HHS Public Access

Author manuscript

Nature. Author manuscript; available in PMC 2023 January 17.

Published in final edited form as:

Nature. 2021 April ; 592(7856): 695–703. doi:10.1038/s41586-021-03307-7.

The central role of DNA damage in the ageing process

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Abstract

Ageing is a complex, multifaceted process leading to widespread functional decline affecting every organ and tissue. Remarkably, it is still unknown if ageing has a unifying causal mechanism or is grounded in multiple sources. Phenotypically, the ageing process is associated with a wide variety of features at the molecular, cellular and physiological level, e.g., genomic and epigenomic alterations, loss of proteostasis, declining overall cellular and sub-cellular function, deregulation of signaling systems. However, the relative importance, mechanistic interrelationships and hierarchical order of those ageing features have not been clarified. Here, we synthesize accumulating evidence that DNA damage affects most if not all aspects of the ageing phenotype making it a most likely unifying cause of ageing. Hence, targeting DNA damage and its mechanistic links with the ageing phenotype will provide a logical rationale for developing interventions to counteract age-related dysfunction and disease in concert.

The ultimate cause of ageing

There is wide agreement that ageing in metazoa is ultimately caused by the declining force of natural selection, once genes have been passed on to the next generation[1]. Hence, mutations that only have adverse effects late in life, are not eliminated by purifying selection and therefore allowed to accumulate in the germline[2]. Pleiotropic mutations

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Author Contributions:

All authors contributed to the writing of the article.

Competing interests:

Jan Vijg is co-founder of SingulOmics Corp., USA

with beneficial effects before, but adverse effects after reproduction, are even positively selected[3]. The consequences of accumulation of such germline mutations only become evident when lifespan is no longer curtailed by extrinsic sources of early mortality, as with modern humans or animals kept in protective environments, explaining the steep rise in multimorbidity at advanced age.

While the evolutionary logic of ageing is clear, surprisingly little is known about its proximate causes, even though ageing is the source of most chronic diseases and the main burden for healthcare in advanced societies world-wide. Does ageing have a sheer infinite number of origins, as predicted by evolutionary theory, or could there be one ancestral cause present from the beginning that with increasing complexity of life was later joined by many secondary causes? In an attempt to better understand ageing, a number of processes that causally contribute to pathologies occurring at old age have been identied[4]. In this perspective we show how the main features of the ageing phenotype, causally and mechanistically, converge onto one factor: DNA damage (Figure 1), rendering this a strong candidate as the primary cause of ageing.

Effects of DNA damage at the molecular level

The inherently instable genome

As the primary template encoding all genetic information, DNA is surprisingly instable. Genome instability can be defined as the tendency of the genome to undergo mutation, i.e., any permanent, transmittable DNA sequence alteration in the genome such as a base substitution, a deletion or insertion, copy number variation, chromosomal aberration or retrotransposition. Mutations generally adversely affect function and are a major cause of cancer and genetic disease. However, in the germ line they are also the substrate of evolution.

Mutations are an inherent characteristic of both nuclear and mitochondrial genomes and a consequence of erroneous replication or repair often starting from DNA damage. In a broader sense, genome instability can refer to the inherent characteristic of DNA to undergo chemical modification, generally termed DNA damage, that alters its structure and functional properties[5]. DNA damage has been a problem from the onset of DNA-based life, given the ubiquitous abundance of DNA-damaging agents, such as UV-rays from the sun, causing lesions that block transcription and replication. DNA damage ranges from spontaneous deamination and hydrolysis to a plethora of chemical alterations including different types of breaks, nicks, gaps, abasic sites, adducts, inter-, intra-strand and DNA-protein crosslinks, subtle chemical modifications, etc. Also, aberrant DNA structures, such as R-loops, G-quadruplexes and persistent single-strand regions or arrested intermediates in DNA transactions such as stalled transcription, replication and recombination complexes should be considered as DNA damage, as they compromise DNA functionality and trigger the same responses. DNA injuries hamper accurate replication, controlled transcription and secure storage of the genetic information. At the apex of the informational hierarchy, nuclear DNA is usually present in only two (distinct) copies and, in contrast to all other biomolecules that can be remade based on instructions carried by the corresponding genes, DNA integrity can only be maintained by constant repair. An elaborate network of highly

sophisticated DNA repair and DNA damage response (DDR) systems counteract the time- and exposure-dependent erosion of the genetic information. Inherited defects in these maintenance systems not only predispose to cancer but also underlie numerous, segmental forms of premature ageing in humans, indicating a tight link between genome integrity, cancer and ageing[6] (Box 1).

During normal ageing, DNA damage occurs continuously on a massive scale, due to numerous exogenous and endogenous genotoxins. The pro-ageing effects of genotoxins are visible during photoaging of the skin but also DNA-damaging chemotherapy accelerates ageing features[7]. Even mechanical stress to tissues can cause genome instability and may contribute to the accelerated ageing in Hutchinson-Gilford Progeria, where mechanical resilience of the nucleus is compromised by a mutation affecting the scaffold protein lamin A[8]. It is estimated that up to 10^5 DNA lesions occur in an active mammalian cell on a daily basis, with spontaneous hydrolysis alone causing ~10^4 abasic (mostly apurinic) sites[5]. Even though most of these lesions are efficiently removed, some escape detection, are irreparable, repaired too late, or repaired in an erroneous way. In time, DNA injuries inevitably accumulate[9] making genome instability a true hallmark of ageing (Figure 2).

Genome instability at dysfunctional telomeres

The discovery in the late 1980s that *S. cerevisiae "*ever shorter telomeres" (EST1) mutants undergo replicative senescence[10] has popularized the concept that progressive telomere shortening drives the ageing process. In mammals, telomeres consist of thousands of TTAGGG repeats covered by the shelterin complex that facilitates formation of a lariat-like T-loop, and thereby hides the telomeric end preventing activation of the DDR sensors[11]. Due to incomplete lagging strand synthesis during DNA replication, the number of repeats decreases with each cell division. In the germline and in some somatic stem cells this loss is compensated by telomerase, which is silenced in most somatic cells during early development, restricting the number of cell divisions until telomeres become critically short. An unprotected telomere resembles a persistent DNA double strand break (DSB) triggering chronic DDR activation resulting in replicative senescence[12]. Even a single DSB suffices to cause full-blown cell cycle blockade[13]. The pathogenicity of telomere shortening in ageing is an antagonistic pleiotropic effect of a trait that must have been selected for its early benefits such as limiting unrestrainted proliferation and hence tumor formation[14].

Genetic defects in telomere maintenance cause human telomeropathies, including dyskeratosis congenita, aplastic anemia, and pulmonary and liver disease exhibiting multiple progeroid features[15]. In mice, segmental premature ageing only manifest in telomerase mutants after several generations, likely because their particularly long telomeric repeats take several generations to become critically shortened and thus dysfunctional[16]. The estimated telomere length in bulk human tissues does not suggest that on average telomeres become critically short in normal ageing, even at old age[17]. However, progressive telomere shortening might alter expression of specific subtelomeric genes[18], the *in vivo* relevance of which during ageing is yet to be determined.

DNA damage-induced epigenetic alterations

The epigenome is comprised of DNA methylation and many histone modifications and is unstable over the lifetime of somatic cells. Some changes are similar between cells in a tissue and are likely adaptive or programmed, others are progressive and/or stochastic, similar to DNA damage and mutations, contributing to intercellular heterogeneity, possibly with important functional consequences.

Chromatin modifications include phosphorylation, methylation, acetylation, ubiquitination, sumoylation, citrullination, and polyADPribosylation (PAR), most of which are also part of the DDR[19]. Age-dependent chromatin modifications include loss of histones[20] and increased “fuzziness” of nucleosomes[21], linked with local and global chromatin remodeling, an imbalance of activating and repressive histone modifications, and transcriptional changes. In humans and experimental animals, diverse sets of age-related alterations in DNA methylation in various tissues have been found to strongly correlate with chronological age and are now used as epigenetic clocks. Because such clocks tick similarly from cell to cell the underlying CpG methylation statuses likely reflecting adaptive changes[22].

Increasing evidence suggests that DNA damage is a major driver of age-associated epigenetic changes. The DNA methyltransferase, Dnmt1, localizes to sites of DNA repair[23] and many chromatin remodelers regulate the assembly of distinct repair machineries, lesion removal, and restoration of the original chromatin state, which may leave epigenetic marks. For example, after the repair of transcription-blocking lesions in *C. elegans*, H3K4me2 deposition facilitates the resumption of transcription of genes regulating protein biosynthesis and homeostasis and consequently promotes longevity[24]. The DDR in human cells leads to loss of H3K27me3, promoting cellular senescence[25]. The phosphorylated histone variant γH2AX forms foci at the site of DSBs. Such foci accumulate in various mouse tissues with ageing[26] indicative of persistent chromatin alterations resulting from DNA damage. ‘DNA segments with chromatin alterations reinforcing senescence’ (DNA-SCARS) have been found enriched in senescent cells. Such DNA-SCARS exemplify persistent local chromatin changes due to irreparable DNA lesions[27]. In cell lines it has been demonstrated that DNA methylation patterns are altered during homologous recombination (HR) repair, followed by further modification weeks later by base excision repair-mediated transcription-associated demethylation[28]. Poly-ADP-ribosylation of histones and the Poly-ADP-Ribose polymerase 1 (PARP1) itself facilitates repair of single-strand breaks serving as a landing platform for proteins in base excision repair. PARylation severely reduces cellular NAD+ pools which may trigger apoptosis or may indirectly inhibit Sirtuin proteins, which in turn affect genome-wide chromatin acetylation, ageing and DNA repair[29] and trigger gene expression changes that resemble those observed in ageing mouse brain[30].

It is thus plausible that continuous DNA damage induction and repair for tens of thousands of lesions daily leave epigenetic marks and thereby contribute to intercellular epigenetic heterogeneity in ageing, particularly since somatic cells do not have to function forever and epigenetic memory is erased in the germline at the start of the next generation. Consistent with these ideas, transcription in aged cells appears far more variable than in young cells[31]. Hence, the DDR likely is a primary cause of epigenetic changes that lead to deterioration

of control of gene expression, which in turn contributes to somatic heterogeneity and time-dependent overall functional decline.

DNA damage-induced proteostatic stress

Proteostatic pathways control the synthesis, folding and degradation of proteins. Several age-related diseases are associated with protein misfolding and aggregation such as Alzheimer (AD) and Parkinson disease (PD). Misfolded proteins can arise when structural alterations affect solubility, thus causing protein aggregates, e.g. upon oxidative, heat, or endoplasmic reticulum stress. Multiple lines of evidence link DNA damage to proteostatic stress. Children with the premature ageing condition Cockayne syndrome (CS), which is caused by a defect in transcription-coupled repair (TCR), show neurofibrillary tangles in the cerebellar cortex[32] occurring decades earlier than in familial early-onset AD. Defective TCR accelerates neurodegeneration in a *C. elegans* model for CS thus further underlining the ancestral role of DNA damage in driving age-related neuronal pathology[33]. DNA damage and altered expression and activity of DNA repair genes have been implicated in the pathogenesis of AD and other dementias[34–38], such as reduced nucleotide excision repair (NER) efficiency in human PD[39]. Several DNA repair mechanisms, particularly mismatch repair, are involved in the repeat expansion underlying Huntington disease[40] and *vice versa* mutant huntingtin has been linked to defects in repairing transcription-associated DNA strand breaks[41].

DNA damage could trigger proteostatic stress, for example, through increased stalling of transcription (transcriptional stress) or (epi)mutation-mediated transcriptional noise. This likely affects assembly, stoichiometry, proper folding and functioning of protein(complexe)s, triggering proteostatic stress and aggregation. Single cell sequencing of human neurons has confirmed that somatic mutations increase during ageing and do so at a higher rate in cells from patients with neurodegenerative diseases[42]. Stochastic transcription-blocking DNA lesions accumulating in post-mitotic tissues such as neurons, which do not dilute DNA damage by replication, likely cause the genome-wide reduced expression preferentially of large genes observed during natural ageing and in an accelerated fashion in progeroid NER/TCR-deficient mice[43]. These DNA-damage-driven mechanisms would explain the decoupling of transcription and protein expression[44] and loss of stoichiometry of protein complexes noted during ageing in different species[45], thus creating proteotoxic stress and protein aggregates.

Defects in chaperones, the ubiquitin proteasome system and autophagy can result in accumulation of misfolded proteins. The DDR itself can strain the proteostatic machineries[46] and IRE1α and transcription factor XBP1 –both key regulators of the endoplasmic reticulum unfolded protein response (UPR^{ER})– are induced in DNA repair defective progeroid mice[43]. Also autophagy is induced by DNA damage signaling and is indeed required for survival amid persistent DNA damage[46]. When unrepaired DNA lesions drive cells into senescence they exert a chronic senescence-associated secretory phenotype[47]; which is thought to strain the UPR^{ER} [48]. In contrast, calorie restriction reduces transcription stress and simultaneously alleviates the UPR^{ER} [43], providing a direct link between DNA-damage-driven transcription stress and proteostatic stress.

Taken together, these observations support a central role of DNA damage and (epi)mutations as major causes of proteotoxic stress with age.

Mitochondrial dysfunction

As the organelles that regulate energy and metabolic homeostasis, mitochondria have since long been associated with ageing, mostly as main source of ROS[49] and linked with ageing diseases, such as PD and sarcopenia[50]. The primary cause of mitochondrial dysfunction has often been sought in ROS-induced damage to mitochondria's own genome, which measuring less than 17 Kb is infinitely smaller than the 3 billion bp of its nuclear counterpart but is present in multiple copies in each of the thousands of organelles in a typical mammalian cell[50].

The most popular hypothesis to explain age-related mitochondrial dysfunction is accumulation of somatic mutations in the mitochondrial genome, as a consequence of errors during replication and the lack of most of the sophisticated repair pathways active in the nucleus. Mice expressing a proofreading-deficient mitochondrial DNA polymerase (POLG) have greatly elevated mtDNA mutations and display multiple symptoms of premature ageing[51,52]. Increased mtDNA mutations have been correlated to loss of Cytochrome C oxidase (COX) activity in aged human skeletal muscle fibers[53,54], substantia nigra and hippocampus of normally aged human brain[55] and various other tissues[56]. However, it is unclear if the frequency of such mtDNA mutations reaches functionally important levels with natural age to ever cause phenotypic effects[57]. More advanced methods, such as digital PCR, indicated fairly low frequencies of mtDNA deletions[58] and ultra-deep sequencing did not show an age-dependent increase of mutations in wild type mice and instead suggested that most somatic mtDNA mutations originate from replication errors during development[59].

An important connection between nuclear DNA damage and mitochondrial dysfunction implicates mitophagy, the selective degradation of mitochondria by autophagy. High levels of nuclear DNA damage, e.g. in cells from aged organisms or DNA repair mutants, lead to prolonged activation of PARP1, a DNA break sensor that upon activation consumes large amounts of NAD+[60]. Inhibition of PARP or supplementation of NAD+ was reported to alleviate some premature ageing phenotypes associated with defects in DNA repair by restoring mitochondrial function and mitophagy[29].

Hence, while the role of mtDNA mutations remains subject to debate, aspects that are not yet well explored are the effect of DNA damage itself (as opposed to mutations) on mitochondrial DNA replication and transcription and damage to the over 1000 mitochondrial genes in the nuclear genome.

DNA damage-driven Cell Fate Decisions

Cellular senescence

Cellular senescence permanently arrests cell proliferation in response to various stresses, most of which DNA-damage-related. Senescence was discovered as a mechanism that limits the number of population doublings in cultured human fibroblasts due to

telomere attrition, triggering DNA-damage-signaled cell cycle arrest[61,62]. Senescence has likely evolved as a mechanism contributing to embryogenesis, regeneration (e.g. wound healing)[63] and cellular defense against overproliferation and thereby cancer. However, senescent cells acquire a "senescence-associated secretory phenotype" (SASP), secreting many pro-inflammatory cytokines, proteases, and growth and angiogenesis factors that can disrupt microenvironments and compromise tissue structure and function thereby contributing to local and systemic ageing-associated pathologies[47] and promote cancer[64]. The proinflammatory mediators can promote sterile inflammation, in this context often referred to as 'inflammaging'. Recently, attention has focused on the effect of senescence *in vivo* where purging p16-positive senescent cells in transgenic mice increased mean lifespan as well as aspects of healthspan[65]. Application of 'senolytic' agents that selectively eliminate senescent cells confirm that they contribute to ageing e.g. in atherosclerotic plaques[66] and osteoarthritic lesions[67].

Cells are driven into senescence by clastogenic compounds, such as bleomycin, doxorubicin, or cisplatin often causing irreparable DNA damage resulting in DNA SCARs[27]. DNA damage is also responsible for oncogene-induced senescence, which involves replication stress and subsequent DSBs as the consequence of hyper-replication associated with activated oncogenes[68]. DDR pathways, including ATR, ATM, and p53 that converge on activation of the cyclin-dependent kinase inhibitors p16, p21, and p27 and hyperphosphorylation of the retinoblastoma protein, trigger the withdrawal from the cell cycle[69]. In addition, cellular senescence can also arise as a consequence of chromosomal aneuploidy[70].

Even the only non-genotoxin related "mitochondrial-dysfunction-associated senescence" (MiDAS)[71] type is most likely also driven by DNA damage given the above described links to mitochondrial dysfunction. Hence, cellular senescence appears a *bona fide* part of the DDR or, as in MiDAS, can be attributed indirectly to DNA damage.

Stem cell exhaustion

Somatic stem cell exhaustion has two components, decline of stem cell number and reduced functional capacity. Different stem cells utilize distinct DDR mechanisms[72]: for instance, quiescent hematopoietic stem cells (HSCs) and hair follicle stem cells (HFSCs) employ fast but less accurate non-homologous end-joining (NHEJ), while cycling HSCs and intestinal stem cells prefer accurate HR or in case of too extensive damage opt for apoptosis, as do embryonic stem cells. In contrast, irreparable damage drives melanocyte stem cells and aged HFSCs into premature differentiation thereby clearing the stem cell pool[73]. Accumulation of DNA damage has been observed in human and mouse HSCs as well as in muscle, intestinal, mesenchymal, neural, skin, and germ stem cells[72]. Various DNA repair deficiencies trigger stem cell exhaustion. Muscle-forming satellite cells in progeroid *Ercc1* repair mutant mice were incapable of following the regular proliferation and differentiation programs[74] and third-generation telomerase-deficient mouse mutants display stem cell insufficiencies in the hematopoietic system, gut, skin and testis[15].

The underlying role of DNA damage has been particularly well documented in HSCs. During ageing, HSCs expand in number but decline in pluripotency, skewing towards

the myeloid lineage[75]. DNA damage increases in aged HSCs[76] likely from replication stress[77]. As most adult stem cells, HSCs reside predominantly in a quiescent state, which offers some protection from endogenous genotoxic stress such as metabolic ROS, but their extended time for accumulating DNA lesions and use of error-prone NHEJ increase mutagenesis[78]. Defective DNA repair limits HSC functionality in ageing and progeroid mice[79]. Thus, time-dependent accumulation of stochastic DNA damage severely hampers stem cell functionality, increasing mutations during human HSC ageing[80], impairing functional properties, promoting clonal expansion of positively selected somatic mutations resulting in loss of clonal diversity[81] or raising the potential for oncogenic transformation. Age-dependent accumulation of somatic mutations has indeed been observed in various cells types[82], such as satellite cells in humans that acquire on average 13 somatic mutations per year[83].

Also the non-cell-autonomous DDR can compromise the stem cell niche and promote stem cell exhaustion. Genome instability amid dysfunctional telomere maintenance or Sirt6 deficiency results in niche-dependent defects in hematopoietic stem cells[84,85]. Notch signaling by the niche regulates the level of p53 in muscle stem cells via Mdm2 repression[86]. With increasing age, fading niche support drives these cells into cell death via mitotic catastrophe upon activation. In *C. elegans* somatic niche cells regulate the DDR in germ stem cells via FGF-like signaling and a similar niche regulation of the p53-mediated DDR was observed in mouse HFSCs[87].

In conclusion, accumulating DNA damage is increasingly recognized to drive stem cell exhaustion during ageing through a combination of apoptotis, premature differentiation, cytostatic DNA damage checkpoint signaling, accumulation of mutations, and DNA damage-driven alterations in intercellular communication affecting stem cell niches.

Systemic effects of DNA damage

Signaling mechanisms impact the ageing phenotype

The importance of signal transduction mechanisms in ageing has become evident since the paradigm-shifting discovery of lifespan-extending mutations in insulin-like signaling (IIS) in *C. elegans*[88]. Consequently, several signaling systems have been shown to regulate longevity in species ranging from yeast to mammals. Interventions such as calorie restriction (CR) at least in part exert their anti-ageing effects by inhibiting signaling cascades such as IIS and the mTOR pathways[89]. In contrast, inflammatory signaling is thought to promote a range of age-related pathologies.

The DDR is a potent activator of inflammatory responses. This is literally obvious in the response to UV-induced DNA damage in the skin where inflammation is counteracted by systemic immunosuppression triggered by Langerhans cells migrating from the skin to the lymph nodes to activate regulatory T cells[90]. As mentioned, DNA-damage-induced senescent cells exert complex non-cell-autonomous effects[63,64], which senolytics aim to curb[66,67]. DNA damage triggers innate immune responses that in *C. elegans* regulate systemic stress signaling[91]. Inflammatory responses have also been observed in DNA-repair-deficient progeroid mice[92], which at the same time attenuate the somatotrophic

(including IIS), thyrotrophic, and lactotrophic hormonal axes, as an anti-aging response, which resembles CR and IGR-1R and other dwarf mutant mice that are long-lived[85,92,93]. Unrepaired transcription-blocking lesions suppress IGF-1 signaling in mouse and human cells resulting in elevated stress resistance[94]. In *C. elegans* IIS attenuation enhanced tissue maintenance amid DNA damage accumulation through the activation of the FOXO transcription factor DAF-16[95]. The paradoxical similarity between responses triggered by DNA damage and interventions delaying ageing suggested that a systemic DDR triggers a 'survival response' to counteract the detrimental consequences of DNA damage.

Taken together, the DDR exerts multiple effects on age-related alterations in local and systemic communication mechanisms by affecting inflammatory and key endocrine signaling components that impact the ageing process.

Anti-ageing responses to nutritional interventions are impacting genome stability

Nutritional interventions impact ageing and lifespan throughout the animal kingdom. Initially observed in the 1930s in rats[96], CR –reduced calorie intake without malnutrition– is the most robust universal health- and lifespan-promoting intervention in species ranging from yeast to mammals. It is thought that CR exerts its lifespan-extending effects through specific nutrient sensing pathways, including IIS, Sirtuins, and the AMP-activated protein kinase (AMPK) regulated mammalian target of rapamycin (mTOR) pathway[97]. In addition to the IIS attenuation in DNA-repair-deficient progeroid mice and worms discussed above, the DDR kinase ATM phosphorylates several key proteins of the IIS–mTOR pathways after DNA damage[98].

CR dramatically delays premature ageing in DNA repair mutant mice likely by decreased levels of ROS and other reactive compounds leading to reduced DNA damage levels[43]. Longevity-promoting changes in nutrient sensing pathways can also stimulate DNA repair itself, suggesting that some of the observed health benefits in normal ageing could be due to improved genome maintenance. mTOR inhibition by rapamycin *in vivo*, which extends lifespan, increases levels of the DNA repair protein O-6-methylguanine-DNA methyltransferase (MGMT)[99]. CR also activates Sirt1 and AMPK[4], promoting DNA damage repair and signaling as an epigenetic regulator[100] and increasing NER capacity[101], respectively. The protein kinase AKT, a central positive regulator of various nutrient sensing pathways, negatively regulates DNA repair and inhibits key DDR factors including Chk1, Topbp1, and p53[102]. FOXO3a, which is activated by reduced IIS, promotes the binding of TIP60 with ATM, optimizing ATM activation after DNA damage[103].

In summary, abundant evidence indicates that DNA damage affects key signaling mechanisms –by impinging on IIS, Sirtuins, AMPK and mTOR– that regulate lifespan and elicit anti-ageing effects of CR in model organisms.

Is DNA damage the primary cause of ageing?

Spontaneous DNA damage thus impinges on all major aspects of the ageing phenotype. Some of the physiological alterations in turn boost genome instability thus amplifying the deterioration of homeostasis during ageing. The strong mechanistic link of DNA damage

with ageing, and the role of DNA as the primary template for all cellular functions, make it a major candidate as the primary cause of ageing. However, at least three important arguments against this conclusion should be addressed.

First, if DNA damage is central to the ageing process, one would expect that improving DNA repair extends lifespan and evidence for this is scarce[104–106]. However, it is important to realize that DNA damage is comprised of a plethora of distinct chemical alterations, the repair of which does not depend on one gene and not even on one pathway. Instead DNA repair involves at least 7 well-balanced multi-enzyme core pathways and many more accessory processes that encompass hundreds of genes, many of which have other roles as well. Hence, the function of DNA repair as a longevity assurance system cannot be generally improved by simply upregulating the activity of one or few genes. It took evolution millions of years improving DNA repair in long-lived species, such as primates. DNA repair capacities have evolved under specific selection conditions largely driven by environmental genotoxins, such as high fluxes of UV or natural compounds. Moreover, apart from DNA repair *per sé*, cellular systems affecting DNA damage generation and outcome, such as metabolism, anti-oxidant defense, cell death, senescence, and mutagenesis are relevant as well.

Second, reliable quantification of spontaneous DNA damage in animal or human tissues appears technically extremely difficult hampering efforts to show an age-related increase to levels that likely impair cellular function and explain age-related pathologies (Text Box 2). However, DNA mutations, a consequence of erroneous DNA repair, can now be accurately determined and have been shown to accumulate with age in humans and mice in a tissue-specific manner[42,107–110]. Nevertheless, while there is no doubt that accumulating mutations cause cancer and, possibly, increased cancer risk with age, it is –as yet– unknown if their frequency is high enough to account for the loss of tissue function and increased disease risk at old age. However, besides causing mutations, accumulating DNA damage also interferes with gene expression and replication causing replication and transcription stress, senescence, functional decline and cell death, all main drivers of ageing (Figure 1).

A third, more recent argument against DNA damage-centric ageing theories is the dearth of DNA repair genes emerging from genome-wide association studies (GWAS) of ageing-related diseases or extreme longevity. However, the utter complexity of the genetics of ageing and longevity makes it highly unlikely to find genetic association with common variants in generally underpowered studies. Extreme longevity is rare and individual age-related diseases often involve genes not necessarily related to systemic ageing, e.g., lipoprotein genes. Nevertheless, in a meta-analysis of over 400 GWAS of five major categories of age-related diseases genome maintenance pathways were found[111] and genome maintenance was also the top pathway found associated with the age of natural menopause[112]. Age of natural menopause is strongly linked with a wide variety of ageing-pathologies, including cardio-vascular disease, type II diabetes and osteoporosis, and importantly with longevity[113]. These findings are consistent with the observation in both humans and mice that the vast majority of rare genetic progeroid syndromes where multiple, *bona fide* ageing-associated diseases develop early in life, is caused by mutations in DNA repair genes[6] (Text box 1).

Hence, while not invalid, all three arguments against a major role of DNA damage in ageing are unconvincing in view of the sheer complexity of DNA repair processes and the abundant evidence that only DNA repair dysfunction, not defects in proteostasis, antioxidant defense, immune response or any other physiological defense system, is associated with systemic premature ageing. Based on all the evidence, DNA damage is by far the most likely molecular driver of ageing. DNA damage and the DDR lead to broad cellular and physiological end points that can explain the entire spectrum of ageing phenotypes, from atrophy to inflammation and cancer (Figure 2). This understanding is far from new: It is known since the 1940s that rodents exposed to radiation show multiple symptoms of premature ageing[114] and the first proposals that DNA damage was the main driver of ageing stem from the 1960s[115]. More recently, the validity of these old observations was dramatically underscored by the notion that the long-term consequences of DNA-targeting chemo- and radiotherapies of cancer are accelerated, multi-organ ageing[7].

The causal relationship between DNA damage and ageing may go back in evolution to the first replicators. When DNA became the genetic material, it was already far more stable than RNA, the presumed initial carrier of genetic information. The subsequent increased length of DNA templates put a premium on faithful replication and repair, which became prerequisites for rejuvenation amid the intrinsic instability of nucleic acids even during early evolution when life was not much more than compartmentalized DNA and well before the various homeostatic alterations of ageing discussed here had evolved. Hence, DNA damage as a primary cause of ageing has probably been with us since the origin of life.

Future prospects

Time-dependent accumulation of DNA damage of endogenous and exogenous origin and its consequences progressively hamper cellular functionality and increase susceptibility to develop the chronic ailments of ageing. Interventions that aim at alleviating the root cause of ageing-associated multimorbidity should therefore be targeted at restoring genome integrity by reducing DNA damage and augmenting DNA repair. Reducing exogenous DNA damage for example through UV protection and avoidance of tobacco smoking has already proven to lower ageing-associated disease risks. Dietary interventions might be able to reign in some endogenous DNA damage sources, but the majority of spontaneous lesions will inevitably occur. Augmenting DNA repair has remained a great challenge due to the intricate complexity of repair machineries. An exception are the highly lesion-specific photolyase repair enzymes, active in many species but not placental mammals. Ectopic expression of this enzyme is indeed sufficient to prevent UV-induced carcinogenesis in mice[116]. However, those one-enzyme reactions are incapable of repairing the myriad of different lesions that require more sophisticated repair systems. Master regulators of DNA repair affecting multiple DNA repair systems have thus far remained elusive but might await discovery. Genetic screens using model organisms might be very suitable for the pursuit of such mechanisms augmenting genome stability.

Since the initial proposals that DNA damage was the main cause and DNA repair the main determinant of ageing[117,118], and the subsequent discovery that DNA repair defects can accelerate the development of a wide range of age-related pathologies[119], great strides have

been made in unraveling the mechanistic links between DNA damage and nearly every aspect of the ageing process. Venturing further into the mechanisms through which DNA damage affects each of the major processes that causally contribute to pathologies occurring at old age opens perspectives to tackle the ageing process at its causal roots and thus counteract all ageing-associated diseases simultaneously.

Acknowledgements:

Research was supported by the Deutsche Forschungsgemeinschaft (SCHU 2494/3-1, SCHU 2494/7-1, SCHU 2494/10-1, SCHU 2494/11-1, KFO 286, KFO 329, GRK 2407 to BS, and CECAD, SFB 829 to BS and JH), Deutsche Krebshilfe (70112899), H2020-MSCA-ITN-2018 (Healthage and ADDRESS ITNs) and John Templeton Foundation Grant (61734) to BS, NIH grants (PO1 AG017242 to JV and JH, and U19 AG056278, U01 ES029519, P01AG047200, U01HL145560, P30AG038072 to JV), European Research Council Advanced Grants DamAge and Dam2Age, ONCODE (Dutch Cancer Society), Memorabel and Chembridge (ZonMW) and BBoL (NWO-ENW) to JP and JH.

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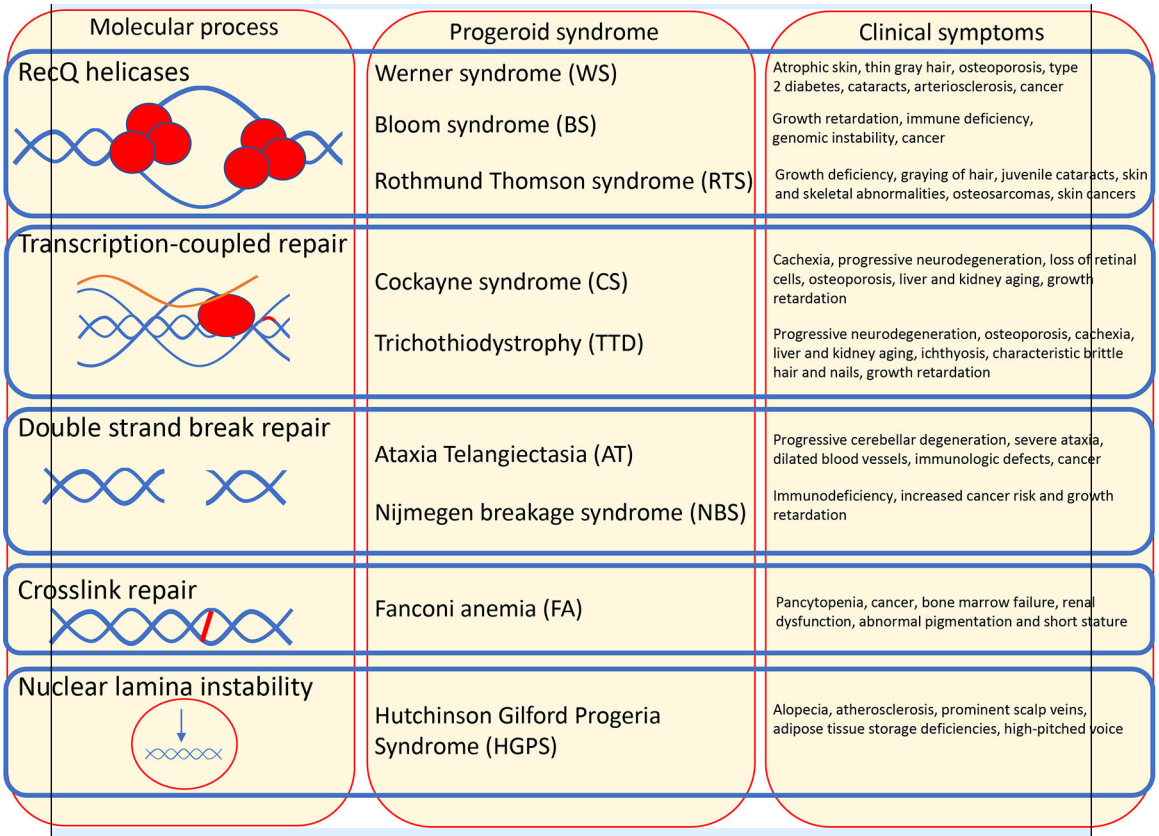
Text Box 1

DNA repair defects accelerate human ageing

Most progeroid ("premature ageing-like") syndromes are caused by mutations in genes involved in maintaining genome stability. Werner syndrome patients display many overt signs of ageing such as hair greying, type 2 diabetes, osteoporosis and cataracts, which often manifest prior to the age of 30. Werner as well as Bloom and Rothmund-Thomson syndromes are caused by mutations in RecQ helicases that function in DNA recombination, replication, repair, and telomere maintenance. Typical ageing-associated pathologies such as neurodegeneration, atherosclerosis and osteoporosis occur in Cockayne syndrome (CS) and trichothiodystrophy (TTD) before the age of 10, caused by impaired transcription-coupled repair. Global-genome nucleotide excision repair defects cause several-thousand-fold increased sun-induced skin cancer susceptibility in xeroderma pigmentosum patients, some of whom also suffer from accelerated neurodegeneration. Defects in DSB repair result in the progeroid conditions Ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS), while DNA crosslink repair deficiencies cause Fanconi anemia (FA). Also nuclear lamina dysfunction that underlies Hutchinson Gilford progeria has been linked to nuclear genome instability[6].

Progeroid syndromes are segmental as a specific DNA repair defect predominantly affects specific tissues, such as hematopoiesis in AT or FA. Neurodegenerative phenotypes occur widespread throughout those progeroid syndromes suggesting that neurons might be particularly sensitive to multiple defects in DNA repair[120]. Premature ageing is also found in long-term cancer survivors that suffer from the long-lasting consequences of genotoxic chemo- and radiotherapy[7]. An additional category of progressive progeroid disorders affecting multiple organs is due to mitochondrial defects[50], which likely involve DNA damage as well (see main text).



Text Box 1 Figure. Examples of progeroid syndromes caused by DNA repair defects.

Text Box 2

Methods to detect DNA damage

A serious challenge for linking DNA lesions to ageing has remained the methodological difficulty of accurately measuring the plethora of chemical alterations in DNA. Key problems are the limited sensitivity and/or specificity of technologies to detect physiological levels of DNA damage and the occurrence of artifacts (e.g. oxidation) during DNA isolation and handling or due to interrupted DNA-metabolizing transactions (e.g. topoisomerases) when cells are lysed. Most lesions can only be determined at semi-quantitative or relative manners or after exposure to unphysiologically high levels of genotoxic agents. Only some lesion types can be directly identified (but not quantified in absolute terms) through lesion-specific antibodies towards CPD, 6–4PP or 8-oxo-dG structures or rough overall DSBs and SSBs assessment through the (variable) COMET assay. HPLC combined with advanced mass spectrometric methodologies can detect specific chemical alterations of nucleosides[121]. There are only few examples of highly sensitive assays reporting reliable quantitation of spontaneous oxidative DNA damage, most notably 8-oxo-dG and cyclopurine lesions. Cyclopurines are endogenous transcription-blocking DNA lesions that were shown to increase from a density of 2 to 4 in young mice to 10–20 per million base pairs in old mice[122]. Indirectly, damaged DNA can be discerned by long range PCR[123], the decline in transcription through large genes resulting in a shift towards mRNAs of small genes in the ageing transcriptome of post-mitotic tissues[43], or detection of transcription-blocking lesions by strand-biased, PCR-based next generation sequencing of DNA protected by elongating RNA polymerases[124]. Specific types of DNA lesions that are amenable either to antibody binding or enzymatic modification have been mapped by high throughput sequencing. Third generation sequencing technologies are rapidly advancing to detect specific DNA modifications even in low amounts of DNA[125]. Also the formation of DNA repair complexes such as foci formation of γH2AX, 53BP1, Rad51 and other repair or signaling proteins at DSB sites and at sites of DNA-damage-induced replication stress are useful indicators. When erroneous repair or lesion bypass during replication results in mutations, sequencing methods can be applied to detect the altered DNA sequence in single cells. Somatic mutations increase linearly during ageing in multiple tissues and species including humans[82]. However, quantitative estimates of the total landscape of spontaneous DNA damage in humans or animals are lacking.

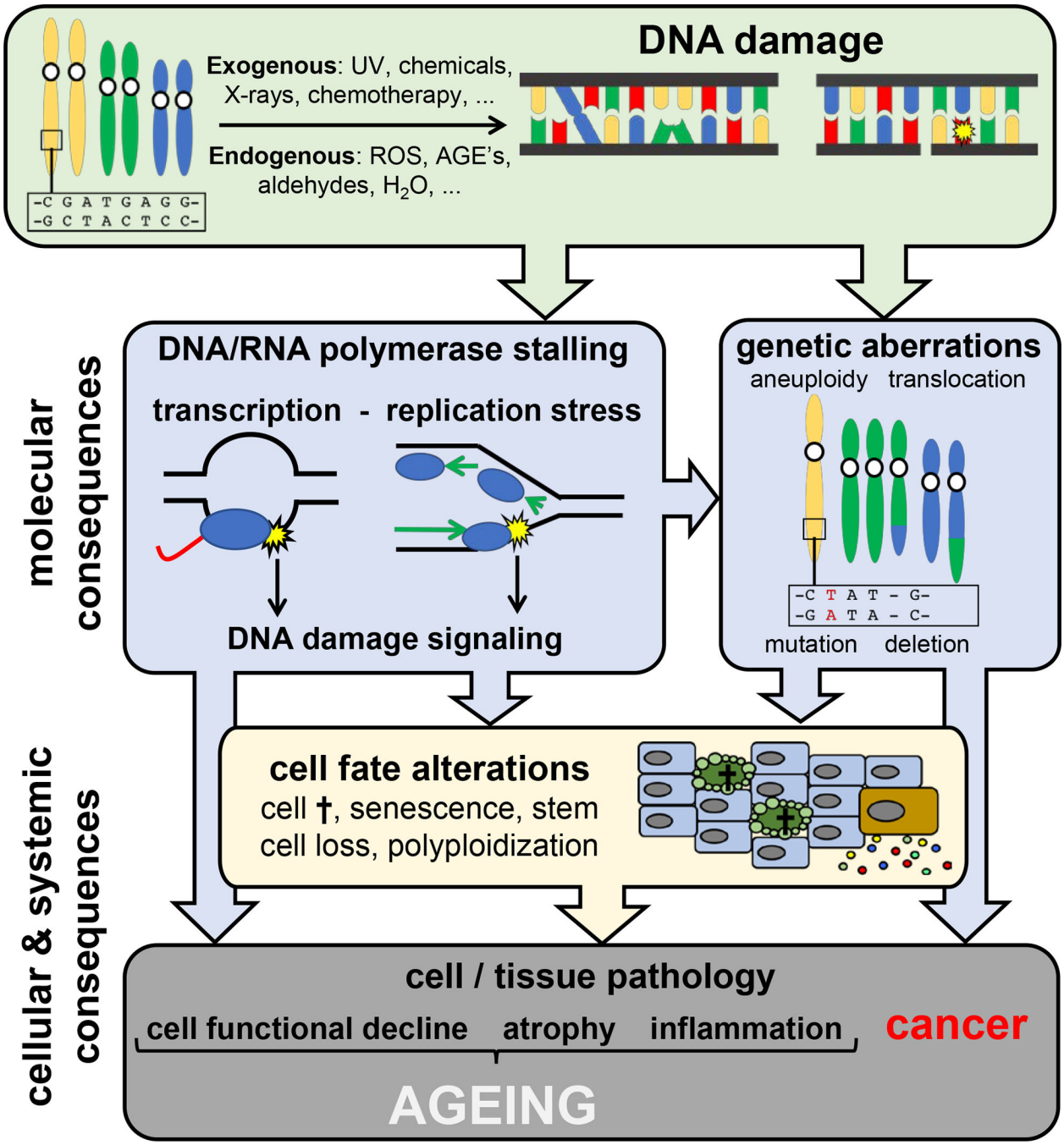


Figure 1. DNA damage is the driver of ageing.

The nuclear and mitochondrial genomes are continuously damaged by exogenous agents (UV, X-rays, chemical compounds in food, water, air), endogenous sources such as reactive oxygen species (ROS), aldehydes and advanced glycation endproducts (AGEs) and spontaneous reactions (hydrolysis). Molecular consequences of time-dependent accumulating DNA damage are: i) genetic aberrations, such as mutations and chromosomal instability, and ii) stalling of RNA and DNA polymerases by DNA lesions, which provokes DNA damage signaling and interferes with primary DNA functioning. Cellular and tissue

consequences of DNA damage include cell fate decisions such as cell death and senescence leading to functional loss of cells and organs, cancer, atrophy and inflammation.

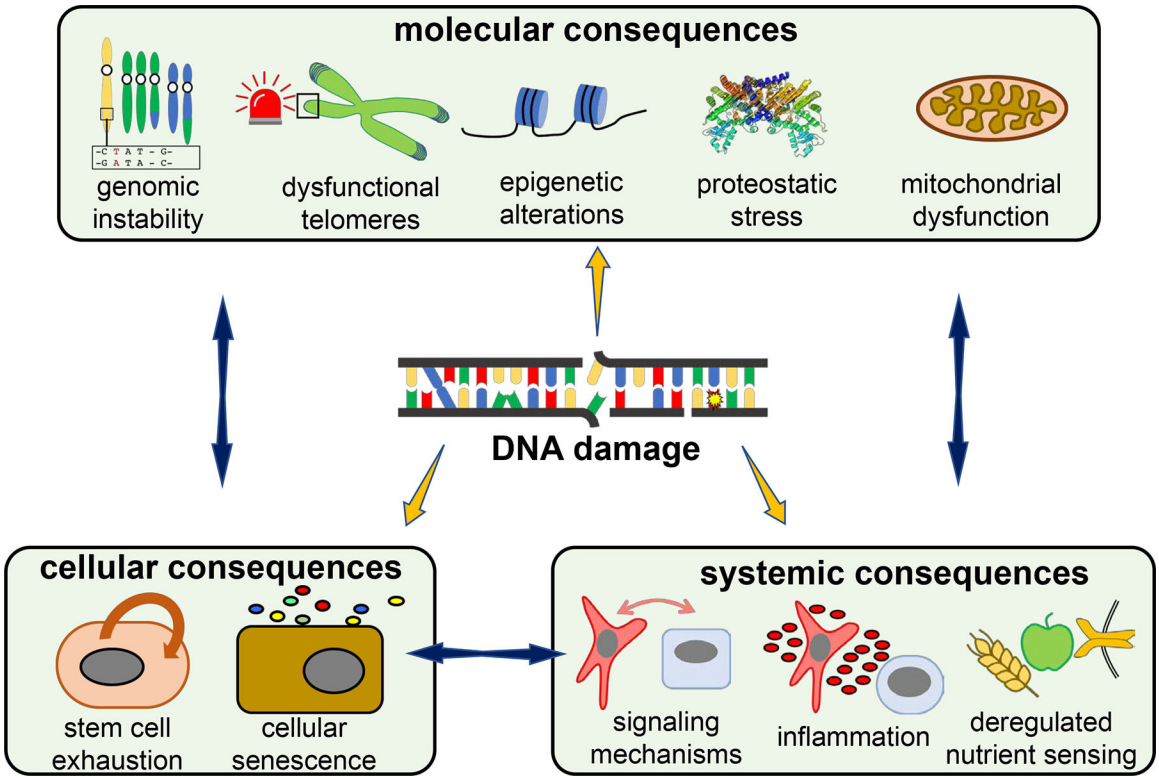


Figure 2. Molecular, cellular and systemic consequences of DNA damage.

DNA damage and the cellular DNA damage response (DDR) can impinge on molecular processes, alter cell fate and deregulate intercellular communication. DNA damage leads to mutations or chromosomal aberrations thus triggering genome instability. Critically shortened telomeres activate the DDR triggering cellular senescence. DNA repair leads to chromatin-remodeling, while the chromatin structure affects DNA damage susceptibility and repair access. The DDR affects autophagy, the UPR^{ER} and leads to a loss of protein complex stoichiometry. Mitochondrial dysfunction is driven by NAD+ deprivation by nuclear DNA repair, DNA damage-induced mitophagy defects, and altered mtDNA polymerase expression that affects mtDNA replication. DNA damage induces dampening of nutrient sensing pathways, which in turn affect DNA damage repair and signaling. Cellular senescence is induced in response to DNA damage. DNA damage causes exhaustion of stem cell pools through DDR-induced apoptosis, senescence, premature differentiation and alterations of the stem cell niche. The DDR impacts intercellular communication through inflammatory cytokines and dampened growth signaling.