containing all 16 exons – are able to elongate telomeres, nor do they exhibit any reverse transcriptase activity (Saebøe-Larssen et al. 2006; Yi et al. 2000). The reverse transcriptase domain of hTERT generates the most common alternatively spliced isoforms: alpha (36 bp in frame deletion in exon 6), beta (truncated form) and alpha-beta. The alpha version is translated into a dominant-negative protein without reverse transcriptase activity (Saebøe-Larssen et al. 2006; Colgin et al. 2000). The α-splice hTERT has abolished telomerase activity through removal of the first 36 bases of exon 6, while the β-splice variant has abolished telomerase activity through the removal of 182 bases between exons 7

and 8, resulting in a transcript with a premature termination codon. The two splice variants can occur simultaneously in one transcript to produce a double-spliced variant, and all three combinations have been shown to occur naturally in humans (Ulaner et al. 1998; Lincz et al. 2008; Hrdlickova et al. 2012). The alpha and beta splice variants were found to be able to efficiently bind hTR and thereby compete with the wild-type protein, suggesting a dominant-negative hTERT function (Colgin et al. 2000; Listerman et al. 2013). The beta splice version which generates a truncated protein has been found predominantly in cancer cells and seems also to regulate the levels of wild-type telomerase. However, this function was insufficient for the complete inhibition of telomerase activity, and stable telomere lengths were maintained in breast cancer cell lines (Listerman et al. 2013). In human embryonic stem cells, it has been recently described that environmental influences such as different oxygen levels are also able to modify the splice pattern of hTERT (Radan et al. 2014).

Post-translational Modification of hTERT

The correlation between TERT mRNA levels and telomerase activity is not consistent, which suggests post-translational TERT regulation (Ulaner et al. 2000; Rohde et al. 2000). Mammalian TERT sequences have sites for phosphorylation (Kang et al. 1999) which implicates at least two kinases in the phosphorylation of hTERT. The ubiquitously expressed tyrosine kinase c-Abl that is activated by DNA double-strand breaks, phosphorylates hTERT and inhibits telomerase activity. In contrast, cells deficient in c-Abl show increase in telomerase activity and telomere lengthening (Kharbanda et al. 2000). A second kinase phosphorylating hTERT is Akt (a serine/threonine kinase) which phosphorylates serine 227 of the hTERT nuclear localisation signal (NLS) (amino acid residues 222–240) and thus promotes nuclear translocation in activated immune cells (Chung et al. 2012). hTERT phosphorylation by Akt at sites Serine243 and 473 increases telomerase activity and is important during the activation in immune cells such as lymphocytes and natural killer cells (Kawauchi et al. 2005; Akiyama et al. 2002, 2003, 2004).

Plunkett et al. (2007) confirmed that hTERT is a substrate for Akt phosphorylation but found that $CD8^+$ cells at senescence (CD28-) were not able to up-regulate their TA despite no changes in hTERT or total Akt levels in any of the $CD8^+$ T-cell subsets. In order for activation of telomerase to occur, Akt must be phosphorylated at both the Ser473 and Thr308 sites. In the $CD8^+CD28^-CD27^-$ T-cell subset, a selective lack of Akt phosphorylation was found at the Ser473 site suggesting that when Akt fails to phosphorylate hTERT on the Ser227, there is a correlation with $CD8^+$ T lymphocyte senescence (Plunkett et al. 2007). This replicative end stage seems to limit the ability of memory $CD8^+$ T cells to proliferate and function properly *in vivo*.

IGF-1 can also activate telomerase activity in various human cell models without changing hTERT transcription but instead increasing the phosphorylation of Akt (Akiyama et al. 2002; Tu et al. 1999). This resulted in phosphorylation and nuclear

translocation of the hTERT protein, thereby increasing telomerase activity (Akiyama et al. 2002).

Epigenetic Regulation of TERT Expression

Chromatin environment and epigenetic modifications of the *hTERT* promoter play an important role in the regulation of *hTERT* transcriptional states as well its differential transcription in different cell types (Zhang et al. 2016a, b, c). Main mechanisms are methylation of the *hTERT* promoter and histone modifications. DNA methylation is involved in general gene silencing. The *hTERT* promoter contains clusters of CpG islands with dense GC-rich regions suggesting that DNA methylation may play a role in the regulation of *hTERT* expression (Devereux et al. 1999). However, the exact relationship between DNA methylation and telomerase regulation is not yet well understood, and contradicting results seem to exist (Ramlee et al. 2016).

In telomerase-expressing cells, *hTERT* expression is associated with hyperacetylation of histone H3 and H4 and methylation of lysine-4 of histone H3 (Atkinson et al. 2005). Induction of the tumour suppressor, AT-rich interactive domain 1A (ARID1A), increases occupancy of H3K9me3 at transcription start site of *hTERT* and decreases acetylated lysine-12 of histone H4 (H4K12Ac) levels at this site. Treatment of telomerase negative cells with the histone deacetylase inhibitor trichostatin A activates the transcription of the *hTERT* gene (Atkinson et al. 2005). Saretzki et al. (2008) reported that both promoters,*hTERT*and *hTR*, were down-regulated during differentiation of human ES cells correlating to HTERT and hTR promoter acetylation. Qing et al. (2016) demonstrated that histone deacetylase (HDAC) inhibition was able to regulate *hTERT* expression and transcription by modulating promoter activity. In contrast, on the protein level, HDAC inhibition reduced TERT protein level by increasing protein degradation. This resulted in decreased telomerase activity and cellular senescence in rat aortic cells (Qing et al. 2016).

Telomere Length Regulation by Binding of Telomerase to and Accessing of Telomeres

Telomeres are not always maintained in cells, despite the presence of active telomerase. One reason can be the inaccessibility of telomeres by telomerase (Counter et al. 1998). In order to successfully extend telomeres, they have to be accessible for the telomerase enzyme. Telomere elongation by telomerase occurs in late S phase when most of the genome has already been replicated. The regulation of accessibility of telomeres for telomerase is not entirely understood. However, one important prerequisite is the regulated opening of the telomere loop and the D-loop making the 3′ telomeric overhang accessible to telomerase as substrate by removing the Pot1 shelterin.

The binding between some shelterin proteins and telomeres prevents telomerase from interacting with the end of chromosomes. Telomere accessibility is regulated

by telomere-associated proteins such as TRF1 and TRF2 that negatively regulate telomere length via a negative feedback mechanism in *cis* by inhibiting the accessibility and action of telomerase at telomeres (van Steensel and de Lange 1997; Smogorzewska et al. 2000). In contrast, a dominant-negative TRF1 caused telomeres to extend in telomerase-positive cells (Karlseder et al. 2002). Since more TRF1 is able to attach to longer telomeres, this results in a negative feedback. This not only regulates telomere length but also provides a potential explanation for why the shortest telomeres are preferentially elongated by telomerase (Steinert et al. 2000; Teixeira et al. 2004).

It is known that POT1 and TPP1 are involved in telomerase-telomere interaction. Association between POT1 and TPP1 at the 3′ end of telomeres prevents telomerase from binding to telomeres (de Lange 2005). It has been suggested that the physical links between the shelterin complex and telomerase are secondary to the affinity of POT1 to telomeric ssDNA and TPP1 with telomerase, which prevents telomerase from binding to telomeres. TPP1 binds to telomeres via TIN2 to regulate the recruitment of telomerase to telomeres (Abreu et al. 2010). The POT1-TPP1 complex promotes telomerase activity at telomeres until a certain threshold of telomere length. In addition, it was shown that the human POT1-TPP1 complex is a processivity factor for telomerase (Wang et al. 2007; Wang and Lei 2011), most likely by terminating telomerase access at some point. The telomere extends to a certain species-specific threshold, by telomerase generating new repeats, to which shelterin complexes bind. However, it is not well understood how this threshold of TL is regulated in mammals including humans. The new 3′ terminus then becomes rebound to POT1-TTP1, preventing further telomere extension (Wang et al. 2007; Nandakumar and Cech 2013). In contrast, telomerase access is blocked by the CST (CTC1, STN1 and TEN1) complex after which event Pol-α is recruited to synthesise the C-strand (Wang et al. 2012). Ku86 was also identified as a negative regulator of telomerase by blocking its access to the telomere (Espejel et al. 2002b). Likewise, DNA-PK also interacts with telomerase to maintain telomeres (Espejel et al. 2002a).

Additionally, binding of TERRA to hnRNPA1 *in vivo* can alleviate the TERRA-mediated inhibition of telomerase. Thus, telomere extension by telomerase may require balanced levels of TERRA and hnRNPA1 at telomeres. Thus, TERRA and hnRNPA1 could function as molecular regulators to turn telomerase on and off at the telomere (Redon et al. 2013).

Mammalian telomere length in normal somatic cells is tightly regulated at a physiological level. Most previous studies have focused on short telomeres that seem to be preferentially elongated and maintained by telomerase activity. Surprisingly, Zheng et al. (2014) demonstrated that hTERT also might be involved in the regulation of excessively long telomeres. The authors demonstrated that hTERT actively shortens excessively long telomeres while at the same time and in the same cell elongating short telomeres maintaining an apparently optimal telomere length at each chromosomal end for efficient protection and capping in primary human as well as cancer cells. This novel function requires enzymatic activity of hTERT, but not the presence of the telomerase RNA component, hTR. The authors found an increase in telomeric circular DNA (t-circles) due to *hTERT*

expression, suggesting an involvement of telomere homologous recombination in the telomere-shortening process, similar to other telomere trimming mechanisms (Pickett et al. 2009, 2011). While "telomere rapid deletion" (Pickett et al. 2009, 2011) involves the occurrence of DNA damage foci on telomeres, this new mechanism apparently is independent of foci formation despite removing large segments of telomeric DNA (Zheng et al. 2014). Thus, both processes seem to be distinct although they have in common the rapidity of the process as well as the formation of t-circles. By simultaneously shortening long and elongating short telomeres, this mechanism seems to be involved in the set-point regulation of telomere length and also decreases the variation in TL within a cell. This novel mechanism adds another level of complexity to the regulation of telomere length homeostasis.

Telomere Length Regulates Telomerase Expression via Loop Formation and Telomere Position Effect (TPE)

The Shay group recently described a phenomenon where genes are regulated by telomere length-dependent loops and telomere position effects over long distances (TPE-OLD) (Robin et al. 2014; Kim et al. 2016). The *hTERT* gene is located around 1.2 Mb from the human chromosome 5p end. The authors found that when telomeres were long, the *hTERT* gene expression was repressed. In contrast, when telomere length was short, at least one *hTERT*allele was spatially separated from the telomere and developed more active histone marks and changes in DNA methylation in the *hTERT* promoter region, resulting in the activation of telomerase. These findings might explain how cells turn off telomerase when telomeres are long and turn it on in cells with short telomeres (Kim and Shay 2018). They also imply that telomere length can be potentially involved in changes in gene expression during ageing and senescence. While the more conventional TPE effect involves a direct effect of telomere shortening on the activation of genes located close to the telomere, there can be also a TPE over long distances (TPE-OLD) which involves the formation of telomere loops (Kim and Shay 2018). The authors demonstrate that the influence of short telomeres on the activation or repression of genes is independent of a DDR and that it can reach over long distances such as several megabases (Kim and Shay 2018).

Consequently, there seems to be a direct feedback loop from short telomeres to the activation of telomerase via an increased transcription of the *hTERT* gene.

Telomerase Regulation by Subcellular Localisation

Subnuclear Localisation of Telomerase

Various subcellular localisations of telomerase have been described, and a nucleolar localisation signal for hTERT has been identified (Etheridge et al. 2002). Tomlinson et al. (2006) found that localisation of telomerase to telomeres peaks at mid-S phase, while at other times telomerase was sequestered to other intranuclear sites away

from telomeres. They also found complex associations of both hTR and hTERT with nucleoli and Cajal bodies, implicating both structures in the biogenesis and trafficking of telomerase. Pinx1 mediates hTERT nucleolar localisation and telomerase enzymatic inhibition as two separate functions of telomerase regulation in human cells (Lin et al. 2007). PinX1 binds directly to both hTERT and hTR, while PinX1 overexpression decreases telomerase activity resulting in telomere shortening (Banik and Counter 2004). Nucleolar localisation and that in Cajal bodies are both implicated in the assembly and maturation of the telomerase complex (Lee et al. 2014b). However, recently it has been demonstrated that telomerase binds to the ribosomal DNA promoter and coding regions in nucleoli and stimulates transcription by polymerase I in a proliferation-dependent manner (Gonzalez et al. 2014). Down-regulating telomerase decreased proliferation: this additional non-canonical function of hTERT could explain its correlation to cellular proliferation in a telomere- and activity-independent way (Gonzalez et al. 2014).

TERT Shuttling Between Nucleus and Cytoplasm

While nuclear translocation of hTERT requires Akt-mediated phosphorylation of the serine 227 of the hTERT nuclear localisation signal (NLS) (amino acid residues 222–240) (Chung et al. 2012), the putative hTERT nuclear export signal (NES) is required to bind the CRM1/exportin-1 to hTERT involving competition for NES binding by the 14-3-3 protein (Seimiya et al. 2000). The authors found that 14-3-3 enhanced nuclear localisation of hTERT by inhibiting the CRM1 binding to the hTERT NES-like motif, while telomerase activity was not changed by either of those factors involved. Xi et al. (2013) found that the serine/threonine-protein phosphatase 2A (PP2A) physically interacts with hTERT and regulates its subcellular distribution by preventing the interaction of hTERT with the 14-3-3θ signalling protein, resulting in cytoplasmic accumulation of hTERT. However, different nuclear exclusion mechanisms seem to exist for hTERT. The molecular mechanism for hTERT nuclear exclusion is induced by both exogenous and endogenous oxidative stress as it arises during senescence (Haendeler et al. 2004). Haendeler and colleagues found that increased oxidative stress such as H_2O_2 treatment induced Src kinase phosphorylation of the hTERT protein on tyrosine 707 resulting in nuclear exclusion (Haendeler et al. 2003). Stress-induced nuclear export of TERT occurred via nuclear pores and was dependent on association with the Ran GTPase. The kinetics of hTERT nuclear exclusion depends on the type and strength of oxidative stress (Ahmed et al. 2008). While H_2O_2 treatment induced a rapid hTERT exclusion from hTERT-overexpressing fibroblasts and cancer cells within around 2–3 h (Ahmed et al. 2008; Singhapol et al. 2013), a more chronic physiological cultivation of *hTERT*-overexpressing fibroblasts resulted in a very slow hTERT exclusion over several months (Ahmed et al. 2008). If the hTERT protein shuttles out of the nucleus to other subcellular localisations, telomeres cannot be maintained (Ahmed et al. 2008). In addition, hTERT protein is also localised outside the nucleus in postmitotic cells such as neurons that don't have telomerase activity when mature (Iannilli

et al. 2013; Spilsbury et al. 2015). Iannilli and co-workers (2013) described a complex between RNA stress particles, p15 and TERT, which can be resolved during increased oxidative stress resulting in p15 translation and allowing hTERT to enter mitochondria.

Mitochondrial Localisation of hTERT

Mammalian TERT has been found to contain a specific N-terminal mitochondrial localisation sequence (MLS) (Santos et al. 2004). It is suggested that this sequence developed late in the enzyme's evolution, as it is not fully formed in plants and is lacking in yeast, ciliates and other simple eukaryotic organisms. When hTERT resides within mitochondria, upon oxidative stress, then less mtDNA damage, ROS and apoptosis are observed (Ahmed et al. 2008; Haendeler et al. 2009). Src kinase also phosphorylates tyrosine 707 of hTERT within mitochondria, resulting in the degradation of hTERT in mitochondria (Büchner et al. 2010).

Both nuclear and mitochondrial telomerase locations have been modelled using organelle-specific localisation vectors (Haendeler et al. 2003, 2009; Singhapol et al. 2013).

Various groups have established the benefits of mitochondrial TERT in differing roles, including improved respiration, decreased mitochondrial superoxide, increased mitochondrial membrane potential and reduced apoptosis and mitochondrial as well as nuclear DNA damage (Ahmed et al. 2008; Haendeler et al. 2009; Kovalenko et al. 2010; Singhapol et al. 2013). Within the mitochondria, hTERT has been found to bind to mitochondrial DNA, such as genes encoding for NADH dehydrogenase subunits 1, 2, 4 and 5, cytochrome oxidase units I and III, subunits 6 and 8 of ATP synthase and mitochondria-specific tRNAs (Sharma et al. 2012). The same group showed that hTERT can bind to mitochondrial RNAs and exert a reverse transcriptase activity in mitochondria. However, the biological consequences of this novel activity remain uncertain.

TERRA and Telomerase

In vitro experiments have demonstrated that TERRA is able to inhibit telomerase activity at telomeres efficiently by base pairing via their 5′-UUAGGG-3′ repeats with the template sequence of telomerase RNA, in addition to contacting the telomerase reverse transcriptase protein subunit (Azzalin et al. 2007). TERRA molecules resemble the telomeric DNA sequence since they contain UUAGGG telomeric repeats which are complementary to the template sequence of telomerase RNA. A fraction of endogenous TERRA has been found bound to human telomerase in cell extracts (Redon et al. 2010). This binding of TERRA to the telomerase reverse transcriptase (TERT) protein subunit occurs independently of hTR. The affinity of telomerase for TERRA is very high and exceeds the affinity of telomerase for

telomeric DNA. In contrast, TERRA is not used as a telomerase substrate. Instead, TERRA acts as a competitive inhibitor for telomeric DNA. TERRA can bind to telomerase even while bound to the telomere substrate (Redon et al. 2010). TERRA is displaced and/or degraded at telomeres by nonsense-mediated decay (NMD) factors which physically interact with telomeric chromatin (Azzalin et al. 2007).

TERRA repeats are not extended by telomerase, but they can associate with the enzyme by binding to the hTERT protein and base pairing to the telomerase RNA template (Redon et al. 2010). However, the TERRA-telomerase interaction can be efficiently abolished by TERRA-binding proteins binding to the (UUAGGG)n telomeric sequence of TERRA molecules (Redon et al. 2013). Thus, in summary, it has been speculated that TERRA could regulate telomerase activity by interacting with its RNA template and also modulate selectivity of telomerase to preferentially extend short telomeres although the mechanisms are not entirely understood yet. Azhibek et al. (2016) demonstrated that TERRA RNA binds better to telomerase compared with its native substrate – the 3′ end of telomere DNA overhang. Consequently, the authors speculate that some specific yet so far unknown factor may exist that regulates the switch of telomerase binding from TERRA to the 3′ telomeric overhang for telomere elongation.

Redon and co-workers showed that a balance between the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) protein that interacts with telomeres and TERRA seems to influence telomerase inhibition by TERRA (Redon et al. 2013). While *in vitro* TERRA molecules are efficient inhibitors of human telomerase, *in vivo* binding of TERRA to hnRNPA1 can alleviate the TERRA-mediated inhibition of telomerase (Redon et al. 2013). Thus, TERRA and hnRNPA1 can function as coordinated molecular regulators to turn telomerase at the telomere on and off.

MicroRNAs Regulate hTERT Expression

MicroRNAs (miRNAs or mir) are small non-coding RNAs that regulate gene expression at post-transcriptional level (Santambrogio et al. 2014). The expression of *hTERT* can be positively or negatively regulated by miRNAs (for detailed review, *see* Farooqi et al. 2018). For example, it has been shown that nuclear localisation of hTERT was indirectly influenced by mir-375 which targeted and regulated an isoform of the 14-3-3 protein (Jung et al. 2014). MiR-34a was demonstrated to inhibit telomerase by targeting the FoxM1/c-Myc signalling cascade. A positive regulation of hTERT by microRNAs has been suggested via the PTEN inhibition and ERK1/2 activation pathways. MiR-21 seems to regulate hTERT expression by the PTEN/PI3K/AKT signalling pathway by directly targeting PTEN, therefore controlling hypertrophic scar fibroblast cell growth (Zhu et al. 2014). The Max dimerisation protein 1 (Mxd1) can be directly inhibited by miR-202 with Mxd1 binding to the *hTERT* promoter and decreasing myc-binding at the same time (Farhana et al. 2015). So far, most of the miRNA- related regulation of hTERT has been shown in cancer cells and might be independent of telomere involvement (Slattery et al. 2016).

Telomeres and Telomerase in Immune Cells and During the Ageing Process

Telomerase in Immune Cells

Telomeres are thought to be good biomarkers for the ageing process. Most studies analyse human telomere length in white blood cells: peripheral blood monocytes (PBMCs), due to the relative ease to obtain blood samples. However, telomere length is influenced by different factors that are important to consider when interpreting results on TL in PBMCs. The immune cell system is unique among human somatic cells in their capacity to up-regulate telomerase in a highly regulated way, upon stimulation, while in resting lymphocyte telomerase activity (TA) is hardly detected (Yamada et al. 1996; Roth et al. 2003; Lin et al. 2015). However, even the presence of telomerase activity in lymphocytes can never completely counteract telomere shortening, but it can delay it in comparison to other somatic cell types without telomerase (see Fig. 9.4b). Telomerase activity is expressed in a highly regulated fashion during human lymphocyte development, differentiation and activation (Hiyama et al. 1995; Weng et al. 1996, 1997a, b; Bodnar et al. 1996; Liu et al. 1999). hTERT transcripts and protein have been identified in all lymphocyte subsets isolated from the thymus and peripheral blood, regardless of the status of telomerase activity (Liu et al. 2001).

While telomerase is widely known to be regulated on the transcriptional level, the hTERT protein is readily available in the cytoplasm of lymphocytes. This is in contrast to other cell types such as most cancer cells where telomerase is mainly located in the nucleus and hTERT is excluded from there upon oxidative stress (Singhapol et al. 2013). Unstimulated T lymphocytes and natural killer (NK) cells contain cytoplasmic telomerase protein hTERT which localises to the nucleus only when the cells are activated (Liu et al. 2001). The authors showed that in normal lymphocytes, stimulation of human $CD4^+$ T cells with anti-CD3/CD28 up-regulated telomerase activity, associated with phosphorylation of the hTERT protein, and its nuclear translocation after 3 days, without any changes in hTERT protein levels. Thus, human T lymphocytes regulate telomerase function independent of hTERT transcriptional activation or changes in protein levels. Instead, phosphorylation and nuclear translocation of hTERT seem to be the dominant mechanisms of regulating telomerase function in human lymphocytes.

Upon stimulation with an antigen or IL-2, the protein is quickly transported into the nucleus and also phosphorylated by Akt which induces telomerase activity (Kawauchi, et al. 2005; Akiyama et al. 2003, 2004; Plunkett et al. 2007). Thus, in lymphocytes, telomerase activity is not primarily regulated on the transcriptional level but rather at the post-transcriptional level. Telomerase activity is necessary for T cells to proliferate following activation, and its level determines the lifespan of T lymphocytes (Roth et al. 2003). In peripheral blood lymphocytes (PBL), telomerase activity and subcellular shuttling are regulated through the PI3K/Akt/NF-κB signalling pathways (Akiyama et al. 2004). Nuclear translocation of hTERT requires Akt-

mediated phosphorylation of the serine 227 of the hTERT nuclear localisation signal (NLS) (amino acid residues 222–240) (Chung et al. 2012).

Reactive oxygen species (ROS) have been implicated in T-lymphocyte differentiation (Moro-García et al. 2018). Interestingly, mitochondrial ROS seem to be involved in T-lymphocyte activation by up-regulating IL-2 and IL-4 expression (Kaminski et al. 2010). Richardson et al. (2018) recently showed that oxidative stress can down-regulate TA and thereby compromise lymphocyte proliferation and increase inflammation. This result of an important influence of oxidative stress on lymphocyte proliferation corresponds well to recent findings from Moro-García et al. (2018) who demonstrated that exposure to high levels of ROS decreased the capacity of activation and T-lymphocyte proliferation, while intermediate levels of oxidation were required for lymphocyte activation, differentiation and effector functions.

TL in different T-cell subsets can be different: longer telomeres were found in naïve T cells compared with memory T cells (Weng et al. 1995; Lin et al. 2015). Undifferentiated ($CD27^+CD28^+$) T lymphocytes have higher TA and longer telomeres than highly differentiated ($CD27^-CD28^-$) T lymphocytes, while intermediate populations ($CD27^-CD28^+$ or $CD27^+CD28^-$) have telomere lengths between both groups (Plunkett et al. 2005). Correspondingly, telomerase activity is higher in the more undifferentiated cells and much lower in the cells with high differentiation. Moreover, differences in telomerase have been found between $CD4^+$ and $CD8^+$ T lymphocytes. $CD4^+$ T lymphocytes had much higher telomerase activity than CD8+ T lymphocytes from the same donor after repeated stimulation (Valenzuela and Effros 2002). This corresponds well to findings of an earlier occurrence of immuno-senescence in $CD8^+$ T lymphocytes than in CD4+ T lymphocytes (Czesnikiewicz-Guzik et al. 2007).

For lymphocytes such as $CD8^+$ T cells, it has been demonstrated that their telomeres get shorter due to lower telomerase activity during the human ageing process, eventually leading to immuno-senescence (Akbar and Vukmanovic-Stejic 2007). Plunkett et al. (2007) have found that when Akt phosphorylation of hTERT on serine 473 fails, telomerase is not activated, and $CD8^+$ T cells could not maintain their telomeres and eventually undergo senescence corresponding to previous *in vitro* findings (Effros et al. 2003). Thus, telomerase activity is an important prerequisite for the proliferation capacity of lymphocytes which can decline during immunosenescence known to be associated with the ageing process. Importantly, replicative senescence in cells including T lymphocytes does not result in a loss of cell viability. Senescent T cells can remain alive and metabolically active for a prolonged period of time but become resistant against apoptosis (Spaulding et al. 1999).

Telomerase activity is highly expressed in haematopoietic stem cells (Morrison et al. 1996) as well as during T-cell development in the thymus and B-cell differentiation in the germinal centre (Norrback et al. 2001; Weng et al. 1997a, b, 1998). T lymphocytes are generated in the bone marrow and then migrate to the thymus for maturation. Naïve T lymphocytes recirculate between blood and secondary lymphoid organs until they encounter their specific antigen. Upon contact, they proliferate and acquire properties to exert an appropriate immune response. After antigen

elimination, a small fraction of these cells remains as memory T cells, while the majority of the effector cells die. The step from naïve T lymphocytes to effector and memory T lymphocytes requires proliferative, metabolic and oxidative adaptations.

In vitro cultivation of T cells stimulates the up-regulation of TA, but during subsequent repeated stimulations *in vitro*, $CD8^+$ T cells become unable to up-regulate telomerase. Ongoing cell division without telomerase activity results in progressive telomere shortening and critically short telomeres signalling senescence. Senescent $CD8^+$ T cells show altered cytokine patterns, resistance to apoptosis and absence of expression of the CD28 co-stimulatory receptor (Effros 2007). Genetic studies on human inherited diseases due to mutations in genes encoding telomerase components identified low telomerase activity levels as an important factor for leukocyte telomere length (Effros 2007). Clinical studies have demonstrated that high proportions of senescent $CD8^+$ T cells strongly correlate with several deleterious physiologic outcomes, including increased pro-inflammatory cytokines.

T-cell receptor (TCR) signalling as well as co-stimulation with other molecules such as CD28 is essential for the induction of telomerase activity. Telomerase activity peaks 4–5 days after the TCR stimulation and then decreases again for 10 days (Plunkett et al. 2005).

In addition, T cell proliferation can also be stimulated by different cytokines without the mediation of TCRs (Moro-García et al. 2018). T-cell proliferation can be activated *in vitro* by IL-7 and IL-15 and was found to be associated with induction of telomerase activity and the prevention of telomere erosion in T lymphocytes (Wallace et al. 2006). In contrast, telomerase activity was inhibited by cytokines such as IFN-α, which increased $CD4^+$ T-cell telomere loss *in vivo* (Reed et al. 2004). *In vitro*, $CD4^+$ T-cell telomerase activity was inhibited by IFN-α secreted by cytomegalovirus (CMV) antigen-stimulated dendritic cells (Fletcher et al. 2005). Akiyama et al. (2004) also showed that in peripheral blood lymphocytes (PBL), cytoplasmic telomerase activity was induced 1 h after TNF-α treatment followed by nuclear translocation of hTERT to the nucleus 2 h after treatment. These authors also demonstrated that activation and nuclear translocation of telomerase are regulated by PI3K/Akt/NF-κB signalling pathways in PBL. Both steps could be inhibited separately. While inhibition of PI3K prevented telomerase activation, blocking the nuclear localisation of NF-κB prevented the TNF-α-induced nuclear translocation of hTERT (Akiyama et al. 2004).

In addition, IL-2 can stimulate hTERT localisation from the cytoplasm into the nucleus in a human natural killer (NK) cell line (Kawauchi et al. 2005). ERK1/2 and Akt kinase (Akt) were activated by IL-2 stimulation, but only the Akt pathway influenced the transcriptional and post-translational activation of hTERT. Interestingly, hTERT co-immunoprecipitated together with Akt, Hsp90, mTOR and p70 S6 kinase (S6K), suggesting that these molecules form a physical complex in the NK cell line as well as primary blood lymphocytes. These results indicate that IL-2 stimulation induced hTERT activation on the transcriptional or post-translational levels and involved regulation through a pathway including PI3K/Akt, Hsp90, mTOR and S6K in NK cells (Kawauchi et al. 2005).

Telomere Lengths Over the Life Course and During Ageing in Immune Cells

Since peripheral blood cells originate from the bone marrow, telomere shortening in haematopoietic stem cells and progenitors correlates well with that of the deriving peripheral blood cells such as lymphocytes (Lansdorp et al. 1997; Kimura et al. 2010). Average TL in PBMCs might reflect the haematopoietic stem cell (HSC) compartment in individuals. Leukocytes all originate from HSCs and then differentiate into different subsets, such as lymphocytes, monocytes and granulocytes (neutrophils, basophils and eosinophils). It is known that telomere length differs between leukocyte subsets (Rufer et al. 1999 a; Lin et al. 2015). Deelen et al. (2014) demonstrated that variation in donor TL was associated with differences in leukocyte subset composition, such as lymphocyte, neutrophil and basophil counts. This result strongly suggests that mean TL is influenced by the composition of the different leukocyte subsets.

Telomeres shorten around 50–100 bp/year in human $CD4^+$ and $CD8^+$ T cells (Weng et al. 1995; Rufer et al. 1999 a) and T-cell populations of old individuals have shorter telomeres than those from young subjects (Hodes et al. 2002; Akbar et al. 2004). This telomere loss could be caused by repeated activation of specific T cells during a lifetime by infections or due to increased oxidative damage during the ageing process. The ratio between other lymphocyte populations such as granulocytes and lymphocytes can also vary. Thus, the cellular composition of peripheral blood cells at a specific time point might vary from other time points. On the other hand, environmental or nutritional influences can target both telomeres directly as well as telomerase activity (Liu et al. 2010).

Naïve T cells divide repeatedly (15–20 times) during an acute immune response in order to generate effector cells. This results in telomere shortening which is not sufficiently counteracted by telomerase activity, in particular during ageing (Plunkett et al. 2007). As a result, memory CD4 and CD8 T cells have a greatly reduced TL compared to naïve T cells, and this also correlates to proliferation capacity (Rufer et al. 1999 a). Ageing of the immune system also affects blood cell TL by a shift occurring from naïve T cells towards memory T cells (Rufer et al. 1998) that have shorter telomeres than naïve cells (Lansdorp 1995). In contrast, experimental overexpression of telomerase is able to extend the replicative lifespan of T cells (Hooijberg et al. 2000; Rufer et al. 2001). Lineage-specific telomere shortening has been detected in CD4+, CD8+ T lymphocytes, B lymphocytes, granulocytes, monocytes and NK cell populations with a different kinetics of telomere attrition (Kaszubowska 2008).

Rufer and co-workers (1999 a) found average telomere shortening rates of 39 bp/year in granulocytes and 59 bp in lymphocytes. However, there was an accelerated telomere shortening early during life during childhood in both cell populations. Until 6 months of age, granulocytes shortened their telomeres by 3 kb, while lymphocytes during 1.5 years up to 1 kb. This corresponds well to findings from others (Frenck et al. 1998). Rufer et al. (1999 a) also discovered a pronounced variation in

TL between individuals with some newborns having a shorter average TL than people over 60. These findings have been also confirmed in various other studies (von Zglinicki et al. 2000; Lin et al. 2015). This observation also stresses the limitation of using TL in cross-sectional studies. Svenson et al. (2011) demonstrated that blood cell TL can fluctuate during a life time and that the actual TL at a defined time point might be influenced by various regulatory mechanisms as well as environmental and lifestyle factors. The study took longitudinal blood samples at 6 months intervals and hypothesised an oscillating TL pattern which might level out at longer follow-up times. Interestingly, the study also found a correlation between absolute TL and telomere shortening rates. Alternatively, some short-term fluctuations in telomere length could be attributed to acute increases in telomerase activity (Epel et al. 2010).

Rufer et al. (1999 a) sorted naïve and memory CD4 T lymphocytes as well as naïve, effector and memory CD8 T cells from over 100 individuals with an age range of 0–90 years using flow-FISH analysis. The study analysed the relative distribution of naïve and memory CD4 and CD8 T-cell subsets in three separate age cohorts, 0–4, 4–35 and 35–90 year. In young children (0–4 years), 70% of T lymphocytes had a naïve phenotype. A continuous decline with age in the proportion of naïve CD4 and CD8 lymphocytes was found that correlated to an increased proportion of memory CD4 and effector CD8 cells and to a lesser extent memory CD8 cells. As a result, naïve CD4 and CD8 T lymphocytes represented a minority after the age of 50 years. Naïve CD4 and CD8 cells had less telomere shortening (TS) (39% and 34%, respectively) compared to 51% and 54% in memory T cells. Consequently, the decrease in telomere length in lymphocytes with age appears to reflect telomere shortening in naïve and memory T cells as well as a gradual shift from a naïve to a memory phenotype. Interestingly, similarities in telomere length were maintained throughout life in both monozygotic (MZ) and dizygotic (DZ) twins. This result suggests that telomere shortening and perhaps even the number of cell divisions in lymphocytes from related individuals are remarkably similar, possibly even inherited (Rufer et al. 1999 a).

Excessive telomere shortening in PBL is also characteristic for various disease conditions due to extensive T-cell proliferation (Effros et al. 1996; Plunkett et al. 2005). Differences in telomere shortening rates between different lymphocyte types could be due to differences in regulation of telomerase activity at the transcriptional and post-transcriptional levels (Weng 2002). Immature thymocytes have much higher telomerase activity than mature cells, while no TA is detected in resting peripheral T cells (Weng et al. 1997a). In T cells, telomerase activity rapidly increases 12–16 h post-stimulation, persists for around 3–5 days and then gradually decreases to undetectable levels again by 15–30 days (Bodnar et al. 1996). *In vivo*, a cross-sectional study demonstrated that memory CD4 and CD8 cells have a higher rate of telomere loss (~50 bp/year) than naïve cells (~37 bp/year), resulting in an average shortening of 31 bp/year in all T cells. Lin et al. (2015) detrmined telomere shortening rates in their longitudinal study for all 4 populations. They found that PBMCs and monocytes shorten by 28bp/year while T cells shorten 25bp/year and B cells 52bp/year.

Immuno-senescent T cells are characterised by shortened telomeres, decreased T cell-specific surface glycoprotein CD28 and T-cell activation antigen CD27, co-receptors for T-cell activation as well as increased β-1, 3-glucuronyltransferase-1 (CD57) and loss of proliferation (Bandres et al. 2000; Weekes et al. 1999; Strioga et al. 2011). Ageing is also characterised by a compromised function of haematopoietic stem cells, reduced amounts of circulating native T cells together with an increased frequency of well-differentiated memory CD28− cells (Deeks 2011).

Acceleration of Telomere Shortening and Immuno-senescence by Viral Infections

Ageing in immune cells is characterised by shorter telomeres in T cells coinciding with a reduced proliferative capacity and a progressive loss of immunological memory. A similar phenotype occurs during chronic viral infection. Chronic viruses known to be responsible for human diseases are Epstein-Barr virus (EBV), hepatitis B/C/D virus (HBV/HCV/HDV), human herpesvirus 8 (HHV-8), human immunodeficiency virus (HIV), human T-cell leukaemia virus type I (HTLV-I), human papillomavirus (HPV), herpes simplex virus-1/2(HSV-1/2) and varicella zoster virus (VZV) (Bellon and Nicot 2017). A chronic and latent virus infection can result in impaired T-cell functions. These dysfunctional memory T cells don't express telomerase anymore and are thus responsible for an ongoing telomere shortening resulting eventually in the premature induction of replicative senescence of virus-specific $CD8^+$ memory T cells (Bellon and Nicot 2017). Accelerated immuno-senescence has also been reported in T lymphocytes due to chronic CMV infection which leads to a higher disease severity in patients with myocardial infarction (as well as increased mortality in older people) (Hoffmann et al. 2015; Spyridopoulos et al. 2016). While senescent T cells are unable to expand and function properly, they can survive for long periods of time and are known to be more resistant to apoptosis (Spaulding et al. 1999). Thus, enhanced T-cell senescence seems to be a common endpoint to chronic viral infections. For further review, see Bellon and Nicot (2017).

Telomeres and Telomerase During Ageing (Population-Based Studies)

Average telomere length declines with age in most mitotic tissues (Hastie et al. 1990; Slagboom et al. 1994; Butler et al. 1998; Frenck et al. 1998; Friedrich et al. 2000; Takubo et al. 2002), while absolute telomere lengths are longer in postmitotic tissue (Benetos et al. 2011).

Various population-based studies have shown a decrease in leukocyte TL (LTL) with increased age (Frenck et al. 1998; Hochstrasser et al. 2012; Sanders and

Newman 2013; Lin et al. 2015). Studies have shown that telomere length dynamics changes during human lifespan and the rate of telomere shortening is highest in early life and slows down at higher age. In humans, the average telomere length at birth is about 10–15 kb in lymphocytes. The fastest decrease in telomere lengths occurs during the first years of life (>1 kb/year, most likely due to fast growth), while during adulthood the rate is around 20–60 bp/year depending on the cell type, their proliferation behaviour and their environment including oxidative stress and inflammatory processes (Frenck et al. 1998; Rufer et al. 1999b; Aviv 2008). Adult cells have a TL of 7–12 kb (de Lange 2005). However, reduction of average TL with age is only an approximation for ageing. It has been demonstrated that a subset of five short telomeres or even one single short telomere was able to trigger senescence (Hemann et al. 2001; Kaul et al. 2012). Consequently, the shortest telomere is a more critical parameter for cellular dysfunction and senescence than average TL (Hemann et al. 2001; Samper et al. 2001).

A decrease of telomere length in peripheral blood mononuclear cells (PBMCs) with age has been demonstrated in various cross-sectional studies (Frenck et al. 1998; Hochstrasser et al. 2012; Sanders and Newman 2013). These studies have suggested a yearly telomere shortening of around 15–50 bp/year in PBMCs or lymphocytes (Nordfjäll et al. 2009; Chen et al. 2011). Many more cross-sectional studies exist, but it is not possible to describe them here comprehensively. In contrast, longitudinal population-based studies are rather rare but very informative (Chen et al. 2011). Lin et al. (2015) performed a longitudinal study in over 200 human donors from the Baltimore longitudinal study aged 20–90 years and assessed them at 3 time points: baseline, 5 and 12 years follow-up. They analysed telomere length and telomerase activity separately in different subpopulations such as lymphocytes and monocytes, as well as changes in composition between different subsets during ageing. However, compared to cross-sectional studies, they did not find a straightforward decline in TL in all individuals over time. While in a part of the study population TL decreased, it did not change in the majority of people, while it increased in others at follow-up time points. The rate and kinetics of telomere change were distinct for T cells, B cells and monocytes in each subject. The study also showed that telomerase activity decreased during ageing in resting T cells and B cells as well as in activated T cells. This result corresponds to that of Plunkett and coworkers (2007) by identifying a declined telomerase activity as the main cause for telomere shortening in $CD8^+$ T cells with age. Another reason for telomere shortening suggested by the study was a decrease in naïve T cells and the change in physiological health conditions such as elevated blood glucose and interleukin (IL)-6 levels (Lin et al. 2015). It is known that numbers and percentages of human lymphocyte subsets and monocytes in the blood change with age (Aviv et al. 2009). These findings suggest that changes in telomere length of the PBMCs with age *in vivo* occur at different rates in individuals and cell types and are influenced by telomerase activity, changes between fractions of naïve and memory T-cell percentage and changes in health conditions such as obesity and inflammation.

This result seems to be in accordance to studies from others reporting a certain fluctuation in PBMC TL when analysed longitudinally at multiple time points in the

same individuals (Aviv et al. 2009; Nordfjäll et al. 2009). Importantly, Nordfjaell and colleagues in their study of almost 1000 individuals at 2 time points around 10 years apart found that individual telomere shortening rate was highly significantly ($P < 0.001$) inversely correlated with TL at a young age. Surprisingly, they found higher telomere shortening rates in individuals with long telomeres at baseline. However, while TL at baseline determined around half of telomere changes, other influences could be lifestyle, oxidative stress, regulation of telomerase activity and telomere accessibility, as well as inflammation. These factors and their influence on TL in human blood lymphocytes have been or will be still described further in this chapter. This observation confirms the differences in inherited telomere lengths as described previously. It also corresponds well to the suggested method of subtracting TL in lymphocytes from that in postmitotic tissue such as muscle, fat, etc., where there is negligible cell proliferation, corresponding to relatively longer TL and thus reflects rather the inherited TL of an individual, as demonstrated in dogs by Benetos et al. (2011). This observation could be in line with the notion that telomerase preferentially maintains the shortest telomeres as already shown previously in mouse models and cell culture (Samper et al. 2001; Teixeira et al. 2004; Ouellette et al. 2000) and corresponding to observations that an increase of telomerase using telomerase activators preferentially decreases the amounts of very short telomeres (Bernardes de Jesus et al. 2011; Salvador et al. 2016). To measure these subtle changes in the quantity of short telomeres, it is not sufficient to measure the average TL using the most common method of quantitative PCR but to employ methods that are able to detect small changes in individual telomeres. Lin et al. (2015) also found that there was a distinct change in TL in the different subsets of PBMCs such as T cells, B cells and monocytes. Moreover, the authors confirmed that memory T cells have shorter TL than naïve T cells corresponding to Plunkett et al. (2007) in $CD8^+$ T cells. In parallel to TS, the authors found a decrease in TA in T cells. While TA, as expected, was very low or even negligible in resting T cells, also the amount of TA after stimulation decreased during ageing thereby explaining decreased TL in older individuals, corresponding again to the study of Plunkett et al. (2007). In contrast, the study of Lin et al. (2015) found no decrease of TA in B cells.

Telomeres and Telomerase During Reproductive Ageing

There are striking differences in reproductive ageing between men and women. Differences in telomere dynamics during ageing of men and women may have evolved due to differences in the risks of ageing on reproduction for men and women (Kimura et al. 2008b; Eisenberg et al. 2012a, b; Prescott et al. 2012). Telomere length decreases in oocytes with increasing age of the female. In women, telomere shortening causes meiotic defects such as synapsis and chiasma, chromosome fusions, embryo arrest and fragmentation, as well as abnormal dysfunctional meiotic spindles. It has been demonstrated that telomere length of polar bodies predicts the fragmentation of human embryos. Telomerase is hardly detected and active in

oocytes and preimplantation embryos. However, during the first cell cycles of embryo development, telomeres are elongated via the recombination-based alternative lengthening of telomeres (ALT). Telomeres are shorter in oocytes from women undergoing *in vitro* fertilisation, resulting in fragmented, aneuploid embryos unable to implant. Strikingly, telomere length increases in sperm cells with human age. In spermatogonia that have a high level of telomerase activity, telomeres elongate in length with age about 57 bp/year (Antunes et al. 2015), although there is individual variation between subjects and individual spermatozoa (Santiso et al. 2010). Faulty telomerase and decreased telomere length in sperm cells might contribute to apoptosis, reduced sperm count and reduced male fertility. For a recent review on telomeres and reproductive ageing, *see* Keefe (2017).

Telomere Length as a Marker for Longevity and Mortality?

A correlation between cellular and organismal lifespan and telomere length has been demonstrated in various *in vitro* systems (Harley et al. 1990a, b; Allsopp et al. 1992, Allsopp and Harley 1995; Hande et al. 1999; Counter et al. 2003) but also *in vivo* in animal models (Blasco et al. 1997; Samper et al. 2001; Herrera et al. 1999) and in cross-sectional and longitudinal observational studies (von Zglinicki et al. 2000; Cawthon et al. 2003; Epel et al. 2008; Bakaysa et al. 2007; Astrup et al. 2010; Chen et al. 2011; Wilbourn et al. 2018).

Telomere length and shortening have been associated to health and longevity. Studies have demonstrated a direct correlation between telomere length and life expectancy, stress, DNA damage and onset of age-related diseases. In cross-sectional and prospective epidemiological studies, decreased telomere length (TL) in leukocytes was associated with increased mortality (Cawthon et al. 2003; Kimura et al. 20008a; Njajou et al. 2007; Martin-Ruiz et al. 2006; Fitzpatrick et al. 2011), although this finding was not consistent (Bischoff et al. 2006; Martin-Ruiz et al. 2005; Bendix et al. 2014). Several studies described that older people with shorter telomeres (in blood) for their age have reduced survival (Bakaysa et al. 2007; Cawthon et al. 2003; Ehrlenbach et al. 2009; Honig et al. 2006; Kimura et al. 2008a).

In humans, average telomere length is thought to reduce with age in a cell-type-specific manner. In addition, critically short telomere lengths or at least a few damaged and/or short and uncapped telomeres are able to induce senescence and are thus also implicated in organismal ageing (Baker et al. 2011, 2016). Several human studies have found a higher mortality among older individuals with shorter blood cell telomere lengths (Cawthon et al. 2003; Goglin et al. 2016; Mons et al. 2017). Importantly, however, this association seems to hold true only until the age of 60–65, while above that no correlation has been found (Martin-Ruiz et al. 2005). One prospective cohort study even found that an increase in LTL after 5 years correlated to decreased mortality (Goglin et al. 2016). By dividing coronary heart patients into three groups, those with telomere loss, those who maintained their TL and those who even extended their TL, the study found a highly significant correla-

tion between the telomere status and mortality, with 39% for those with short, 22% for maintained and 12% for those who extended their telomeres. The latter group was more than 50% less likely to die (Goglin et al. 2016). This shows a good prognostic factor for even average TL.

Another study analysed female twin pairs and correlated TL and physical functioning in order to establish whether TL predicts the level of physical functioning over an 11-year follow-up period in 386 older mono- and dizygotic twin sisters. The study found a correlation between TL and physical activity and concluded that TL is a good biomarker for the prediction of disability development in older people (Sillanpää et al. 2016).

However, other studies have not found telomere length as the predominant factor for longevity of centenarians, but other factors such as inflammation were important. While centenarians from the Tokyo Centenarians Study and their offspring were able to maintain long telomeres, telomere length was not a predictor of successful ageing in centenarians and semi-supercentenarians (Arai et al. 2015). The authors concluded that inflammation was an important malleable driver of ageing up to extreme old age in humans.

There is still a debate ongoing about the value of telomere length as a predictor of health, longevity and mortality (Aviv 2008; Boonekamp et al. 2013; Bendix et al. 2014; Simons 2015). Some studies have not found any correlation between telomere length and mortality in larger longitudinal studies over a decade and stress the important influence of lifestyle factors (Bendix et al. 2014).

A recent study found that telomere length in early life is a better predictor of vertebrate lifespan (Heidinger et al. 2012). This finding assumes that individuals with longer telomeres at birth have a relatively long lifespan, while those with short telomeres from birth have a shorter lifespan (Heidinger et al. 2012). Although this study was performed in birds (zebra finches), the fact that there is also the largest amount of telomere shortening during the first years of life in humans suggests a similar relationship between body growth and telomere length.

However, another important parameter to consider is the different rates of shortening for telomeres which can depend on genetic make-up, such as the expression of antioxidant enzymes (von Zglinicki et al. 2000) or nutrition and life history factors (Bendix et al. 2014; Tricola et al. 2018). Others have reported a relationship between telomere attrition and components of fitness (Dantzer and Fletcher 2015). However, most of these studies have been performed in rodents or birds and could not always be reproduced in human studies (de Rooij et al. 2015). Additionally, cellular and organismal longevity is also dependent on the accumulation of dysfunctional telomeres and TAFs, which can be independent of absolute telomere length (Hewitt et al. 2012).

Telomere dysfunction was recently described as being determined by the frequency of critically short telomeres (Vera et al. 2012). The study found that an increased rate in the frequency of critically short telomeres determined longevity in mice and not the general rate of telomere shortening. Moreover, it is possible that specific organs or cell types such as cardiac stem cells are more susceptible to telomere shortening than other cell types (Matsumoto et al. 2018).

A study on offspring of Ashkenazi Jewish centenarians found that they had longer mean TL compared with controls from the general population (Atzmon et al. 2010). Interestingly, neither the centenarians nor their offspring showed any expected decrease in TL with age as in controls. The authors suggested that better telomere maintenance may be a feature in long-lived families. However, they did not measure telomerase activity as an important telomere maintenance mechanism in lymphocytes, and other studies could not confirm this association (Deelen et al. 2014; Arai et al. 2015). It is important to emphasise that a mere association of shorter telomeres with increased prospective mortality cannot necessarily be explained by a causal effect of TL on health conditions.

Several genetic loci have been identified recently that were suggested to influence TL in Western populations (Mangino et al. 2012; Codd et al. 2013). These loci include genes known to be involved in the regulation of telomere biology genes including TERC and TERT that could be responsible for around 1% of variation in TL. Deelen et al. (2014) utilised data from the Leiden Longevity Study where TL was measured in 870 nonagenarian siblings, as well as their offspring and their spouses. These families showed a survival benefit with a 30% decreased mortality risk in three generations compared to the general Dutch population (Schoenmaker et al. 2006). Interestingly, previous studies had shown that the offspring from the nonagenarian siblings had a better health status, various healthy metabolic parameters and a lower prevalence of age-related diseases such as type 2 diabetes, cardiovascular disease and hypertension compared to their spouses (Westendorp et al. 2009; Slagboom et al. 2011).

Deelen et al. (2014) found that subjects with long telomeres indeed displayed a survival advantage compared to subjects with shorter telomeres. Importantly, the survival benefit was independent of the analysed immune-related markers or associated genetic variants. The authors concluded that average telomere length in lymphocytes might be a good marker for prospective mortality in humans. However, the lack of association of TL with known telomere-associated genetic variants and familial longevity in middle age means that there is no causality of telomere lengths contributing to variation in human lifespan. In contrast, the correlation of TL between spouses rather emphasises the importance of non-genetic but environmental effects (Broer et al. 2013; Bendix et al. 2014). Intriguingly, while there were no major differences in telomere length between the offspring of the nonagenarian siblings and their spouses, the offspring still had a significantly better health status. This finding strongly suggests that the association between TL and mortality seems to be independent of the familial properties that influence metabolic health in these families at middle age. The authors draw the final conclusion that telomere lengths seems to meet the majority of criteria they proposed for a biomarker of healthy ageing (Deelen et al. 2013). The authors conclude that TL is associated with chronological age and morbidity as well as mortality in prospective studies. Additionally, mean TL measured in PBMCs seemed to be influenced by the frequency of the different leukocyte subsets. However, when the authors adjusted the prospective analysis of mortality for these counts, the effect of LTL on prospective mortality still remained (Deelen et al. 2014).

The Influence of Inflammation on Telomere Length and Telomerase Activity

Ageing is well known to be associated with chronic inflammation characterised by immune system dysregulation with increased inflammatory cytokine production (Alonso-Arias et al. 2011; Abedin et al. 2005).

Chronic low-level inflammation is known to be an important factor during general ageing and has been shown to be better correlated with longevity than TL (Arai et al. 2015). In addition to senescent lymphocytes, age-related inflammation can also be due to chronic activation of macrophages and the production of pro-inflammatory cytokines (Franceschi et al. 2000). In contrast to any acute inflammation due to infections, ageing is characterised by a chronic, low-grade inflammation. The immune system exhibits characteristic changes in older people compared to younger ones: that is, immuno-senescence. Cellular senescence is characterised by an increased secretion of inflammatory cytokines and chemokines as well as matrix-remodelling factors – the senescence-associated secretory phenotype (SASP) (Coppe et al. 2008, 2010; Young and Narita 2009; Kuilman et al. 2010; Rodier and Campisi 2011).

Importantly, conditions such as inflammation and oxidative stress might be involved in the development of various age-related chronic diseases such as cardiovascular and neurodegenerative diseases as well as cancer (Perry et al. 2007).

It has not yet been analysed in detail how telomerase activity in lymphocytes is regulated under conditions such as oxidative stress (Richardson et al. 2018) or inflammation (Gizard et al. 2011). Richardson et al. (2018) recently showed that TA and proliferation *in vitro* in mouse splenocytes are down-regulated under chronic oxidative stress. The same group had reported a substantial down-regulation of TA in patients with acute ST elevation myocardial infarction (STEMI), a state of high inflammation (Bennaceur et al. 2014). Corresponding to an improvement of the disease condition, after 3 months TA was back to the levels of healthy donors, showing the dynamics of changes in TA. Unfortunately, the authors did not measure TL.

Various factors can contribute to age-related inflammation, for example, tissue damage, a dysfunctional immune system, pro-inflammatory cytokines secreted as part of SASP by senescent cells, enhanced NF-κB activation and a defective autophagy (Salminen et al. 2012). Jurk and colleagues used a genetic mouse model of chronic low-grade inflammation to demonstrate that it increases ROS and as a result exacerbates telomere dysfunction and induces cellular senescence (Jurk et al. 2014). The transcription factor nuclear factor kappa B (NF-κB) has been shown to regulate telomerase and interact with the shelterin protein RAP1 that is involved in telomere homeostasis (Ghosh and Tergaonkar 2010). Likewise, ROS has also been shown to contribute to NF-κB increase during chronic inflammation (Sarkar and Fisher 2006).

Up-regulation of the inflammatory NF-κB pathway is an important hallmark of the ageing process (Lopez-Otin et al. 2013). NF-κB can be a master regulator of the inflammatory process, with downstream inflammatory molecules such as TNF-α, interleukins and cyclooxygenase (COX) (Baeuerle and Baltimore 1996; Schreck et al. 1992).

Mitochondria are also involved in inflammatory pathways. Mitochondrial dysfunction is another important hallmark of ageing (Lopez-Otin et al. 2013; Wallace et al. 2010). One critical mechanism underlying the mitochondrial dysfunction is the expansion of mutations and deletion of mitochondrial DNA (Greaves et al. 2014), partly due to decreased autophagy and mitophagy (Green et al. 2011). ROS promote further mitochondrial dysfunction, oxidative stress and release of DNA into the cytosol where this can activate the NLRP3 inflammasome (Sandhir et al. 2017).

Increased inflammation in PBMCs has been shown to increase TA (Rentoukas et al. 2012). These authors investigated the association between telomerase activity and inflammation due to impaired endothelial function in patients with metabolic syndrome. They found higher telomerase activity, TNF-α, IL-6 and ADMA in PBMC of the patients compared to healthy volunteers. PBMC telomerase was negatively correlated with high-density lipoproteins (HDL) and correlated with ADMA, but no association between TNF-α and IL-6 was observed. However, no direct correlation between TA in PBMC and systemic inflammatory markers was observed.

It is largely unknown whether telomerase is activated or rather repressed during inflammatory processes or what conditions lead to the different directions of regulation.

Gizard et al. (2011) analysed the induction of *hTERT* expression and TA in a human macrophage model (differentiated U937 cells) using atherogenic stimuli such as oxidised low-density lipoprotein (LDL), LPS and different cytokines *in vitro*. They found a significant short-term up-regulation of *hTERT* expression and TA in human macrophages 3 h after treatment with inflammatory stimuli such as LPS, TNF-α and oxidised LDL of IL-1β. They also showed a greatly increased hTERT staining in macrophages of human arteriosclerotic plaques and highly increased TA in aortic tissue of a mouse model of atherosclerosis. Importantly, the authors identified a NF-κB response element within the TERT promoter. Upon inflammation, NF-κB binds to it and induces hTERT transcription. Thus, hTERT seems to be a direct downstream target of NF-κB (Gizard et al. 2011).

In addition, others have described noncanonical functions of the hTERT protein in atherosclerosis by improving vasodilation in arterioles from patients with coronary artery disease (Beyer et al. 2016). However, while the TERT protein was markedly decreased in tissue from left ventricles, there was no telomere shortening observed in the patients. The authors interpret this as a non-canonical mechanism for TERT action independent of TL while they did not measure TA. Instead, they related hTERT levels to a decrease of mitochondrial ROS and increase in nitric oxide-related vasodilation, linked to blood flow due to higher eNOS activity (Beyer et al. 2016).

A feedforward regulation loop between hTERT and NF-κB had been suggested previously (Akiyama et al. 2004). Nuclear translocation of hTERT in activated lymphocytes is mediated through its interaction with the NF-κB p65 subunit, suggesting a possible link between telomerase and inflammatory signalling (Akiyama et al. 2003).

At the same time, telomerase can directly regulate NF-κB-dependent transcription (Ghosh et al. 2012). The authors found that hTERT is recruited to a subset of promoters regulated by NF-κB via its interaction with the NF-κB p65 component

and thereby regulates gene expression of NF-κB targets such as IL-6, IL-8 and TNF-α that are prominently involved in inflammatory processes (Ghosh et al. 2012). In the same study, the authors also demonstrated a reduction of IL-6 levels upon telomerase inhibition in primary leukaemic cells from cancer patients. At the same time, binding of hTERT to the NF-κB subunit p65 influenced its binding specificity to NF-κB target genes. The authors also demonstrated that both hTERT and NF-κB can partially substitute for each other in processes such as cell proliferation, resistance to cell death and regulation of cellular immunity. In summary, there exists a mutual regulation of the important inflammatory transcription factor NF-κB and telomerase which could also be tissue specific.

The Relationship of TERRA and Inflammation

While it is well known that senescence is associated with a pro-inflammatory phenotype or SASP, the direct involvement of dysfunctional telomeres in the induction of inflammation has not been addressed in much detail. Interestingly, analysis of RNA-Seq expression data showed that the NF-κB-p50 subunit binding motif was over-represented at more than 80% of TERRA proximal promotors (Porro et al. 2014). This observation seems to suggest a potential interaction between the master inflammatory transcription factor NF-κB and the transcriptional activity of telomeres.

While most TERRA molecules are associated with telomere DNA, some of it can be found outside the nucleus in exosomes (Wang and Lieberman 2016). Wang et al. (2015) described a cell-free form of TERRA that has been secreted into extracellular exosomes purified from the medium of cultivated lymphoid cells. This specific form of TERRA is shorter than intracellular TERRA with around 200nt and is more stable and associated with histones (Wang et al. 2015). Incubation of cfTERRA-containing exosomes with peripheral blood mononuclear cells stimulated the transcription of several inflammatory cytokine genes, including TNF-α, IL-6 and CXC chemokine 10 (CXCL10) (Wang et al. 2015). Such cfTERRA-containing exosomes can also be detected in human serum and are able to induce cytokines and an inflammatory response. As described above, TERRA seems to be predominantly generated from short and potentially perhaps also from dysfunctional telomeres (Porro et al. 2014). Wang et al. (2015) thereby defined a telomere-associated molecular pattern (TAMP) as a novel form of inflammation inducers and thus uncovered a previously unknown mechanism of communication between telomeres and the innate immune system in tissue microenvironment. Due to its properties, one could suggest the use of cfTERRA as a potential biomarker for telomere dysfunction and associated human diseases (Wang and Lieberman 2016). The same group has recently shown (Wang et al. 2017) that TERRA responds to viral infections. Virus infections can induce cellular remodelling events and stress responses, including telomere-specific modifications. DNA viruses, such as adenovirus, herpes simplex virus, cytomegalovirus, varicella zoster and an RNA influenza virus, were found to increase TERRA generation (Deng et al. 2014). HSV-1 was particularly fast and consistent in activating TERRA. In addition

to TERRA activation, HSV-1 infection produced also other changes in telomere maintenance such as the dissociation of shelterin from telomeric DNA, the formation of single-stranded DNA throughout telomeres and the eventual degradation of telomeric repeats (Deng et al. 2014).

Intriguingly, exosomes from irradiated cells were shown to induce telomere shortening in bystander cells in an RNA-dependent manner (Al-Mayah et al. 2017). Perhaps this mechanism could also underlie the observation that telomeres in antigen-specific, terminally differentiated $CD8^+$ T cells are significantly shorter eventually resulting in immuno-senescence and compromised immune-competency (Hoffmann et al. 2015).

Telomeres and Telomerase in Age-Related Diseases

Telomere shortening and cellular senescence in dividing cells during the ageing process have been suggested to contribute to human diseases and pathologies including cardiovascular, autoimmune diseases, chronic obstructive pulmonary disease (COPD) and metabolic disorders such as diabetes. Often, these diseases display inflammatory parameters, underlining the tight link between telomeres and inflammation (*see* above).

Increased oxidative stress together with shortened leukocyte telomeres is a hallmark for type 1 and type 2 diabetes (Sampson et al. 2006) as well as obesity (Hulsegge et al. 2016; Zhao et al. 2016). Obesity has been associated with shorter leukocyte telomere length (Ma et al. 2013; Muezzinler et al. 2016; Wulaningsih et al. 2016).

Chronic inflammation is a major risk factor for many age-associated diseases, including COPD, neurodegeneration, obesity and vascular disease (Moro-García et al. 2018). Shorter telomere length in peripheral blood lymphocytes is common in these diseases. An increased risk of cardiovascular disease (CVD) was associated with shorter telomere length (Chang et al. 2016a, b; Gebreab et al. 2017). Goglin et al. (2016) even suggested that changes in TL predict the mortality risk in patients with CVD. However, longitudinal and well-controlled studies of telomere length, inflammatory and oxidative stress markers are necessary to better understand the link between TL and various age-related diseases.

Through the mechanism of telomere shortening beyond a critical protective length, it is thought that the accumulation of senescent cells contributes to age-related tissue deterioration and disease phenotypes (Campisi et al. 2001; Stewart and Weinberg 2006). Numerous studies have been performed in order to establish associations between average telomere length in lymphocytes and many age-related diseases. Since reviewing them all in detail would be far beyond the scope of this chapter, here only some general statements will be given.

Telomere length is considered an emerging biomarker for age-related diseases, particularly chronic diseases in tissues with high cellular turnover (Hahn and Weinberg 2002; Huzen et al. 2010). Oxidative stress, that is able to experimentally

induce accelerated telomere shortening (von Zglinicki et al. 1995), is a common feature for various conditions and diseases where shorter telomeres have been found in patients compared to controls. Such conditions include abnormal blood lipid levels and diabetes but also chronic psychological stress (Babyyashak et al. 2010; Houben et al. 2008; Epel 2009). Shorter telomeres have been associated with an increased risk of various age-related diseases such as cardiovascular disease (Fitzpatrick et al. 2007; Hoffmann et al. 2015), hypertension (Demissie et al. 2006) and cancer (Willeit et al. 2011). It has been demonstrated that telomere shortening in lymphocytes can result in deregulation of immune homeostasis in age-related diseases (Andrews et al. 2010). However, in addition to telomere shortening, increasing interest and attention has been paid to telomere damage instead of shortening (Birch et al. 2015, 2016).

Defects and mutations in genes regulating telomere length homeostasis are known to cause various diseases, named telomeropathies (Townsley et al. 2014; Martínez and Blasco 2017). These diseases can be caused by mutations in telomerase or telomere-related genes and are usually characterised by decreased telomerase activity and accelerated telomere shortening. Patients often display exhaustion in highly proliferative tissues such as bone marrow, premature ageing and increased risk of cancer and CVD, highlighting the importance of telomere homeostasis in human health.

It is known that processes such as oxidative stress and inflammation are major components of many age-related diseases and can result in accelerated telomere shortening and cellular senescence. Cellular senescence and the ageing process are known risk factors for age-related chronic diseases, such as neurodegenerative, metabolic and cardiovascular disorders. These diseases are often associated with increased oxidative stress, inflammation, mitochondrial dysfunction, DNA damage, and telomere dysfunction/shortening. However, the mechanistic causes underlying these processes are often not well understood. Xiong et al. (2015) have shown how genes and pathways involved in biogenesis of mitochondria such as the master regulator PGC-1α can also influence telomere integrity as well as telomerase expression. Similar *in vitro* associations between mitochondria, DNA damage and telomeres have been shown by others previously (Passos et al. 2010).

Accumulation of senescent $CD28^-$ T cells with shorter TL and undetectable telomerase activity have been described in inflammatory conditions such as atherosclerosis and autoimmune diseases (Liuzzo et al. 2000; Moosig et al. 1998; Valenzuela and Effros 2002). As already described previously, these senescent cells are pro-inflammatory themselves due to SASP being an important hallmark of the phenotype. Thus, they could even further promote inflammation. Short telomeres and low telomerase activity have also been found in PBMCs of patients with autoimmune disorders including systemic lupus erythematosus (Beier et al. 2007; Tarhan et al. 2008) and rheumatoid arthritis (Colmegna et al. 2008; Fujii et al. 2009).

Several studies associate shorter TL in patient's lymphocytes to disease risk for cardiovascular disease (Huzen et al. 2010; Spyridopoulos et al. 2016; Mazidi et al. 2018), obesity (Valdes et al. 2005) and type 2 diabetes (Adaikalakoteswari et al. 2007). In addition, association of LTL with disease risk factors such as oxidised

LDL (Nawrot et al. 2010), hypertension (Yang et al. 2009), insulin resistance (Gardner et al. 2005), psychological stress (Epel et al. 2004) and the ApoE4 gene variant (Takata et al. 2012) shows potential mechanisms for the role of telomeres in the development of these diseases. For example, arterial tissues with atherosclerotic lesions have shown to display shorter TL than surrounding tissues (Nzietchueng et al. 2011; Ogami et al. 2004). Telomere shortening, the accumulation of senescent cells and increased levels of inflammatory markers, including IL-8, MCP1, Hu-GRO-α and ICAM-1, were found in lung tissues obtained from COPD patients (Kordinas et al. 2016).

However, it now also becomes obvious that non-canonical functions of telomerase/hTERT and telomere damage without changes in TL can be involved in human age-related diseases. The Passos lab has been pioneering this research and showed the involvement of telomeric damage (in the form of TAFs) and cellular senescence related to it in human ageing and various age-related diseases of the lung, heart and liver (Birch et al. 2015, 2016, 2018; Anderson et al. 2017; Ogrodnik et al. 2017; Schafer et al. 2017).

Beyer et al. (2016) recently described a novel non-canonical hTERT function for maintaining microcirculation in patients with coronary artery disease (CAD). Endothelial cells are some of the few human somatic cell types that express telomerase activity. While senescence down-regulates TA, decreased oxygen concentration increases TA and telomere maintenance in human vascular endothelial cells (Jakob and Haendeler 2007; Guan et al. 2012). In addition to telomerase activity, the TERT protein is also excluded from the nucleus during endothelial senescence due to increased endogenous oxidative stress that can be prevented using antioxidants (Haendeler et al. 2004). Increased telomerase activity has been shown to protect against reactive oxygen species (ROS)-induced endothelial dysfunction (Minamino et al. 2002). Endothelium-dependent dilation in response to increased shear stress is normally mediated by nitric oxide (NO) but in CAD by hydrogen peroxide (H_2O_2) released from mitochondria (Beyer et al. 2016). These authors showed that patients with CAD have greatly reduced hTERT protein levels in microvessels from left ventricular tissue while, interestingly, telomere length was not changed in samples from CAD patients. Cobsequently, oxidative stress and ROS were increased when hTERT levels were inhibited pharmacologically (Beyer et al. 2016). Various groups have shown that mitochondrial localisation of hTERT decreases intracellular oxidative stress (Ahmed et al. 2008; Haendeler et al. 2009; Kovalenko et al. 2010; Singhapol et al. 2013; Sharma et al. 2012). Using a telomerase activator (AGS499), increased hTERT levels significantly and improved endothelial function by stimulating endothelial nitric oxide synthase (eNOS) (Beyer et al. 2016). Thus, telomerase up-regulation seemed to shift the balance back from pro-inflammatory H_2O_2 to beneficial vasoprotective NO. However, since the authors did not measure telomerase activity, it is hard to say whether it was exclusively the non-telomeric function of hTERT that contributed to this change.

This novel research shows that there is more to telomeres than just shortening and more to telomerase than its telomere-related function.

Environmental and Lifestyle Influences on Telomerase Activity and Telomere Length

Telomeres and Telomerase Activity in Relation to Social Status

A lower socio-economic status at birth, frequent consumption of alcohol (specifically consumption of spirits), a history of cancer and a lower self-reported health status were reported to be significantly associated with shorter leukocyte telomere length (De Rooij et al. 2015). Low socio-economic status has been linked to worse health outcomes, which may arise from a higher likelihood of an unhealthy lifestyle and adverse events, while having a limited access to health benefits (Adler and Rehkopf 2008). Several studies have found short telomere lengths to be associated with various factors related to low socio-economic status (Batty et al. 2009; Cherkas et al. 2006; Shiels et al. 2011), in particular when experienced during early life (Chen et al. 2014; Kananen et al. 2010; Kiecolt-Glaser et al. 2011; Steptoe et al. 2011; Needham et al. 2012; Shalev et al. 2013a, b; Surtees et al. 2012). An unhealthy lifestyle including smoking, obesity and lack of exercise seem to have an important impact on telomere length since they could be translated into oxidative stress and in that way contribute to decreased telomerase activity and telomere shortening. However, not all studies found a correlation between TL and socio-economic status (Adams et al. 2007), while others reported even a shorter telomere length in men of higher socio-economic status (Woo et al. 2009).

Intriguingly, Zalli et al. (2014) found that in older men a phenotype of shorter telomeres but higher telomerase activity in PBMCs correlated to delayed recovery after mental stress exposure (two computer-related tasks in a laboratory setting that was used to induce a stress reaction in systolic blood pressure, heart rate variability and monocyte chemoattractant protein-1, as well as a reduced responsivity in diastolic BP, heart rate and cortisol), in comparison to men with longer telomeres or men with shorter telomeres and low TA. At the same time, shorter telomeres with high TA were also associated with reduced social support, lower optimism, higher hostility and greater early-life adversity. However, these effects were independent of age, socio-economic status and body mass index. Importantly, the differences in biology were not apparent in measures taken at rest but only when physiological regulation was challenged by mental stress. In addition, all these associations were only found in men, not in women. The authors concluded that these results might suggest that the combination of shorter leukocyte telomeres with high telomerase activity was associated with stress-related impairment of biological and psychological functions.

The Influence of Life and Psychological *Stress on Telomerase Activity and Telomere Length*

In addition to genetic factors, environmental influences such as diet, physical activity, lifestyle and stress are also known to modulate physiological parameters of stress and inflammation and might thereby influence health and longevity as well as telomere dynamics. The influence of environmental effects on telomere length is supported by findings that increased oxidative stress can accelerate telomere shortening in cultured fibroblasts (von Zglinicki et al. 1995) and decrease telomerase activity in lymphocytes *in vitro* (Richardson et al. 2018). These findings are supported at the population level by a correlation of TL between spouses (Broer et al. 2013). Lifestyle factors can include physical exercise, diet, micronutrient supplementation, mindfulness meditation or yoga mediation. Several studies have found an association between telomerase activity and psychological stress and lifestyle factors. Three studies found decreased telomerase activity in subjects under chronic psychological stress. In contrast, one study found that acute psychological stress significantly increased telomerase activity (Damjanovic et al. 2007).

Several studies have linked reduced telomerase activity with unhealthy lifestyles. For example, chronic psychological stress seems to be associated with decreased telomere length and reduced telomerase activity (Epel et al. 2004; Daubenmier et al. 2012; Shalev et al. 2013a, b). Epel and co-workers pioneered the connection between psychological stress, telomeres and telomerase (Epel et al. 2004). The authors analysed 39 caregiving mothers of chronically ill children as well as controls. Analysing TA in the caregiver group found that those under high perceived psychological stress had significantly lower telomerase activity than those with lower stress. This result corresponded well to lower telomere length in the caregiver-group with low TA. The chronicity of stress also correlated well with a lower TL. In 2006 the same group showed a connection between lower TA and the ability of individuals to cope with an acute laboratory stress such as preparing and giving a videotaped presentation. The authors measured other physiological parameters such as heart rate variability in response to acute stressors. A reduced variability is regarded as an indicator of psychological stress vulnerability. Interestingly, women with lower telomerase activity excreted more nocturnal stress hormones, epinephrine and norepinephrine (Epel et al. 2006). Similar results were obtained with 41 caregivers of Alzheimer's disease (AD) patients who showed increased depressive symptoms compared to aged-matched controls (Damjanovic et al. 2007). Caregivers had significantly lower T-cell proliferation but higher production of immune-regulatory cytokines (TNF-α and IL-10) than controls in response to stimulation *in vitro*. Corresponding to other studies, they found an accelerated telomere shortening but, interestingly, a higher resting/basal telomerase activity that the authors interpreted as a non-successful response to shorter telomeres in PBMCs and T cells, while no differences were observed after PBMC activation *in vitro* (Damjanovic et al. 2007).

Exposure to psychosocial stress has been directly associated with increased oxidative stress and inflammation (Wilson et al. 2013). Even stress experienced during childhood can influence LTL in adults (Kananen et al. 2010; Tyrka et al. 2010). Children that were exposed to stressful life events had shorter leukocyte telomeres when reaching middle age (Osler et al. 2016). A study by Entringer et al. (2013) even reported shorter telomeres in children born to mothers stressed during pregnancy. Likewise, adults exposed to psychosocial stress have similarly reduced telomeres length (van Ockenburg et al. 2012). However, there are also contradictory observations found in this area of research. For example, telomerase activity in stressed rats was elevated by 54% (Beery et al. 2012). The effect of stress on acutely increasing the level of telomerase activity has been replicated in a human trial involving exposing both dementia patients and their caregivers to acute psychological stress (Epel et al. 2010). However, it is not clear and was not analysed in both studies whether increased TA in turn modifies telomere length.

Psychological stress is usually accompanied by an increase in stress hormones such as cortisol. Higher levels of nocturnal cortisol expression, a hormone associated with chronic stress, are related to shorter telomeres, while *in vitro* a 3-day exposure of human T lymphocytes to cortisol significantly decreased telomerase activity as well as *hTERT* expression (Daubenmier et al. 2012).

Since stress elevates levels of cortisol and this results in a depressed immune function as well as decreased levels of telomerase in $CD4^+$ and $CD8^+$ T cells (Choi et al. 2008), it is possible that this mechanism increases inflammation and can thus translate into telomere shortening. It is possible that the responses of telomerase activity and telomere length to stress depend on the amount and endurance of the stressor. Effects of daily life stress can correlate to physical health over a long period (Leger et al. 2018).

In 2010 Epel and colleagues found that TA can increase rather quickly under acute laboratory psychological stress an hour after the stress. Independent on the basal TA levels, it increased after an acute stressor (an interview-like situation). However, it was not analysed how long this increase in TA persisted, and one could suggest that it decreased quickly after the event and thus most likely was not associated with any measurable telomere changes.

It is possible that transient stress induces a kind of hormesis effect, while an ongoing chronic stress might be more harmful.

Lifestyle Interventions

A pioneering study on the effect of an integrative lifestyle intervention on telomerase activity was carried out on 30 men with low-risk prostate cancer (Ornish et al. 2008). The intervention consisted of 3 months of comprehensive lifestyle changes including a diet low in fat and carbohydrates, high in fruits, vegetables, unrefined grains and legumes, moderate aerobic exercise, such as light walking every day as well as moderate daily yoga-based stretching, breathing and meditation. Telomerase

activity in the PBMCs of participants increased significantly after the 3-month intervention. This increase in telomerase activity was significantly associated with decreased perceived psychological stress and low-density lipoprotein cholesterol levels (Ornish et al. 2008). Unfortunately, no controls were included in this small study, and no TL was measured in order to evaluate whether higher TA translated into longer or more stable telomeres. However, often changes in telomere length can only be detected several years after the implementation of the intervention (Ornish et al. 2013). Thus, the response of telomerase activity could be much faster than that of telomere length. However, it is not clear how well changes in TA are indeed translated into changes in TL since, as previously described, telomerase has to access telomeres and this accessibility is regulated by multiple factors that are not yet well understood.

An increased level of IL-6 together with downstream signalling pathways may be a potential mechanism behind the elevated telomerase activity in response to acute psychological stress and physical exercise. TNF-α can activate the NF-κB subunit p65, which directly binds to hTERT protein in T lymphocytes and promotes hTERT translocation from the cytoplasm to the nucleus in these cells, thus inducing a fast activation of telomerase (Akiyama et al. 2003; Ghosh et al. 2012).

Exercise, Telomeres and Telomerase

The influence of physical activity and exercise has been intensely studied over the last decades and has been found beneficial for the maintenance of telomeres (Cherkas et al. 2008; Puterman et al. 2010).

Werner et al. (2008) found that 6 months of running up-regulated cardiac telomerase activity and increased TERT protein levels compared to sedentary controls and reduced expression of senescence-associated mediators such as Chk2, p53 and p16 in mice. While myocardial and leukocyte telomere length did not change in sedentary or running mice, telomerase activity and *TERT* expression were persistently increased after 6 months, and the expression of Chk2, p53 and p16 remained low. The importance of telomerase/TERT for this effect was confirmed by the lack of any beneficial exercise effects in *TERT*–/– mice (Werner et al. 2008). Werner et al. (2009) showed that 3 weeks of exercise up-regulated telomerase activity in the thoracic aorta and in PBMCs compared with sedentary controls in mice. Such preconditioned mice also showed a marked reduction in lipopolysaccharide (LPS)-induced aortic endothelial apoptosis. This result suggests that increased TA and *TERT* expression improved cellular survival of cells and decreased their sensitivity against adverse stimuli. The same study also measured TA and TL in long-term endurance athletes and found that old and young athletes had higher TA than their sedentary counterparts. However, only in old athletes there was an increased TL compared to sedentary controls, while TL was identical in young athletes and controls. This could mean that higher TA is only important while TL decreases in higher age, while in young subjects TA did not change TL.

However, other studies did not confirm changes in TA in cardiac or liver tissue in mice with short telomeres after long-term wheel running of almost 1 year but only found some TA increase in muscle tissue (Ludlow et al. 2012). The same group also found no differences in PBMC TA in athletes but detected a dependence of TA activity level on an *hTERT* promoter gene polymorphism although the general TA level was extremely low in this study (Ludlow et al. 2008).

A study from Melk et al. (2014) demonstrated that TA increased continuously during 6 months of exercise of sedentary middle-aged men, while TL increased only between 3 and 6 months of exercise in middle-aged sedentary males. The latter kinetics correlated with a decrease in the senescence marker p16, suggesting a decrease of senescence in PBMCs. Importantly, individuals with an inflammatory process such as periodontitis did not show beneficial changes in TL and senescence markers (Melk et al. 2014).

Recently, Dimauro et al. (2017) analysed the effect of exercise on control and diabetes type 2 individuals and found in both groups a similar beneficial effect on PBMC telomere length. At the same time, PBMCs from exercised individuals showed lower oxidative damage and apoptosis (Dimauro et al. 2017). These results have been widely confirmed (Shadyab et al. 2017; Saßenroth et al. 2015; Du et al. 2012; Krauss et al. 2011; Cherkas et al. 2008), while some studies did not find a correlation between telomere length and exercise (Ogawa et al. 2017; von Känel et al. 2017; Gardner et al. 2013).

Loprinzi and Loenneke (2018) examined the association between leukocyte telomere length (LTL) and mortality when considering physical activity, using data from over 6000 participants of the 1999–2002 National Health and Nutrition Examination Survey aged 20–85 years. They found that longer telomeres were associated with increased survival in physically active men. Importantly, Soares-Miranda et al. (2015) found that physical fitness (PF) and activity (PA) improved health and decreased telomere shortening rate even at an old age of around 73 years in a cross-sectional study on 582 adults. Prospective analyses demonstrated that changes in PA and PF were associated with differences in changes in TL. Thus, even at a high age, changes in some PA and PF parameters could still be associated with changes in TL (Soares-Miranda et al. 2015). However, Gardner and co-workers did not find a correlation between telomere length and similar parameters of physical fitness and activity in their meta-analysis of a large cohort (several thousand participants) aged between 53 and 80 years old. These different results might be due to the difference in age-range in both studies.

Interestingly, Simpson and co-workers (2010) aimed to provide a biological explanation for longer telomeres after rigorous exercise, which is known to mobilise memory $CD8^+$ T cells expressing the senescence-related cell surface marker KLRG1. These authors showed that after 1 h of rigorous exercise, $KLRG1^+$ senescent $CD8^+$ T cells are preferentially mobilised immediately after exercise, returning to baseline after 1 h. Telomeres in $CD8^+$ T cells displayed a longer telomere length immediately after exercise, whereas no change occurred in other T-cell subsets. The authors speculate that this preferential mobilisation of senescent cells could result in their death and disappearance making space for naïve T cells with longer

telomeres (Simpson et al. 2010). However, the kinetics of these changes is not clear, but the analysis of changes in T-cell subsets is certainly important (Lin et al. 2015).

Mindfulness and Meditation

Meditation training has been reported to decrease psychological stress and to promote well-being (Jacobs et al. 2011). The authors analysed the effects of a 3-month Buddhist meditation retreat on telomerase activity in PBMCs in 17 participants. Telomerase activity after 3 months was significantly higher than in a randomised control group. Unfortunately, no baseline data were available for the participants (Jacobs et al. 2011).

Rao and co-workers (2015) demonstrated that a 3-week mind and body treatment (MBT) programme increased TA telomerase activity in around half of all participants in this group. The study also looked for the level of blood stem cells and found a substantial increase in stem cell counts in 90% of participants after the MBT. These results suggest that increased telomerase activity and stem cell counts in peripheral blood from an MBT retreat programme could result in health benefits, improved quality of life and perhaps even increased longevity.

Another randomised control trial was conducted to test the effect of a lifestyle intervention of mindfulness-based stress reduction with improved eating awareness on telomerase activity in overweight or obese women (Daubenmier et al. 2012). While telomerase activity increased significantly in both intervention (n = 24) and controls (n = 23) compared to baseline levels, no significant difference in telomerase activity was detected between intervention and control groups. However, there was a clear correlation between increases in telomerase activity and improvements in psychological stress, eating behaviour and metabolic health (Daubenmier et al. 2012). Again, no TL was measured in all these studies.

Another study examined the effect of a mindfulness-based stress reduction on telomere biology of breast cancer survivors (Lengacher et al. 2014). Participants in the intervention group were trained by a psychologist and performed formal mindfulness including meditation, a body scan, gentle yoga or every-day relaxed walking. The study found that telomerase activity in PBMCs increased continuously over 4 months in the intervention group (n = 74), while there was no change in the control group (n = 68). Again, a more favourable psychological state was associated with higher telomerase activity while no differences in telomere length were detected within the given time frame.

A related intervention is that of using yoga. Caregivers of dementia patients were exposed to yoga meditation and telomerase activity measured (Lavretsky et al. 2013). After 8 weeks the intervention group showed a significant increase in PBMC telomerase activity compared with baseline levels, while the control group who just listened to relaxing music for 12 minutes per day did not show any increase. In the meditation group, higher telomerase activity was associated with improved mental health scores, while that association was not significant in the control group (Lavretsky et al. 2013).

The Influence of Nutrition on Telomere Length and Telomerase

Diet might be able to influence telomere/telomerase homeostasis. Short telomeres and a decrease in telomerase activity have been reported in patients with a high body mass index, higher circulating glucose levels and abdominal fat, while a healthy lifestyle with the intake of many antioxidants, fruit/vegetables, less processed meat and more exercise has been linked with longer telomeres (Crous-Bou et al. 2014; Boccardi et al. 2013). Importantly, it has been demonstrated that interventions in eating habits can increase telomerase activity and telomere length. Studies on Mediterranean diet found an association between this diet and increased telomerase activity and longer telomeres in PBMC as well as a generally better health status (Daubenmier et al. 2012; Crous-Bou et al. 2014; Boccardi et al. 2013). Telomerase activity in people on a Mediterranean diet was negatively correlated to inflammatory markers, while a higher dietary fat intake correlated to more inflammation and to shorter telomeres (Chan et al. 2010). Intake of Chinese tea in a study on 2000 older (65 years or more) Chinese people revealed a good correlation to longer average telomere length, equating to a gain in lifespan of 5 years in men (Chan et al. 2010). Similarly, García-Calzón and co-authors (2015a) found in a cross-sectional and longitudinal study on 500 people with an average age of 67 years in Spain that an anti-inflammatory diet was consistent with longer telomeres and a slower telomere shortening rate within 5 years.

Although there are accumulating data that suggest an association between diet, lifestyle, telomeres/telomerase and inflammation, the details and mechanisms underlying this possible connection are still rather vague and not well understood (Daubenmier et al. 2012; Boccardi et al. 2013; Rafie et al. 2016). Likewise, the striking fact that many correlations of lifestyle factors seem to be more significant in men than in women is not well understood. For a recent meta-analysis on telomeres and gender effects, *see* Gardner et al. (2014). For a recent update and summary of trials on the influence of nutrition on telomeres, *see* Freitas-Simoes et al. (2015).

Nutritional Intervention

Zhu et al. (2012) supplemented 19 African overweight people with Vitamin D for 4 months and found an increase in TA in PBMCs compared to the control group. Boccardi et al. (2013) analysed the effect of Mediterranean diet on TA in over 200 older subjects. Telomerase activity in PBMCs in the group with high adherence to Mediterranean diet was higher than in other groups with median or low adherence to Mediterranean diet. Moreover, telomerase levels were also associated with general health status independently of age, gender and smoking habits (Boccardi et al. 2013).

For 66 middle-aged healthy women, the effect of a 12-week micronutrient supplementation complex on anti-ageing biomarkers was examined, including telomerase activity (Balcerczyk et al. 2014). The supplementation consisted of omega-3 acids, ubiquinone, astaxanthin, lycopene, lutein palmitate, zeaxanthin palmitate, l-selenomethionine, cholecalciferol and α-tocopherol. The study showed that the supplementation significantly increased PBMC telomerase activity together with an improvement in antioxidant capacity (Balcerczyk et al. 2014).

A study using supplementation with omega-3 fatty acids, thereby decreasing the ratio of dietary omega-6 with pro-inflammatory activity to omega-3 fatty acids, on anti-inflammatory activity on middle-aged and old men and women, found reduced oxidative stress and increased telomere length in PBMCs (Kiecolt-Glaser et al. 2013). This effect could be caused by a decrease in pro-inflammatory cytokines and thus reduce the rate of attrition of telomere length. A diet rich in monounsaturated fatty acids such as in olive oil and the Mediterranean diet have also been shown to reduce leukocyte telomere shortening. In healthy Japanese adults, enhanced levels of β-carotene and α-tocopherol protected buccal mucosa cell from telomere shortening (Yabuta et al. 2016). Similarly, in an Australian stroke prevention study, enhanced plasma concentrations of lutein, zeaxanthin and vitamin C were associated with longer leukocyte telomeres (Sen et al. 2014). A middle-aged (35–55 years old) human population that received a dietary multivitamin supplementation containing omega-3 fatty acid, carotenoids, coenzyme Q10, selenium, vitamin D and α-tocopherol for 12 weeks showed an increased telomerase activity in PBMCs, but no change in telomere length was detected during this time frame (Balcerczyk et al. 2014). Probably 3 months is not long enough for increased telomerase activity to translate into longer telomeres, as shown in an exercise intervention study analysing 3-month and 6-month time points (Melk et al. 2014) where TA was increased already at 3 months, but TL only increased at 6 months. However, there are also other studies that did not find significant effects of diet and nutrition or supplements on telomeres (Balcerczyk et al. 2014). Thus, this is a field of ongoing intense research.

Alcohol consumption has also been described to associate with shorter telomere length in PBMC and oesophageal epithelium. In PBMCs telomere shortening correlated with the amount of drinks per day (Pavanello et al. 2011). A study of 3660 young adults in the USA found that increased serum levels of carotenoids were significantly associated with longer leukocyte telomeres (Min and Min 2017). This could be due to the antioxidant properties of carotenoids decreasing oxidative stress and protecting telomere length. A Spanish study in children and adolescents confirmed the beneficial effect of a high dietary total antioxidant capacity on telomere length, while a poor diet such as too much white bread has been associated with shorter telomere length (García-Calzón et al. 2015b). Similarly negative effects of specific dietary components were reported by Kark and colleagues in a longitudinal observational study of 600 young adults who found that a high intake of omega-6 fatty acids with pro-inflammatory activity correlated with an increased rate of telomere shortening in leukocytes between the age of 30 years and 43 years (Kark

et al. 2012). Interestingly, the authors also found an inverse relationship between caloric intake and telomere lengths in men, which is consistent with data on general beneficial effects of calorie restriction on ageing biomarkers (Wang et al. 2010; Ishaq et al. 2016; Ogrodnik et al. 2017). At the same time, smoking, which is known to be associated with increased oxidative stress and inflammation, seemed to abrogate the protective effects of lower caloric intake on LTL (Kark et al. 2012).

Modulating TA and TL with Telomerase Activators

Restoring telomerase activity in T lymphocytes would have a great impact on human health and longevity by ameliorating telomere shortening and preventing immunosenescence. Several studies have shown that when telomerase activity and telomere length are preserved, replicative senescence can be delayed (Dagarag et al. 2004; Fauce et al. 2008). One possible solution to achieve this would be to eliminate senescent T lymphocytes from the bloodstream or to induce apoptosis in them using senolytics (Chang et al. 2016b; Kirkland et al. 2017). Another approach is the use of telomerase activators that have been developed to physiologically activate TA without any risks for cancer development which usually involves genetic changes. Mouse studies supplementing older animals with a plant-based telomerase activator (TA65) demonstrated health benefits and a significant increase in lymphocyte TL (Bernardes de Jesus et al. 2011). Importantly, the study analysed preferentially changes in very short telomeres with low fluorescence signals, compared to a method that analyses just global average TL. A similar method did not find significant changes in TL in a human study using different supplements including TA65 (the so-called Patten protocol) (Harley et al. 2011). However, a more recent study with 100 participants using a high-throughput sensitive telomere FISH measurement found some differences in TL at one concentration of TA65, although there were quite substantial variations during the 1-year period of intervention (Salvador et al. 2016). More studies are currently underway, and one study, although not analysing telomere lengths, showed a significant functional improvement of age-related macular degeneration over a 1-year intervention with TA65 (Dow and Harley 2016). A synthetic telomerase activator on the basis of cycloastragenol (GRN510) counteracted the effects of idiopathic lung fibrosis in a mouse model, with telomerase activity increased in bone marrow progenitor cells (Le Saux et al. 2013).

Beyer et al. (2016) demonstrated that the synthetic telomerase activator AGS499 (Eitan et al. 2012) increased hTERT protein levels significantly in small vessels and thereby improved endothelial function by stimulating endothelial nitric oxide synthase (eNOS). Taken together, these developments seem encouraging in an attempt to find easy and low-risk interventions aimed at increasing telomerase activity, together with stabilising the shortest telomeres which are known to induce senescence preferentially.

Conclusions

Although it has been suggested that telomere length may be a suitable biomarker of human ageing, health and longevity, this is still heavily disputed. Due to individually inherited telomere length, differential and inducible telomerase expression which can be modulated by environmental factors as well as inflammation and diseases, telomere length might not be a good biomarker for ageing. Telomeres are able to respond to different stress inducers, including viral infection (Caslini et al. 2009; Deng et al. 2014), reactive oxygen species (von Zglinicki et al. 1995) and DNA damage signalling (Tutton et al. 2016). In addition, the rate of individual telomere shortening depends on many different parameters, such as genetically determined antioxidant make-up and thus resistance of cells against oxidative stress (von Zglinicki et al. 2000), the number of infections an individual undergoes during its life time and on internal conditions such as disease, inflammation, food, psychological stress and more.

However, telomeres might still be considered good markers of cellular senescence and ageing independently of their lengths. It recently transpired that telomeric damage could serve as an age-related biomarker. Various groups have shown this in different species and tissues (Herbig et al. 2006; Hewitt et al. 2012; Fumagalli et al. 2012).

The appearance of telomere-associated foci (TAFs) or telomere dysfunction-induced foci (TIFs) (Takai et al. 2003) has been tightly associated with cellular senescence and ageing. However, due to the high technical demand of a combined DNA damage staining together with a telomere fluorescence in situ hybridisation (FISH) which requires the analysis of co-localisation of both signals using a fluorescence microscope, this parameter has not been adapted for the high throughput of samples required for large-scale population-based studies and is thus so far rather restricted to research laboratories and small sample numbers. However, the technical development that makes it possible to even do telomere-FISH on individual chromosomes/telomeres in a multiwell format (Salvador et al. 2016) is progressing rapidly. The background for preferring this technique to measuring an average telomere length using telomere restriction fragment analysis (TRF, Southern blot based) or qPCR is that quite subtle changes on the shortest telomeres are detectable with a higher resolution with such a technique (Bernardes de Jesus et al. 2011). However, to add a DNA damage marker and co-localise it to telomeres is still another technical challenge.

Importantly, telomere homeostasis in PBMCs to a large degree also depends on the presence, activation and nuclear localisation of telomerase. However, telomerase activity as well as telomere accessibility and actual elongation by telomerase are regulated on multiple molecular levels. Telomerase activity is predominantly regulated at the transcriptional level of the *hTERT* gene (Cong et al. 2002; Gomez et al. 2012; Poole et al. 2001). However, in PBMCs and lymphocytes, additional regulation levels such as post-translational modification of hTERT, mainly phosphorylation, and subsequent nuclear translocation of the hTERT protein are the predominant

processes affecting telomerase activity *in vivo* (Gladych et al. 2011; Liu, 1999). In other cell types, for example endothelial cells, the telomerase protein can be excluded from the nucleus by oxidative stress and senescence, shifting telomerase away from telomere maintenance while conducting telomere-independent functions (Saretzki 2014; Beyer et al. 2016).

Conditions such as oxidative stress (Richardson et al. 2018) and inflammation are able to modify telomerase activity in different cell types including immune cells (Gizard et al. 2011), while the telomerase protein hTERT can also function as a transcription factor for inflammatory factors such as NF-κB, thereby directly influencing inflammation processes (Ghosh et al. 2012). Telomerase activity can be upregulated acutely (within minutes) or chronically by external factors such as psychological stress (Epel et al. 2004, 2010), and it is not known to what extent this is translated into changes in telomere length and/or telomere damage. While it is normally assumed that telomerase preferentially extends the shortest telomeres, preferential shortening or trimming of longer telomeres has been reported. However, all these regulatory mechanisms and their multiple interactions are not well understood to date. It seems that not all telomeres are extended in the same way and that shorter telomeres are extended by telomerase preferentially (Teixeira et al. 2004). This has initially been shown in late-generation telomerase knockout mice, which were backcrossed to heterozygotes in order to achieve a limited dose of telomerase (Samper et al. 2001). This preferential stabilisation of the shortest telomeres in lymphocytes can also be achieved using natural or pharmacological activators of telomerase (Bernardes de Jesus et al. 2011; Salvador et al. 2016). This can only be detected with very sensitive fluorescence in situ hybridisation (FISH) techniques that measure the lengths of individual telomeres rather than a summary of average telomeres, as in PCR or Southern blot-based techniques. However, it could be a way forward to actively counteract the ageing process.

New and exciting aspects and components of telomere biology are emerging constantly, such as telomere position effects (TPE) between short telomeres and *hTERT* transcription (Kim and Shay 2018), trimming of long telomeres by TZAP or hTERT (Li et al. 2017; Pickett et al. 2009, 2011), telomere transcription (TERRA) (Azzalin and Lingner 2015) and even cell-free TERRA (cfTERRA) molecules, mediating a communication between telomeres and innate immune signals (Wang et al. 2015). In addition, telomerase localisation and its regulation of telomere access could be different in various cell types such as stem cells (embryonic and adult) as well as mitotic and postmitotic somatic cells. In neurons it has been demonstrated that there is no telomerase activity in adult neurons and the TERT protein is localised predominantly outside the nucleus, in the cytoplasm (Iannilli et al. 2013; Spilsbury et al. 2015). In contrast, telomerase localisation and activation is completely different in human lymphocytes. There hTERT protein is shuttled into the nucleus upon activation, and telomerase activity is increased by Akt phosphorylation (Akiyama et al. 2002, 2003, 2004; Kawauchi et al. 2005). Telomerase activity causally contributes to the proliferation of lymphocytes, and both are downregulated under oxidative stress (Richardson et al. 2018).

Revealing all these different processes in discrete cell types will certainly improve our understanding of telomere and telomerase biology as well as the association of telomeres in PBMCs to health, ageing and age-related diseases. At the same time, it also demonstrates the intricate and extremely complex relationship of the different components and processes active at the telomere.

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Chapter 10
Nuclear DNA Damage and Ageing

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Abstract Although the links between defects in DNA repair and cancer are well established, an accumulating body of evidence suggests a series of functional links between genome maintenance pathways, lifespan regulation mechanisms and age-related diseases in mammals. Indeed, the growing number of DNA repair-deficient patients with progeria suggests that persistent DNA damage and genome caretakers are tightly linked to lifespan regulating circuits and age-related diseases. Here, we discuss the impact of irreparable DNA damage events in mammalian physiology highlighting the relevance of DNA repair factors in mammalian development and aging.

Keywords DNA damage · DNA repair · Accelerated ageing · GH-IGF1 axis · Calorie restriction

The Adverse Consequences of Genome Instability

DNA damage is essentially random in nature, ubiquitous and unavoidable for most, if not all, living organisms. Moreover, the mammalian genome must be preserved for relatively long periods of time so that it is faithfully passed into the progeny. This, and the fact that DNA has an inherently vulnerable physicochemical stability, have pushed cells to evolve mechanisms to efficiently counteract DNA damage. Such mechanisms often involve a battery of programmed responses i.e. delicate DNA damage sensors, signaling cascades and overlapping DNA repair mechanisms that continuously scan the genome to detect DNA lesions, surmount a proper DNA damage response (DDR) and repair the myriads of (structural) DNA modifications

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that may hamper DNA-templated transactions. Depending on the developmental stage or the cell type involved, similar or identical DNA lesions may lead to dramatically different outcomes; for instance, certain types of cells, such as the neurons are more transcriptionally active than others. Others, such as the adipocytes may differ in their metabolic demands and certain tissues, including the liver, have different regenerative or replicative capacities or as in the case of lung are exposed to distinct intrinsic and environmental hazards. Such attributes become further profound when one considers the sharp differences between the immortal germ line and the disposable soma; persistent DNA lesions may obstruct DNA replication leading to mutations in progenitor cells that are transmitted to daughter cells, thereby compromising the integrity of the cell lineage with detrimental consequences for organismal development. Alternatively, DNA damage may interfere with the process of RNA synthesis, thus hampering the proper execution of developmental gene expression programs. Indeed, the accumulation of DNA lesions in the genome of distinct cell types, tissues or developmental time points is expected to give rise to a perplexing array of tissue-specific pathologies ranging from various degrees of neurodegeneration to severe immunodeficiency, growth retardation and systemic metabolic abnormalities, including cancer. The latter may also explain how random DNA damage events trigger the intricate repertoire of specific pathological outcomes. More complex scenarios are seen when one considers the multiple functional roles that DNA repair genes maintain beyond genome maintenance, including transcription, and chromatin architecture (Chatzinikolaou et al. 2017; Kamileri et al. 2012b) or of genes that are involved in one or the other DNA repair mechanism but whose primary function lies well beyond DNA repair or the canonical DNA damage response (Kamileri et al. 2012a, b).

DNA Repair Mechanisms and the DNA Damage Response

Upon DNA damage, a battery of highly conserved DNA damage checkpoints delay cell cycle progression and activates repair processes. Recognition of DNA damage relies on protein kinases, such as the ATM (Ataxia telangiectasia, mutated) and ATR (ATM- and Rad3-related) that sense DNA damage or stalled replication forks or transcription and amplify the signaling cascade (Abraham 2001; Shiloh and Kastan 2001). Shortly after the induction of e.g. DNA double strand breaks (DSBs), ATM which exists as a homodimer becomes autophosporylated; intermolecular phosphorylation of the subunits triggers the dissociation of the monomers which are then free to phosphorylate other substrates (Bakkenist and Kastan 2003); indeed, over 700 human and/or mouse proteins are now known to contain a consensus ATM and ATR phosphorylation motif and are phosphorylated in response to DNA damage (Matsuoka et al. 2007); the exact function of these proteins remains to be established, but they are thought to be required for effective DDR. Depending on the type of damage inflicted on DNA's double helix, DNA damage is then dealt by a

battery of partially overlapping, DNA repair mechanisms to restore the original information of DNA.

DNA double strand breaks (DSBs) are induced by both endogenous (i.e. metabolic byproducts) and exogenous (e.g. ionizing irradiation) sources and are considered to be the most deleterious DNA lesions threatening cell viability (for reviews see (Dudas and Chovanec 2004). Inaccurate repair of DSBs disrupts both DNA strands interrupting the continuity of the DNA molecule, undermines chromatin stability and challenges the repair machinery because an intact template strand is lacking to assist restoration of DNA sequence integrity. DNA DSBs are repaired through either of two distinct biochemical pathways, homologous recombination (HR) or non-homologous end joining (NHEJ). HR is an error-free mechanism that requires the generation of single-stranded DNA molecules, which are then used for homology searching within adjacent sister chromatids. HR uses a sister chromatid as template DNA to achieve proper repair; it functions preferentially in the S and G2 phases. Instead, NHEJ is active throughout the cell cycle; it requires minimal processing of the damaged DNA by nucleases and restores DNA integrity by joining the two DNA ends. As such the DNA sequence is only accidentally preserved rendering NHEJ prone to errors. HR fully relies on the Mre11-Rad50-Nbs1 (MRN) complex to detect DSBs (Grenon et al. 2001). After incision, the 3′ end single-stranded DNA coated with Replication Protein A (RPA) and Rad51 invades into a homologous recipient DNA duplex. Two Holliday junctions are then formed, each between four strands of DNA that are then converted into recombination products. Instead, NHEJ requires the Ku70/80 DNA-binding complex and the DNA-dependent protein kinase (DNA-PK); NHEJ is initiated by the recognition and binding of the Ku protein, a heterodimer of Ku70 and Ku80, to the broken DNA ends that forms the DNA-binding component of DNA-dependent protein kinase (DNA-PK). Ku aligns the DNA ends followed by DNA polymerases and nucleases that fill in or trim off the single-stranded DNA overhangs, respectively to generate the two DNA blunt ends. Next, DNA-PK and the XRCC4/DNA ligase IV ligation complex facilitate re-joining of broken non-compatible DNA ends. Besides DSBs, DNA interstrand crosslinks (ICLs) comprise another highly cytotoxic type of DNA lesion caused by a variety of endogenous metabolites, environmental exposures, and cancer chemotherapeutic agents; DNA ICLs covalently connect two nucleotide residues from the same DNA strand i.e. intrastrand DNA crosslinks or from opposite strands i.e. DNA interstrand crosslinks, thereby blocking any DNA templated process requiring strand separation, including DNA replication or transcription. Whereas DNA intrastrand crosslinks are readily removed by the highly conserved nucleotide excision repair pathway (NER; see below), DNA ICLs are dealt by several enzymes, including structure-specific endonucleases (e.g. XPF), recombinases (e.g. Rad51 and BRCA2), translesion DNA polymerases (e.g. Rev1 and DNA pol zeta), and a family of proteins that is known to enhance resistance to crosslinking agents i.e. the FANC family of proteins (McHugh et al. 2001). Base mismatches are commonly generated during DNA replication and are primarily dealt by the evolutionarily conserved process of Mismatch repair (MMR) (Pena-Diaz and Jiricny 2012); AG or TC mismatches are recognized by two heterodimers, MUTSα or

MUTSβ that discriminate between the parental and the newly synthesized strand, remove the mismatched nucleotide and allow the replication machinery to use the original DNA template to restore the damaged DNA strand back to its native form. MMR is highly relevant for long repetitive DNA sequences, such as the microsatellites; microsatellites are particularly prone to errors as they can be replicated inaccurately due to frequent strand misalignment followed by inefficient proofreading (Kunkel and Erie 2005). Small, non-helix distorting DNA lesions that could otherwise lead to mutations or DNA breaks during replication are dealt by base excision repair (BER); the mechanism is initiated by DNA glycosylases to detect and remove DNA lesions through hydrolysis, resulting in abasic sites (Krokan and Bjoras 2013). Abasic sites are then cleaved by an apurinic/apyrimidinic endonuclease resulting in DNA SSBs, which are repaired by either a short- or a long-patch repair mechanism whether one or more nucleotides are replaced, respectively. DNA ligase III and X-ray repair cross-complementing protein 1 catalyzes the nick-sealing step in short-patch BER. DNA ligase I ligates the DNA SSB in long-patch BER. DNA polymerase β is typically involved during the DNA synthesis step. Bulkier helix distorting DNA adducts, such as those induced by the Ultraviolet component of sunlight, are repaired by the highly conserved nucleotide excision repair (NER) (Kamileri et al. 2012a). Two subpathways of NER can be distinguished that differ primarily in how the damage is initially recognized: the global genome repair (GGR) subpathway is responsible for the removal of lesions from the entire genome. A major limitation of this system, however, is that certain types of damage (like UV-induced CPDs) are less well recognized and accordingly less efficiently repaired. To avoid that such lesions hamper transcription by stalling RNA polymerase II, a distinct NER subpathway has evolved, called Transcription-Coupled Repair (TCR). This system directs the repair machinery preferentially to the template strand of actively transcribed DNA and operates as a fast backup system for lesions that are slowly repaired by GGR. In GGR, it is the XPC-RAD23-CETN2 complex (Masutani et al. 1994; Nishi et al. 2005) and the UV-damaged DNA-binding protein (UV-DDB; DDB1-DDB2-containing E3-ubiquitin ligase complex) that scan the genome for bulky DNA adducts (Lagerwerf et al. 2011). Unwinding of the DNA around the DNA lesion requires the multisubunit complex TFIIH containing XPB, p62, p52, p44, p34, p8, and XPD in addition to the Cdk-activating-kinase complex [22] and XPG (Egly and Coin 2011). Next, the XPG and ERCC1-XPF, structure-specific endonucleases cleave the 3′ and 5′side of the ~30-nucleotide fragment containing the damaged DNA, respectively. The single-strand DNA gap is then filled by DNA polymerases δ and ε or the translesion DNA polymerase κ and the nascent DNA fragment is sealed by DNA ligase III-XRCC1 (Moser et al. 2007) and DNA ligase I (Araujo et al. 2000; Mocquet et al. 2008). Unlike GGR, damage recognition in TCR requires RNA polymerase II that is stalled at the damaged template (Fousteri et al. 2006; Laine and Egly 2006a). CSB binds stalled RNAPII and triggers the assembly of the NER machinery and the DNA lesions is then removed by the core NER reaction (Laine and Egly 2006b).

DNA Repair Defects: From Developmental Abnormalities to Accelerated Ageing and Cancer

A number of inborn mutations in genes associated with the DDR or DNA repair in humans lead to a growing number of syndromes with severe developmental abnormalities and/or the premature onset of age-related diseases, including cancer. Patients with defects in ataxia telangiectasia-mutated (AT patients), Nibrin (Nijmegen breakage syndrome e), MRE11, Rad50, Artemis, DNA ligase IV and Cernunnos-XRCC4-like factor are hypersensitive to ionizing irradiation and exhibit immunodeficiency along with a spectrum of partially overlapping pathological features, including enhanced cancer predisposition. AT patients present with clinical manifestations that range from progressive cerebellar ataxia to oculocutaneous telangiectasia, gonadal sterility, growth retardation and a high incidence of lymphoid tumors; interestingly, AT is gradually perceived to represent a highly heterogeneous syndrome with dramatic pleomorphic manifestations (Teive et al. 2015). Patients with Nijmegen breakage syndrome represent a rare autosomal recessive syndrome that presents with short stature, microcephaly and characteristic facial appearances and are particularly prone to respiratory tract infections and highly susceptible to B-cell lymphomas (Chrzanowska et al. 2012). Fanconi anemia patients (affected proteins FANC, BRCA2) are clinically highly heterogeneous but share a number of phenotypic similarities with NBS patients; the great majority of FA patients present with major birth defects ranging from short stature and developmental delay to cutaneous, skeletal, craniofacial, and genital anomalies along with bone marrow failure and myelodysplasia; about 80% of children and adults with FA are reported with a least one endocrine defect, including GH deficiency, abnormal glucose or insulin metabolism and dyslipidemia or hypothyroidism (Petryk et al. 2015). Likewise, patients with Bloom (BLM) or Rothmund-Thomson (affected protein: RECQL4) syndromes all exhibit growth defects and pathological features ranging from cataracts and diabetes to renal dysfunction and immunodeficiency. The relevance of NER in man is highlighted by three major syndromes namely xeroderma pigmentosum (XP), Cockayne syndrome (CS) and Trichothiodystrophy (TTD) (Bootsma et al. 1998, 2001). Similar to other DNA repair-deficient syndromes, the phenotypes are highly heterogeneous ranging from freckling in sun-exposed skin areas and, if left unprotected, multiple skin cancers (as in XP) to severe progeroid defects but no tumors as in CS, a rare multisystem disorder characterized by cachectic dwarfism, nervous system abnormalities and features of premature aging. (Nance and Berry 1992). Besides the characteristic sulphur-deficient brittle hair phenotype, TTD patients also manifest similar features to those seen in CS, including accelerated ageing and neurological abnormalities of varying severity (Itin et al. 2001).

Genome Maintenance and Lifespan Regulation: The DNA Damage Link

Mouse mutants with inborn defects in NER closely mimic their human counterparts and display severe developmental abnormalities and short lifespan (Schumacher et al. 2008). Previous work has shown that the onset of progeroid defects in *Csb*$^{m/m}$/*Xpa*$^{-/-}$, *Xpd*^{TTD}/*Xpa*$^{-/-}$ and *Ercc1*$^{-/-}$ NER mutants are intimately linked to changes in gene expression and physiological parameters, including the suppression of the lifespan regulator growth hormone (GH)/insulin growth factor (IGF)-1 somatotrophic axis, the suppression of lactotrophic and thyrotrophic processes as well as the dampening of oxidative metabolism (Garinis et al. 2008; Niedernhofer et al. 2006; van de Ven et al. 2006; van der Pluijm et al. 2007). These changes are accompanied by reduced serum glucose and insulin levels, along with a marked propensity to store glycogen and triglycerides, indicating an attempt by the organism to trigger a self-imposed dietary restriction program and withhold, rather than utilize, primary energy resources. Strikingly, a very similar response is also seen in mice with constitutive defects in single genes that are involved in the GH/IGF1 endocrine signaling, i.e. the Ames and Snell dwarfs (Andersen et al. 1995; Li et al. 1990) the little mouse (*Ghrhr*$^{lit/lit}$) (Flurkey et al. 2001), the growth hormone receptor/binding protein (*Ghr/bp*$^{-/-}$) (Zhou et al. 1997), or the heterozygous IGF1 receptor (*Igf1r*$^{+/-}$) (Holzenberger et al. 2003). Also, knockout mice and the Klotho-overexpressing mice (Kurosu et al. 2005) show suppression of the GH/IGF1 axis that is often paralleled with reduced levels in glucose, suppressed thyrotroph and lactotroph functions, lower body temperature and diminished generation of ROS; improved antioxidant defenses and stress resistance, enhanced storage of primary carbon sources, delayed development, reduced fertility and increased average and maximal life span (Bartke and Brown-Borg 2004). Interestingly, the great majority of these changes are also seen upon calorie restriction (CR) and there might be additional, yet unknown pro-longevity processes to be identified (Fig. 10.1); for instance CR is known to further extend lifespan in long-lived Ames dwarfs (Bartke et al. 2001). Intriguingly, attenuation of the Insulin/IGF1 somatotropic axis and oxidative metabolism occurs in naturally aging mice and the elderly as well (Niedernhofer et al. 2006; van de Ven et al. 2006; van der Pluijm et al. 2007). Similar to CR, one of the presumed mechanisms linking suppression of the IGF1/insulin signaling to longevity in dwarf rodent models is the consistent ability to protect against spontaneous tumors (Bartke and Brown-Borg 2004) or resist tumor induction following exposure to chemical carcinogens (Ramsey et al. 2002). Conversely, overexpression of GH in GH-transgenic mice causes severe kidney pathology, a higher frequency of liver adenomas and carcinomas, fibrotic alterations and a markedly shortened lifespan (Bartke et al. 2002). In agreement, injection of high GH doses in rats results in hepatomegaly and a 20% higher mortality rate (Groesbeck et al. 1987). Increasing IGF1/insulin signaling also appears to dampen the (antioxidant) stress responses in GH-transgenic mice (Brown-Borg and Rakoczy 2000) or long-lived Ames dwarf mice (Brown-Borg and Rakoczy 2003). Besides the suppression of GH/IGF1 axis in

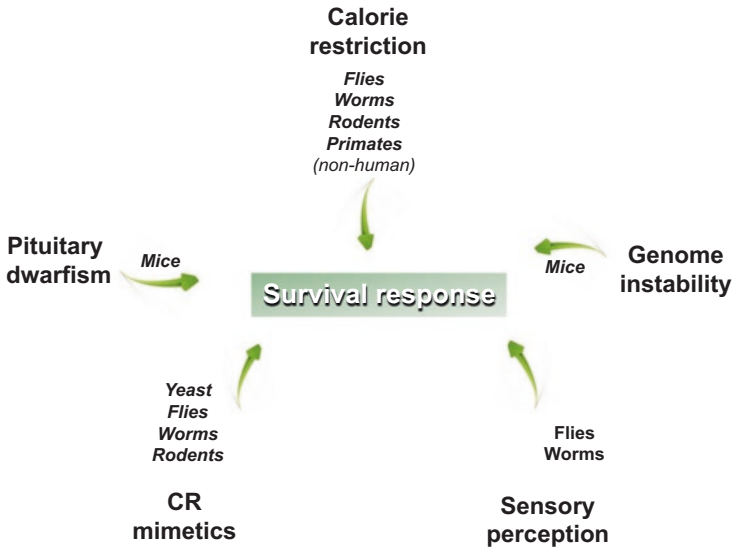

Fig. 10.1 Instigators of the survival response

the NER-defective animals, more recent work has revealed that persistent DDR activation in *Ercc1*$^{-/-}$ mice also triggers a tissue-specific auto-inflammatory response leading to severe fat depletion and metabolic complications associated with advanced old age (Chatzinikolaou et al. 2014; Karakasilioti et al. 2013). In support, pharmacologic inhibition of NF-κB dramatically delays a wide range of pathological symptoms in DNA repair-deficient progeroid mice (Tilstra et al. 2012).

At first sight, it may seem paradoxical that NER progeroid mice showing accelerated aging features have similar responses to animals that live substantially longer than their wild-type littermates. However, this apparent discrepancy may be better rationalized when one considers the fine balance between growth and maintenance during early and late stages of organismal life. During mammalian development, the growth-promoting action of GH and IGF1 may well fuel cellular metabolism, further promoting tissue growth and function (Bartke 2003; Carter et al. 2002; Chandrashekar et al. 2004). However, the latter occurs at the expense of higher oxygen consumption (Carter et al. 2002) and likelihood of increased ROS through the parallel increase of mitochondrial electron transport, peroxisomal fatty acid metabolism and/or microsomal cytochrome *P-450* enzymes (Beckman and Ames 1998). A defect in NER is expected to further aggravate the increased burden of DNA damage in the transcribed strand of active genes. It has, therefore, been hypothesized that this probably triggers an adaptive response, i.e. reduction of metabolic activity through down-regulation of the GH/IGF1 axis, to relieve the pressure on their genome. This response has often be named as a "survival response" aimed at shifting resources from growth to maintenance at times of stress, including the presence of irreparable DNA damage accumulation seen in the NER-defective animals or with advanced old age. Other animals

carrying defects in DNA damage signaling such as mouse models for the Seckel syndrome with hypomorphic mutations in the ATR protein kinase (Murga et al. 2009), ATM KO mutants (Hishiya et al. 2005) all of which display many symptoms of accelerated aging, tend to shift their expression towards growth suppression and upregulated anti-oxidant defenses as observed in the 'survival' response. Similarly, mouse models for Hutchinson Gilford progeria, which suffer from a defect in nuclear Lamin A rendering nuclei particularly sensitive to, for example, mechanical stress, reveal expression changes with clear hallmarks of the survival response (Marino et al. 2008). This likely applies to the human situation as well, as suggested by the suppression of growth which is characteristic of many of the corresponding human progeroid repair syndromes, such as Cockayne syndrome, Trichothiodystrophy, Seckel syndrome, Ataxia telangiectasia, Hutchinson Gilford progeria and variants, XFE syndrome, etc. (Schumacher et al. 2008). Thus, it seems that excessive cytotoxic DNA damage either caused by compromised repair or deficient damage signaling not only accelerates aging in the affected organs and tissues presumably by increased cell death and cellular senescence but at the same time triggers the protective 'survival' response, which intends to alleviate the negative effects by boosting available defenses, adjusting metabolism and suppressing growth (Fig. 10.2).

Importantly, wt mice chronically exposed to subtoxic doses of oxidative damaging agents were found to shift their expression profile towards suppression of the IGF1/GH somatotrophic axis and upregulation of anti-oxidant systems, which are key components of the CR response, indicating that continuous (DNA) damage is able to persuade a healthy organism in the direction of the 'survival' mode (van der Pluijm et al. 2007). As for humans, it is notable that the growth and development of children is temporarily arrested, during a period in which they experience serious

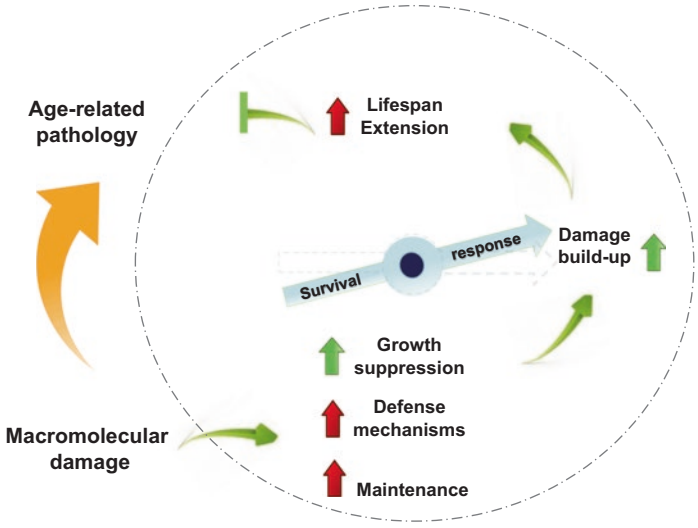

Fig. 10.2 Macromolecular damage and the survival response

illnesses, likely a reflection of the instalment of the 'survival' response. Finally, even with normal aging a systemic suppression of the IGF1/GH somatotrophic and thyrotrophic axes is registered (van den Beld and Lamberts 2002). In light of the above, this might be a consequence of age-dependent accumulated damage inducing stress, which in turn triggers the somatotrophic suppression indicative of the survival response. Since the accumulated damage and consequent stress are in this case constitutive, the growth suppression is also permanent, which may explain the negative growth curve of very old people. On the other hand, the CR and dwarf mutants, which express major elements of the survival response in the absence of damage, show that constitutive induction of the survival response over long periods promotes longevity and healthy aging although it was never selected for this purpose in evolution, as healthy aging occurs only post reproduction.

Consistent with the existence of one dominant mode of the survival response is the observation that this response offers a broad protection against a variety of stresses (Grifantini 2008; Heydari et al. 2007; Koubova and Guarente 2003), including ischemia-reperfusion, which is associated with high levels of oxidative stress Mitchell et al. 2009). Counterintuitively, even DNA repair-deficient progeroid mice subjected to ischemia reperfusion induced by kidney clamping were found to be more resistant than wild-type animals, whereas *a priori* a greater sensitivity would have been coined (van Ginhoven et al. 2009). Presumably, the protection offered by the survival response exceeds the sensitivity predicted on the basis of the DNA repair deficiency. All the above points argue in favor of the presence of a broad specificity, powerful core survival response that arose early in evolution as an answer to a range of life-threatening challenges. Experiments involving fasting of mice suggest that the survival response in some mammals may show protective effects, e.g. in ischemia reperfusion already 24 to 48 hrs. After initiation of fasting (Mitchell et al. 2009). In Drosophila, the maximal benefits on longevity are obtained within 1–3 days after switching to a CR diet (Mair et al. 2003), suggesting that kinetics of induction of the survival response may be as quick in mammals as in flies.

The notion of a common survival response (Fig. 10.3) triggered by various environmental threats closely resembles hormesis, a toxicology term that refers to the activation of generally-favorable biological responses to low dose challenge with a toxin. It remains to be identified, however, whether CR (a non-toxic stimulus per se) or persistent DNA lesions that, unlike a single dose exposure, are continually present in the genome can trigger similar beneficial responses to those observed upon low exposures to toxins.

The Survival Response: A Cell Autonomous Adaptive Response

The systemic suppression of the hormonal regulatory axes suggests a master endocrine regulator e.g. the hypothalamus and pituitary gland or that single cells exposed to various stressors are unable to surmount a similar type of response. However,

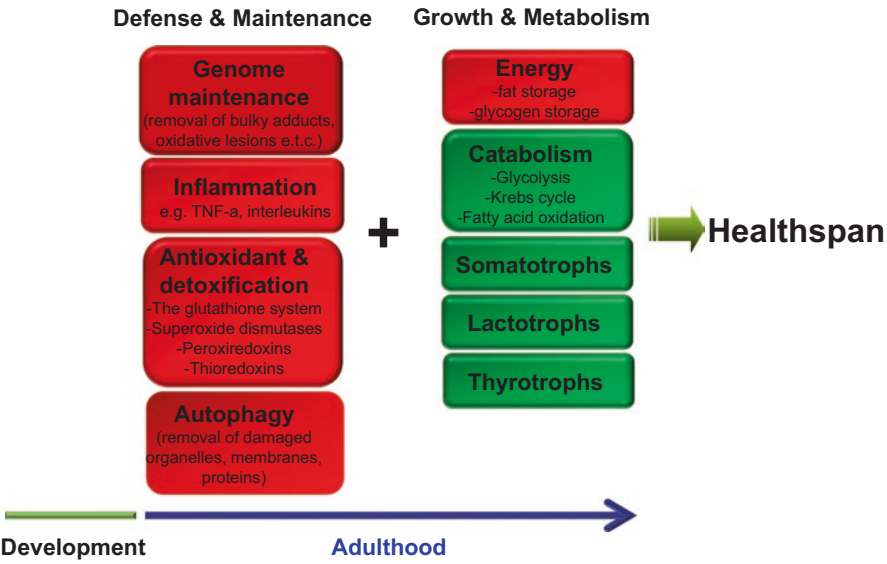

Fig. 10.3 The survival response in mammals

exposure of several types of cultured cells to Ultraviolet (UV)-induced DNA lesions, such as the cyclobutane pyrimidine dimers (CPDs) were recently shown to trigger a battery of dose-dependent expression changes that closely mimic the responses seen in mice carrying defects in NER as well as upon DR (Garinis et al. 2009). These findings indicate a cell-autonomous rather than systemic origin of the survival response. It also explains how individual cells markedly differing in developmental origin adjust their energy, growth and metabolic circuits to cope with macromolecular damage. Interestingly, CPD lesions can be removed by photolyases (Garinis et al. 2005). Photolyases are ubiquitously expressed and highly conserved enzymes that can selectively remove a distinct type of UV-induced DNA lesion (Garinis et al. 2006); however, these enzymes are not found in placental mammals. Using photolyase transgenic mice that efficiently repair CPD lesions, it was possible to alleviate the survival response highlighting CPDs (and likely other DNA bulky adducts) as the main culprit in the case of UV-induced damage (Garinis et al. 2009). The findings also highlight the causal contribution of DNA damage in triggering the survival response. Importantly, DNA damage could trigger the suppression of the GH/IGF1 axis in proliferating, quiescent as well as post-mitotic cells allowing for flexibility in terms of heterogeneity across different organisms or even organs and distinct types of tissues. The hypothalamus/pituitary hormonal axis is expected to adjust the balance between any systemic components of the adaptive survival response and the individual status of compromised cells.

At present, any mechanistic insights on how the survival response is triggered remain unknown. Indeed, the main stressors known to promote such responses include CR, fasting and DNA damage. For instance, DNA lesions are known to

interfere with transcription and transcription stress i.e. RNA Pol II blockage at sites of DNA damage sites could represent the primary triggering mechanism. Importantly, several DNA repair factors, including some within the NER machinery are known to be involved, in addition to DNA repair, in transcription activation of genes involved in growth (Kamileri et al. 2012b). It remains, therefore, to be seen how such proteins prioritize their function in DNA repair and transcription or whether at times of excessive, irreparable DNA damage accumulation such DNA repair factors are only dealt with DNA repair, thereby compromising the process of mRNA synthesis (Garinis et al. 2009). Similarly, energy shortage triggered by CR or fasting may compromise transcription or hamper translation. DNA replication is likely less critical as quiescent and post-mitotic cells are also able to surmount the survival response.

Future Perspectives

At present, there is solid evidence that DNA repair mechanisms (and by inference persistent DNA damage) are functionally linked to lifespan regulatory circuits and adaptive cellular responses aimed at prolonging healthspan (Garinis 2008; Garinis and Schumacher 2009; Schumacher et al. 2009; van de Ven et al. 2007). The latter also suggests the possibility to develop a series of rationalized intervention strategies. For instance, besides the rather straightforward avoidance of any source of exogenously derived cytotoxic types of DNA damage, recent strategies are also focused on alleviating the continuous source of damage that primarily originates from ROS and subsequent byproducts during mitochondrial respiration and lipid peroxidation. In this respect, the use of nutraceuticals e.g. resveratrol (Baur and Sinclair 2006; Ingram et al. 2006), rapamycin or other compounds that act as CR mimetics (albeit individual side effects) look promising paving the way to exploit further strategies that would exploit the full potential of the survival response in the absence of harmful DNA lesions or CR.

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Chapter 11
Signal Transduction Pathways in Ageing

Cathy Slack and Jennifer Tullet

Abstract It is now widely recognised that ageing and its associated functional decline are regulated by a wide range of molecules that fit into specific cellular pathways. Here, we describe several of the evolutionary conserved cellular signalling pathways that govern organismal ageing and discuss how their identification, and work on the individual molecules that contribute to them, has aided in the design of therapeutic strategies to alleviate the adverse effects of ageing and age-related disease.

Keywords Insulin signalling · mTOR · AMPK · Ras/MAPK · Ageing

Introduction

Despite its complexity, recent advances in the field of Biogerontology have shown that ageing and its associated functional decline are not simply consequences of the passage of time but are driven by underlying biological processes. Several key molecular regulators of organismal ageing have now been identified by studying the effects of single gene mutations in model organisms. These mutations not only extend lifespan, but in many cases also delay the onset of age-related pathology. Many of these mutations affect the activity of integral components of highly conserved cell signalling pathways. These pathways do not operate in isolation and extensive cross-talk exists between them via multiple nodes of reciprocal regulation. Moreover, they often converge on common signalling effectors. The net result is a highly complex and interconnected cell signalling network, the activity of which

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determines both lifespan and age-related health in evolutionarily distant species, from yeast to mammals.

Here, we describe some of the signal transduction pathways that contribute to this regulatory network, focusing on those that have been shown to affect ageing across multiple species. We highlight their ability to promote healthy lifespan, or healthspan, and discuss their potential to offer cellular targets for pharmacological interventions to improve age-related health.

Pro-longevity Signalling Pathways

Insulin/IGF-1-like Signalling

The insulin/IGF-1-like signalling (IIS) pathway was the first example of a cellular signalling pathway that could determine lifespan in an animal model. Single gene mutations that dramatically extended lifespan in the nematode, *Caenorhabditis elegans*, were mapped to the DAF-2 insulin receptor (Kenyon et al. 1993; Kimura et al. 1997) or the phosphoinositide 3-kinase (PI3K) AGE-1 (Friedman and Johnson 1988; Morris et al. 1996) thus identifying the IIS pathway as a key modulator of animal ageing.

Signal transduction via the IIS pathway is triggered by the binding of insulin/insulin-like peptides (ILPs) to an insulin receptor expressed on the cell surface of target tissues. This initiates an intracellular kinase cascade ultimately resulting in the phospho-activation of the kinase, AKT. Activated AKT then phosphorylates the FOXO (Forkhead bOX-containing protein, subfamily O) transcription factors, sequestering them within the cytoplasm away from their target gene promoters thereby rendering them inactive.

In *C. elegans*, inhibition of IIS either via reduced activity of the DAF-2 insulin receptor or inhibition of its downstream signal transduction pathway extends worm lifespan (Kenyon 2011), an effect which requires the worm FOXO orthologue, DAF-16 (Lin et al. 2001; Ogg et al. 1997). Interestingly, activation of the IIS transcriptional response by direct expression of DAF-16 itself is also sufficient to elicit longevity (Alic et al. 2014b; Bansal et al. 2014; Henderson and Johnson 2001; Kwon et al. 2010; Qi et al. 2012). These effects of reduced IIS activity on lifespan are conserved in the fruit fly, *Drosophila melanogaster.* Genetic perturbation of the expression or availability of ILPs by deletion of three of the seven ILP genes (Grönke et al. 2010), ablation of the neuroendocrine cells that in which they are produced (Broughton et al. 2005) or over-expression of an ILP binding protein that reduces ILP availability in the hemolymph (Alic et al. 2011b), all induce longevity in the fly. Similarly, decreasing the activity of the insulin receptor (Slack et al. 2011; Tatar et al. 2001) or components of its downstream signalling cascade (Clancy et al. 2001; Slack et al. 2011) all extend *Drosophila* lifespan. The requirement for FOXO transcriptional regulation in this IIS-mediated longevity response is also conserved in the fly as deletion of the *Drosophila* FOXO orthologue prevents the ability of

reduced IIS to increase lifespan (Slack et al. 2011). Similarly to the situation in worms, increasing FOXO levels in flies is also sufficient to extend their lifespan (Giannakou et al. 2004; Hwangbo et al. 2004).

In mammals, the somatotropic axis which regulates the release of growth hormone and insulin-like growth factors (IGFs) also influences animal lifespan. Growth hormone induces the release of IGF-1 from the liver that stimulates IIS transduction in several tissues via activation of the IGF-1 receptor. Several spontaneous mutations have been identified, including the Ames dwarf, Snell, and Little mouse, that result in growth hormone deficiency and reduced circulating IGF-1 levels, all of which display exceptional longevity (Bartke et al. 2000; Brown-Borg et al. 2009). Reducing the bioavailability of circulating IGF-1 by inhibiting the proteolytic cleavage of the IGF binding protein, IGFBP-4, which normally binds to circulating IGF-1 and sequesters it away from its target tissues, also extends lifespan in mice (Conover and Bale 2007). Similarly, over expression of Klotho, a transmembrane protein that is thought to be cleaved releasing an IGF-1 inhibitor, results in long-lived mice (Kurosu et al. 2005). Moreover, targeted disruption of insulin/IGF-1 receptor activity (Bluher et al. 2003; Holzenberger et al. 2003) or components of the downstream signalling pathway including the insulin receptor substrates, IRS1 and IRS2 (Selman et al. 2007; Taguchi et al. 2007), PI3K (Foukas et al. 2013) or AKT (Nojima et al. 2013) or over-expression of PTEN, a negative regulator of IIS (Ortega-Molina et al. 2012), have all been shown to increase lifespan in murine models.

There is a great deal of interest in extending findings in model organisms to human ageing. Around one quarter of the variability in human lifespan can be attributed to genetic factors (Christensen et al. 2006). Interestingly, the FOXO family member, FOXO3A, an established downstream mediator of IIS transduction in mammals, has been implicated in human longevity. Genome-wide association studies of exceptionally long-lived human populations have revealed associations between single nucleotide polymorphisms (SNPs) in the *FOXO3A* gene and the attainment of extreme old age (Pawlikowska et al. 2009; Sun et al. 2015; Willcox et al. 2008; Zhao et al. 2014).

mTOR Signalling

The mechanistic Target of Rapamycin (mTOR) is an atypical serine/threonine kinase that is inhibited by the macrolide compound, rapamycin, and forms part of two structurally and functionally distinct complexes, mTORC1 and mTORC2 (Saxton and Sabatini 2017). mTORC1 is composed of mTOR, Raptor, GβL, and DEPTOR and is inhibited by rapamycin. It integrates diverse nutritional and environmental cues, including growth factors, cellular energy levels, stress, and nutrients, to control cellular growth through the regulation of protein translation, ribosome biosynthesis, lipid synthesis and autophagy (Ben-Sahra and Manning 2017). mTORC2 contains mTOR, Rictor, GβL, Sin1, PRR5/Protor-1 and DEPTOR. mTORC2 promotes cellular survival through activation of AKT, regulates

cytoskeletal dynamics by activating PKCα, and controls ion transport and growth via SGK1 phosphorylation (Gaubitz et al. 2016). mTORC2 is not directly inhibited by rapamycin although prolonged exposure to rapamycin can indirectly inhibit this complex in some cell types (Sarbassov et al. 2006).

A role for mTOR in ageing has been well established with the pro-longevity effects of both genetic and pharmacological inhibition of mTOR by rapamycin now documented in yeast, worms, flies, and mice (Saxton and Sabatini 2017). mTORC1 regulates protein translation and growth through the phosphorylation of two key downstream effectors, the ribosomal protein S6 kinase (S6K) and the eukaryotic translation initiation factor 4E-binding protein (4E-BP) (Showkat et al. 2014). Studies in *Drosophila* have suggested that rapamycin-dependent lifespan extension requires inhibition of S6K but that 4E-BP activity is dispensable for rapamycin to extend fly lifespan (Bjedov et al. 2010). Moreover, genetic modifications that alter the expression or activity of S6K have also been shown to increase lifespan in different species. Deletion of the kinase, SCH9, in *S. cerevisiae,* which shows sequence and functional similarity to mammalian S6K1, extends both chronological and replicative lifespan (Kaeberlein et al. 2005) while worms mutant for S6K (*rsks-1*), flies expressing a dominant negative form of S6K and mice carrying a genetic deletion for *S6K1* are all long-lived (Hansen et al. 2007; Kapahi et al. 2004; Pan et al. 2007; Selman et al. 2009; Vellai et al. 2003).

Eukaryotic translation initiation factor 4E-binding protein (4E-BP), represses cap-dependent mRNA translation initiation by sequestering the eukaryotic translation initiation factor 4E (eIF4E) away from the translation initiation complex. Despite the lack of interaction between 4E-BP and rapamycin-induced longevity in *Drosophila* (Bjedov et al. 2010), activation of 4E-BP specifically in fly muscle prevents age-dependent muscle deterioration and extends lifespan (Demontis and Perrimon 2010). Similarly, increased 4E-BP activity in mouse skeletal muscle also demonstrates positive effects in older animals by protecting against age-induced metabolic dysfunction and insulin resistance through increased mitochondrial respiration and enhanced translation of PPARγ coactivator-1α (PGC-1α) (Tsai et al. 2015).

Ras/MAPK Signalling

In mammals, the oncogenic Ras protein is an established signalling intermediary of the IIS pathway (White 1997). Ras is a small GTPase, which cycles between an inactive GDP-bound form and an active GTP-bound form mediated by the activity of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The activation of Ras initiates a signal transduction cascade via the extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK). Interestingly, the two Ras homologues in *S.cerevisiae*, RAS1 and RAS2, were the first proteins shown to influence lifespan in yeast (Jazwinski 1999). Deletion of *RAS1* extends replicative lifespan while deletion of *RAS2* extends chronological

lifespan (Fabrizio et al. 2003; Sun et al. 1994). Replicative lifespan determines the number of daughter cells produced by an individual mother cell whereas chronological lifespan measures the survival time of non-dividing cells in the stationary phase. While replicative and chronological lifespan are clearly linked, the relationship between the two is not yet fully understood and it remains to be determined why *RAS1* and *RAS2* deletion have different effects on ageing depending on the method used to measure lifespan.

Recent studies also implicate cell signalling via Ras as a key modulator of animal ageing. In *Drosophila,* direct genetic inhibition of either Ras itself or the downstream kinase, ERK, can induce longevity (Slack et al. 2015). Furthermore, reduced Ras signalling accounts for at least part of the increased longevity incurred by reducing IIS in flies. Two key downstream effectors of Ras signal transduction during development in the fly are the two ETS transcription factors, Pointed (Pnt) and Anterior open (AOP). Pointed is a transcriptional activator which is stimulated in response to Ras activation while AOP is a transcriptional repressor that is inhibited by Ras activation. Both ETS transcription factors bind to the same regulatory elements and influence the expression of the same target genes but with opposing outcomes (Brunner et al. 1994; Halfon et al. 2000; O'Neill et al. 1994). Inhibition of AOP activation can abrogate the longevity effects of both reduced IIS and Ras/MAPK signalling suggesting that AOP is also a key effector of signal transduction through these pathways during ageing (Slack et al. 2015). Furthermore, in an analogous manner to FOXO, expression of an activated form of AOP is sufficient to extend fly lifespan (Alic et al. 2014a). Interestingly, AOP and FOXO share many of the same transcriptional targets suggesting that they may regulate the expression of a common set of pro-longevity genes (Alic et al. 2014a).

Although the canonical Ras signalling pathway is conserved in *C. elegans*, its role in worm ageing is somewhat different to that in flies. In worms, the Ras orthologue, LET-60 plays an important role in the development of the vulva and excretory systems, and is involved in sex-myoblast migration (Sternberg and Han 1998). LET-60 has also been implicated in DAF-2 dependent lifespan extension. However, LET-60 activation rather than its inhibition was required for *daf-2* mutation to extend lifespan (Nanji et al. 2005). The ETS transcription factor, LIN-1, is a downstream effector of LET-60 signalling in worms and is potentially regulated by MAPK phosphorylation (Tan et al. 1998). But here, the LET-60 signal transduction cascade lacks an AOP orthologue and this key difference in the downstream effectors of Ras between worms and flies may explain the differential effects of Ras signalling on ageing in the two models.

In mammals, there are four members of the Ras protein family, N-RAS, H-RAS, K-RAS4A and K-RAS4B, and apart from *K-RAS4A,* which does not seem to affect lifespan (Plowman et al. 2006), the effects of direct genetic inhibition of *H-RAS* or *N-RAS* on lifespan are yet to be determined. Disruption to Ras signal transduction, at least in certain tissues, may confer longevity in mice as animals deficient for the tissue specific Ras-GEF, *RasGrf1*, which is predominantly expressed within the pancreatic islets, hippocampus and hypothalamus, are long-lived (Borras et al. 2011). The RasGrf1 protein stimulates the dissociation of GDP from Ras promoting

the active GTP-bound form of Ras (Fernandez-Medarde and Santos 2011). However, RasGrf1 also shows affinity for other ligands including Rac, Rho, microtubules, PI[4,5]P2, and fasfatidic acid and so RasGrf1-dependent longevity may not be a direct result of Ras inhibition (Mirisola and Longo 2011). Yet, activation of the downstream kinase, ERK, has also been linked to mammalian longevity. Fibroblasts isolated from long-lived species of mammals and birds as well as long-lived mouse mutants show altered kinetics of ERK phosphorylation in response to stress (Elbourkadi et al. 2014; Sun et al. 2009). Ras/MAPK signalling has a well-established role in tumorigenesis but RasGrf1 deficiency in mice did not simply extend lifespan by preventing cancer as age-related survival was also increased in tumour-free animals. Old RasGrf1 deficient animals also had lower circulating IGF-1 levels, elevated expression of SIRT1 and enhanced oxidative stress resistance, all of which may have contributed to their longer lifespan (Borras et al. 2011).

In humans, mutations in *HRAS* are associated with symptoms of premature ageing such as osteoporosis and osteopenia in patients with Costello Syndrome, a rare multi-systemic disorder characterised by a failure to thrive, short stature, developmental delay or intellectual disability, soft skin and distinctive facial features (Rauen 2007). Genetic variants of *HRAS1* have also been associated with both exceptional longevity and healthy ageing in humans (Jazwinski et al. 2010) implicating Ras signal transduction in human aging.

AMPK

AMP-activated kinase (AMPK) is a highly conserved cellular energy sensor that is activated by elevated levels of AMP/ADP in response to cellular ATP hydrolysis (Salminen and Kaarniranta 2012). Activation of AMPK reduces macromolecule biosynthesis and increases catabolic processes thus coordinating energetically demanding cellular processes with energy availability (Carling 2017). In mammals, AMPK is a serine/threonine protein kinase composed of a catalytic α subunit and regulatory β and γ subunits. Several upstream kinases including Ca2+/calmodulin-dependent protein kinase kinase β (CaMKKβ), serine/threonine kinase 11 (LKB1) and transforming growth factor-β-activated kinase 1 (TAK1) can activate AMPK by phosphorylating the catalytic α subunit at Thr172 (Carling 2017). Phosphorylated AMPK is then inactivated by protein phosphatases such as PP2A, PP2Cα and Ppm1E (Hardie 2016).

AMPK activity has been linked to ageing in several model systems. In mammals, the responsiveness of AMPK activation declines with increased age (Salminen and Kaarniranta 2012). Moreover, AMPK activity may determine the lifespan effects of other signalling pathways. For example, in *C. elegans,* the longevity effects of reduced IIS or mTOR activity as observed in *daf-2* or *rsks-1* mutants, respectively, are dependent on the activity of the worm AMPK orthologue, AAK-2 (Apfeld et al. 2004; Curtis et al. 2006; Selman et al. 2009). Also, a specific form of dietary restriction in worms that restricts bacterial food intake induces longevity in an AAK-2

dependent manner (Greer et al. 2007). Moreover, direct over expression or activation of AMPK in worms can also cause modest increases in lifespan (Apfeld et al. 2004; Greer et al. 2007; Mair et al. 2011). Activation of AMPK may also modulate ageing in other animal models. In *Drosophila*, gain-of-function mutations in *lkb-1*, encoding an upstream activator of AMPK or modulating the expression of AMP biosynthetic enzymes to artificially alter the AMP/ADP ratio, both of which lead to AMPK activation, also extend lifespan in flies (Funakoshi et al. 2011; Stenesen et al. 2013). Furthermore, flies with tissue specific induction of AMPK activation by over expression of the catalytic α subunit specifically in neuronal tissue were longer lived (Ulgherait et al. 2014) and, in mice, *S6K1* knockouts which are long-lived show elevated AMPK activity (Selman et al. 2009).

The downstream targets of AMPK that mediate its longevity effects have been intensively sought. One candidate pathway is the cAMP responsive element binding protein (CREB) and its associated cytoplasmic co-activator, cAMP-regulated transcriptional coactivators (CRTCs). AAK-2/AMPK directly phosphorylates CRTCs in *C. elegans*, rendering them inactive (Mair et al. 2011). Conversely, dephosphorylation of CRTCs by protein phosphatases such as protein phosphatase 2B or calcineurin allow CRTC to translocate to the nucleus where it can bind to CREB factors and promote CREB target gene expression. In worms, AAK-2/AMPK signalling via inhibition of the CRTC-CREB pathway extends lifespan (Mair et al. 2011). This pro-longevity effect of CRTC inhibition may also be conserved in mammals. There are three mammalian CRTC proteins and CRTC1 which is predominantly expressed in neuronal tissues regulates CREB-dependent expression of calcitonin gene related peptide (CGRP), a secreted neuropeptide that inhibits insulin secretion and is detrimental to murine lifespan (Riera et al. 2014). However, a direct link between AMPK and CRTCs in mammalian ageing remains to be shown.

Pro-longevity Signalling Pathways: Cross-Talk and Convergence

When studying an individual signalling pathway, it is tempting to view them as discrete, linear units with a beginning, middle and end-point. Many pro-longevity signalling pathways actually converge onto common downstream effectors that mediate their longevity effects. Moreover, there is considerable co-regulation between them forming a highly connected and complicated cell signalling network (Fig. 11.1). Such cross-talk can be referred to as feedback and, in a biological system, this is defined as a response within a system (molecule, cell, organism, or population) that influences the continued activity or productivity of that system. Feedback regulation is traditionally divided into two categories: it can act to down-regulate the activity of a pathway (i.e. negative feedback) or it acts to keep a process or pathway switched on (i.e. positive feedback). To achieve homeostasis, the positive and negative outputs of each signalling pathway need to be balanced and feedback mechanisms can be employed at any point in the signalling cascade to achieve

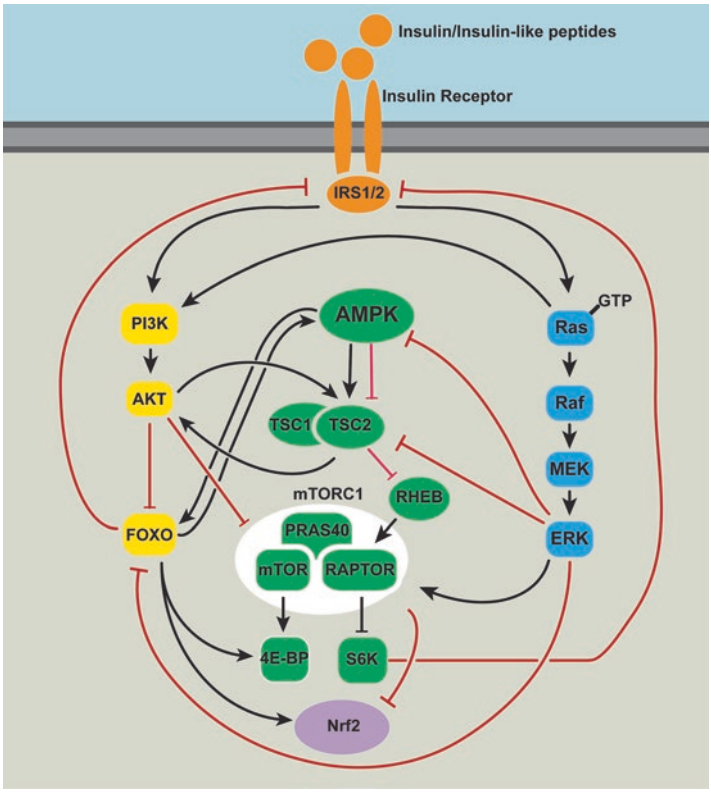

Fig. 11.1 Cross-talk and convergence within the pro-longevity cell signalling network. The pro-longevity signalling molecules and signal transduction pathways described within this chapter form an intricately connected cell signalling network with multiple nodes of co-regulation and convergence onto common downstream effectors. Positive regulatory interactions are indicated by black arrows. Negative regulatory interactions are shown as red blunt-ended lines

this. Many of the molecules discussed in the previous section are subject to feedback regulation and in most cases, this molecular communication serves to self-regulate the pathways. In the context of a whole organism, this feedback provides an optimum output in terms of organismal health. Indeed, in some cases perturbations in the pathways that lead to extended lifespan are a direct result of altering the feedback mechanisms between them.

Studying Feedback Mechanisms

Studying feedback loops in biological systems is inherently challenging because the purpose of feedback is to maintain homeostasis. As a result, there is no single point readout for measuring its occurrence. In very simple organisms such as yeast and bacteria it is possible to alter an experimental parameter, for example a sensory cue,

and examine the effect of that change on growth over time by sampling from a synchronised culture. The growth conditions of these simple organisms are such that very short time intervals can be measured in this way. Using these techniques, gene regulatory feedback in relation to transcription factor networks have been elegantly characterised in unicellular organisms such as *S. pombe*, (Alon 2007). However, this time-sensitive sampling, particularly at short intervals, gets much less tractable as organisms become more complex. Some attempts to adopt similar strategies have been made using *C. elegans* where animals were synchronised and harvested at short intervals following a gene knockdown (Tullet et al. 2014). However, in mammals such time-dependent sampling is neither experimentally or financially practical. Other techniques must therefore be utilised to further understand the regulatory relationships between cell signalling molecules during ageing.

Genetic epistasis studies are the classical method for examining molecular interactions using model organisms. Genetic manipulations that affect the activity of molecules within different cell signalling pathways are combined and the resulting animals are studied for overlapping or additive phenotypic effects. Data from epistasis studies can prove a powerful tool but should be interpreted with caution (Gems et al. 2002). Alternatively, genome-wide mRNA expression or chromatin profiling can be used to examine the relationships between signalling pathways through the analysis of the genome-wide effects caused by signalling perturbations.

The complexity of cross-talk between signalling pathways in higher organisms is also compounded by the existence of tissue-specific signalling effects and the occurrence of both cell autonomous and non-autonomous effects. For instance, the majority of worm mRNA profiling work is carried out from mRNA extracts of whole animals. Whilst this gives a good overall picture of gene expression changes and should capture both cell autonomous and non-autonomous effects, it inevitably leads to an under-representation of expression changes from less abundant cells or tissues. For *C. elegans* researchers, newer techniques that allow tissue specificity at the level of mRNA isolation or post-array bioinformatic analysis could improve our understanding of feedback in worms. In flies and mice, the use of isolated tissues for mRNA preparation is more straightforward and negates part of the problem, but also means that the signal from cell non-autonomous effects are lost.

Thus, when considering feedback in the context of organismal longevity it is important that researchers gather evidence from multiple lines of investigation. By using a combination of these techniques, we have gained significant insight into regulatory cross-talk between the pro-longevity cell signalling pathways.

Positive and Negative Feedback Within the IIS Pathway

Signalling via the IIS pathway culminates in the phosphorylation and subsequent nuclear localisation of the FOXO transcription factor (Kenyon 2010) and so FOXO is often considered to be the end-point of the IIS pathway. The biological actions of FOXO are so broad, and its impact on the cell and subsequently the organism so

extreme that it should be considered more as a central node of IIS. As a transcription factor, FOXO regulates the expression of a wide variety of target genes (Tullet et al. 2014) many of which underpin the feedback mechanisms both within and between signalling pathways. The pro-longevity effects of reduced IIS are dependent on FOXO transcriptional activity and so a great deal of effort has been spent identifying the targets of this transcription factor. By describing the FOXO transcriptional response, individual biochemical processes regulated by FOXO activity could be targeted to recapitulate the positive effects of reduced IIS on lifespan and age-related health.

By cross-referencing the data sets from a combination of both mRNA and chromatin profiling techniques, many targets of FOXO have now been identified in *C. elegans* and *Drosophila* (Alic et al. 2011a; Schuster et al. 2010). In combination with genetic deletion or inactivation of FOXO, such studies have led to the identification of both direct FOXO target genes in which FOXO binds directly at the promoter leading to a change in target gene expression and indirect FOXO target genes, in which FOXO elicits a change in gene expression via affecting the expression of second tier of transcription factors (Alic et al. 2011a; Schuster et al. 2010).

While activation of FOXO is both necessary and sufficient to increase lifespan, too much FOXO can lead to toxic effects and so it is important that the IIS pathway can self-regulate. Several direct FOXO target genes encode upstream components of the IIS pathway itself. In worms, several DAF-16/FOXO direct targets encode proteins whose actions have a negative effect on IIS such as the kinases *akt-1/2 and pdk-1*, and the worm insulin receptor substrate homologue *ist-1* (Murphy et al. 2003; Schuster et al. 2010). A similar situation has been described in *Drosophila*, where FOXO was observed directly bound to the promoters of genes encoding the insulin receptor substrates *chico* and *Lnk* as well as *akt* and *Sos* adaptor proteins (Alic et al. 2011a). FOXO activation in *Drosophila* also leads to increased expression of *Imp-L2*, which encodes a *Drosophila* IGF-binding protein homologue and is a negative regulator of IIS (Alic et al. 2011b; Honegger et al. 2008) alongside decreased expression of the insulin-like peptide, *dilp3*, and *pdk1*, both of which are positive regulators of IIS in flies. Intrinsic regulation of IIS signalling by mammalian FOXO has also been described: activation of murine FOXO up-regulates expression of the Insulin Receptor in both liver and skeletal muscle (Puig and Tjian 2005) as well as the insulin substrate protein, IRS2, therefore promoting IIS at more than one point (Tsunekawa et al. 2011).

There is also evidence that FOXO activity mediates positive feedback onto IIS. Although not a direct target of DAF-16/FOXO, expression of *ins-7* encoding an insulin peptide homologue in *C. elegans*, is down-regulated in response to DAF-16/FOXO activity (Murphy et al. 2007). Worms have 40 insulin like peptides, each of which is expressed in a tissue-specific manner and acts differently on the DAF-2/insulin receptor (Fernandes de Abreu et al. 2014; Ritter et al. 2013). They can act as either agonists or antagonists and *ins-7* functions as a DAF-2 agonist (Fernandes de Abreu et al. 2014; Murphy et al. 2007). Thus, DAF-16 activity seems to maintain insulin sensitivity by positive feedback onto *ins-7* expression.

Cross-Talk Between IIS and mTOR

In both mammals and flies there is considerable co-regulation within the signalling output of these two conserved signalling pathways. Some of this cross-talk occurs as a result of upstream kinase and phosphatase activity. Additionally, IIS and mTOR both converge onto FOXO and their co-regulation occurs in response to transcriptionally induced changes initiated by FOXO activation. Signal transduction via the IIS pathway can activate mTOR because AKT can activate TORC1 via direct phosphorylation of TSC2. Under normal conditions, TSC2 interacts with TSC1 to inhibit mTOR signalling but AKT-dependent phosphorylation of TSC2 disables this TSC1/2 interaction leading to an increase in mTOR activity (Huang and Manning 2009). Conversely, TORC2 can stimulate the IIS pathway by directly phosphorylating AKT at Serine 473 (Bhaskar and Hay 2007; Hay 2011). The downstream effector of mTOR signalling, S6K, has a negative effect on IIS. In mammals, when mTORC1 is constitutively active, for example in TSC1/2 null cells, and therefore independent of growth factors and AKT, AKT levels are decreased (Chandarlapaty et al. 2011). This reduction in AKT expression is due to the inhibitory effects of S6K on insulin receptor substrates (IRS1 or IRS2) and a subsequent downregulation of IIS (Chandarlapaty et al. 2011).

In flies, FOXO acts as a transcriptional regulator of several elements of the mTOR pathway. mTOR itself is a direct target for FOXO regulation and FOXO activity is required to maintain expression of mTOR in adult flies (Alic et al. 2011a). Additionally, the two key outputs of mTOR signalling, S6K and 4E-BP, are also directly regulated by FOXO transcriptional activity (Junger et al. 2003; Puig et al. 2003; Teleman et al. 2005).

In worms, however, the interaction between IIS and mTOR is different. Worm AKT cannot activate TORC1 as the worm genome does not have identifiable homologues of TSC1/2 (Long et al. 2002). However, AKT can still impact on TOR signalling via the activity of DAF-16/FOXO, which is responsible for transcriptionally down-regulating expression of the Raptor gene (Jia 2004). When AKT phosphorylates DAF-16/FOXO and inactivates it, this increases expression of DAF-15, the *C. elegans* Raptor homologue, and leads to increased mTOR signalling. Interestingly, activating DAF-16/FOXO by a different kinase, JNK-1, in response to stress has the opposite effect (Hay 2011) implying that different cellular responses require different balances of IIS and mTOR signalling to maintain fitness. In addition, Chen and co-workers showed that although reductions in either IIS or mTOR signalling leads to lifespan extension, mutations in both *daf-2* and *rsks-1,* encoding the worm orthologue of S6K, together showed additive effects on lifespan (Chen et al. 2013) again indicating reciprocal regulation between these two pathways.

All of these examples of cross-talk between IIS and mTOR signalling illustrate the high level of interdependence between these two pathways for their proper regulation. However, the relevance of each of these feedback mechanisms to ageing is yet to be fully determined.

A Feedback Loop Between FOXO and AMPK

Another interesting feedback loop exists between FOXO/DAF-16 and AMPK. As described earlier, AMPK is a heterotrimeric complex comprised of α catalytic, β linker and γ regulatory subunits (Hardie 2014). Some animal species possess multiple forms of each subunit which are expressed in a tissue-specific manner. In *C.elegans*, DAF-16 transcriptionally regulates the expression of four of the AMPK subunits (*aak-2* (α), *aakb-1*(β), *aakg-4* (γ) and *aakg-5* (γ)) (Schuster et al. 2010). Moreover, the regulation of at least one of these subunits, the γ subunit *aakg-4*, is direct (Chen et al. 2013; Tullet et al. 2014). Thus, under conditions of reduced IIS, DAF-16 acts to promote formation of AMPK complexes. Notably, one of AMPK's targets is DAF-16 itself and phosphorylation of DAF-16 by AMPK allows DAF-16 to accumulate in the nucleus where it is able to access chromatin and promote transcription of its target genes. This suggests the presence of a positive feedback loop i.e. DAF-16 is effectively propagating its own action via the stabilization of AMPK. These feedback loops could act to accelerate induction or increase expression of other FOXO target genes, or increase stability of the activated AMPK molecule. This is supported by recent work showing the existence of such a loop acting downstream of reduced IIS, to increase the rate of DAF-16-dependent transcription, and contribute to DAF-2 dependent longevity (Tullet et al. 2014). Although knockdown of the direct DAF-16 target *aakg-4* does not fully recapitulate the effect of *daf-16* mutation on *daf-2* lifespan, its involvement in gene regulatory loops and ageing phenotypes supports a model whereby DAF-16 acts to regulate a number of other different regulatory molecules. These regulators then act in feedback loops, and each factor makes a small but significant contribution to lifespan.

Similar FOXO-AMPK feedback loops may also be present in flies and mammals. In *Drosophila*, expression of the AMPK γ subunit, SNF4Aγ, is up-regulated in flies expressing a dominant negative form of the insulin receptor, although direct binding of FOXO to the SNF4Aγ promoter has not yet been detected in whole flies (Alic et al. 2011a). In humans, FOXO3 and FOXO4 can directly activate LKB1 expression *in vitro* (Bakker et al. 2007). As LKB1 is an upstream activator of AMPK, this suggests the existence of a similar FOXO-AMPK feedback loop in mammals. However, to date no FOXO-dependent changes in the expression of AMPK γ subunits have been detected in murine RNA profiling studies (Bakker et al. 2007; Eijkelenboom et al. 2013; Greer et al. 2007; Paik et al. 2007; Tothova and Gilliland 2007) although such studies have only been performed using a limited number of tissues.

AMPK Communicates with Both IIS and mTOR

AMPK, IIS and mTOR are all nutrient- and energy-dependent signalling pathways and so it is perhaps not all that surprising that they communicate with one another at both the cellular and organismal level. We have previously discussed the relationship between AMPK and DAF-16/FOXO but the effect of AMPK is broad. Moreover, its

impact on mTOR signalling is also instrumental to how IIS and mTOR interact with each other. In mammals, when ATP levels decline and AMP levels increase, the increased activity of AMPK leads to phosphorylation of TSC2. This direct phosphorylation event results in TORC1 inhibition (Inoki et al. 2003). In addition mammalian AMPK can inhibit TORC1 itself via the inhibitory phosphorylation of Raptor (Gwinn et al. 2008). This latter mechanism may also be applicable to flies but the AMPK phosphorylation sites in TSC2 are not fully conserved in *Drosophila*.

Lowering AMPK activity can also impact on TORC1 activity in an IIS-dependent manner. One of the cellular functions of AKT is to maintain ATP levels. Thus, active AKT reduces AMPK activity which can then activate TORC1. Another indirect link between AMPK, IIS and mTOR occurs via the conserved family of Sestrin proteins, originally identified as antioxidants (Budanov and Karin 2008; Lee et al. 2010). In flies, FOXO transcriptionally up-regulates the expression of *sestrin* genes, which once expressed activate AMPK. This active AMPK then phosphorylates TSC2 and inhibits TORC1 activity. A similar relationship between mammalian FOXO1 and Sestrin proteins has also been observed in mammalian cells where FOXO1 directly activates transcription of Sestrin 3 (Chen et al. 2010).

As discussed earlier, mutation of both *daf-2* and *rsks-1* show additive effects on lifespan in worms (Chen et al. 2013). These worms also show an increase in their expression of the AMPK γ subunit and *aakg-4*, the latter of which is required for *daf-2; rsks-1* mutants to extend lifespan (Chen et al. 2013). Under normal conditions, *rsks-1* acts to block AMPK activity and phosphorylation of the AMPK alpha subunit is decreased (Chen et al. 2013). Thus, the exceptional longevity of these *daf-2; rsks-1* double mutant animals relies on feedback between DAF-16/FOXO and AMPK. In both worms and mice, the longevity phenotype of *rsks-1* mutant worms and *S6K* mutant mice requires AMPK activity (Selman et al. 2009; Sheaffer et al. 2008) but the regulation by DAF-16/FOXO of AMPK links the two pathways.

An interesting twist to this feedback mechanism is that AMPK can also phosphorylate either Raptor or the TSC1/2 complex either of which leads to the inhibition of mTORC1 activity (Huang and Manning 2009; Laplante and Sabatini 2012). A similar relationship is maintained in *C. elegans* with DAF-15 (the worm Raptor homologue) also being a target for AMPK (Erdogan et al. 2016). Taken together, cross-talk between AMPK and mTOR is the main process that regulates energy sensing. Downregulation of mTOR because of increased AMPK activity leads to the decreased activity of S6K. Based on the interactions observed in *C. elegans,* this would lead to further activation of AMPK via a positive feedback loop.

Interplay Between Ras Signalling and Other Pro-longevity Signalling Pathways

Oncogenic Ras signalling is an integral component of the mammalian IIS pathway. Upon activation, the mammalian insulin receptor recruits activated Ras via insulin receptor substrate (IRS) proteins that couple the receptor to the Ras-GEF, SOS, by

binding to the Grb2 adaptor protein (White 1997). In *Drosophila*, extension of lifespan by mutation of the insulin receptor substrate, *chico,* requires the activity of the Ras-responsive transcription factor, AOP. Moreover, mutation of Chico within the selective domain that initiates signalling via Ras/MAPK is sufficient to extend fly lifespan (Slack et al. 2015). Therefore, in *Drosophila* inhibition of Ras/MAPK signalling is at least in part responsible for the longevity effects of reduced IIS.

Extensive cross-talk occurs between the two signalling branches downstream of the insulin receptor. For instance, activated Ras directly binds to and activates the catalytic subunit of PI3K (Castellano and Downward 2011), an interaction that is required for maximal activation of PI3K during growth (Orme et al. 2006). In addition, ERK has been shown to phosphorylate FOXO3A, targeting it for ubiquitination and subsequent proteasomal degradation (Yang et al. 2008). Phosphorylation of Raf by AKT negatively regulates ERK activation by sequestering ERK away from Ras and MEK within the cytosol (Zimmermann and Moelling 1999). Thus, downstream of the insulin receptor, Ras/MAPK and PI3K/AKT signalling show extensive co-regulation via both stimulatory and inhibitory mechanisms although the full implications of this cross-talk for lifespan regulation are not yet fully understood.

Ras/MAPK activity also regulates mTOR signalling. Activated ERK phosphorylates RAPTOR thereby promoting mTORC1 activation and phosphorylation of its downstream target, 4E-BP (Carriere et al. 2011). ERK activity also inhibits the ability of the TSC1/2 complex to function as a GAP for the small GTPase, RHEB, leading to increased mTORC1 activity.

Lastly, Ras/MAPK signalling can also influence the activation of AMPK activity. AMPK activity is regulated by phosphorylation with both positive and inhibitory outcomes. One such inhibitory site on AMPK, Ser485, is directly phosphorylated by ERK (Lopez-Cotarelo et al. 2015) and stress-induced ERK activation can promote the translocation of AMPK from the cytoplasm to the nucleus (Kodiha et al. 2007). Conversely, the kinase suppressor of Ras (KSR), which acts as a scaffold for ERK at the cell membrane facilitating the colocalisation of ERK with its upstream kinases, also positively regulates AMPK activation (Costanzo-Garvey et al. 2009).

Convergence on Nrf2 Transcription Factors

We have already discussed the DAF-16/FOXO transcription factor as an effector of cell signalling during ageing from several divergent pro-longevity signalling pathways. A similarly pervasive effector of cell signalling in the context of ageing is the nuclear factor (erythroid-derived 2)-like 2, or Nrf2, transcription factor, a key transcriptional regulator of the cellular antioxidant defence response. When cellular stress levels are low, Nrf2 is sequestered away from the nucleus by forming an inactive cytoplasmic complex with the Kelch-like ECH-associated protein 1 (Keap1) which is bound to the actin cytoskeleton. Keap1 also targets Nrf2 for polyubiquitination by a Cullin3-based E3 ligase complex and subsequent degradation via the 26S proteasome. Thus, under basal conditions Nrf2 expression and activity are

maintained at low cellular levels (Itoh et al. 1999). In response to a wide range of different cellular stressors, including exposure to reactive oxygen species (ROS) and xenobiotics, modifications to key redox-sensitive cysteine residues in the Keap1 protein result in conformational changes to its structure that prevent Nrf2 ubiquitination and degradation. In addition, proteins such as p21 and p62 can bind to Keap1 or Nrf2 inhibiting their interaction and thus preventing Nrf2 ubiquitination and degradation (Kansanen et al. 2013). Once free from Keap1-mediated inhibition, Nrf2 translocates to the nucleus where it dimerises with a member of the small Musculoaponeurotic fibrosarcoma oncogene (small Maf) family of proteins and binds to antioxidant response elements (ARE) located within promoter regions of its target genes, initiating their expression (Itoh et al. 1997; Katsuoka et al. 2005b).

In recent years, Nrf2 has emerged as a key modulator of lifespan with multiple studies suggesting that changes in Nrf2 activity may contribute to organismal ageing and its associated pathologies (Sykiotis and Bohmann 2010). Increased age in mammals is associated with decreased Nrf2 activity including reduced Nrf2-ARE binding activity and diminished Nrf2 transcriptional activity resulting in reduced expression of Nrf2 target genes. Such age-related changes in Nrf2 activity are thought to underlie the increased oxidative damage, ROS production and glutathione depletion observed in ageing mammals, including humans (Sykiotis and Bohmann 2010). A prominent role for Nrf2 in ageing is supported by genetic studies of Nrf2 and Keap1 loss- and gain-of-function. Mutation of the gene encoding the worm orthologue of Nrf2, SKN-1, results in shortened lifespan and sensitivity to oxidative stress (An and Blackwell 2003). Conversely, moderate over expression of a constitutively active form of SKN-1 increases lifespan and confers increased resistance to oxidative stress (An et al. 2005; Tullet et al. 2008) although recent evidence suggests that these effects may be mediated via distinct mechanisms (Tullet et al. 2017). Furthermore, SKN-1 activity contributes to the lifespan extension incurred by several genetic and environmental manipulations in *C. elegans* (reviewed in (Blackwell et al. 2015). For example, the longevity effects of *daf-2* mutation are dependent on SKN-1 activity, at least in part (Tullet et al. 2008) while dietary restriction in worms not only activates SKN-1 expression but SKN-1 activity is required in a subset of neuronal cells for dietary restriction to extend lifespan (Bishop and Guarente 2007). SKN-1 has also been proposed to participate in a feedback loop with mTOR signalling as genetic inhibition of TORC1 in *C. elegans* activates SKN-1/Nrf2-dependent expression of cytoprotective genes to increase stress resistance and longevity alongside upregulation of TORC1 pathway gene expression (Robida-Stubbs et al. 2012).

In *Drosophila*, the functional homologue of SKN-1 and the vertebrate Nrf2 proteins is encoded by the *cap n' collar (cnc)* gene. The *cnc* locus produces three protein isoforms of which CncC is the longest and contains a unique N-terminus with homology to the Keap1-binding domain of Nrf2 (McGinnis et al. 1998). CncC activity in flies is induced by several oxidants including paraquat and hydrogen peroxide (Sykiotis and Bohmann 2008). Furthermore, *Drosophila* possess a Keap1 ortholog that functions as an inhibitor of CncC and ARE activity (Sykiotis and Bohmann 2008). Genetic mutation of *Keap1* in flies induces Nrf2 activation and

extends lifespan (Sykiotis and Bohmann 2008). Interestingly, treatment of flies with Keap1 inhibitors alleviates some of the motor dysfunction associated with a fly model of Alzheimer's Disease (Kerr et al. 2017) indicating the real potential of modulating Nrf2 to improve age-related health.

Enhanced Nrf2 activity is also associated with longer lifespan in mammalian models. The naked mole rat is an exceptionally long-lived species, living on average four times longer than other similarly sized rodents (Buffenstein 2008). Naked mole rats also do not succumb to many of the deleterious effects of ageing and so do not show typical increases in cancer incidences, neurodegeneration or metabolic dysfunction with age (Edrey et al. 2012; Liang et al. 2010). Naked mole rats show elevated levels of Nrf2 protein expression, Nrf2-ARE binding activity and Nrf2 target gene expression compared to other rodent models yet paradoxically, they also display high levels of oxidative damage throughout their lifespan (Andziak and Buffenstein 2006). Interestingly, they also show elevated clearance of damaged macromolecules which may underlie their exceptional longevity and may be regulated by their elevated Nrf2 activity (Bruns et al. 2015).

In mammalian models of caloric restriction, many of the beneficial effects of reduced food intake have been attributed to Nrf2 activation. Caloric restriction can prevent the age-dependent decline of Nrf2 activity. Moreover, old mice under a calorically restricted regime display higher levels of Nrf2 activity than young fully-fed mice (Csiszar et al. 2014). It remains to be determined whether Nrf2 deletion will abrogate the beneficial effects of caloric restriction on lifespan. Studies so far have proved somewhat controversial. Calorically restricted mice were protected from cancer in an Nrf2 dependent manner but no significant decline in longevity was observed in calorie restricted animals in the absence of Nrf2 (Pearson et al. 2008). However, the absence of key controls in this study suggest that a role for Nrf2 in the longevity response to caloric restriction remains possible (Sykiotis and Bohmann 2010).

Nrf2 activity has also been linked to exceptional longevity in humans. Evidence suggests that centenarians may be able to better respond to cellular stress due to constitutively up-regulated Nrf2 activity (Davinelli et al. 2012). Furthermore, exercise stimulates Nrf2 activation and is one of the most effective interventions to prevent chronic disease in the elderly (Muthusamy et al. 2012).

Nrf2 regulates the expression of over two hundred target genes including those encoding key antioxidant enzymes such as superoxide dismutase, peroxiredoxins, glutathione S-transferases and glutamate cysteine ligase modifiers (Motohashi and Yamamoto 2004), pro-inflammatory mediators including cytokines, chemokines, cell adhesion molecules, matrix metalloproteinases, cyclooxygenase-2 and inducible nitric oxide synthase (Kim et al. 2010) as well as integral mediators of autophagy (Dodson et al. 2015). The promoters of the genes encoding Keap1 and MafG, a small Maf protein, also contain AREs and so are expressed in response to Nrf2 activity (Katsuoka et al. 2005a). Nrf2 also regulates its own expression. The 5-prime flanking region of the *Nrf2* promoter contains two ARE-like motifs that mediate

induction of *Nrf2* gene expression upon Nrf2 activation (Kwak et al. 2002). Thus, Nrf2 activation promotes its own expression in an example of positive feedback regulation. Determining how Nrf acts to promote longevity remains a key point for future studies.

Is Ageing Caused by Aberrant Signal Transduction?

With such an intricately linked cell signalling network, perturbations in signal transduction via any individual pathway is likely to have significant impact on the others and drastic consequences for physiological outcomes. Are pathologies associated with ageing therefore caused by inappropriate signal transduction through this network? There is certainly evidence that associates increased age with aberrant signal transduction. Mis-regulation of both PI3K/AKT and Ras/MAPK signalling often accompanies premature ageing (reviewed in (Steelman et al. 2011)). Ras-dependent activation of ERK declines with age (Hutter et al. 2000) while several studies have shown that signalling downstream of AMPK also declines during ageing suggesting that AMPK inhibition may contribute to the ageing process (Salminen and Kaarniranta 2012). However, while advanced age is associated with perturbed signal transduction a direct causal link between the two has yet to be established.

The biological purpose of the signalling pathways described within this chapter is to promote growth and development whilst supporting reproduction. Reducing signalling via cellular pathways like IIS and mTOR leads to an extension of lifespan suggesting that they normally function to inhibit longevity. Back in 1957, George Williams proposed the theory of antagonistic pleiotropy, that genes required to promote growth and reproduction during development are detrimental in later life, in an effort by a species to eliminate individuals that are no longer useful (Williams 1957). There are, however, few examples of such genes. More recently, it has been proposed that biological processes that contribute to early life health and fitness simply continue into adulthood in an unregulated fashion (Blagosklonny 2006, 2007, 2008). This is referred to as quasi-programming, developmental run-on or hyperfunction theory (Fig. 11.2) (Blagosklonny 2006; Gems and de la Guardia 2013) and distinguishes itself from the traditional description of antagonistic pleiotropy by arguing that the adult run-on of these processes is un-programmed. Diseases associated with ageing in mammals are often associated with increased or inappropriate growth or hypertrophy e.g. cardiovascular disease, type 2 diabetes and perhaps most obviously cancer (reviewed in Gems and Partridge 2013). It is also observed that simpler organisms such as worms exhibit late-life hypertrophic pathologies associated with the inappropriate action of developmental pathways running on to adulthood. One well characterised example in *C. elegans* is yolk production. As a hermaphrodite, the adult worm produces its own sperm and oocytes. To support oocyte development, the worm synthesises large quantities of yolk in the intestine

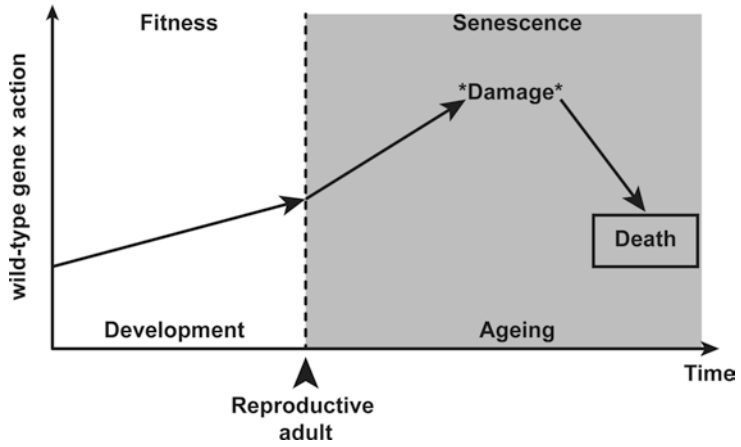

Fig. 11.2 Ageing as a consequence of developmental run-on (hyperfunction). The wild-type function of gene x promotes fitness during developmental stages. However, once the organism matures past the reproductive adult stage, the normal function of gene x becomes excessive (hyperfunction) leading to molecular and cellular damage, organismal decline and ultimately death. During ageing, the hyperfunction of gene x therefore promotes senescence. (Adapted from Blagosklonny 2012)

and then shuttles it across the body wall cavity to the gonads once oocyte development is initiated. Fertilisation of the oocytes is limited by a finite number of sperm but even after the sperm has been depleted and oocyte fertilization ceases, yolk is continuously produced and collects in pools in the body cavity (Herndon et al. 2002). This yolk accumulation is not observed in long-lived IIS pathway mutants suggesting that this developmental run on may be detrimental to lifespan and contribute to ageing (Herndon et al. 2002). Other examples of hypertrophy have been described during ageing in *C. elegans* including neurite outgrowth, thickening of the cuticle, ectopic deposition of lipids in the body wall muscle, oocyte stacking in the gonad and endoreduplication of oocytes leading to tumour like masses in the uterus (Golden et al. 2007; Herndon et al. 2002; Jud et al. 2007; Pan et al. 2011; Tank et al. 2011).

Ageing *Drosophila* may also exhibit phenotypic characteristics consistent with hypertrophy. Male flies show increased incidence of tumours within the testis and gut as they age (Salomon and Rob Jackson 2008). Neoplastic transformation in the intestinal epithelium of the ageing fly is associated with over-proliferation of the intestinal stem cells (ISCs) and improper differentiation of their daughter cells resulting in loss of tissue homeostasis (Biteau et al. 2008, 2010). Therefore, rather than aberrant signal transduction, ageing might simply result from the converging effects of multiple different developmental run-on programs each causing different pathologies in organisms as they get older, the combined result of which is ageing and ultimately death.

Pharmacological Intervention of Ageing by Targeting Cell Signalling Pathways

As described above, genetic studies in laboratory models have now identified pro-longevity cell signalling pathways many of which are conserved in mammals. The observations in mammalian models that similar genetic interventions can not only increase lifespan but also delay the onset of age-associated diseases raises the intriguing possibility that pharmacological strategies targeting components of the pro-longevity signalling network could be employed as preventative measures for multiple age-related pathologies.

Several small molecule inhibitors of the signalling pathways we have described have now been used in ageing studies to demonstrate their efficacy in delaying age-related mortality, many of which are drugs already approved for human use. Perhaps the most successful in terms of its translation to human clinical trials is the mTOR-specific inhibitor, rapamycin. Pharmacological inhibition of mTOR extends lifespan in organisms as distantly related as yeast and mice. In addition to increasing their lifespan, rapamycin treatment in mice can also protect against multiple age-related pathologies. For example, rapamycin has been shown to reduce age-related cancer incidence (Anisimov et al. 2011), reverse cardiac decline (Flynn et al. 2013), improve cognitive function (Halloran et al. 2012; Majumder et al. 2012), recover stem cell function (Chen et al. 2009) and augment muscle function (Bitto et al. 2016) in old animals. Furthermore, starting rapamycin treatment in middle aged mice produces comparable extension of lifespan to life-long treatment (Harrison et al. 2009). Low-dose administration of rapamycin in mice generally extends lifespan in the absence of significant deleterious effects although long-term rapamycin treatment has been associated with impaired wound healing, immunosuppression, increased cataract and testicular degeneration (Wilkinson et al. 2012). A recent study in which healthy middle-aged companion dogs were treated with low-dose rapamycin for 10 weeks found no significant clinical side effects but did observe improvements in cardiac function (Urfer et al. 2017). Furthermore, a clinical trial in elderly people found that 6 weeks of treatment with a rapamycin derivative improved their immune response to influenza vaccine with no significant side effects reported (Mannick et al. 2014).

Another compelling candidate for a pharmacological intervention that targets ageing is the anti-diabetic drug, metformin. Metformin can delay ageing and extend lifespan in *C. elegans* as well as several rodent strains including outbred wild-type strains and models of age-related disease. Several benefits to age-related health associated with metformin treatment were also reported within these studies including improved activity and metabolic functions along with better cognitive performance and reduced cancer incidence. At a cellular level, metformin induces changes in signal transduction within the pro-longevity signalling network including decreased insulin levels, impaired IGF-1 signalling, mTOR inhibition and activation of AMPK.

Metformin is the most widely prescribed treatment for type 2 diabetes worldwide. Interestingly, data from several observational studies as well as from randomised clin-

ical trials has suggested that metformin administration is associated with decreased incidence of age-related diseases in those diabetic patients receiving metformin treatment to regulate their blood glucose. Thus, metformin has been associated with improved cardiovascular health, decreased cancer incidence, better cognitive function and perhaps even delayed mortality (reviewed in Barzilai et al. 2016). The mechanism of action through which metformin improves age-related health outcome is yet to be determined and it remains unclear whether metformin exerts its effects on ageing through multiple cellular targets or via a single mechanism.

Conclusions

Genetic analyses of lifespan in laboratory organisms has identified a network of highly connected cell signalling pathways that show evolutionary conserved effects on animal ageing (Table 11.1). Our understanding of the cell signalling network that

Table 11.1 Signalling molecules implicated in the ageing process and their counterparts in different species

	Species			
	Yeast	Worm	Fly	Mammals
Insulin/IGF-1-like signalling		DAF-2	InR	Insulin receptor
		IST-1	CHICO	Insulin receptor substrates
		AGE-1	Dp110	PI3 kinase
		DAF-18	PTEN	PTEN
		PDK-1	PDK-1	PDK1
		AKT	AKT	AKT
		DAF-16	dFOXO	FOXO
TOR	TOR1/TOR2	CeTOR	dTOR	mTOR
	KOG1	DAF-15	dRAPTOR	Raptor
	LST8	CeLST8	dLST8	mLST8
	Tco89p	–	–	–
	–		–	Deptor
	–		–	PRAS40
	TOR2	CeTOR	dTOR	mTOR
	AVO1	SINH1	dSIN1	mSIN1
	AVO2	–	–	–
	AVO3	RICT1	dRICTOR	Rictor
	LST8	CeLST8	dLST8	LST8
		–	–	Deptor
	Bit61p	–	–	Protor1
	–	–	d4E-BP	4E-BP
	Sch9	RSKS-1	S6 Kinase	S6 kinase

(continued)

Table 11.1 (continued)

	Species			
	Yeast	Worm	Fly	Mammals
RAS	RAS1 and RAS2	LET-60	RAS	RAS
		MPK-1	ERK	ERK
		LIN-1	–	ETS transcription factor
		ETS-4	–	ETS transcription factor
		–	Pointed	ETS transcription factor
		–	Anterior Open	ETS transcription factor
Nrf2	YAP1	SKN-1	Cap'n'collar	Nrf2
		–	Keap1	Keap1
AMPK (α subunit)	SNF1p	AAK-1/2	SNF1A	AMPK α 1 and 2
AMPK (β subunit)	Sip1p	AAKB-1/2	dmAMPK β	AMPK β 1 and 2
AMPK (γ subunit)	SNF4p	AAKG-1-5	SNF4Aγ	AMPK γ 1–3

determines lifespan and associated age-related health is still in its infancy but has nonetheless already led to the identification of cellular targets for pharmacological interventions that stimulate pro-longevity pathways or diminish pro-ageing pathways. By aiming future therapeutic strategies at the process of ageing itself, broad spectrum treatments for the diseases associated with ageing could revolutionise geriatric medicine by combating a wide-range of co-morbities associated with advanced age thereby improving lifelong health on a global scale.

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Chapter 12
The Gut Microbiota and Ageing

Claire Maynard and David Weinkove

Abstract Understanding how the human gut microbiota might influence ageing is challenging. The gut microbiota is a hugely complex ecology of organisms that varies greatly with individuals and time, making age-related changes difficult to measure. However, elderly and younger populations do show differences in gut microbe composition. The key question is whether these differences only reflect age-related changes in host physiology and diet, or if microbes can drive host ageing? Model organisms allow this question to be addressed. Longitudinal analyses in the fruit fly *Drosophila melanogaster* show that changes in microbial composition precedes intestinal and host ageing, and antibiotic treatment increases lifespan, implicating microbes in accelerating ageing. Antibiotics also extend the lifespan of middle-aged killifish but additional transplantation of gut microbes from young killifish extends lifespan further, suggesting a positive effect of microbes associated with young animals. Microbes from old, but not young, mice induce inflammation when added to germ-free mice suggesting that microbes become more harmful to the host with age. These studies implicate broad classes of bacteria, particularly members of the phylum Proteobacteria, as drivers of ageing in a feed-forward loop with intestinal degradation and inflammation. The nematode *Caenorhabditis elegans* can be associated with single strains of genetically-tractable bacteria, and this simplified system has revealed specific interventions in bacterial metabolism, such as inhibition of bacterial folate synthesis, that extend animal lifespan. Transferring this understanding to the human microbiota is challenging but promises to reveal how manipulation of the gut microbiota might be a route to maintain health in old age.

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Structure and Function of the Human Gut Microbiota

Large collaborative studies, such as the Human Microbiome Project (Turnbaugh et al. 2007) and the Metagenomics of the Human Intestinal Tract (MetaHIT) (Qin et al. 2010) have characterized the human gut microbiota. Although the gut microbiota also contains archaea, viruses, fungi and other eukaryotes, it is dominated by obligate anaerobic bacteria of the phyla Firmicutes and Bacteroidetes, in addition to species belonging to the Proteobacteria and Actinobacteria phyla at much lower abundances. Species-level composition in adulthood is highly individually specific, probably due to differences in diet, geography and host genetics, making the 'healthy' adult gut microbiota difficult to define (Backhed et al. 2012). It has been proposed, that despite species-level differences, the human gut microbiota is conserved in function along the gastrointestinal tract by proximal- distal gradients of pH, oxygen, nutrient availability and the host secretion of antimicrobial peptides and bile acids that drive the colonization of microbes with specific functions into distinct spatial niches (Tropini et al. 2017).

The small intestine is inhabited mainly by fast-growing facultative anaerobes that compete with host epithelial transporters for dietary nutrients (Donaldson et al. 2016). The large intestine has a higher species diversity, is more densely populated than the small intestine (Booijink et al. 2010), and is inhabited mainly by obligate anaerobic species that metabolize the indigestible components of our diet and generate easily absorbable, high energy short-chain fatty acids (Macfarlane and Macfarlane 2003). Inhabitants of the colon synthesize essential amino acids and vitamins, including B12 and folate (Flint 2012; Magnusdottir et al. 2015). Bacteria which colonize the mucal layers of the gut lumen, created by the secretion of mucin by the host epithelia, are perhaps the most intimately associated with the host, and are thought to modulate the host immune system by exterior signaling structures and the secretion of specific molecules and peptides (Pereira and Berry 2017). Such bacterial species also moderate the expansion of opportunistic pro-inflammatory bacteria, termed 'pathobionts' (Rampelli et al. 2013). Pathobionts are primarily of the Proteobacteria phylum and reside in the healthy adult gut microbiota, kept in check in low abundances by microbe-microbe and host-microbe signaling.

The Human Gut Microbiota in Disease

Perturbations to the composition of the gut microbiota have been associated with several inflammatory and metabolic diseases, including inflammatory bowel disease (IBD) (Chassaing and Darfeuille-Michaud 2011; Marteau and Chaput 2011; Matsuoka and Kanai 2015), obesity (Ley et al. 2006), insulin resistance and type 2

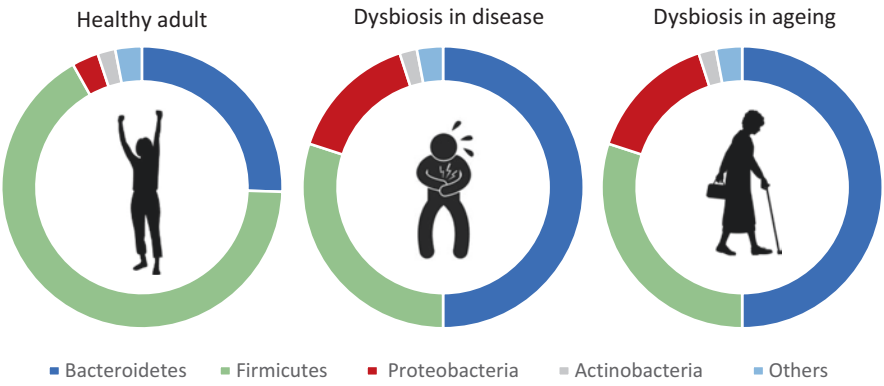

Fig. 12.1 Phylum-level composition of the human gut microbiota in health, disease and ageing. The gut microbiota of a healthy adult is conserved at the phylum level, with Bacteroidetes and Firmicutes predominating and Proteobacteria and Actinobacteria in much lower abundances. In association with certain diseases and in old age, the relative abundances of these four main phyla changes; a shift in the ratio of Bacteroidetes (increase) and Firmicutes (decrease) is observed and Proteobacteria increase. These changes are termed dysbiosis

diabetes (Cani et al. 2007b, 2012) and colorectal cancer (O'Keefe et al. 2009). These changes in composition are commonly viewed as an imbalance between microbes of different functional groups and has been termed 'dysbiosis' (Peterson et al. 2015). Due to a large degree of variability in gut microbe composition associated with the same diseases between individuals, dysbiosis is difficult to define. Its use as a descriptive term for the state of the microbiota is therefore somewhat limited to meaning a change from the 'normal' state that is associated with disease. Nevertheless, studies agree that on the phylum level, dysbiosis is generally characterized by an overall decrease in bacterial diversity, an increase in Bacteroidetes and decrease in Firmicutes, and an increase in the abundance of pro-inflammatory pathobionts (Fig. 12.1) (Shin et al. 2015; Mukhopadhya et al. 2012; Nagao-Kitamoto et al. 2016). Dysbiosis is associated with activation of the immune system and increased inflammation, perhaps due to the dysregulation of homeostatic host-microbe immuno-modulation. Despite efforts to understand the molecular mechanisms underpinning dysbiosis, it is not clear whether dysbiosis is a cause or consequence of inflammation and human disease.

Compositional Changes in the Elderly Human Gut Microbiota

Compositional differences in specific bacterial species have been identified in the elderly human gut microbiome compared to the young adult microbiome (Claesson and O'Toole 2010). These early studies used standard microbiological and molecular techniques and were therefore limited to the detection of culturable bacterial species. Advances in sequencing and metagenomic technologies has enabled

studies using culture-independent techniques, however, these techniques rely on fecal samples and therefore results are skewed towards changes in the human colon and do not discriminate between luminal and mucosal bacteria. The over-riding theme emerging from these studies, however, is an overall loss of diversity and stability in the elderly gut microbiota, combined with a large degree of inter-individual species-level variability. Despite this variability, similarities in studies include a shift in the proportion of Bacteriodetes (increase) and Firmicutes (decrease) with age, primarily due to changes in *Clostridium* groups, a decreased abundance of 'health-associated' bacteria, such as Bifidobacteria, and an increased abundance of Proteobacteria, particularly Gammaproteobacteria, compared to younger adults (Biagi et al. 2010; Claesson et al. 2011). Microbial composition in old age was found to correlate to measures of ill-health and frailty, (Claesson et al. 2012; Jeffery et al. 2016), inflammation (Biagi et al. 2010) and co-morbidity (Claesson et al. 2012). In contrast, a study examining the gut microbiota of extremely long-lived people reported that the guts of supercentenarians were colonized by a high abundance of 'health-associated' taxa (Biagi et al. 2016; Biagi et al. 2017). For a recent review, see (O'Toole and Jeffery 2018).

Age-associated changes in the gut microbiota resemble those associated with dysbiosis (Fig. 12.1). Several extrinsic factors correlate with these age-associated changes, which have already been implicated as factors that drive dysbiosis and disease: dietary changes, such as the decreased consumption of fibrous foods (Donini et al. 2009), increased antibiotic administration, and changes in living conditions (co-habitation in nursing homes) (Claesson et al. 2012). Furthermore, both ageing and dysbiosis share inflammation as a common hallmark and inflammation is a known risk factor for the progression of several age-related diseases (Franceschi 2007). Together, this has led to the speculation that age-associated dysbiosis may be a causative factor of ageing, however, it is not possible to assign causality based on these studies.

Using Model Organisms to Understand Age-Associated Changes in Microbiota

In order to delineate the order of events, longitudinal studies of the microbiota with age are required. Model organisms with short lifespans, less complex microbiotas and established biomarkers of ageing make this easier to study. For example, the integrity of the intestinal epithelium/barrier has been shown to decline in function with age in *C. elegans* (McGee et al. 2011), *Drosophila* (Rera et al. 2012) and mice (Thevaranjan et al. 2017), and has also been associated with human ageing (Man et al. 2015). The intestinal epithelium provides a physical barrier against the invasion of pathogens, whilst also allowing the selective absorption of dietary components and bacterial metabolites. It also plays a key role in the innate and adaptive host immune response (Macpherson and Uhr 2004). The 'leaky gut' hypothesis proposes that as the intestinal barrier breaks down, the epithelium becomes

permeable and allows the unregulated leakage of bacterial cells, their secreted pro-inflammatory toxins, peptides and metabolites, as well as undigested food particles, into the blood stream, thus activating the immune system and causing inflammation (Ahmad et al. 2017). The question is whether age-associated changes in the microbiota occur before or after barrier breakdown.

Microbiota Changes Precede Age-Associated Intestinal Barrier Permeability in Drosophila melanogaster

The fruit fly, *Drosophila melanogaster,* hosts up to approximately 30 bacterial taxa (Broderick and Lemaitre 2012) and has a short lifespan and cheap husbandry costs compared to mice. Age-associated intestinal epithelial disruption is associated with an increased load of intestinal microbes (Broderick et al. 2014; Buchon et al. 2009; Rera et al. 2011). Age-related changes in the composition of the *Drosophila* gut microbiota, namely an increase in the abundance of Proteobacteria, mostly *Gammaproteobacteria,* and a decrease in the proportion of Firmicutes, were found to precede cellular changes which compromised intestinal barrier integrity and levels of pro-inflammatory markers (Clark et al. 2015). The subsequent expansion of *Alphaproteobacteria* was thought to cause further host immune activation and shorten lifespan. Moreover, flies raised in the absence of microbes showed delayed intestinal barrier dysfunction (Clark et al. 2015). This study provided the first evidence that age-related changes in the gut microbiota, which resemble dysbiosis, precede changes in intestinal dysfunction and drive inflammation and mortality (Clark et al. 2015).

Age-Associated Dysbiosis and Inflammation in Mice

The ageing mouse gut microbiome has been shown to undergo distinct compositional changes, similar to those observed in humans, which correlate with age and frailty (Langille et al. 2014). Age-associated intestinal dysfunction and inflammation is reduced in germ-free mice compared to those with a gut microbiota (Thevaranjan et al. 2017). Further, co-housing young germ-free mice with old wild-type mice resulted in colonization of the young mice with an ‘old-like’ microbiota and significantly increased intestinal permeability and markers of inflammation. This suggests the microbiota can ‘age’ or it changes with host age, and that chronic age-associated inflammation might depend on the microbiota. However, when old germ-free mice were used as recipients, the age of the donor significantly influenced intestinal permeability, suggesting that host tissue ageing is also an important factor (Thevaranjan et al. 2017). These intriguing experiments need to be repeated with larger sample sizes, but similar to what had been found in fruit flies (Clark et al.

2015) they suggest that age-associated changes in the mouse microbiota may be a hallmark of ageing, a potential cause of age-related inflammation and therefore a risk factor for several age-related diseases.

Microbiota Transplant and Ageing in African Turquoise Killifish

The naturally short-lived African turquoise killifish is a useful model vertebrate for studying host-microbe interactions. Killifish ageing is associated with a decrease in gut bacterial diversity, an increase in the abundance of Proteobacteria and an increase in systemic inflammation (Smith et al. 2017). By recolonizing middle-age fish (after antibiotic treatment) with the gut microbiota from young fish, Smith and colleagues found that microbiota composition resembled a diverse, young-like composition which persisted into old age. This change was associated with a significant increase in lifespan and a delay in age-dependent activity decline. However, markers of inflammation were not measured. Microbiota transfer was found to extend lifespan more than antibiotic treatment in middle-age, suggesting that young-associated bacteria have an active beneficial impact on host health. Recolonizing young fish with the microbiota from middle-aged fish did not shorten lifespan (Smith et al. 2017), suggesting an 'older' microbiota does not accelerate ageing, though this experiment used a small sample size, potentially hiding an effect.

Transcriptomic analysis revealed significant up-regulation of genes involved in responding to pathogenic bacteria in middle-age killifish colonized with a young microbiota, compared to middle-age fish with a middle-age microbiota (Smith et al. 2017). This result may be due to the higher abundance and diversity of bacteria in the young microbiota. Interestingly, the metabolism of hyaluronic acid, a key extracellular matrix component associated with inflammation (Cho et al. 2017), cancer (Tian et al. 2013) and skin ageing (Papakonstantinou et al. 2012), was increased in fish transplanted with the middle-age microbiota (Smith et al. 2017). Whilst only correlative, this transcriptomic data provides candidates by which microbes might influence host ageing.

Potential Mechanisms: A Feed-Forward Cycle of Bacteria Induced Inflammation

These studies generally support a model whereby age-associated dysbiosis increases intestinal permeability, which in turn activates a host inflammatory immune response. Inflammation impacts the composition of the microbiota, further promoting dysbiosis, leading to a feed-forward loop which maintains the inflammatory response (Clark and Walker 2018) (Fig. 12.2). It has been shown that changes in gut

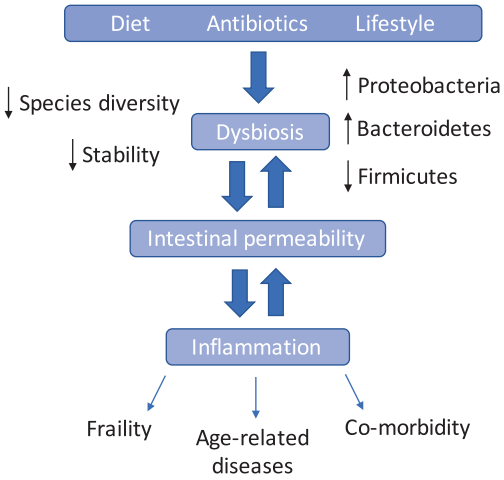

Fig. 12.2 A feed-forward cycle of age-associated dysbiosis. Changes in diet, increased antibiotic usage and changes in living conditions with age correlate with changes in the composition of the gut microbiota. This change in state is described as dysbiosis and, whilst there is a high degree of inter-individual variability, it is characterized by decreased species diversity and stability, and at the phylum level by a decreased abundance of Firmicutes, an increase in Bacteroidetes and a bloom in Proteobacteria. Dysbiosis impairs the integrity of the gut epithelium and increases intestinal permeability. This activates the host immune response which is thought to create a favourable environment for the further expansion of Proteobacteria, and thus a sustained inflammatory response. Chronic inflammation leads to increased frailty, the onset of age-related diseases and co-morbidity

microbiota composition and density can influence systemic inflammation (Belkaid and Naik 2013). Widespread inflammation is a key factor in causing tissue damage and the functional decline associated with ageing (Franceschi 2007). It thereby follows that by severing this feed-forward loop by preventing dysbiosis, we may decrease age-associated inflammation and maintain a healthy microbiota into old age, perhaps delaying age-related diseases.

One potential mechanism for how bacteria cause chronic inflammation in disease conditions is through lipopolysaccharide (LPS), a complex molecule on the outer membrane of gram-negative bacteria (Shin et al. 2015). Most research on LPS is focused on *Enterobacteriaceae*, a family of Proteobacteria that includes known pathogens, and is associated with dysbiosis. LPS is recognized by the host toll-like receptor-4 (TLR-4), and activation of this receptor results in increased expression of inflammatory cytokines (Park and Lee 2013). LPS in the plasma, known as endotoxemia, has been proposed as a potential cause of chronic inflammation in obesity and insulin resistance (Cani et al. 2007a). Monocolonization of germ-free mice with the LPS-producing *Enterobacter cloacae* strain, B29, isolated from the gut of an obese patient, was able to induce obesity on a high fat diet, thus demonstrating a potential mechanism for a microbe in potentiating chronic disease (Fei and Zhao 2013). Furthermore, Theveranjan and colleagues provided evidence that chronic inflamma-

tion caused by leakage of LPS from the mouse intestine was a leading cause of age-related functional decline (Thevaranjan et al. 2017). Together, this provides a mechanism by which changes in bacterial composition in disease and ageing may actively drive chronic inflammation. See Belkaid and Hand for a thorough review discussing potential mechanisms underpinning the impact of the gut microbiota on systemic inflammation (Belkaid and Hand 2014).

An interesting study has reported that *Escherichia coli* is able to use the nitrate generated from host inflammation as an alternative electron acceptor for anaerobic respiration (Winter et al. 2013). In a mouse model, the use of host-derived nitrate was found to confer a growth advantage to *E. coli* and allowed it to outcompete the obligate anaerobic Firmicutes and Bacteroidetes (Winter et al. 2013). A subsequent metagenomics study by the same group has further corroborated the role of nitrogen dehydrogenases in the successful colonization of *Enterobacteriaceae* in the inflamed gut (Hughes et al. 2017). Thus, there are potential molecular mechanisms underlying the blooms of Proteobacteria associated with chronic inflammation and ageing. Indeed, Proteobacteria have been described as the 'microbial signature of dysbiosis' (Shin et al. 2015).

C. elegans- E. coli as a Model to Understand How Bacteria Influence Ageing

Research using the short-lived nematode worm, *Caenorhabditis elegans,* has approached the question of how bacteria might influence ageing from an entirely different perspective. Though it is exposed to, and associated with, a myriad of microbes in the wild, *C. elegans* is maintained in the lab on a bacterial monoculture that provides nutrition to the animal (Brenner 1974). Lab strains of *E. coli*, particularly the B strain OP50, are most commonly used, but *C. elegans* can grow on a wide range of bacteria which differentially impact *C. elegans* physiology and life traits (Brooks et al. 2009; MacNeil et al. 2013; Reinke et al. 2010). Bacterial growth is supported by a growth media which can be chemically defined and controlled and supplemented with exogenous compounds. Genetic manipulation of both *C. elegans* and *E. coli* combined with pharmacological intervention allows investigation into the processes underpinning bacterial impact on ageing in this host-microbe model (Fig. 12.3) (Yilmaz and Walhout 2014; Cabreiro and Gems 2013).

Treating *E. coli* with UV, heat or antibiotics extends *C. elegans* lifespan (Garigan et al. 2002; Gems and Riddle 2000), demonstrating that *E. coli* accelerates ageing. These findings, together with the observation that old worms often have large accumulations of *E. coli* in their intestines (Portal-Celhay et al. 2012; Garigan et al. 2002), led to the hypothesis that the treatments increased lifespan by preventing bacterial proliferation in the intestine. More recent work has challenged this view. Inhibition of *E. coli* folate synthesis with the sulfonamide antibiotic, sulfamethoxazole (SMX), extends *C. elegans* lifespan without influencing bacterial growth and with

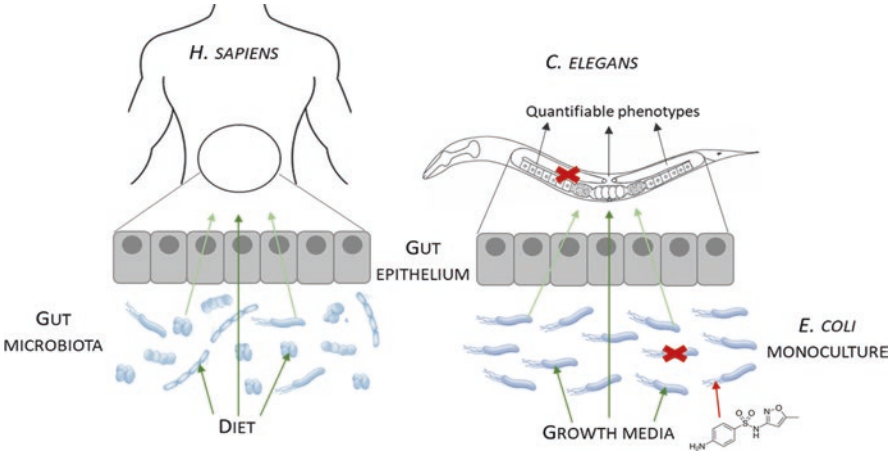

Fig. 12.3 Model of *C. elegans- E. coli* host-microbe-diet interaction. *C. elegans* feeds on a monoculture of *E. coli* which provides it with essential macro- and micronutrients. *E. coli* growth and proliferation is supported by a growth medium, which can be chemically defined and altered with the addition of certain dietary components. *C. elegans* may also uptake some components directly from the growth medium. Drugs may target either *E. coli* or *C. elegans,* or both. Either organism may be targeted with genetic techniques (red crosses in diagram) allowing reverse genetic screens for genes involved in ageing

identical effects on survival as found with kanamycin treatment (Virk et al. 2012). So how do *E. coli* influence *C. elegans* ageing? First it is important to understand the structure and function of the *C. elegans* intestine.

The *C. elegans* Gastrointestinal Tract: How It Ages and Its Interaction with Bacteria

The *C. elegans* GI tract consists of a mouth opening that feeds into the pharynx, a tube-like muscular structure that ends in the grinder, where bacteria are broken up by fast regular movements at around 200 times per minute (pharyngeal pumping). From the pharynx, the ingested material passes into the intestinal lumen, which is formed from 16 pairs of cells with an apical surface with microvilli extending into the lumen (Fig. 12.4). Contractions of surrounding muscles occur every 50 s as part of the defecation cycle, passing the intestinal contents out through the anus. The GI tract shows rapid decline with age, both functionally, with slowed pharyngeal pumping and defecation, and morphologically, with intestinal cells losing cell volume and structure, and damage to the pharynx (Herndon et al. 2002; McGee et al. 2011; Zhao et al. 2017). With this decline in function, it is probably no surprise that bacteria can accumulate in the intestine and in the pharynx of old worms. The question is whether these bacteria accelerate ageing and if they do, is accumulation how they

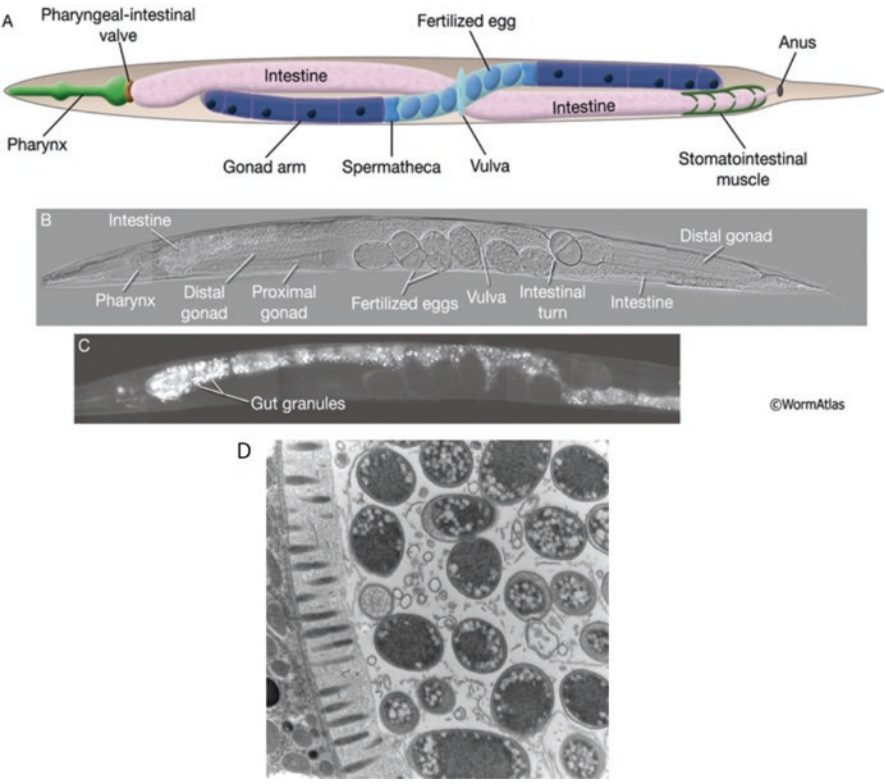

Fig. 12.4 The anatomy of the *C. elegans* gastrointestinal tract shown in (**a**) Diagrammatic form (**b**) DIC light microscopy (**c**) autofluorescence of gut granules within intestine cells (from Worm Atlas) (**d**) transmission electron microscopy of the worm lumen showing microvilli and partially-digested *E. coli* (Photo Christine Richardson, Durham University)

exert their effect on lifespan? An alternative explanation is that bacteria influence lifespan through other means, and bacterial accumulation is a consequence rather than a cause of ageing.

We have found that SMX reduced bacterial accumulation but that many worms on the untreated control died without visible accumulation and that accumulation was delayed in a similar time frame to the delay in the decline in motility, another indicator of health (Virk et al. 2016). Thus, this data is consistent with bacterial accumulation being a symptom of ageing, but it is also very possible that bacterial accumulation enhances animal ageing further in a feedback loop; for example, accumulation may enhance proximity of bacteria to the intestine. It is clear from both light and electron microscopy studies that it is very rare for *E. coli* to invade *C. elegans* tissues. A good explanation for this finding is the lack of the surface O-antigen in *E. coli* strains used for *C. elegans* experiments, where the restoration of O-antigen to these strains results in invasion of intestinal cells (Browning et al. 2013).

Genetics of *E. coli* Help Understand Mechanisms

A major advantage of the *C. elegans: E. coli* system is that *E. coli* can be easily genetically manipulated and compounds can be added to the system by controlling the composition of the growth media. The first two *E. coli* mutants found to increase *C. elegans* lifespan were found serendipitously. A mutant in *ubiG*, required to make coenzyme Q was used to understand how the biology of the *C. elegans clk-1* mutant, which disrupted *C. elegans* coenzyme Q production, extends lifespan (Larsen and Clarke 2002). Using a unique method to supplement *C. elegans* directly with Q, the *E. coli ubiG* mutant was found to extend *C. elegans* lifespan not by restricting dietary Q, but by interfering with bacterial respiration (Saiki et al. 2008). The second mutant to be found was *aroD*, which disrupts synthesis of aromatic compounds in *E. coli*. Addition of only one of these compounds, para-amino-benzoic acid (PABA), a precursor of folate, brings *C. elegans* lifespan back to normal (Virk et al. 2012). *C. elegans* depends on *E. coli* for folate, so a decrease in animal folate was a possible mechanism for lifespan extension (Virk et al. 2012). However, folate supplementation of the worm did not prevent lifespan extension, and together with other experiments, it was shown that inhibiting bacterial folate synthesis prevents an unknown bacterial activity that accelerates ageing (Virk et al. 2016).

Screens Demonstrate That Only Specific Bacterial Mutants Extend *C. elegans* Lifespan

We screened 1000 single-gene deletion mutants from the Keio *E. coli* mutant collection for effects on *C. elegans* lifespan (Virk et al. 2016). Only 9 mutants were found that robustly extended lifespan and across the dataset, no correlation was found between bacterial growth and lifespan. Two of these, deletions of *pabA* and *pabB*, slowed bacterial folate synthesis (Virk et al. 2016). A subsequent screen of all 3983 deletions of non-essential genes in the Keio collection found similar results (Han et al. 2017). Using a slightly different methodology, this study identified 27 mutants that extended *C. elegans* lifespan, including *pabB* and *aroD* but not *pabA*, suggesting that other mutants were missed. Mutants were classified into several groups by whether they could extend a panel of *C. elegans* mutants involved in known longevity pathways. This analysis suggested that there are at least 5 ways that *E. coli* can extend *C. elegans* lifespan; for example, the *pabB* and *aroD* mutants could not extend a *rict-1* mutant, suggesting an interaction with the *C. elegans* TOR pathway. The majority of mutants that extended lifespan had no influence on bacterial survival or accumulation in *C. elegans*, consistent with the idea that bacterial accumulation is not the major mechanism by which *E. coli* influence *C. elegans* ageing (Han et al. 2017).

One interesting group of mutants was *lon* and *hns*. Both resulted in overproduction of colanic acid. Addition of colanic acid was found to increase lifespan,

whilst moderately increasing bacterial growth, but with no effect on bacterial accumulation in the intestine or pathogenicity (Han et al. 2017). Experiments with both *E. coli* and *C. elegans* mutants were consistent with the induction of the mitochondrial unfolded protein response, a known mechanism to increase lifespan (Durieux et al. 2011). Colanic acid is produced by bacteria under environmental stress (Chen et al. 2004). It was hypothesized that colanic acid production may act as a signal from the microbiota under conditions of nutrient deprivation, allowing the host to generate a suitable physiological response (Han et al. 2017). Unlike inhibition of bacterial folate synthesis that prevents a pro-ageing activity, these bacterial mutants seem to enhance a bacterial activity that slows ageing.

Potential Applications of *C. elegans* Research to the Human Microbiota

Research in *C. elegans* has shown that microbes might not just influence ageing by protecting or damaging the intestine, but might influence systemic ageing. Further work will reveal more about the genetics of these interactions. Most research in *C. elegans* has been undertaken with single lab strains of *E. coli,* enabling a genetically amenable system. In the wild, where *C. elegans* lives in rotting fruit and other vegetation, it associates with a broad spectrum of bacterial strains (Berg et al. 2016; Dirksen et al. 2016; Samuel et al. 2016). When cultured with single strains, the lifespan of *C. elegans* varies between strains. For example, lifespan is shorter on the wild strain *Comamonas* DA1877 than on *E. coli* OP50, but interestingly *C. elegans* develop faster (MacNeil et al. 2013). Lifespan on the gram-positive *Bacillus subtilis* is longer than on OP50 (Garsin et al. 2003). This result was originally used to argue that OP50 had a lifespan-shortening property but the genetic screens discussed above suggest that each bacterial strain could have many positive and negative influences on ageing. There are some attempts to recreate a natural 'microbiota' for *C. elegans* in the lab, though in the wild, this might change rapidly. Interestingly, experiments have shown that a 'benign' strain of bacteria can evolve to protect *C. elegans* from a more pathogenic strain, providing a simplified model for the microbiota (King et al. 2016).

C. elegans, Bacteria and Drugs That Slow Ageing

Orally-administered pharmaceuticals have the capability of interacting with gut microbiota, and *C. elegans* has been shown to be a powerful system to assess the bacterial genetics of drug interactions. The drug metformin, used to treat diabetes is thought to slow ageing in humans (Bannister et al. 2014) and there is a current

human trial to test this hypothesis. Metformin causes a strain-specific increase in *C. elegans* lifespan, most likely mediated through changes in bacterial metabolism (Cabreiro et al. 2013). Two screens have identified bacterial genes involved in metabolism and activity of metformin-related drugs (García-González et al. 2017; Scott et al. 2017). We have recently found that the synthetic supplement called "folic acid", an oxidized folate not in nature, can only be taken up by *C. elegans* via a bacterial pathway. *E. coli* cannot take up folates but can take up a breakdown product that is found in folic acid supplements. This uptake can increase *E. coli* folate synthesis and decrease *C. elegans* lifespan (Maynard et al. 2018).

Inhibition of Bacterial Folate Synthesis as a Potential Therapy

It remains to be determined whether inhibiting bacterial folate synthesis is a conserved mechanism to extend longevity; further studies in model organisms with more complex microbiotas are required in order to address this question. Intriguingly, an early study reported the use of the sulfonamide, sulfadiazine, as an effective means to extend lifespan in rodents. Although this study did not regard the impact of sulfadiazine on the microbiota, the addition of the bacterial folate precursor, PABA, was found to reverse lifespan on sulfadiazine, implicating bacterial folate synthesis as the mediator of longevity (Hackmann 1958). A more recent metagenomics study comparing the functionality of the gut microbiome of babies and adults reported that genes involved in folate synthesis were upregulated in the microbiomes of babies compared to adults, whereas genes involved in folate salvage were upregulated in the adult gut microbiome (Yatsunenko et al. 2012). Thus, in healthy ageing, folate synthesis in the gut may be controlled, however, there may be certain cases where this balance is altered.

Indeed, we have seen that ageing is associated with a bloom of Proteobacteria, 71% of which (gut-associated species only) are able to synthesize folate *de novo* (Magnusdottir et al. 2015). It is therefore not unlikely that ageing is associated with an increase in folate levels in the gut. This may cause increased folate-dependent toxicity that may damage host tissues, cause inflammation, and drive ageing. In support of this hypothesis is the association of small intestinal bacterial overgrowth with both inflammation and increased serum folate levels (Camilo et al. 1996; Dukowicz et al. 2007). Furthermore, the sulfonamide drug, sulfasalazine, which targets bacterial folate synthesis, is administered to patients with Crohn's disease and ulcerative colitis to alleviate inflammation (Peppercorn 1990). This is interesting considering the association of these disorders with dysbiosis and an increased abundance of Proteobacteria (Matsuoka and Kanai 2015; Shin et al. 2015). Together, this points to bacterial folate as a potential driver of ageing, however, further mechanistic work in model organisms and the analysis of folate levels in the elderly human gut in relation to measures of health are required.

Conclusions and Future Perspectives

The advent of metagenomic sequencing technologies has allowed us to gain a much clearer understanding about our microbial counterpart in health, disease and ageing. It has become apparent that the human gut microbiota undergoes distinct changes in old age, resembling a composition associated with inflammatory and metabolic diseases. Studies in model organisms have demonstrated that these changes are indeed a causative factor of ageing, as they precede the breakdown of the intestinal barrier that then allows the leakage of bacterium and bacterial-derived endotoxins and metabolites which triggers an inflammatory response. Several studies have indicated that this response has a propagating impact on the composition of the microbiota, allowing further expansion of pro-inflammatory bacteria, namely Proteobacteria, and increased inflammation.

However, as the studies which have demonstrated causative roles of the microbiota on ageing have been conducted in model organisms, it remains unclear whether dysbiosis has the same handle over human ageing. Indeed, the 'leaky gut' hypothesis remains controversial in humans (Valentini et al. 2014), but a study by Man and colleagues reported that elderly adults showed increased levels of intestinal epithelial cytokines and increased intestinal permeability compared to younger adults (Man et al. 2015). Nevertheless, this body of work provides promising evidence for the potential of the gut microbiota to be targeted in order to improve health in old age and delay the onset of age-related disease. The data discussed in this review suggests that there are three main avenues by which this may be achieved: (1) Reversing or preventing age-associated dysbiosis so as to reduce/prevent intestinal permeability and associated inflammation; (2) Inhibiting the generation of harmful and/or toxic metabolites and; (3) Promoting the production of beneficial bacterial components.

In relation to the first method, studies in model organisms have indicated that the transfer of a young-like gut microbiota into an older host has the potential to alleviate symptoms of inflammation and prolong lifespan (Smith et al. 2017). Indeed, fecal transplantation is proving successful in the clearance of *Clostridium difficile* infections associated with ageing, which is also concomitant with the re-establishment of a young-like microbiota, showing an overall increase in diversity, a decrease in Proteobacteria and increase in Firmicutes and Bacteroidetes (Lagier and Raoult 2016). Furthermore, investigation into the mechanisms governing the expansion of Proteobacteria following chronic inflammation are providing molecular targets which may enable prevention of the reciprocal feed-back cycle that propagates dysbiosis in ageing (Winter et al. 2013; Hughes et al. 2017). A significant challenge now facing scientists is the design of genetically engineered microbes targeting specific enzymes, which can proliferate within and colonize the human intestine as a means to prevent or specifically inhibit processes responsible for the propagation of dysbiosis (Ni et al. 2017).

The use of *C. elegans- E. coli* as a host-microbe model system has enabled high-throughput investigation into the impact of bacterial genes and metabolites on *C.*

elegans longevity. These studies have provided us with several novel mechanisms by which the microbiota may be impacting human ageing, via the production of both beneficial and detrimental metabolites. The engineering of microbes to overexpress genes involved in, for example, colanic acid production, or the use of synthetic analogs as dietary supplements has the potential to improve health and longevity. Moreover, the inhibition of bacterial folate synthesis in the gut is also a promising target to maintain a healthy gut microbiota and promote healthy ageing (Virk et al. 2012; Virk et al. 2016). However, further studies are required in order to test whether these molecular mechanisms uphold in model organisms with more complex microbiotas, where microbe-microbe and host-microbe interactions may perhaps temper the mechanism of action of the specific metabolite.

Perhaps more simply, age-associated dysbiosis may also be prevented in the first instance by making modifications to our diet. Diet is widely appreciated as one of the biggest determinants of microbiota composition and a driver of dysbiosis (Pallister and Spector 2016). The high-fat and meat-dominated diet of Western cultures has been correlated with dysbiosis and disease, with studies in model organisms indicating causative mechanisms (Backhed et al. 2007; Cani et al. 2007a; Cani et al. 2008). In contrast, the Mediterranean diet has been shown to promote the persistence of a healthy gut microbiota and promote health (De Filippis et al. 2016). Increased consumption of dietary fiber has been heralded as a key dietary factor which can promote healthy ageing by ensuring the continued colonization of commensal bacteria which ferment fiber to generate SCFAs in the elderly gut microbiota (Keenan 2015). Together, this holds promise that the diet may be a viable method to manipulate our microbiota and promote healthy ageing, however, studies examining the impact of these dietary changes on the microbiota and measures of health are required.

The studies discussed in this review have together uncovered a plethora of potential mechanisms by which the human gut microbiota may impact the trajectory of our health as we enter old age. Although this field is in its infancy, the gut microbiota provides a promising target for novel interventions to promote healthy ageing. Owing to advances in genome editing technologies, such as CRISPR/Cas9, these interventions are likely to be based on targeting single bacterial species involved in the propagation of dysbiosis, or the production of toxic metabolites. Advances in this area may enable a reduction in the use of broad-scale antibiotics, which is known to increase with age. Ironically, antibiotic administration is a known causative factor for dysbiosis and is suspected as a cause of age-related *H. pylori* (Pilotto et al. 2004) and *C. difficile* infection (Jump et al. 2014) and age-related inflammatory diseases, such as IBD (Nimmons and Limdi 2016). Therefore, as well as having an obvious beneficial impact on the global antibiotic resistance crisis, the design of specific interventions, or 'alterbiotics', to target the gut microbiota in old age is likely to have a beneficial impact on the prevention of dysbiosis, and therefore the progression of ageing and age-related disease.

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Chapter 13
Nutrition and Ageing

Fiona C. Malcomson and John C. Mathers

Abstract The ageing trajectory is plastic and can be slowed down by lifestyle factors, including good nutrition, adequate physical activity and avoidance of smoking. In humans, plant-based diets such as the Mediterranean dietary pattern are associated with healthier ageing and lower risk of age-related disease, whereas obesity accelerates ageing and increases the likelihood of most common complex diseases including CVD, T2D, dementia, musculoskeletal diseases and several cancers. As yet, there is only weak evidence in humans about the molecular mechanisms through which dietary factors modulate ageing but evidence from cell systems and animal models suggest that it is probable that better dietary choices influence all 9 hallmarks of ageing. It seems likely that better eating patterns retard ageing in at least two ways including (i) by reducing pervasive damaging processes such as inflammation, oxidative stress/redox changes and metabolic stress and (ii) by enhancing cellular capacities for damage management and repair. From a societal perspective, there is an urgent imperative to discover, and to implement, cost-effective lifestyle (especially dietary) interventions which enable each of us to age well, i.e. to remain physically and socially active and independent and to minimise the period towards the end of life when individuals suffer from frailty and multi-morbidity.

Keywords Plant-based diets · Dietary patterns · Mediterranean diet · Dietary energy restriction · Hallmarks of ageing

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Introduction

In this chapter, we use three perspectives to review the evidence that nutrition influences ageing. We begin by considering evidence, largely from observational studies in humans, which links nutrients, foods and eating patterns with ageing and risk of age-related disease. Secondly, using data from intervention studies in humans, we seek for causal evidence that diet modulates the ageing trajectory. This area of research is handicapped by the lack of reliable biomarkers of (healthy) ageing which can be used as surrogate endpoints (Lara et al. 2015) and so most these studies have used age-related disease or mortality as outcomes. Finally, in an attempt to understand the mechanisms by which dietary factors may accelerate or retard the ageing process, we use the "Hallmarks of ageing" (Lopez-Otin et al. 2013) concept to structure consideration of evidence from cell systems, animal models and, more limited, human studies.

Evidence That Nutrition Influences the Ageing Trajectory

Dietary Restriction – Energy Restriction

2017 Marked the centenary of ground-breaking studies by Osborne and colleagues which showed that rats that were nutritionally deprived in early life had increased lifespan (Osborne et al. 1917). These observations were extended by McCay and colleagues who reported that when white (laboratory) rats were subjected to a reduced intake of nutritionally-adequate food, sufficient to lower their growth rates substantially, they lived longer (McCay et al. 1989). This counter-intuitive finding has stimulated multiple studies of dietary restriction (DR). It is now clear that DR increases lifespan in a wide range of species from yeast to mammals and that it lowers the risk of several common age-related diseases including diabetes, cancer, and cardiovascular disease (Fontana et al. 2010). However, DR does not enhance median or maximum lifespan in all rodent strains and, for example, in the ILSXISS strain 114 of mice, DR reduced lifespan (Swindell 2012). This evidence for genotype-environment interactions may have important implications for understanding the mechanisms through which DR can extend lifespan and improve health during ageing (Mitchell et al. 2016) and for the development of personalised, or stratified, interventions.

For both ethical and practical reasons it is unlikely that randomised controlled studies of DR in humans that have lifespan or incidence of age-related disease as primary outcomes will be attempted. However, three independent trials of DR (restricting energy intake whilst ensuring adequate intake of nutrients) have been carried out in rhesus monkeys (*Macaca mulatta*) in the USA. This first of these studies, carried out at the University of Maryland, was not designed primarily to examine effects of DR and allocated few animals to this treatment regime (Bodkin

et al. 2003). The other two studies, conducted at the National Institute on Aging (Mattison et al. 2012) and at the University of Wisconsin Madison (Colman et al. 2009, 2014), were larger and have yielded more robust data (Mattison et al. 2017). Both studies showed that DR appeared to slow the ageing process and to reduce the incidence of age-related disease, but only the University of Wisconsin study detected an increase in lifespan. A recent analysis by both research teams suggests that differences in study design, particularly age at which DR was initiated, genotype (or geographical origin) of the rhesus monkeys and details of the imposed diet, may explain the differences in observed outcomes (Mattison et al. 2017). These authors concluded that the age-related increase in disease vulnerability in primates is malleable and that ageing itself is a potential target for intervention (Mattison et al. 2017). However, a contemporary commentary on outcomes from both rodent and non-human primate studies concluded that "*the belief that the rate of aging is directly proportional to caloric intake (with obvious limits at the higher and lower ends of the spectrum) has now been shown to be incorrect*" (Vaughan et al. 2017). This reinforces the message that details of the design of investigations can have profound effects on subsequent outcomes and that translation of findings from model organisms to humans should proceed with care.

Dietary Restriction – Protein Restriction and Restriction of Specific Amino Acids

Lower dietary energy intake can be achieved by reducing the intake of any macronutrient (fats, carbohydrates, protein and alcohol) than can be metabolised by the cell to generate ATP. This has complicated interpretation of outcomes of DR in model organisms where reduced energy intake was imposed by reducing the intake of the total diet or by restricting intake of one or more specific macronutrient – the latter has consequences for the relative intake of other macronutrients. However, there is growing evidence that restricted intake of protein, or of specific amino acids, *per se* may be beneficial in extending both length of life and health-span (Fontana and Partridge 2015). Using data from 6,381 adults aged ≥50 who participated in NHANES III, a nationally representative, cross-sectional study in the United States of America (USA), Levine and colleagues observed that high protein intake was associated with 5-fold greater risk of death from diabetes during the following 18 years (Levine et al. 2014). For this purpose, high protein intake was assessed as ≥20% of dietary energy from protein (Levine et al. 2014). However, for other mortality outcomes, the effects were age-dependent. In those aged 50–65 who reported high protein intake, overall mortality was increased by 75% and there was a 4-fold increase in cancer mortality. In addition, there was evidence that this adverse effect of high protein intake was associated with protein from animal sources only and that there was no relationship with intake of plant proteins (Levine et al. 2014). These findings are in accord with the observation that plant food-based

diets are associated with better health in later life (Kiefte-de Jong et al. 2014) – see below.

The potential importance of amount and dietary source of proteins on ageing and risk of age-related disease has been confirmed in a study of 29,017 initially healthy, middle-aged women (aged 55 and 69 years at baseline) resident in Iowa, USA who were followed prospectively for 15 years (Kelemen et al. 2005). Kelemen and colleagues found that isoenergetic substitution of vegetable protein for animal protein (whilst keeping constant intakes of carbohydrate, total energy, and potential confounding factors) reduced coronary heart disease mortality (Kelemen et al. 2005). Further, analysis of prospective data from 131,342 US health care professionals showed that higher intake of animal protein was associated with higher cardiovascular mortality whereas higher intake of plant protein was associated with lower all-cause and cardiovascular mortality (Song et al. 2016a). However, it would be naive to assume that animal proteins are necessarily inhibitory to healthy ageing since Song and colleagues observed that these associations were confined to participants with at least one unhealthy lifestyle factor (Song et al. 2016a). The authors speculate that the adverse effects of higher intakes of animal protein may occur only in those with some underlying inflammatory or metabolic disorder (Song et al. 2016a). In addition, not all animal protein sources appear to have similar effects since those participants with unhealthy lifestyles ate more red meat, whereas the healthy-lifestyle group consumed more fish and chicken (Song et al. 2016a).

In contrast with the apparent benefits of lower protein intake in those aged <65 years, the NHANES III-based study showed that high protein intake was associated with reduced cancer and overall mortality among those aged ≥65 years (Levine et al. 2014). This suggests that low protein intakes may be disadvantageous in older people. Data from the Newcastle 85+ Study show that low protein intake (defined as < 0.8 g of protein per adjusted body weight (aBW) per day) is common in the very old – reported by 28% of the cohort of 85 year olds – and risk of low protein intake is higher among those with lower intakes of meat and meat products and higher intakes of cereal and cereal products and of non-alcoholic beverages (Mendonca et al. 2017). Low protein intake among the very old appears to be functionally important and those consuming <1 g protein/kg aBW/d had lower muscle strength and physical performance in late life, especially in older women, independent of major covariates (Granic et al. 2017).

Dietary Restriction – Fasting and Related Strategies

Continuous access to attractive, nutritious food at affordable prices is a relatively recent experience for humankind and for much of evolutionary time humans have had to cope with periods of food shortage. In common with other large mammals, humans have evolved efficient mechanisms for storing excess energy, primarily as fat, and metabolic, endocrine and nervous systems that enable not only survival but also high levels of physical and mental performance when fasted (Mattson et al.

2017). Often for religious reasons, periods of voluntary abstinence from eating and drinking (fasting) have been practiced by many cultures over the millennia (Patterson et al. 2015). At present there is considerable interest in the health effects of intermittent fasting (IF) which can be implemented in several ways including alternate day fasting, time-restricted feeding (*ad libitum* food intake within a specific time window, daily), religious (e.g. Ramadan) fasting (Patterson et al. 2015) and periodic fasting (PF) for ≥3 days every 2 or more weeks (Longo and Mattson 2014). A popular variant is the 5:2 diet which involves fasting, or substantially reduced food intake, for 2 days per week and *ad libitum* intake for the other 5 days (Harvie et al. 2011). Such IF regimes have been successful in supporting weight loss through reduced energy intake (Harvie et al. 2011) and for the management of hypertension and inflammatory diseases (Longo and Mattson 2014). However, more recently, attention has focussed on potential generic, long-term benefits and it has been argued that such fasting may delay aging, and optimize health, because it suppresses inflammation and lowers insulin concentration (Patterson et al. 2015), results in ketogenesis and enhances cellular processes including mitochondrial function, stress resistance, DNA repair and autophagy (Longo and Mattson 2014; Mattson et al. 2017).

Dietary Patterns and Healthy Ageing

Epidemiological studies of populations living longer and healthier lives highlight the likely importance of lifestyle factors including dietary choices (Appel 2008). In general, dietary patterns that are plant food-based and nutritionally adequate are associated with better health in later life (Kiefte-de Jong et al. 2014). Those consuming vegetarian diets tend to be leaner (lower body mass index (BMI; mass (kg)/height (m^2)), have lower prevalence of age-related diseases, including type 2 diabetes (T2D) and hypertension, and experience lower all-cause mortality (Orlich and Fraser 2014; Orlich et al. 2013). In contrast, a recent analysis involving >1 million years of follow-up of United Kingdom (UK) participants in two longitudinal cohorts to age 90 years found no difference in mortality between vegetarians and non-vegetarians (Appleby et al. 2016).

The most extensively studied dietary pattern is the so-called Mediterranean dietary pattern that was "*found in olive-growing areas of the Mediterranean region in the late 1950s and early 1960s, when the consequences of World War 2 were overcome but fast-food culture had not yet reached the area*" (Trichopoulou 2004). Such diets are low in meat and meat products, confectionery and desserts but high in vegetables, fruits, nuts, legumes, fish and olive oil with moderate consumption of alcohol, usually as red wine (Trichopoulou 2004). A meta-analysis of data from prospective cohort studies showed that adherence to a Mediterranean dietary pattern was associated with significantly reduced total mortality, reduced incidence and mortality from cardiovascular diseases (CVD) and cancer (Sofi et al. 2010). Analysis of prospective data from the EPIC-Norfolk study showed that greater adherence to

the Mediterranean diet was associated with lower CVD incidence and mortality (Tong et al. 2016) which illustrates the potential importance of this dietary pattern in reducing risk of age-related disease in a non-Mediterranean setting. In addition, recent evidence shows that adherence to a Mediterranean dietary pattern is associated with lower CVD mortality in a developing country setting (Mohammadifard et al. 2017).

Ageing is accompanied by loss of brain volume (brain atrophy) (Resnick et al. 2003) and increased rates of brain atrophy are associated with greater risk of dementia (Scahill et al. 2002). Quantification of changes in brain volume using magnetic resonance imaging (MRI) over 3 years in approximately 400 older people (73 years at baseline) showed that those with lower adherence to a Mediterranean dietary pattern had significantly faster rates of brain atrophy (Luciano et al. 2017). This finding is in keeping with the observation of better cognitive performance (Petersson and Philippou 2016) and reduced neurodegenerative disease in those with higher adherence to a Mediterranean dietary pattern (Sofi et al. 2010; Anastasiou et al. 2017). The specific components of a Mediterranean diet which enhance cognition during ageing are poorly understood but data from the PREvención con DIeta MEDiterránea (PREDIMED) Study suggest that higher intakes of polyphenol-rich foods are associated with better cognitive function in older people at raised CVD risk (Valls-Pedret et al. 2012).

To a large extent, it appears that other diet-quality indices including the Healthy Eating Index-2010, the alternative Healthy Eating Index 2010, the alternate Mediterranean Diet Score and the Dietary Approaches to Stop Hypertension (DASH) capture similar aspects of dietary patterns, at least in respect of associations with mortality (Harmon et al. 2015). In a US study of White, African American, native Hawaiian, Japanese American and Latino adult participants (n = 215,782) in the Multi-ethnic Cohort, higher adherence to each of these dietary patterns was associated with lower risk of all-cause mortality and mortality from CVD and cancer (Harmon et al. 2015). Each of these dietary patterns emphasises higher intakes of plant foods (including vegetables, fruits, (whole) grains, legumes/pulses and potatoes), higher intakes of fish and restricted consumption of red meat and sugar-rich foods (Kiefte-de Jong et al. 2014).

Obesity, Ageing and Age-Related Diseases

Large-scale, prospective epidemiological studies provide strong evidence of progressive increases in death from age-related disease with higher levels of adiposity measured, usually, as BMI (Berrington de Gonzalez et al. 2010). In an analysis of almost 4 million adult never-smokers without known chronic disease at baseline, all-cause mortality was significantly higher for those who were overweight (25< BMI <30) and obese (BMI>30). The relationships between adiposity and mortality were broadly similar across different geographical regions (Europe, North America, East Asia, and Australia and New Zealand) (Global et al. 2016) suggesting that this

may be a universal human phenomenon. In 2010, 3.4 million deaths (equivalent to 4% of total lives lost) and 4% of disability-adjusted life-years (DALYs) were attributable to overweight and obesity (Lim et al. 2012). Importantly, the adverse effects on ageing and on age-related disease are greater in those with more severe obesity (Keating et al. 2014).

The prevalence of obesity has been increasing globally for 3–4 decades (Ng et al. 2014) and, more than a decade ago, it was suggested that this secular rise in obesity prevalence in the USA will stop the rise in life expectancy which has been observed over the last two centuries (Olshansky et al. 2005). A recent analysis of NHANES data has reported that the rising levels of BMI between 1988 and 2011 has prevented the USA from achieving the improvements in life expectancy that have been enjoyed by international comparators (Preston et al. 2018). The global increase in BMI is apparent at all life-stages, including in childhood (Ng et al. 2014), and the legacy of childhood obesity may be particularly important for ageing because childhood obesity tracks into adulthood – the heavier children are at early ages, the greater the likelihood of their becoming obese adults (Ward et al. 2017). Indeed, recent simulations using nationally representative data for the USA predict that, given the current level of childhood obesity, the majority (57%) of today's children will be obese at age 35 year (Ward et al. 2017). Further, the main behavioural factors (poor diet and lack of physical activity) which result in obesity track from childhood into adulthood (Craigie et al. 2011) and are also significant determinants of less healthy ageing (Kiefte-de Jong et al. 2014).

Among women who were free from major diseases in mid-life (mean age 50 years) and who lived to >70 years, greater weight gain between age 18 years and mid-life was associated with reduced likelihood of having healthy survival after age 70 i.e. no history of 11 major chronic diseases and have no substantial cognitive, physical or mental limitations (Sun et al. 2009). Similarly, weight gain in adulthood, especially when that was associated with great abdominal adiposity, increased the risk of colorectal cancer (another age-related disease) (Keum et al. 2015; Song et al. 2016b).

Interventions in Humans to Enhance Healthy Ageing and Reduce the Risk of Age-Related Disease

Although there is ample evidence from observational epidemiological studies (summarised above) and from studies in animals that links dietary factors with ageing and risk of age-related disease, evidence for causal relationships in humans requires human intervention studies. To date, most studies of this kind have reported outcomes for age-related disease, mortality or disease-related risk factors because of the difficulty of measuring ageing *per se* (Lara et al. 2015).

Healthier Dietary Patterns

The Lyon Diet Heart Study was a landmark randomized secondary prevention clinical trial which demonstrated that a Mediterranean-type diet reduced substantially the rate of recurrence after a first myocardial infarction when compared with a prudent Western-style diet (de Lorgeril et al. 1999). This protective effect was evident after 27 months of follow-up and continued up to at least 46 months (de Lorgeril et al. 1999). Since cardiovascular disease (CVD) is one of the commonest age-related diseases, this was powerful evidence that the Mediterranean dietary pattern may slow the ageing process, at least in those at high CVD risk.

More recently, the PREDIMED Study tested the efficacy of a Mediterranean dietary pattern in a primary prevention trial in Spanish participants who were at raised cardiovascular risk, but with no CVD at enrolment. Participants were randomised to one of three diets: a Mediterranean diet supplemented with extra-virgin olive oil, a Mediterranean diet supplemented with mixed nuts, or a control diet (advice to reduce dietary fat) and the primary outcome was the rate of cardiovascular events (Estruch et al. 2013). The trial was planned to last 6 years but, on the basis of an interim analysis, was stopped early (median follow-up 4.8 years) when there was clear evidence of fewer cardiovascular events in those randomised to either Mediterranean diet (Estruch et al. 2013). The Mediterranean dietary intervention in the PREDIMED Study resulted in multiple improvements in factors related to cardiovascular health including 24-hour ambulatory BP, total cholesterol, and fasting glucose (Domenech et al. 2014) and was associated with increased serum markers of atheroma plaque stability which may explain, at least in part, the observed protection against ischemic heart disease (Casas et al. 2014). The benefits of the dietary intervention were not restricted to the cardiovascular system and further analyses of the PREDIMED Study data showed a beneficial effect of a Mediterranean diet supplemented with extra-virgin olive oil in the primary prevention of breast cancer – the first randomized trial demonstrating an effect of a long-term dietary intervention on this age-related malignancy (Toledo et al. 2015). Following the failure of yet another pharmaceutical agent to improve cognitive function in those with mild to moderate Alzheimer Disease (AD) (Atri et al. 2018), research priorities are shifting to focus on interventions that may delay or prevent development of dementia. In that context, the evidence that randomisation to the Mediterranean diets in the PREDIMED Study improved measures of cognition (Martinez-Lapiscina et al. 2013; Valls-Pedret et al. 2015) is particularly encouraging.

Taken together, these findings from intervention studies confirm the associations between higher adherence to the Mediterranean diet and reduced prevalence of several age-related diseases, as discussed above. In terms of improving public health, it will be important to build on this evidence to develop Mediterranean diet intervention modalities which can be delivered cost-effectively, at scale, and that have demonstrated utility in enhancing healthy ageing, especially in non-Mediterranean countries. Using a participatory co-design approach (O'Brien et al. 2016), we have developed an internet-based intervention platform which facilitates older individuals

to adopt a more Mediterranean-style eating pattern and which showed feasibility and acceptability in a pilot study (Lara et al. 2016).

Complex Interventions – Diet and Physical Activity

Increased insulin resistance occurs commonly during ageing and is associated with both obesity and multiple age-related diseases and conditions (Cleasby et al. 2016). In particular, chronically raised insulin resistance (impaired glucose tolerance or pre-diabetes) is a marker of risk of T2D and individuals with pre-diabetes show accelerated ageing (Szoke et al. 2008). The outcomes from ground-breaking intervention studies which randomised middle-aged participants with pre-diabetes have shown consistently that improved lifestyle (healthier diet and more physical activity) reduces the risk of conversion to diabetes (Tuomilehto et al. 2001; Knowler et al. 2002; Kosaka et al. 2005). Further analysis of pooled data from 3 European diabetes prevention trials, that used a similar lifestyle intervention in comparable participants, showed that the likelihood of avoiding T2D was strongly related to sustained weight loss (Penn et al. 2013).

A recent systematic review and meta-analysis of randomised clinical trials reported that, at the end of the active intervention period (range, 0.5–6.3 years), lifestyle-based intervention studies and drug treatments were equally effective in preventing diabetes (Haw et al. 2017). However, with longer-term follow-up (mean follow-up, 7.2 years; range, 5.7–9.4 years), the lifestyle-based interventions continued to show significantly reduced T2D risk whilst there was no sustained risk reduction with the drug treatments (Haw et al. 2017). This is likely due to sustained improvements in diet, greater physical activity and reduced adiposity, in those randomised to the lifestyle interventions.

In individuals diagnosed with T2D, rapid and sustained weight loss induced by bariatric surgery normalises glycaemia and causes diabetes remission in the large majority of initially obese T2D patients (Sjostrom et al. 2014; Meijer et al. 2011). The likelihood of diabetes remission is greater for those who have been diagnosed for a shorter time and in those who achieve larger weight loss following surgery (Steven et al. 2015). Rapid and extensive weight loss appears to be key to normalisation of glycaemia and diabetes remission. Indeed, dietary energy restriction alone causes normalisation of beta cell function and of hepatic insulin sensitivity in obese T2D patients (Lim et al. 2011; Henry et al. 1985). A recent RCT carried out in overweight and obese T2D patients in primary care in the UK has demonstrated that rapid weight loss followed by weight maintenance resulted in nearly 50% being in remission after 1 year (Lean et al. 2017). Whilst the long-term effect on T2D remission remains to be determined, this study has shown the potential of weight loss in those who are overweight and obese to improve a key metabolic feature of the ageing process, even in those with a T2D diagnosis.

The Finnish Geriatric Improvement Study to Prevent Cognitive Impairment and Disability (FINGER) reported that lifestyle changes may improve or maintain

cognitive functioning in older people (Ngandu et al. 2015). The FINGER Trial recruited 1260 people aged 60–77 years who were at higher CVD risk and who had mean or slightly lower cognition than for age and randomised them (1:1) to a multidomain intervention of diet, exercise, cognitive training and vascular risk monitoring or to a control group (general health advice). After 2 years, those randomised to the active intervention showed significant improvement in a neuropsychological test battery (Ngandu et al. 2015). With such a complex intervention, it is difficult to separate out which components of the intervention had most effect and, indeed, to discover whether the totality of the intervention is essential to maximise efficacy and to address inter-individual differences in intervention needs (Celis-Morales et al. 2015). Further analysis showed that the intervention implemented in the FINGER Trial prevented age-related decline in diet quality (assessed using both food groups and nutrients) (Lehtisalo et al. 2017) and this may have contributed to the improvement in cognition.

Interventions with Specific Foods/Nutrients

In contrast with the generally positive effects on ageing of improved dietary patterns and of weight loss (in those who are overweight or obese), interventions that have tested the effects of single foods or nutrients on ageing-related outcomes have often shown little or no benefit. For example, evidence from observational studies suggests that higher intakes of β-carotene, and other carotenoids, from fruits and vegetables are associated with lower cancer risk (Riboli and Norat 2003) and of lung cancer, specifically (Vieira et al. 2016). However, interventions with supplemental β-carotene in those at higher risk of lung cancer (because of smoking and/or exposure to asbestos) showed no benefit. In an outcome that shocked the research community, supplementation with β-carotene (with or without α-tocopherol) in male smokers in Finland led to higher incidence of lung cancer and higher total mortality (Alpha-Tocopherol 1994). Similarly, in the Beta Carotene and Retinol Efficacy Trial, supplementation with β-carotene and its metabolic derivative vitamin A appeared to increase both incidence and risk of death from lung cancer and from CVD in smokers and workers exposed to asbestos (Omenn et al. 1996). Such findings undermined confidence in the safety of interventions with large doses of single nutrients and contributed to the conclusion that dietary supplements are not recommended for cancer prevention (WCRF/AICR 2007).

Circulating concentrations of homocysteine increase with age (Miller 2003) and raised plasma homocysteine concentration is associated with greater risk of multiple age-related conditions including CVD, dementia and osteoporosis (Kuo et al. 2005). There is convincing evidence that, through its role as a methyl donor, folic acid alone (Homocysteine Lowering Trialists 2005) or in combination with other B vitamins involved in one-Carbon metabolism (Lonn et al. 2006) lowers homocysteine concentration very effectively. However, the benefit of B vitamin supplementation in CVD prevention trials has been controversial with some trials showing little or no

effect. A recent systematic review and meta-analysis of RCTs found that such supplementation had no effect on coronary heart disease risk but lowered risk of stroke by 10% (Li et al. 2016). In addition, a meta-analysis of data for 22,000 participants who participated in homocysteine-lowering trials using supplemental B vitamins showed no effect on individual cognitive domains, global cognitive function or cognitive ageing (Clarke et al. 2014). The conclusion that these findings mean that supplemental B vitamins do not reduce dementia risk was challenged by Garrard and Jacoby (2015) who pointed to methodological weaknesses in Clark's meta-analysis and to the evidence of biological and neuropsychological benefit from B vitamins interventions in those with mild cognitive impairment (MCI) (Garrard and Jacoby 2015; Smith et al. 2010; Douaud et al. 2013; de Jager et al. 2012).

Docosahexaenoic acid (DHA) is a long-chain polyunsaturated fatty acid (PUFA) of the n-3 family and is the most abundant PUFA in brain tissue (Kim 2007). Most pre-formed DHA and eicosapentaenoic acid (EPA; the other major long-chain n-3 PUFA) in the human diet is derived from oily fish and data from cohort studies show that populations with higher intakes of oily fish or fish-derived PUFA have lower risk of MCI and of dementia (Zhang et al. 2016a). A meta-analysis of data from RCTs conducted before December 2014 in older people showed that supplementation with n-3 PUFA slowed the rate of cognitive decline (measured using the Mini-Mental State Examination, MMSE) (Zhang et al. 2016b). This finding remains controversial and more recent data from a large trial in older (70+ years), community-dwelling people showed no effect of supplementation with DHA + EPA for 3 years on cognitive decline (Andrieu et al. 2017). In the double-blind RCT by Quinn and colleagues, supplementation of patients with mild to moderate Alzheimer Disease with DHA alone for 18 months had no significant effect on the primary outcome, cognitive decline, or on the secondary outcome, loss of brain volume (Quinn et al. 2010). However, a pre-specified exploratory analysis indicated that DHA supplementation slowed the rate of cognitive decline in those who were *APOE ε4* negative but had no effect in those who were *APOE ε4* positive (Quinn et al. 2010). These findings suggest that there may be merit in identifying nutrient-gene interactions that could be used to develop personalised, or stratified, intervention (Celis-Morales et al. 2015).

Vitamin C (ascorbate) is found widely in fruits and vegetables and observational studies have suggested that higher intakes of this antioxidant nutrient are associated with lower risk of CVD and of other age-related diseases (Moser and Chun 2016). Higher intakes of vitamin C may enhance healthy ageing and, in particular, ageing of the cardiovascular system though its roles as an antioxidant, by nitric oxide-sparing, by lipid-lowering and by blood pressure-lowering (Ashor et al. 2016). In addition, it has been discovered recently that ascorbate is a co-factor for ten-eleven translocation (TET) dioxygenases that catalyse oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) that leads, ultimately, to the removal of the epigenetic methyl group from 5-mC residues in DNA (Young et al. 2015). As a consequence, vitamin C status may have a wide influence on gene expression and, therefore, on functional dysregulation during ageing. Despite this strong epidemiological and mechanistic rationale, a recent systematic review of intervention studies with supplemental vitamin C (with or without other so-called antioxi-

dant vitamins) concluded that there is no evidence to suggest that vitamin C supplementation is effective in primary prevention of CVD in healthy participants or in those at increased CVD risk (Al-Khudairy et al. 2017).

Dietary (Energy) Restriction

For ethical, logistical and budgetary reasons, it is unlikely that RCTs testing the effects of dietary energy restriction on ageing and on lifespan, of the kind that has been carried out in multiple model organisms and in non-human primates, will be attempted in humans. However, shorter-term studies of dietary energy restriction which assess ageing-related functional and other surrogate end-points may provide important information about the potential safety and efficacy of such interventions. The pioneering Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy (CALERIE) study has attempted to test the hypothesis that dietary energy restriction in healthy people results in adaptive changes analogous to those seen in rodents subjected to similar dietary energy restriction (Rochon et al. 2011). The intervention study was carried out for 2 years with 218 healthy men and women (aged 21–51) who were randomised to achieve 25% dietary energy restriction by dietary and behavioural changes (CR) or to *ad libitum* feeding (AL). In the first 6 months of the intervention, the CR group achieved close to 20% dietary energy restriction. However, the degree of energy restriction reduced substantially over the subsequent 18 months and averaged 11.7% over the whole intervention period (Ravussin et al. 2015). As a consequence, mean body weight fell by 7.1 kg at 6 months and remained approximately 8 kg below baseline for the remainder of the trial (Ravussin et al. 2015). Given that the mean BMI of participants at baseline was 25.1, the mean BMI after the intervention was well within the normal range. The CALERIE Study Group concluded that this degree of dietary energy restriction, in initially non-obese individuals, was safe and well-tolerated (Romashkan et al. 2016) although 8 participants were withdrawn (3 permanently) from the intervention because of safety concerns, principally loss of bone mineral density and treatment-resistant anaemia (Ravussin et al. 2015). Relative to the AL group, 2 years of dietary energy restriction (CR) reduced circulating concentrations of inflammatory markers but had no effect on *in vivo* markers of cell-mediated immunity (Meydani et al. 2016). The CR group self-reported significantly improved mood, reduced tension, and improved general health and sexual drive and relationship (Martin et al. 2016). In addition, the greater the weight loss at 24 months, the greater were the reported improvements in vigour, mood and general health (Martin et al. 2016). It is difficult to generalise the effects of this trial because the participants were highly selected, not least in their willingness to undergo such substantial weight loss under controlled conditions when this was not mandated by their initial BMI or for health reasons.

In an attempt to determine whether the dietary energy restriction implemented in the CALERIE study influenced biological ageing, Belsky and colleagues applied two different algorithms for assessing biological age in cross-sectional studies to data from the intervention study (Belsky et al. 2017). This analysis suggested that the CR group experienced reduced biological ageing and, surprisingly, the authors claimed that this slowing of biological ageing was not accounted for by weight loss during the intervention (Belsky et al. 2017). It is not known whether this diminution of rate of ageing can be maintained in the longer-term but analysis of data from the relatively small proportion (39 of the original 218) of participants in the CALERIE study who continued in a follow up study after the intervention period, showed that about half of the original weight loss was sustained 2 years later, probably as a consequence of enduring effects on behaviours and dietary restraint (Marlatt et al. 2017).

From a public health perspective, there is likely to be limited societal benefit in respect of ageing and age-related disease from heroic efforts to initiate and sustain dietary energy restriction in those who are non-obese. The burden of age-related disease and the adverse effects on lifespan increase with increasing BMI (Keating et al. 2014; Global et al. 2016) so that public health interventions which reduce the development of obesity, especially childhood obesity (Mathers 2015), and that are effective, and cost-effective, in inducing sustained weight loss in those who are obese are likely to yield bigger public health returns on investment (Ahern et al. 2017; Briggs et al. 2017).

Mechanisms Through Which Nutrition Modulates Ageing

The Hallmarks of Ageing

Across multiple species, ageing is characterised by progressive functional decline and in humans is associated with increased risk of common complex diseases including cancer, dementia and CVD. The universality of the cellular and molecular changes associated with ageing was summarised by Lopez-Otin and colleagues in their influential proposal for nine hallmarks of ageing (Lopez-Otin et al. 2013). In this section, we review the evidence on the potential mechanisms through which nutrition may modulate the ageing trajectory with reference to each of these hallmarks. For example, energy and protein restriction may affect several of these hallmarks including nutrient-sensing pathways and oxidative stress (Fig. 13.1).

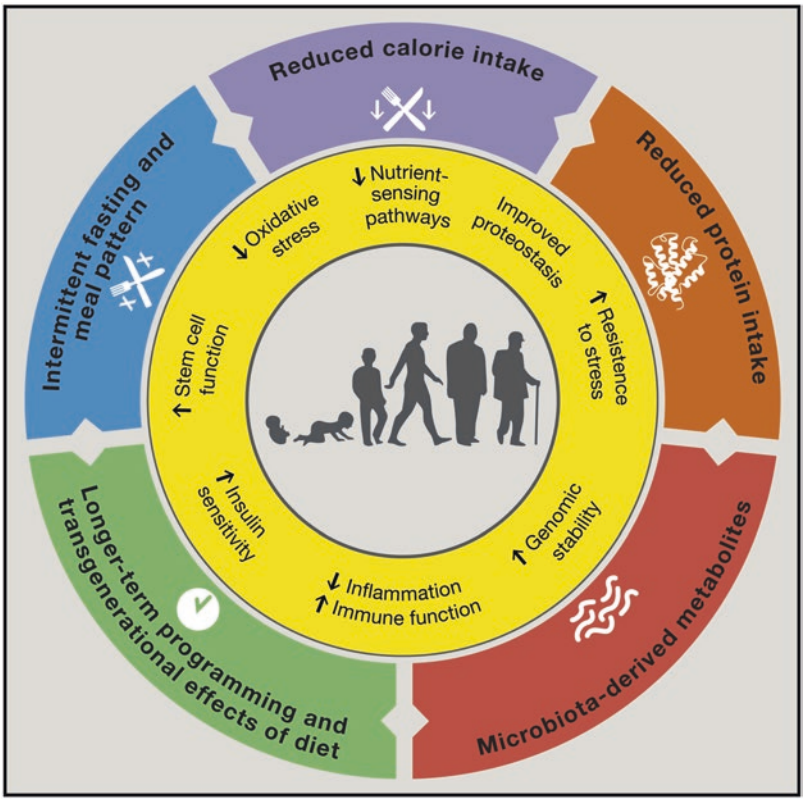

Fig. 13.1 Effects of dietary factors and patterns, such as dietary energy restriction, on mechanisms associated with healthy ageing and longevity, including lower oxidative stress, improved genomic stability and increased insulin sensitivity. (Reproduced with permission from Fontana and Partridge 2015)

Epigenetics

Epigenetics describes heritable changes to the genome without changes to the DNA sequence itself. Epigenetic mechanisms involve a consortium of marks and molecules including DNA methylation, histone modification and the enzymes and other proteins which enable the reading, writing and erasing of these marks. In addition, patterns of expression of microRNAs (miRNAs) and other non-coding RNAs contribute to the epigenetic mechanisms that modulate gene expression and, consequently, cellular and tissue function (Malcomson and Mathers 2017). The ageing process is associated with epigenetic changes, including a decrease in global methylation levels (DNA hypomethylation) (Bollati et al. 2009) and gene-specific hypermethylation which may lead to gene silencing. Ageing is also associated with histone modifications such as histone acetylation which results in transcriptional activation. Nutrients and other food constituents modify epigenetic marks and

molecules and this may be an important mechanism through which nutrition influences ageing and the risk of age-related diseases such as cancer (Park et al. 2017). At birth, the epigenomes of monozygotic twins are very similar but epigenetic patterning in twin pairs diverges during ageing, especially in those twins who have lived apart, which suggests effects of environmental factors, including nutrition (Fraga et al. 2005). *In vitro* and animal studies have provided further evidence of causal relationships (Mathers 2006).

The most studied dietary factors modulating the epigenome, in particular DNA methylation, are one-carbon donors such as folate which determine the availability of S-adenosyl methionine, the methyl donor for DNA methylation. For example, erythrocyte/serum folate concentration is correlated with global DNA methylation in the colonic mucosa of healthy adults (Pufulete et al. 2003, 2005). In older (>63 years) postmenopausal women, a low folate diet (118 micrograms folate per day – about half of the recommended intake) for 7 weeks led to leukocyte DNA hypomethylation which was not reversed following 7 weeks folate repletion (Rampersaud et al. 2000). In contrast, in younger (49–63 years) post-menopausal women, Jacob and colleagues showed that global DNA hypomethylation was reversed by folate repletion (Jacob et al. 1998) which suggests that such epigenetic responses to dietary change may be delayed or blunted during ageing. Several plant secondary metabolites, including polyphenols, are modulators of DNA methylation and histone modifications (Link et al. 2010; Vanden Berghe 2012). For example, (-)-epigallocatechin-3-gallate (EGCG) from green tea inhibits DNA methyltransferases (DNMTs) by binding in the catalytic pocket of the enzyme and restores expression of genes silenced by hypermethylation in cancer cells (Fang et al. 2003). In contrast, some dietary factors, such as alcohol, may have detrimental effects on ageing and age-related diseases by causing global DNA hypermethylation (Thapar et al. 2012).

Histone modifications, including acetylation and methylation of lysine residues, alter the packaging of DNA within the nucleus and, consequently, affect access of the transcriptional machinery to DNA. Dietary factors modulate histone modifications, primarily through alterations to the enzymes which catalyse these modifications, including histone acetyl transferase (HAT) and histone deacetylases (HDACs). Histone modifications contribute to brain ageing and the development of neurodegenerative diseases such as Alzheimer's disease and dietary energy restriction restored, partially, the histone modifications, including H3K27me3 and H3R2me2, that occur with ageing in the mouse brain (Gong et al. 2015). Similarly, in a mouse model of diet-induced obesity, *Glut4* expression was reduced whilst 30% caloric restriction rescued this phenotype by increasing acetylation of histone 4 at the *Glut4* gene promoter (Wheatley et al. 2011). These findings suggest a role for chromatin remodelling in mediating the effects of dietary energy restriction.

Several other dietary factors have effects on post-translational modifications of histones. The flavonoid quercetin, found in fruits, vegetables and tea, may exert its anti-inflammatory effects in intestinal epithelial cells by inhibiting HAT activity and reducing phosphorylation and acetylation at histone H3 associated with inflammatory genes (Ruiz et al. 2007). Butyrate, a short-chain fatty acid (SCFA) end-product

of dietary fibre fermentation in the large bowel, is a well-established histone deacetylase inhibitor (HDACi) and one of the earliest identified epigenetic modifiers (Candido et al. 1978). At physiological concentrations, butyrate inhibits the removal of acetyl groups from histones, resulting in a more open chromatin structure that promotes gene expression by facilitating access by the transcriptional machinery (Kurdistani et al. 2004). Butyrate also has effects on miRNA expression which may contribute to its potential chemoprotective effects against colorectal cancer, a cancer strongly associated with age (Louis et al. 2014).

miRNA are small, non-coding RNA, approximately 22 nucleotides long, which regulate gene expression by inhibiting translation or causing messenger RNA degradation. Thousands of miRNA have been identified which regulate the expression of up to 60% of protein-coding genes (Friedman et al. 2009) and, consequently have effects on multiple physiological processes such as cell proliferation, differentiation and apoptosis. Altered miRNA expression or function has been linked with ageing and risk of age-related diseases, including cancers and neurodegenerative diseases (Jung and Suh 2012). In particular, these miRNAs have been shown to modulate components of nutrient-sensing pathways, such as mTOR and the insulin/IGF-1 pathway (discussed later). In a randomised controlled trial, supplementation with butyrylated resistant starch (a butyrate precursor) restored the expression of some oncogenic members of the *miR-17-92* cluster that had been dysregulated by high red meat intake, a risk factor for CRC (Humphreys et al. 2014). In addition, the rescued miRNA expression was associated with restored levels of colonic crypt cell proliferation, a well-recognised biomarker of CRC risk (Mills et al. 1995, 2001). Supplementation of healthy human participants with non-digestible carbohydrates, that are fermented in the large bowel to produce SCFA including butyrate, increased expression of *miR-32*, which could be one of the mechanisms through which non-digestible carbohydrates and butyrate regulate processes such as cell proliferation in that organ (Malcomson et al. 2017).

In *C.elegans*, expression of the miRNAs *lin-4*, *miR-71* and *miR-1* modulate lifespan and age-related functional decline (Boehm and Slack 2005; de Lencastre et al. 2010; Pincus et al. 2011; Ibanez-Ventoso et al. 2006). In rhesus monkeys, differential expression of multiple miRNAs, including downregulation of *miR-181b* and upregulation of *miR-144,* has been observed in skeletal muscle from young versus old monkeys (McGregor et al. 2014) and in humans, miRNA expression in skeletal muscle of older men was reduced compared with that in younger men (Rivas et al. 2014). Dietary energy restriction in monkeys restored some of these age-related changes in miRNA expression by upregulating *miR-1323* (downregulated in older tissues) and reversing *miR-181b* and *miR-489* downregulation. Resistance exercise altered the expression of approximately 20 miRNAs in young, but not older, men, suggesting that abnormal miRNA expression may play a role in the reduced response of muscle to exercise with ageing. In particular, *miR-126,* which modulates signal transduction in response to IGF-1, was associated with reduced lean mass in older participants (Zhang et al. 2008). Interestingly, this miRNA has been associated with reduced IRS-1 protein expression, resulting in impaired insulin signalling, in a model of mitochondrial dysfunction (Ryu et al. 2011). Further studies are required

to investigate the role of these abnormally expressed miRNAs in ageing and the effect of dietary interventions that are known to influence longevity and the risk of age-related diseases.

Dietary polyphenols modulate the expression of multiple miRNAs including those that are involved in the regulation of inflammation, apoptosis and carcinogenesis (Milenkovic et al. 2013). For example, the phenolic acid, 5-O-caffeoylquinic acid found in coffee upregulates *miR-122* expression in a mouse hepatoma cell line, with subsequent effects on lipid metabolism. The anti-proliferative effects of eIlagitannin, another phenolic acid, may be mediated via modulation of miRNA expression (Wen et al. 2009). In lung adenocarcinoma patients, a quercetin-rich diet was associated with differential expression of several miRNAs including the tumour suppressor *let-7* and the oncogenic *miR-17* (Lam et al. 2012). Curcumin has antioxidant, anti-inflammatory and pro-apoptotic effects that may benefit health. In human rectal and colon cells, curcumin treatment downregulated the expression of oncogenic miRNAs including *miR-17, miR-20a* and *miR-21* (Mudduluru et al. 2011; Gandhy et al. 2012). The potentially beneficial effects of the polyphenol resveratrol on inflammation, mitochondrial function and cell death (Delmas et al. 2011) may also result from the modulation of miRNA expression (Lancon et al. 2012). In human THP-1 monocytic cells, resveratrol upregulates *miR-663,* an anti-inflammatory and tumour suppressor miRNA, and blunts the upregulation of *miR-155* in response to lipopolysaccharides, with effects on the immune response and inflammation (Tili et al. 2010a). Resveratrol also reduces the expression of oncogenic miRNAs, including *miR-17, -21, -25* and *-146a*, and increases expression of the tumour suppressor *miR-663* (Tili et al. 2010b). These miRNAs have a role in the regulation of TGFβ signalling, a potential mechanism for the chemoprotective effects of resveratrol. However, such results should be interpreted with caution since most of the data for epigenetic effects of plant secondary metabolites have been obtained from *in vitro* studies, often using transformed cells lines, where the observed effects may have been influenced by the applied dose (often high concentrations compared with physiological) and treatment duration and where the chemical form of the tested compound may not match that observed *in vivo*.

Dietary energy restriction, one of the most studied dietary modulators of ageing, has effects on DNA methylation and histone modifications. In rhesus monkeys, 30% dietary energy restriction for 7 years attenuated age-related methylation drift (gene-specific hypermethylation and global hypomethylation), resulting in a blood methylation age estimated to be 7 years younger than the animals' chronological age (Maegawa et al. 2017). More pronounced findings were observed in mice given 40% CR, which may have resulted from (i) a greater degree of CR and (ii) a longer relative duration (almost the entire lifespan) of CR. DNA methylation correlated with gene expression levels and the age-related change in methylation was inversely correlated with lifespan, suggesting that the effects of CR on this change may have beneficial effects on longevity. Short-term CR (4 weeks) has also been shown to ameliorate age-related methylation changes (hypermethylation of promoter regions of disease-related genes) in old rats (Kim et al. 2016).

In overweight or obese men, 8 weeks of dietary energy restriction resulted in differential methylation of 170CpG sites in peripheral blood mononuclear cells, assessed by microarray analysis, including specific CpG sites within the *ATP10A* and *WT1* genes, and effects on DNA methylation were greatest in those who responded best to the intervention (with a weight loss over 5%) (Milagro et al. 2011). Also in peripheral blood mononuclear cells, TNF-alpha promoter methylation was significantly reduced in obese men with a 5% or more body weight loss following eight-weeks of dietary energy restriction (Campion et al. 2009). Similar effects of response to DR have been observed in abdominal subcutaneous adipose tissue from overweight or obese post-menopausal women. A significant difference in DNA methylation at three loci (on chromosomes 1q36, 4q21 and 5q13) was observed between low and high-responders (≥3% loss in body fat) in abdominal subcutaneous adipose tissue following a 6 month CR (Bouchard et al. 2010).

Nutrient Sensing Pathways

Because of its centrality in facilitating function, cells need to monitor and respond appropriately to their nutritional environment. Nutrient sensing pathways detect concentrations of intra- and extra-cellular nutrients, such as sugars and lipids, and consequently elicit a response (Efeyan et al. 2015). From both genetic and dietary energy restriction-based studies of the plasticity of ageing, it has been established that interventions that modulate ageing positively do so via effects on nutrient sensing pathways (Fontana et al. 2010). These core pathways include the mechanistic target of rapamycin (mTOR), the insulin/insulin-like growth factor (IGF-1) and sirtuin pathways which regulate processes such as cell growth and metabolism (Aiello et al. 2017). The inhibitory effects of dietary energy restriction on mTOR signalling as a mechanism of increased lifespan is conserved across multiple species (Kapahi et al. 2010; Kaeberlein et al. 2005; Vellai et al. 2003; Bjedov et al. 2010). Furthermore, mTORC1 inhibition is associated with enhanced mitochondrial and stem cell function and maintenance of proteostasis (Johnson et al. 2013), three other hallmarks of ageing that are discussed later in this chapter. mTOR inhibition reduces mitochondrial membrane potential and oxygen consumption and, consequently, lowers the production of reactive oxygen species (ROS) (Schieke et al. 2006) which may reduce ROS-related macromolecular damage during ageing. mTOR is hyperactive in many cancers (Populo et al. 2012) and, for example, the dietary polyphenol EGCG, a competitive inhibitor of mTOR at physiological concentrations, leads to reduced cell proliferation, which is a potential mechanism for its chemoprotective effects (Van Aller et al. 2011). Similar observations have been made for quercetin and resveratrol which also inhibit mTOR (Bruning 2013; Park et al. 2016).

The insulin/IGF-1 signalling pathway is activated by the binding of insulin or of IGF-1 to insulin and IGF-1 receptors, respectively. Downstream targets of this pathway include transcription factors such as FOXO3A, which is involved in healthy ageing, and activation of this pathway can also activate mTOR signalling (Siddle

2011). As expected, dietary energy restriction and fasting reduce insulin/IGF-1 signalling (Fontana and Partridge 2015) and result in improved insulin sensitivity and reduced inflammation (Hine et al. 2015). Dietary effects on insulin/IGF-1 signalling are not restricted to effects of energy intake and the protein content of the diet is also important (Levine et al. 2014; Fontana et al. 2008). Serum IGF-1 concentrations correlate inversely with protein intake (Fontana et al. 2006) and restriction of protein and energy for 3 weeks produced a significant reduction in serum IGF-1 concentrations (Fontana et al. 2008). Similar findings have been observed in sedentary adults consuming a vegetarian low-protein, low-calorie diet and were associated with improved plasma insulin, C-reactive protein and leptin levels compared with those eating a Western diet (Fontana et al. 2006). The type of protein may also be important in determining the impact of protein intake with diets low in animal protein, or higher in vegetable sources such as beans, tofu and nuts, having beneficial effects (Verburgh 2015) that are associated with improved IGF-1 and insulin concentrations (Levine et al. 2014). Women consuming vegan diets had significantly lower concentrations of IGF-1 compared with meat-eaters and lacto-ovo-vegetarians (Allen et al. 2002).

Earlier in this chapter, we have reviewed the evidence for positive effects of the Mediterranean diet on ageing and risk of age-related disease and such effects may be mediated by downregulation of the insulin/IGF-1 pathway (Vasto et al. 2014). A Mediterranean diet may activate *FOXO3A* and the consequent activation of transcription of homeostatic genes may promote longevity through reduced cell proliferation (via reduced *ERK* expression) and by lower transcription of inflammatory genes (via reduced activation of NF-KB) (Vasto et al. 2014). However, a 6 month intervention with a Mediterranean diet in 69 healthy women aged 25–59 years had no effects on measures of insulin sensitivity including plasma insulin concentrations, *IGF-1* or *IGF-BP3* expression (Djuric et al. 2009). Although the PREDIMED Study did not investigate effects of a Mediterranean Diet intervention on insulin/IGF-1 signalling *per se*, a sub-group analysis reported lower diabetes risk in participants at enhanced risk of CVD (Salas-Salvado et al. 2014).

Telomere Attrition

Telomeres are regions of repeated nucleotide sequences that cap the ends of chromosomes and protect them from degradation and end-to-end fusion. Telomeres shorten during ageing because of the gradual loss of these repetitive sequences with each round of cell division. In older humans (60+ years), those with shorter telomeres had poorer survival due to higher rates of cardiovascular and infectious disease (Cawthon et al. 2003). Telomere attrition is accelerated by inflammation and other DNA damaging processes (Paul 2011) and shorter telomeres are observed in smokers and those who are obese (Valdes et al. 2005). In a study of 200 very old participants, higher adherence to the Mediterranean diet was associated with longer leukocyte telomeres and greater telomerase activity, which may have been due to

dampened inflammation and lower oxidative stress (Boccardi et al. 2013). Among Korean older adults, consumption of a plant-based dietary pattern characterised by high intake of whole grains, seafood, legumes, vegetables and seaweed at baseline was associated with increased telomere length at 10 years follow up (Lee et al. 2015). Similarly, a vegetable-rich dietary pattern was associated with longer telomeres in Chinese women but not men (Gong et al. 2017). In the Nurses' Health Study, healthier lifestyles (healthy diet, low alcohol consumption, not-smoking, regular physical activity and a healthy body weight), and, in particular, higher intakes of cereal fibre and wholegrains, were associated with longer telomere length in leukocytes whereas polyunsaturated fatty acid intake and waist circumference were inversely correlated with telomere length (Sun et al. 2012; Cassidy et al. 2010). Some of these protective effects may have resulted from reduced inflammation (Lopez-Garcia et al. 2004). In the FINGER Trial, leukocyte telomere length at baseline was positively associated with healthy lifestyle (Sindi et al. 2017). In addition, the cognitive response, particularly for executive functioning, to the multi-domain intervention was greater in those with shorter telomeres at baseline (Sindi et al. 2017).

Ornish and colleagues conducted a 3 month pilot study to investigate the effects of another multi-domain lifestyle change intervention, including a low-fat (10% fat), plant-based diet rich in fruits, vegetables, unrefined grains, and soy, fish oil, vitamin E, selenium and vitamin C supplements, moderate aerobic exercise, stress management and weekly group support sessions, on telomerase activity in peripheral blood mononuclear cells from 30 low-risk prostate cancer patients (Ornish et al. 2008). Immediately following the intervention, telomerase activity increased by almost 30% and this was associated with positive effects on additional health-related markers including low-density lipoprotein cholesterol. At 5 years follow-up, men who completed the lifestyle intervention had significantly increased telomere length, and those in the control group had significantly reduced telomeres compared with baseline (Ornish et al. 2013). Although telomerase activity decreased at 5 years follow-up in both intervention and control groups, this decrease was smaller in those completing the lifestyle intervention (Ornish et al. 2013).

Higher blood concentrations of lutein, zeaxanthin and vitamin C concentrations were associated with longer leukocyte telomeres in older adults enrolled in the Austrian Stroke Prevention Study (Sen et al. 2014). This is likely to result from the anti-oxidative properties of these micronutrients which are associated with improved antioxidant defence and reduced oxidative stress (Song et al. 2015; Chen et al. 2001). Intakes of food-derived polyphenols may also affect telomere length. For example, habitual tea drinkers (>3 cups per day) had longer telomeres than those in the lowest quartile of tea consumption, and this difference corresponded to approximately 5 years of life (Chan et al. 2010). Higher maternal vitamin D concentrations and lower maternal protein intake during pregnancy are associated with greater telomere length in leukocytes (Kim et al. 2017) and in the aorta (Tarry-Adkins et al. 2008) of the offspring. The influence of such maternal nutritional factors on telomere length in later life is not known but sustaining longer telomeres could help

mediate the effects of maternal nutrition on ageing predicted by the Developmental Origins of Adult Health and Disease (DOHaD) hypothesis (Gluckman et al. 2005).

On the contrary, unhealthy diets are associated with faster telomere shortening. For example, folate deficiency is associated with shorter telomeres probably due to enhanced DNA damage resulting from reduced thymidylate synthesis (requires folate and/or other one-carbon donors) and consequent mis-incorporation of uracil instead of thymidine in DNA (Blount et al. 1997). Zinc deficiency is also associated with telomere shortening via stimulation of oxidative stress and DNA damage, perhaps, via effects on telomerase activity (Nemoto et al. 2000).

Obesity, as assessed by raised BMI, waist-to-hip ratio and body adiposity index, is also associated with shorter telomere lengths (Buxton et al. 2014). In white blood cells, ageing was associated with a reduction of 27bp telomere length per year and telomeres were 240bp shorter in obese compared with lean females, highlighting the role of obesity in accelerating hallmarks of ageing (Valdes et al. 2005). Bariatric surgery increased telomere length up to 10 years post-surgery, suggesting a potential beneficial effect of surgery-induced weight loss on telomere length and protection against telomere attrition (Laimer et al. 2016), which could contribute to the lower risk of age-related disease in those who have undergone bariatric surgery (Sjostrom et al. 2007).

Stem Cell Function

Ageing is associated with a decline in stem cell function and results in reduced regenerative potential of tissues (Lopez-Otin et al. 2013). Dietary energy restriction of 25% for 24 months in BALB mice improved haematopoietic stem cell function to levels better than those observed in younger (3 months old) mice (Chen et al. 2003) and improvement in haematopoietic stem cell function was also observed with short-term (5 months) dietary energy restriction. Similar positive findings of dietary energy restriction on stem cell function have been observed in other tissues including skeletal muscle (Cerletti et al. 2012) and the central nervous system (Park et al. 2013). However, the effects of dietary energy restriction on stem cell function are not universal and may depend on genotype (Ertl et al. 2008). These observations are in accord with the heterogeneity in lifespan responses to dietary energy restriction (Vaughan et al. 2017) as discussed earlier in this chapter. As would be expected from its role in accelerating ageing, obesity, and also obesity-associated diseases such as type 2 diabetes, are associated with reduced stem cell function (Mihaylova et al. 2014). Feeding a high fat diet for 4 months reduced stem cell numbers and neurogenesis (the production of neurons from neural stem cells) (Li et al. 2012). On the other hand, short-term feeding of a high-fat diet (60% fat) induced neurogenesis by four-fold in adult mice (Lee et al. 2012).

To date, relatively little is known about the effects of specific foods or their constituents on stem cell function during ageing. A dietary supplement containing blueberry, green tea, vitamin D2 and carnosine induced proliferation of human bone

marrow and haematopoietic stem cells *in vitro* and stimulated neurogenesis in aged rats (Acosta et al. 2010; Bickford et al. 2006). In addition, spirulina (an alga rich in proteins, vitamins, minerals and amino acids) may have protective effects against age-related reduction in neurogenesis and cognitive decline since feeding a diet supplemented with 0.1% spirulina protected against lipopolysaccharide-induced inflammation and rescued stem cell proliferation (Bachstetter et al. 2010).

Genomic Instability

Genomic instability describes alterations to the genome resulting from the accumulation of genetic damage that compromises DNA integrity and stability. Genomic instability is implicated in the pathogenesis of several diseases, particularly cancer, as well as in the aetiology of accelerated ageing (Vijg and Suh 2013). Diet is likely to play a key role in the maintenance of genomic stability through its role in multiple pathways, including those involved in DNA repair, DNA synthesis and apoptosis (Fenech and Ferguson 2001). The majority of studies investigating the effects of dietary factors on genomic stability have focussed on DNA damage, which is the most important factor that induces genomic instability, but also include studies on telomere length and function and mitochondrial DNA integrity (discussed in other sections within this chapter). In addition, the sophisticated and overlapping systems for DNA repair counter-act genomic damage and variability in the efficacy of these systems contributes to inter-individual differences in disease risk and in the ageing trajectory (Hoeijmakers 2009).

Dietary energy restriction (25%) for 6 months in healthy, but overweight, sedentary adults reduced DNA damage as assessed by DNA fragmentation (Heilbronn et al. 2006; Fromm and Robertson 1975). A similar study performed in non-diabetic, obese adults found that restricting energy intake to 1000kcal (4.184 MJ) per day for 4 weeks reduced ROS generated by leukocytes and oxidative damage to lipids, proteins and amino acids (Dandona et al. 2001). A sustained energy deficit induced by 20% dietary energy restriction or increased exercise for 1 year lowered oxidative DNA and RNA damage in white blood cells by approximately 50% (Hofer et al. 2008). Similar findings have been reported in rhesus monkeys where dietary energy restriction reduced accumulation of oxidative damage in skeletal muscle (Zainal et al. 2000).

Given its functional role in multiple proteins including zinc finger proteins and other proteins that are critical for interactions with DNA and RNA (Cassandri et al. 2017), it is unsurprising that zinc is a key trace element involved in the maintenance of genomic stability through the regulation of cellular processes such as DNA repair (Sharif et al. 2012). Zinc is also essential for the maintenance of genomic integrity via its association with proteins involved in antioxidant and DNA damage response, for example copper/Zn super oxide dismutase, poly(ADP-ribose) polymerases (PARP) and p53 (Dreosti 2001; King and Cidlowski 1998; Petrucco and Percudani 2008). Zinc deficiency can induce DNA damage and repair, impair the methionine

cycle and increase oxidative stress, which are associated with a genomic instability phenotype (Song et al. 2009b, 2017). In healthy men, dietary zinc depletion increased DNA strand breaks in peripheral blood cells, which were restored to baseline levels with zinc repletion, supporting the role for zinc in the maintenance of DNA integrity (Song et al. 2009a).

DNA repair mechanisms including base excision repair, nucleotide excision repair and DNA mismatch repair play major roles in the identification and repair of damage to DNA molecules (Hoeijmakers 2009). Such damage, if not corrected, leads to the accumulation of damage to the DNA structure and contributes to the development of cancer and other age-related disease (Hoeijmakers 2009). This likely critical role of such DNA repair systems in influencing the ageing trajectory has been established in a mouse model deficient in the *Ercc1* DNA excision-repair gene that shows multiple features of accelerated ageing and lives for only 4–6 months (Vermeij et al. 2016). Importantly, the plasticity of ageing in response to diet has been shown clearly in this model. When dietary energy intake by $Ercc1^{\Delta/-}$ mice was restricted by 30%, their remaining lifespan was tripled and there were improvements in several features of the ageing phenotype including retention of more neurones and maintenance of good motor function into old age (Vermeij et al. 2016). The mechanism(s) responsible for this dramatic effect of dietary energy restriction have not been elucidated in detail but it is apparent that the intervention leads to reduced accumulation of γH2AX DNA damage foci and preserved genomic stability (Vermeij et al. 2016).

Through their antioxidant, anti-mutagenic and anti-inflammatory properties, polyphenols have beneficial effects on genomic stability (Ferguson 2001). For example, vanillic acid may protect against mutagens by promoting DNA replication and repair (Takahashi et al. 1990; Ohta et al. 1988). DNA repair mechanisms are also influenced by other polyphenols such as myricetin (a flavonoid found in tea and berries) that induces the DNA repair enzyme DNA polymerase in response to genotoxicity (Abalea et al. 1999). However, most studies in this area have been performed *in vitro* or in animal models and may not have used physiological concentrations of the appropriate polyphenol metabolites.

In mice, methylation of base excision repair genes, such as *Ogg1*, increases with ageing and is associated with 20% lower base excision repair capacity in the brain and an increase in oxidative lesions (Langie et al. 2017). Adult offspring mice from mothers fed a folate-depleted diet during pregnancy and lactation that were weaned on to a high fat diet had decreased base excision repair capacity in several brain regions after 6 months (Langie et al. 2013). This double nutritional insult appeared to reduce the animals' capacity to repair DNA damage but the long-term effects on the ageing phenotype have yet to be established.

To date, there have been a few intervention studies in humans that have examined the effects of foods and specific nutrients on base excision repair (that corrects small single base lesions such as those caused by oxidative damage) or on nucleotide excision repair (which repairs bulky DNA adducts such as those caused by UV light). For example, in a randomised, cross-over trial, supplementation with kiwi fruits for 3 three-week periods increased base excision repair capacity but had no effects on

expression of *OGG1* or *APE1*, two base excision repair-related genes (Collins et al. 2003). Similarly, supplementation with 600g of fruits and vegetables or of a supplement containing the same amounts of vitamins and minerals for 24 days had no effect on the expression of *ERCC1* or *OGG1* DNA repair genes (Moller et al. 2003). In healthy participants, supplementation with a mixture of α-carotene and β-carotene or with cooked carrots increased DNA repair activity significantly (Astley et al. 2004). This is in line with an earlier study which reported that repair of induced damage was increased in healthy volunteers supplemented with carotenoids, zinc and nicotinamide for 7 weeks (Sheng et al. 1998). In healthy young participants from the DNA damage And Repair Trial (the DART Study), the capacity for base excision repair and nucleotide excision repair differed greatly (11-fold range) between individuals and correlated inversely with age (Caple et al. 2010; Tyson et al. 2009). In addition, there was an inverse correlation between nucleotide excision repair capacity and adiposity (Tyson et al. 2009). Whilst there were no overall effects of supplementation with an antioxidant complex containing selenium and vitamins A, C and E for 6 weeks on DNA repair, supplementation reduced levels of endogenous DNA damage and that induced by oxidative challenge in those in the highest tertile of DNA damage at baseline (Caple et al. 2010).

Cellular Senescence

Cellular senescence describes a permanent arrest of cell division and is a defence mechanism that occurs in response to stressors. The prevalence of senescent cells within tissues increases with age and senescence is implicated in age-related loss of function, and in risk of age-related diseases as well as in physiological processes such as tissue repair (Childs et al. 2015; van Deursen 2014). Recent studies have shown that the removal of senescent cells by genetic or pharmacological means improves function in older animals (Baker et al. 2011, 2016).

To date, there has been little systematic study of the impact of dietary factors on senescence. However, obesity increases the accumulation of senescent cells in adipose tissue and this may be associated with obesity-associated diseases such as diabetes (Tchkonia et al. 2010; Minamino et al. 2009; Kirkland 2010). Using transgenic mice that express enhanced green fluorescence protein (EGFP) in response to activation of the senescence-associated p16(INK4a) promoter, feeding an unhealthy diet (a fast-food diet with 40% fat) for 4 months increased markers of senescence including p16 and EGFP and led to the development of a characteristic senescence-associated secretory phenotype in mouse visceral adipose tissue (Schafer et al. 2016). As expected, these mice had higher body weight and fat mass and exhibited additional detrimental effects on multiple functional outcomes including cardiovascular and metabolic function. The increase in senescence and adverse health effects induced by the fast-food diet were abrogated by exercise for 14 weeks post-diet (Schafer et al. 2016).

In contrast, in mice, feeding a low fat diet reduced cellular senescence in white adipose tissue, evidenced by reduced activin A (a protein released by senescent fat cell progenitors) and lower numbers of beta-galactosidase-stained cells (List et al. 2016). An earlier study by this group demonstrated that mice on the low fat diet and those undergoing a weight loss cycle diet had a significantly increased lifespan compared with those on the high fat diet (List et al. 2013).

Dietary factors also have effects on senescence in other tissues. For example, Wang and colleagues showed that short-term (3 months) dietary energy restriction reduced senescence in the small intestinal epithelium and liver of mice (Wang et al. 2010). These effects on cellular senescence were also accompanied by other markers of healthier ageing including improved telomere maintenance (increased telomere length in enterocytes) and reduced cumulative oxidative stress markers (Wang et al. 2010).

Mitochondrial Dysfunction

Ageing and age-related diseases are associated with a decline in the number of mitochondria and in mitochondrial function (Harman 1972) and the ability of the mitochondria to respond to energy demands is compromised (Tauchi and Sato 1968). Whilst the mitochondria in older individuals are larger in size, they are less efficient and are associated with increased production of free radicals and oxidative damage (Rustin et al. 2000). The fact that the production of energy by the mitochondria is influenced by nutrient availability makes mitochondrial function a strong candidate for the modulation of the ageing trajectory and age-related diseases by nutrition. Furthermore, nutrients with antioxidant properties may protect the mitochondria from free radicals, thus reducing the free radical production associated with ageing.

One of the mechanisms through which dietary energy restriction promotes health in later life and longevity may be through improving mitochondrial function and maintaining mitochondrial dynamics as a result of the regulation of upstream pathways such as mTOR (Ruetenik and Barrientos 2015) as described earlier. For example, PGC-1α activation by dietary energy restriction stimulates the transcription of genes such as *Nrf1* involved in mitochondrial biogenesis and regulation. Regulation of mitochondrial damage through mitophagy (selective degradation and recycling of the components of damaged mitochondria) occurs following activation of autophagy, for example by SIRT1. In humans, long-term dietary energy restriction improved insulin sensitivity and increased levels of adiponectin, which are associated with increased mitochondrial fatty acid oxidation (Hamdy 2005; McKee Alderman et al. 2010). Dietary energy restriction may also preserve mitochondrial function without increasing mitochondrial biogenesis (Hancock et al. 2011; Miller et al. 2012; Lanza et al. 2012) e.g. by reducing oxidative damage and increasing antioxidant defences. The underlying mechanism may be improved mitochondrial function e.g. OXPHOS efficiency and reduced ROS production (Knight et al. 2011)

and there is strong evidence that the beneficial effects of dietary restriction on mitochondrial function are highly conserved from yeast to humans (Ruetenik and Barrientos 2015).

In addition to total energy intake, the ratio of macronutrients within the diet may be important in modulating lifespan (Solon-Biet et al. 2014) and these effects may involve altered mitochondrial function. Inhibition of TOR signalling in *Drosophila* by amino acid restriction was associated with increased translation of the OXPHOS consortium of genes (Grandison et al. 2009; Zid et al. 2009). In the liver of *ad libitum*-fed mice with a low protein intake (Solon-Biet et al. 2014), mitochondrial number correlated with protein intake and was inversely correlated with mitochondrial respiration and hydrogen peroxide production. Similarly, methionine restriction by 40% for 7 weeks reduced the production of ROS in heart mitochondria and reduced oxidative damage in rats (Sanchez-Roman et al. 2011).

As discussed earlier in this chapter, DHA is the most abundant PUFA in brain tissue (Kim 2007) and there is evidence that DHA and other long chain omega-3 PUFAs may delay age-related mitochondrial dysfunction in the brain. PUFAs administrated in fish oil restored the age-related decrease in respiration, evidenced by improved complex I + II and IV activity of the mitochondrial respiration system and greater ATP production, in brain cells from mice (Afshordel et al. 2015). PUFA treatment also improved membrane fluidity and increased levels of neuroprotective compounds such as secreted amyloid precursor protein-α and neuroprotection D-1 (Afshordel et al. 2015).

Deficiencies in any macro- or micronutrient required for synthesis or activity of any component of the respiratory chain will lead to mitochondrial dysfunction and thus, accelerate ageing. For example, inadequate intakes of iron, zinc, biotin or pantothenic acid will reduce heme biosynthesis, leading to diminished heme-a, oxidant leakage and mitochondrial decay (Ames et al. 2005). In addition, inadequate intakes of selenium, a component of the antioxidant enzyme glutathione peroxidase, are associated with altered structure of the mitochondria and with functional effects including impaired oxidation and increased ROS production (Rani and Lalitha 1996). Adequate selenium status also appears to have protective effects via activation of mitochondrial biogenesis (Mehta et al. 2012).

Vitamin E is a key antioxidant nutrient which becomes incorporated into membranes and disruption of mitochondrial structure is an early symptom of vitamin E deficiency (Wang and Quinn 2000). Rats fed a vitamin E-deficient diet for 12 weeks have significantly increased lipid peroxidation and reduced cytochrome oxidase and NADH CoQ1 reductase activity (Rafique et al. 2004). With increasing age in rats, there is evidence of hippocampus and frontal cortex mitochondrial dysfunction, with lower rates of tissue and mitochondrial respiration and lower activities of complexes I and IV and of mitochondrial nitric oxide synthase (Navarro et al. 2011). Vitamin E supplementation of rats from 9 to 12 months was associated with restored mitochondrial respiration, improvement in the activities of complex I and IV and of nitric oxide synthase and blunting of the increase in oxidation products (Navarro et al. 2011).

Loss of Proteostasis

Proteostasis (protein homeostasis) is the maintenance of the proteome, including tightly-coordinated cellular systems and pathways that regulate the synthesis, folding, degradation and repair of proteins that ultimately lead to a balance in protein turnover. The maintenance of normal proteostasis requires a consortium of chaperones that help newly-synthesized proteins and unfolded proteins to adopt their mature, stable folded state and two proteolytic systems, the ubiquitin-proteasome and the lysosome-autophagy systems (Kaushik and Cuervo 2015). The effectiveness of these systems declines during ageing and cells from older organisms contain more proteins with oxidative modifications, including carbonylation, oxidized methionine, and glycation, as well as accumulating cross-linked and aggregated proteins, and less catalytically active enzymes (Taylor and Dillin 2011). This leads to disturbances in proteostasis, including protein misfolding, that contribute to the development of multiple age-related diseases notably neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Kaushik and Cuervo 2015). This ageing-related disturbance in proteostasis is due to the accumulation of ROS and oxidative damage which lead to misfolding of proteins and the accumulation of protein aggregates that disrupt cellular function (Kaushik and Cuervo 2015) .

Obesity has been associated with significantly greater whole body protein turnover and this was enhanced in those with a greater degree of obesity (Henderson et al. 2010). In obese individuals, dietary restriction for 30 days (1400 kcal deficit with maintained protein intake) significantly reduced three markers of proteostasis, leucine rate of appearance, non-oxidative leucine disposal and leucine disposal (Henderson et al. 2010). Similar to the effects of dietary energy restriction, fasting causes reduced whole-body protein turnover (Nair et al. 1989) but whether this relates directly to altered proteostasis is not clear. Age-dependent changes in the abundance of specific proteins in heart muscle including fewer proteins involved in mitochondrial function, electron transport chain, citric acid cycle, and fatty acid metabolism and higher amounts of proteins involved in glycolysis and oxidative stress response have been demonstrated by quantitative proteomics (Dai et al. 2014). This age-dependent cardiac proteome remodelling was reversed by either short-term dietary energy restriction or by treatment with the anti-ageing agent rapamycin (Dai et al. 2014). In addition, use of stable isotope labelling is beginning to reveal some of the mechanisms through which dietary energy restriction and rapamycin treatment counteract the age-increased accumulation of long-lived aggregating proteins that characterise ageing and increase disease risk (Basisty et al. 2017).

Dietary and other environmental factors which damage the endoplasmic reticulum (ER) may exacerbate age-related loss of proteostasis because of the central role of the ER in folding, modifying and trafficking secretory and membrane proteins to the Golgi compartment (Back and Kaufman 2012). For example, high-fat diets which result in increased free fatty acids in plasma (lipotoxicity), and obesity, cause ER stress and lead to cellular dysfunction and cell death (Prentki and Nolan 2006). Similar effects are observed with glucotoxicity resulting from hyperglycaemia

(raised blood glucose concentrations) (Back and Kaufman 2012). High-fat diets stimulate protein synthesis and this can exceed the protein processing capacity in the ER and can activate the unfolded protein response pathway (Back and Kaufman 2012). These effects of high-fat diets have been observed in animal models and have yet to be reproduced in humans.

Altered Intercellular Communication

Ageing is associated with changes in cell-to-cell communication through endocrine, neuroendocrine and neuronal routes (Lopez-Otin et al. 2013). Inflammation is one of the most widely studied, and one of the most significant, intercellular communication processes in ageing and in the development of age-related diseases. Age-associated inflammation, known as 'inflammaging', drives the effects of ageing on intercellular communication and is associated with several age-related diseases including atherosclerosis, cancers and T2D (Salminen et al. 2012). Potential causes for inflammaging include increased production of ROS, increased secretion of pro-inflammatory cytokines and increased activation of the NF-κB pathway (Szarc vel Szic et al. 2015).

Obesity is accompanied by low-level systemic inflammation that results from activation of an inflammatory programme early in adipose expansion and which leads to permanent skewing of the immune system towards a pro-inflammatory phenotype (Saltiel and Olefsky 2017). This may be exacerbated when the increased adiposity leads to abdominal obesity (Barzilai et al. 2012; Jura and Kozak 2016) which is a critical player in the aetiology of the metabolic syndrome and in the development of common age-related diseases (Despres and Lemieux 2006). Mimamino and colleagues proposed that excess adiposity results in oxidative stress which leads to macrophage accumulation, the secretion of pro-inflammatory cytokines such as leptin, inhibition of adiponectin and, eventually, causes insulin resistance (Minamino et al. 2009). BMI and body fat percentage correlate positively with circulating concentrations of inflammatory markers, such as interleukins 6 and 8, and are inversely correlated with markers of muscle size, structure and strength (Erskine et al. 2017). Weight loss following bariatric surgery reduced circulating concentrations of C-reactive protein and leptin and reduced expression of the pro-inflammatory gene *COX-1* in the colo-rectal mucosa (Afshar et al. 2017). In addition, the widely-used anti-inflammatory agent, aspirin, increases lifespan in mice (Strong et al. 2008) and reduces the risk of death from cancer in humans (Rothwell et al. 2011). Importantly, aspirin abrogates the obesity-related increase in bowel cancer risk seen in those at high genetic risk of the disease (Movahedi et al. 2015).

The Mediterranean diet appears to be protective against inflammaging and this may be one of the mechanisms through which this dietary pattern lowers the risk of age-related diseases such as atherosclerosis (Estruch et al. 2013). In the PREDIMED Study, participants randomised to the Mediterranean Diet had lower circulating concentrations of inflammatory biomarkers including C-reactive protein and

interleukin-6 and this reduction was amplified when the Mediterranean Diet was supplemented with nuts (Casas et al. 2014). The specific foods, or nutrients, within a Mediterranean dietary pattern that are responsible for these anti-inflammatory effects are poorly understood but virgin olive oil consumption for 3 weeks reduced markers of inflammation (IL-6 and C-reactive protein) in patients with stable coronary heart disease (Fito et al. 2008). This was not observed when the patients were supplemented with refined olive oil suggesting that it is the content of phenolic compounds such as hydroxytyrosol that exert these anti-inflammatory and cardioprotective effects (Fito et al. 2008). Since multiple foods and dietary constituents affect markers of inflammation, positively or negatively, it has been proposed that an integrated measure of the "inflammatory potential" of whole diets could be characterised by derivation of a Dietary Inflammatory Index (DII) (Cavicchia et al. 2009). DII has been shown to correlate with changes in inflammation markers (Cavicchia et al. 2009) and a recent meta-analysis has shown that DII has a significant positive correlation with colo-rectal cancer risk (Shivappa et al. 2017). Analysis of data from the PREDIMED Study showed both cross-sectional and longitudinal associations between DII and telomere shortening in individuals at high CVD risk (Garcia-Calzon et al. 2015). In addition, DII predicted CVD incidence in PREDIMED participants (Garcia-Arellano et al. 2015). However, a recent systematic review and meta-analysis of human intervention studies conducted in overweight and obese individuals found only weak evidence for causal links between dietary fat and markers of inflammation (Telle-Hansen et al. 2017), which suggests that other dietary factors e.g. plant-derived polyphenols may be of greater importance in this respect.

The composition of the gut microbiome changes during ageing and, in older individuals, composition is correlated with measures of frailty, co-morbidity, nutritional status and markers of inflammation (Claesson et al. 2012). Therefore, some effects of ageing on inflammation may be mediated by alterations to the microbiome, leading to greater inter-individual variation in microbiota species e.g. lower abundance of bacteria from the *Clostridium* cluster XIVA and *Faecalibacterium prausnitzzi*. Ottaviani and colleagues have suggested that it may be possible to promote longevity by modulating the gut microbiota (Ottaviani et al. 2011). In *C. elegans,* feeding with *Lactobacilli* or *Bifidobacteria* extended lifespan significantly and protected against host infection compared with feeding with standard bacteria (Ikeda et al. 2007). One of the mechanisms proposed for the effects of the microbiome on ageing is via effects on inflammaging (Ottaviani et al. 2011; Fransen et al. 2017). Diet is a major determinant of the amount, composition and metabolic activity of the gut microbiota with changes in microbiota associated with cancer risk (O'Keefe et al. 2015). Therefore, changes in dietary intake could be a potential route to reduce systemic inflammation and to delay ageing. Feeding a diet based on whole grains, traditional Chinese medicinal foods and prebiotics to 93 obese participants for 9 weeks (Xiao et al. 2014) resulted in improvements in insulin sensitivity and lipid profiles, increased adiponectin and reductions in inflammatory markers such as TNF-α and IL-6. In addition, this intervention resulted in a significant

reduction in potentially pathogenic bacteria, such as *Enterobacteriaceae* and an increase in beneficial bacteria such as *Bifidobacteriaceae* (Xiao et al. 2014).

Carbohydrates that resist enzymatic hydrolysis in the small intestine and flow to large bowel are the major constituents of dietary fibre and provide substrates for fermentation by the commensal microbiota. Resistant starch (RS) is a component of dietary fibre which is fermented to produce butyrate (and other SCFAs) that has anti-inflammatory properties and other anti-neoplastic effects in the gut (Williams et al. 2003). The anti-inflammatory actions of butyrate appear to occur primarily via inhibition of NF-κB activation in human colonic epithelial cells and may be a consequence of butyrate's action as an epigenetic regulator via HDAC inhibition (Canani et al. 2011). These anti-inflammatory effects may be one of the mechanisms that lead to improved symptoms in inflammatory bowel disease and that protect against bowel cancer (Higgins and Brown 2013). In patients with metabolic syndrome, supplementation with RS for 12 weeks modulated the abundance of 71 bacterial operational taxonomic units, including increasing members of the *Bacteroidies* species and decreasing pathogenic *Enterococcus casseliflavus*, and raised faecal SCFA concentrations (Upadhyaya et al. 2016). This intervention was also associated with improvements in markers of inflammation including IL-6 and TNF-α, fasting glucose and adiposity. In 18 rural Malawian children, supplementation with 8.5g RS type 2 daily for 4 weeks modulated the diversity of the microbiota, including an increase in Lactobacillus and a reduction in Roseburia, and changed faecal SCFA concentrations. Surprisingly, RS significantly increased faecal calprotectin concentrations, suggesting an increase in local inflammation (Ordiz et al. 2015).

Ageing-associated loss of gastric acid production (achlorhydria) results from multiple causes including *Helicobacter pylori* infection, gastric cancer and use of multiple drugs such as antacids, H2-receptor antagonists and proton pump inhibitors. Achlorhydria increases the likelihood of small bowel bacterial overgrowth which can lead to malnutrition and reduced gut barrier function which promotes inflammation (Dukowicz et al. 2007). To test whether probiotics could improve this condition, Schiffrin and colleagues fed 23 elderly people with suspected small intestine bacterial overgrowth and 12 controls a probiotic yoghurt containing *Lactobacillus johnsonii La1* for 4 weeks (Schiffrin et al. 2009). Although the probiotic yoghurt did not affect markers of gut integrity, concentrations of endotoxins were reduced in both groups suggesting a reduction in inflammation. Further, in 53 patients with chronic liver disease, randomization to probiotic therapy (6 different organisms) for 4 weeks was effective in alleviating the bacterial overgrowth and in improving and clinical symptoms, but was ineffective in improving intestinal permeability (Kwak et al. 2014).

Concluding Remarks

There is now ample evidence that the ageing process is due to the stealthy, pervasive, and stochastic accumulation of multiple types of molecular damage which is responsible, eventually, for the key characteristics of the ageing phenotype including increased risk of frailty, disability, disease and death (Kirkwood 2005). Secondly, it is clear that the ageing process is highly individual and that the specific features of the ageing phenotype experienced at any given chronological age, or life-stage, differ between people (Belsky et al. 2015). Thirdly, the ageing trajectory is plastic and can be slowed down by lifestyle factors, including good nutrition, adequate physical activity and avoidance of smoking. In humans, plant-based diets such as the Mediterranean dietary pattern are associated with healthier ageing and lower risk of age-related disease whereas obesity accelerates ageing and increases the likelihood of most common complex diseases including CVD, T2D, dementia, musculoskeletal diseases and several cancers. As yet, there is only weak evidence in humans about the molecular mechanisms through which dietary factors modulate ageing but evidence from cell systems and animal models suggest that it is probable that better dietary choices influence all 9 hallmarks of ageing (Lopez-Otin et al. 2013). It seems likely that better eating patterns retard ageing in at least two ways including (i) by reducing pervasive damaging processes such as inflammation, oxidative stress/redox changes and metabolic stress and (ii) by enhancing cellular capacities for damage management and repair.

From a societal perspective, there is an urgent imperative to discover, and to implement, cost-effective lifestyle (especially dietary) interventions which enable each of us to age well i.e. to remain physically and socially active and independent and to minimise the period towards the end of life when individuals suffer from frailty and multi-morbidity. Such interventions are likely to be more acceptable, and to deliver greater cost-effectiveness, when they are implemented before individuals begin to experience significant age-related functional impairment and we have suggested that the period around retirement may represent an important window for such interventions (Celis-Morales et al. 2015). Research on the development and testing of intervention designed to enhance ageing are limited by the lack of reliable biomarkers of ageing which could be used as surrogate endpoints in such trials (Lara et al. 2015). Building on the concept of the healthy ageing phenotype (Franco et al. 2009), we have proposed a panel of (bio)markers of physiological and metabolic health, physical capability, cognitive function, social wellbeing and psychological wellbeing that may have utility as outcomes measures in lifestyle-based intervention studies (Lara et al. 2013). In addition, in pilot studies, we have shown that an internet-based platform which delivers personalised advice and support to improve behaviours, including changing eating patterns towards the Mediterranean dietary pattern (O'Brien et al. 2016), in combination with use of this panel of outcome measures, is acceptable to people in the retirement transition (Lara et al. 2016). The next stage will be to attempt to deliver such lifestyle-based interventions at scale and to determine their uptake, efficacy and cost-effectiveness.

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Chapter 14
Role/s of 'Antioxidant' Enzymes in Ageing

Elizabeth Veal, Thomas Jackson, and Heather Latimer

Abstract Reactive oxygen species (ROS), generated externally and during aerobic metabolism, are a potent cause of cell damage. Oxidative damage is a feature of many diseases and ageing, including age-associated diseases, such as diabetes, cancer, cardiovascular and neurodegenerative diseases. Indeed, this association helped lead to the widely expounded 'Free Radical Theory of Aging', proposing that the accumulation of ROS-induced damage is the underlying cause of ageing. In the last decade, it has become apparent that ROS play more complex roles in ageing than simply causing damage. This includes the induction of signalling pathways that protect against/repair cell damage. Cells encode a variety of enzymes that metabolise ROS, some of which reduce them to less reactive species. In this chapter, we review the evidence that manipulating the levels of these enzymes has any effect/s on ageing. We will also highlight a few examples illustrating why it is an oversimplification to describe the activities of some of these enzymes as 'antioxidants'. We discuss how these studies have helped refine our view of how ROS and ROS-metabolising enzymes contribute to the ageing process.

Keywords Peroxiredoxins · Superoxide dismutase · Catalase · Signal transduction · Hydrogen peroxide · Yeast · Flies · Worms · Mice · Antioxidants

Introduction

The photosynthesis-driven rise in atmospheric oxygen and subsequent evolution of oxygen-dependent eukaryotes, resulted in a new danger to life, in the form of reactive oxygen species (ROS). Generated by the reduction of oxygen, both in

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response to external factors, such as irradiation, and as an unavoidable feature of aerobic metabolism, ROS readily oxidise cellular macromolecules causing potentially cytotoxic levels of cell damage. Accordingly, exposure to ROS has provided a strong selection pressure driving the evolution of enzymatic and non-enzymatic 'antioxidants' to remove ROS before they cause irreversible damage to cell components. However, the prevalence of oxidative cell damage as a feature of ageing and age-associated diseases suggests that ROS defences can be inadequate, and led to the proposal in the 1950s, of a Free Radical Theory of Aging, with ROS-induced damage as a highly plausible 'cause' of ageing (Harman 1956). Consistent with this theory, was the epidemiological evidence that diets high in plant sources of non-enzymatic 'antioxidants', protect against heart disease and cancer. This suggested that specifically increasing 'antioxidants' might be a means to reduce the risk of these diseases, and even slow the aging process itself. This stimulated extensive investigation of the potential of nutritional supplements, such as vitamins C and E and carotenoids, to augment ROS defences preventing oxidative damage and disease. However, while a plant-based diet, high in fruit and vegetables, does protect against some diseases, there remains little evidence that dietary antioxidant supplements are an effective strategy to delay aging, or reduce the risk of age-associated diseases, such as cancer (Fortmann et al. 2013). In fact, in some studies increased intake of antioxidants has been found to increase risk of certain cancers (Klein et al. 2011). One possible explanation for the failure of these studies is that it reflects the impossibility of ensuring these antioxidants reach the cellular location, at the point at which they are needed, to neutralise ROS. Thus, in recent decades, attention has moved towards the possibility that manipulating the levels of endogenous ROS-detoxifying 'antioxidant' enzymes might be a more effective way to protect against ROS-induced damage. In fact, it has been proposed that the beneficial effects of plant-rich diets or small doses of some dietary 'antioxidants' could reflect their potential to induce increased expression of these enzymes (Chikara et al. 2018). Genetic studies examining the *in vivo* function of these enzymes have been used to explore these possibilities, and test the Free Radical Theory of Ageing. In this chapter we will review these studies, which have examined the roles of the various enzymes involved in ROS metabolism in ageing. We will discuss how some unexpected findings from these studies have contributed to the prevailing view that, although oxidative damage is a cause of loss of tissue function, ROS are not the primary 'cause' of aging. Indeed, ROS can have physiological roles as signalling molecules, and even mediate the pro-longevity effects of certain regimes (For reviews *see* Ristow and Zarse 2010; Veal and Day 2011). As we will discuss, rather than simply removing ROS, growing evidence suggests that one family of ROS-metabolising enzymes, the peroxiredoxins, may actually be important for transducing these ROS signals and that this may underlay some of their pro-longevity functions.

Reactive Oxygen Species and ROS Metabolism

In vivo *Sources and Properties of Reactive Oxygen Species*

Reactive oxygen species is a broad term used to refer to various reduced forms of oxygen. These include the three main ROS that are generated endogenously by aerobic metabolism; peroxides($O_2\bullet^{2-}$), hydroxyl radicals($OH\bullet$) and superoxides($O_2\bullet^-$) (Fig. 14.1). However, it is important to note that there is an enormous range in the reactivity of these ROS, as well as in their other biophysical properties. As we will discuss, this means that they have very different potentials for causing oxidative damage and also for the types of defences/protective mechanisms that cells employ against this damage. Superoxide anions are produced by the addition of one electron to oxygen. A major source of $O_2\bullet^-$ is unavoidable leakage of electrons from the electron transport chain, but $O_2\bullet^-$ is also generated deliberately by NADPH oxidases (NOX), Lipoxygenase and Xanthine oxidase. $O_2\bullet^-$ is highly reactive and readily oxidises cellular macromolecules, obtaining an additional

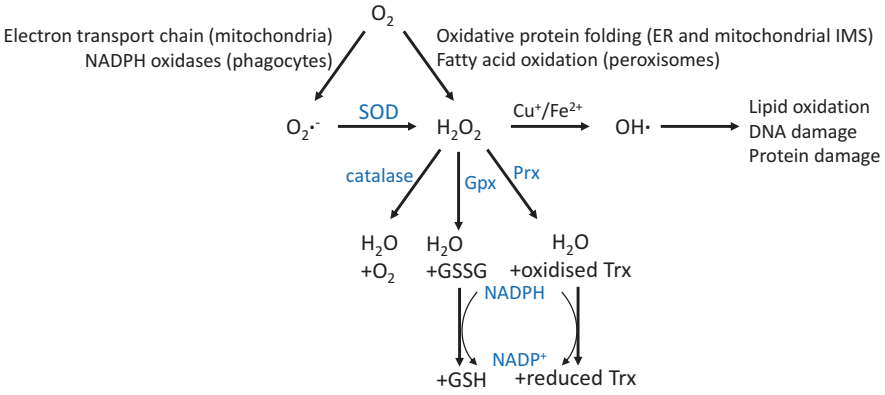

Fig. 14.1 Some of the biological sources of reactive oxygen species and the enzymatic systems reducing them. In aerobic metabolism oxygen is used as an electron acceptor. This can result in the formation of hydrogen peroxide (H_2O_2) during oxidative protein folding (Ero1-driven formation of protein disulphides in the ER and Erv1-driven formation of protein disulfides in the mitochondrial intermembrane space) and during peroxisomal fatty acid oxidation. The partial reduction of oxygen during the electron transport chain can produce superoxide anions ($O_2\bullet^-$). Superoxide anions are also produced by the action of NADPH oxidases activated by pathogens in innate immune cells or by growth factors/cytokines in other cell types. Superoxide anions are reduced to less reactive hydrogen peroxide by the action of superoxide dismutases (SODs). Hydrogen peroxide is reduced by catalase, glutathione peroxidases (Gpx) and Peroxiredoxins (Prx). However, the Fenton reaction with reduced iron or copper causes it to be split to generate highly reactive hydroxyl radicals ($OH\bullet$) which will oxidise the first cellular molecule they encounter, often generating further radicals and thus causing significant free radical-mediated cell damage. Glutathione or thioredoxin is oxidised during the reduction of peroxides by Gpx or Prx. Subsequently oxidised Trx and glutathione are reduced by the action of glutaredoxins, glutathione reductase or thioredoxin reductase using electrons provided by NADPH

electron, that converts it to peroxide ion ($O_2\bullet^{2-}$). Thus high levels of NOX-generated superoxide are an important part of the weaponry of innate immune cells, but lower levels of NOX-generated superoxide are also used in signalling. At physiological pH, $O_2\bullet^{2-}$ can also become protonated, forming hydrogen peroxide (H_2O_2). H_2O_2 is much more stable/limited in its reactivity than superoxide, enabling it to diffuse over a larger distance. H_2O_2 is also apolar, allowing it to pass through membranes. H_2O_2 is produced through a dismutation reaction, catalysed by superoxide dismutase (SOD) but also by the very many metabolic reactions in which oxygen is used as an electron acceptor, including fatty acid oxidation and oxidative protein folding in the ER. The main possibility for H_2O_2-caused damage to DNA, proteins or lipids, comes following its reduction to hydroxyl radicals (OH•) by reduced iron or copper ions (Fe^{2+} or Cu^{+}). Hydroxyl radicals are so highly reactive that they will abstract electrons from the nearest available molecule, often generating radicals to propagate further damaging oxidation reactions, for example, in the lipid peroxidation of membranes. Hence, cell defences specifically against hydroxyl radicals are largely limited to mechanisms that prevent their generation or repair the damage they cause. This includes strictly limiting the availability of reduced copper and iron ions, by sequestering these metals with chaperone proteins, as well as a host of enzymes that remove peroxides (Faulkner and Helmann 2011). A few of the sources of ROS and the mechanisms by which they are metabolised are illustrated in Fig. 14.1.

H_2O_2 is also reactive with deprotonated cysteine thiols. However, the low pH in the cytoplasm means that most protein cysteine thiols are protonated and hence relatively resistance to oxidation by H_2O_2. Amongst the exceptions to this, are the active site cysteines intimately involved in the catalytic activity of certain enzymes (For a review *see* Winterbourn 2008). These include metabolic enzymes, such as glyceraldehyde phosphate dehydrogenase (Grant et al. 1999), protein tyrosine phosphatases (Meng et al. 2002) and ubiquitin/ubiquitin-like conjugating enzymes (Bossis and Melchior 2006; Doris et al. 2012; Veal and Day 2011). Accordingly, these enzymes are sensitive to inactivation by H_2O_2. Indeed, the sensitivity of these enzymes to inactivation by H_2O_2-induced oxidation can be an important protective cell response to increased H_2O_2 (Day et al. 2012; Peralta et al. 2015). Moreover, H_2O_2 is utilised as a signalling molecule to regulate diverse cellular processes, including cell division, migration and differentiation (Fig. 14.2). Indeed, H_2O_2 signals have now been shown to be involved in regulating many fundamental biological responses to changes in internal and external environment, including those affecting ageing (For reviews *see* Sanz 2016; Veal and Day 2011). Thus, there is a growing appreciation that the enzymes that metabolise different types of ROS not only remove damaging ROS, but can impact on these H_2O_2–dependent signalling processes in both positive and negative ways.

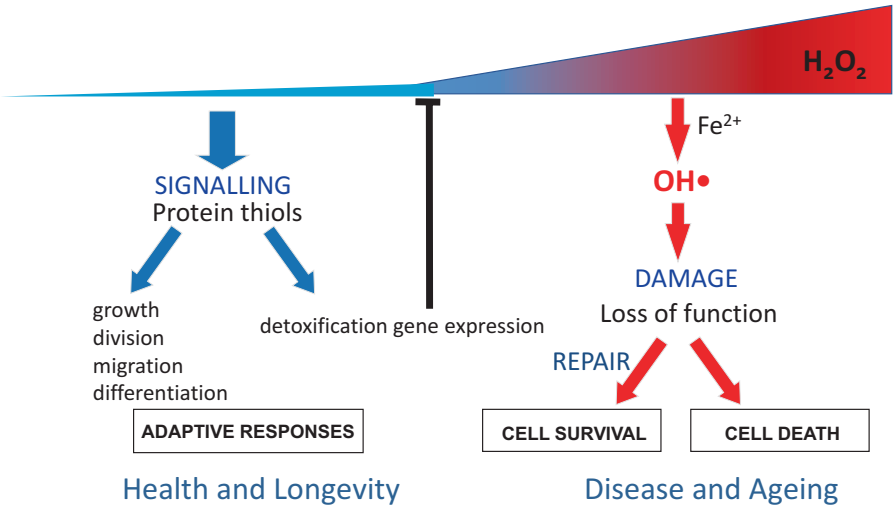

Fig. 14.2 Roles of ROS in physiology and ageing. Although a potential source of damage, hydrogen peroxide (H_2O_2) also has important signalling functions, mediated through the oxidation of protein thiols. These include adaptive transcriptional responses to small increases in hydrogen peroxide that upregulate detoxification and repair enzymes, protecting against the damaging effects of higher levels. Maintenance of redox homeostasis, by regulation of these responses and repair mechanisms, is important for normal health and longevity

ROS-Metabolising Enzymes

ROS-metabolising enzymes can be broadly sub-divided, based on the particular ROS that they reduce, into superoxide-metabolising and peroxide-metabolising. Here we have further subdivided the peroxidases into two groups, heme peroxidases and thiol peroxidases based on the source of electron donors for the reduction of peroxide. In addition, when considering how these enzymes impact on ROS metabolism, it is vital to consider a number of other factors: [1] their proximity to the ROS source; although H_2O_2 can cross membranes, both by diffusion and transported by aquaporins, superoxide will have a much more limited diffusion. Accordingly, ROS-metabolising enzymes will likely only impact on ROS generated in the compartment in which they are located. [2] The levels of essential cofactors, NADPH or reduced glutathione and recycling enzymes in the compartment in which the 'antioxidant' enzymes are located. This is particularly relevant for thiol peroxidases, which work as a series of redox couples and therefore actually promote the oxidation of glutathione, thioredoxin and NADPH, such that if any of these are limiting their activity will inhibit the activity of other enzymes requiring these cofactors and promote the oxidation of proteins (Brown et al. 2013; Day et al. 2012).

Superoxide Dismutases

Superoxide dismutases (SODs) catalyse the conversion of superoxide ($O_2\bullet^-$) to less-reactive H_2O_2. There are multiple SOD isoforms, grouped according to their metal centre (Manganese or Copper/Zinc) and amino acid constituency. There are at least two intracellular SODs in most eukaryotic cells; MnSOD which functions in the mitochondria, and Cu/Zn SOD, which function in the cytoplasm. In addition, there are also SODs that are targeted to the extracellular compartment (For a review s*ee* Valko et al. 2006). Although SOD activity is critical for removing damaging superoxide anions, as generators of H_2O_2, SOD can also potentially promote H_2O_2–signalling and increase hydroxyl radical production.

Heme Peroxidases; Catalase, Cyt C Peroxidase

Heme peroxidases describes a diverse group of peroxidases that use heme-based electron transfers to reduce peroxides in a variety of ways. In some cases, such as myeloperoxidase (MPO), an important neutrophil-based contributor to innate immune defences, the enzymatic activity generates even more cytotoxic chemicals, hypochlorous acid and tyrosyl radicals as anti-microbicides. However, other heme peroxidases, for example, catalase, have a ROS-protective activity, reducing peroxides to water or alcohols. In mammals, the majority of catalase is targeted to peroxisomes, organelles where metabolic reactions, particularly the oxidation of fatty acids, lead to the generation of hydrogen peroxide. However, catalase is also targeted to other organelles and found in the cytosol. CytC peroxidase (CCP) in yeast, has been shown reduce ROS levels in the mitochondrial intramembrane space where it is located (Martins et al. 2013, 2014).

Thiol Peroxidases; Glutathione and Thioredoxin Peroxidases

Thiol peroxidases are ubiquitous peroxidases that use reversibly oxidised cysteines (peroxiredoxins), or selenocysteines (some glutathione peroxidases), in the reduction of peroxides. The common feature of their mechanism is a peroxide-reacting/peroxidatic cysteine/selenocysteine that is deprotonated and therefore highly susceptible to oxidation by peroxide. In the case of cysteine-based thiol peroxidases, the initial oxidation to a sulfenyl group is then stabilised by formation of a disulphide with another cysteine. This ‘resolving’ cysteine may be found elsewhere in the same polypeptide (e.g. atypical 2-Cys Prx), in another protein or in glutathione. This disulfide bond is subsequently reduced using electrons from NADPH. Thioredoxin and thioredoxin reductase or glutaredoxin are required to transfer the electrons from NADPH to reduce the oxidised form of the thiol peroxidase and restore it to its original state. Thus it may be more appropriate to think of thiol peroxidases as the composite of a series of redox couples rather than enzymes (Pillay et al. 2009) (Fig.14.1).

Broadly speaking thiol peroxidases can be separated into different classes, thioredoxin peroxidases and glutathione peroxidases. Although, ostensibly, this separation is based on the reductant used in the catalytic cycle, in reality enzymes classified, based on their structure/*in vitro* evidence, with some 'glutathione peroxidases' actually reduced by thioredoxin *in vivo*. In animals glutathione peroxidase is one of a handful of proteins in which the 21st amino acid, selenocysteine, is incorporated. The underlying reason why animals have evolved to incorporate selenocysteine rather than cysteine into their glutathione peroxidases has been elusive. However, recent evidence supports the hypothesis that the presence of selenocysteine may be important to inhibit 'over-oxidation' to inactive forms (Ingold et al. 2018).

Peroxiredoxins (Prx) are a family of extremely abundant, highly conserved thioredoxin peroxidase antioxidant enzymes (Wood et al. 2003b), involved in the detoxification of H_2O_2, peroxynitrite and other hydroperoxides. The peroxiredoxin family of thiol peroxidases contains many sub-families, classified based on the absence of a resolving cysteine (1-Cys Prx) and whether the resolving cysteine is located in the same (atypical 2-Cys *e.g.*Prx5) or a different (typical 2-Cys *e.g.* Prx1,2 3 and 4) peroxiredoxin molecule. 2-Cys Prx can be further subdivided based on sequence features, some of which determine their localisation; Prx1 and 2 (cytoplasm), Prx3 (mitochondrial) and Prx4 (ER and secreted) (Wood et al. 2003b).

Peroxiredoxins

The largest group of Prx, typical 2-Cys Prx, employ two catalytically-active cysteine residues in their role to reduce H_2O_2. Under non-stressed conditions, non-covalent dimers of typical 2-Cys Prx interact to form decamers. Once the peroxidatic cysteine thiol has become oxidised by H_2O_2 to a sulphenic acid, the sulphenic acid then reacts with the resolving cysteine of a neighbouring Prx molecule and becomes stabilised due to the formation of an intermolecular disulphide bond. The 2-Cys Prx is restored to its original active redox state through the thioredoxin system; the disulphide bond between the two catalytic cysteines becomes reduced by thioredoxin (Trx) (Wood et al. 2003b).

In response to higher concentrations of H_2O_2, the sulphenic acid derivative of eukaryotic, but not most prokaryotic, 2-Cys Prx is readily 'hyperoxidised' to sulphinic and sulphonic acid derivatives (Wood et al. 2003a). In contrast, atypical 2-Cys Prx, Prx5, and 1-Cys Prx, Prx6 are much less sensitive to hyperoxidation of their peroxidatic cysteine. Hyperoxidation inactivates the thioredoxin peroxidase activity of the 2-Cys Prx. The hyperoxidised cysteine cannot be reduced by thioredoxin, however, almost all eukaryotes encode 'sulphiredoxin' which specifically reduce the sulphinylated peroxidatic cysteine of 2-Cys Prx, restoring their catalytic activity (Biteau et al. 2003). Srx1 specifically catalyses the reduction of hyperoxidised cysteine residues in typical 2-Cys Prx, as sulphinic acids in other proteins, such as atypical 2-Cys Prx, 1-Cys Prx and GAPDH, cannot be reduced by Srx1 (Woo et al. 2005). Intriguingly, one of the most commonly used models for studying

aging, *C. elegans* has lost the sulfiredoxin gene and is unable to reduce the hyperoxidised forms of its 2-Cys Prx (Olahova et al. 2008; Thamsen et al. 2011).

The discovery that eukaryotic 2-Cys Prx have evolved to be sensitive to inactivation of their thioredoxin peroxidase activity by peroxide, was accompanied by a proposal that this may have co-evolved with the utilisation of hydrogen peroxide as a signalling molecule. Accordingly, it was proposed that in eukaryotes 2-Cys Prx acted as a 'Floodgate', preventing peroxides from reacting with other cell components, and that their sensitivity to hyperoxidation was important to allow peroxide to accumulate/persist long enough to oxidise signalling proteins (Wood et al. 2003a). Although there is now much more evidence that H_2O_2 is used as a signalling molecule, regulating a range of biological responses, there remains no good evidence to support this 'Floodgate model'. Instead, there is evidence that hyperoxidation of 2-Cys Prx is important to limit the damage to other proteins and promotes cell/organismal survival, particularly under acute stress conditions (Day et al. 2012; Hanzen et al. 2016; Jang et al. 2004; Olahova et al. 2008). There are at least 2 ways in which Prx hyperoxidation can protect cells; by preserving thioredoxin, for the reduction of oxidative damaged proteins, and by promoting alternative 'chaperone' activities of 2-Cys Prx that protect against protein aggregation (Day et al. 2012; Hanzen et al. 2016; Jang et al. 2004) (for a review *see* Veal et al. 2018). Indeed, it is important to remember that, as prevalent thioredoxin substrates, although Prx activity detoxifies peroxides, it also promotes the oxidation of thioredoxin family proteins. Accordingly, Prx activity actually promotes protein oxidation, particularly if thioredoxin reductase activity is limiting (Brown et al. 2013; Cao et al. 2014; Dangoor et al. 2012; Tavender et al. 2010; Zito et al. 2010) (Fig. 14.3).

Roles of ROS-Metabolising Enzymes in Ageing

Models for Studying Aging

The ability of yeast cells to survive long periods (weeks) in stationary phase/quiescence, then resume growth and proliferate to form a colony has allowed unicellular yeast to become an accepted model of chronological ageing. Of course, there are likely to be differences between some of the mechanisms governing the survival of yeast and animal cells. However, as comparative studies in the highly divergent budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* have allowed the identification of key features of other processes, such as cell division, there is the expectation that these studies will reveal conserved and species-specific aspects of cellular ageing. Notably, the asymmetric cell division of *S. cerevisiae* leads to 'scars' on the cell surface following the budding of a daughter cell. Remarkably, it has been established that each mother cell undergoes a finite (20–30) number of cell divisions before it is unable to undergo further replications. Thus many factors which influence this 'replicative lifespan' have also been identified. Although some of the mechanisms involved are yeast-specific, nevertheless, the

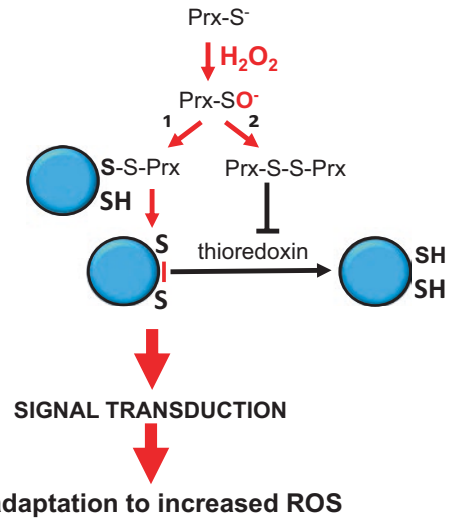

Fig. 14.3 2-Cys peroxiredoxins play multiple roles in promoting peroxide signal transduction. 2-Cys peroxiredoxins (Prx) have been shown to positively promote the oxidation of redox-sensitive signalling proteins, such as the example shown in blue, by 2 mechanisms: 1. the sulphenylated Prx acts as a direct redox transfer protein e.g. Sty1 MAPK (Veal et al. 2004), Ask1 MAPKKK (Jarvis et al. 2012) and STAT3 transcription factor 2 (Sobotta et al. 2015). Prx disulphides competitively inhibit thioredoxin-mediated reduction of the oxidised signalling protein e.g Pap1 (Brown et al. 2013)

maintenance of replication-competence is an important aspect of ageing in animals, for example, in maintenance of competent stem cells to repair tissues during ageing. The presence of mutant libraries and a wealth of robust quantitative data continues to facilitate advances in genetic studies of ageing using both yeast models.

Like many other areas of research, research into ageing has also benefitted enormously from work undertaken in the invertebrate model animal, the nematode worm *C. elegans*. *C. elegans* genetic homogeneity (predominantly reproducing as a self-fertilising hermaphrodite) and short lifespan (a few weeks under standard laboratory conditions) continues to allow the rapid investigation of how genetic and environmental changes affect the rate of ageing of this simple animal. The discovery that ageing is a genetically regulated process, with mutations that impair the function of mitochondria, insulin-signalling or mimic dietary restriction, often leading to dramatic increases in *C. elegans* lifespan, has revealed potential for positively manipulating the ageing process. This has also motivated substantial efforts to understand the underlying cause/s of ageing. A key component underpinning efforts to answer this question has been the utilisation of multiple model systems to study the causes of ageing. These models, ranging from unicellular yeast to long-lived naked mole rats, continue to provide researchers with great tools to test various ageing theories. Importantly, the use of multiple, diverse models has enabled researchers to test whether various theories hold true for all systems, or only a sub-

set. The role/s of free radicals/ROS in ageing have been intensively studied in many of these models. Here we review the evidence provided by studies investigating the effects on ageing/lifespan of genetically manipulating the levels of different ROS-metabolising enzymes in yeast, worms, flies and mice that has led to a more nuanced view of the role of ROS in ageing.

Superoxide Dismutases

The potential reactivity of superoxide with cellular components and the prevalence of oxidative damage in aged tissues/disease suggested that SODs may protect against this damage and against ageing. Further evidence to suggest this was the discovery that *sod-3*, encoding a mitochondrial MnSOD, was amongst the genes that are strongly upregulated in long-lived mutant *C. elegans* in which insulin-signalling is reduced (Honda and Honda 1999; Libina et al. 2003). Hence, each SOD-encoding gene has been systematically disrupted and over-expressed in *C. elegans*, and a host of other models (Table 14.1). There are some cases where loss of SOD activity is deleterious to viability/lifespan (Longo et al. 1996) (Table 14.1). However, notably, loss of all 5 SODs in *C. elegans* causes no significant reduction of lifespan (Van Raamsdonk and Hekimi 2012). Moreover, unexpectedly, in several cases, genetic loss of SOD-encoding gene/s has been shown to actually increase lifespan (Table 14.1). Overall, these studies reveal a complex picture, with no consistent correlation between SOD levels and longevity. One possible underlying cause of these complex effects is that genetically manipulating SOD levels will inevitably impact on the levels of H_2O_2 (Fig.14.1). For example, loss of SOD activity may reduce the levels of H_2O_2 that has important signalling functions. Indeed, although loss of SOD1 activity in mice has been shown to precipitate increases in age-associated muscle loss, the absence of this phenotype in mice where only expression of SOD1 in muscle is ablated, suggests that this progeric phenotype is due to cell non-autonomous effects on signalling rather than directly increasing ROS-induced damage (Sakellariou et al. 2018). Moreover, as loss of a gene with an important function may reduce lifespan for a number of reasons, not necessarily related to ageing, in addition to biomarkers of aging, such as loss of muscle/impaired movement, it has become accepted that a better test of a pro-longevity 'geronto-gene' is whether its increased expression can cause an increase in lifespan. This has provided some evidence for an increase in lifespan in animals expressing increased levels of SOD (Table 14.1). For instance, overexpression of either *C. elegans sod-1* or *sod-2* has been shown to increase *C. elegans* lifespan. However, despite increasing lifespan, overexpression of *sod-1* in these studies was also associated with increased H_2O_2 levels and oxidative protein damage (Cabreiro et al. 2011). Indeed, evidence suggests that the increased lifespan of these animals reflects increased activation of stress-protective transcriptional responses, rather than increased SOD-mediated protection against ROS-induced damage (Cabreiro et al. 2011). The links between increased SOD activity and longevity have also been extensively

Table 14.1 Summary of some of the genetic studies in model organisms investigating the role of superoxide dismutase enzymes on lifespan/survival

Species	Gene	Genetic manipulation	Effect on lifespan/ageing compared with wild-type	References
S. cerevisiae	*Sod1 Sod2*	Overexpression	Increased both chronological and replicative lifespan in the presence of high copper. Reduced chronological and replicative lifespan in normal conditions.	Harris et al. (2005)
S. cerevisiae	*Sod2*	Overexpression	Increase in chronological lifespan.	Harris et al. (2003)
			Shortened replicative lifespan.	
S. cerevisiae	*SOD1*	Knockout	reduced chronological lifespan with *SOD1SOD2* double mutant the most severe defect	Longo et al. (1996)
	SOD2			
S. cerevisiae	*SOD1*	Knockout	Replicative life span decreased by 40% for *SOD1*Δ and 72% for *SOD2*Δ mutants.	Unlu and Koc (2007)
	SOD2			
S. pombe	*sod2*	Knockout	Reduced chronological lifespan	Ogata et al. (2016)
C. elegans	*sod-1*	Transgenic overexpression	*sod-1*: *33%* increase in median lifespan dependent on *daf-16* and partially dependent on *hsf-1*. *sod-2*: 25% increase in mean and maximum lifespan dependent on *daf-16* and partially dependent on *hsf-1*	Cabreiro et al. (2011)
	sod-2			
C. elegans	*sod-1*	RNA interference	*sod-1*: slight reduction in lifespan	Yang et al. (2007)
	sod-2		*sod-2*: no change in lifespan	
C. elegans	*sod-2*	*sod-2 sod-3* double mutants	No effect	Gruber et al. (2011)
	sod-3			
C. elegans	*sod-1 sod-2 sod-3, sod-4 sod-5*	Loss of function mutant alleles	No effect, including *sod-1sod-3sod-5* triple mutant and *sod-1sod-2sod-3sod-4sod-5* quintuple mutant lacking any SOD activity, except *sod-2* mutant and *sod-2;sod-3;sod-5* and *sod-1;sod-2;sod-4* mutant worms were all long-lived.	Van Raamsdonk and Hekimi (2009, 2012)
C. elegans	*sod-1 sod-2 sod-3, sod-4, sod-5*	Loss of function mutant alleles and transgenic overexpression	No effect except loss of *sod-1* caused 15–31% decrease in mean lifespan and overexpression of *sod-1* slightly increased lifespan.	Doonan et al. (2008)
D. melanogaster	*Sod1*	Overexpression	No effect	Seto et al. (1990)) and Orr and Sohal (1993)

(continued)

Table 14.1 (continued)

Species	Gene	Genetic manipulation	Effect on lifespan/ageing compared with wild-type	References
D. melanogaster	*Sod1*	Overexpression	Up to 48% increase in mean lifespan	Sun and Tower (1999)
D. melanogaster	*Sod2*	Overexpression	15% increase in mean and maximum lifespans	Sun et al. (2002)
D. melanogaster	*Sod1*	Null mutant	Reduced lifespan	Phillips et al. (1989)
D. melanogaster	*Sod2*	Null mutation	Flies with no *sod-2* activity survived no longer than 36 h.	Duttaroy et al. (2003)
D. melanogaster	*Sod2*	Mutant alleles	Flies expressing 50% *sod-2* activity had mean and maximum life spans reduced by 20–24%.	Paul et al. (2007)
			Flies expressing only 25% *sod-2* activity had shortened mean and maximum life spans by 38–43%.	
D. melanogaster	*Sod2*	Knockdown in all or specific tissues	Ubiquitous or muscle-only knockdown: more than 50% reduction in maximum and median lifespan. Nervous system-only knockdown: less severe lifespan reductions.	Martin et al. (2009)
D. melanogaster	*Sod2*	RNA interference	76–84% reduction in lifespan.	Kirby et al. (2002)
Mus Musculus	*sod1*	Targeted inactivation	30% reduction in lifespan	Elchuri et al. (2005)
Mus Musculus	*sod2*	Overexpression	No effect	Jang et al. (2009)
Mus Musculus	*sod1 sod2*	Overexpression	No effect	Perez et al. (2009)
Mus Musculus	*sod1*	Knockout	*sod1*: shortened lifespan	Sentman et al. (2006)
	sod3 (EC-SOD)		*sod3*: no effect even in *sod1* mutant background	

investigated in flies, but with some conflicting results (Seto et al. 1990; Sun and Tower 1999). In mice the picture is also unclear, with some reporting that genetically increasing the expression of SOD genes increases lifespan, whereas others finding no significant effect (Hu et al. 2007; Jang et al. 2010; Perez et al. 2009) (Table 14.1). Thus, although mutations and dietary changes that increase lifespan are associated with increased *sod* gene expression, it seems that increased SOD activity alone is insufficient to consistently delay ageing.

Catalase

Many studies have investigated whether manipulating the levels of catalase, alone or in conjunction with SOD activity affects lifespan (Table 14.2). Unexpectedly, it was found that loss of the *S. cerevisiae* catalase gene, *CTL1*, increases chronological lifespan (Mesquita et al. 2010; Weinberger et al. 2010). Although there has been a high throughput screen of the *S. pombe* gene deletion library for mutants that affect chronological lifespan (survival in quiescence) a mutant lacking the single catalase, *ctt1*, was not detected amongst either the mutants that were longer or shorter-lived than their counterparts (Sideri et al. 2014). *C. elegans* encode 3 catalases; predicted cytoplasmic catalases, CTL-1 and CTL-3, and peroxisomal CTL-2. Although loss/reduced expression of these genes has a minimal effect on the lifespan of wild-type animals, RNAi targeting either *ctl-1* or *ctl-2* significant reduces the long-lifespan of mutants with defective insulin-signalling, suggesting that the increased expression of these genes in these animals may contribute to their long lifespan (Murphy et al. 2003). Interestingly, overexpression of all 3 catalase *C. elegans* genes causes a large proportion of animals to rupture. The dependence of this phenotype on the presence of *sod-2*, suggesting that there may be a positive role of H_2O_2 in maintenance of tissue structure. However, overexpression of *ctl-1, ctl-2 and ctl-3* had no effect on lifespan, even when combined with overexpression of *sod-2* (Cabreiro et al. 2011). Similarly, overexpression of catalase alone had little effect on the lifespan of Drosophila (Table 14.2). Thus, while catalase may be important for the increased longevity associated with reduced insulin signalling, in general, overexpression of catalase alone is insufficient to increase lifespan. In mammals, targeted overexpression of catalase in different cellular locations has provided some evidence that overexpression in the mitochondria can increase the lifespan of mice, whereas increasing the concentration of catalase in other organelles has little, if any, effect on ageing (Schriner et al. 2005). However, it remains undetermined whether increased mitochondrial catalase extends lifespan by removing peroxides or by triggering pro-longevity stress responses, such as the mitochondrial UPR (Shpilka and Haynes 2018).

Notably, overexpressing both SOD and catalase has been shown to extend lifespan in yeast and flies, suggesting that it may be important to balance the production and removal of H_2O_2 (Fabrizio et al. 2003; Orr and Sohal 1994). However, more recent studies, suggest that differences in the genetic background may also have contributed to differences in lifespan in flies, highlighting that it is important to eliminate the possibility of genetic background differences when investigating the effects of any specific gene or genes on lifespan (Sun and Tower 1999). Moreover, overexpression of combinations of SOD and catalase genes does not appear to increase the lifespan of mice suggesting that the levels of these ROS-metabolising enzymes do not limit the lifespan of mammals (Perez et al. 2009).

Table 14.2 Results of some of the studies that have investigated the effect of catalase on lifespan by genetically manipulating catalase expression in model organisms

Species	Genes	Genetic manipulation	Effect on lifespan/ageing	References
Hansenula polymorpha	*CAT*	Gene knockout	Small increases or decreases in the chronological lifespan depending on the growth conditions	Kawalek et al. (2013)
Saccharomyces cerevisiae	*CTA1, CTT1*	Gene knockout	Increased chronological lifespan.	Mesquita et al. (2010)
Caenorhabditis elegans	*ctl-1, ctl-2, ctl-3*	Overexpression in *sod-1* overexpressing worms	Small reduction in lifespan	Doonan et al. (2008)
Caenorhabditis elegans	*ctl-1, ctl-2*	RNA interference in long-lived *daf-2* mutant worms	Reduction in lifespan	Murphy et al. (2003)
Caenorhabditis elegans	*ctl-1, ctl-2*	Null mutant alleles	*ctl-1* no effect 16% reduction in mean lifespan of *ctl-2* mutant	Petriv and Rachubinski (2004)
Drosophila melanogaster	*Cat*	Overexpression	No effect on lifespan	Orr and Sohal (1993) and Sun and Tower (1999))
Drosophila melanogaster	*Cat*	Overexpression in *Sod2*-overexpressing background	No effect on lifespan	Sun et al. (2002)
Drosophila melanogaster	*Cat*	Overexpression of mitochondria-targeted catalase	reduced mean lifespan by between 4–18%.	Mockett et al. (2010)
Drosophila melanogaster	*Cat*	Overexpression	Catalase overexpression suppressed lifespan extension conferred by mild mitochondrial stress.	Owusu-Ansah et al. (2013)
Mus Musculus	Human catalase	Overexpression in particular cell compartments	Peroxisomal and nuclear overexpression no effect Expression of mitochondrially-targeted catalase increased mean and maximum lifespan.	Schriner et al. (2005)
Mus Musculus	Human catalase and SOD genes	Overexpression	Overexpression of catalase no effect, either with or without Cu/ZnSOD and MnSOD co-overexpression	Perez et al. (2009)

Glutathione Peroxidase

S. cerevisiae encodes 3 glutathione peroxidases. Of these, only *GPX1* has been shown to affect ageing, with loss of *GPX1* reducing replicative lifespan, compared with wild-type (Schleit et al. 2013). *S. pombe* encodes a single glutathione peroxidase. Gene expression of *gpx1* is strongly induced as cells enter stationary phase and the survival of *gpx1* mutant cells in stationary phase in rich media has been shown to be greatly impaired, suggesting that this induction is required to inhibit chronological ageing (Lee et al. 2008). However, *gpx1* mutants were also one of the long-lived mutants identified by a high throughput screen of the *S. pombe* gene deletion library for genes; under competitive growth conditions, increasing cell survival in quiescence induced by low nitrogen (Sideri et al. 2014). There are a number of possible explanations why opposite effects on lifespan should have been observed in these studies. For instance, differences in the growth media (rich versus defined), the means to promote exit from growth phase (glucose depletion or nitrogen limitation) and conditions under which 'chronological lifespan' was determined (survival in homogenous single allele cultures or competitive cultures). In addition, the screen by Sideri et al. (2014) was carried out in a prototrophic genetic background, removing any possible confounding effects associated with the presence of auxotrophic markers in the wild-type or *gpx1* mutant. In contrast, in the earlier study, the wildtype and *gpx1* mutant both had mutant alleles conferring adenine and leucine auxotrophy, but only the wild-type strain was also auxotrophic for uracil, with the *gpx1* gene replaced with a gene to restore uracil biosynthesis in the *gpx1*Δ mutant (Lee et al. 2008; Sideri et al. 2014). Nevertheless, it remains unclear whether Gpx1 promotes or inhibits the chronological ageing of *S. pombe* and whether its role differs according to different growth conditions. In *C. elegans* there are 8 Gpx genes, all of which utilise cysteine rather than selenocysteine. Although the extent of redundancy between these genes is unclear, a mutant lacking 4 of these genes has been shown to be short-lived (Sakamoto et al. 2014). Gpx genes have been individually overexpressed and knocked out in mice. However, there are currently no reports that loss of function of any Gpx gene shortens lifespan, or that overexpression has an anti-ageing effect. Indeed, although *gpx4* is essential for mouse fertility, mutants in which 1 copy of the gene is lost, reducing Gpx4 levels by about 50%, are actually slightly longer-lived than their littermates (Ran et al. 2007).

Peroxiredoxins

As described above, the effects of manipulating levels of catalase, glutathione peroxidase and SODs on lifespan have been shown to vary widely between species, assay conditions and even between different genetic backgrounds. In contrast, studies in yeast, flies, worms and mice have relatively consistently shown cytoplasmic members of the 2-Cys peroxiredoxin subfamily of peroxiredoxins to function as

pro-longevity genes. In the budding yeast *S. cerevisiae* there are two 2-Cys Prx, Tsa1 and Tsa2, likely arising from the partial genome duplication that occurred during the evolution of *S. cerevisiae*. Tsa1 is the more abundant of these two 2-Cys Prx and has been demonstrated to have non-redundant roles. For instance, loss of *TSA1* has been shown to have a profound effect on genome stability; increasing the rate of mutations, chromosomal rearrangements, recombination and telomere lengthening (Huang and Kolodner 2005; Lu et al. 2013). Despite increased telomere length, but in accordance with some of these phenotypes, loss of *TSA1* shortens the replicative lifespan of *S. cerevisiae* (Molin et al. 2011; Schleit et al. 2013). TSA2 is expressed at much lower levels and no effect of loss of *TSA2* on either the replicative or chronological lifespan of *S. cerevisiae* has been reported. In *S. pombe,* loss of the single 2-Cys Prx, Tpx1, has a profound effect on cell growth, particularly under aerobic conditions. However, some of these phenotypes may reflect the inability of Δ*tpx1* mutant *S. pombe* to activate transcriptional responses to H_2O_2 (Veal et al. 2004).

In *C. elegans* there are two 2-Cys Prx; PRDX-2, and the mitochondrial matrix-targeted PRDX-3. *C. elegans* lifespan is highly influenced by the temperature at which the worms are maintained, with mean and maximum lifespan inversely correlated with temperature. For convenience, most lifespan assays are carried out at 20 or 25 °C, to expedite the results. Although loss of *prdx-3* has no effect on ageing, intriguingly, loss of PRDX-2 leads to premature ageing, as determined by accumulation of lipofuscin and loss of mobility, and a shortened lifespan. These phenotypes are particularly prominent at 15 °C. However, the lifespan of *prdx-2* mutants is remarkably similar to wild-type lifespan at 25 °C (Isermann et al. 2004), suggesting that PRDX-2 is required for the extension of lifespan at lower temperatures. *prdx-2* mutant animals are killed by exposure to levels of peroxides that are normally sub-toxic to wild-type animals (Olahova et al. 2008). This suggests that PRDX-2 plays an important role in protecting against oxidative stress. In contrast, loss of PRDX-2 renders *C. elegans* hyper-resistant to the toxicity of arsenite and cadmium, most likely by virtue of increased expression of phase 2 detoxification enzymes and increased levels of glutathione, that likely result from their lower levels of secreted insulin which normally limits the expression of these genes (Olahova and Veal 2015; Tullet et al. 2008). PRDX-2 is expressed in a wide range of tissues, including the intestine, a tissue that may even have evolved as a primary defence against exogenous stress (An and Blackwell 2003; Olahova et al. 2008). Intriguingly, while expression of PRDX-2 from an intestine-specific promoter is sufficient to restore wild-type peroxide-tolerance to *C. elegans*, and further increases the arsenite resistance of *prdx-2* mutants, it does not increase the lifespan of these animals (Olahova et al. 2008). As well as disconnecting PRDX-2's function in resistance to acute stresses from its role in ageing, this suggests that PRDX-2 is required in other tissues for its pro-longevity effects. PRDX-2, and orthologous 2-Cys Prx in yeast and humans, have important, positive roles in signal transduction. For example, *C. elegans* PRDX-2 is required for activation of the p38-related MAPK, PMK-1, in response to oxidative stress (Olahova et al. 2008). Although it is not clear in which tissues PRDX-2 acts to promote PMK-1 activation, neuronal PRDX-2 has been shown to be required for a light-sensing mechanism, involving H_2O_2 as a second

messenger, that regulates feeding behaviour (Bhatla and Horvitz 2015). Accordingly, it is possible that role/s in transducing ROS signals, may underlay some of PRDX-2's effects on ageing and stress resistance, rather than PRDX-2's ability to remove peroxides.

Drosophila encode three 2-Cys Prx, each targeted to a different cellular location; ER/secreted, mitochondrial and cytoplasmic. Notably, ectopic expression of Jafrac1 (the orthologue of human Prx1/2) in neuronal tissues has been shown to extend lifespan (Lee et al. 2009). This is amongst the strongest evidence to date that increased 2-Cys Prx levels can promote longevity. There is also evidence that limited overexpression of the ER-targeted 2-Cys Prx, Prx4, or the atypical 2-Cys Prx, Prx5, increases the lifespan of flies (Klichko et al. 2016; Radyuk et al. 2009).

Mammals have 2 orthologous cytoplasmic 2-Cys Prx, Prx1 and Prx2. It is currently unclear the extent of the redundancy between these Prx. However, mouse knockouts indicate that they do have some non-redundant functions. Prx1 mutant mice are predisposed to an increased incidence of certain tumours, particularly as the mice age, and have a shortened lifespan (Neumann et al. 2003). Prx2 mutant mice suffer from haemolytic anemia and some indications of accelerated ageing (Lee et al. 2003; Cha et al. 2018; Park et al. 2018). This suggests that both Prx1 and Prx2 are important for longevity. However, it is possible that this could reflect an essential role for Prx1 and 2 in redox-signalling/homeostasis or as chaperones protecting against loss of proteostasis, rather than an 'antioxidant' function. Indeed, peroxide is now established as an important signalling molecule and 2-Cys Prxs have been shown to be vital for peroxide-induced oxidation of signalling proteins in yeast, worms and mammals (Bozonet et al. 2005; Jarvis et al. 2012; Sobotta et al. 2015) (Fig. 14.3). Moreover, Prxs have also been shown to have a chaperone activity that facilitates the refolding of aggregated proteins (Jang et al. 2004). Therefore, the conserved pro-longevity activity of 2-Cys Prx may not be due to their activity in removing peroxides and preventing ROS-induced damage, but instead be linked to their ability to transduce redox signals and protect against protein aggregation (Hanzen et al. 2016; Stocker et al. 2018).

Regulation of ROS-Metabolising Enzyme Activity and Ageing

Thioredoxin, Thioredoxin Reductase, Glutaredoxins, Glutathione and NADPH in Ageing

Peroxiredoxins and other thiol peroxidases ultimately derive their reductive power from NADPH. However, thioredoxins, glutaredoxins and glutathione mediate this transfer, restoring active forms of these thiol peroxidases. Thioredoxin family proteins also reduce disulphides that form during the catalytic cycles of other enzymes, or following ROS-mediated oxidation of cysteine residues. Although most cytoplasmic cysteines are protonated at cellular pH, rendering them relatively resistant to

oxidation, strong oxidants, pH changes and changes in the flux of carbon through the pentose phosphate pathway, that reduces NADP to NADPH, will all influence the oxidation of protein cysteine-thiols. Indeed, there is evidence to suggest that reversible oxidation of cysteine-thiols on the surface of proteins could itself provide an effective, limited capacity to remove peroxides (Hansen et al. 2009; Tomalin et al. 2016). Accordingly, any changes in the levels of NADPH, thioredoxin and glutathione will have implications for recovery and repair of oxidative damage following exposure to ROS. Notably, a fall in NADPH levels has been shown to precede many of the changes occurring in aged yeast cells (Brandes et al. 2013). The activity of many enzymes, including thiol peroxidases, but also enzymes involved in repair of oxidised methionines (methionine sulfoxide reductase) or damaged DNA (deoxyribonucleootide reductase) are ultimately dependent on the levels of reduced thioredoxin. There are many additional thioredoxin family proteins, based on the presence of the thioredoxin fold and WGCPC motif. The thioredoxin activity of these proteins is generally confirmed by their ability to reduce disulphides to insulin *in vitro*. However, it is likely they will show varying degrees of substrate specificity for the protein disulphides they reduce (Berndt et al. 2015). However, there is significantly less redundancy in the enzymes that mediate the reduction of these thioredoxin family proteins in each cell compartment. Accordingly, thioredoxin reductase and glutaredoxin/glutathione, which recycle thioredoxins, have a pivotal role in preventing/repairing ROS-induced damage.

Transcriptional Regulation of ROS-Metabolising Enzymes and Ageing

One issue with drawing conclusions from studies manipulating the levels of one or two ROS-metabolising enzymes is that even simple eukaryotes contain multiple enzymes and systems that contribute to the detoxification of ROS and repair of ROS-induced damage. Indeed, a battery of genes encoding ROS-protective enzymes are transcriptionally upregulated in response to increased stress (Chen et al. 2003; Gasch et al. 2000). In the case of unicellular organisms, these transcriptional responses afford vital protection against environmental sources of ROS, as well as plant and animal innate immune defences, which employ ROS as toxic weapons against invading pathogens.

Conserved transcriptional regulators are activated under stress conditions, promoting increased expression of these ROS-detoxifying and damage repair genes. Although the transcriptional regulators involved in activating these responses in yeast are well-established, the role of these transcriptional responses in maintaining replicative or chronological lifespan in yeast is less clear. However, in animals, several families of transcriptional regulators identified as important for longevity promote increased expression of ROS-metabolising genes. Indeed, part of the correlative evidence in support of ROS as a cause of ageing has been the finding that increased expression of ROS-metabolising enzymes and oxidative stress resis-

tance are a common feature of many of the regimes or genetic mutants that extend lifespan. For example, the long-lifespan of *C. elegans* mutants with reduced insulin signalling has been shown to require the activity of the FOXO transcription factor, DAF-16 and the CnC transcription factor, SKN-1 which both promote the expression of ROS-metabolising enzymes and oxidative stress resistance (Lin et al. 1997; Tullet et al. 2008). Indeed, amongst other genes, the FOXO family of Forkhead transcription factors, FOXO (mammals and flies) and DAF-16 (*C.elegans*), promote the expression of superoxide dismutases, catalase and other peroxidases (for a review *see* Murphy 2006). CnC family transcription factors, Nrf2(mammals), CNC(flies) and SKN-1(*C. elegans*), also activate ROS-metabolising enzymes; playing a vital role in promoting the expression of Phase 2 detoxification genes, which include glutathione synthesising enzymes and glutathione transferases (for reviews *see* Blackwell et al. 2015; Kensler et al. 2007; Pitoniak and Bohmann 2015). Moreover, DAF-16, SKN-1 and the *C. elegans* PPARalpha/HNF4-related nuclear hormone receptor, NHR-49 are all important for the increased longevity associated with loss of germ-line signalling (Lin et al. 2001; Ratnappan et al. 2014; Steinbaugh et al. 2015). Although NHR-49 is an established regulator of lipid metabolism it has recently been found to be important for an oxidative stress-protective transcriptional response to increased peroxide levels and fasting (Goh et al. 2018). Thus, although these pro-longevity transcriptional regulators regulate many genes, the presence of ROS-metabolising and stress-protective activities amongst the genes they upregulate, does suggest that increased protection against ROS may contribute to their pro-longevity function. In some cases, the increased expression of particular ROS-metabolising enzymes is important for associated increases in longevity. For example, upregulation of *sod-3* and *sod-5* is important for the long-lifespan of *isp-1* mutant *C. elegans* (Dues et al. 2017).

Interestingly, many of the transcriptional activators that promote increased expression of ROS-metabolising enzymes are actively inhibited under normal growth conditions, implying that elevated expression of ROS-metabolising/reductive enzymes can be deleterious under these conditions. For instance, many of these stress-protective responses are upregulated to promote survival of quiescent cells. However, increased activity of these transcription factors alone is not sufficient to extend lifespan, as the shortened lifespan of *prdx-2* mutant of *C. elegans*, which has elevated activity of both DAF-16 and SKN-1, indicates (Olahova and Veal 2015). Indeed, the constitutive activation of Nrf2 is lethal in mice, suggesting that excessively reducing conditions can interfere with normal growth and development (Wakabayashi et al. 2003). Similarly, while overexpressing SKN-1 at low levels can increase oxidative stress resistance and lifespan, constitutive activation of SKN-1 can lead to severe developmental delay/impaired growth (Tang and Choe 2015; Tullet et al. 2008). Moreover, the life-extending effects of caloric restriction and manipulation of mitochondrial activity require associated increases in ROS (Yang and Hekimi 2010; Zarse et al. 2012). These findings suggest that maintaining an appropriate redox environment for normal physiological ROS functions is also important for healthy ageing (Fig. 14.2). Indeed, a growing number of studies suggest that ageing is associated with a reduced ability to regulate this redox environment,

including a reduced ability to increase ROS defences in response to increased ROS. Accordingly, as others have suggested, maintaining highly responsive ROS-signalling systems may prove key to healthy ageing (Van Raamsdonk 2018).

Conclusions

The idea that increased ROS cause ageing and that treatments that remove or prevent ROS increases would therefore have anti-ageing effects was logical, particularly based on the evidence that ROS-induced damage is associated with ageing and so many diseases. However, as we have learned more about the genetic and dietary factors that influence aging, the positive signalling functions of certain ROS, and the complexity of mechanisms controlling the redox state of different cells/subcellular locations, it has become apparent that this simple approach is inadequate. Nevertheless, as we have discussed, some of the enzymes that generate and remove ROS, do have effects on ageing. Moreover, the finding that regimes that extend lifespan are often associated with upregulation of different selections of ROS-metabolising enzymes suggests that, collectively, these enzymes do contribute positively to healthy ageing (Fig. 14.2). Therefore, it remains plausible that, with a better understanding of the mechanisms by which these enzymes influence redox-signalling/homeostasis, we could seek to manipulate these processes in a more targeted way. Indeed, it is possible that the age-associated decline in the inducibility of protective transcriptional programmes, may actually be responsible for the subsequent increases in oxidative damage and loss of tissue function. Perhaps uncovering a way to ensure the sensitivity of these ROS-adaptive, protective responses persists into old age will be the key to future success.

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Chapter 15
B Vitamins and Ageing

Kathleen Mikkelsen and Vasso Apostolopoulos

Abstract Vitamin B contributes to the overall health and wellbeing, including that of energy metabolism, methylation, synthesis and DNA repair and proper immune function. Deficiency in B vitamins has been linked to neurocognitive disorders, mitochondrial dysfunction, immune dysfunction and inflammatory conditions. In ageing populations B vitamin deficiency has been linked to cardiovascular disorders, cognitive dysfunction, osteoporosis and methylation disorders and can increase the risk of developing degenerative diseases, particularly cardiovascular disease, cognitive diseases and osteoporosis. Optimization of B vitamin status in the elderly may prove beneficial in the prevention of degenerative diseases. Here we discuss broadly the role of B vitamins in ageing.

Keywords Vitamin B · Ageing · Niacin · Thiamine · Folate · Cobalamin · Riboflavin · Pyridoxine

Introduction: Old Age Is a Privilege Denied to Many

The perception of successful ageing in modern society is often referred to as an optimization of longevity whilst minimizing physical and mental deterioration and disability (Bowling and Dieppe 2005). Successful ageing, however, can rely on a variety of factors, including genetic background, and sociocultural and lifestyle choices. These choices may include whether or not to exercise, smoke cigarettes, drink alcohol or consume a healthy diet. Along with dietary, pharmacological and lifestyle interventions to promote health and longevity, certain nutritional interventions may serve to delay the onset or prevent some degenerative diseases and cognitive decline in the ageing population (Porter et al. 2016).

There is a growing body of literature that recognizes the fundamental role by which the B vitamins contribute to overall health and proper physiological and

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biochemical function. This includes energy metabolism, methylation, production of monoamine oxidase, synthesis of DNA, DNA repair, maintenance of phospholipids, immune function and steroid hormone activity (Mikkelsen et al. 2016a). There is a clear link between vitamin B deficiency and its contribution to a number of diseases such as pellagra, beriberi, Wernicke Korsakoff and pernicious anaemia. In addition, vitamin B deficiency contributes to neurocognitive disorders including Alzheimer's disease, dementia, depression, stress and anxiety (Jerneren et al. 2015; Mikkelsen et al. 2016b; Mitchell et al. 2014; Pan et al. 2016), as well as mitochondrial dysfunction (Abdou and Hazell 2014; Du et al. 2014; Fu et al. 2014), immune dysfunction, inflammatory conditions (Eyre and Baune 2012; Kiykim et al. 2015; Mikkelsen et al. 2017b; Pariante 2015; Slavich and Irwin 2014), liver damage, insulin sensitivity, lethargy, peripheral neuropathy, anaemia and fatigue (Mikkelsen et al. 2016a). Low vitamin B status amongst elderly people has been linked particularly to cardiovascular disorders, cognitive dysfunction, osteoporosis and methylation disorders and can increase the risk of developing degenerative diseases, particularly cardiovascular disease, cognitive diseases and osteoporosis. Furthermore, deficiencies in B vitamins coupled with genetic polymorphisms (e.g. MTHFR 677C-T) can cause complications involving one carbon metabolism (Porter et al. 2016). Optimization of B vitamin status in the elderly may prove beneficial in the prevention of degenerative diseases (Fig. 15.1). Currently 8% of the world's population is aged 65 and over, and this is predicted to increase to 16% by the year 2050 (Beard and Bloom 2015). One quarter of the global cost of disease is carried by the elderly which means the maintenance of health during ageing becomes a public health priority.

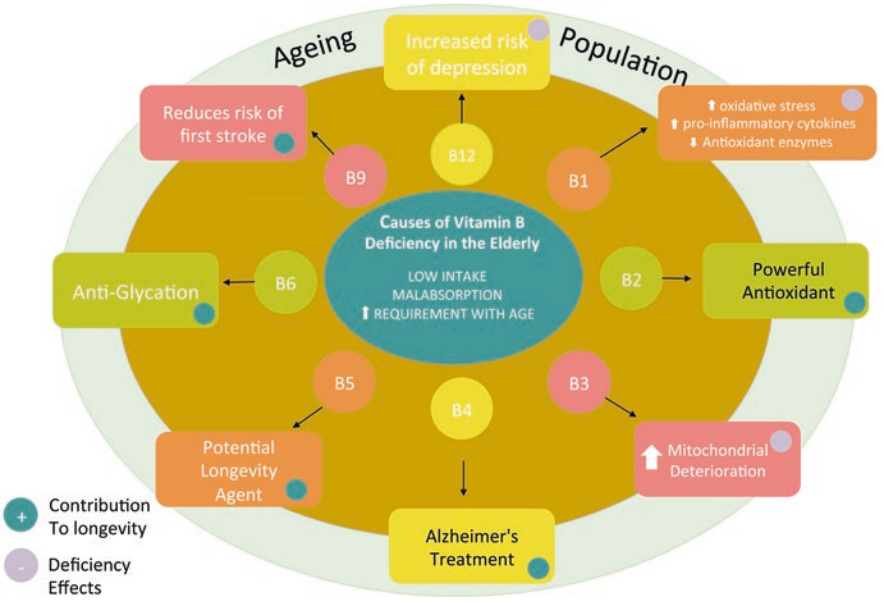

Fig. 15.1 Summary of vitamin B complex contributing to ageing

Improving nutritional outcomes may prevent or delay the progression of declining health due to ageing. The chemical structure of the B vitamins discussed in this chapter is given in Fig. 15.2.

The B Vitamins

B vitamins are a group of eight essential water-soluble vitamins which work individually and together to facilitate the physiological functioning of the body. B vitamins include B1, B2, B3, B5, B6, B7, B9 and B12 (Fig. 15.1). Choline, also known as B4, is not considered part of the B vitamins complex but was officially recognized as an essential nutrient by the Institute of Medicine in 1998 (Zeisel 1992).

B vitamins are crucial for the adequate functioning of the methylation cycle and the synthesis and repair of DNA, RNA protein and phospholipids. They participate in the functioning of the immune system and inflammatory balance as well as cell metabolism and repair and energy production. B vitamins are essential for maintaining a healthy nervous system, balancing mood and maintaining cognitive function and are essential components of neurotransmitters. B vitamins act as coenzymes in many enzymatic reactions and together are involved in the metabolism of carbohydrate, protein, lipids, other vitamins, minerals and drugs.

B vitamins are found in animal and dairy sources such as meat, poultry, fish eggs and milk; they are also, however, plentiful in plant-based diets in fresh fruit, vegetables, nuts, seeds, grains, legumes, soy products and fortified cereals. B12 is synthesized by bacteria and archaea and found in small quantities in plants and soil, often via contamination with bacteria usually from faecal origin (Herbert 1988). The main source of B12 in the human diet comes from animal origin, and the presence of B12 in animal flesh is mostly dependent on the process of bio-magnification through food chains (Rizzo et al. 2016). Yeast-based spreads are a rich source of vitamin B, and certain spreads are fortified with B12 (Mikkelsen et al. 2018). Nutritional yeast is another rich source of B12 and is often consumed by people following a vegetarian or vegan diet as a nonanimal form of B12 supplementation. Vitamin B deficiency is commonly found in the elderly, in particular, the metabolically related B vitamins B6, B9 and B12. This is often due to malabsorption, poor intake or higher requirements

Causes of B Vitamin Deficiency in the Ageing Population

There are a number of causes for B vitamin deficiency within the ageing population. The most common cause of B2 and B9 deficiency is low intake, whilst B12 deficiency is mostly caused by malabsorption and B6 deficiency from increased requirement with ageing (Porter et al. 2016). Other factors contributing to B vitamin deficiency include drug-nutrient interactions, genetic disorders and certain medical

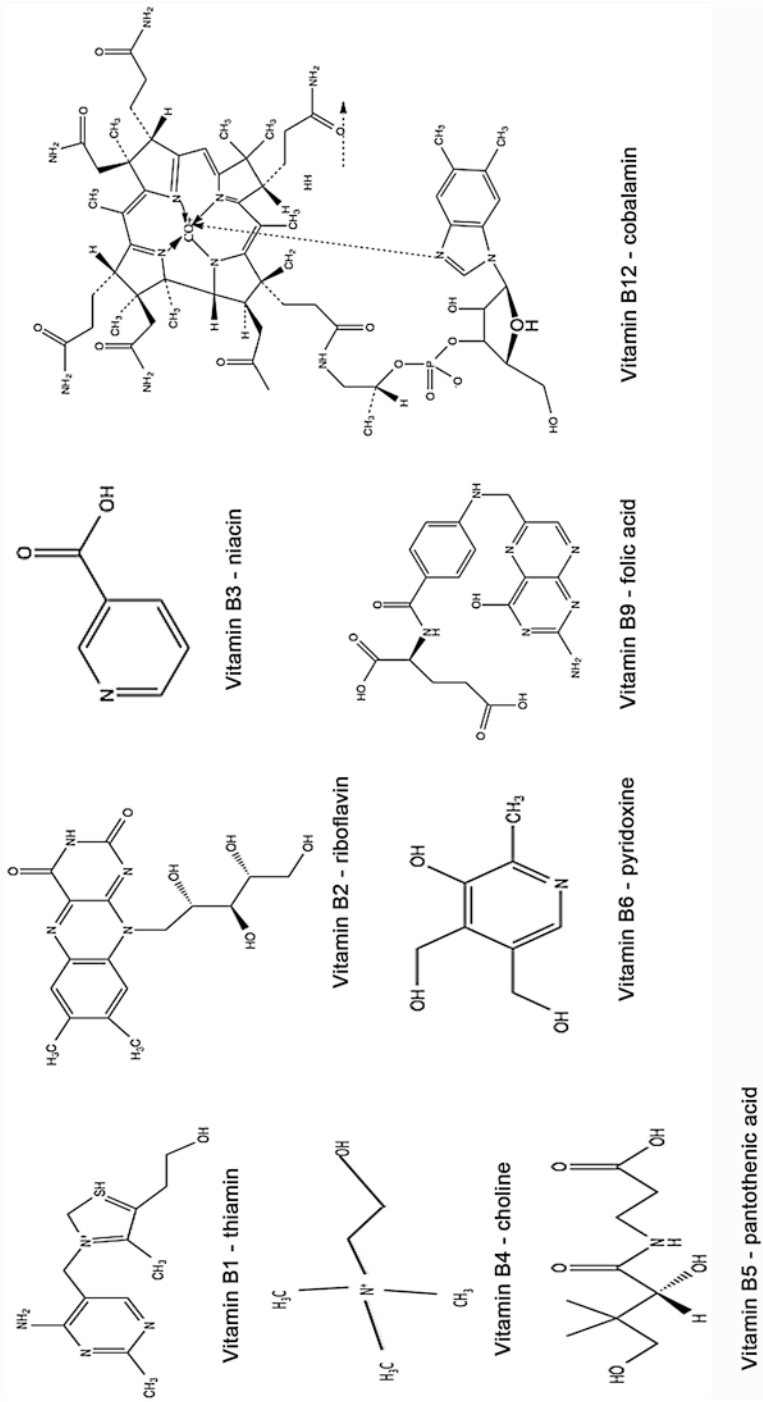

Fig. 15.2 Chemical structures of the B Vitamins

conditions. The natural process of ageing can also affect how B vitamins are absorbed, transported and metabolized within the body. Dietary factors play a major role in the pathogenesis of many diseases suffered by the ageing population. Amongst these, micronutrient status plays a significant part. Micronutrient deficiency often occurs due to reduced food intake or lack of variety in the diet (WHO 2017). Malabsorption of micronutrients is a common problem, and the elderly are particularly at risk for B12 deficiency due to malabsorption (Fig. 15.1).

Vitamin B12 absorption happens in several steps and is dependent on several factors to ensure this happens successfully. Initially B12, which is often bound to animal protein in the diet, is ingested and broken down in the stomach by pepsin and hydrochloric acid. It is then released as free B12 whereby it forms another complex with R protein secreted from bile. Pancreatic enzymes break this complex down releasing B12 once again. Parietal cells in the duodenum release intrinsic factor which binds to the free B12, and this travels undisturbed down into the ileum where it binds to mucosal cell receptors. Finally it is carried via the portal system by transcobalamin, a transport protein, which delivers it to all somatic cells (Wong 2015). There are a few pathophysiological changes that can occur with ageing to prevent B12 absorption along this pathway. Hypochlorhydria, which can occur due to atrophic gastritis or the use of certain drugs such as proton-pump inhibitors and histamine H2 blockers, can interfere with the release of B12 from binding protein. Pernicious anaemia, gastrectomy and atrophic gastritis can interfere with the release of intrinsic factor, preventing B12 from travelling to the ileum. Exocrine pancreatic insufficiency impairs the secretion of pancreatic enzyme which prevents the release of B12 from B12-R protein. Within the ileum, Crohn's disease, ileum resection, bacterial overgrowth and the use of certain drugs including metformin and cholestyramine can all cause interference with B12 absorption. Finally, transcobalamin deficiency can prevent B12 from being carried within the plasma to somatic cells (Wong 2015).

Thiamine (B1)

Vitamin B1 or thiamine is part of the coenzyme thiamine pyrophosphate. It is responsible for the generation of nerve impulses and the synthesis of neurotransmitters, nucleic acids, fatty acids, steroids and complex carbohydrates. B1 is crucial for the effective functioning of the nervous system, and deficiency can lead to mood disorders, anxiety and depression as well as severe cardiovascular complications and heart failure (Mikkelsen et al. 2016a, b, 2017a, b, 2018; Nemazannikova et al. 2017).

Thiamine deficiency (TD) can cause neurological damage leading to the production of free radicals, increased oxidative stress, axonal damage, improper myelin production and glutamate-mediated excitotoxicity. The immune effects of thiamine deficiency include an increase in neuroinflammation, T-cell infiltration and increased production of pro-inflammatory cytokines including IL-1, TNF-alpha and IL-6 as well as an overproduction of CD40 and CD40 ligand by microglial cells and astro-

cytes. Thiamine binds to serum proteins in the blood, mostly albumin. It is absorbed in the small intestine in low concentration by passive transport and in high concentrations by active transport and excreted in the urine as thiamine and acid metabolites (Abdou and Hazell 2014; Carney et al. 1979; Read and Harrington 1981; Zhang et al. 2013).

Thiamine is an essential nutrient for brain metabolic and cellular functioning and production of neurotransmitters including acetylcholine. TD can contribute to impairment of oxidative metabolism, neuroinflammation, endoplasmic reticulum stress, autophagy and neurodegeneration. These factors play a role in the pathogenesis of ageing-related diseases such as dementia, Alzheimer's disease, Parkinson's disease and Huntington's disease (Meldrum 2000; Pavlin et al. 2015; Schmitt and Falkai 2015; Serafini and Amore 2015).

Thiamine-Deficient Neurodegeneration Mechanisms

It is clear from *in vivo* and *in vitro* cell studies that TD increases reactive oxygen species production resulting in oxidative stress in the brain and neuronal tissues (Liu et al. 2017; Wang et al. 2017). This may be due to several factors including mitochondrial dysfunction. Mitochondria are a key source of ROS and a target for TD. Mitochondrial dysfunction can cause an increase in binding site densities for the translocator protein, and this increase is noted in the brains of TD animals (Leong et al. 1994).

Other studies have indicated that TD can interfere with the antioxidant system within the brain. TD has shown to significantly reduce antioxidant enzymes such superoxide dismutase, catalase and glutathione peroxidase in animal models (Sharma et al. 2013). TD causes an increase in pro-inflammatory cytokines, chemokines and transcription factors which contribute to neuro-inflammation. Neuroinflammation in turn can alter mitochondrial function and increase oxidative stress. Autophagy plays an important role in maintaining healthy cells by degrading, transporting and recycling damaged and dysfunctional organelles and is often found to be impaired in neurodegenerative diseases (Shibata et al. 2006). TD can induce an accumulation of autophagosomes and upregulate autophagy marker expression. Thiamine supplementation however has been shown to reverse this process (Liu et al. 2017). Endoplasmic reticulum stress is another mechanism which may contribute to neurodegeneration. Amongst the many roles of the endoplasmic reticulum are protein folding, modification and transport. Endoplasmic reticulum stress response triggers a condition known as the unfolded protein response, which is a homeostatic adaptive response to endoplasmic reticulum stress. If the unfolded protein response does not result in alleviating the endoplasmic reticulum stress, then apoptotic cell death can occur. Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis all commonly show the occurrence of protein aggregates in the brain which signify neuronal loss. Evidence points to endoplasmic reticulum stress as a contributing factor to central nervous

system damage (Begum et al. 2013; Mota et al. 2015; Wang et al. 2017). Although there is much evidence linking TD with aspects of cognitive decline, there is far less current evidence linking thiamine supplementation with improvement of cognitive symptoms in the ageing population. Although high doses of thiamine supplementation in patients with newly diagnosed Parkinson's disease were effective in reversing symptoms (Costantini et al. 2013).

Riboflavin (B2)

Vitamin B2 (riboflavin) is a powerful antioxidant which acts as a coenzyme in a number of reactions and is involved in energy and iron metabolism. The two active forms of riboflavin are flavin mononucleotide and flavin adenine dinucleotide, and both function as cofactors in essential metabolic reactions. Flavin adenine dinucleotide forms part of complex II of the electron transport chain and is used in phosphorylation, oxidation of pyruvate and fatty acid oxidation. Flavin mononucleotide is required for conversion of tryptophan to niacin (vitamin B3) in complex I of the electron transport chain and reduces glutathione by glutathione reductase in complex II. It is also an important component of the coenzyme form of vitamin B6. The resulting pathology of B2 deficiency often manifests as anaemia, inflammation, cognitive dysfunction and depression and during embryonic development can result in congenital malformations (Mikkelsen et al. 2016a, b). In older adults, B2 deficiency is linked to reduced cognitive outcome, depression, adverse personality changes such as aggression and distinct alterations within the central nervous system (Mikkelsen et al. 2016a, b). Within the immune system, riboflavin plays an essential role in the function of mucosal-associated invariant T cells. The mucosal-associated invariant T cells are the only T cells activated by MR1-bound riboflavin metabolite derivatives. Free riboflavin is bound to albumin and certain immunoglobulins in the blood and is absorbed in the upper part of the small intestine. B2 is excreted in the urine as riboflavin or other metabolites (Foraker et al. 2003; Massey 2000; Miyake et al. 2006; Murakami et al. 2008; Powers 2003; Sheraz et al. 2014).

Riboflavin and Oxidative Damage

The free radical theory of ageing proposes that aerobic metabolism causes cumulative oxidative damage to body cells and tissues over time (Wickens 2001). If this proposal is sound, then in theory, antioxidants should aid in extending life span. Based on this idea, there has been much research into the effect of endogenous antioxidants and how they relate to the life span of various organisms (Sadowska-Bartosz and Bartosz 2014). Some of the more popular and commonly studied antioxidants include vitamin C, vitamin E, resveratrol, curcumin, tocopherol and coenzyme Q and have all been shown to prolong the life span of modelled

organisms (Sadowska-Bartosz and Bartosz 2014). Riboflavin is known for its antioxidant abilities yet is neglected within the realm of studies proposing antioxidants and longevity. Riboflavin acts as an antioxidant via two mechanisms: (i) it prevents lipid peroxidation and (2) attenuates reperfusion oxidative injury (Ashoori and Saedisomeolia 2014). Recently, it was noted in 400 fruit flies (*Drosophila melanogaster*) that the life span was extended by 14.1% in the group supplemented with riboflavin compared to the control group (hydrogen peroxide to stimulate oxidative stress). Furthermore, SOD-1, an antioxidant enzyme that is made in the body and declines with age, was found to be enhanced in the riboflavin group compared to the control group (Zou et al. 2017). Based on these findings, further research is warranted to establish the link between riboflavin and slowing the human ageing process.

Niacin (B3)

Vitamin B3 or niacin (nicotinic acid and nicotinamide) is precursor of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADPH). NAD is phosphorylated to NADPH by the enzyme NAD+ kinase. NAD is required for catabolism of fat, carbohydrate, protein and alcohol, cell signalling and DNA repair. NADPH is required for fatty acid and cholesterol synthesis. Niacin deficiency manifests in cognitive symptoms such as tension, anxiety and depression as well as manic depression and schizophrenia. Low niacin levels have been linked to pellagra, a disease which can cause inflamed skin, dermatitis, diarrhoea, dementia and sores around the mouth (Mikkelsen et al. 2016a, b). Within the immune system, niacin has been shown to reduce pro-inflammatory cytokines and dampen the effects of inflammation as shown in atherosclerosis (Digby et al. 2012; Lipszyc et al. 2013). B3 is absorbed in the stomach and small intestine and stored in the liver with excessive amounts secreted in the urine. It is interesting to note that tryptophan can also synthesize NAD via the kynurenine pathway (Davison and Kaplan 2012; Fu et al. 2014; Li et al. 2010; Smesny et al. 2010).

NAD and Mitochondria

Energy metabolism is one of the key duties of NAD. Ageing has been associated with diminished levels of NAD, and this in turn has been implicated in mitochondrial deterioration (Lanza and Nair 2010). Mitochondrial decay is a key player in ageing and can be reversed with dietary supplements that increase cellular levels of NAD (Gomes et al. 2013). Nicotinamide riboside is a precursor to NAD and effective in boosting NAD levels. In fact a study by demonstrated that treatment with nicotinamide riboside for 1 week was adequate to boost NAD levels sufficiently to restore muscle health and mitochondrial homeostasis in 22-month-old mice to

levels comparable to those seen in 6-month-old mice (Gomes et al. 2013). In addition, nicotinamide riboside restores NAD+ levels in the mitochondria of mice bred with Cockayne syndrome, a neurodegenerative accelerating ageing disorder (Scheibye-Knudsen et al. 2014). In humans nicotinamide riboside supplementation has also shown to increase NAD levels. NAD has been shown to work synergistically with a group of enzymes called sirtuins. Sirtuins have been implicated as key players in longevity and have an overall benefit in good health. NAD and sirtuins work together to induce formation of new mitochondria and ensure proper mitochondrial function. When NAD levels drop, as commonly noted in ageing, the activity of SIRT1 also drops. This can affect signalling level from cell nuclei to mitochondria causing mitochondrial dysfunction and neurodegenerative disease. Further studies may find a mode of combining the action of sirtuin and NAD to provide an effective anti-ageing intervention regulating health and increasing life span (Imai and Guarente 2014).

Choline (B4)

Choline, although classed with B vitamins because of its chemical structure, is not necessarily defined as one of the B vitamins. Choline plays numerous roles and is involved with cell signalling and the functioning of membranes. It is present in phospholipid components such as phosphatidylcholine and sphingomyelin. It is the basic constituent of lecithin and present in cell membrane structure and plasma lipoproteins. Within tissues, choline prevents fat deposits in the liver and assists the movement of fat into the cells. Choline is also responsible for the synthesis of the neurotransmitter acetylcholine which is important for numerous functions, including memory and muscular control (Nemazannikova et al. 2017).

Choline and Alzheimer's Disease

Choline plays an important role in the treatment of Alzheimer's patients. Past studies have shown that cholinergic neurons are involved in learning, memory and cognition and that both muscarinic and nicotinic acetylcholine receptors are involved in the encoding of new memories. Cholinergic brain function relies on the neurotransmitter acetylcholine, and a reduction of acetylcholine means a reduction in cholinergic function (Bird et al. 1983; Fu et al. 2004). There are numerous pathological changes which occur within the brains of people suffering from Alzheimer's disease. The loss of brain tissue and the presence of neurofibrillary tangles and harmful deposits known as senile amyloid-beta plaques are often found. Much of this damage has been attributed to the effect of oxidative damage and inflammation. Choline acetyltransferase (ChAT) is the enzyme responsible for catalysing the synthesis of acetylcholine in cholinergic neurons. In Alzheimer's patients there is a

specific loss of cholinergic neurons in the basal forebrain and a reduction of ChAT activity which correlates with the severity of the disease (Fu et al. 2004). In a mouse model of Alzheimer's disease, recombinant TAT-choline acetyltransferase fusion protein was injected into the brains of mice and found to improve memory and cognitive function (Fu et al. 2004). One of the most widely accepted Alzheimer's disease treatments involves drugs which inhibit the action of acetylcholinesterase. It is this enzyme which breaks down acetylcholine. By inhibiting this enzymatic activity, the amount of acetylcholine available to the brain neurons is effectively increased. These drugs are not curative but can halt the progression of the disease for a short time or slow down its progress; however, this therapy is expensive and can have unpleasant side effects. A safer more natural supplement that is being trialled in Alzheimer's disease studies is a precursor to acetylcholine called CDP-choline or citicoline. CDP-choline can increase acetylcholine levels in the brain and has the potential to aid neural repair in conditions such as Alzheimer's disease (Arenth et al. 2011) and other conditions involving degenerative and vascular cognitive decline (Gareri et al. 2015).

Pantothenic Acid (B5)

Vitamin B5 or pantothenic acid (pantothenate) is part of the chemical structure of coenzyme A and an important component of the Krebs cycle dealing with energy metabolism and amino acid metabolism, fatty acid and glycogen synthesis and the production of steroid hormones melatonin and acetylcholine. B5 aids in the normal development of the central nervous system, and deficiency, although rare, can lead to fatigue and depression, insomnia and irritability, vomiting, nausea, stomach cramps, hypoglycaemia and increased sensitivity to insulin (Mikkelsen et al. 2016a, b; Mitchell et al. 2014). Vitamin B5 is bound to proteins such as acyl carrier protein and must be converted to free pantothenic acid in the intestine before absorption can take place in the small intestine. B5 is excreted intact in the urine (Jansen et al. 2013; Spry et al. 2013).

Pantothenic Acid and Longevity

Humans have long sought a magic potion for longevity. There is some evidence to suggest that pantothenic acid may play some role. As early as 1948, Gardner conducted experiments on *Drosophila melanogaster* (fruit flies) by feeding them with royal jelly. He noted that the flies that had been fed royal jelly had an increased life span compared to those who did not have royal jelly. Gardner researched the constituents of royal jelly and noted it to be one of the highest sources of pantothenic acid known (Gardner 1948). Over 50 years later, mice were fed high doses of royal jelly, and their life span increased by 25% when compared to control mice, with

reduced DNA damage, possibly as a result of a reduction in oxidative damage (Inoue et al. 2003). In addition, royal jelly extends the life span of *C. elegans* (Honda et al. 2011). It is largely unknown however which constituent of royal jelly is responsible for its longevity effects, although it is assumed that B5 may play a role. In fact, mice fed with calcium pantothenate (B5) (added to the drinking water) lived on average of 653 days compared to control mice which lived on average 549 days. More recently, however, a study claimed that the longevity effects of royal jelly may be due to the proteins called major royal jelly proteins (Xin et al. 2016).

Pyridoxamine (B6)

Vitamin B6 is found in three naturally occurring forms, pyridoxine, pyridoxal and pyridoxamine, and has many and varied essential functions within the endocrine, neurological and immune systems (Mikkelsen et al. 2017b). The active coenzyme form, pyridoxal-5 phosphate, is a biologically active form of B6 and, as a cofactor, aids the synthesis of the neurotransmitters serotonin, dopamine, epinephrine and GABA. It aids the conversion of tryptophan to niacin or serotonin and is involved in steroid hormone activity (Mikkelsen et al. 2016a, b). Pyridoxal-5 phosphate also breaks down amino acids and transports amine groups and is involved in glucose and lipid metabolism and facilitates the synthesis and catabolism of sphingolipids. Vitamin B6 is the vitamin mostly associated with pathogenesis of depression (Mikkelsen et al. 2016a). Vitamin B6 deficiency can result in high homocysteine levels effecting methylation and has been associated with seizures, migraines and irritability, confusion and depression (Mikkelsen et al. 2016a). Within the immune system, B6 has been shown to downregulate NF-kappa B activation by macrophages in mice and along with B12 deficiency can enhance the inflammatory response in dendritic cells (Mikkelsen et al. 2017b). The conversion of the inactive forms of B6 (pyridoxal, pyridoxine, pyridoxamine) to the active form pyridoxal phosphate is catalysed by pyridoxal kinase and requires zinc for full activation. Vitamin B6 is absorbed in the jejunum and ileum by passive diffusion and excreted in the urine as 4-pyridoxic acid. When vitamin B6 levels are high, a small amount is also excreted in the faeces (Balk et al. 2007; Malouf and Grimley Evans 2003; Miyake et al. 2006; Murakami et al. 2008; Richard 2014; Wang and Kuo 2007; Wu and Lu 2012).

Pyridoxine and Glycation

Vitamin B6 in the form of pyridoxamine has been touted as playing its own unique role in the war against ageing. Amongst the different theories of ageing is the cross-linking theory which deals with how proteins react with sugars to form a protein/glucose complex, often yellow brown in colour, which includes many cross-linked structures. Food chemists were the first to study this process, known as the Maillard

reaction, which is used in cooking (commonly called browning or caramelization) as a source of flavour, colour and texture. It was discovered in the 1970s–1980s that this process also occurs within the body and is also known as advanced glycation (Tamanna and Mahmood 2015). The products of advanced glycation or AGEs (advanced glycation end products) have been linked to the increase of the production of free radicals and implicated in causing complications associated with ageing, via tissue damage. AGEs can be deposited anywhere within the body, including the skin (Bailey 2001), eyes (Jansirani and Anathanaryanan 2004), lungs, kidneys, blood vessels (King 2001) and brain (Shimizu et al. 2013). Deposits in any of these areas can cause abnormal function of that organ or tissue. AGEs can cause cataracts if deposited in the eyes, in the tissues arthritis and in the kidneys nephrosis and kidney failure. Within the vascular system, they can cause micro- and macrovascular complications. AGE formation within the vascular walls is particularly damaging and can result in plaque formation, thickening of basement membrane and the loss of elasticity with the vessels leading to heart disease. Vitamin B6 in all forms has the ability to function as an anti-glycation agent although pyridoxamine appears to be the most potent of the three (Voziyan and Hudson 2005). Pyridoxamine can inhibit the formation of AGEs by trapping reactive carbonyl groups from the by-products of sugar and lipid degradation. It also plays a part in inhibiting certain steps of the glycation reaction by binding catalytic redox metal ions and the trapping of reactive oxygen species. Vitamin B6 may prove to be a valuable AGEs inhibitor although the association between these factors needs to be investigated in further studies.

Folic Acid (B9)

Vitamin B9 (folate, folic acid) plays a vital role in many functions within the body. Folate in its primary coenzyme form tetrahydrofolate is important in facilitating a series of one-carbon transfer reactions during metabolism. It is an important component together with B12 and B6 in methylation for recycling homocysteine into methionine. B9 assists the synthesis, repair and methylation of DNA and is integral for the process of cell division; in fact without folate, cells cannot divide and function. Folate in the form of 5-methyl tetrahydrofolate assists regulation of monoamine neurotransmission and breakdown of norepinephrine, dopamine and synthesis of both these, plus serotonin (Mikkelsen et al. 2016a). B9 also helps convert B12 to a coenzyme form, whilst B12 is needed to convert B9 to a coenzyme form. Folate deficiency can result in anaemia, neural tube defects in the foetus as well as neurocognitive effects such as behaviour disorders, cognitive decline and depression (Mikkelsen et al. 2016a). Furthermore, low B9 levels have been linked to dementia and Alzheimer's disease (Mikkelsen et al. 2016b). Symptoms of folate deficiency include mental confusion, weakness, fatigue, shortness of breath, irritability, headache, a smooth red tongue, depressive symptoms and elevated homocysteine levels (Fava and Mischoulon 2009; Loria-Kohen et al. 2013; Walker et al. 2012). Patients who suffer with megaloblastic anaemia, which is the clinical form of folate

deficiency, have been shown to suffer from an impaired immune response, which is reversible with folic acid supplementation. Folates are present in food in the form of polyglutamates and need to be broken down to folate monoglutamates in the intestine before absorption can occur. Excretion of vitamin B9 occurs via the urine (Balk et al. 2007; Du et al. 2014; Fava and Mischoulon 2009; Jerneren et al. 2015; Loria-Kohen et al. 2013; Mitchell et al. 2014; Walker et al. 2012).

Folic Acid, Stroke and Neurodegenerative Disease

Folic acid is well known for its role in preventing neural tube defects in the developing foetus (Pitkin 2007). As a preventative measure to this, some countries employed the use of folic acid food fortification; in fact 80 countries around the world currently have mandatory folic acid fortification policies. An unexpected result of this, however, besides a decrease in neural tube defects at birth, was an improvement in stroke mortality. A study undertaken by Yang et al. (2006) noted that this improvement occurred after the mandatory folic acid fortification policy was implemented in Canada and the USA. In contrast, there was no improvement in the same period, in England and Wales, where folic acid food fortification was not implemented (Yang et al. 2006). A more current study of note in relation to folic acid and stroke was undertaken in China by Huo et al. (2015). This study set out to find whether therapy with enalapril (a drug used to treat high blood pressure) and folic acid proved more effective in reducing primary stroke than enalapril alone in a cohort of Chinese adults suffering hypertension. The results of this study showed that the use of folic acid supplementation significantly reduced the risk of first stroke (Huo et al. 2015).

It is thought that the reason folic acid decreases stroke risk is because it helps to reduce homocysteine levels, and elevated homocysteine is correlated with increased risk of stroke (Casas et al. 2005; Holmes et al. 2011), whilst lower blood homocysteine levels are associated with reduced risk for stroke (Saposnik et al. 2009). Homocysteine is a by-product of the methylation cycle. Methylation reactions, a mechanism by which the body deals with stress, infection and toxic metabolites, are involved in almost every endogenous chemical reaction. Occasionally this reaction can fail to proceed effectively, and the result can be an increase in homocysteine levels. This in turn can lead to metabolic impairment and certain neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, depression and dementia (Bottiglieri et al. 2000). In order for methylation to proceed effectively, an adequate amount of folate is required in the diet. Folate is a key part of the methylation cycle and essential for the conversion of homocysteine to methionine and also in the synthesis of s-adenosyl-methione required for the methylation of DNA, protein and lipids (Mikkelsen et al. 2016b; Saposnik et al. 2009). In fact, the methylation cycle is not only reliant on folate but also requires B1, B2, B6 and B12 all working in synergy. Insufficient levels of these vitamins due to low intake, malabsorption or increased requirement result in the elevation of homocysteine levels and the pathogenesis of many neurocognitive disorders associated with ageing (Tucker et al. 2005; Xiu et al. 2012).

Cobalamin (B12) and Depression

The B12-related compounds are characterized by a cobalt-centred corrin nucleus. Cyanocobalamin is the metabolically active form of B12, but other forms found naturally in biological systems include methyl cobalamin, cob(I)alamin, 5′-deoxyadenosylcobalamin and hydroxycobalamin. The reactive C-Co bonds in B12 participate in isomerase and methyltransferase reactions which help to extract energy from proteins and fats and participated in methylation. B12 is required for proper functioning of the nervous system, nerve cell maintenance, cell synthesis and breakdown of fatty acids and amino acids (Mikkelsen et al. 2016a, b, 2017b). A close relationship exists between B12 and folate as each depends on the other for activation. Vitamin B12 is synthesized by bacteria, and interestingly, human colon bacteria make large amounts of vitamin B12, but it is not absorbed through the colon. A fascinating study was undertaken in the 1950s whereby vegan patients suffering from vitamin B12 deficiency were fed water extracts of their own stools which subsequently cured their B12 deficiency. Clinical symptoms of B12 deficiency include weakness and fatigue, loss of appetite, weight loss and constipation, peripheral tingling and soreness of the mouth and tongue (Mikkelsen et al. 2016a, b, 2017b; Nemazannikova et al. 2017).

B12 deficiency has been attributed to severe depression, suicidal behaviours, reduced cognition, mental fatigue, low mood, mania, psychosis and intense agitation. B12 deficiency also interferes with the body's ability to produce high turnover cells such as red blood cells which can result in megaloblastic or pernicious anaemia. When B12 deficiency is present, it can cause folate to be trapped as its inactive form instead of its active form which in turn can interfere with DNA production. Within the immune system, vitamin B12 deficiency has been correlated with a reduction in CD8+ and natural killer cells and increased TNF-alpha production by macrophages, whilst IL-6 and TNF-alpha deviations were corrected with vitamin B12 supplementation (Fava and Mischoulon 2009; Loria-Kohen et al. 2013; Walker et al. 2012). Within the body B12 is bound to protein in food, and hydrochloric acid and proteases within the stomach help its release. Parietal cells within the stomach secrete intrinsic factor which forms a complex with B12 allowing it to be absorbed by the ileum. This absorption is reliant on the capacity of the intrinsic factor. After absorption B12 is transferred into cells via plasma transporter transcobalamin II where it is degraded by lysosomal activity and free B12 moves into the cytoplasm. B12 is excreted mostly in the bile and then reabsorbed and stored in the liver with a small amount entering the faeces. B12 taken in larger amounts is excreted in the urine (Balk et al. 2007; Du et al. 2014; Fava and Mischoulon 2009; Jerneren et al. 2015; Loria-Kohen et al. 2013; Mitchell et al. 2014; Walker et al. 2012).

The normal function of the brain and nervous system is reliant on adequate levels of vitamin B12 being obtained in the diet or via supplementation. Vitamin B12 deficiency can result in development of severe depression, irritability, mania, psychosis and suicidal behaviours (Almeida et al. 2015; Mitchell et al. 2014; Petridou et al. 2016; Sengul et al. 2014; Seppala et al. 2013; Syed et al. 2013). The link between

vitamin B12 and neurotransmitter synthesis provides some insight as to why B12 deficiency can lead to depression. Low vitamin B12 status in the elderly is common. Ageing-related disturbances in absorption, transfer and metabolism of B12 contribute to deficiency status. Methylation reactions in the brain include one carbon metabolism which is responsible for the production of monoamine neurotransmitters such as dopamine, serotonin and norepinephrine. Vitamin B12 deficiency reduces the activity of methionine synthase. This blocks the formation of tetrahydrofolate which causes folate to become trapped as 5-methyl tetrahydrofolate. The interrelatedness of the methionine pathway with the purine and thymidylate cycles plays a critical role in the synthesis of neurotransmitters, and any disturbances in one pathway can cause an imbalance within the others, which in turn can affect the production of neurotransmitters leading to depression (Dayon et al. 2017; Mitchell et al. 2014).

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Chapter 16
Gene Expression, Epigenetics and Ageing

Babukrishna Maniyadath, Namrata Shukla, and Ullas Kolthur-Seetharam

Abstract As the popular adage goes, all diseases run into old age and almost all physiological changes are associated with alterations in gene expression, irrespective of whether they are causal or consequential. Therefore, the quest for mechanisms that delay ageing and decrease age-associated diseases has propelled researchers to unravel regulatory factors that lead to changes in chromatin structure and function, which ultimately results in deregulated gene expression. It is therefore essential to bring together literature, which until recently has investigated gene expression and chromatin independently. With advances in biomedical research and the emergence of epigenetic regulators as potential therapeutic targets, enhancing our understanding of mechanisms that 'derail' transcription and identification of causal genes/pathways during ageing will have a significant impact. In this context, this chapter aims to not only summarize the key features of age-associated changes in epigenetics and transcription, but also identifies gaps in the field and proposes aspects that need to be investigated in the future.

Keywords Ageing · Senescence · Gene expression · Epigenetics · Transcriptional Noise · Chromatin · Histone Variants · DNA methylation · 5hmC · SAHF

Introduction

Genetic and epigenetic information that is inherited across evolution and across life history in any given individual is essential to determine the expressability of genes, which then contribute to diverse physiological outputs. Therefore, it is not surprising that numerous studies have aimed at profiling transcripts to provide target pathways or genes that could be causal to a particular phenotype. Parallel efforts to unravel chromatin structure and functions have indeed catapulted our efforts at

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understanding how signalling to chromatin, including environmental influences, shape gene expression patterns.

Ageing is a continuum of physiological processes, which over the lifespan of an organism, contributes to fitness that is essential for the survival of individuals. In fact ageing, which also ensures removal of unfit individuals, contributes to the overall fitness of the species (Croft et al. 2015; Flatt and Schmidt 2009; Parsons 2007; Zhang and Hood 2016). Ageing also increases the risk of diseases such as diabetes, obesity, cancer and neurodegeneration (Campisi 2013; Frasca et al. 2017; Niccoli and Partridge 2012; Wyss-Coray 2016).

Given this, the quest to identify molecular mechanisms and pathways that drive healthy ageing has motivated researchers to investigate causal genes. Efforts to profile alterations in gene expression and the underlying chromatin based mechanisms have therefore provided vast amount of information. In this article, we have attempted to not only review the current status of the field, but also raise open questions or gaps in the field that need to be addressed in the future. Specifically, we have tried to link functional outputs vis-à-vis gene expression with altered chromatin status during ageing, some of which are otherwise disparate.

Transcriptome and Ageing

The physiological state of cells or tissues is directly affected by gene expression and is therefore likely to impact ageing as well. It is also well established that changes in gene expression are contributed by chromatin structure (Sproul et al. 2005; Venkatesh and Workman 2015). Although, chromatin is known to be dynamic, alterations in gene expression are more variable. Similar to studies on chromatin (discussed below), most of the transcriptome profiling has been done on liver and brain in addition to senescent primary cells in culture. In this section, we have highlighted the alterations in transcriptome including the non-coding RNAs, which have been profiled in various studies.

Global Alterations in Gene Expression

Extensive analyses of transcriptional changes have been done on various model organisms and across ages. While, most of these studies have highlighted global alterations, whether these are causal or consequential to ageing is still unclear. Alterations in gene expression have been addressed at multiple levels: (a) turning on and off of specific genes or pathways that could contribute to ageing, (b) global deregulation vis-à-vis variance and an increase or decrease in noise and (c) expression of non-coding or repetitive elements in the genome. Although most of the

studies have addressed the first aspect, very little is known about the other two. Given that the non-coding component of the genome increased during evolution, it remains to be seen if expressability of such regions correlate with or contribute to organismal longevity.

While all of these studies have clearly demonstrated that young and old cells or tissues display distinct transcriptional profiles, very few studies have addressed the time window during ageing when these changes begin to manifest. Attempts at identifying age-wise changes in gene expression have been done in fruit flies, rodents and humans (Thomas et al. 2002). It is interesting to see that in humans, differential transcription is more pronounced and varied in middle-aged individuals, at least in the brain cortex. Individuals below 42 years and above 71 years of age were seen to be more homogenous and also showed complete switch in the expression vis-à-vis up- and down-regulated genes (Lu et al. 2004). In rat liver, gene-expression changes seem to be progressive, starting from 6–8 weeks of age and possibly suggest a higher mid-life variance (Kwekel et al. 2010). In contrast, although the pattern of heterogeneity in gene expression starts early in *Drosophila,* by the age of 13 days post eclosion, it changes little thereafter (Kim et al. 2005). Studies by both Lui and Finkielstain and their colleagues draw interesting correlations between the transcriptional programs during early post-natal development and ageing. It is shown that the transcriptional modules, which govern deceleration in growth in post-natal stages, are remarkably similar to those seen in aged tissues (Finkielstain et al. 2009; Lui et al. 2010). To our best knowledge there are no studies, which have systematically investigated short-lived and long-lived individuals in any given species or compared long-lived versus short-lived evolutionarily diverse organisms to even hypothesize if the overall lifespan impinges on rate of change of gene expression or vice versa.

There are very few studies that have highlighted sexual dimorphism in terms of both temporal and amplitudinal changes. Results from Kwekel et al. (2010) clearly demonstrate that male and female rats show maximal differences in their gene expression patterns between the ages of 15–52 weeks, which interestingly also marks the period of highest reproductive fitness. Intriguingly, a subset of DEGs (differentially expressed genes) showed age-dependent inversion by being highly expressed in young females but not males and were induced more in old males when compared to old females. Similar trends have been observed in ageing murine hearts, hinting at a conserved mechanism of sexual dimorphism across organs (Vijay et al. 2015). Studies in humans showed higher global gene expression in aged male brains than their female counterparts (Berchtold et al. 2008). These clearly suggest that age-dependent transcriptional controls are different in males and females. However, some studies do indicate that in extremely aged individuals the differences between the sexes disappear and the transcriptional pattern looks more feminized (Kwekel et al. 2010), a concept that is otherwise gaining ground.

Non-coding RNAs Associated with Ageing

Based on the literature accumulated over the past two to three decades it is now evident that non-coding RNAs, which include long non-coding (lncRNAs), retroviral transcripts and short non-coding RNAs such as microRNAs, circular RNAs, snoRNAs, tRNAs and rRNAs are now known to control the expression of genes transcribed from the coding part of the genome (Bonasio and Shiekhattar 2014; Kaikkonen et al. 2011; Patil et al. 2014; Rinn and Chang 2012). Importantly, ncRNAs themselves have turned out to be regulated both in terms of their expression and turnover across biological contexts and evolutionarily diverse organisms (Kutter et al. 2012; Spurlock et al. 2016). It is therefore not surprising to find that ncRNAs have also been associated with ageing.

Small Non-coding RNAs in Ageing

Studies in leukocytes showed that progressive differential expression of small non-coding RNAs (miRs and snoRNAs) during ageing seems to be well correlated with differentially expressed coding genes. Interestingly, these changes in ncRNAs are more pronounced around mid-life (Munoz-Culla et al. 2017).

Regulation of gene expression by microRNAs is well known (He and Hannon 2004; Valinezhad Orang et al. 2014) and there are several candidate-based studies, which have highlighted their roles in ageing (Harries 2014; Smith-Vikos and Slack 2012). Given the extremely diverse physiological contexts, and the genes and pathways that have been analyzed, it is difficult to comment on the overall directionality of miR changes that affect ageing from these reports. Nevertheless, it is clear that miR levels show drastic changes during ageing and their targets control some of these miRs, in a feed-back manner (Smith-Vikos and Slack 2012; Tsai et al. 2011). However, a few studies on non-coding RNAs, detailed above, have attempted to uncover global changes in miR expression and as mentioned changes in miRs and mRNAs do show correspondence.

One of the reasons for dysregulation of microRNA profiles in ageing individuals has been attributed to abnormal levels of circularRNAs (circRNA) (Panda et al. 2017b). Known for their miR-sponging function (Hansen et al. 2013; Memczak et al. 2013), increased levels of circRNA have been reported across species. For example, in mice and flies abundance of circRNAs increased in neurons, specifically in the cortex and hippocampus of mice (Gruner et al. 2016; Westholm et al. 2014). Although, these studies seem to suggest that this could be a tissue specific phenomenon, it remains to be seen if this is indeed true and whether it is present in humans as well. Reduction in the levels of a particular circRNA, circPVT1 in senescing human fibroblasts has been shown to affect the proliferative capacity of the cells. This has been attributed to upregulation of its target microRNA let-7, which antagonizes proliferative genes such as IGF2BP1, KRAS and HMGA2 (Panda et al. 2017a). It is important to note that these transcripts have indeed been shown to be down-regulated during ageing, by several independent reports

(Hansen et al. 2004; Nishino et al. 2008). circRNAs have also been recently implicated in fecundity or fertility. It was observed that oocytes retrieved from the granulosa cells of women above 38 years of age, who registered for *in vitro* fertilization, showed higher expression of circRNAs, which was inversely co-related with number of healthy zygotes obtained (Cheng et al. 2017).

Long Non-coding RNAs and Ageing

It is rather intriguing to note that there have been fewer studies that have analyzed lncRNAs during ageing. A whole transcriptome analysis in the liver by White et al. (2015) has revealed that several pseudogenes along with lncRNAs are differentially expressed between young and old tissues. It is interesting to note that their results indicate more ncRNAs were up-regulated during ageing than those, which had reduced expression. Moreover, highlighting a possible locus specific control of lncRNA transcription Meg3, Rian and Mirg, which are adjacent and are imprinted, were shown to be up-regulated. Similarly, this study also reported two novel lncRNAs, which flank the cdkna2 gene to be up-regulated. In this context, we would like to highlight that cdkna2 itself has been independently shown to be deregulated during ageing (Lui et al. 2010). Efforts to unravel differentially expressed genes in the cerebral cortex at 6, 12 and 28 months in mice seems to suggest that ncRNA expression, at least in this tissue, could be more pronounced during early ageing (Wood et al. 2013).

This raises the need to carry out a comprehensive analyses of changes in coding transcripts, which are contributed by both transcriptional (chromatin dependent) and post-transcriptional mechanisms (mediated by ncRNAs). Another aspect that needs to be addressed in the future is the involvement of ncRNAs in affecting chromatin structure and function, which will potentially influence transcription during ageing.

rDNA Expression, Energetics and Ageing

Involvement of rDNA and rRNA in ageing was first seen in yeast, which led to the identification of *Sir2*, whose orthologues and paralogues across species are known to be major determinants of organismal longevity (Banerjee et al. 2012; Gottlieb and Esposito 1989; Sinclair and Guarente 1997; Smith and Boeke 1997). Since there are excellent reviews on the role of Sirtuins and the mechanisms of ageing in yeast, we encourage the reader to refer to them (*see* Guarente 2007; Wierman and Smith 2014). Nevertheless, emerging studies including those from yeast clearly point out that ribosomal transcription has a major impact on ageing, which in part has been ascribed to the energetic cost of maintaining and transcribing rRNA genes (Larson et al. 2012; Sinclair and Guarente 1997; Warner 1999). Maintenance of nucleolar chromatin and structure that directly impacts rDNA expression has been speculated to be a key piece in the puzzle that link energetic cost of transcription to ageing.

Age Associated Transcriptional Deregulation of Pathway Genes

The main motivation to profile gene expression changes during ageing, as in any other physiological context, has been to identify pathways that are differentially regulated. Specifically, the goal is to discover either upstream transcriptional regulators or their target genes, which maybe causal to ageing and hence, manipulating them would potentially mitigate age-related pathophysiology. Here again, several global and candidate based analyses, across models, have provided a vast amount of information and we have attempted to summarize this to give a systems-level perspective (Fig. 16.1).

While efforts to catalogue age associated DEGs in lower metazoans have enabled us to fine map alterations as a continuum, they have lacked resolution vis-à-vis tissue specificity, with a few exceptions (Girardot et al. 2006; Golden and Melov 2004; Zhan et al. 2007). Despite this limitation, such whole organismal studies have unraveled genes or pathways, which are deregulated at a global level indicating their importance in contributing towards ageing (Girardot et al. 2006; Golden and Melov 2004; Zhan et al. 2007).

Intuitively, major classes of genes that were dysregulated, at least in some tissues, exemplified cell type specific alteration in physiology. For example, a com-

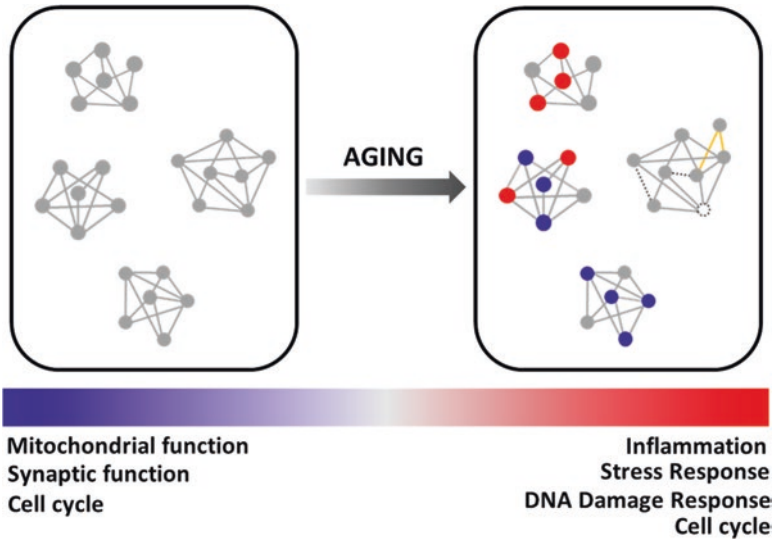

Fig. 16.1 Transcriptional networks and ageing. Hierarchical/functional clustering of differentially expressed genes (DEGs) have clearly indicated that pathways, which contribute to overall fitness to be majorly deregulated. Decreased expression of 'protective' genes and hyper-induction of genes that cause exaggerated activation of stress response pathways is consistently observed in aged individuals. In addition, silencing or induction of key regulatory factors could lead to a restructuring of the gene network and hence the physiological output from the pathway

mon signature of altered liver physiology during ageing is seen by the up-regulation of stress response genes. Studies across species have shown a general trend towards increase in expression of genes involved in xenobiotic metabolism, oxidative stress and DNA damage. Genes such as for cytochrome P450, glutathione S transferase, superoxide dismutase *etc* have been regular candidates that are over-represented (Kwekel et al. 2010; Thomas et al. 2002). Genes coding for proteins important for synaptic plasticity, vesicle release and recycling including synapsin II, RAB3A *etc* have been seen to be down-regulated in ageing brain (Girardot et al. 2006; Lu et al. 2004). Another common feature associated with ageing is diminishing visual capacity (Owsley 2016). Fruit fly studies have shown several genes that are associated with the photo-response are progressively down-regulated in ageing *Drosophila*. The list includes guanylate cyclase, neurotransmitter secretion related protein, CAMKIIa as well as genes involved in DNA damage like mre11, rad50 and Ku80 (Hall et al. 2017; Kim et al. 2005). This hints towards not just a progressive decline in photo-responsiveness but also additional accumulation of DNA damage that ultimately may lead to poor vision in old age. Similarly, up-regulation of ECM genes like type 5 collagen and alpha-2 macroglobulin in the kidney (Rodwell et al. 2004) could contribute to fibrosis associated with old age.

Cell cycle genes are one of the most prominent that seem to be differentially expressed in aged tissues, as seen from both mice and human studies. This may not be surprising since *in vitro* systems have indeed pointed towards reduced proliferative potential as a major contributing factor for cellular senescence (Carlson and Conboy 2007; Kim et al. 2008; Maedler et al. 2006). Interestingly, this was observed in tissues such as liver, lung, pancreas, muscles and kidney and was conspicuously absent in brain (Enge et al. 2017; Lui et al. 2010; Welle et al. 2003; White et al. 2015). We surmise that this may be due to relatively fewer regenerative cells in the brain, which are also known to drastically decrease during ageing (Raz et al. 2004). It is interesting that genes required for progression, as well as those which inhibit cell cycle, were dysregulated within same tissues. For example, CDKN2A, which inhibits G1/S progression has been shown to be up-regulated across tissues in mammalian systems, wherein cell cycle progression genes like cdc5b, cdk6 and cyclin b1 are up-regulated. On the other hand the E2F family of transcription factors are also down-regulated (Baker et al. 2008; Lui et al. 2010; White et al. 2015). This discord could possibly be explained by one of the following: (a) a deregulation or lack directionality, (b) some of these genes may have cell cycle independent functions and (c) distinct cellular populations, with increased mitotic potential and enhanced senescent feature in these tissues could result in such heterogeneous outputs.

Across numerous studies, ontological analyses or functional clustering of genes that display maximal changes revealed enrichment in metabolic and mitochondrial genes. This is not surprising since inability to maintain metabolic fitness or altered dietary inputs have been well established as major drivers of ageing and age-related diseases (Banerjee et al. 2012; Barzilai et al. 2012; Lopez-Otin et al. 2013). Despite the tissue- and organism-specific signatures, genes involved in lipid, amino acid and carbohydrate metabolism have been seen to have abnormal expression in older

individuals. Some of these genes include glycolytic genes such as PFK, GAPDH1; TCA cycle components like PDH, malate dehydrogenase, fumarate hydratase *etc*; also those involved in mitochondrial electron transport chain and ATP synthesis, and those important for amino acid metabolism as well as for cholesterol biosynthesis such as HMGCR are also downregulated (Boisvert et al. 2018; Golden and Melov 2004; Kim et al. 2005; Welle et al. 2003; Zhan et al. 2007).

In a similar vein, nuclear encoded mitochondrial genes that modulate organelle biogenesis also emerge as a major subclass that is altered in an age-dependent manner (Hebert et al. 2010; Joseph et al. 2012; Ungvari et al. 2008). Even here, transcription of genes that are necessary for mitochondrial energetics and ROS homeostasis are affected predominantly (Thomas et al. 2002). Although mitochondria have now emerged as key signalling organelles, one exciting aspect that still needs careful analyses is the importance of retrograde signalling that originate in the mitochondria and impinge on nuclear transcription during ageing (Couvillion et al. 2016; Latorre-Pellicer et al. 2016; Richter-Dennerlein et al. 2016). One area of research, which has otherwise made significant progress but has not been thoroughly explored is that of global and tissue specific changes in mitochondrial transcription during ageing. Mitochondrial dysfunctions and the concept of nuclear-mitochondrial synchrony, which have been shown to be affected in ageing and in various diseases, needs to be investigated in light of mitochondrial cues controlling chromatin modifications and transcription (Jazwinski et al. 2017).

Similar to metabolic factors, genes that govern immune and inflammatory pathways are enriched in both up- and down-regulated categories from multiple studies, again across models. This is not surprising since many diseases and ageing have been traced to these pathways. Increased expression of genes such as for immunoglobulins and complement system components illustrate this feature (de Magalhaes et al. 2009; Rodwell et al. 2004). Elevated expression of antimicrobial peptides in fruit flies, which are a sign of activated immune system, is also commonly observed, especially in the gut, in old cohorts (Zhan et al. 2007). Inflammatory genes such as CTSS, FCGR2B, IGJ, C3, C1QA and C1QB have been shown to be up-regulated during ageing (de Magalhaes et al. 2009).

Reiterating the gender dependent alterations, gene expression pathways were shown to be deregulated differentially in males and females. While males show a down-regulation in overall metabolic and energetic pathway associated genes, immune pathways are more up-regulated in females (Berchtold et al. 2008).

As described above, classical approaches to group genes in well-defined pathways have clearly yielded high dividends in terms of enlisting them as being deregulated during ageing or in age-related diseases. However, it is now given that both upstream inputs/signals and downstream effectors that belong to all these pathways show a high degree of cross-talk. In this context, future studies should be focused at analyzing such changes at a systems level, which could include investigating chromatin contexts of loci that harbor these genes.

Elevated Transcriptional Noise

One of the characteristic features of ageing is the loss of transcriptional control, often manifested either in the form of aberrant gene expression or increase in heterogeneity within populations (at cellular and organismal levels). Studies across species have shown that with increasing age, gene expression tends to become more stochastic, as seen by the increased level of 'noise' in transcriptional profiles (Bahar et al. 2006; Martinez-Jimenez et al. 2017). This decrease in signal to noise ratio has been proposed as one of the leading causes of ageing and age-related diseases. There are reports, which argue in favor or against this hypothesis and most of these differences could be attributed to either tissue specific effects (Warren et al. 2007) or those stemming from experimental approaches that lack high sensitivity. However, emerging literature seems to indicate a significant correlation between transcriptional noise and ageing (Enge et al. 2017; Martinez-Jimenez et al. 2017; Southworth et al. 2009; Wang et al. 2011).

One of the key questions that remain to be comprehensively addressed is that of defining periods during ageing when noise becomes dominant and whether this precedes physiological attributes of ageing. In this regard, a multi-specie study published by Wang et al. (2011) highlighted the time windows during which the noisy gene expression begins to manifest. While deregulated transcription seemed to emerge around mid-life (~30 days) in *Drosophila*, in rodents this was observed by 10–15 months of age. Interestingly, similar to sex-specific onset in global transcriptional change, described above, emergence of transcriptional noise also seemed to be sexually dimorphic. Male mice were shown to be more predisposed to display this behavior, which occurred as early as 6 months of age, across tissues. On the other hand, noisy gene expression in females showed up only by 16 months of age.

It is interesting that pathways that show most dysregulation with age such as mitochondrial function, ribosomal biogenesis, inflammation *etc*. are also the ones that show maximum variation with age (Southworth et al. 2009). Southworth and colleagues also found that genes, which showed decreased correlation were clustered together on the same chromosome. This points towards a potential contribution by chromatin architecture or epigenetic mechanisms, which might result in increased noise in gene expression.

Moreover, with age, many genes in the same functional group have been shown to change expression in opposing direction, a phenomenon known as transcriptional drift, which has been shown to occur across tissues. Interestingly, lifespan expanding paradigms such as attenuation of insulin signalling and calorie restriction have been shown to suppress transcriptional drift (Murphy et al. 2003; Rangaraju et al. 2015).

It is also worthwhile to note that many of these studies indicate asynchrony in terms of different organ-systems or tissues, which show transcriptional noise, at least at the ages that were tested. This raises an extremely important question specifically in the context of deregulated transcription as a causal mechanism for ageing,

which is as follows: does the differential emergence of noise in different tissues contribute to tissue specific ageing and whether at an organismal context, there exists hierarchy for organ-system dependent transcription causing ageing?

Chromatin, Epigenetic Landscape and Ageing

It is not surprising to see that chromatin and ageing have created much interest amongst researchers, which is reflected by a vast number of papers and reviews in the field. Several review articles by others and ourselves have reflected the state of the field. Given this, we have restrained from reiterating some of the details, which have been already described in these reviews and we encourage the readers to consult them (*see* Feser and Tyler 2011; Fraga and Esteller 2007; Lazarus et al. 2013; O'Sullivan and Karlseder 2012; Pal and Tyler 2016). However, we have tried to provide a perspective based on some of the recent studies and link these chromatin changes with the functional or physiological attributes of chromatin.

Chromatin Compaction

The state of chromatin compaction is one of the best correlates of architectural change that is associated with ageing. For example, global changes in chromatin compaction have been studied across various tissues namely liver and brain in mice, and in primary cells in culture (Bochkis et al. 2014; Dell'Orco and Whittle 1982; Gaubatz et al. 1979; Ishimi et al. 1987; Thakur et al. 1999; Zongza and Mathias 1979). Classical nuclease digestion assays have revealed that there seems to be a general trend towards more compact chromatin in aged tissues (Gaubatz et al. 1979; Zongza and Mathias 1979). *In vitro* models of senescence specifically using human fibroblasts also showed that there is an age-dependent reduction in nuclease sensitivity (Dell'Orco and Whittle 1982; Ishimi et al. 1987). However, intriguingly the decrease in accessibility has been noted for both inter-nucleosomal and nucleosomal DNA depending upon the tissue or cells that were employed (Gaubatz et al. 1979; Ishimi et al. 1987; Thakur et al. 1999; Zongza and Mathias 1979). Although, some of these changes were found to be more or less pronounced in specific tissues or cell types, these early studies nevertheless indicated that ageing was indeed associated with hetero-chromatinization. These also hinted that the age-dependent changes could be brought about by alterations in nucleosomal occupancy and contributions made by non-histone proteins (Zongza and Mathias 1979).

With the advent of next generation sequencing platforms, recent studies have now unraveled loci that show differential nuclease sensitivities. It is interesting to note that maximal differences in nucleosomal occupancy, both gain and loss, were seen at distal regulatory elements (Bochkis et al. 2014). While it is probable that any differences in these studies could reflect biological heterogeneity between cells or

tissues, it is also likely to be a result of differential nuclease treatment itself. A recent report by Mieczkowski and colleagues clearly demonstrated that sequencing reads could be influenced by the extent of nuclease digestion (Mieczkowski et al. 2016).

Although, the field generally accepts the fact that chromatin may undergo compaction during ageing, loci or region specific changes have only recently begun to be unraveled. Moreover, interpreting chromatin to be more compact based on reduced nuclease accessibility is rather too simple. It is likely that some of these nucleosome bound regions still display high dynamicity and are amenable to transition to an open chromatin conformation under different physiological conditions, even in aged cells or tissues.

Chromatin Composition

It is obvious that any structural change in chromatin will be associated with histone and DNA modifications. Identification of DNA methylation and histone modifications, which define chromatin as being open or closed (hetero- or euchromatin), enabled the field to investigate if some of these were associated with global and/or loci specific alterations during ageing. It is important to note that given the recent advances in the field vis-à-vis novel modifications and along with differential deposition of histone variants, our current understanding is likely to expand immensely in the future. Further, given that some of these modifications are directly impacted by alterations in metabolism, it will be interesting to investigate if dietary inputs can encode chromatin states (Dai et al. 2014; Fan et al. 2015; Goudarzi et al. 2016; Li et al. 2016; Lu and Thompson 2012; Sabari et al. 2015; Sabari et al. 2017). This is relevant since modulations in diets have been well established to alter fitness and also contribute to organismal longevity (Banerjee et al. 2012; Fontana and Partridge 2015; Newman et al. 2017; Peleg et al. 2016).

DNA methylation

DNA methylations are well documented to influence gene expression via various mechanisms, depending on their abundance and whether they occur on inter-genic or intra-genic regions. Most of the current studies on young and old cohorts have largely investigated changes in CpG methylations (mCpG). Two prevalent views have emerged from the studies on various model systems: (a) that a global increase in promoter methylations could lead to gene expression changes in old age and (b) that a loss of DNA methylations at repeat elements seems to agree with the heterochromatin loss model of ageing (Kwabi-Addo et al. 2007; Pal and Tyler 2016; Rath and Kanungo 1989; Takasugi 2011; Wilson et al. 1987). However, more recent efforts indicate that both loci and age specific alterations in 5-methyl cytosine (5-mC) marks are more complex, wherein global bi-directional methylation changes

are variable within a population or an individual across tissues (Bjornsson et al. 2008; Christensen et al. 2009; Maegawa et al. 2010).

Studies in human peripheral blood cells have provided insights into age-dependent alterations in DNA methylation. Bjornsson et al. (2008) have reported global alterations and that the most prevalent differential methylations occurred near the transcription start site (TSS) of a number of immune-modulatory genes in aged human unfractionated peripheral blood cells. Efforts to identify age associated differentially methylated regions (aDMRs) revealed both hyper-aDMRs and hypo-aDMRs, most of which cluster around the TSS and is consistent with the previous study (Christensen et al. 2009; Rakyan et al. 2010). Further, addressing such changes in myeloid derived monocytes and lymphoid derived T-cells uncovered cell type changes (Rakyan et al. 2010). Studies have also revealed no specific bias towards enrichment in methylation at non-/CpG islands. Highlighting tissue/cell type specific variations, while the global DNA methylation did not alter significantly between young and old human epidermis, there were loci specific hyper and hypo methylated DMRs (Raddatz et al. 2013).

A relatively comprehensive analysis of CpG methylations in multiple human tissues revealed that genes associated with ageing show similar trends towards hypermethylation (Christensen et al. 2009). Intriguingly, while a positive correlation emerged between methylation and ageing for loci in the CpG islands (CGI), those that are not associated with CpG islands showed significant loss of methylation with age in solid tissues (Christensen et al. 2009). A comparative analysis of DNA methylation from young and old intestines, both in humans and in mice (Maegawa et al. 2010), showed that telomeres had maximum hypermethylation and it was least in centromeric regions. The physiological implications of these, both in terms of mechanisms and functional outcomes are still unclear. Intriguingly, while hypermethylation in human and mice intestines showed some concordance, there was no conservation for hypomethylated regions. However, consistent with the aforementioned study, a candidate based approach revealed that CpGs at specific gene-loci such as *ESR1, CDKN2A, P2RX7*, DMR1/2 of *IGF2 etc*, showed similar trends for either hyper- or hypo-methylation, across tissues in aged mice (Maegawa et al. 2010). While the results indicated that genes involved in development and differentiation were hyper-methylated, surprisingly, no such clustering was apparent for hypo-methylated loci (Maegawa et al. 2010).

Replicative senescence in mesenchymal stem cells (MSCs) was also shown to be associated with alterations in methylation at CGIs. Similar to ageing in tissues, even in the absence of a global change, methylations at specific CpGs showed senescence associated directional changes (Schellenberg et al. 2012). Comparative analysis between MSCs from adipose tissue and bone marrow showed tissue specific methylation changes and the regions that were differentially methylated during senescence showed enrichment for metabolic genes (Schellenberg et al. 2012).

It is not surprising to find that chromatin regulators are themselves under the control of epigenetic changes. In this context, age associated methylation changes were observed in various telomere maintenance and epigenetic regulatory genes

including TERT, ERCC1, DNMT3B *etc*. It was interesting to see the CpG methylation of the *de novo* DNA methyl transferase (DNMT3B) was itself significantly reduced during ageing, across tissues (Christensen et al. 2009). These suggest that epigenetic alterations at such loci could lead to a cascading effect resulting in global changes.

Recently identified 5-hydroxy methyl cytosine marks on DNA brought about by TET enzymes have enhanced our understanding about DNA modifications and their functional and regulatory roles (Pfeifer et al. 2013; Tan and Shi 2012). Unlike in other physiological contexts, the role of TET proteins and association of 5-hmC marks along with the process of cellular/organismal ageing has not been comprehensively investigated. Global changes in 5hmC marks have been assayed during ageing in specific regions of mice brain (Chen et al. 2012; Chouliaras et al. 2012a; Chouliaras et al. 2012b; Szulwach et al. 2011). A global increase in hippocampal 5hmC content in aged mice was seen, independent of any changes in TET expression (Chen et al. 2012). On the contrary, genome wide analysis and comparison of 5hmC marks in mice and human brain samples showed that these marks were relatively stable across development and ageing (Szulwach et al. 2011). Nevertheless, there were localized differentially hydroxy methylated regions (DhMRs) at the CGIs or TSS, indicating that loci specific 5hmC could be further acquired or lost with age (Szulwach et al. 2011).

Since oxidation of 5mC results in 5hmC, it will now be interesting to compare the regions that show age associated anti-correlative changes in these marks. Specifically at loci that show loss of methylation, a gain of 5hmC could indicate directional switch during ageing. Further, a meta-analysis of transcripts emerging from such regions might provide information about the importance of DNA methylation and de-methylation (to 5hmC) in regulating gene expression in aged tissues.

Histones and Modifications

Early studies, which assayed for nuclease sensitivity and nucleosomal packaging, as mentioned previously, displayed age-associated changes. These further led the field to check if overall nucleosomal content was altered during ageing. For example, a severe loss of nucleosomes owing to a reduction in core histone protein levels has been shown to be associated with replicative ageing in budding yeast (Dang et al. 2009; Feser et al. 2010; Hu et al. 2014). Although, studies in higher organisms have shown a prevalent decrease in the levels of histones H3 and H4 (but not of H1) during ageing (Liu et al. 2013; O'Sullivan et al. 2010), there are no reports to indicate altered nucleosomal deposition. Deregulated transcriptional, post-transcriptional and translational mechanisms have been attributed to this loss in histone proteins (Liu et al. 2013; O'Sullivan et al. 2010). Based on these observations, one could speculate that at least in higher eukaryotes, reduced histones along with altered

expression/activity of histone chaperones (O'Sullivan et al. 2010) may not necessarily lead to altered nucleosomal content, but may actually result in lower turnover and contribute to closed chromatin structures.

Histone Variants

In addition to changes in total histone levels, there is ageing dependent differential deposition of histone variants, which further strengthens the concept of altered nucleosomal composition during senescence. Independent studies have demonstrated a global decline in H3.1 and H3.2, the replication dependent H3 variants in higher organisms (Maze et al. 2015; Pina and Suau 1987; Rogakou and Sekeri-Pataryas 1999). Increase in H3.3 (a replication independent and constitutively expressed variant) in senescent cells/tissues, which no longer are mitotic seemed rather predictable (Duarte et al. 2014; Maze et al. 2015; Piazzesi et al. 2016; Pina and Suau 1987; Rogakou and Sekeri-Pataryas 1999). In senescent human fibroblasts and melanocytes, elevated H3.3 and its N-terminally cleaved forms (H3.3cs1/2) were associated with repressive marks and lead to down-regulation of cell cycle progression genes (Duarte et al. 2014). Piazzesi et al. (2016) showed that even at an organismal level, ageing leads to heightened levels of H3.3 in worms.

Histone H2A and its variants have been well documented to impact transcription, both positively and negatively. Specifically, H2A.Z at +1 and − 1 nucleosomes is considered as a hallmark of actively transcribing loci. H2A.Z levels were enhanced with ageing in human cells and mice brain (Rogakou and Sekeri-Pataryas 1999; Stefanelli et al. 2018), which was mostly seen at TSS of genes involved in transcription and chromatin regulation (like Crebbp, Dnmt3a *etc*) and ubiquitin mediated proteosomal degradation (Stefanelli et al. 2018). The canonical H2A variants, H2A.1 and H2A.2, showed opposing changes during ageing. Wherein the levels of H2A.1 showed progressive reduction during the course of senescence in human fibroblasts and rat cortical neurons, H2A.2 levels were elevated (Pina and Suau 1987; Rogakou and Sekeri-Pataryas 1999). However, functional specializations of the canonical variants have not been characterized to-date. Variant macroH2A1 showed a consistent age associated increase in its isoforms (mH2A1.1 and mH2A1.2) across higher mammals and during replicative senescence in cells (Chen et al. 2015; Kreiling et al. 2011; Zhang et al. 2005). Enhanced macroH2A1 was seen to be associated with facultative heterochromatin and lead to up-regulation of Senescence Associated Secretory Phenotype (SASP) genes in fibroblasts (Chen et al. 2015; Zhang et al. 2005).

Apart from core nucleosomal histones, variants of the linker histone H1 (H1.3, H1A, H1B and H1^{o}) also showed alterations in aged chromatin. Although, cellular senescent models showed a loss of total H1 levels in senescent cells (Funayama et al. 2006), a global increase in H1 variants was seen in murine tissues during ageing (Medvedev and Medvedeva 1990). Altogether, age dependent elevation of histone variants that are primarily associated with heterochromatin formation or maintenance could aid in increased chromatin compaction (Table 16.1).

Table 16.1 Age-associated changes in histone variants

Variant	Model Organism	Tissue/Cell type	Change	Change Localised to	References
H3.1	Human	Lung fibroblasts	Decrease	Overall	Rogakou et al. (1999)
	Rat	Brain Cortical Neurons	Decrease	Overall	Pina and Suau (1987)
	Mouse and Human	NeuN+ Chromatin and whole brain	Decrease	Overall	Maze et al. (2015)
H3.2	Human	Lung fibroblasts	Slight Increase	Overall	Rogakou et al. (1999)
	Rat	Brain Cortical Neurons	Decrease	Overall	Pina and Suau (1987)
	Mouse and Human	NeuN+ Chromatin and whole brain	Decrease	Overall	Maze et al. (2015)
H3.3	Human	Lung fibroblasts	Increase	Overall	Rogakou et al. (1999)
	Human	Fibroblasts, Melanocytes	Increase in H3.3 and its cleaved forms	Euchromatic regions	Duarte et al. (2015)
	Mouse and Human	NeuN+ Chromatin and whole brain	Increase	Overall	Maze et al. (2015)
	Rat	Brain Cortical Neurons	Increase	Overall	Pina and Suau (1987)
	Worms	Whole Body, Nerve Ring, Foregut	Increase		Piazzesi et al. (2016)
H2A.1	Human	Lung fibroblasts	Decrease	Overall	Rogakou et al. (1999)
	Rat	Brain Cortical Neurons	Decrease	Overall	Pina and Suau (1987)
H2A.2	Human	Lung fibroblasts	Increase	Overall	Rogakou et al. (1999)
	Rat	Brain Cortical Neurons	Increase	Overall	Pina and Suau (1987)
H2A.Z	Human	Lung fibroblasts	Increase	Overall	Rogakou et al. (1999)
	Rat	Brain Cortical Neurons	No Change	Overall	Pina and Suau (1987)
	Mouse	Hippocampus	Increase	TSS, +1 and -1 nucleosome	Stefanelli et al. (2018)
H2AX	Human	Lung fibroblasts	Decrease	Overall	Rogakou et al. (1999)
	Rat	Brain Cortical Neurons	Increase	Overall	Pina and Suau (1987)

(continued)

Table 16.1 (continued)

Variant	Model Organism	Tissue/Cell type	Change	Change Localised to	References
macroH2A1	Human	IMR90-Fibroblasts	Increase in mH2A1.1 and mH2A1.2	Facultative Heterochromatin	Chen et al. (2015)
	Human	W138-Fibroblasts	Increase in mH2A1.2	Heterochromatin Foci	Zhang et al. (2005)
	Human	Fibroblasts	Increased but variable cell to cell intensity	Heterochromatin Foci	Kreiling et al. (2011)
	Mouse	Lung, Liver, Muscle	Increase in proportion of cells with higher intensity	Overall	Kreiling et al. (2011)
	Baboon	Muscle, Dermal Fibroblasts	Increase in proportion of cells with higher intensity in muscle, subtle increase in skin	Overall	Kreiling et al. (2011)
H1	Human	IMR90, MRC-5, BJ fibroblasts	Decrease	Overall	Funayama et al. (2006)
H1 A	Rat and Mice	Liver	Increase	Overall	Medvedev et al. (1990)
H1B	Rat and Mice	Liver	Increase	Overall	Medvedev et al. (1990)
H1°	Rat and Mice	Liver, Kidney	Increase	Overall	Medvedev et al. (1990)

Modifications

Ourselves and others have reviewed histone modifications and their association with cellular and organismal ageing extensively in the past (Lazarus et al. 2013; Pal and Tyler 2016). Hence, we have now tried to provide a more elaborate picture based on recent studies in this section, specifically in relation to alterations in gene expression. Histone modifications have also been implicated in the formation of senescence-associated heterochromatin foci (SAHF), which have been observed in senescing cells and are described in a later section.

Given the diversity of histone modifications, it is not surprising to see that some of these have been studied in a candidate based manner and have alluded to their roles in regulating expression of a subset of genes during ageing. Based on multiple studies to unravel global alterations in histone modifications, the findings can be grouped as below: (a) general euchromatic versus heterochromatic marks possibly involved in mediating chromatin architecture, (b) specific activatory or inhibitory marks mostly associated with immediate upstream regions, (c) distal regulatory regions such as silencers or enhancers and (d) at repeat and retroviral elements (Table 16.2).

Table 16.2 Age-dependent alterations in histone modifications

Modification	Model Organism	Tissue/Cell type	Change	Change Localised to	Assay Platform	References
Activatory Marks						
H3K4me3	Mouse	Hematopoietic stem cells	Spread of H3K4me3	TSS +1 and -1 nucleosome	Chip-seq	Sun et al. (2014)
	mouse	muscle stem cells	No change, reduced intensity	TSS +1 and -1 nucleosome	Chip-seq	Liu et al. (2013)
	Drosophila	Head	Overall reduction	TSS and downstream	ChIP-chip and WB	Wood et al. (2010)
	Human	Dermal Fibroblasts	Decreased but biphasic with increasing PDL	Overall	WB	Sullivan et al. (2010)
	Human	Fibroblast	Decrease	Overall	WB	Duarte et al. (2015)
	Worms	Whole Worms	No change	Overall	WB	
H3K9ac	Mice	Brain	Increase	Overall	WB	Rodrigues et al. (2014)
	Rat	Liver	Decrease	Overall	WB	Kawakami et al. (2009)
	Human	Dermal Fibroblasts	Slight increase	Overall	WB	Sullivan et al. (2010)
H3Ac	Rat	Pancreatic islets	Decrease	Enhancer at the Hnf4a Locus	ChIP	Sandovici et al. (2011)
H4K5Ac	Human	Dermal Fibroblasts	Increased with PDL	Overall	WB	Sullivan et al. (2010)
H3K18Ac	Human	IMR90 Fibroblast	Decrease	Overall	WB	Duarte et al. (2015)
H3K14ac	Human	IMR90 Fibroblast	Decrease	Overall	WB	Duarte et al. (2016)
	Rat	Liver	No change	Overall	WB	Kawakami et al. (2009)
H4K12Ac	mice	Hippocampus	No increase in response to Fear Conditioning	TSS and gene body downstream	WB and microarray and ChIP-seq	Peleg et al. (2010)
H4K16Ac	Human	Dermal Fibroblasts	Increased with PDL	Overall	WB	Sullivan et al. (2010)
	Yeast	Budding yeast cells	Increase	Overall	WB	Dang et al. (2009)
H3S10p	Rat	Liver	Increase	Overall	WB	Kawakami et al. (2009)
	Human	Dermal Fibroblasts	Decreases	Overall	WB	Sullivan et al. (2010)
H3K56Ac	Human	Dermal Fibroblasts	Decreased with PDL	Overall	WB	Sullivan et al. (2010)
	S.cer	Budding yeast cells	Decrease	Overall and at Telomeres	WB	Dang et al. (2009)
H3K36me3	Drosophila	Head region	Reduction majorly in the gene body	Mid gene body	ChIP-chip and WB	Wood et al. (2010)
N-Ac-H10	Rat and Mouse	Liver	Increase with age	Overall		Lindner et al. (1999)
H10 Asn-3/Asp-3		Kidney	Major Increase with age			

(continued)

Table 16.2 (continued)

Modification	Model Organism	Tissue/Cell type	Change	Change Localised to	Assay Platform	References
Inhibitory marks						
H3K27me3	Mouse	muscle stem cells	Majorly Increase but decrease at specific loci	Spread in to gene body and upstream	Chip-seq	Sun et al. (2014)
	mouse	QSC and ASC of muscle	Spread but no overall change in levels	Less Focussed and spread around TSS	Chip-seq	Liu et al. (2013)
	Human	Embryonic Fibroblasts	Reduction across PDLs	TSS and promoter of INK4b-p16-ARF cluster	ChIP-PCR	Bracken et al. (2006)
	Human	Dermal Fibroblasts	Increased in specific cell cycle stage-leading to overall increase	Overall	WB	Sullivan et al. (2010)
	Mouse	b-cells	Increases	Near proximal promoter region	ChIP-Seq	Avrahami et al. (2015)
	Human	IMR90 Fibroblast	Increase	Overall	WB	Duarte et al. (2015)
	Rat	Pancreatic islets	Increases	Enhancer/Promoter at Hnf4a locus	ChIP	Sandovici et al. (2011)
H3K9me3	Mice	Brain	Slight Reduction	Overall	WB	Rodrigues et al. (2014)
	Drosophila	Head region	No overall change, loss w.r.t to HP1 was more	Overall	ChIP-chip and WB	Wood et al. (2010)
		Fat body	On WB there is an increase	Spread by IF	IF	
	Rat	Liver	No change	Overall	WB	Kawakami et al. (2009)
	Human	Multiple skin fibroblasts	Overall decrease with age but increase with PDL in cells derived from aged donors	Overall	IF	Scaffidi (2006)
	Rat	Pancreatic islets	Increases	Enhancer/Promoter at Hnf4a locus	ChIP	Sandovici et al. (2011)
H3K9me1, me2	Human	Dermal Fibroblasts	Decreases	Overall	WB	Sullivan et al. (2010)
			Increases with PDL			
uH2A and uH2B	Mice	Brain	Increase	Overall	WB	Morimoto et al. (1993)
H4K20me3	Human	Dermal Fibroblasts	Decreases	Overall	WB	Sullivan et al. (2010)
H4K20me2	Rats	Liver, Kidney	Increase	Overall	Mass-spec	Sarg et al. (2002)
			No Change			
H4K20me1			Increase			

(continued)

Table 16.2 (continued)

Modification	Model Organism	Tissue/Cell type	Change	Change Localised to	Assay Platform	References
Distal Regulatory Marks						
H3K4me1	Rat	Pancreatic islets	Decrease	Enhancer at Hnf4a locus	ChIP	Sandovici et al. (2011)
	Human	Dermal Fibroblasts	Decreased but biphasic in cell cycle with increasing PDL	Overall	WB	Sullivan et al. (2010)
At Repeat Elements						
uH2B	Yeast	Budding yeast cells	Increase with age	At the telomeres	WB	Ho Rhie et al. (2013)
H4K16Ac	Yeast	Budding yeast cells	Increase with age	At telomeres and rDNA loci	ChIP	Dang et al. (2009)
H4K16Ac	Human	Dermal Fibroblasts	Increase with PDIs	At the telomeres	ChIP and Dot Blot	Sullivan et al. (2010)
H4K20me2	Human	Dermal Fibroblasts	No change in levels but shift in cell cyce phase	At the telomeres	ChIP and Dot Blot	Sullivan et al. (2011)
H3K79me2	Human	Dermal Fibroblasts	Increase with PDIs	At the telomeres	ChIP and Dot Blot	Sullivan et al. (2010)

The prominent euchromatin marks like K4me3, K9Ac, K14Ac, K36me3 on histone H3 and acetylations on K5, K12 and K16 of histone H4 have been widely assayed in the context of ageing (Duarte et al. 2014; Kawakami et al. 2009; Liu et al. 2013; O'Sullivan et al. 2010; Sun et al. 2014; Wood et al. 2010). Several studies across tissues and cell types have reported an overall reduction in the transcriptional activation mark H3K4me3, at the proximal promoter regions (Duarte et al. 2014; Liu et al. 2013; O'Sullivan et al. 2010; Sun et al. 2014; Wood et al. 2010). The H3K4me3 peak at the TSS is now considered as a bona-fide mark of active transcription. ChIP-Seq analysis in young and old hematopoietic stem cells indicated that, while some regions had decreased H3K4me3, these were much fewer when compared to the percentage of genes, which showed an age associated gain in this mark (Sun et al. 2014). Interestingly, both reduced levels and spread of H3K4me3 (away from its typical +1 and − 1 nucleosomes) have been reported specifically in old stem cells (Liu et al. 2013; Sun et al. 2014). In addition to the findings from Sun and colleagues on promoter switching and expression of isoforms in old HSCs (Sun et al. 2014), this spread of K4me3 raises the possibility that TSS in aged cells could be altered or ill defined (Fig. 16.2). Efforts to investigate the H3K4-me1/me2/me3 in cellular models of senescence added a layer of complexity with regards to cell cycle phase dependent alterations at a global level (O'Sullivan et al. 2010). It remains to be seen if such changes are found in other cell/tissue types and whether

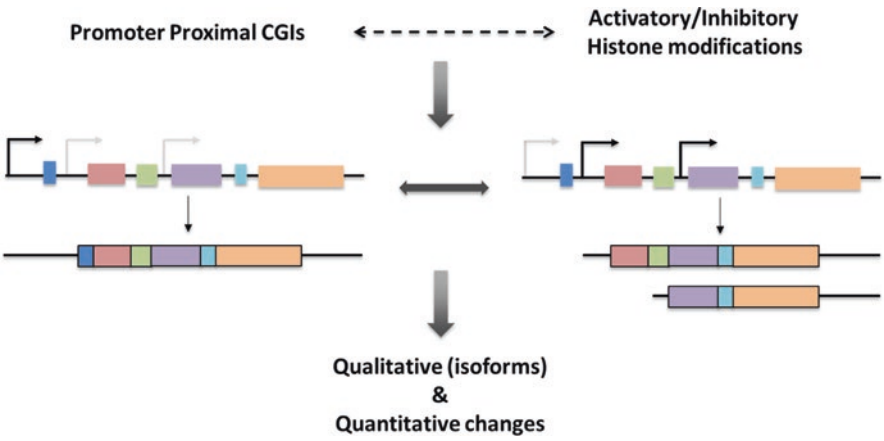

Fig. 16.2 Alternate start site specification. Loss of 5mC at CpG islands (CGIs) and spilling of H3K4me3, beyond the typical di-nucleosomal context, could lead to specification of novel or aberrant transcription start sites (TSS). Specification of such TSS have been proposed to generate alternate transcripts, which could have differential localizations, stabilities and in some instances code for different isoforms of proteins

these lead to increase in noise vis-à-vis both abundance and type of transcripts produced during ageing (Fig. 16.3).

Limited numbers of studies that have investigated the changes in histone H3 lysine-9 acetylation, an open chromatin signature, illustrate it to be more variable across cell/tissue types during ageing (Kawakami et al. 2009; O'Sullivan et al. 2010; Rodrigues et al. 2014). In aged rat liver there was a global decrease in K9Ac, whereas it showed an overall increase in nuclei from old mice brains (Kawakami et al. 2009; Rodrigues et al. 2014). Although there seems to be a very slight accumulation of K9Ac in senescent human fibroblasts, the changes were also cell cycle dependent (O'Sullivan et al. 2010). In the absence of genome wide studies to define loci-specific changes in H3K9Ac, at present, there is poor concordance between organismal ageing and replicative senescence.

Across models, other activatory modifications on H3 showed a general trend towards decrease during ageing. For example, reduced global H3 acetylation (Sandovici et al. 2011), K18Ac, K14Ac (Duarte et al. 2014) K56Ac (Dang et al. 2009; O'Sullivan et al. 2010) and K36me3 (Wood et al. 2010) were observed. On the contrary, activatory marks on histone H4 such as K5Ac and K16Ac were shown to increase with age (Dang et al. 2009; O'Sullivan et al. 2010). In addition to H4 marks, deamidation and N-terminal acetylation of H1 also showed a substantial increase with age in mice and rat tissues (Lindner et al. 1999). Even though this

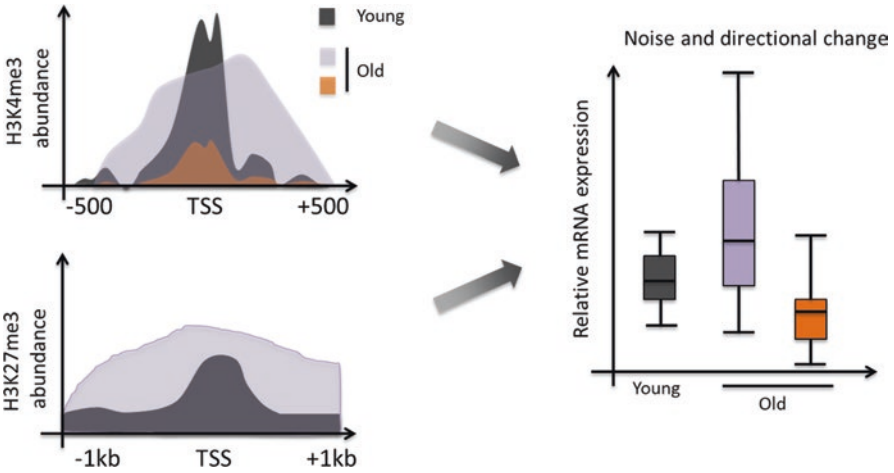

Fig. 16.3 Epigenetics as causal mechanism for age associated transcriptional deregulation: Perturbations in histone modifications around the upstream cis-regulatory elements lead to transcriptional noise and altered expression. An increase in the repressive mark H3K27me3 and a decrease in the active mark H3K4me3 occur during ageing. Intuitively, these could result in both up- and down-regulation of genes, which have been seen independently. In addition to these, H3K4me3 that is typically present in −1/+1 nucleosomes also seems to spread around the TSS. Similarly, H3K27me3 is also shown to spread in aged tissues/cells. It is therefore plausible that altered regulatory histone modifications cause the age-associated elevation in transcriptional noise

seems to indicate a non-directional perturbation, whether these changes happen at same or different loci needs to be addressed. Irrespectively, this once again points towards deregulated signatures that may contribute to aberrant or noisy transcription.

With the general understanding that heterochromatin content increases during ageing, there has been a special effort to unravel the importance of repressive histone modifications in this regard. H3K27me3, a polycomb dependent repressive chromatin mark, showed consistent increase during ageing, both in stem cells and differentiated cells (Avrahami et al. 2015; Duarte et al. 2014; Liu et al. 2013; O'Sullivan et al. 2010; Sandovici et al. 2011; Sun et al. 2014). More detailed analysis at specific loci across the genome revealed that unlike in younger cells, methylation at this residue tend to be less focused around the promoter proximal sites and spread in to upstream and downstream regions (with reference to TSS) in older cells (Liu et al. 2013, Sun et al. 2014). Heterochromatic H3K9me3 and H4K20me3 marks showed an overall decrease in fibroblasts with higher population doubling

levels (PDLs). Interestingly, this was associated with a concomitant increase in mono- and/or di-methylations (O'Sullivan et al. 2010; Rodrigues et al. 2014; Sarg et al. 2002; Scaffidi and Misteli 2006). On the contrary, aged tissues such as *Drosophila* heads and rat liver showed no apparent change in total K9me3 levels (Kawakami et al. 2009; Wood et al. 2010). But fat body and pancreatic islets from aged *Drosophila* and rats, respectively, showed enhanced tri-methylation (Kawakami et al. 2009; Wood et al. 2010). Assaying for H3K9me3 by immune-fluorescence in *Drosophila* fat body showed an increased intensity and dispersed localization of this mark in old flies (Wood et al. 2010).

Ubiquitination on H2A, a co-signature of polycomb associated K27me3 mark, and on H2B also increased with age and this seemed to be consistent across cell/tissue type (Morimoto et al. 1993; Rhie et al. 2013). Although, these observations raise the possibility of combinatorial/interdependent histone marks to be similarly regulated during ageing, this requires further comprehensive analysis. Taken together, the preponderance of inhibitory chromatin marks are in concordance with increased chromatin compaction and heterochromatinization in aged cells/tissues. This notion is also supported by the observation that both H3K27me3 and H3K9me3 showed a general trend of dispersed localization on chromatin during the process of ageing (O'Sullivan et al. 2010; Wood et al. 2010). Albeit that some of these studies highlight a lack of correlation regarding either an increase or decrease in repressive modifications, it is important to note that assaying for these by biochemical or cell biological methods does not provide the required resolution. Hence, a thorough high-throughput based analysis will provide information about regions that show a gain or loss of heterochromatic marks.

There is enough evidence in the literature demonstrating interdependence of DNA methylations and histone modifications, that eventually result in different chromatin states (Balasubramanian et al. 2012; King et al. 2016; Morselli et al. 2015; Rose and Klose 2014). With this premise, studies that focused on aged chromatin have tried to draw correlations between bi-directional changes in the levels of DNA methylation and states of chromatin (w.r.t. histone marks). Across cell types, age dependent hypo-DMRs were strongly correlated with histone modifications such as H3K4me1/3 and K27Ac that mark actively transcribing regions or active enhancers (Avrahami et al. 2015; Raddatz et al. 2013; Schellenberg et al. 2012; Sun et al. 2014). Not surprisingly, heterochromatin marks like H3K9me3 and K27me3 were highly associated with hyper-aDMRs (Raddatz et al. 2013; Rakyan et al. 2010; Schellenberg et al. 2012; Sun et al. 2014). Interestingly, poised/bivalent regions that harbor both H3K27me3 and H4K4me3 marks at the promoters showed elevated CpG methylation during ageing (Raddatz et al. 2013; Rakyan et al. 2010). These results indicate that the intricate crosstalk between modifications on DNA and histones (Table 16.3) bring about chromatin remodeling, with an overall trend towards heterochromatinization, during ageing (Fig. 16.4). Furthermore, it is still not clear as to which of these modifications drive ageing associated chromatin compaction.

Physiologically, key cellular and molecular dysfunctions ranging from stem cell fatigue to DNA damage and mitochondrial/metabolic outputs have been shown to be major drivers of ageing (Balaban et al. 2005; Beerman et al. 2014; Bratic and

Table 16.3 Association of DMRs with histone modifications

DNA methylation status	Model Organism	Tissue/Cell type	Associated Histone marks	Loci	References
Hypo-DMRs	Mouse	β-cells	H3K4me1, H3K27Ac	Active Loci	Avrahami et al. (2015)
	Human	MSC-AT and ESCs	H3K4me3	Active Loci	Schellenberg et al. (2011)
	Human	Epidermis	H3K27Ac, H3K4me1	Enhancers	Raddatz et al. (2013)
	Human	HSCs	H3K4me3, H3K27me3	Promoters and Poised	Sun et al. (2014)
Hyper DMRs	Human	CD4+ T Cells	H3K9me3, H3K27me3, H3K4me3	Heterochromatic and poised	Rakyan et al. (2010)
	Human	MSC-AT and ESCs	H3K9me3, H3K27me3,	Heterochromatic	Schellenberg et al. (2011)
	Human	Epidermis	H3K27me3, H3K4me3, H3K27Ac	Poised Promoters	Raddatz et al. (2013)
	Human	HSCs	H3K27me3	Heterochromatic	Sun et al. (2014)

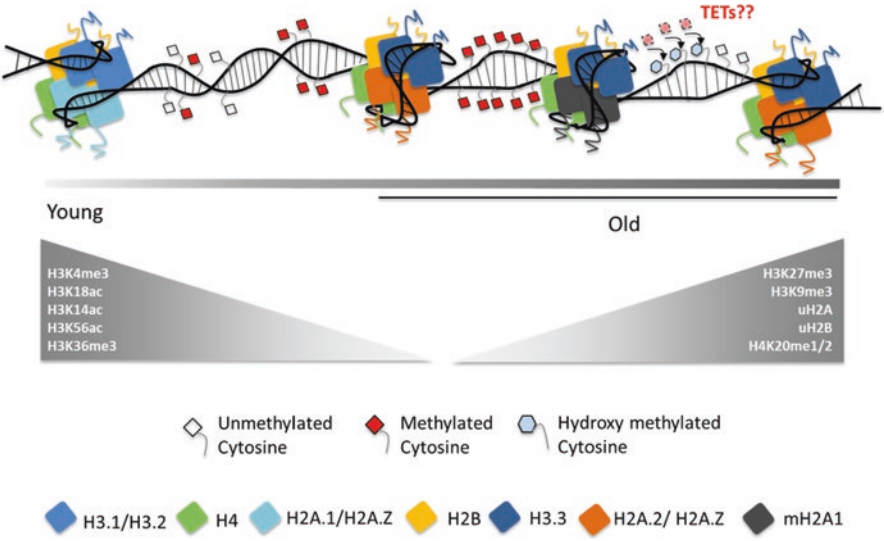

Fig. 16.4 Chromatin composition and modifications drive global compaction during ageing. Globally, chromatin gets compacted during ageing. This is associated with altered nucleosomal composition and a gain in heterochromatic marks, as detailed in the figure and the article. Some of these alterations are also evident in the form of senescence associated heterochromatin foci (SAHF) that are mostly seen in *in vitro* models of ageing. It is still unclear if the loss of 5mC at some regions correlates with a gain in 5hmC at the very same loci. Meta-analyses of recruitment of chromatin modifiers, along with these marks and in an age-dependent manner, will be critical to assess the progression in altered chromatin architecture

Trifunovic 2010; Chambers et al. 2007; Chen et al. 2007; Di Micco et al. 2008; Finkel 2015; Latella et al. 2017; Pollina and Brunet 2011). In this regard, very few existing and emerging studies have tried to link the changes in chromatin to these, possibly mediated by altered gene expression patterns and deregulated DNA damage response (Burgess et al. 2012; Gorbunova and Seluanov 2016; O'Sullivan et al. 2010). Independently, age dependent reduction in genomic integrity and alterations in histone modifications that affect DNA damage response are well documented (Oberdoerffer et al. 2008; Pan et al. 2014; Schotta et al. 2008; Van Meter et al. 2014). However, very few studies have linked these together at phenomenological and mechanistic levels during ageing. Recent reports illustrate that both DNA and histone modifications are visibly dissimilar in young and old stem cells. Modifications such as H3K4me3, K9me3 and K27me3, which are central determinants of gene expression and genome integrity, show dramatic alterations during ageing that together could influence proliferation and differentiation potentials (Liu et al. 2013; Schellenberg et al. 2012; Sun et al. 2014). Here again, combining transcriptome data with ChIP-seq analyses for histone modifications will be crucial to unravel the importance of chromatin structure in driving age-associated transcriptional changes.

Senescence Associated Heterochromatin Foci (SAHF)

Formation of dense and compact DNA foci, now called Senescence Associated Heterochromatin Foci (SAHFs) is considered to be one of the better-characterized correlates of irreversible chromatin changes during senescence. Therefore, it is not surprising that several review articles, including ours (Lazarus et al. 2013), have elaborated on the identification and composition vis-à-vis histone variants, modifications and non-histone proteins (Adams 2007; Funayama and Ishikawa 2007; Narita 2007; O'Sullivan and Karlseder 2012; Pal and Tyler 2016; Rastogi et al. 2006; Sadaie et al. 2013) (Table 16.4). In this light, we have only reviewed more recent studies and specifically highlight aspects that provide novel insights.

SAHFs have been mostly observed and described for *in vitro* models of senescence and there are no studies about their presence in aged tissues as far as our literature survey goes (Funayama et al. 2006; Narita et al. 2006; Narita et al. 2003; Zhang et al. 2007; Zhang et al. 2005). However, in a report by Kreiling et al. (2011) it seemed as if mH2A, a component of SAHFs, was enhanced and strongly associated with heterochromatic foci in aged liver and muscle. Although, the authors of the study do not comment if these could be typical SAHFs, this needs further investigation in the future.

It is important to note that very few reports to-date, have tried to address the functional relevance, if any, of formation of these foci. Further, there is no clear understanding of the regions that contribute to the formation of these foci, although heterochromatinization itself is a generic event during ageing. Given that heterochromatin signatures like H3K9me2/3 and H3K27me3 are enriched in SAHFs, a study by Chandra and colleagues methodically demonstrated the spatial

Table 16.4 Senescence-associated heterochromatin foci from *in vitro* models

Model Organism	Tissue/Cell type	Associated Histone Variants	Associated Histone Modifications	Associated Proteins	Additional Observations	Genes Affected	Ref	Paradigm
Human	IMR90, WI38 fibroblasts		H3K9me3	HP1α,β,γ		Up: p21 Down: E2F responsive genes like cyclin E	Narita et al. (2003)	Ras induced and late passage
Human	WI38 fibroblasts	macroH2A1.2	H3K9me3	HP1α,β,γ, HIRA, ASF1a	ASF1a H3 binding function is necessary		Zhang et al. (2005)	Ras induced and late passage
Human	WI38 fibroblasts		Marks are distinct from mitotic/apoptotic condensed chromatin		Whole chromosome condense in to SAHF		Funayama et al. (2006)	Ras induced senescence
Human	IMR90, BJ fibroblasts		H3K9me3	HMGA1, HMGA2		Down regulation of Cyclin A, MCM3	Narita et al. (2006)	Ras induced senescence
Human	MCF7			Prohibitin, HP1γ	Prohibitin was indispensable & associated with HP1	Represses E2F mediated Transcription	Rastogi et al. (2006)	Adriamycin induced Senescence
Human	WI38 fibroblasts	macroH2A1.2	H3K9me2/3 HP1γS9p	HP1α,β,γ, HIRA, ASF1a	Each chromosome condenses in to SAHF focus		Zhang et al. (2007)	Ras induced senescence
Human	IMR90 fibroblasts		H3K9me2/3, H3K27me3, HP1γ		K9me3 limited to the core of SAHF, K9me2 formed a layer around it, K27me3 formed the outer shell	Down regulation of CCNA2, Up regulation of p16	Chandra et al. (2012)	Ras induced senescence
Human	WI38 fibroblasts				Reduced intra-TAD interactions increased inter-TAD interactions		Chandra et al. (2015)	WI-38hTERT/GFP-RAF1-ER moel
Human	IMR90 fibroblasts				Reduced LaminB1 in K9me3 regions and Increase at K27me3 regions		Sadaie et al. (2013)	Ras induced senescence

organization and senescence-associated repositioning of these modifications (Chandra et al. 2012). Interestingly, H3K27me3 that formed the 'outermost shell' of the repressive core in these SAHFs (Chandra et al. 2015; Chandra et al. 2012) could act as potential barriers to regions of transcriptional activity.

Attempts to unravel if specific regions of chromosomes or entire chromosomes are packaged into SAHFs have been scarce. Early studies have shown that whole chromosomes condense to form a single SAHF (Funayama et al. 2006; Zhang et al. 2007). But recent observations with inactivated X chromosome in senescent cells showed that involvement of whole chromosomes might not be necessary for SAHF remodeling (Chandra et al. 2012). However, whether this is true for all chromosomes and cells in a population and the heterogeneity associated, have not been investigated thus far.

Unlike euchromatin, quite expectedly heterochromatin forms a dense structure, which has led researchers to investigate Topologically Associated Domains (TADs) in SAHFs. A very recent study demonstrated the architectural changes in the genome during cellular senescence (Chandra et al. 2015). With no apparent change in global chromosomal interactions, these results revealed striking changes in the strength of intra-TAD interactions. While they saw a loss of interactions within TADs, senescent cells seemed to gain cross boundary interactions across TADs. Domains, which lost boundary strength with ageing, were enriched in H3K9me3 and Lamin Associated domains (LADs). Whereas the TAD boundaries that attained strength with age, showed enrichment in H3K36me3 marks. Very interestingly, the study demonstrated that a LAD that drifted away from the nuclear periphery during senescence was in close proximity to the *CDKN2A* locus, expression from which has been well documented to be altered during ageing (Chandra et al. 2015). These findings have defined yet another layer of complexity to the mechanisms that can possibly lead to altered gene expression and thus the pathophysiology of ageing.

Concluding Remarks

Interactions between several transcription factors and plasticity of chromatin have only recently begun to be appreciated. Such interactions are likely to be more complex during ageing since physiological manifestations are a summation of both short term and long term impact on mechanisms that determine gene expression. This brings in the burden of ensuring accuracy and responsivity throughout the lifespan of an individual.

While most of the current literature provides a general picture, it will be essential to investigate such changes in gene expression and chromatin architecture in response to altered environmental inputs. This will be key, because ageing is affected by a plethora of factors. Moreover, such analyses will also likely provide information vis-à-vis memory and plasticity of gene expression mechanisms. As pointed out earlier, a systems level approach, including help from mathematical modeling, will be critical in analyzing both existing and newly generated high-throughput data.

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Chapter 17
Glycobiology of Aging

Fabio Dall'Olio

Abstract Glycosylation is one of the most frequent post-translational modification of proteins. Many membrane and secreted proteins are decorated by sugar chains mainly linked to asparagine (N-linked) or to serine or threonine (O-linked). The biosynthesis of the sugar chains is mainly controlled by the activity of their biosynthetic enzymes: the glycosyltransferases. Glycosylation plays multiple roles, including the fine regulation of the biological activity of glycoproteins. Inflammaging is a chronic low grade inflammatory status associated with aging, probably caused by the continuous exposure of the immune system to inflammatory stimuli of endogenous and exogenous origin. The aging-associated glycosylation changes often resemble those observed in inflammatory conditions. One of the most reproducible markers of calendar and biological aging is the presence of N-glycans lacking terminal galactose residues linked to Asn_{297} of IgG heavy chains (IgG-G0). Although the mechanism(s) generating IgG-G0 remain unclear, their presence in a variety of inflammatory conditions suggests a link with inflammaging. In addition, these aberrantly glycosylated IgG can exert a pro-inflammatory effect through different mechanisms, triggering a self-fueling inflammatory loop. A strong association with aging has been documented also for the plasmatic forms of glycosyltrasferases B4GALT1 and ST6GAL1, although their role in the extracellular glycosylation of antibodies does not appear likely. Siglecs, are a group of sialic acid binding mammalian lectins which mainly act as inhibitory receptors on the surface of immune cells. In general activity of Siglecs appears to be associated with long life, probably because of their ability to restrain aging-associated inflammation.

Keywords Glycosylation in aging · Inflammaging · Hypogalactosylated antibodies · Siglecs · Plasmatic glycosyltransferases

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The gene names and enzyme names are according to HUGO nomenclature rules (https://www.genenames.org/)

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Abbreviations

DAMPS	danger-associated molecular patterns
DC-SIGN	dendritic cell-specific ICAM-grabbing non-integrin
Fuc	fucose
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetylglucosamine
ITIM	immunoreceptor tyrosine-based inhibition motif
Man	mannose
MBL	mannose binding lectin
PAMPS	pathogen-associated molecular patterns
RA	rheumatoid arthritis
ROS	reactive oxygen species
SASP	senescence-associated secretory phenotype
SHP	Src-homology 2 domain (SH2)-containing phosphatese
TLR	toll-like receptor

Essentials of Glycosylation

Proteins or lipids to which a sugar portion is covalently attached are referred to as glycoproteins and glycolipids and, together, as glycoconjugates. Their biosynthesis is mediated by glycosyltransferases, enzymes which catalyze the addition of single monosaccharides to proteins, lipids or other sugars. Glycosylation (not to be confused with the non-enzymatic addition of glucose to lysine residues, which is referred to as glycation) is one of the most frequent and functionally relevant post-translational modification of proteins. A significant percentage of the human genes encode proteins related with biosynthesis, function and degradation of the sugar chains. The two main kinds of sugar-protein linkages are referred to as N- and the O-linkages. In the first, the reducing N-acetylglucosamine (GlcNAc) of the glycan is linked to the nitrogen of asparagine. N-linked chains are pre-assembled as dolichol-linked glycans comprised of 2 GlcNAc, 9 mannose (Man) and 3 glucose (Glc) residues in the rough endoplasmic reticulum and *en bloc* transferred on a Asn-X-Ser/Thr sequence (where X is any aminoacid, except proline) of the nascent protein (Kornfeld et al. 1985) (Fig. 17.1, and a list of the enzymatic activities involved in this process is provided in Table 17.1). Successively, during the transit of the glycoprotein along the exocytic pathway, this immature N-linked glycan undergoes trimming of 3 Glc and a total of 6 Man residues. Next, residues of GlcNAc, Gal, sialic acid (Sia) and fucose (Fuc) are added, resulting in various structures mounted on 2–4 branches (*antennae*) which form "complex type" glycans. The classical trisaccharide unit which forms an *antennae* is comprised of Sia-Gal-GlcNAc-R. When a GlcNAc residue is β1,4-linked to the innermost Man residue (Fig. 17.1), it is referred to as

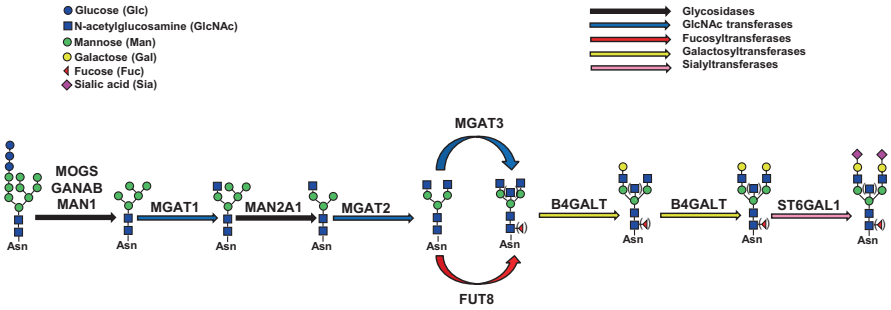

Fig. 17.1 Schematic representation of N-glycan biosynthesis. A structure comprised of 2 GlcNAc, 9 Man and 3 Glc residues is transferred *en bloc* to an Asn residue of the nascent polypeptide chain. Through the activity of α-glucosidases (MOGS and GANAB) and of α-mannosidase-I (MAN1), 3 glucose and 4 mannose residues are removed, leading to the GlcNAc2, Man5 structure, which serves as an acceptor for GlcNAc transferase-I (MGAT1). Then, two additional Man residues are removed by α-mannosidase-II (MAN2A1), forming the substrate structure for GlcNAc transferase-II (MGAT2). To the resulting structure, galactose residues can be added by B4GALT with or without the concomitant addition of core fucose and/or bisecting GlcNAc. The last step is the addition of sialic acid residues. ST6GAL1 is the only sialyltransferase able to catalyze α2,6-sialylation of N-linked chains, while the α2,3 sialylation can be mediated by multiple enzymes (not shown). Sugar residues in bracket can be present or absent in the structure

Table 17.1 Nomenclature of glycosidases and glycosyltransferases described in the text

Enzyme name	Aliases	Abbreviations	Gene name
Mannosyl-Oligosaccharide Glucosidase-I	Glucosidase I	Glcase-I	*MOGS*
Glucosidase-II α-Subunit	Glucosidase II	Glcase-II	*GANAB*
Class 1 α1,2 mannosidases	Mannosidase I	Man ase-I	*MAN1*
Mannosidase Alpha Class 2A Member 1	Mannosidase-II	Man ase II	*MAN2A1*
Mannosyl (α1,3)-glycoprotein β1,2-N-acetylglucosaminyltransferase	GlcNAc transferase-I	GnT-I GlcNAc-T1	*MGAT1*
Mannosyl (α1,6)-glycoprotein β1,2-N-acetylglucosaminyltransferase	GlcNAc transferase-II	GnT-II GlcNAc-T2	*MGAT2*
β1,4-galactosyltransferase 1	β1,4-galactosyltransferase-I	β4Gal-T1	*B4GALT1*
Mannosyl (β1,4)-glycoprotein β1,4-N-acetylglucosaminyltransferase	Bisecting GlcNAc transferase	GnT-IV	*MGAT3*
	GlcNAc transferase-IV		
Fucosyltransferase 8	α1,6 fucosyltransferase 8	FucT-VIII	*FUT8*
ST6 β-galactoside α2,6-sialyltransferase 1	α2,6 sialyltransferase 1	α2,6ST, SiaT1	*ST6GAL1*

The gene names and enzyme names are according to HUGO nomenclature rules *(*https://www.genenames.org/)

"bisecting GlcNAc" and is not elongated further. The presence of a fucose residue α1,6-linked to the innermost GlcNAc is referred to as "core fucosylation" (Fig. 17.1). In O-linked chains, the first non-reducing sugar is linked to the hydroxyl group of serine or threonine. Although different sugars, such as GlcNAc, Man or Fuc can be linked to serine or threonine, the most classical and frequent type of O-glycosylation involves the addition to serine or threonine of N-acetylgalactosamine (GalNAc).The biosynthesis of this type of sugar chain begins in the *cis*-Golgi apparatus and involves the stepwise addition of single monosaccharides along the exocytic pathway. The addition of the first GalNAc residue to the peptide can be mediated by 20 different peptide:GalNac transferases with subtle substrate differences (Bennett et al. 2012). While the biosynthesis of nucleic acids and proteins is a deterministic process, glycosylation is a probabilistic process, regulated mainly by the relative abundance of the glycosyltransferases, of their substrates and of catabolic enzymes (glycosidases) which remove single monosaccharide units. Due to its probabilistic nature, glycosylation exhibits microheterogeneity, which means that the structure of the sugar chains attached to a specific glycosylation site in a given glycoprotein displays a certain degree of variability. For example, the core fucose or the bisecting GlcNAc can be present on some but not all the N-linked chains attached to Asn_{297} of IgG.

Glycoconjugates serve a variety of biological roles (Varki 2017; Ohtsubo and Marth 2006; Hart and Copeland 2010) and often act as a "fine tuning" of cellular and molecular interactions. Glycosylation undergoes profound changes in pathological conditions, including cancer [reviewed in (Dall'Olio et al. 2012; Pinho and Reis 2015)], inflammatory and autoimmune diseases and in aging (Dall'Olio et al. 2013). Characteristic aging-associated changes of N-linked glycans present in serum and body fluids (N-glycome) have been detected by analyzing samples from a large number of individuals using high-throughput methods of analysis. Aging-associated structures are often similar to those associated with inflammatory and autoimmune diseases, in particular the structures linked to Asn_{297} of immunoglobulin G heavy chains (Maverakis et al. 2015).

A Link Between Aging and Inflammation: The Inflammaging

The long life exposure to proinflammatory stimuli of microbial, environmental and endogenous origin is at the basis of a chronic, low-grade, asymptomatic inflammatory status known as inflammaging (Franceschi 2007; Franceschi et al. 2007). Some of the stimuli putatively triggering inflammaging are depicted in Fig. 17.2. They include (but are not limited to) the pro-inflammatory cytokines released by senescent cells displaying the "senescence associated secretory phenotype" (SASP) (Rodier and Campisi 2011), the danger-associated molecular patterns (DAMPS), released by necrotic or damaged cells, the pathogen-associated molecular patterns (PAMPS) associated with microorganisms and the advanced glycation end-products (AGEs) released by glycated proteins. Senescent cells are more numerous in elderly people, while DAMPS, PAMPS and AGEs stimulate various receptors of the innate immune system, resulting in its chronic low-grade activation.

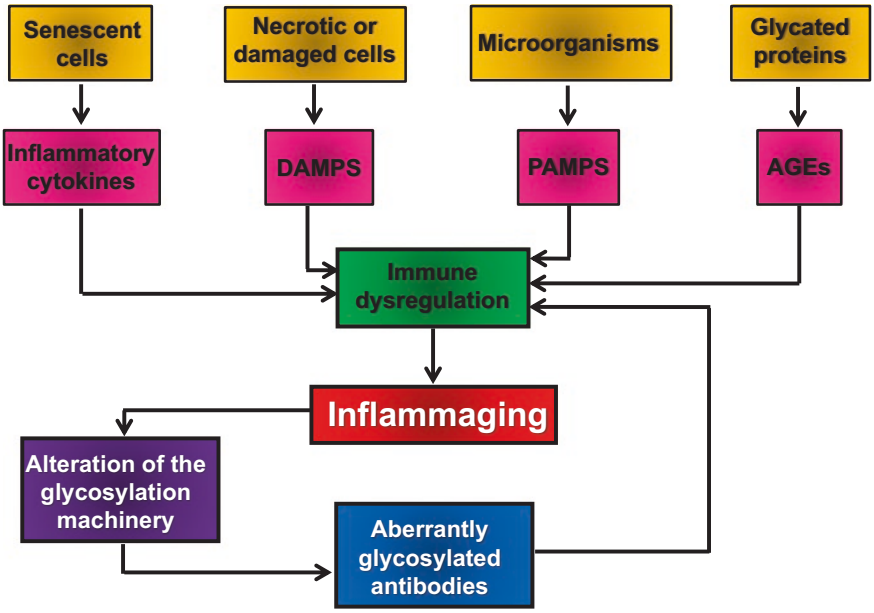

Fig. 17.2 Proposed model linking the basis of inflammaging and IgG-G0 generation. Aging is associated with accumulation of senescent cells which secrete inflammatory cytokines (SASP phenotype), necrotic or damaged cells which release DAMPS, life long exposure to microbial PAMPs and AGEs. These are among the plausible causes of inflammaging. Through poorly understood mechanisms, inflammaging alters the glycosylation machinery, generating aberrantly glycosylated antibodies which, in turn, contribute to the systemic inflammatory status

Glycomic Changes in Aging and Inflammatory Diseases

In this section we will describe the major N-glycomic changes associated with inflammatory conditions and aging which are highly similar, suggesting that inflammaging may be a link between the two conditions.

Inflammatory Diseases

The Asn_{297} residue of the heavy chains of IgG bears a biantennary N-linked chain (Arnold et al. 2007) whose two branches are terminated by a variable number of sialic acid and galactose residues (Tsuchiya et al. 1998), while the core structure can be substituted by bisecting GlcNAc and/or core-linked fucose (Figs. 17.1 and 17.3). Only a minority of the chains are terminated by sialic acid, which is usually α2,6-linked (Anthony et al. 2008b). Owing to its peculiar position in the cavity between the two IgG heavy chains, these N-linked chains regulate the interaction of IgG with recognition molecules (Krapp et al. 2003; Barb and Prestegard 2011), resulting in the mediation of IgG stability (Yamaguchi et al. 2006) and effector functions (Raju 2008).

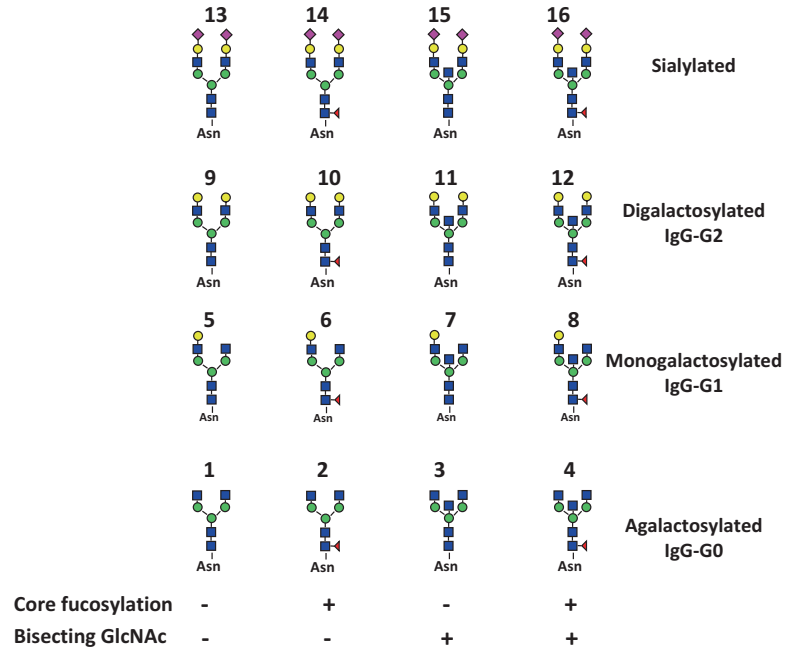

Fig. 17.3 Diantennary N-glycans usually decorating Asn_{297} of IgG heavy chains. These structures differ mainly for the number of galactose residues and for the presence/absence of bisecting GlcNAc and core fucosylation. A minority of the sugar chains carries sialic acid, usually in α2,6-linkage

A large body of evidence indicates that Asn_{297} -linked chains undergo glycosylation changes in inflammatory conditions and in aging. These glycosylation changes include a reduced presence of galactose and sialic acid on the branches, leaving GlcNAc in terminal position; an increased expression of bisecting GlcNAc and an increased core fucosylation (Fig. 17.3). Among these changes, the level of branch galactosylation is certainly the most relevant. Three glycotypes of IgG, called IgG-G0 (no galactose, structures 1–4 in Fig. 17.3), IgG-G1 (galactose on one arm, structures 5–8 in Fig. 17.3) and IgG-G2 (galactose on both arms, structures 9–12 in Fig. 17.3) have been defined on the basis of the level of branch galactosylation (Jefferis et al. 1990; Wormald et al. 1997). A seminal study published in 1985 (Parekh et al. 1985), reported for the first time an increased presence of N-linked chains terminated by GlcNAc in IgG (IgG-G0) of serum from rheumatoid arthritis (RA) and primary osteoarthritis patients [reviewed in (Parekh et al. 1989; Rademacher et al. 1988; Rademacher 1991)]. Increased levels of agalactosylated N-linked chains were successively reported in IgG of several autoimmune diseases (Axford et al. 2003; Isenberg 1995; Pilkington et al. 1995; Martin et al. 2001). Increased IgG-G0 levels were found also in several mouse models of inflammatory conditions (Rook et al. 1991a; Yagev et al. 1993; Kuroda et al. 2001; Bodman et al. 1994; Endo et al. 1993). In RA patients, the increased IgG-G0 level is reverted in conditions ameliorating the disease, such as anti-TNF treatment (Van

Beneden et al. 2009; Croce et al. 2007; Pasek et al. 2006), pregnancy (Rook et al. 1991b; Alavi et al. 2000; van de Geijn et al. 2009; Pekelharing et al. 1988) and fasting (Kjeldsen-Kragh et al. 1996). Hypogalactosylation of N-linked chains is specific for the heavy chain linked to $Asn_{297}$of IgG, not involving their light chains (Mimura et al. 2007; Holland et al. 2006; Youings et al. 1996) nor IgA (Field et al. 1994). However, hypogalactosylation of N-linked chains in RA patients is detectable even in whole serum (Nakagawa et al. 2007). Increased biantennary agalactosylated structures and decreased di-galactosylated structures were also associated with risk factors for cardiovascular diseases, such as high body fat and blood pressure (Knezevic et al. 2010), conditions linked to a low grade inflammatory status (Rocha and Libby 2009).

Human Aging

The first indication of an increased expression of IgG-G0 in aging came from a pioneering study published in 1988 (Parekh et al. 1988). According to this study, in both genders the percentage of IgG-G0 starts increasing after the age of 25, while IgG-G2 showed a concomitant inverse relationship and the level of IgG-G1 remained constant (1988). A large body of successive studies have confirmed the close association of IgG-G0 with age (Yamada et al. 1997), although other structures decorating the N-linked chains attached to Asn_{297}, such as bisecting GlcNAc (Figs. 17.1 and 17.3), also show age dependence (1997). In a study published 1 year later, it was confirmed the age dependent increase of agalactosylated structures, although only in females, and of bisecting GlcNAc in both genders (Shikata et al. 1998). Since then, the age-associated accumulation of IgG-G0 structures has been confirmed by many other investigations. The development of high throughput approaches has allowed the detailed analysis of a very large number of plasma or serum specimens of various cohorts. The high-throughput techniques used include the use of a DNA-sequencer for the separation of glycans (DSA-FACE) (Callewaert et al. 2001), the hydrophilic HPLC (Royle et al. 2008) and the use of mass-spectrometry-based techniques (Ruhaak et al. 2010). Studies by DSA-FACE of cohorts of Italian and Belgian individuals, revealed in people over 60 an increase of core-fucosylated, agalactosylated biantennary N-linked chains with or without a bisecting GlcNAc and a concomitant decrease of core-fucosylated di-galactosylated structures (Vanhooren et al. 2007, 2008, 2009). These investigators proposed as a good indicator of the age-associated N-glycan shift the GlycoAge test (Vanhooren et al. 2010), which is the Log of the ratio between two core-fucosylated diantennary N-glycans: the first with two terminal GlcNAc residues, the second with two terminal galactose residues (structures 2 and 10 in Fig. 17.3). Similar patterns were observed in both IgG and IgG-depleted serum glycoproteins. If it is considered that the vast majority of plasma proteins are of hepatic origin while IgG are produced by plasma cells, this data suggests either a common regulatory biosynthetic step in hepatocytes and plasma cells or the existence of mechanisms affecting the glycosylation of plasma glycoproteins acting after the canonical cell-associated glycosylation. People above 90 exhibit an increase of agalactosylated structures and a decrease of di-galactosylated structures

more pronounced than people aged 60–90, while young people affected by progeroid Werner or Cockaine syndromes display a galactosylation pattern similar to that of centenarians. However, in a cohort of children the GlycoAge test was not lower than that of young adults (Catera et al. 2016), indicating that the increase of the GlycoAge test starts with adulthood. A quite different pattern of age-associated glycomic changes was reported in a cohort of Japanese semisupercentanarians (mean age 106.7 years), showing an increase of multi-branched and highly sialylated N-glycans as well as agalacto- and/or bisecting N-glycans and a decrease of biantennary N-glycans (Miura et al. 2015; Miura and Endo 2016).

In patients who underwent liver transplantation, the GlycoAge test is lower (younger) than that of the same patients before transplantation, but also lower than that of age-matched healthy controls (Capri et al. 2017). While the "rejuvenation" of the plasma N-glycome of transplanted individuals can be explained by their improved general health, the reason why their GlycoAge test is lower than that of healthy controls remains to be established. In Down syndrome, which displays signs of accelerated aging, some plasma N-glycomic changes (such as hypogalactosylation and reduced α2,3 sialylation) were shared with aging people, while α2,6 sialylated glycans were reduced in Down syndrome patients but increased or unchanged in aging people (Borelli et al. 2015). An index based on the relative abundance of three biantennary N-linked structures (with/without core fucose, bisecting GlcNAc and terminal galactose) derived from IgG, allows to explain up to 58% of variance in chronological age, more than telomere length (Kristic et al. 2014). These data indicate a very strong link between alterations of serum glycome and physiological or pathological aging.

Extreme longevity is a heritable tract. Thus, it is of crucial importance to understand whether the presence of plasma glycomic markers allows to identify people with propensity to long life. To this purpose, it is very useful the study of cohorts of centenarians, of their offspring who share with centenarians about half of the genetic background and partners of the offspring who serve as controls because they share with offspring the environment but not the genetic background. In a MALDI-TOF mass spectrometry study of IgG glycopeptides, it was confirmed the age-association of hypogalactosylated IgG with or without bisecting GlcNAc (2010). Interestingly, the level of agalactosylated structures with a bisecting GlcNAc was lower in the offspring below 60 than in their partners, suggesting that a low level of this marker in relatively young people predicts a genetic propensity to longevity (2010). A link between genetic propensity to longevity and level of plasma glycomic markers was confirmed by successive HPLC studies on N-glycans from total serum glycoproteins (Ruhaak et al. 2011). Strong indications about the relative contribution of genome and environmental factors to a given trait can be obtained by the study of monozygotic and dizygotic twins. This approach has established that about two thirds of the plasma glycomic traits have an additive genetic component, while the remainder are influenced mainly by environmental factors (Menni et al. 2013). Polymorphisms in the genes encoding the glycosyltransferases which synthesize aging-associated glycans are plausible candidates to explain the genetic bases of the aging-associated glycomic shift. However, genome wide association studies failed to identify associations between agalactosylated sugar structures in human blood and single nucleotide polymorphisms (SNPs) in the glycosyltransferase genes

potentially involved in the biosynthesis of aging-associated structures, such as MGAT3 or B4GALNT1, although it was found an association with a SNP in an intron of the FUT8 locus (Lauc et al. 2009), encoding for the fucosyltransferase which mounts core-linked fucose (Yanagidani et al. 1997; Lauc et al. 2010). Linkage with agalactosylated biantennary N-linked chains was displayed also by the locus encoding estrogen receptor β (ESR2).

How variable is the glycomic pattern among individuals of the same age class and how stable is the plasma glycome of an individual over short periods of time? The majority of individuals display a "normal" N-glycomic pattern, while in a limited number of outliers the pattern is different (Pucic et al. 2010). In particular, people with reduced galactosylation or with increased core-fucosylation were found to be healthy (Pucic et al. 2010).The glycome of healthy individuals undergoes very little or no changes over few days, while minor changes can be observed in healthy individuals over a 1 year long period (Gornik et al. 2009). Altogether, available data indicate that the human serum glycome is influenced by the genetic background, although not at the level of glycosyltransferase polymorphisms and by environmental conditions, including pathophysiological processes (Knezevic et al. 2009, 2010). As a consequence, plasma glycome reflects age as well as the health status of a person. Although the notion that the glycomic shift observed in aging is somehow related to inflammaging is highly plausible, observations in humans (Ruhaak et al. 2011) and in animal models (Vanhooren et al. 2011), support the possibility that the age-associated N-glycomic shift is regulated by metabolic pathways, independently of the inflammatory status.

Aging in Animal Models

Increased IgG-G0 were also observed in mouse models of aging. In fact, a marked tendency to age-dependent increase of IgG-G0 was observed in all seven mice strain examined between 2 and 8 months of age. In mice, 30–40% caloric restriction is associated with 20–50% increased lifespan compared with *ad libitum* fed mice (Weindruch et al. 1988). The increase of agalactosylated N-glycans in serum glycoproteins observed during aging from 3 to 26 months in mice was reverted by caloric restriction (Vanhooren et al. 2011).

Functional Significance of Age-Associated Glycosylation Changes of IgG

Aberrantly glycosylated antibodies are the result of aging and inflammatory conditions but at the same time they can fuel the inflammatory process, triggering a kind of vicious loop. Conflicting results have been reported on the mechanisms leading aberrantly glycosylated IgG to inflammation. A proof of principle of the pathogenic effect of IgG-G0 was provided by showing that IgG treated with galactosidase

displayed increased ability to induce arthritis if inoculated in healthy mice (Rademacher et al. 1994). At least four mechanisms can explain the pathogenicity of aberrantly glycosylated IgG; (1) interaction with the mannose binding lectin (MBL), the first component of the complement lectin pathway; (2) interaction with lectin receptors of macrophages and dendritic cells; (3) interaction with Fcγ receptors of leukocytes; (4) formation of antibody/antibody aggregates. However, the relative contribution of these mechanisms to the pathogenesis of inflammatory diseases and aging is far from clear.

Lectin Pathway of Complement

Hypogalactosylated IgG possess higher complement activation activity than normally glycosylated IgG, because of a stronger interaction with MBL, the first component of this pathway (Malhotra et al. 1995; Ezekowitz 1995) [reviewed in: (Rudd et al. 2001; Arnold et al. 2006)]. However, in both MBL-deficient RA patients (Stanworth et al. 1998) and mice with MBL deficiency, the pathogenic activity of IgG-G0 antibodies was not impaired (Nimmerjahn et al. 2007).

Lectin Receptors of Antigen Presenting Cells

Macrophages and dendritic cells are decorated by various lectin receptors, including the mannose-binding receptor and DC-SIGN. It has been reported that the uptake of IgG-G0 by mannose-binding receptor (Dong et al. 1999) and DC-SIGN (Yabe et al. 2010) is increased. The intravenous administration of high doses IgG (IVIG) induces a transient anti-inflammatory effect which is beneficial for several autoimmune diseases (Baerenwaldt et al. 2010). Although the molecular bases of this phenomenon have not been fully understood, it appears that the interaction of α2,6-sialylated IgG with DC-SIGN is required (Kaneko et al. 2006; Anthony et al. 2008a, b). It is reasonable to hypothesize that IgG-G0, which obviously lack sialic acid, do not contribute and probably inhibit the anti-inflammatory effects of α2,6-sialylated IgG (Nimmerjahn et al. 2007).

Fcγ Receptors

Glycosylation of IgG at Asn_{297} glycosylation plays a major role in regulating the binding of IgG to activating and inhibitory Fcγ receptors (Nimmerjahn et al. 2007; Nimmerjahn and Ravetch 2006, 2008; Albert et al. 2008; Li et al. 2017). In particular, the absence of galactose, the α2,6-sialylation (Scallon et al. 2007), the core fucosylation (Niwa et al. 2005; Shields et al. 2002; Shinkawa et al. 2003; Satoh

et al. 2006; Iida et al. 2006, 2009; Shibata-Koyama et al. 2009) and bisecting GlcNAc (Umana et al. 1999; Davies et al. 2001) play the most relevant effects. However, the impact of IgG glycosylation on the FcγR function is far from clear. In fact, a study reported that the ability of IgG-G0 to interact with low affinity FcγRII is undistinguishable from that of normally glycosylated antibodies (Groenink et al. 1996), while other studies reported that the binding to FcγR of asialyl- (Adler et al. 1995) or agalactosyl IgG (Kumpel et al. 1995) was impaired.

Anti IgG Antibodies

Anti IgG antibodies whose presence characterizes rheumatoid arthritis and other inflammatory diseases are, in some cases, directed against IgG-G0 (Nishijima et al. 2001; Das et al. 2004; Maeno et al. 2004). Several lines of evidence support the notion that rheumatoid factors bind better to hypogalactosylated IgG (Matsumoto et al. 2000; Soltys et al. 1994; Imafuku et al. 2003).

Molecular Basis of N-Glycosylation Changes

The molecular bases of IgG-G0 production have been investigated in human inflammatory conditions and in murine experimental systems, mainly utilizing total lymphocyte populations or isolated B lymphocytes. No studies with lymphocytes of elderly people have been published so far, to my knowledge. These studies have provided conflicting results, and conclusive evidence on the formation of IgG-G0 and other aging/inflammation-associated glycans is still lacking. These studies have been intrinsically limited by the fact that the primary source of circulating antibodies are not B-lymphocytes but plasma cells, which are morphologically and functionally very different from the B-lymphocytes from which they are derived. Consequently, it cannot be assumed that the glycosylation machinery remains the same during B-cell differentiation to plasma cells. In two studies in which the level of sialyltransferase ST6GAL1 (Table 17.1) was measured in plasma cells, have shown that α2,6-sialylated IgG are produced by plasma cells against T-independent antigens (Hess et al. 2013) and by cooperation with T-lymphocytes in tolerogenic conditions (Oefner et al. 2012). IgG-G0 glycans can be produced *in vitro* by lymphocytes from RA patients (Bodman et al. 1992). Some studies reported a consistent inverse relationship between IgG-G0 and the level of B4GALT activity (Table 17.1), putatively responsible for galactosylation of IgG (Axford et al. 1987, 1992; Alavi and Axford 1995). However, according to other studies no differences were observed between the galactosyltransferase level in B lymphocytes of RA patients and healthy controls (Furukawa et al. 1990; Keusch et al. 1998; Delves et al. 1990; Jeddi et al. 1996). Studies in murine models have also provided conflicting results on the relationship between IgG-G0 and galactosyltransferase

expression. The level of B4GALT1 transcript in total splenic lymphocytes was reduced in the arthritis-prone MLR lpr/lpr mice (Jeddi et al. 1994, 1996). However, the galactosyltransferase activity was found to be lower in peripheral but not in splenic B lymphocytes in mouse models of arthritis (Axford et al. 1994; Alavi et al. 1998). Structural studies on antibodies of monoclonal origin indicate that B4GALT activity is lower in hybridomas producing rheumatoid factors (RF) than in hybridomas secreting irrelevant antibodies. Altogether, available data suggest that big differences exist between the galactosyltransferase levels of lymphocyte populations of the same organism. In considering the increased level of bisecting GlcNAc associated with aging, it should be kept in mind that the agalactosylated biantennary N-linked chains which accumulate in aging are a preferred substrate for MGAT3 (Narasimhan 1982), which is the only enzyme mediating the addition of bisecting GlcNAc (Ihara et al. 1993). Thus, the biosynthesis of this structure can be favored simply by the increased presence of its precursor substrate.

Glycosylation Changes Associated with Cellular Senescence

Little is known on the changes of cellular glycoconjugates associated with aging. It is known that late passage fibroblasts showed decreased expression of α2,6-sialylated N-glycans, because of reduced ST6GAL1 activity (Tadokoro et al. 2006). Consistently, it has been recently reported that late passage fibroblasts and fibroblasts obtained from older individuals display reduced α2,6 sialylation and α2,3 sialylation, with a concomitant increase in galactose exposure (Itakura et al. 2016). On the other hand, muscle sialylation increases in aging mice (Hanisch et al. 2013).

Other Glycomic Markers of Aging

Plasmatic Glycosyltransferases

Glycosylation has long been considered an exclusively intracellular process because it was thought that only inside the cells, glycosyltransferases and donor substrates reached the sufficient concentration for enzymatic reactions. However, this paradigm has recently been challenged by the demonstration that glycosylation can take place extracellularly, thanks to the plasmatic glycosyltransferases and sugar nucleotide donors contained in platelets and other microvesicular plasma components (Wandall et al. 2012; Jones et al. 2012; Lee et al. 2014; Nasirikenari et al. 2006, 2010, 2014). In particular, extracellular sialylation of IgG can be mediated by plasmatic ST6GAL1 in mice (Jones et al. 2012, 2016), mainly in inflammatory conditions (Manhardt et al. 2017). This points to the possible role of plasmatic glycosyltransferases in regulating the plasma glycome and, in particular, the glycosylation of IgG. Owing to the unclear molecular bases of increased IgG-G0

expression in aging and inflammatory diseases, it is reasonable to propose a plasmatic glycosyltransferases model for the glycosylation of IgG and other plasma glycoproteins. For this reason, we have compared the plasmatic level of two key glycosyltransferases, namely ST6GAL1 and B4GALT1, with the glycosylation of IgG and other plasmatic glycoproteins in cohorts of aging people. The two enzymes are putatively involved in the addition of α2,6-linked sialic acid and β1,4-linked galactose to antibodies and other plasmatic glycoproteins. We found that the two enzymes undergo relevant variations in aging. In particular, plasmatic B4GALT1 activity exhibits a linear increase from infancy to centenarians, whereas ST6GAL1 is higher in children and above the age of 80. However, no relationship was observed between the level of the plasmatic glycosyltransferases and that of their putative cognate structures on IgGs, suggesting that extracellular glycosylation does not play a major role in IgG glycosylation in humans. In rodents, ST6GAL1 behaves as an acute phase protein (Dalziel et al. 1999; Kaplan et al. 1983) and probably exerts an anti-inflammatory role. In fact, the extracellular sialylation of mice hematopoietic stem cells by plasmatic ST6GAL1 limits neutrophil production (Appenheimer et al. 2003; Jones et al. 2010, 2012, 2016) and plasmatic ST6GAL1-mediated extracellular α2,6-sialylation of mice IgG leads to the biosynthesis of anti-inflammatory α2,6-sialylated IgGs. We observed that the level of plasmatic ST6GAL1 was higher in the offspring of centenarians than in the offspring of non long-lived parents. If the data obtained with experimental models of murine inflammation are translated to old humans, it can be hypothesized that high levels of plasmatic ST6GAL1represent an attempt of the body to limit the adverse effects of inflammation, consistent with the higher level of plasmatic ST6GAL1 observed in the offspring of centenarians, who are at lower risk of inflammatory diseases associated with aging.

Galectins

Galectin-3 is a member of the Galectin family, mammalian lectins involved in a variety of roles, including immune regulation, cancer cell growth and apoptosis (Boscher et al. 2011). The levels of plasma Galectin-3 are lower in healthy centenarians than in people aged 70–80 years, suggesting that a low level of Galectin-3 is a marker of successful aging (Sanchis-Gomar et al. 2016).

Role of Siglecs in Aging

Siglecs are sialic acid-binding lectins of the immunoglobulin superfamily, which are mainly expressed by cells of the immune system (Macauley et al. 2014). The majority of Siglecs are inhibitory receptors with immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic portion and inhibit inflammation mediated by DAMPs and PAMPs by binding to sialylated ligands expressed in the

same cells or by responding to pathogen-derived sialo-glycoconjugates (Pillai et al. 2012). Binding of PAMPS derived from microorganisms or DAMPS derived from damaged tissues to TLR expressed on membranes of the immune cells triggers TLR signaling, which results in transcription of inflammatory cytokine genes (Fig. 17.4a). Binding of sialylated structures expressed by pathogens to Siglecs, recruit them in proximity of TLR, resulting in down-regulation of their signaling through the activity of associated SHP phosphatases (Fig. 17.4b). It has been reported that through the sialylated GPI-anchored CD24 molecule, cells of the innate immune system can discriminate between PAMPs and DAMPs. In the proposed model (Fig. 17.4c) (Liu et al. 2009), DAMPs bind to both TLR and CD24. Through its sialylated glycans, CD24 binds and recruits Siglec 10 (human)/Siglec-G (mouse) and its associated SHP phosphatase which inhibits TLR signaling. Through these mechanisms, Siglecs exert a general down-regulation of inflammatory and immune responses. This notion is confirmed by the fact that the presence of Siglec-G ligands on both T-dependent and T-independent immunogens down-regulates antibody production and induces B-cell tolerance (Chen et al. 2014) and by the fact that destruction of the CD24/Siglec-G interaction by bacterial sialidases exacerbates the inflammation associated with sepsis (Chen et al. 2011).

In aging mice, Siglec-G deficiency leads to autoimmunity with high autoantibody production and higher number of activated CD4 T cells (Muller et al. 2015). A significant correlation exists between the number of genes encoding Siglecs of the CD33 family and maximum lifespan in mammals. In addition, mice KO for Siglec-E, the main member of the CD33 family, exhibit shorter lifespan and signs

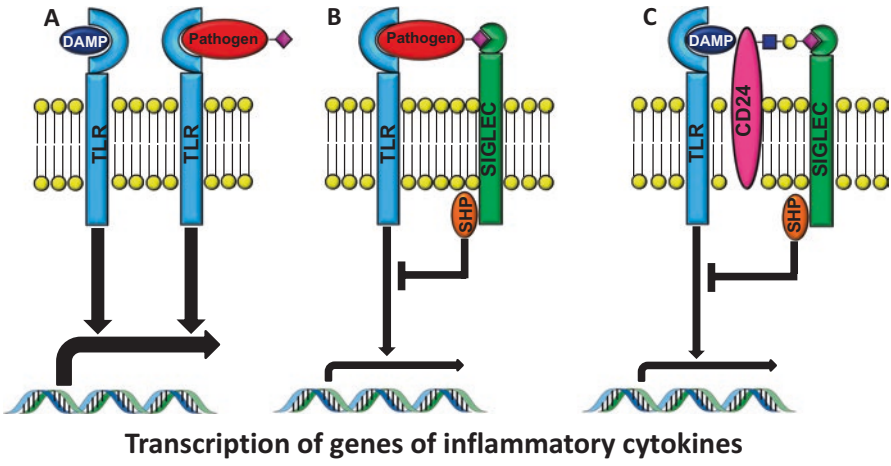

Fig. 17.4 Role of Siglecs in the inhibition of the immune response. (**a**): in the absence of Siglecs, pathogens or DAMPS activate TLR which ultimately stimulate transcription of inflammatory cytokine genes. (**b**): sialylated pathogens recruit Siglecs whose associated SHP phosphatase down-regulates TLR signaling. (**c**): sialylated CD24 binds directly or indirectly to DAMPs, recruiting Siglecs whose associate SHP phosphatase down-regulates TLR signaling. This mechanism has been proposed to discriminate DAMPS from PAMPS signaling

of accelerated aging with DNA damage, oxidized aminoacids and isolated foci of liver, kidney and lung inflammation (Bordon 2015; Schwarz et al. 2015). It has been proposed that these effects are related to both an unbalanced metabolism of reactive oxygen species (ROS) and impairment in detoxification of reactive molecules. Although the role played by the different members of the Siglec family can be very different, these results are consistent with an important role of Siglecs interaction with sialylated molecules in reducing ROS and antibody production and inflammatory status, resulting in slow down of the aging process (Fig. 17.5).

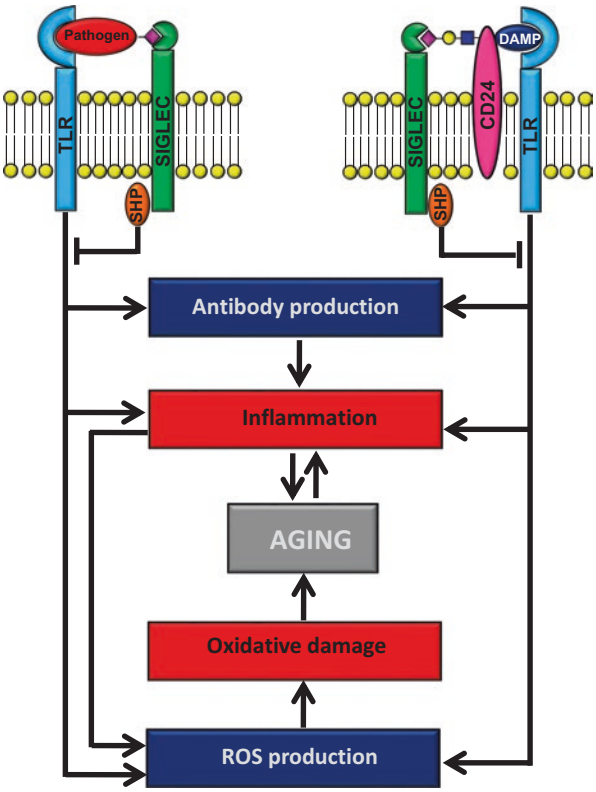

Fig. 17.5 Inhibition by Siglecs of the inflammatory and oxidative stimuli generated by PAMPs and DAMPs. Pathogens and their associated PAMPs as well as PAMPs interact with TLR and other membrane or cytoplasmic receptors of the innate immunity (not shown), stimulating cytokine production and triggering antibody production. Inflammation activated through these mechanisms leads to ROS generation and oxidative damage. Together, these conditions lead to aging and its associated inflammatory status (inflammaging). These processes can be partially counteracted by some Siglecs which, through recognition of sialic acid residues, recruit their associated SHP phosphatase in proximity with TLR, resulting in a down-regulation of their signaling

Concluding Remarks

A major goal of research in the field of glycobiology of aging is the identification of markers which enable establishment of the biological age of a person, rather than his/her calendar age, for which a sophisticated glycomic analysis is not necessary. By contrast, to assess the overall health conditions of a person and the prevalence of specific aging-associated traits (inflammation, metabolic diseases, sarcopenia, cognitive impairment, atherosclerosis), the availability of glycomic markers would be extremely useful. This aim, which requires the comprehension of the intimate mechanisms linking the glycosylation machinery with the multifaceted aspects of aging and the use of high-throughput systems of glycomic analisis, would allow clinicians to monitor the health process, with adjustment of life style to achieve the ultimate goal of successful aging.

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