The impact of oxidative DNA damage and stress on telomere homeostasis

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ABSTRACT

Telomeres are dynamic nucleoprotein-DNA structures that cap and protect linear chromosome ends. Because telomeres shorten progressively with each replication, they impose a functional limit on the number of times a cell can divide. Critically short telomeres trigger cellular senescence in normal cells, or genomic instability in pre-malignant cells, which contribute to numerous degenerative and aging-related diseases including cancer. Therefore, a detailed understanding of the mechanisms of telomere loss and preservation is important for human health. Numerous studies have shown that oxidative stress is associated with accelerated telomere shortening and dysfunction. Oxidative stress caused by inflammation, intrinsic cell factors or environmental exposures, contributes to the pathogenesis of many degenerative diseases and cancer. Here we review the studies demonstrating associations between oxidative stress and accelerated telomere attrition in human tissue, mice and cell culture, and discuss possible mechanisms and cellular pathways that protect telomeres from oxidative damage.

1. Introduction

Telomeres address two critical problems posed by the organization of mammalian genomes into linear chromosomes, thereby placing them at the forefront of aging and cancer research. First, the inability to complete DNA replication to the very chromosome ends leads to gradual loss of about 20–50 base pairs every cell division. Thus, progressive chromosome shortening occurs during cell replication, and is observed with aging (Harley et al., 1990). Germ cells, some stem cells, and most cancer cells prevent shortening by expressing the enzyme telomerase, which lengthens chromosomes by adding consecutive 5′-TTAGGG-3′ repeats. In humans, telomeres consist of approximately 10–15 kilobases of TTAGGG duplex repeats and end in a single stranded 3′ tail, which serves as the substrate for telomerase (Palm and de Lange, 2008). However, most human somatic cells lack telomerase, and when the telomeres reach a critically short length, they can no longer perform the second crucial function – chromosome end protection. Critically short telomeres lack sufficient binding sites for the six-member protein complex, termed shelterin, which remodels telomeres into a "capped" structure. Loss of the telomere "cap" exposes the chromosome end to potential degradation, and false recognition by the DNA damage response proteins as chromosome breaks (d'Adda di Fagagna et al., 2003; de Lange, 2009). In normal cells this triggers senescence and loss of replicative capacity. Senescent cells accumulate with age, secrete inflammatory cytokines, and have well-established roles in promoting degenerative diseases and pathology with aging (reviewed in (Campisi et al., 2011)). However, pre-malignant cells lacking a functional p53 pathway bypass senescence and continue dividing. The uncapped ends are processed by the DNA double strand break repair machinery leading to chromosome fusions and rampant chromosomal instability, which kills most cells, but drives malignant transformation in the survivors (Artandi et al., 2000). Most survivors upregulate telomerase to maintain telomeres for unlimited proliferation, but about 15% use the alternative lengthening of telomeres (ALT) pathway (reviewed in (Opresko and Shay, 2017)). Given the critical roles of telomeres in cancer and aging, the rate at which telomeres shorten can profoundly influence genome stability, as well as the overall health and well being of an organism (i.e. healthspan).

Are telomeres "rusting" as we age? While numerous genetic and environmental factors are associated with accelerated telomere shortening, the most commonly cited underlying mechanism is oxidative stress. Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and cellular antioxidant defenses, and contributes to the pathogenesis of numerous human diseases including cancer. These include neurological, pulmonary, and cardiovascular diseases, along with atherosclerosis, diabetes, obesity and arthritis (reviewed in (Hegde et al., 2012; Lonkar and Dedon, 2011; Malinin et al., 2011; Mao et al., 2017; Reuter et al., 2010)) (Fig. 1). For most of these diseases the primary source of ROS is inflammation and/ or mitochondrial dysfunction, since ROS is generated by immune cells

Examples of reactive oxygen species:
$^{\bullet}OH$, $^{\bullet}O_2^-$, H_2O_2, $^1O_2$

Cancer   Diabetes

Autoimmune   Arthritis

Oxidative Stress
Chronic Inflammation
Telomere Shortening

Lung disease
Asthma
COPD, IPF

Cardiovascular
Atherosclerosis

Ulcerative colitis
Barrett's esophagus
hepatitis

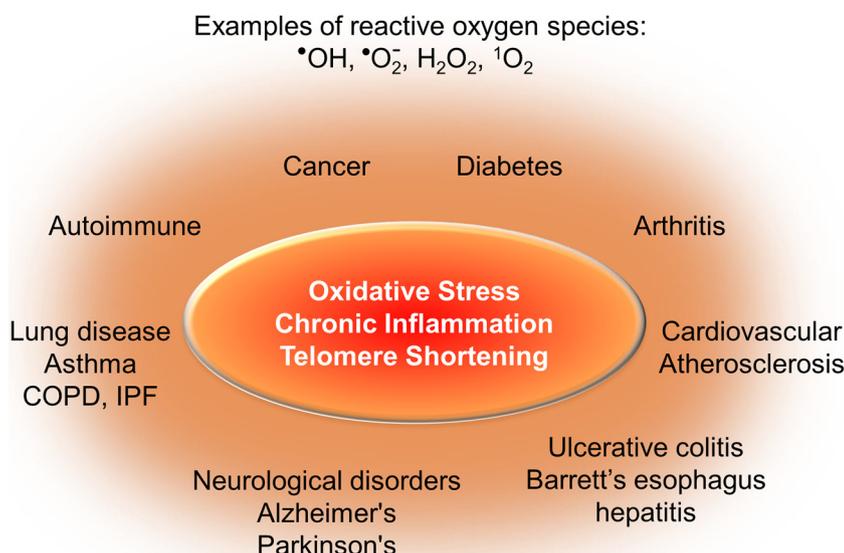Neurological disorders
Alzheimer's
Parkinson's

Fig. 1. Diseases associated with oxidative stress, chronic inflammation and shortened telomeres. The schematic summarizes various diseases that are characterized by elevated reactive oxygen species as well as shortened average telomere lengths as measured either in white blood cells or in the affected/inflamed tissues, compared to unaffected individuals or tissues. See text for details and citations.

in response to infection or injury and from oxygen metabolism. ROS is elevated at sites of chronic inflammation, and is prevalent in chronic inflammatory diseases such as ulcerative colitis, Barrett's esophagus and hepatitis (Lonkar and Dedon, 2011). Chronic inflammation is proposed to cause more than 20% of cancers based on epidemiology studies, and is a major risk factor for aging-related diseases (Bouvard et al., 2009; Lonkar and Dedon, 2011). Oxidative stress is also associated with numerous environmental exposures including radon, cigarette smoke, pollution, toxic metals, ultraviolet light and pesticides (Aseervatham et al., 2013; Baltazar et al., 2014; Poljsak and Fink, 2014). Here again, inflammation and mitochondrial dysfunction are often proposed to be responsible for the elevated ROS. Numerous studies have shown that most of the degenerative and inflammatory diseases, and many cancers, characterized by oxidative stress are also associated with accelerated telomere shortening (Sanders et al., 2011; Valdes et al., 2005; Zhang et al., 2016) (Fig. 1). These studies are based on measurement of average telomere length in white blood cells (WBC), inflamed tissues and tumors from patients (see below). In addition, many of the environmental exposures that lead to ROS elevation, are also associated with shortened telomeres in epidemiology studies based on WBC analysis (Martens and Nawrot, 2016; Valdes et al., 2005; Zhang et al., 2013). In this review we will discuss the evidence from studies in human tissues, animal models and cell culture that oxidative stress accelerates telomere shortening and dysfunction. We will then elaborate on possible mechanisms, and will focus primarily the processing of oxidative damage to DNA bases in telomeres.

2. Oxidative stress and accelerated telomere shortening or dysfunction

2.1. Evidence from human studies

A recent review documented that 6 out of 8 human population studies reported markers for oxidative stress correlated with shorter average telomere lengths, as measured in WBCs (Reichert and Stier, 2017). Similar correlations were observed in populations with higher perceived psychological stress and higher inflammatory loads (Epel et al., 2004; O'Donovan et al., 2011). While interesting, it is difficult to derive mechanistic information from these types of correlative studies. More convincing evidence for direct roles of oxidative stress in telomere length homeostasis comes from studies of inflamed tissues. Chronic inflammatory diseases are characterized by elevated ROS and development of pre-neoplastic lesions that can progress to cancer. Ulcerative colitis (UC) and Barrett's esophagus patients present with shortened

telomeres in affected mucosa compared to normal stroma and controls from unaffected individuals, as revealed by telomere quantitative fluorescence *in situ* hybridization (qFISH) or high resolution Southern blot (Finley et al., 2006; Letsolo et al., 2017; O'Sullivan et al., 2002). Shorter telomeres correlate with greater chromosomal instability and elevated infiltrating leukocytes (i.e. inflammation) (Baird et al., 2003; Risques et al., 2011). Shorter telomeres are observed in inflamed livers from chronic hepatitis and liver cirrhosis, and in atherosclerotic lesions, compared to unaffected tissues (Aikata et al., 2000; Nzietchueng et al., 2011; Rey et al., 2017). Hyper-proliferation of cells can contribute to telomere shortening. However, in a recent review Graham and Meeker argue that at normal telomere shortening rates, prostate cells would require ~100 years of proliferation to achieve the critical telomere lengths observed in prostate cancer (Graham and Meeker, 2017). They propose oxidative stress in prostatic inflammatory atrophy and precancerous prostatic intraepithelial neoplasia (PIN) cooperates with increased proliferation to accelerate telomere shortening.

Finally, critically short telomeres have a well-recognized role in both sporadic and familial pulmonary fibrosis. Familial forms are caused by mutations in telomere maintenance genes (Armanios and Blackburn, 2012). Mechanisms of telomere shortening in sporadic disease are not understood, but may relate to oxidative stress (Kliment and Oury, 2010). Shortened telomeres are observed in alveolar epithelium from sporadic IPF lungs compared to controls, and in alveolar type 2 cells from fibrotic areas compared to non-fibrotic areas (Alder et al., 2008; Snetselaar et al., 2017). In addition, pulmonary vascular endothelial cells from patients with chronic obstructive pulmonary disease (COPD) showed shorter telomeres compared to normal controls, and telomere length correlated with inflammatory loads (Amsellem et al., 2011). Numerous examples of telomere length analysis in chronic inflammatory diseases consistently reveal shortened telomeres in inflamed tissues, compared to normal tissue from the same patient or from unaffected individuals. More mechanistic studies are required to determine how inflammation and oxidative stress accelerate telomere shortening.

2.2. Evidence from mouse models

Studying factors that influence telomere length in mice is challenging because laboratory inbred mice have very long telomeres (~20–40 kb). To overcome this barrier, studies have used late generation telomerase deficient mice bred to harbor shorter telomeres, or the wild-derived CAST/Ei strain which possess telomere lengths similar to humans. Chronically exposing CAST/Ei mice to L-buthione
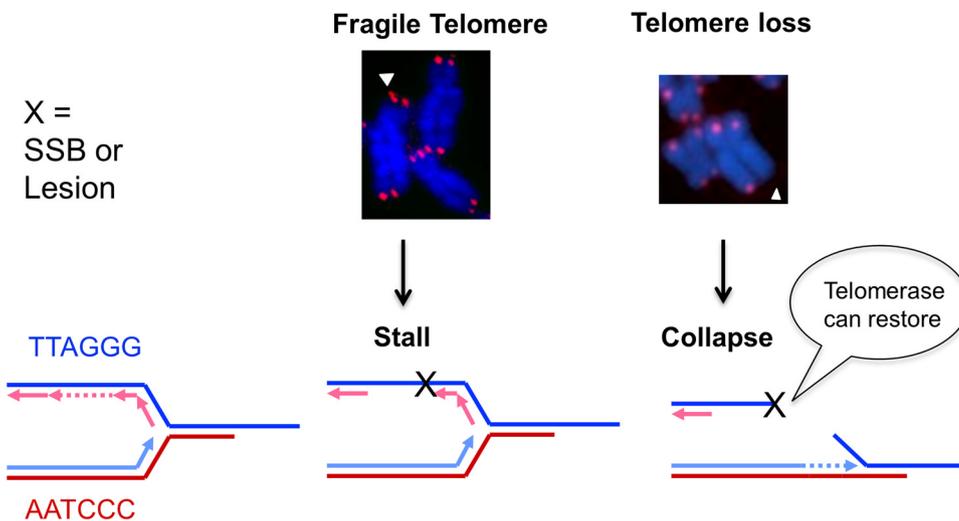
Fig. 2. Consequences of replication fork stalling and blocks at telomeres. The schematic shows a model for how telomere fragility or telomere losses arise from DNA lesions that stall or block replication fork progression, respectively. DNA replication fork encounters with single strand breaks (SSBs) can cause the fork to collapse into a double strand break. Fragile telomeres manifest as multi-telomeric foci at a chromatid end, and are proposed to result from uncondensed regions arising from accumulated unreplicated ssDNA. Telomeres losses manifest as chromatid ends lacking sufficient telomeric DNA for detection with a telomeric probe. Telomerase can suppress telomere losses by extending a pre-maturely truncated telomere.

sufoximine (BSO), which depletes glutathione antioxidant, accelerated telomere shortening in fat, skin, tail, and testis, and increased the level of oxidized proteins (Cattan et al., 2008). Other tissues, including lung, heart and liver, did not show BSO-induced changes in average telomere length. The authors suggested some tissues may have higher antioxidant capacities. Alternatively, average telomere length measurement by Southern blot may lack the sensitivity required to detect telomere changes. The few critically short telomeres that are sufficient to trigger senescence (Kaul et al., 2012) may not alter average telomere lengths. A recent study provides evidence that chronic inflammation in mice leads to telomere dysfunction and premature aging. Jurk and colleagues examined *Nfkb1* knock out mice that lack NF-κB proteins p105 and p50, which regulate inflammatory gene expression (Jurk et al., 2014). These mice showed inflammatory and premature aging phenotypes, as well as increased cell senescence and telomere dysfunction, that were rescued with anti-inflammatory and antioxidant treatments (Jurk et al., 2014). Telomere dysfunction was marked by localization of the DNA damage response protein 53BP1 to telomeres, while changes in average telomere length were not observed. Lack of detectable length changes may be due to the very long telomeres in this inbred mouse strain.

Studies in telomerase deficient mice ($Tert^{-/-}$ or $Terc^{-/-}$) that are bred for shortened telomeres have revealed interesting links between telomere dysfunction and mitochondrial dysfunction in highly proliferative organs as well as more quiescent organs. Analysis of hematopoietic cells, heart, and liver in these mice showed hallmarks of mitochondria dysfunction including decreased mitochondrial biogenesis, reduced oxidative phosphorylation and ATP generation, and increased ROS (Sahin et al., 2011). The mitochondrial effects were mediated by suppression of transcriptional co-activators PGC-1α/β through telomere dysfunction-induced activation of p53 (Sahin et al., 2011). Importantly, because dysfunctional mitochondria generate more ROS, this sets up a vicious cycle in which telomeres may suffer further insult from oxidative damage. Recent studies confirm that the cross-talk between telomere dysfunction and mitochondrial dysfunction impacts the heart. Duchenne muscular dystrophy (DMD) can be recapitulated in transgenic mice lacking both dystrophin and telomerase that are bred to possess shortened telomeres, resembling human telomere lengths (Chang et al., 2016). Loss of dystrophin alone is insufficient to induce DMD symptoms in mice with long telomeres, indicating that "humanized" telomere lengths are essential for fully recapitulating DMD. Telomere shortening occurred in the non-proliferating cardiomyocytes, and critically short telomeres activated p53-mediated repression of PGC-1α/β, triggering mitochondrial dysfunction, which was suppressed with a mitochondrial anti-oxidant (Chang et al., 2016). Whether protecting the mitochondria also prevents telomere shortening in this model has yet to be examined. More work is required to determine how oxidative stress promotes telomere shortening in the absence of proliferation, and whether this effect extends to other non-proliferating cells such as neurons.

2.3. Evidence from cell culture

Numerous studies have reported that oxidative stress and exposure to ROS-generating agents accelerate telomere shortening in cultured human cells. A review of 22 studies consistently showed that mild oxidative stress accelerated telomere shortening in cultured normal human fibroblasts and endothelial cells, whereas antioxidants and free radical scavengers decreased shortening rates and increased proliferative lifespan (von Zglinicki, 2002). In these studies oxidative stress was achieved by culturing cells at 20% O_2 or by exposing to oxidants such as hydrogen peroxide. Subsequent studies confirmed that culturing cells at 20% O_2 accelerates telomere shortening, compared to physiological 3–5% O_2 (Coluzzi et al., 2014; Forsyth et al., 2003; Richter and von Zglinicki et al., 2007; Wang et al., 2010). Other studies in mesenchymal stem cells showed oxidative stress induces stochastic telomere losses, indicated by an increase in critically short telomeres (Harbo et al., 2012). Just five critically short telomeres can trigger senescence in normal cells (Kaul et al., 2012). Finally, oxidative stress induced by mitochondrial dysfunction in cultured cells also accelerates telomere shortening and/or causes telomere dysfunction, even in cells expressing telomerase (Ahmed et al., 2008; Passos et al., 2007; Saretzki et al., 2003). These studies further support the link between mitochondrial and telomere dysfunction.

3. Mechanisms of oxidative stress induced telomere changes

Several mechanistic models have been proposed to explain how oxidative stress accelerates telomere shortening. One possibility is that oxidative stress triggers cell death and/or senescence, and to compensate the survivors undergo more cell divisions, leading to increased telomere shortening. However, this does not explain accelerated telomere shortening observed under non-cytotoxic and mild oxidative stress conditions (von Zglinicki, 2002). One widely cited model suggests that ROS induces single strand breaks (SSB)s at telomeres directly, or as intermediates in lesion repair, leading to replication fork collapse and telomere loss (Fig. 2) (von Zglinicki, 2002). Alternatively, lesions that impede telomere replication can cause an accumulation of unreplicated ssDNA, and manifest as multi-telomeric foci at chromatid ends termed fragile telomeres (Fig. 2) (Sfeir et al., 2009). Replication interference does not explain how oxidative stress affects telomeres in non-proliferative cells. Other possibilities are that oxidative lesions interfere with shelterin binding or transcription at telomeres into TERRA
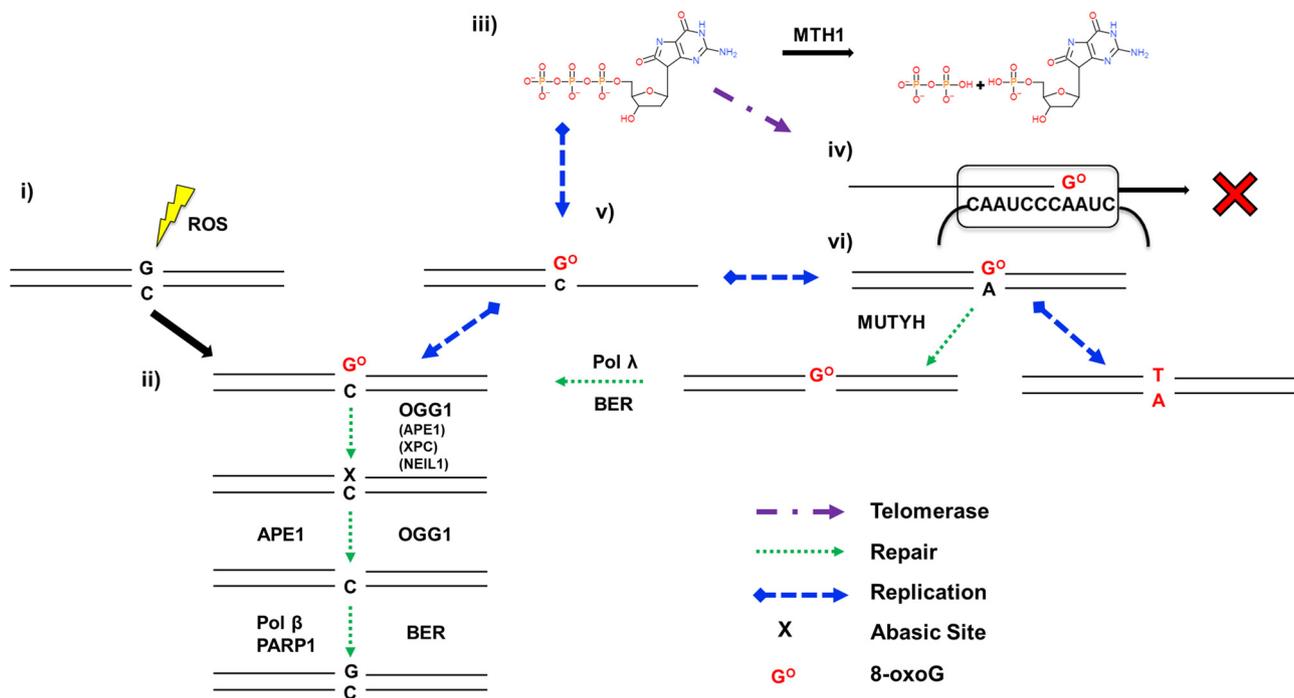
Fig. 3. Processing of 8-oxoG. When 8-oxoG forms opposite C by direct oxidation (i), it is recognized by OGG1 (ii) which removes the modified base. OGG1 or APE1 then processes the abasic site to make it a suitable substrate for the remaining steps of BER. When dGTP is oxidized to 8-oxodGTP (iii), and escapes hydrolysis by MTH1, incorporation by telomerase results in termination of telomere synthesis (iv). If a DNA polymerase incorporates 8-oxodGTP opposite C and it escapes BER, a subsequent round of replication can result in incorporation of A opposite 8-oxoG (vi). MUTYH is able to excise the A, producing a gap filled in by Pol λ or Pol β, avoiding a G:T/ C:A transversion mutation. Proteins in parentheses can stimulate OGG1 activity.

transcripts. Finally, processing of oxidative lesions may lead to changes in telomere repeat number. These possibilities are explored in more detail below, along with evidence from studies that support these models.

3.1. ROS-induced damage to telomeric DNA

Is oxidative damage to telomeric DNA responsible for accelerated telomere shortening under oxidative stress? When various ROS react with DNA they can generate upwards of 100 different types of oxidatively damaged bases (Cadet and Wagner, 2013). These lesions include damaged pyrimidines and purines, as well as SSBs and abasic sites. Guanine is the most susceptible of the natural bases to oxidation, commonly generating 8-oxoguanine (8-oxoG), which is even more sensitive to oxidation, ultimately giving rise to hydantoin lesions (Fleming and Burrows, 2017; Luo et al., 2001). Biochemical studies show TTAGGG repeats are preferred sites for iron binding and iron mediated Fenton reactions, which generate hydroxyl radicals that induce cleavage 5′ of GGG (Henle et al., 1999; Oikawa and Kawanishi, 1999; Oikawa et al., 2001). Consistent with this, several studies reported more SSBs or 8-oxoG lesions in telomeres compared to microsatellite repeats and bulk genomic DNA, after cellular exposures to oxidizing agents (Coluzzi et al., 2014; Petersen et al., 1998; Rhee et al., 2011; Wang et al., 2010). Antioxidant peroxiredoxin 1 (PRDX1), which scavenges H_2O_2, is enriched at telomeres and PRDX1 loss leads to preferential damage at the telomeres (Aeby et al., 2016). Whether the preferential accumulation of oxidative damage at telomeres is due to increased damage susceptibility and/ or decreased repair is unresolved. Indeed, 8-oxoG cannot be repaired in the context of folded telomeric G-quadruplex structures (Zhou et al., 2013). Cellular studies suggest shelterin protein TRF2 may interfere with base excision repair (BER) (Richter et al., 2007), whereas biochemical studies indicate shelterin proteins enhance BER *in vitro* (Miller et al., 2012). More work is required to determine how efficiently oxidative base damage is repaired

at telomeres, compared to elsewhere in the genome.

3.2. Base excision repair of oxidative base damage

Most oxidative lesions are repaired by BER, which is essential for genome stability and for preserving telomeres. These lesions can be cytotoxic or mutagenic, and thereby promote carcinogenesis (for review see (Wallace et al., 2012)). In BER, a mono-functional DNA glycosylase recognizes and removes specific DNA lesions, thereby generating an abasic site. APE1 endonuclease cleaves 5′ of the abasic site to generate an SSB with a 3′hydroxyl and a 5′ sugar phosphate. DNA polymerase (Pol) β removes the 5′ sugar phosphate via its lyase activity, and fills the gap by templated DNA synthesis. DNA ligase I or III seals the nick (Srivastava et al., 1998). While these core steps are conserved, several variations in BER (sub-pathways) exist, assisted by additional proteins. In mammalian cells the five glycosylases that recognize oxidative base damage include OGG1, NTHL1, NEIL1, NEIL2 and NEIL3 (Krokan and Bjoras, 2013; Wallace, 2013). They are bi-functional glycosylases, meaning that they both remove the damaged base and cleave the DNA backbone 3′ to the abasic site. The resulting 3′ blocking sugar or phosphate group is removed by APE1 or polynucleotide kinase, respectively (Krokan and Bjoras, 2013; Wallace, 2013). BER normally involves the replacement of a single nucleotide, but if the 5′end is refractory to Pol β processing, then Pol δ or ε adds several nucleotides, generating a displaced DNA flap that is cleaved by FEN1 endonuclease. BER intermediates can be cytotoxic and therefore, efficient hand-off to each downstream processing enzyme is essential (Sobol et al., 2000). XRCC1 scaffolding protein and modifying enzyme Poly(ADP)-Ribose Polymerase 1 (PARP1) have important roles in coordinating and recruiting proteins involved in SSB repair and BER.

3.3. Processing of 8-oxoG at telomeres

Given that 8-oxoG is one of the most common oxidative lesions,

multiple pathways exist to deal with this form of damage. When 8-oxoG forms opposite C it is recognized by OGG1 glycosylase, which removes the lesion, generating an abasic site (Fig. 3ii). Biochemical studies show OGG1 glycosylase activity is stimulated by APE1, XPC, and NEIL1 (Hill et al., 2001; Mokkapati et al., 2004; Vidal et al., 2001). OGG1 can further process this site with its AP-lyase function, however OGG1 has high affinity for abasic sites, and becomes trapped by its own product (Hill et al., 2001; Morland et al., 2005). More likely, APE1 endonuclease removes the deoxyribose moiety, generating an SSB. However, OGG1 cannot remove 8-oxoG when present in ssDNA or a folded G-quadruplex structure (Zhou et al., 2013). This raises the question of how 8-oxoG is repaired in the ssDNA regions of the telomeric overhang or the t-loop/D-loop structure. Remarkably, an unbiased screen in yeast for genes that alter telomere length revealed that *ogg1* deletion strains had longer telomeres than wild type (Askree et al., 2004). An independent study confirmed this result and reported that the lengthening was partly telomerase dependent (Lu and Liu, 2010). The longer telomere phenotype is recapitulated in $Ogg1^{-/-}$ mice *in vivo*, but when the cells from these mice are cultured in pro-oxidant conditions *in vitro* they exhibit increased telomere shortening, losses and aberrations compared to wild type (Wang et al., 2010). This may represent a hormesis situation, in which low levels of 8-oxoG at telomeres promotes lengthening, whereas high amounts cause telomere losses and aberrations. Mechanistically, we showed that a single 8-oxoG in telomeric ssDNA disrupts the folded G-quadruplex structures that impede telomerase loading, thereby promoting telomere elongation (Fouquerel et al., 2016a). In contrast, how oxidative stress synergizes with unrepaired 8-oxoG lesions to induce telomere losses in $Ogg1^{-/-}$ cells is unclear, since this lesion is not a strong block to DNA replication or transcription. The increased 8-oxoG at telomeres may lead to shelterin disruption (Lu and Liu, 2010; Opresko et al., 2005) or synergize with other ROS-induced lesions (i.e. SSBs, oxidized pyrimidines) to disrupt telomere processing and/or function.

If 8-oxoG in the DNA escapes repair, a round of DNA replication can result in misincorporation of A opposite 8-oxoG (Fig. 3vi). This is because 8-oxoG miscodes for A. Changes in the telomeric sequence would disrupt shelterin binding. MUTYH glycosylase excises A opposite 8-oxoG, producing a gap to be filled in by Pol λ or Pol β in BER (Maga et al., 2007; Markkanen, 2017). If this error is not corrected, such mispairing would convert TTA(8-oxoG)GG repeats to TTATGG variants,

which are not commonly observed (Lee et al., 2014) (Fig. 4B). This suggests that either 8-oxoG is efficiently repaired at telomeres prior to replication, or that MUTYH efficiently removes A opposite 8-oxoG at telomeres to prevent mutations. Sequencing telomeres from oxidative stress conditions in OGG1 and MUTYH singly and doubly deficient cells, is needed to fully elucidate the mutagenic potential of 8-oxoG at telomeres, and the roles for these glycosylases in preserving telomeric repeats.

Free dNTPs are even more susceptible to oxidative damage than bases in duplex DNA and chromatin. As such, cells possess sanitases including MTH1, which hydrolyze oxidized dNTPs before they can be incorporated into the genome by DNA polymerases during replication (Fig. 3) (Rudd et al., 2016). Oxidized dNTPs may be even more deleterious to telomeres than oxidative base damage within the duplex DNA. First, the preferential insertion of 8-oxodGTP opposite A during replication of the 5–15 kb telomere duplex would convert TTAGGG repeats to GTAGGG and TGAGGG repeats (Fig. 4A). Interestingly, these are the most common variant repeats reported from telomere sequencing studies (Lee et al., 2014). Second, telomerase can incorporate 8-oxodGTP during telomere extension, and like most DNA polymerases, it preferentially misincorporates the oxidized dGTP opposite rA (Aeby et al., 2016; Fouquerel et al., 2016a). Lee et al. reported that GTAGGG variants occur more frequently in distal telomere locations in telomerase positive cells, suggesting they are generated by telomerase errors (Lee et al., 2014) (Fig. 4C). However, 8-oxodGTP is a telomerase chain terminator *in vitro* (Aeby et al., 2016; Fouquerel et al., 2016a) (Fig. 3iv). Consistent with this, acute MTH1 depletion increases telomere losses and cell death in telomerase positive cancer cells harboring critically short telomeres, but not in cancer cell with longer telomere reserves (Fouquerel et al., 2016a). This suggests that 8-oxodGTP, and potentially oxidized versions of dATP, which are normally removed by MTH1, inhibit telomerase restoration of critically short telomeres. Some cancer cell lines are more sensitive to MTH1 inhibition, compared to normal cells, which may be due to differences in the reliance on telomerase activity for short-term survival (Gad et al., 2014). Whether MTH1 inhibition leads to mutagenesis at telomeres remains to be determined. Although, 8-oxodGTP is a telomerase chain terminator, factors such as POT1-TPP1, may assist telomerase extension following misincorporation of damaged dNTPs during telomere extension in cells. Specialized DNA polymerases promote DNA synthesis from damaged
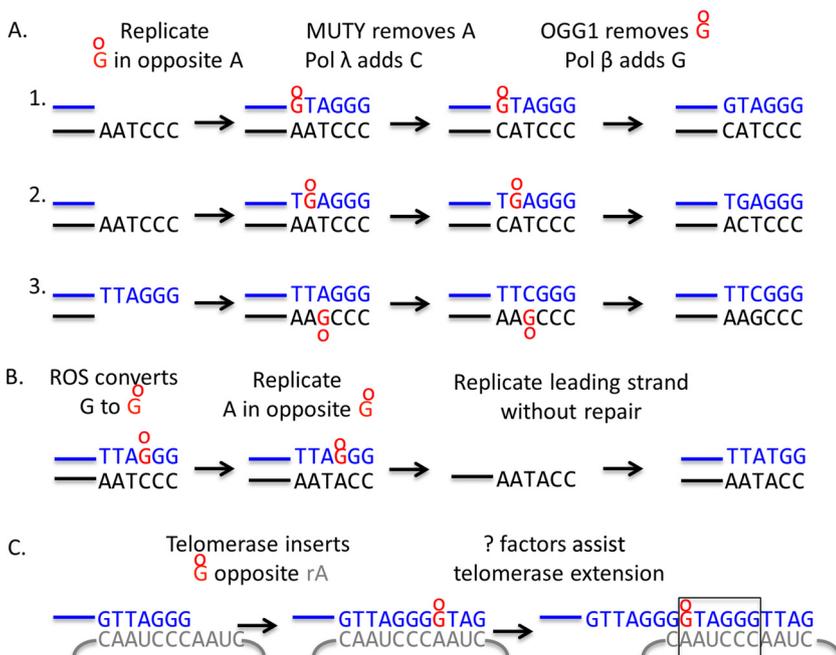

Fig. 4. Models for 8-oxoG induced mutations at telomeres. A) The schematic shows three possible scenarios of 8-oxodGTP insertion opposite A during telomere replication and the resulting change in telomeric repeat sequence. B) If 8-oxoG forms directly in telomeric repeats, the misincorporation of dATP during telomere replication would alter the telomeric repeat to TTATGG, TTAGTG or TTAGGT depending on the lesion position. C) The variant GTAGGG repeat can arise if telomerase misincorporates 8-oxodGTP opposite rA during telomeric DNA synthesis, and the mispair is extended.

bases to enable continued replication (Sale et al., 2012). Whether a similar mechanism exists to assist telomerase extension from a terminal 8-oxoG remains unknown.

3.4. Processing of other oxidized purines

While 8-oxoG is primarily mutagenic, other oxidized purines are more cytotoxic due to their ability to block DNA replication and transcription. Since 8-oxoG has a lower redox potential than the normal unmodified bases, it can be further oxidized to other lesions including spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) (Luo et al., 2001). These distorting hydantoin lesions impede DNA replication and transcription (Henderson et al., 2003; Kolbanovskiy et al., 2017), but can be removed by any of the three NEIL glycosylases (reviewed in (Wallace, 2013)). Interestingly, NEIL1 and mNeil3 remove Sp and Gh from telomeric G-quadruplex structures and ssDNA, whereas 8-oxoG cannot be removed (Zhou et al., 2013). How BER proceeds in the absence of a templating base is unclear, but the processing of lesions in telomeric ssDNA and G-quadruplexes has important implications for telomere integrity. Cellular studies show NEIL3 localizes to telomeres during the S/G2 cell cycle phase, and NEIL3 depletion increases telomere loss, fusions and aberrations, and consequent anaphase DNA bridges (Zhou et al., 2017). The offending lesion(s) responsible for telomere loss is difficult to define because NEIL glycosylases remove several lesion types including ring opened 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and oxidized pyrimidines (see below). Interestingly, both $Ogg1^{-/-}$ mice and $Neil2^{-/-}$ mice show increased susceptibility to LPS and oxidative stress induced inflammation (Aguilera-Aguirre et al., 2014; Chakraborty et al., 2015). Culturing cells derived from $Neil2^{-/-}$ mice at 20% O_2 increases telomere losses compared to controls, similar to $Ogg1^{-/-}$ cells (see above), as observed by telomere FISH (Chakraborty et al., 2015). Since NEIL2 is implicated in BER during transcription, unrepaired hydantoin lesions may interfere with generation of telomeric TERRA transcripts. Roles for NEIL1 at telomeres have not been examined. However, $Neil1$, $Neil2$, and $Neil3$ triple knock out mice did not exhibit telomere shortening as measure by qPCR (Rolseth et al., 2017). Analyses using more sensitive measurements such as qFISH could be more revealing. Collectively, these studies suggest that NEIL glycoslyases have important roles in protecting telomeres against ROS-induced base damage, particularly to preserve DNA replication and transcription at telomeres.

3.5. Processing of oxidized pyrimidines

Pyrimidine bases are also susceptible to damage by free radicals, giving rise to various lesions including thymine glycol (Tg), 5-hydroxycytosine, and 5-hydroxyuracil. Tg is the most common oxidized thymine lesion and is cytotoxic because it can block DNA replication (McNulty et al., 1998). These modified bases are removed either by NTHL1 glycosylase in duplex DNA, or by NEIL glycosylases in duplex or ssDNA (reviewed in (Krokan and Bjoras, 2013; Wallace, 2013)). However, only Neil3 can excise Tg from a telomeric G-quadruplex and shows a strong preference for excising Tg from telomeric versus non-telomeric duplex DNA (Zhou et al., 2013). Therefore, unrepaired Tg lesions may be partly responsible for the telomere defects observed in Neil3 and/or Neil2 deficient cells (Chakraborty et al., 2015; Zhou et al., 2017). $Nth1^{-/-}$ mice exhibit increased fragile telomeres *in vivo*, and cells derived from these mice show increased telomere shortening, losses, dysfunction and aberrations when cultured at 20% oxygen, compared to wild type controls (Vallabhaneni et al., 2013), similar to $Ogg1^{-/-}$ cells (see above). Furthermore, telomerase deficiency exacerbates the telomere shortening and telomere loss phenotypes in $Nth1^{-/-}$ cells, suggesting telomerase is able to partly restore telomeres rendered critically short by replication failures (Vallabhaneni et al., 2013) (Fig. 2). Telomeres from $Nth1^{-/-}$ mice show a higher frequency of oxidized pyrimidines. Biochemical and biophysical studies indicate that

unlike 8-oxoG, the presence of a single Tg only slightly disrupts telomeric G-quadruplexes (Lee et al., 2017). However, Tg alters the structural conformations and dynamics of the telomeric G-quadruplex in a manner that favors telomerase binding (Lee et al., 2017). These studies reveal that Tg likely causes telomere defects through interference with telomere replication rather than telomerase inhibition.

4. Roles for other repair proteins in processing oxidative base damage

While the members of the BER pathway are well known, studies show proteins from other repair pathways also promote the repair of oxidative lesions. For example, Nek7 kinase is recruited to telomeres after the localized induction of superoxide anion, and stabilizes shelterin TRF1 (Tan et al., 2017). Xeroderma Pigmentosum (XP) complementation group C protein, which is an integral component of nucleotide excision repair (NER), stimulates the activity of both OGG1 and thymine DNA glycosylase *in vitro* (D'Errico et al., 2006; Shimizu et al., 2003; Shimizu et al., 2010). Additionally, XPG, a structure-specific endonuclease, and CSB (Cockayne Syndrome Type B), stimulate the activity of NTH1 and NEIL2 glycosylases respectively (Aamann et al., 2014; Klungland et al., 1999). Treatment of cell lines deficient in XPC, XPB, XPD, and CSB with oxidants demonstrated an oxidized purine repair defect, and/or increased cell death and mutagenesis compared with normal cells (D'Errico et al., 2006; Gopalakrishnan et al., 2010; Melis et al., 2013; Tuo et al., 2003). Similarly, several NER proteins have been shown to protect telomeres against oxidative DNA damage. $Xpc^{-/-}$ mouse fibroblasts grown at 20% O_2 display a fragile telomere phenotype, that can be rescued at 3% O_2 (Stout and Blasco, 2013). Human cells deficient in XPB or XPD show elevated levels of chromosome ends lacking telomeric DNA following H_2O_2 treatment (Gopalakrishnan et al., 2010). Interestingly, multiple studies reported that XPA does not affect general or telomere specific oxidative repair (D'Errico et al., 2006; Gopalakrishnan et al., 2010; Klungland et al., 1999). Whether these proteins protect telomeres by stimulating BER, or by removing oxidative lesions through NER, remains to be determined.

Mismatch repair also contributes to oxidative damage repair in human cells. Loss of mismatch repair proteins MSH2 or MLH1 elevates both the basal and H_2O_2 treated levels of 8-oxoG in mouse and human cell lines (Colussi et al., 2002). This effect was reduced when MTH1 was overexpressed, indicating MMR processes 8-oxoG misincorporated during replication or repair events. Interestingly, the MMR complex MutSα recognizes 8-oxoG opposite A or C very poorly, but has strong affinity for 8-oxoG opposite T or G (Larson et al., 2003; Mazurek et al., 2002). While these mispairings are unlikely, 8-oxoG opposite C is the preferred substrate of OGG1, and A opposite 8-oxoG is the preferred substrate of MUTYH. Therefore, it makes sense that cells would have evolved a mechanism for dealing with the remaining two 8-oxoG pairings, via MutSα. These studies also raise the possibility that MMR proteins are also important for protecting telomeres from oxidative base damage.

5. Consequences of defects during repair of oxidative lesions

Processing of oxidative DNA damage can be more detrimental than the initial damage, if toxic intermediates arise in the repair pathway that are not properly resolved. PARP1/2 and poly(ADP-ribosyl)ation (PAR) function in BER following base damage conversion to an SSB (Fig. 3). However, roles in preserving telomeres under oxidative stress remains controversial. Two studies reported extensive telomere shortening, as well as telomere losses and chromosomes fusions in cells derived from $PARP1^{-/-}$ mice (d'Adda di Fagagna et al., 1999; Tong et al., 2001). Pharmacologic or genetic inhibition of PARP1 in HeLa and hamster cells, presumably cultured at 20% O_2, corroborate these observations (Beneke et al., 2008). Similarly cultured $PARP2^{-/-}$ mouse cells show an increased frequency of telomere losses (Dantzer et al.,

2004). Interestingly, telomerase activity was not altered in $PARP1^{-/-}$ or $PARP2^{-/-}$ cells, or in HeLa cells treated with a PARP1 inhibitor, ruling out modulation of telomerase activity by PARP enzymes in these experimental conditions. In contrast, an independent study reported no telomere shortening or increased chromosome fusions in cells derived from $PARP1^{-/-}$ mice generated by disrupting a different gene exon (Samper et al., 2001). However, these cells displayed a significant increase in chromosome end-to-end fusions after 26 population doublings. This suggests culture conditions, such as oxygen levels that can cause oxidative stress, may be responsible for the increased telomere aberrations in the $PARP1^{-/-}$ cells in which toxic BER intermediates such as SSBs can accumulate. Consistent with this, $PARP1^{-/-}$ cells from the above studies show increased telomere losses, end-to-end fusions and telomere shortening, compared to wild type cells, after exposures to ROS-generating agents including H_2O_2, X-rays or arsenite (Gomez et al., 2006; Poonepalli et al., 2005). Conversely, depletion of poly(ADP-ribose) glycohydrolase (PARG), which is responsible for PAR degradation, protects against irradiation-induced telomere defects (Ame et al., 2009). Finally, PARP1 and PARP2 interact with shelterin TRF2, which may serve to regulate PARP1/2 at damaged telomeres (Dantzer et al., 2004; Gomez et al., 2006). Taken together, these studies reveal that the telomere defects observed in absence of PARP1 or PARP2 most likely arise from aborted BER of base damage or SSBs.

As mentioned above, if the ssDNA intermediates that arise during BER persist or accumulate, they can lead to replication fork collapse and subsequent DSB formation (Ebrahimkhani et al., 2014; Sobol et al., 2000) (Fig. 2). Failures in repair of SSBs leads to numerous neurological disorders (reviewed in (Rulten and Caldecott et al., 2013)). The impact of SSBs or processing of oxidative base damage in telomeres in neuronal cells remains to be examined. The repair of closely spaced damaged bases (i.e. clustered lesions) on opposing strands can also lead to a DSB (Cannan et al., 2014). Importantly, the efficiency and accuracy of DSB repair at the telomeres may be comprised or altered by the presence of shelterin proteins and the highly repetitive nature of the sequence (reviewed in (Doksani and de Lange, 2014)). Finally, the repair of 8-oxoG though OGG1 mediated BER causes the pathogenic CAG expansion in a mouse model of Huntington's disease (Kovtun et al., 2007). This raises the possibility that BER processing of 8-oxoG in telomeric repeats may also lead to changes in telomeric repeat number. Similar to CAG repeats, telomeric TTAGGG repeats are also capable of forming alternate structures (i.e. G-quadruplexes) that could impact repair processing (reviewed in (Fouquerel et al., 2016b)).

6. Perspective

Accumulating evidence indicates that oxidative stress correlates with accelerated telomere shortening and dysfunction in studies from human tissues, mouse models, cell culture and biochemical experiments. As discussed in this review, several mechanisms have been proposed to explain how elevated ROS alters telomere length homeostasis; the most prominent being oxidative DNA damage. However, many questions remain. Given that ROS can damage numerous cellular components, it is difficult to determine whether changes in telomere length and integrity under oxidative stress conditions are due to indirect factors, or direct damage to the telomeres. Tools to selectively induce oxidative base damage at telomeres will be useful for elucidating how the formation and processing of oxidative lesions impacts telomere maintenance, cellular function, as well as organism health and aging. Answering the question of whether telomeres are more susceptible to oxidative base damage in cells requires more accurate tools and methods to measure and quantify various types of oxidative lesions at telomeres. This is particularly challenging due to the highly repetitive telomeric sequence, their location at chromosome ends, and their low abundance. Analytical methods such as HPLC-MS require micrograms of DNA. Telomeres are ~0.025% of the genome, meaning 10,000 microgram s of DNA is required to obtain just 2.5 micrograms of

telomeres. Possible oxidation of DNA during isolation procedures confounds the ability to accurately measure DNA lesions. The ability to measure different lesion types will be important since cytotoxic lesions, mutagenic lesions, single strand breaks, and repair intermediates can lead to different telomere outcomes. The same features that make damage detection in telomeres difficult also make sequencing telomeres extremely challenging. Advances in next generation sequencing tools and protocols should help overcome these barriers, and will be required to determine whether oxidative base damage leads to mutations in telomeric DNA and accumulation of variant repeats. Future studies are also required to address how oxidative base damage, and the processing of damage by repair enzymes, impact non-proliferating cells including neurons. Activation of DNA repair at the telomeres has the potential to dramatically alter telomere lengths even in the absence of replication. Understanding how the formation and processing of oxidative damage alters telomere length homeostasis and integrity will be valuable for developing intervention strategies that protect telomeres in the face of oxidative stress, and promote healthy aging.

Conflict of interest statement

The authors declare no conflict of interest

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