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DNA damage processing at telomeres: The ends justify the means

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Abstract

Telomeres at chromosome ends are nucleoprotein structures consisting of tandem TTAGGG repeats and a complex of proteins termed shelterin. DNA damage and repair at telomeres is uniquely influenced by the ability of telomeric DNA to form alternate structures including loops and G-quadruplexes, coupled with the ability of shelterin proteins to interact with and regulate enzymes in every known DNA repair pathway. The role of shelterin proteins in preventing telomeric ends from being falsely recognized and processed as DNA double strand breaks is well established. Here we focus instead on recent developments in understanding the roles of shelterin proteins and telomeric DNA sequence and structure in processing genuine damage at telomeres induced by endogenous and exogenous DNA damage agents. We will highlight advances in double strand break repair, base excision repair and nucleotide excision repair at telomeres, and will discuss important questions remaining in the field.

Keywords

Telomeres; DNA damage; DNA repair; Double strand break repair; Nucleotide excision repair; Base excision repair; Shelterin proteins; G-quadruplex

1. Introduction

Telomeres are dynamic nucleoprotein-DNA structures that cap and protect linear chromosome ends. They preserve genome stability, survival, and proliferation on a cellular level, and prevent degenerative diseases and cancer on an organism level [1,2]. Dysfunctional telomeres contribute to aging-related pathologies and carcinogenesis [3,4]. In humans telomeres consist of long (10–15 kb) tandem arrays of 5′-TTAGGG-3′ repeats on the lagging strand and 5′-CCCTAA-3′ on the leading strand. The G-rich strand terminates in a single stranded 3′ overhang of about 50–200 nucleotides [5]. This overhang can

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invade the telomeric duplex to form a large lariat-like t-loop [6,7]. Current models suggest t-loop formation resembles the initial steps in homologous recombination (HR), whereby overhang pairing with the duplex displaces a portion of the G-rich strand and forms a D-loop. This structural rearrangement of the chromosome end requires a complex of six proteins collectively termed shelterin (reviewed in Ref. [8]) (Fig. 1). TRF1 and TRF2 form homodimers and bind to TTAGGG repeats in duplex DNA through a Myb domain. POT1 binds to single stranded TTAGGG repeats, and interacts with TPP1 protein, which recruits telomerase to telomeres [9]. TIN2 connects the shelterin proteins through interactions with POT1, TRF1 and TRF2, and RAP1 interacts with TRF2.

Telomeres solve two problems at chromosome ends. The first is the end replication problem due to the inability of the DNA replication machinery to copy the very ends of the lagging strand, which leads to chromosome shortening each time a cell divides. Telomerase expression in germ cells and the majority of cancer cells, compensates for shortening by adding TTAGGG repeats to the telomeric overhang, which is then partly converted to duplex DNA by the lagging strand replication machinery [10]. Telomerase is a reverse transcriptase that uses an integral RNA template to synthesize single stranded TTAGGG repeats. The second is the end protection problem, the study of which has revealed fascinating connections between telomeres and the DNA damage response (DDR) and repair machineries. This refers to the inherent similarity of chromosome ends to DNA double strand breaks (DSBs), which can cause the telomeres to be falsely recognized by cellular DDR and repair proteins. Shelterin loss activates DDR at telomeres and triggers cell senescence or apoptosis, or in the absence of functional p53 leads to chromosome end-to-end fusions by non-homologous end joining (NHEJ) or alternative end joining (A-EJ), or leads to telomere alterations by homologous recombination (HR) [11]. TRF2 suppresses ATM kinase activation and POT1 suppresses ATR kinase activation at telomeres to prevent DDR signaling. Mechanisms for how shelterin interacts with various DSB repair proteins to protects telomeres and prevent inappropriate repair have been extensively reviewed elsewhere [12–14]. However, shelterin regulation of DNA repair enzymes to solve the end protection problem raises interesting questions about how shelterin influences the processing of genuine DNA damage at telomeres, including chromosome breaks and DNA lesions, and is the topic of this perspective. Additionally, we focus our discussion on the most recent developments regarding how telomeric sequence and structure impact the processing of various types of damage at telomeres.

2. Processing of telomeric chromosome breaks

Shelterin prevents the false recognition of telomeres as DSBs by promoting alternate structure (i.e. t-loop), but also by regulating DNA repair enzymes, which could affect the processing of genuine DSBs at telomeres (Fig. 2). Exposure of non-dividing fibroblasts to ionizing radiation induces persistent DDR foci, marked by 53BP1 and phosphorylated histone H2AX (γH2AX), of which 30% co-localized with telomeres [15]. This is impressive considering that telomeres represent less than 0.025% of the genome, and suggests genuine DSBs at telomeres may be refractory to repair, at least in non-cycling cells. Consistent with this, the placement of telomeric repeats adjacent to an inducible DSB site in yeast, or at linear DNA ends in an end-joining assay using human cell extracts, inhibits DSB

repair [15,16]. The inhibitory activity was ascribed to TRF2-RAP1 [16], and was confirmed by tethering a TRF2 fusion protein to an inducible I-*Sce*I chromosomal break in mouse cells [15]. In another study the analysis of I-*Sce*I-induced breaks at a sub-telomeric site in human tumor cells suggested that TRF2 suppresses normal DSB repair by promoting aberrant processing of broken ends, rather than by inhibiting ATM kinase signaling of the DSB [17]. Results from two studies examining how TRF2 prevents end joining at dysfunctional telomeres, provide plausible mechanisms for how TRF2 may inhibit repair of genuine breaks in telomeres. The first study discovered that TRF2 prevents RNF168 protein from recruiting 53BP1 protein; a factor required for NHEJ. Ubiquitin chains recruit RNF168 to damage, and TRF2 is proposed to prevent ubiquitin-dependent signaling by recruiting the de-ubiquitinating enzyme BRCC3 via interaction with the MRE11 complex [18]. The second study found TRF2 binds to a region of Ku70 required for Ku heterotetramerization, and this may prevent Ku-mediated bridging of DNA ends in NHEJ [19]. These studies provide evidence that shelterin interactions with proteins involved in DSB repair, cause chromosomal breaks at telomeres to be processed differently than breaks elsewhere in the genome.

The other major pathway for processing DSBs, homologous recombination (HR), is also differentially regulated at telomeres compared to elsewhere in the genome (Fig. 2). Telomeres are thought to be hyper-recombinogenic due to the presence of a 3′ overhang and highly homologous sequence. Shelterin represses aberrant HR at telomeres that can lead to telomere loss and/or dramatic telomere shortening via unequal sister chromatid exchanges (SCE) (reviewed in Ref. [14]). TRF2 binds SLX4, and prevents telomere loss by preventing endonucleases SLX1-SLX4, MUS81 and GEN1 from cleaving at the telomeric t-loop, and suppresses SCEs partly by preventing these endonucleases from cleaving HR intermediate structures including D-loops and Holliday Junctions (HJ) [20–23]. SCEs are prevented when HJ structures are disrupted and/or dissolved by helicases, rather than cleaved by endonuclease (reviewed in Ref. [24]). Whether HR can be invoked to recover from genuine chromosome breaks at telomeres, and whether TRF2 influences how the consequent HJ repair structures are processed, are not fully understood.

Evidence suggests that defective HR regulation at telomeres occurs in cells that use the alternative lengthening of telomeres (ALT) pathway to maintain telomere lengths for sustained cell proliferation in about 15% of cancers (reviewed recently in Ref. [25]). Cells that use ALT lengthen telomeres by homology directed DNA synthesis and HR-mediated mechanisms rather than by telomerase, and therefore, studies of ALT have advanced understanding HR repair at telomeres. To investigate the ALT mechanism, the Greenberg lab induced enzymatic breaks specifically at telomeres by expressing TRF1 fused to the FokI nuclease. TRF1-FokI induces γH2AX and 53BP1 foci that co-localize with telomeric DNA, covering a few hundred kilobases, in both ALT positive U2OS cells and HEK293 telomerase positive cells [26–28]. TRF1-FokI cleavage imparts telomere mobility and clustering, and Rad51 filament formation between recombining telomeres in ALT cells, but not in telomerase positive cells [27]. These data provide evidence that enzymatically generated DSBs at telomeres are processed by recombining telomeres in ALT cells. These mechanisms are suppressed in telomerase positive cells where it is uncertain whether or how the enzymatic DSBs are repaired. The number of DSBs induced by TRF1-FokI

at a telomere is also unclear. A similar approach to target DNA damage to telomeres fused the photosensitizer KillerRed to TRF1, which generates damaging superoxide radical upon activation with visible light [29]. KillerRed-TRF1 activation induced γH2AX foci at telomeres in ALT positive U2OS cells that decreased with recovery time [28]. However, γH2AX is not specific for DSBs and also marks stalled replication forks and other defects [30]. While KillerRed-TRF1 activation also induced telomeric 53BP1 foci, recovery was not reported [28]. Importantly, it is difficult to distinguish whether DDR foci at telomeres represent uncapped telomeres or genuine DBSs, further complicating interpretation. The induction and detection of chromosomal breaks at telomeres remains a challenge, and much work is still required to uncover how genuine telomeric DSBs are processed, and how the telomere maintenance status (i.e. absent, ALT, or telomerase) influences the response and potential repair mechanism.

2.1. Processing of replication-induced DSBs and stalled replication forks

DSBs can also arise from DNA replication fork collapse. How shelterin and telomeric sequence influence the processing of defective replication forks at telomeres is an active area of investigation (Fig. 2). Telomeres are highly sensitive to replication stress induced by replication fork slowing or stalling due to obstacles or decreased DNA synthesis (recently reviewed in Refs. [31,32]). Cells under replication stress frequently exhibit increased telomere aberrations that appear on metaphase chromosomes as multi-telomeric signals at a chromatid end (fragile telomere) or a telomere signal-free end (telomere loss) [33]. The terms ‘fragile telomere’ and ‘telomere loss’ throughout this perspective refer to these two telomeric phenotypes. While the precise mechanisms are unknown, fragile telomeres are thought to arise from uncondensed regions at unreplicated ssDNA, and telomere loss is thought to result from fork collapse into DSBs and failed telomere replication [34] (Fig. 3C). Paradoxically, the TRF2-inhibited endonucleases SLX4-SLX1, MUS81 and GEN1 prevent telomere fragility [21,22]. Perhaps their activities are required to process stalled forks, but must be restrained by TRF2. These findings, along with evidence that shelterin regulates HR mechanisms (see above), suggest that replication fork demise at telomeres may be particularly detrimental.

2.2. Fork remodeling and lesion bypass at telomeres

TRF1 and TRF2 preserve telomere replication and prevent telomere fragility by recruiting specialized helicases BLM and RTEL1, respectively [34,35] (Fig. 2). Both helicases are proposed to unwind G-quadruplex (G4) structures on the G-rich strand, and RTEL1 is proposed to unwind t-loops [23,34]. G4 is a secondary structure that can block DNA polymerases on the lagging strand, and thereby uncouple leading and lagging strand replication [36]. Fragile telomeres arise from lagging strand replication in BLM deficient cells [34], suggesting BLM unwinds G4 DNA to preserve replication. However, RTEL1, BLM and other RecQ helicases (i.e. WRN) that are implicated in telomere maintenance function in various pathways of replication fork recovery involving HR and/or fork regression, which enable bypass of obstacles to prevent fork collapse (reviewed in Refs. [24,37]). Replication fork regression and remodeling are known responses to replication stress caused by fork stalling, DNA lesions and secondary structures, and telomeres are highly prone to fork regression [38]. Therefore, helicase roles in fork remodeling may be

critical for processing blocked forks at telomeres, perhaps at G4 structures and/or DNA lesions (Fig. 3). Fork remodeling by RAD51, BRCA1 and BRCA2 proteins prevents lagging strand telomere fragility caused by exposure to ligands that stabilize G4 DNA [39]. These data together with RAD51's role in fork regression [40], support the model that replication fork remodeling is critical for bypassing obstacles during telomere replication.

Telomerase is emerging as an exciting new player in telomere replication stress. The Blackburn lab found in budding yeast that telomerase inactivation prior to critical telomere shortening accelerates aging, which can be prevented by increasing dNTP pool concentrations [41]. This suggests replication stress contributes to the accelerated aging, and the authors propose that telomerase may be recruited to regressed replication forks to lengthen the exposed G-rich single strand (Fig. 3B). Fill in by lagging strand synthesis and fork restoration would enable bypass of lesions or structures at the stalled replication fork. Alternatively, telomerase may restore a truncated telomere caused by fork breakage (Fig. 3C). Two recent studies found that ATM and ATR kinase signaling are required for telomerase recruitment and elongation at telomeres, and at sites of newly seeded telomeres [42,43]. Furthermore, the induction of replication fork stalling by aphidicolin-mediated polymerase inhibition increases telomerase recruitment to telomeres, and induces telomere lengthening [33,43]. These data provide evidence that telomerase is recruited to damaged replication forks, and it will be interesting to determine if this extends to replication blocking lesions such as UV photoproducts. Finally, the human CST complex (CTC1, STN1, and TEN1) regulates telomerase activity by interacting with telomerase partners POT1-TPP1, and promotes lagging strand synthesis of the C-rich strand after telomerase extension of the G-rich strand [44,45]. Interestingly, CST also promotes fork progression through telomeric regions and functions in replication fork restart genome wide following replication stress [46]. CST is thought to recruit polymerase α-primase to prime synthesis to restart replication at dormant origins [46], but may also prime synthesis on remodeled replication forks in cooperation with telomerase (Fig. 3B). Much work is still required to understand how damaged and blocked replication forks are processed at telomeres.

Consistent with the idea that lesion bypass may be particularly important at telomeres, translesion DNA polymerase η is required to prevent telomere loss and telomere fragility induced by cellular exposures to UV irradiation and hexavalent chromium [47]. These agents induce bulky DNA lesions that can impede DNA replication and lead to HR repair of DSBs at broken forks and consequent sister chromatid exchanges (SCE) [48]. Whether lesions can induce HR repair and SCEs at telomeres is unknown, but shelterin has been shown to suppress telomeric SCEs [12,14]. Therefore, mechanisms that prevent fork collapse into DSBs may be preferred mechanisms for processing damaged forks at telomeres. Potential roles for other translesion polymerases in telomere preservation remain to be tested.

3. Bulky lesions at telomeres

3.1. Frequency of bulky lesion formation at telomeres

Bulky lesions are chemical modifications that distort the DNA double helix, impede DNA replication and transcription, and arise through exposure to UV light, environmental

genotoxicants and many anti-cancer drugs (Fig. 2). The abundance of di-pyrimidine sites in 5′-TTAGGG/5′CCCTAA repeats predicts that telomeres are targets for UV light-induced reactions that covalently link adjacent pyrimidines. Cyclobutane pyrimidine dimers (CPD) form more frequently at 5′TTs, while pyrimidine (6–4) pyrimidine photoproducts (6–4 PP) form more frequently at 5′CC sites [49]. However, both lesions can form in 5′TT, CC, or CT sequences indicating that both could arise on either the G-rich and/or C-rich telomeric strands [49]. A few studies have attempted to quantify UV photoproducts at telomeres *in vivo* and *in vitro* [50–52]. UVC (20 J/m^2) exposure of primary human fibroblasts induced more CPDs in telomeric DNA, compared to portions of the p53 gene or 28S ribosomal DNA [51]. 6–4PPs were not reported in this study. Curiously, the CPD frequency was higher on the 5′CCCTAA strand, compared to the 5′TTAGGG strand despite the CPD preference for TTs [51]. In this study CPD-containing denatured and fragmented DNA was immunoprecipitated from UV (20 J/m^2) exposed cells with a CPD-antibody, and damaged DNA was identified by quantitative PCR [51]. In a more recent study, telomere sensitivity was compared to the bulk genome by using a reverse approach in which telomeric DNA was first isolated from UV-exposed (10 J/m^2) telomerized human fibroblasts and then analyzed for CPDs and 6–4PPs by immunospot blotting with antibodies against each lesion. The blots revealed approximately 2-fold fewer CPDs and 6–4 PPs in telomeres compared to the bulk genome [52]. Therefore, telomeres may be more sensitive to UV-induced damage compared to specific genomic loci, but are less sensitive than the genome overall.

Numerous factors beyond telomeric sequence likely influence the formation of UV photoproducts and other bulky lesions at telomeres. *In vitro* UVC (100 J/m^2) exposure of purified DNA followed by immunospot blotting revealed more CPDs in duplexes containing ten TTAGGG repeats [51], but similar amounts of CPDs and 6–4PPs in long duplexes containing 270 TTAGGG repeats [52], compared to non-telomeric controls. Therefore, the number of telomeric repeats may influence damage susceptibility. Furthermore, when purified and irradiated *in vitro*, telomeres are as susceptible to UV-induced CPD and 6–4PP formation as the bulk genome, but telomeres are less sensitive than the bulk genome when irradiated *in vivo* [52]. This suggests the intimate interactions of shelterin with telomeric DNA likely influence lesion formation at telomeres. TRF1 binding to telomeric DNA *in vitro* reduced the amount of UVC-induced CPDs and 6–4PPs compared to naked telomeric DNA [52]. How might shelterin proteins protect telomeres from UV damage? There are several possibilities. First, amino acids in TRF1 and TRF2 specifically interact with the dipyridimineresidues on both strands, which may alter the propensity for dimer formation [53]. Protein binding to DNA may trigger a local change of the DNA conformation rendering the adjacent pyrimidines less susceptible to UV-induced covalent linkage. TRF2 binding induces DNA compaction, and novel Dual-Resonance-frequency-enhanced Electrostatic force microscopy (DREEM) reveals that there are two levels of compaction: (1) by DNA wrapping around individual TRFH domains in TRF2 and (2) by compacting DNA inside large TRF2 complexes resulting in protruding DNA loops [54–56]. How shelterin interacts with telomeric DNA to modulate susceptibility to various types of DNA damaging agents has yet to be fully understood, and requires further investigation.

3.2. Bulky lesion removal at telomeres

Helix distorting lesions are primarily removed by nucleotide excision repair (NER) (Fig. 2). This versatile mechanism involves over 30 distinct proteins that coordinate various steps culminating in the release of a 24–32mer oligonucleotide containing the lesion (reviewed in Ref. [57]). Damage recognition proteins initiate the global genome repair (GGR) and RNA polymerase blockage initiates the transcription coupled repair (TCR) pathways of NER. Helicases XPB and XPD generate a ~30 nucleotide bubble by separatingstrands around the lesions, and XPA and RPA proteins stabilize the bubble. Endonuclease XPF-ERCC1 incises the strand 5′ to the lesion and XPG incises 3′ to lesion. DNA polymerase fills the gap and ligase seals the nick.

Investigation of NER at telomeres has yielded variable results. One study reported a lack of CPD removal from telomeric DNA in UVC irradiated primary human fibroblasts up to 48 h post exposure [51]. In this study damaged DNA was immunoprecipitated with a CPD antibody, and telomeric DNA in the precipitate was measured by qPCR. A more recent study using the reverse approach of isolating telomeres from UVC irradiated telomerized fibroblasts, followed by immunospot blotting showed nearly complete repair of CPDs and 6–4PPs in telomeres by 48 and 6 h, respectively [52]. These results are inline with the first report of photoproduct measurement in telomeres from UVC irradiated primary human fibroblasts [50]. Here CPDs were detected as T4 endonuclease cleavage sites in telomere restriction fragments, which were analyzed by denaturing gel electrophoresis and blotting with telomeric probes. Conflicting results may be due to differences in how the telomere lesions were measured, or perhaps differences in cell lines. It will be important to determine whether telomerase influences repair at telomeres, although repair occurs in both telomerase positive and negative cells [50,52]. UV photoproduct removal from telomeres is abolished in cells lacking NER protein XPA, indicating that NER is active at telomeres [52]. Unlike for base excision repair (see below), there is a lack of biochemical data describing how telomeric sequence or shelterin impacts NER at telomeres. XPF-ERCC1 is recruited to telomeres through interaction with TRF2 and SLX4 proteins, and promotes telomere shortening in cells overexpressing TRF2 [58–60]. However, TRF2 prevents XPF-ERCC1 from cleaving the telomeric 3′ single strand overhang [58]. While shelterin regulation of XPF-ERCC1 is important for telomere preservation, how this interaction impacts NER at telomeres remains unexamined.

What role does NER have in telomere preservation? Previous studies showed that cellular exposures to UVC or Cr(VI) induce an increase in fragile telomeres and telomere loss, that is exacerbated in the absence of translesion polymerase η [47]. This provides evidence that lesion interference with telomere replication causes telomere aberrations. Biochemical studies showed that a CPD lesion within telomeric DNA strongly inhibits TRF1 binding [52]. Given that TRF1 loss causes telomere fragility [33], the disruption of shelterin provides another mechanism by which unrepaired lesions may compromise telomere replication and integrity. Information regarding telomere integrity in NER deficient cells is limited. Chronic UVB exposure of *Xpc*$^{-/-}$ mice induces greater telomeres shortening in shaved skin, compared to wild type [61]. Curiously, cells derived from these mice show increased telomere fragility prior to UV exposure that is partly prevented by decreasing oxidative

stress [61]. This may be due to a potential role for XPC and/or NER in processing endogenous oxidative lesions [62]. XPB and XPD deficient human cells show increased telomere loss and telomeric fusions, compared to wild type, that is exacerbated with hydrogen peroxide [63]. Cyclopurine adducts caused by hydroxyl radical reaction with dA or dG are repaired by NER and have been implicated in neurodegenerative disease (reviewed in Ref. [64]). However, much work remains to determine the importance of NER and removal of bulky lesions in preserving telomere integrity.

3.3. Transcription coupled NER at telomeres

As mentioned above NER is divided into two sub-pathways. GGR removes bulky lesions in the entire genome, while the more rapid TCR pathway removes lesions from the transcribed strand of actively transcribed regions (reviewed in Ref. [57]). XPA deficient cells lack telomeric repair [52], but XPA is required for both GGR and TCR, so whether both pathways act at telomeres remains unknown. The telomeric C-rich strand is transcribed into long non-coding G-rich RNAs called TERRA that initiate in the sub-telomere and function in telomere maintenance (reviewed in Ref. [65]). Therefore, CPDs on the telomeric C-rich strand are good candidates for TCR. This may partly explain the 1.5 fold faster rate of CPD removal in telomeric DNA, compared to the bulk genome, while 6–4PP repair rates are similar in telomeres and the bulk genome [52]. TCR of 6–4PPs is often obscured because they are rapidly removed by GGR [66]. The impact of UV irradiation or DNA lesions on telomere transcription is unknown. TERRA transcription is increased at short and uncapped telomeres, as well as upon treatment with DSB generator zeocin or TRF2 depletion, to promote chromatin modification or recruit telomerase to damaged telomeres [67,68]. Cells lacking CSB, a protein that functions in TCR and transcriptional regulation, show decreased TERRA transcripts even in the absence of exogenous DNA damage [69]. CSB localizes to a subset of telomeres and interacts with TRF2 [69], but whether TRF2 influences CSB's role in recruiting downstream TCR factors to the stalled RNA polymerase is unknown [57]. Finally, CSB deficient cells show increased telomere loss and fragile telomeres, suggesting that CSB and/or TCR preserve telomere replication [69]. CSB may mediate TCR removal of oxidative lesions at telomeres [62,64] or the processing of the R-loops at the stalled RNA polymerase which can block replication [70]. R-loops may be particularly detrimental at telomeres because the displaced DNA strand can form G-quadruplex (Fig. 4A). It will be important to determine whether TCR is active at telomeres, and whether this pathway and TCR factors CSA and CSB defend telomeres against bulky DNA lesions. The processing of bulky lesions at telomeres remains largely under-investigated.

4. Base damage and single strand breaks

4.1. Frequency of base damage at telomeres

Small chemical modifications to the DNA bases can occur by exposure to reactive oxygen species, ionizing radiation or alkylating agents (Fig. 2). Telomeric sequence is clearly more prone to 8-oxoguanine (8-oxoG) formation *in vitro*, partly due to the lower oxidative potential of guanine when present in G runs [71] and preferential binding of Fe^{2+} to telomere repeats which allows for hydroxyl radical production [72]. It is less clear if oxidative lesions arise more frequently at telomeres compared to the bulk genome *in vivo*.

An early attempt to measure lesion frequency in telomeres used S1 nuclease to convert single stranded breaks (SSB)s and gaps (which are also repair intermediates) to DSBs, which could be detected as cleaved fragments on non-denaturing gels by electrophoresis. A higher frequency of H_2O_2 or alkylating agent-induced SSBs or gaps were detected in telomeric DNA, compared to microsatellite or bulk genomic DNA from exposed fibroblasts [73]. A variation of this assay used Fapy DNA glycosylase to convert oxidized guanines to SSBs, and the cleaved telomeric fragments were detected on denaturing gels. This assay was used to confirm an increase in 8-oxoG in telomeres from *Ogg1*$^{-/-}$ repair deficient mice, and revealed that menadione induces similar lesion frequencies in telomeric DNA compared to mini-satellite DNA, but the telomeric lesions persisted after 6 h [74,75]. In constrast, oxidation *in vitro* yielded 50% more lesions in naked telomeric DNA compared to mini-satellite DNA, suggesting shelterin may shield telomeres from damage *in vivo* similar to UV damage [52,75]. Another approach quantitated lesions based on decreased PCR amplification of telomeric DNA or genetic loci harboring Fapy-cleaved lesions, and revealed more lesions in telomeric DNA compared to the 36B4 gene following cellular H_2O_2 exposures [76]. Whether oxidative stress induces more lesions at telomeres compared to the bulk genome remains unclear. The approaches mentioned above to measure lesion frequency are semi-quantitative and indirect. More direct and precise methods for quantitating lesions, such as HPLC or mass spectrometry, are challenging due to the low abundance of telomeric DNA, but will be important for addressing questions regarding the frequencies, types and persistence of base damage at telomeres.

4.2. Base damage influences telomere length homeostasis

Evidence indicates that the processing of base damage at telomeres impacts telomere length. Numerous studies reported that the generation of oxidative stress by various means including chronic inflammation and H_2O_2 is associated with accelerated telomere shortening and/or telomere dysfunction in cell culture and *in vivo* ([77] and reviewed in Ref. [78]). Induction of telomeric SSBs by oxidative stress or alkylating agents correlates with accelerated telomere shortening [71,79], but precisely how SSBs might shorten telomeres remains to be demonstrated. Paradoxically, an unbiased screen in *S. cerevisiae* for genes involved in telomere length homeostasis revealed that *ogg1* deletion strains have longer telomeres than wild type [80]. This was confirmed in an independent study that also found the longer telomere phenotype was dependent on telomerase or HR [81]. How 8-oxoG may promote telomerase or telomeric HR remains unknown. The longer telomere phenotype was recapitulated in *Ogg1*$^{-/-}$ mice *in vivo* and when fibroblasts from these mice were cultured at 3% oxygen, but not when they were cultured at 20% oxygen [74]. Pro-oxidant conditions accelerated telomere shortening in Ogg1 deficient cells compared to wild type [74]. This raises the paradox that persistent 8-oxoG lesions promote telomere lengthening, while general oxidative stress promotes telomere shortening. Future studies are needed to determine whether ROS-induced products other than 8-oxoG, such as other lesion types, SSBs, oxidized nucleotides or proteins, may be responsible for accelerating telomere shortening. Similar to 8-oxoG, persistent uracil residues at telomeres also promote telomere lengthening *in vivo*, while oxidized pyrimidines cause no obvious length change [82,83]. Uracil can arise in DNA from deamination of cytosine to uracil or polymerase misincorporation of dUTP. Defining how the formation and processing of various base

lesions impact telomere length homeostasis will require new technologies for inducing specific forms of damage at telomeres, and techniques for precisely measuring the load of different base modifications at telomeres.

4.3. Base damage removal at telomeres

Processing of base damage at telomeres has been studied by investigating the consequences of depleting base excision repair (BER) proteins on telomere integrity (Fig. 2). BER restores chemically damaged DNA bases and is essential for the repair of SSBs and abasic sites. BER is initiated by various DNA glycosylases that recognize and remove specific damaged bases thereby generating an abasic site that is converted by APE1 endonuclease to an SSB. Polymerase β (Pol β) removes the 5′ dRP residue and fills the single nucleotide gap, and ligase seals the nick. Variations in BER are numerous and include long-patch repair in which the gap is filled by several nucleotides and the displaced 5′ flap is cleaved by FEN1 (reviewed in ref. [84]). PARP1 recognizes the SSB generated after glycosylase and APE1 activities, and facilitates the recruitment of Pol β and the scaffold protein XRCC1 [85–87]. PARP1 also binds TRF2 [88], and shelterin interacts with many other proteins in BER [89].

Studies in glycosylase deficient mice support the importance of removing base damage at telomeres. The loss of most key BER enzymes is embryonic lethal in mice, however, the loss of DNA glycosylases is generally tolerated [90]. Deficiency in removal of 8-oxoG and oxidized pyrimidines in *Ogg1*$^{-/-}$ mice and *Nth1*$^{-/-}$ mice, respectively, did not reveal any obvious telomere dysfunction *in vivo*, which is consistent with the general lack of disease in these mice [74,82,90]. This may be due to some redundancy among glycosylases. While fragile telomeres are elevated in *Nth1*$^{-/-}$ mice *in vivo*, a corresponding health consequence for the mice is not obvious [82]. However, when cells derived from *Ogg1*$^{-/-}$ mice and *Nth1*$^{-/-}$ mice are cultured at 20% O_2, they show an increase in telomere shortening, loss, dysfunction and aberrations, along with alterations in telomeric SCEs compared to wild type [74,83]. These studies suggest that BER is critical for preserving telomeres under elevated oxidative stress conditions. Interestingly, APE1 deficiency also leads to an increase in telomere loss, telomere dysfunction, and telomeric end-to-end fusions in cultured human cells [91]. Assuming these cells were cultured at atmospheric 20% O_2, the abasic sites could have arisen from oxidative base damage that was removed by glycosylases and could not be further processed without APE1. It will be important to determine if the telomeric defects in APE1 depleted cells are modulated by oxidative stress conditions, similar to Ogg1 and Nth1 deficiency. Finally, a recent study reported no significant telomere dysfunction in mice from *Ung*$^{-/-}$ mice lacking uracil DNA glycosylase, also similar to *Ogg1*$^{-/-}$ and *Nth1*$^{-/-}$ mice. Curiously, UNG deficiency increased fragile telomeres and telomeric SCEs in the absence of telomerase; both defects associated with replication stress [83]. Telomerase deficiency also exacerbated the telomeric defects in *Nth1*$^{-/-}$ cells [82]. These findings suggest that telomerase may protect against replicative stress at telomeres induced by unrepaired lesions, and are consistent with new evidence that telomerase acts in recovery from replication stress (see Section 2.2). It will be important to examine telomeres in cells depleted for other BER enzymes and whether any telomeric defects are exacerbated by telomerase deficiency.

Biochemical studies of BER in the context of telomeric DNA structure and/or shelterin have revealed interesting features of damage processing at telomeres, which remain to be fully understood *in vivo*. The reconstitution of BER *in vitro* requires relatively few proteins compared to other DNA repair pathways such as NER, and has allowed for detailed analysis of how shelterin and telomeric structure impacts various aspects of the BER process. The interactions of shelterin proteins TRF1, TRF2 and POT1 with BER proteins (FEN1, APE1, Pol β) appear to stimulate various enzymatic steps of the BER pathway [89]. This contrasts with cellular evidence that BER may be less efficient at telomeres compared to other genomic regions [28,73,75], and suggests factors other than shelterin may compromise BER efficiency at telomeres.

5. Repair in the context of telomeric G-quadruplex

Recent studies show that telomeric sequence and G4 structures influence BER. Single stranded telomeric TTAGGG repeats can fold into various conformations of G4 structures, in which four guanines pair by Hoogsteen bonds to form a planar tetrad [92]. Stable intra-molecular G4 folding requires a minimal sequence of $GGG(TTAGGG)_3$ which forms a stack of four planar tetrads (Fig. 4). In addition to OGG1 and NTH1 glycosylases, the NEIL1, NEIL2 and NEIL3 glycosylases also remove oxidized bases, while NEIL1 and NEIL3 prefer lesions in ssDNA compared to duplex DNA (reviewed in Ref. [84]). This preference is very interesting given the presence of ssDNA at telomeres (Fig. 1). Biochemical studies reveal that among these glycosylases, mouse Neil3 and human NEIL1 uniquely exhibit increased removal of damaged bases in telomeric duplex, compared to non-telomeric duplex [93]. The mechanism by which telomeric sequence enhances activity is unknown. The Wallace and Burrows labs examined the ability of OGG1, NTH1 and NEIL glycosylases to remove various oxidative lesions from telomeric G4 DNA including 8-oxoG, guanidinohyantoin (Gh), spiroiminodihydantoin (Sp), and thymine glycol (Tg). These lesions do not prevent a $AGGG(TTAGGG)_3$ molecule from folding into G4 DNA, but they decrease the G4 stability in a manner dependent on the lesion location [93–95]. When the lesion was within G4 DNA none of the glycosylases could remove 8-oxoG, while only mNeil3 could excise Tg and both mNeil3 and NEIL1 could excise Gh and Sp [93]. In contrast, all the NEIL glycosylases exhibited poor activity when the lesions were placed at the preferred site of oxidation (5′Gs) [95]. These data suggest that G4 formation in telomeres may inhibit repair of oxidized bases, especially the common 8-oxoG base. Biophysical studies show that increasing the number of telomeric repeats from four to seven greatly increases the oligonucleotide's structural dynamics and accessibility to proteins [96]. Consistent with this, all three NEIL glycosylases showed improved activity when the Gh lesion (5′G) was within a five repeat oligonucleotide [95]. However, this improvement may be due to exclusion of the lesion from the G4 such that it occurs in a single stranded loop that connects the tetrads. OGG1 still could not excise 8-oxoG from a five repeat substrate since it is inactive on ssDNA [95]. The mNeil3 and NEIL1 preference for excising lesions from ssDNA and G4 DNA suggests they may have critical roles in repair at telomeres. Cells derived from *Neil2*$^{-/-}$ mice show telomere loss [95], while telomeres in NEIL1 and NEIL3 deficient cells remain to be examined.

The generation of ssDNA during the processing of DNA damage, including chromosome breaks, collapsed replication forks, and bulky lesions, raises the possibility that pathways

other than BER are also influenced by G4 formation in telomeres. The ssDNA present in an NER bubble and/or at a stalled RNA polymerase during TC-NER is susceptible to G4 formation since only 21 nucleotides [GGG(TTAGGG)$_3$] are required to form a highly stable G4 structure [96]. Whether G4 folds influence enzymatic NER processing, or whether UV photoproducts within G4 DNA can be excised by NER is unknown. *In vitro* studies indicate that UVB irradiation of telomeric G4 DNA at physiological K^+ concentrations induced CPD formation between adjacent Ts, but also non-adjacent Ts in the loops of specific G4 conformations [97] (Fig. 4B). This raises the interesting possibility that UV irradiation may "lock" telomeric G4 in place, thereby preventing their unwinding. Furthermore, biophysical studies show that stable G4 formation in telomeric ssDNA inhibits RAD51 loading, which is required for initiation of HR [96]. Whether RAD51 loading factors, such as BRCA2, can overcome this inhibition remains to be determined. Although there is strong evidence that G4 influences telomere replication [23,33], very little is known regarding how G4 formation impacts DNA damage processing at telomeres both *in vitro* and *in vivo*. The development of G4 specific antibodies and small ligands that stabilize G4 structures [98,99], should facilitate study of how DNA damage formation influences G4 folding at telomeres, and how G4 stability influences repair at telomeres.

6. Conclusion

Studies in recent years have greatly advanced understanding of how telomeric shelterin proteins commandeer DNA repair enzymes for remodeling the chromosome ends into telomeric caps, and how shelterin controls repair enzymes to prevent inappropriate processing that leads to telomere loss and chromosome fusions. Much less is known about how shelterin interactions with DNA repair proteins influence how well these enzymes function in the processing of genuine DNA damage at telomeres, including stalled replication forks, chromosome breaks and DNA lesions (Fig. 2). While recent evidence indicates that bulky lesions and damaged bases are removed at telomeres, the efficiency of repair is less well understood, and is likely influenced by telomere structure and shelterin. The efficiency of double strand break repair at telomeres remains unresolved, and very little is known regarding how DNA interstrand crosslinks and mismatched bases are processed at telomeres. Finally, while it is clear that telomeres are highly sensitive to replication stress, how stalled and blocked forks are processed at telomeres remains poorly understood. We have highlighted these and other important unanswered questions throughout this perspective. Advances in understanding damage processing at telomeres will likely require mechanisms and technologies for confining damage to the chromosome ends, to avoid indirect effects of collateral damage elsewhere in the genome on telomere maintenance. Furthermore, methods for detecting and quantifying various types of damage and DNA lesions at telomeres will be critical for assessing how the damage is processed, but remains a challenge given the highly repetitive nature of telomeric DNA, their location at chromosome ends, and their low abundance. A better understanding of how DNA damage is processed at telomeres will be valuable for advancing therapies and interventions that preserve telomere function to maintain healthy cells and promote healthy aging, or that conversely deplete telomeres to arrest proliferating cancer cells.

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Abbreviations:

6-4PP	pyrimidine (6–4) pyrimidine photoproducts
ALT	alternative lengthening of telomeres
BER	base excision repair
CPD	cyclobutane pyrimidine dimer
DDR	DNAdamage response
DSB	DNA double strand break
dsDNA	double stranded DNA
G4	G-quadruplex
GGR	global genome repair
γH2AX	phosphorylated histone H2AX
HJ	Holliday junction
HR	homologous recombination
NER	nucleotide excision repair
NHEJ	nonhomologous end joining
ROS	reactive oxygen species
SCE	sister chromatid exchange
ssDNA	single stranded DNA
SSB	single strand break
TCR	transcription coupled repair
UV	ultraviolet light

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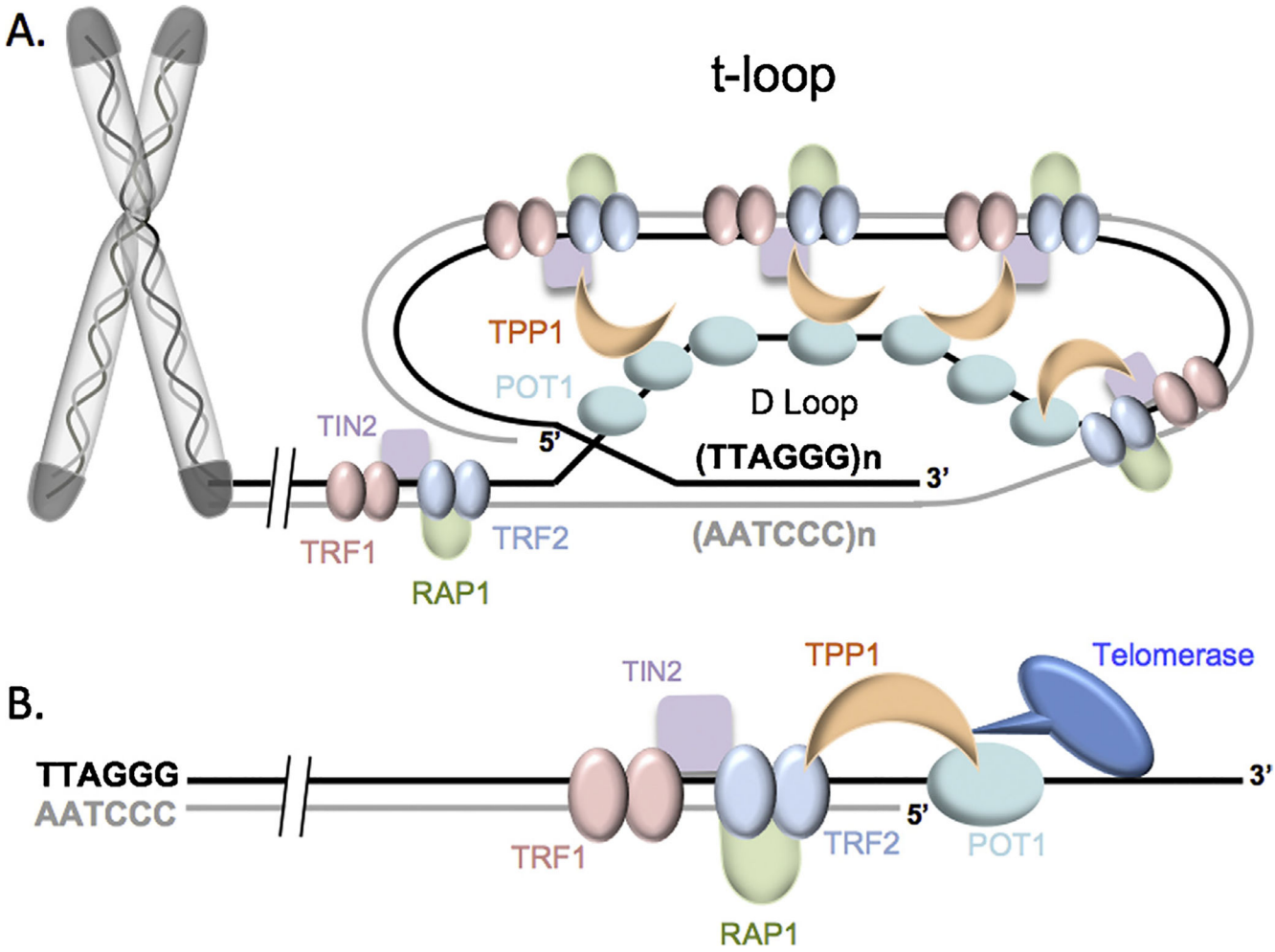

Fig. 1.

Schematic of the telomeres in a closed and open conformation. Telomeres comprise of tandem 5′TTAGGG hexameric repeats and are coated with a complex of six proteins termed shelterin. TRF1 and TRF2 bind duplex repeats and POT1 binds single stranded TTAGGG repeats. (A) In the closed and capped conformation the telomeric ssDNA overhang can wrap around and invade duplex sequence to form a D-loop that stabilizes that large t-loop. (B) in an uncapped and open conformation the telomeric 3′ overhang is accessible to lengthening by the enzyme telomerase which is recruited through interactions with TPP1.

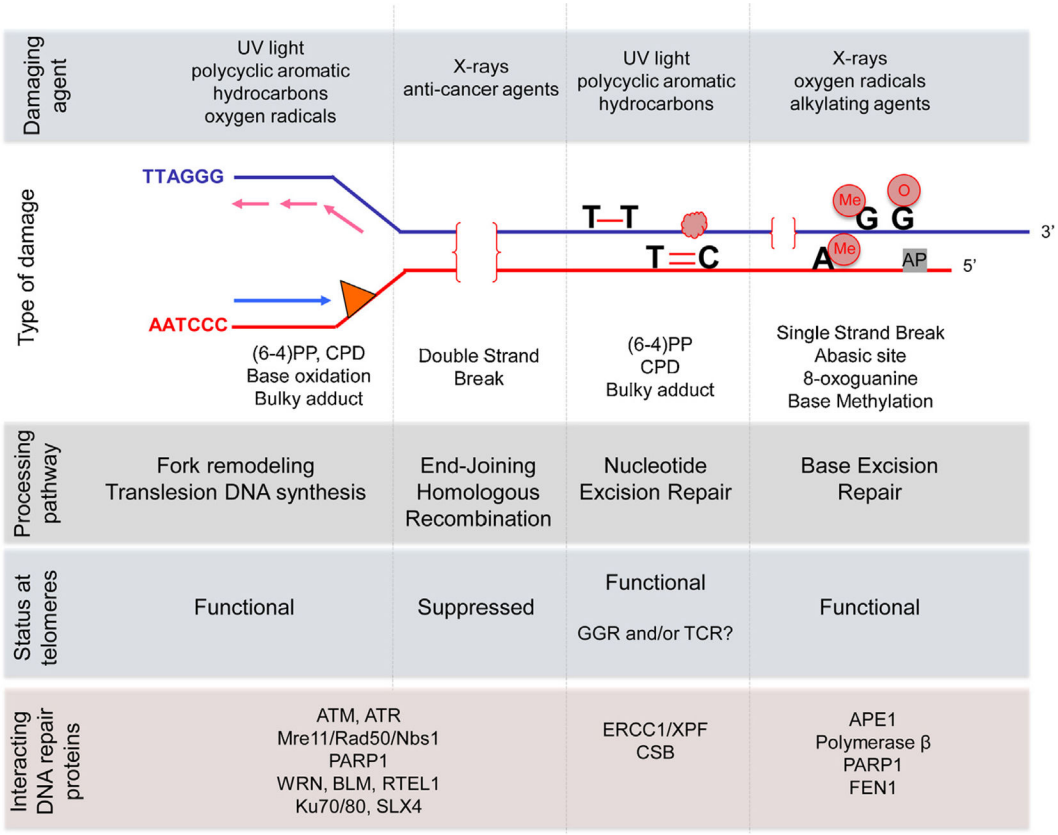

Fig. 2.

Summary of DNA Repair Pathways at Telomeres. Common DNA damaging agents are listed in the top panel followed by the resulting DNA lesions and damage types listed below. The most relevant damage tolerance or repair pathways for processing the damage are listed under each damage type, followed by whether the pathway is functional or suppressed at telomeres based on current published evidence. The last panel lists proteins in the relevant DNA repair or tolerance pathway that interact with shelterin proteins. Since many of the proteins that function in HR also function in replication fork remodeling they are listed under both double strand break repair pathways and fork remodeling.

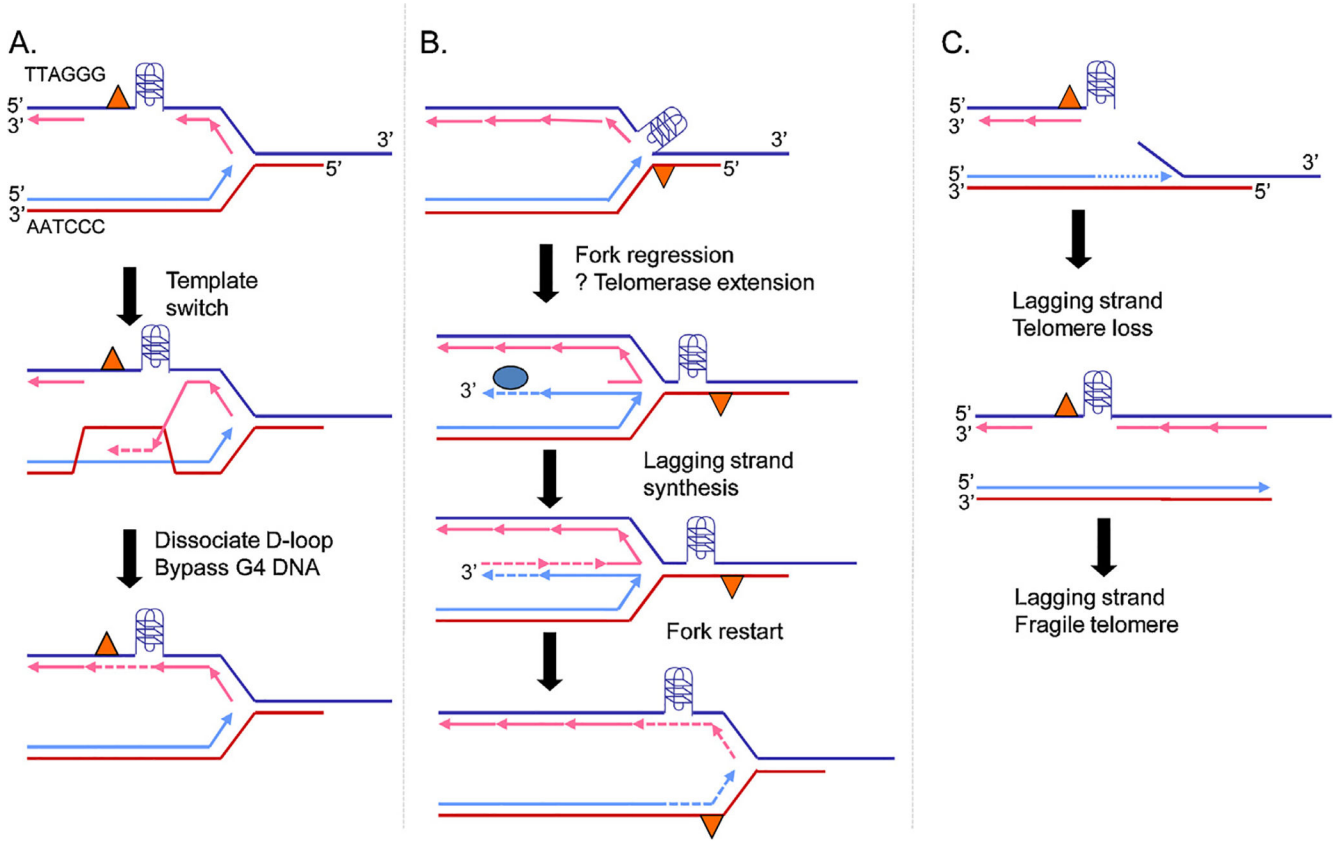

Fig. 3.

Examples of replication fork remodeling pathways to bypass obstacles at stalled or blocked replication forks. G-quadruplex (G4) represents an example of a structural block, and the orange triangle represents an example of a blocking DNA lesion, such as a UV photoproduct. (A) obstacles may be bypassed by strand invasion and use of the sister chromatid as template for DNA synthesis followed by D-loop dissociation. (B) replication fork regression and annealing of the newly synthesized strands followed by extension and fork restart also allows for bypass of obstacles. Telomerase is proposed to lengthen the G-rich strand in the regressed fork. (C) model for replication associated telomere aberrations. Failure to recover from a collapsed and broken replication fork leads to sister chromatid telomere loss, and failure to recover DNA synthesis at a stalled replication fork leads to unreplicated single stranded DNA that manifests as fragile telomeres (also termed multi-telomeric signal).

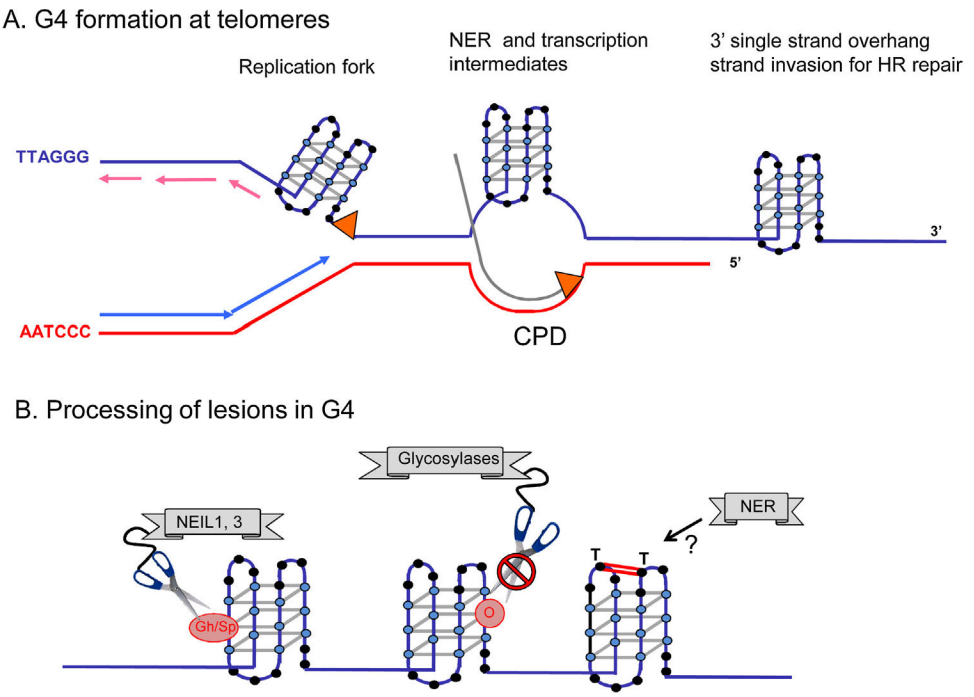

Fig. 4.

Model for how G-quadruplex formation in telomeric DNA influences damage processing. The G4 “chair” conformation is selected as an example, since CPDs can form in this structure, although several G4 conformations can fold in single stranded telomeric DNA and likely exist in dynamic equilibrium. The orange triangle denotes a blocking lesion. (A) G4 could potentially form when ssDNA is generated at blocked replication forks, at bubble intermediates formed during NER, R-loops formed at a blocked RNA polymerase during TC-NER and transcription, and after resection to generate ssDNA for HR repair of chromosome breaks. (B) Repair of lesions in telomeric G4 DNA. NEIL1 and mNeil3 can excise Gh and Sp lesions from telomeric G4, while 8-oxoG is refractory to excision from G4 DNA. CPDs can form in the loops of telomeric G4, but whether they can be repaired is unknown.