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Review

A review and appraisal of the DNA damage theory of ageing

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ABSTRACT

Given the central role of DNA in life, and how ageing can be seen as the gradual and irreversible breakdown of living systems, the idea that damage to the DNA is the crucial cause of ageing remains a powerful one. DNA damage and mutations of different types clearly accumulate with age in mammalian tissues. Human progeroid syndromes resulting in what appears to be accelerated ageing have been linked to defects in DNA repair or processing, suggesting that elevated levels of DNA damage can accelerate physiological decline and the development of age-related diseases not limited to cancer. Higher DNA damage may trigger cellular signalling pathways, such as apoptosis, that result in a faster depletion of stem cells, which in turn contributes to accelerated ageing. Genetic manipulations of DNA repair pathways in mice further strengthen this view and also indicate that disruption of specific pathways, such as nucleotide excision repair and non-homologous end joining, is more strongly associated with premature ageing phenotypes. Delaying ageing in mice by decreasing levels of DNA damage, however, has not been achieved yet, perhaps due to the complexity inherent to DNA repair and DNA damage response pathways. Another open question is whether DNA repair optimization is involved in the evolution of species longevity, and we suggest that the way cells from different organisms respond to DNA damage may be crucial in species differences in ageing. Taken together, the data suggest a major role of DNA damage in the modulation of longevity, possibly through effects on cell dysfunction and loss, although understanding how to modify DNA damage repair and response systems to delay ageing remains a crucial challenge.

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1. Introduction

Ageing is a widespread process, occurring in most animal species and in all human beings fortunate to live long enough to suffer the effects of ageing. A few animal species, such as Hydra, certain fish and some turtles, however, do not appear to undergo ageing, the reasons for which are far from understood [1]. Ageing can be defined as a progressive deterioration of physiological function, accompanied by an increase in vulnerability and mortality with age [2]. A major motivation for ageing research is that age is the greatest risk factor for many diseases, including most types of cancer. The gradual "greying" of the world's population makes research into the mechanisms of ageing a pertinent medical, social and economic problem. Despite its importance and considerable progress in this area in the last few decades, however, ageing is still a mysterious process, whose fundamental causes are still strongly debated.

One of the reasons why the mechanisms of ageing are poorly understood is the difficulty in discerning cause from effect and focusing on the underlying processes of ageing rather than its manifestations [3]. Because it is impossible to quantify ageing accurately, in spite of some efforts [4], longevity is often used as a readout. Nonetheless, longevity, which can be defined as how long an organism lives and can be quantified for experimental cohorts as average or maximum longevity, can be influenced by many factors independent of ageing. Mutations that trigger specific diseases, for example, may decrease longevity without impacting on ageing. Therefore, it is crucial, even if often hard, to interpret experimental results in light of how they inform the ageing process [4].

Given the central role of DNA in life, and how ageing can be seen as the gradual and irreversible breakdown of living systems, it is intuitive to think that alterations to the DNA with time are a key process in ageing, perhaps even the primary, underlying cause of ageing [5]. The first suggestion that ageing could derive from mutations to the DNA was by Failla in 1958 [6] with the work of physicist Leo Szilard a year later also widely cited [7]. With technical advances permitting detection of new forms of DNA damage and mutations, the DNA damage theory of ageing has changed over the years [5]. The goal of this review is to provide an overview and analysis of the evidence suggesting DNA damage and its imperfect repair play a major role in human ageing.

2. The DNA damage theory of ageing

DNA can be subject to mutations and damage [8]. Mutations are changes in the nucleotide sequence, involving deletions, insertions, substitutions or rearrangements of base pairs, and can lead to dysfunctional proteins. In contrast, DNA damage refers to physical or chemical alterations in the structure of the double helix. In other words, mutations change the informational content of a DNA molecule, whilst damage modifies the structure of a DNA molecule. Early theories by Failla and Szilard focused on the role of mutations in ageing, yet the focus later shifted to DNA damage which can be seen as a broader theoretical framework since DNA damage can lead to mutations [5,9,10].

The DNA damage theory of ageing postulates that the main cause of the functional decline associated with ageing is the accumulation of DNA damage and ensuing cellular alterations and disruption of tissue homeostasis [7,8]. Although damage to other kinds of molecules found in cells may also influence ageing, DNA damage is particularly important because, unlike other cellular components which can normally be replaced, DNA must last the lifetime of the cell [11]. Damage to the DNA can have multiple effects, depending on the type of damage and genomic region affected [5,9,10]. In particular, DNA damage can dysregulate gene expression and cell function, impair transcription, cause cell cycle arrest and (if the damage is too serious) trigger programmed cell death (apoptosis). DNA damage can also lead to mutations when the DNA is repaired and/or replicated. Hence, the DNA damage theory of ageing can be interpreted in different ways, depending on how one interprets the relative contribution of each of these effects of DNA damage on the ageing process.

Although the focus of our review is on damage to the nuclear DNA (nDNA), a role of damage to mitochondrial DNA (mtDNA) in ageing has also been proposed. The mtDNA is much more prone to damage than nDNA, since mtDNA is not protected by histone proteins and it is close to the site of reactive oxygen species (ROS) generation in the mitochondrial membrane. In addition, overall the repair of mtDNA is less efficient than the repair of nDNA. However, the mtDNA encodes only 37 genes and the relative importance of mtDNA damage for ageing is still controversial and less supported by experimental evidence than damage to nuclear DNA [3]. As concluded by Khrapko and Vijg in a recent review of this subject [12]: "...the study of mitochondrial DNA mutations has not reached a stage at which clear, definitive conclusions can be drawn regarding causal relationships." Hence, in this review we focus on nDNA damage, which accounts for about 99% of cellular DNA.

Because the effects of disruption of certain DNA repair pathways in accelerating ageing are arguably the strongest evidence to date supporting the DNA damage theory of ageing this is initially reviewed herein. Major sources and types of DNA damage as well as the main DNA repair pathways associated with ageing are then described, before putting the pieces together and discussing how DNA damage may lead to cell dysfunction and loss and finally to organismal ageing.

3. Progeroid syndromes

There are many types of diseases in which patients show signs of accelerated ageing. Such diseases are called *premature ageing syndromes* [13] or *progeroid syndromes* [14]. Most of these diseases are caused by defects in DNA repair genes [15–17], supporting the idea that the balance between DNA damage and repair determines the rate of ageing.

Table 1
Summary of major human progeroid syndromes originating in single-gene defects.

Syndrome	Genetic defect and main processes affected	Mean lifespan (years)	Predisposition to cancer?
Werner	RecQ-like DNA helicase and exonuclease, involved in DNA repair	47	Yes
Hutchinson–Guilford	Lamin A, involved in DNA replication, transcription, nuclear organisation	13	No
Trichothiodystrophy	TFIIH helicase, involved in DNA repair and transcription	10	No
Cockayne	CSA or CSB gene, involved in DNA repair and transcription	12–20	No
Ataxia telangiectasia	ATM protein kinase, involved in DNA damage response	20	Yes
Rothmund–Thomson	RecQ-like DNA helicase	Normal?	Yes
Xeroderma pigmentosum	XPA–XPG genes, involved in DNA repair	Lower than normal?	Yes

Adapted from [8,14,15].

Table 1 shows the major human progeroid syndromes, which are then discussed below. Only progeroid syndromes caused by single-gene defects are included because the causal mechanisms are better understood. For example, Down's syndrome can be classified as a progeroid syndrome [18], but its molecular mechanisms are more poorly understood.

3.1. An overview of progeroid syndromes

3.1.1. Werner syndrome (WS)

This is widely considered the progeroid syndrome that shows the most symptoms of normal ageing [16]. WS patients are usually normal during childhood, but stop growing during the teenage years and go on to develop a variety of signs of premature ageing [19]. The following ageing symptoms have been described [14,20]: premature greying of the hair and baldness, skin and muscular atrophy, hypogonadism, poor wound healing, atherosclerosis, osteoporosis, soft-tissue calcification, juvenile cataracts, a tendency towards diabetes, and an elevated cancer frequency [8,21]. On the other hand, WS patients show no increased tendency for neurodegeneration, and the immune system remains normal.

WS is caused by one of a variety of mutations in a single gene (*WRN*) coding for a protein that is a member of the RecQ DNA helicase family [21,22]. The WRN protein is involved in several important biological processes, related to DNA replication, recombination, apoptosis and telomere metabolism, but its major function seems to be the re-initiation of stalled replication forks. The cells of WS patients show significant chromosomal abnormalities, increased frequency of deleterious mutations and accumulation of DNA double-strand breaks [19,23]. WS fibroblasts reach the stage of replicative senescence considerably faster than normal fibroblasts [21,22].

3.1.2. Hutchinson–Gilford progeroid syndrome (HGPS)

This progeroid syndrome has an onset in childhood, much earlier than the onset of WS. HGPS patients show the following symptoms [8,24]: premature loss of hair and subcutaneous fat (starting in the first year), postnatal growth is severely disturbed, osteolysis, decreased joint mobility from the second to third year, thinning of the skin, limited sexual development and severe vascular problems in the brain and elsewhere – strokes occur at the median age of 9 years. The vast majority of patients have a normal cognitive development.

HGPS is caused by a point mutation in the gene for lamin A (*LMNA*), a type of protein that forms a network of filaments beneath the inner nuclear membrane called the nuclear lamina [25]. A-type lamins can directly bind to DNA and to chromatin, but because they are involved in a variety of processes, the exact molecular mechanism of HGPS remains unclear. Although WS and HGPS patients have little overlap of clinical symptoms, at the cellular level both these progeroid syndromes are associated with increased genomic instability [25].

3.1.3. Trichothiodystrophy (TTD)

TTD patients show the following symptoms [15,26]: neurodegeneration (including cerebellar ataxia), skeletal degeneration, impaired sexual development, cachexia, osteoporosis, cataracts, brittle hair and nails. TTD is caused by point mutations in the *XPD* gene, which encodes one of the two core transcription factor IIH (TFIIH) helicases [15]. Different mutations in this gene can give rise to TTD, xeroderma pigmentosum or Cockayne syndrome. The helicase encoded by the *XPD* gene is involved in both DNA repair and transcription initiation [26].

3.1.4. Cockayne syndrome (CS)

CS is caused mainly by mutations in either the *CSA* or *CSB* gene. In addition, as mentioned in the previous section, CS can be caused by a mutation in *XPD*. CS patients show the following symptoms [13,15]: neurodegeneration, growth retardation, cachexia, thin hair, retinal degeneration, hearing loss, and cataracts – which can be seen at birth in the most severe cases. Almost all CS patients are mentally retarded. Despite chromosomal instability, patients show no predisposition to cancer. Note that TTD and CS have several symptoms in common [26].

3.1.5. Ataxia telangiectasia (AT)

AT is caused by a loss-of-function mutation in the *ATM* (ataxia-telangiectasia mutated) gene. The term ataxia refers to the shaky and unsteady limb movements of patients due to dysfunction of the cerebellum. In particular, ATM is involved in cell cycle progression and checkpoint response to DNA damage, including oxidative damage to DNA [27]. AT can be diagnosed by a cytogenetic test that detects a high-level of chromosome breakage after ionising radiation, which reflects DNA damage repair pathways [28].

AT patients show the following main symptoms [13,27,29]: progressive neurodegeneration (with cerebellar ataxia becoming apparent when the patient begins to walk), telangiectases with onset typically between 3 and 5 years of age, immunodeficiency, genomic instability, strong cancer predisposition and sensitivity to radiation, accelerated telomere loss, and often growth retardation.

3.1.6. Rothmund–Thomsom (RT) syndrome

RT is caused by a mutation in a gene (*RECQL4*) coding for a RecQ-like DNA helicase [27,15]. Patients typically exhibit the following symptoms [13]: skin changes starting in the first year of life and leading to poikiloderma, growth retardation, a variety of skeletal and ocular abnormalities, including osteoporosis and corneal/retinal atrophy, as well as juvenile cataracts. Malignancies have also been reported, and delayed or immature sexual development has been reported for about 28% of the patients. Most patients have normal intelligence. Surprisingly, RT patients seem to have a normal lifespan.

3.1.7. Xeroderma pigmentosum (XP)

This is a disease due to a defect in one of seven proteins (XPA–XPG) required for nucleotide excision repair (discussed in Section

5.2). XP victims show dramatically accelerated ageing only in areas of skin exposed to the sun and a skin cancer rate more than a thousand times greater than normal, and frequently exhibit neurodegeneration [15,19].

3.1.8. Progeroid syndromes in mice

In addition to human progeroid syndromes, a number of mouse models have been created through genetic manipulation that exhibit evidence of accelerated or premature ageing with varying degrees of severity. As reviewed by many authors [3,11,15], a significant proportion of genes in which mutations appear to accelerate ageing in mice are also involved in DNA repair. In fact, many of the genes and pathways involved in human progeroid syndromes also result in progeroid syndromes when mutated in mice. These include *Lmna* [30] and associated laminopathy-based premature ageing syndromes [31], *Xpa* and *Xpd* [26]. Interestingly, *Wrn* mutations only result in accelerated ageing in mice with short telomeres [32]. Atm-deficient mice exhibit most of the symptoms of the human disease [27]. Atm disruption in mice with short telomeres, however, results in symptoms of accelerated ageing [29].

Taken together, the results from progeroid mice confirm the observations from human progeroid syndromes and thus will not be presented in detail here (some are discussed further ahead in the context of specific DNA repair pathways in Section 5). Readers are referred to one of the many excellent reviews on this topic for more detailed information [15,33]. Overall, ample evidence suggests that disruption of genes involved in DNA repair and/or DNA metabolism can result in a premature ageing phenotype.

3.2. On the relevance of progeroid syndromes to the study of human ageing

The relevance of the study of progeroid syndromes for the understanding of normal ageing is controversial [34]. A major point of criticism is that patients or animal models of progeroid syndromes show just a subset of the symptoms of normal ageing (and are called "segmental progeroid" by many authors). In addition, Miller [34] points out that it is relatively easy to make a short-lived animal by introducing a defect in some crucial DNA repair gene, but it is much harder to show that the defect is really accelerating ageing.

Counter-arguments to Miller's criticism have been provided in refs. [21,35]. Hasty and Vijg [35] point out that the "segmental" nature of progeroid syndromes does not invalidate their relevance for the study of normal ageing, because every individual who undergoes normal ageing exhibits a segmental ageing phenotype. In addition, at least WS is considered to have symptoms which significantly overlap with the symptoms of normal ageing [18,21].

Our opinion is that the fact that no progeroid syndrome is a perfect phenocopy of ageing is not surprising considering the multitude of factors that can influence ageing in different tissues. The ability of a single-gene disruption to accelerate multiple aspects of human ageing, as in WS, is remarkable in itself given the complexity of the ageing process. Therefore, progeroid syndromes due to defects in DNA repair genes offer strong support to the idea that a major causal factor of ageing is the accumulation of DNA damage and mutations with time [15].

4. Sources and types of DNA damage

4.1. Major sources of DNA damage

Damage to the DNA can originate in multiple extrinsic and intrinsic sources [5,9,11]. Extrinsic sources comprise chemicals and radiations, such as UV damage, and viruses. Intrinsic sources include spontaneous chemical reactions and reactive oxygen species (ROS). It has been long argued that the predictable patterns of ageing (e.g., in setting a range of species-specific maximum lifespan limits) supports the greater importance of endogenous causes of DNA damage [5].

A common cause of DNA damage is exposure to ROS, which has long been hypothesized to be involved in ageing. ROS include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. Oxidized DNA can produce several kinds of DNA damage, e.g., oxidized bases, abasic sites and single- and double-strand breaks [36,37]. Organisms have several defence mechanisms to cope with ROS, including antioxidant enzymes that eliminate ROS or convert them to less harmful molecules [38]. However, the production of ROS can be so overwhelming that those defence mechanisms are not enough, resulting in oxidative stress.

ROS can produce many different kinds of damage and mutations in DNA. For instance, the cytosine base alone can undergo oxidative damage producing at least 40 different modified species [37]. Some oxidatively modified bases block DNA replication, whilst others are mispaired and lead to base substitutions in the DNA. Interestingly, some of the progeroid syndromes caused by defective DNA repair discussed earlier – such as XP and AT – are associated with a high amount of 8-hydroxydeoxyguanosine (8-oxo-dG), a measure of oxidant-induced DNA damage [36]. First observed in rats via measurements of 8-oxo-dG [39], it is now widely accepted that oxidative damage to DNA tends to increase with age in mammalian tissues. Although normally a by-product of oxygen metabolism in mitochondria, ROS can have exogenous sources such as UV radiation [40]. Therefore, although which types of DNA damage are more important contributors to ageing is not known at present, ROS could be an important source of DNA damage in the context of ageing.

4.2. An overview of major types of DNA damage

4.2.1. Abasic (AP) sites, depurination and depyrimidination

An abasic site, also called an "apurinic or apyrimidinic" (AP) site, is formed when a base is lost from the DNA by cleavage of a N-glycosyl bond, leaving the sugar-phosphate chain intact [38]. At normal physiological conditions, it has been estimated that 50,000–200,000 AP site lesions persist at a steady-state level in mammalian cells [41].

Abasic sites are potentially mutagenic and can be produced by spontaneous depurination and depyrimidination reactions. Depurination involves the loss of purine bases (adenine and guanine) from DNA. In spontaneously occurring depurination reactions, the N-glycosyl bond to deoxyribose is broken by hydrolysis, leaving the DNA's sugar–phosphate chain intact, producing an abasic site. Depyrimidination involves the loss of pyrimidine bases (cytosine and thymine) from DNA. Depyrimidination is much less common than depurination, however, since the N-glycosyl bond between a pyrimidine base and the deoxyribose is more stable than the corresponding bond for purine bases [42]. Abasic sites can also be produced by ROS [41,43], as well as being produced in intermediate steps of the base excision repair pathway (to be discussed later in Section 5.1). Inefficient or incomplete base excision repair might leave abasic sites in DNA.

4.2.2. Deamination

Deamination involves the loss of amino groups from DNA bases. Almost all DNA bases undergo deamination in spontaneous reactions, with the exception of thymine – which does not have an amino group. Most types of deaminations produce a base that does not naturally occur in DNA (the only exception is the deamination of 5-methylcytosine), and this facilitates the identification and excision of the deaminated base by a DNA glycosylase enzyme.

The most common type of deamination event in cells is deamination of cytosine into uracil. This event occurs at a rate of about 100–500 bases per cell per day in mammalian cells, in spontaneous deamination reactions [38]. Interestingly, one possible reason why the genetic code, which is thought to have been initially carried in RNA bases (A, C, G, U), was replaced by the current code carried in DNA bases is so a deaminated C converted to a U can be easily recognized as damage [44].

DNA can also contain the base 5-methylcytosine, which base pairs with guanine and is involved in silencing gene expression at CpG sequences. The deamination of 5-methylcytosine into thymine leads to the formation of a G–T base pair, which is potentially mutagenic. Interestingly, although only about 3% of the C bases in human DNA are methylated, GC → AT transitions at the sites of those methylated cytosines account for about one-third of the single-base mutations in inherited human diseases [44,45].

4.2.3. DNA strand breaks

Some strand breaks are produced in intermediate steps of natural reactions. As an example, the process of V(D)J recombination during lymphocyte development is initiated by a kind of programmed double-strand break between two recombining variable-region gene segments and their flanking sequences [46,47]. However, some strand breaks are a serious form of DNA damage and inhibit DNA replication, leading to the activation of DNA repair mechanisms. DNA strand breaks can be caused by oxidative damage to DNA [48] or by ionizing radiation [38]. Double-strand breaks can also result from the blockage or pausing of DNA replication – which can lead to replication fork collapse and free double-stranded ends [49].

It is interesting to note that disruption of pathways involved in single-strand break repair often results in neurological diseases rather than carcinogenesis or progeria. Because ROS are one of the major causes of single-strand breaks, one possible explanation is that the oxygen consumption in the nervous system makes it more susceptible to defects in single-strand break repair. Therefore, single-strand breaks may contribute to neurological decline [50].

Misrepaired double-strand breaks lead to genomic rearrangements, a common and serious problem in ageing organisms [51]. A considerably increased frequency of DNA double-strand breaks is observed in patients of some progeroid syndromes discussed earlier, such as WS and AT [52]. The number of single- and double-strand breaks in the neurons of rat cerebral cortex has been shown to considerably increase with age [53].

4.2.4. Cyclobutane pyrimidine dimers (CPDs)

CPDs are characterized by covalent linkages between adjacent pyrimidines in the same DNA strand, and they are the most frequent type of photoproduct produced when DNA is exposed to UV-B [40] or to UV-C radiation [38]. The type of CPD most frequently found in DNA consists of a thymine dimer, which is known to be mutagenic in mammalian cells [54]. The formation of CPDs can also enhance the deamination of cytosine [38].

5. Major DNA repair pathways associated with ageing

In this section we review three major types of DNA repair pathways, namely base excision repair (BER), nucleotide excision repair (NER) and the repair of DNA double-strand break via the non-homologous end joining (NHEJ) pathway. Out of those three pathways, BER seems the least associated with ageing, whilst the evidence for association with ageing is considerably stronger for the NER and NHEJ pathway. Note that this section does not cover some types of DNA repair that, although important, are not thought to be relevant for ageing. For instance, it does not cover mismatch repair [55,56], since this pathway has been mainly associated with cancer [56,57], rather than ageing. It also does not cover mechanisms that repair single-strand breaks as these have been associated with neurological disorders rather than broader aspects of ageing [50].

5.1. Base excision repair (BER)

The BER pathway corrects small alterations in a DNA strand that do not distort the overall structure of the DNA helix, such as a base altered by deamination or a missing base due to a depurination reaction. The base alterations targeted by BER may or may not block transcription and normal replication, but they frequently lead to changes in the DNA sequence, being therefore potentially mutagenic [55]. BER is the main pathway to repair oxidative damage [58].

The BER pathway can be categorized into two sub-pathways, namely short-patch BER, where only one nucleotide is replaced; or long-patch BER, where 2–13 nucleotides are replaced [51]. The decision between performing a short-patch or long-patch repair is modulated by PARP1 and PARP2 (poly(ADP-ribose) polymerases) [59]. One difference between these two sub-pathways is that in the long-patch pathway the WRN protein interacts physically and functionally with several other proteins such as PCNA and RPA, which is not the case in the short-patch pathway [22,53].

The BER pathway is particularly important in the brain [52,60], for at least two reasons. First, BER is the primary pathway to repair oxidative DNA damage, and this is the most likely kind of damage to occur in brain tissue, which is metabolically very active [53]. Secondly, neurons are non-dividing cells, and thus in principle other DNA repair pathways such as homologous recombination and mismatch repair are not important in neurons.

There is good evidence that, overall, the level of BER activity is reduced with age. In particular, the activity of polβ – an important component of the BER pathway – has been shown to be considerably reduced with age in mice in many investigations, e.g., in refs. [53,60–62]. The activity of polγ – which performs the gap-filling step of BER in mitochondrial DNA [63] – has also been observed to decrease with age [61]. There are, however, studies reporting that some BER enzymes have an increased expression with age – see e.g., [64]. This seems likely to be a response to increased levels of oxidative DNA damage with age, although the response might not be effective due to the aforementioned decrease in polβ activity.

Another line of evidence linking BER to ageing comes from mice deficient in Sirt6 (sirtuin 6) which exhibit signs of premature ageing 2–3 weeks after birth, accompanied by genomic instability and evidence of a BER defect, though it is unknown how Sirt6 affects BER [65]. That said, there is limited evidence from genetic mutants linking BER to ageing, though it has been pointed out that most BER proteins are necessary for embryonic development which hinder an accurate assessment of the role of BER in ageing via genetic manipulation experiments [58].

5.2. Nucleotide excision repair (NER)

The NER pathway copes with lesions in a DNA strand that distort the DNA double helix. This kind of lesion interferes with base pairing and usually blocks transcription and normal replication [55]. NER is considered the most versatile DNA repair pathway in terms of the variety of lesions that it can recognize – it recognizes several types of bulky lesions, produced, for instance, by ultraviolet light and carcinogens.

The NER pathway is usually classified into two types, namely global genome NER (GG-NER), which occurs everywhere in the genome, and transcription-coupled NER (TC-NER), which occurs in the transcribed strand of active genes [51,66]. In GG-NER, the first step is the recognition of the DNA damage by the XPC–HR23B

complex. In contrast, in TC-NER the repair process is believed to be triggered by a stalled RNA polymerase, and initiation of the repair requires the proteins CSB and CSA [51,55]. Afterwards, GG-NER and TC-NER seem to proceed in an identical way. The presence of damage is verified by XPA; the XPB (ERCC3) and XPD (ERCC2) helicases in complex with the TFIIH transcription factor open the DNA double helix around the damage; RPA (replication protein A) stabilizes the open DNA by binding to the undamaged strand; the endonucleases XPF and XPG cleave the borders of the open segment in the damaged strand; the damaged segment is then removed, and the repair is completed by DNA polymerase and DNA ligase.

There have been many experiments investigating whether or not NER efficiency in repairing UV-induced damage decreases with age, with conflicting results [19]. For instance, NER efficiency was observed to decrease with age in refs. [54,67,68], but observed not to decrease with age in ref. [69]. It seems likely that these different results are due to the use of different experimental procedures and different types of damages being investigated.

As evidence for an association between NER and the ageing process, inherited defects in NER cause three major types of progeroid diseases in humans: XP, CS, and TTD. The severity of the symptoms in XP varies significantly across its different types – associated with defects in different genes – and in general the more the mutation affects the NER pathway, the more severe the symptoms are [70]. Moreover, multiple mutations in NER genes have been shown to result in dramatically accelerated ageing phenotypes in mice [11,51,71,72]. In addition, XPD-mediated NER has been observed to have a significant role in maintaining the functional capacity of long-term reconstituting haematopoietic stem cells (LT-HSCs) with age, by helping to preserve the proliferative capacity and to prevent apoptosis under stress [73].

It should also be noted that XP – which is associated with a dramatic increase in skin cancers – is mainly caused by a defect in GG-NER; whilst the progeroid syndromes CS and TTD – which show no evidence of increased risk cancer – are caused mainly by defects in TC-NER [71]. This is because GG-NER is responsible mainly for repairing pre-mutagenic DNA lesions, preventing carcinogenesis; whilst TC-NER is responsible mainly for repairing DNA lesions that block transcription [74].

A particularly interesting gene for the study of the NER pathway is *XPD* because different point mutations in this gene are associated with different phenotypes: cancer (XP), the TTD progeroid syndrome, or a combination of cancer and a progeroid syndrome, namely XP combined with CS (XPCS) or XP combined with TTD (XPTTD) [72]. Hence, many mouse models have been created with mutations in the *Xpd* gene, as follows. First, inactivation of the *Xpd* gene led to embryonic lethality [75]. Later, the same group generated mice carrying an *Xpd* point mutation found in TTD patients, which produced mice with several symptoms of TTD, including cachexia [26]. The authors proposed that the observed premature ageing of TTD mice is due to the accumulation of DNA damage, which leads to impaired transcription, apoptosis, functional decline, and depletion of cell renewal capacity. They also crossed TTD mice with $Xpa^{-/-}$ mice, which greatly increased the NER defect. This produced mice with increased neonatal lethality and extreme cachexia.

The multiple effects of Xpd have also been exploited to create mouse models of "progeroid NER syndromes", by combining different mutant *Xpd* alleles with a $Xpa^{-/-}$ background [72]. The authors observed that such progeroid NER mice share many similarities with long-lived dwarf and calorie-restricted mice, in particular reduced postnatal growth and small size. They argued that this is likely due to an adaptive response to genomic instability during postnatal development, which involved dampening of the somatotropic GH/IGF-1 (growth hormone/insulin-like growth factor 1) axis, rather than due to the proliferative defects associated with premature cell senescence – a common explanation for this progeroid phenotype.

In another work, a mouse model was created with a mutation in the *Xpd* gene that exhibits strong signs of progeroid TTD [76], and the Xpd^{TTD} mice were also observed to have reduced body and organ weight. In this work the rate of apoptosis exceeded the rate of cell proliferation, resulting in homeostatic imbalance, and this imbalance was associated with decreased energy metabolism and reduced IGF-1 signalling. Hence, similarly to [72], the authors [76] concluded that the reduced energy metabolism is likely an adaptive response to the increased DNA damage in those mouse mutants. It is possible, however, that decreased GH/IGF-1 signalling is a disease (or disease-response) mechanism rather than an adaptive response targeting the ageing process.

5.3. Repair of double-strand breaks via non-homologous end joining (NHEJ)

First, let us briefly discuss the difference between the NHEJ and the homologous recombination (HR) pathway, which are two basic pathways for the repair of DNA double-strand breaks. For a discussion of variants of those basic pathways, see refs. [38,49].

In the HR pathway the undamaged chromosome is used as the template for the repair of the broken chromosome. This type of repair involves the two sister DNA molecules that exist in each chromosome in cells that have replicated their DNA but not divided yet – i.e., in phases S and G2 of the cell cycle [55]. In contrast, the NHEJ pathway is mainly used in phase G1 of the cell cycle, before DNA replication, when there is no sister copy of DNA to be used for homologous recombination, although NHEJ seems to occur throughout the cell cycle [11]. The type of double-strand break repair also depends on the tissue or cell type. For instance, in non-dividing cells like neurons, it seems that HR is not an option, and double-strand breaks have to be repaired by NHEJ [77]. The NHEJ pathway simply links the ends of a double-strand break together, without using any strand as a template. This pathway is more error prone than the HR pathway, and it tends to insert deletions or insertions in DNA strands. Nonetheless, in mammalian cells NHEJ seems to be the main pathway for the repair of double-strand breaks resulting from ionizing radiation [47].

The NHEJ repair process starts with the binding of the KU heterodimer (consisting of KU70 and KU80 subunits) to the broken DNA strand ends, which recruits DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) [51,55,78]. KU is one of the most abundant proteins in human cells, it associates with telomeres and telomerase [79], and it also forms a complex with WRN, and with PARP1, suggesting that these proteins act together as "caretakers" of genome integrity [80].

Evidence for the KU complex's role in ageing has been shown in several studies with mice knockouts, as follows. In experiments carried out around the late 1990's, $Ku80^{-/-}$ mice exhibited signs of premature ageing without significantly increased cancer [81,82]. Surprisingly (considering that Ku70 and Ku80 form a complex), $Ku70^{-/-}$ mice exhibited instead a significant incidence of thymic lymphoma [83,84]. However, more recently, Li et al. [85] reported that those differences were likely due to differences in genetic background and/or environment. Their experiments with three types of mice cohorts, consisting of $Ku70^{-/-}$, $Ku80^{-/-}$, and $Ku70^{-/-}/Ku80^{-/-}$ double-mutant mice, showed that all these cohorts exhibit a premature ageing phenotype and lower cancer levels than previously reported for $Ku70^{-/-}$ mice. However, a different type of phenotype is obtained when combining the deletion of *Ku70* and/or *Ku80* with the deletion of the well-known *p53* gene, a transcription factor which, among other functions, acts as a tumour suppressor. Recently, Li et al. [86] have shown that, surprisingly, $Ku70^{-/-}/p53^{-/-}$ mice live significantly longer than

either *Ku80*$^{-/-}$/*p53*$^{-/-}$ mice or *Ku70*$^{-/-}$/*Ku80*$^{-/-}$/*p53*$^{-/-}$ triple mutant mice, due to a much lower incidence of pro-B-cell lymphoma in the former cohort.

There is also evidence that NHEJ activity is considerably reduced with age in rat cortical neurons [48,77]. This decreased NHEJ activity cannot be trivially explained as a consequence of a reduced number of double-strand breaks, because the number of double-strand breaks in the neurons of rat cerebral cortex has been shown to considerably increase with age [53]. Also, genetic defects in the NHEJ pathway have been shown to reduce hematopoietic stem function in an age-dependent manner under conditions of stress in mice [73].

Turning to human ageing, the level of mRNA expression of KU70 was observed to decrease considerably with age in human hematopoietic stem and progenitor cells [87]. Furthermore, the levels of the KU70 protein and of MRE11 (another DNA repair protein involved in NHEJ) were observed to significantly decline with age [88]. In addition, KU70 expression was significantly higher in a particular community known to have the highest average lifespan in South Korea when matched to other individuals of the same age, similar life patterns and same region. Hence, the authors suggested that KU70 expression in lymphocytes may be considered a biomarker of ageing [88]. Moreover, a systematic analysis of DNA repair proteins associated or not with ageing – based on data mining methods – indicated that NHEJ is central to proteins associated with ageing [89].

6. From DNA damage to ageing: networks, cells and evolution

In this section we first interpret the aforementioned relationship between DNA repair pathways and ageing in context of interactions between different molecular pathways, their effects on cellular processes and how the complexity of DNA damage response systems impacts on our understanding of the role of DNA damage in ageing. Secondly, we discuss the DNA damage theory of ageing in the context of evolution and of how differences in DNA repair could have contributed to known differences in longevity and ageing rates between species.

6.1. Complex interactions between molecules, pathways and cells

Human and mouse progeroid syndromes show that defects in DNA repair can accelerate the ageing phenotype, possibly by impacting to some degree on the ageing process. Not all mouse mutants with defective DNA repair genes show signs of accelerated ageing, however. Hasty and Vijg [35] point out that deletion of some crucial DNA repair genes leads to embryonic death or cancer at an early age, so that there is no time for the ageing phenotype to appear. This shows that DNA repair is crucial for survival, but this is not incompatible with the fact that DNA repair is also important for ageing. Nonetheless, as discussed above (Section 5), some DNA repair pathways clearly have been more strongly linked to ageing than others, suggesting that specific types of damage or those that trigger particular downstream events are crucial for ageing. However, one prediction of the DNA damage theory of ageing is that improved DNA repair should lead to slower or postponed ageing, ultimately leading to longer lifespan and this has not been demonstrated to date [8].

Chevanne et al. [90] compared the efficiency with which cells from young, old and centenarian subjects repair DNA strand breaks caused by sublethal concentrations of hydrogen peroxide. They observed that cells from centenarians are about as efficient in that kind of repair as the cells from young subjects, and both types of cell were considerably more efficient in that task than the cells of old subjects. They also observed that the expression level of PARP1 was significantly decreased in the cells of old subjects, but not in the cells of young and centenarian subjects. In addition, centenarians have significantly higher levels of the KU70 protein. Although these results support the hypothesis that improved DNA repair systems may lead to longer lifespan, they are correlative in nature and far from conclusive.

Recent studies have shown associations between human longevity and DNA repair genes. Polymorphisms in ATM are one of such case. Interestingly the ATM polymorphism associated with longevity affects ATM expression, yet it is not the variant associated with high or low ATM expression that is associated with longevity but rather the polymorphism associated with moderate expression [91]. Similarly, ERCC2 polymorphisms associated with low ERCC2 expression are associated with longevity [92].

Findings from human progeroid syndromes have been validated in mouse models through disruption of DNA repair mechanisms that appear to accelerate ageing, but again the opposite is far from proven. One study showed that *Drosophila melanogaster* with one or two extra copies of a DNA repair gene had a slightly extended lifespan [93]. There have been attempts to optimize DNA repair mechanisms in mammals through genetic manipulations but by and large these have been unsuccessful [3]. A classic example is the *p53*$^{+/m}$ mouse strain which has an activated p53 yet surprisingly displays signs of premature ageing [94]. Given the complexity of DNA repair pathways, perhaps upregulating a single DNA repair protein merely shifts the rate-limiting step to another protein and fails to have an impact on ageing. Cellular responses to DNA damage involve a large number of proteins. ATM and ATR (ataxia telangiectasia and Rad3 related) are among the key mediators of the signal transduction pathway in response to DNA damage. A large-scale proteomic analysis revealed over 700 proteins phosphorylated by ATM and ATR in response to DNA damage and painted a picture of a highly interconnected network [95]. This work further exemplifies the complexity of the pathways involved and how incomplete our understanding of these networks still is.

Possibly the only mouse model hinting that improving DNA repair may delay ageing comes from cancer-resistant mice with telomerase constitutively expressed. Cancer-resistant mice with enhanced expression of p53 and other tumour suppressors, p16 and p19^{ARF}, have a normal ageing process [96,97]. When also overexpressing telomerase their median lifespan increases by up to 40% [98]. It is not clear whether ageing is delayed in these animals or whether DNA repair is improved but these findings do point towards some level of protection from age-related degeneration via optimization of pathways associated with cancer and DNA damage responses. Although it is clear that DNA damage and mutations increase with age, the molecular, cellular and physiological mechanisms leading to degeneration are poorly understood. One emerging hypothesis is that alterations in DNA damage or in DNA repair pathways impact on cellular processes that either limit cell division or increase cell loss (Fig. 1). In fact, a number of mutations resulting in premature ageing in humans and mice are associated with cellular phenotypes, such as premature replicative senescence or increased apoptosis, as reviewed in ref. [16]. Biomarkers of DNA damage and telomere dysfunction – more precisely, human orthologs of proteins secreted from telomere-dysfunctional bone-marrow cells of late generation telomerase knockout mice – have also been observed in ageing humans [99]. Dysfunctional telomeres, in turn, activate DNA damage responses which trigger cell cycle arrest [100]. A more specific hypothesis of the above is that DNA damage accumulating in stem cells has a particular strong contribution to ageing alterations as these will be more easily propagated in tissues and hence impair tissue regeneration. For example, disruption of ATR in adult mice results in stem cell loss and premature ageing [101]. Similarly, it has been suggested that the premature ageing observed in the aforementioned *p53*$^{+/m}$ mice may be caused by loss of cellularity due to stem

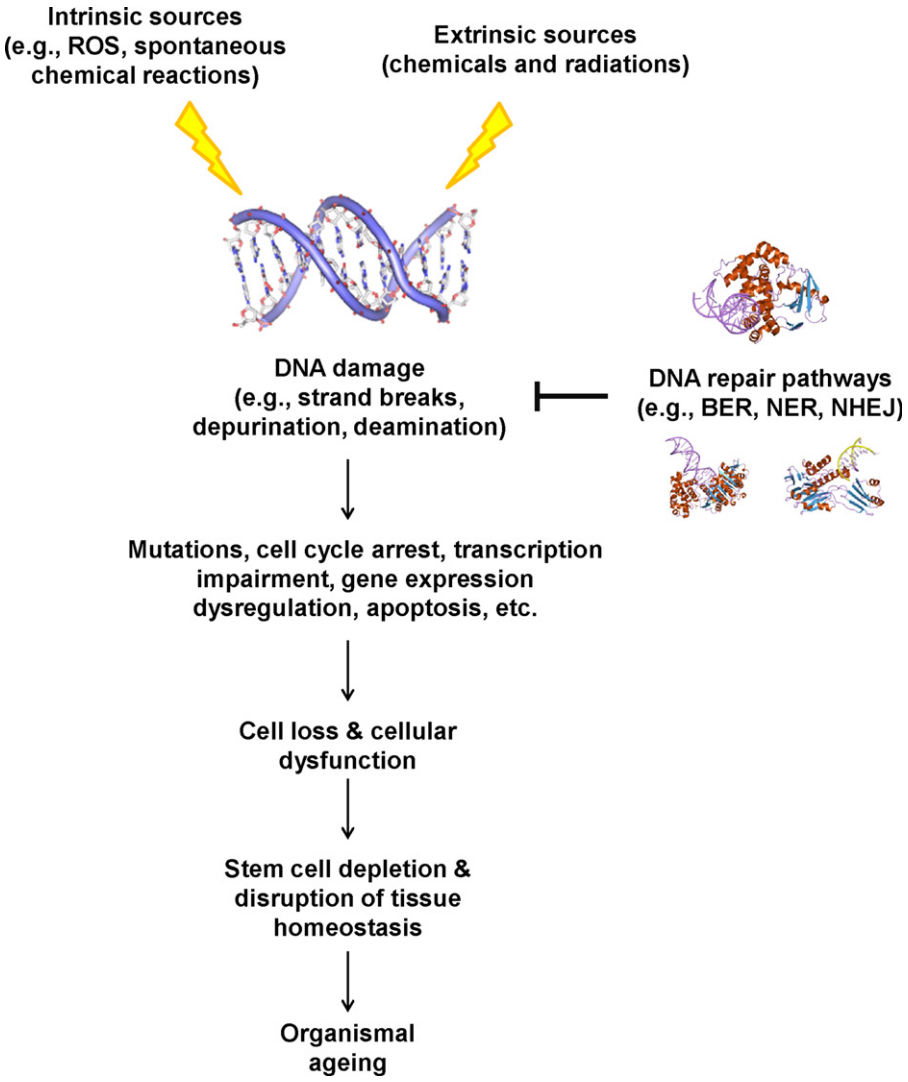

Fig. 1. Overview of the DNA damage theory of ageing. A variety of intrinsic and extrinsic sources can result in DNA damage. An array of complex DNA repair mechanisms evolved to repair DNA damage, yet these are not perfect. DNA lesions in cells can lead to mutations, cell cycle arrest, blocked transcription, apoptosis and many other problems which in turn result in loss of cell function and cell death. With biological time, the accumulation of DNA damage in an increasing number of cells may lead to loss of stem cells and disruption of tissue homeostasis which causes ageing of the organism. Protein structures by the EBI (http://www.ebi.ac.uk/).

cells undergoing premature replicative senescence [94]. In haematopoietic stem cells DNA damage has been shown to accumulate with age and contribute to functional decline [73]. Therefore, stem cell ageing caused by DNA damage accumulation remains one powerful downstream mechanism of DNA alterations with age [16,102].

Overall, the path from DNA damage to ageing involves multiple interacting molecular and cellular processes (Fig. 1). Given the fine-tuning necessary in DNA damage responses and during interactions between pathways associated with DNA repair, including cell cycle, perhaps it is not too surprising that researchers are yet to develop a mouse model of enhanced DNA repair that delays ageing. Likewise, the findings that it is not the highest expression of ATM or ERCC2 that is associated with human longevity need to be put in context of the hundreds of other associated proteins, as well as cellular processes, though the fact that polymorphisms in genes involved in DNA repair are associated with human longevity is noteworthy.

6.2. Species differences in ageing and DNA repair

Although ageing is observed in most animal species, and in all studied mammals, there is a great variance in the rate of ageing changes across different species [103,104]. If DNA damage is the main underlying mechanism of ageing then one hypothesis is that the optimization or enhancement of DNA repair contributed to the evolution of long-lived species [104].

Some correlations have been reported between DNA repair mechanisms and longevity in mammals [105–107]. It has been argued, however, that such correlations may be an artefact of long-lived species being on average bigger since, independently of lifespan, larger animals are expected to have higher DNA repair rates [108].

Whilst earlier studies focused on NER, more recent studies have focused on BER, though results have been mixed [104]. One study reported a correlation between PARP1 activity and longevity of mammals [109]. To test the hypothesis that PARP1 optimization contributed to the evolution of longevity, mice with human PARP1 ectopically expressed were generated, though it resulted in impaired DNA repair and a short lifespan [110]. Of course, because DNA repair is a complex process, it could be that ectopic expression of PARP1 disrupted the fine balance of DNA repair proteins, disrupting DNA repair. Recent progress in large-scale sequencing methods is resulting in a growing number of sequenced genomes which can be employed for detecting genes associated with the evolution of longevity [104,111]. By employing comparative

genomics to detect proteins with patterns of selection specific to long-lived lineages, we recently detected that some DNA repair proteins are associated with the evolution of longevity in mammals (Li and de Magalhaes, unpublished results). Whilst these correlations are based on computational analyses, they provide evidence that at least some DNA repair proteins are selected for during the evolution of long lifespans, which might be due to an optimization of DNA repair pathways. Because DNA repair systems tend to be largely conserved evolutionary, it could also be that species differences in ageing are due to the way cells respond to DNA damage. This could involve cell decisions on how to repair the damage (i.e., which pathways to activate) or even decisions at the level of whether to repair the damage or let the damaged cell die. There are complex pathways affecting these decisions and these may be involved in species differences in ageing. We speculate that short-lived species, which tend to grow fast, would favour responses that optimize growth even if that leads to a build-up of damaged cells and consequently a higher cancer incidence and a faster ageing process later in life. On the other hand, longer-lived species can "afford" to eliminate damaged cells and grow slower, resulting in a slower build-up of damage, lower cancer incidence and slower ageing. According to this model, trade-offs in the evolution of DNA damage responses would be important for species differences in ageing rather than DNA repair *per se*.

Studies of long-lived mammalian species are necessary to address these questions and identify the specific cellular, genetic and molecular mechanisms involved in species differences in ageing and cancer. One emerging model in ageing research is the naked mole–rat (*Heterocephalus glaber*). Capable of living over 30 years, *Heterocephalus* is the longest-lived rodent and is exceptionally resistant to neoplasia [112]. Studies of DNA repair and, perhaps even more importantly, cellular DNA damage responses in *Heterocephalus* and in other long-lived species are likely to augment our understanding of the adaptations necessary for species to evolve a long lifespan and cancer resistance.

7. Conclusions and perspectives

It is undeniable that alterations to DNA can have a profound impact on cellular functions and even lead to cell death. As such, a broad array of mechanisms evolved to maintain genome integrity. These mechanisms combine processes for assuring that the DNA of each cell is maintained unchanged (including copying the DNA and repairing it) and replacing genomes damaged beyond a given threshold by cell self-destruction mechanisms. It is equally clear that DNA changes accumulate with age in multiple tissues and are likely to contribute to the increased cancer incidence observed with age, even though which DNA lesions are more important contributors to broader aspects of ageing remains unknown. One major hypothesis is that DNA damage activates signalling pathways that, perhaps through cell function disruption or cell loss, result in a depletion of stem cell stocks which then contributes to ageing. As such, the idea that DNA damage accumulation with age is the primary cause of ageing remains an intuitive and powerful one.

Human progeroid syndromes clearly show that disruption of DNA repair pathways can accelerate the ageing phenotype. Although it is not clear how representative of normal ageing the changes observed in progeroid syndromes are, the breadth of age-related changes observed prematurely in certain progeroid syndromes suggests that at least some aspects of ageing are the same. The fact that disruption of DNA repair can accelerate ageing is not proof by itself that DNA damage causes ageing but remains a strong argument. Recent results showing that optimization of DNA repair pathways can extend mouse lifespan, even if it remains questionable whether ageing was delayed, hint that indeed the balance between DNA damage and repair sets the pace of the ageing process, at least to some degree.

The complexity of responses to DNA damage, which involve networks of interacting processes, including DNA repair mechanisms, cell cycle checkpoints and apoptotic pathways, means that the cellular processes of DNA damage responses and DNA repair and how these impact on organismal processes like ageing are only partly understood. Elucidating the evolution of these mechanisms and how adaptations in them could have contributed to species differences in ageing also remains a promising but daunting task. Advances in data integration, modelling and high-throughput approaches have the potential to elucidate the complex interplay between DNA damage, DNA repair, and their interacting networks, but there is still a long road ahead. Ultimately, however, such approaches can lead to solving ageing, one of the greatest biological riddles of our time.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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