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Unraveling secrets of telomeres: One molecule at a time

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ABSTRACT

Telomeres play important roles in maintaining the stability of linear chromosomes. Telomere maintenance involves dynamic actions of multiple proteins interacting with long repetitive sequences and complex dynamic DNA structures, such as G-quadruplexes, T-loops and t-circles. Given the heterogeneity and complexity of telomeres, single-molecule approaches are essential to fully understand the structure–function relationships that govern telomere maintenance. In this review, we present a brief overview of the principles of single-molecule imaging and manipulation techniques. We then highlight results obtained from applying these single-molecule techniques for studying structure, dynamics and functions of G-quadruplexes, telomerase, and shelterin proteins.

1. Introduction to telomeres

Telomeres play important roles in maintaining the stability of linear chromosomes [1–5]. The telomeric structure allows a cell to distinguish between natural chromosome ends and double-stranded DNA breaks. As such, telomeres prevent the inappropriate activation of DNA damage signaling pathways, which can lead to cell cycle arrest, senescence, or apoptosis [6]. Loss of telomere function can activate DNA repair processes, leading to nucleolytic degradation of natural chromosome ends and end-to-end fusions. Telomere dysfunction and associated chromosomal abnormalities have been strongly associated with age-associated degenerative diseases and cancer [7,8]. Great progress has been made in the last 20 years in understanding telomere biology in model systems, including ciliates, yeast, *Drosophila*, plants, and mouse [9–11]. Due to space limitations, this review focuses mainly on the human telomere system (Fig. 1).

In a typical human somatic cell, the length of tandem repeats of telomeric DNA TTAGGG is ~2–15 kb with a 3′ overhang of ~100–200 nt [8,12]. This 3′-overhang serves as a substrate for the reverse transcriptase telomerase, which replicates the telomeric sequence by using an internal RNA subunit as a template to direct DNA synthesis (Fig. 1) [1,4,13]. A specialized protein complex, called shelterin or telosome, binds to and protects telomeres at chromosome ends (Fig. 1) [14]. In humans, this complex consists of six core proteins: duplex TTAGGG-repeat binding factor-1 (TRF1) and -2 (TRF2), the single-stranded telomeric DNA binding protein protection of telomeres-1 (POT1), TRF1-interacting nuclear protein 2 (TIN2), POT1- and TIN2-organizing protein (TPP1), and transcriptional repressor/activator protein RAP1 [4,14,15]. The RPA-like CTC1–STN1–TEN1 complex binds to ssDNA and protects telomeres independently of the POT1 protein, and acts as a terminator of telomerase [16,17]. Shelterin proteins interact with numerous protein factors, including proteins involved in DNA recombination and repair, such as ERCC1-XPF, WRN, BLM, and DNA-PK (Fig. 1) [14,18–20]. Adding to the complexity of telomere structures, telomeric repeat-containing RNA (TERRA) was identified as an integral part of telomeric heterochromatin [21,22]. TERRA is associated with TRF2 and a large number of RNA-binding proteins, and is implicated in telomere structural maintenance and heterochromatin formation [23,24].

Telomeres can adopt different types of open or closed (capped) conformations. Telomeric DNA from humans, as well as from several other organisms, can be arranged into T-loops, in which the 3′ overhang invades the upstream double-stranded region (Fig. 1) [25–29]. Telomeres can be maintained by the recombination-dependent alternative lengthening of telomeres (ALT) pathway in telomerase-negative tumors. The ALT pathway is accompanied by

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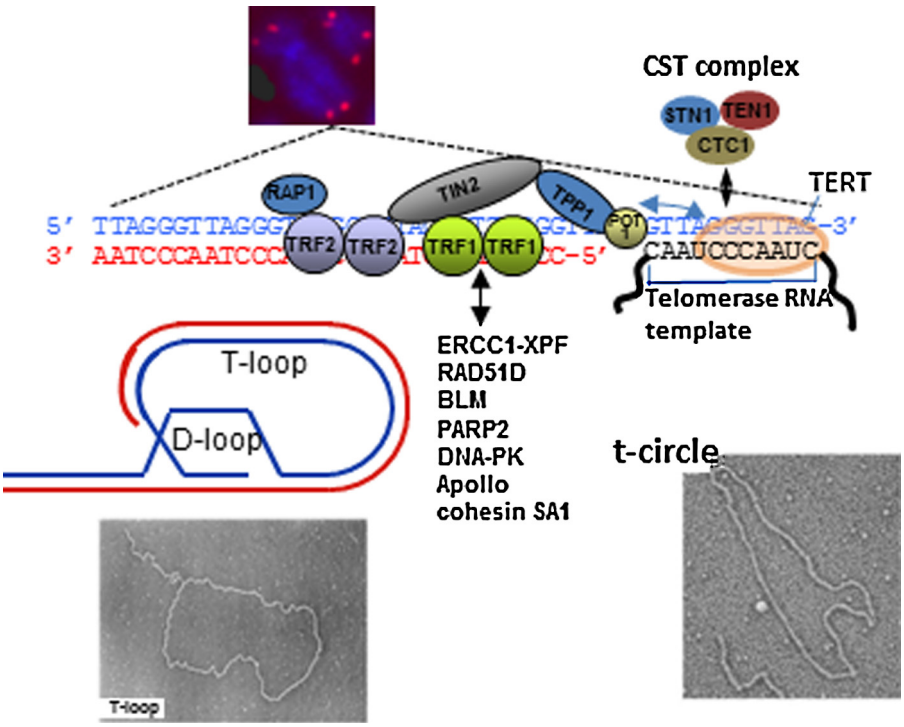

Fig. 1. Human telomeres. Top: Shelterin proteins, including TRF1, TRF2, TIN2, RAP1, TPP1, and POT1, interact with many proteins involved in cell cycle progression and DNA repair [14]. The telomerase complex is regulated by shelterin proteins and DNA structures at telomeres, such as T-loop and G-quadruplexes. Telomeric DNA can be modeled into a T-loop structure (bottom left) [25] or exists as extrachromosomal telomeric circles (t-circles, bottom right) [137].
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the generation of duplex or single-stranded DNA circles formed from telomeric repeat sequences (t-circles) (Fig. 1) [30]. G-rich sequences have been shown to form discrete four-stranded structures termed G-quadruplexes *in vitro* [31]. Studies using an engineered, structure-specific G-quadruplex antibody provided evidence that G-quadruplex DNA exists at telomeres *in vivo* [32–34]. Stable G-quadruplexes have been detected in both the telomere and sub-telomere regions. G-quadruplex DNA plays important roles in the regulation of telomere extension and organization, as well as pairing of homologous chromosomes [31].

2. Single-molecule techniques for studying telomeres

Cell-based and biochemical assays have brought exciting discoveries regarding telomere structure and function, but have also left many unanswered questions. Telomere maintenance involves dynamic actions of multiple proteins on a long complex DNA structure. Given the heterogeneity and complexity of telomeres, single-molecule approaches are essential to fully understand the structure–function relationships that govern telomere maintenance. Single-molecule techniques gather information on large populations of individual molecules. Therefore, single-molecule studies can provide additional information on biomolecules compared to that obtained from bulk biochemical and biophysical studies, which analyze the average behavior and properties of the whole population. In addition, single-molecule techniques allow us to observe biologically important rare events or conformations that would not be detectable in bulk assays. Single-molecule manipulation enables direct investigation of the forces associated with biological molecules and multistate folding of single proteins and nucleic acid structures. Single-molecule imaging and manipulation techniques, such as electron microscopy (EM), atomic force microscopy (AFM), single-molecule Förster (fluorescence) resonance energy transfer (smFRET), optical tweezers, and magnetic tweezers, have revealed many secrets of telomeres [35]. We will provide a brief overview and comparison of several commonly used single-molecule techniques, followed by discussions of results obtained from these techniques (Section 3). For more detailed descriptions of single-molecule imaging and manipulation techniques and their applications, readers are encouraged to refer to several excellent reviews [36–41].

2.1. Electron microscopy (EM) and atomic force microscopy (AFM)

Since the first direct visualization of DNA using electron microscopy (EM), EM has become a gold standard in imaging of protein–DNA complexes [42]. Typical sample preparation for imaging of DNA and protein samples involves fixation of samples using glutaraldehyde or formaldehyde, glow charging the supporting carbon film/foil grid, and contrast enhancement by heavy metal shadowing or staining. Different from typical optical microscopes and EM, AFM generates an image of a surface by scanning with a sharp sensor tip attached to a cantilever [38,43–46] (Fig. 2A, left). Many protein–protein and protein–DNA complexes have been imaged in air and under solution at nanometer resolutions, establishing AFM as a versatile imaging tool for studying these biological systems [38,44–46]. Recent technical advances have enabled high-speed AFM imaging at high spatiotemporal resolution in liquids [47]. Both AFM and EM have been used to determine the mass of protein complexes free in solution and assembled onto DNA or RNA [38,48,49]. The oligomerization states of a protein and protein–protein dissociation constants can be determined from the established standard curve that correlates the volume measured from AFM images with the molecular weight of proteins [38]. In AFM force spectroscopy, the same imaging cantilever can be used as a force sensor, which can be manipulated in the vertical direction. The cantilever deflection x in response to tip-sample interactions is

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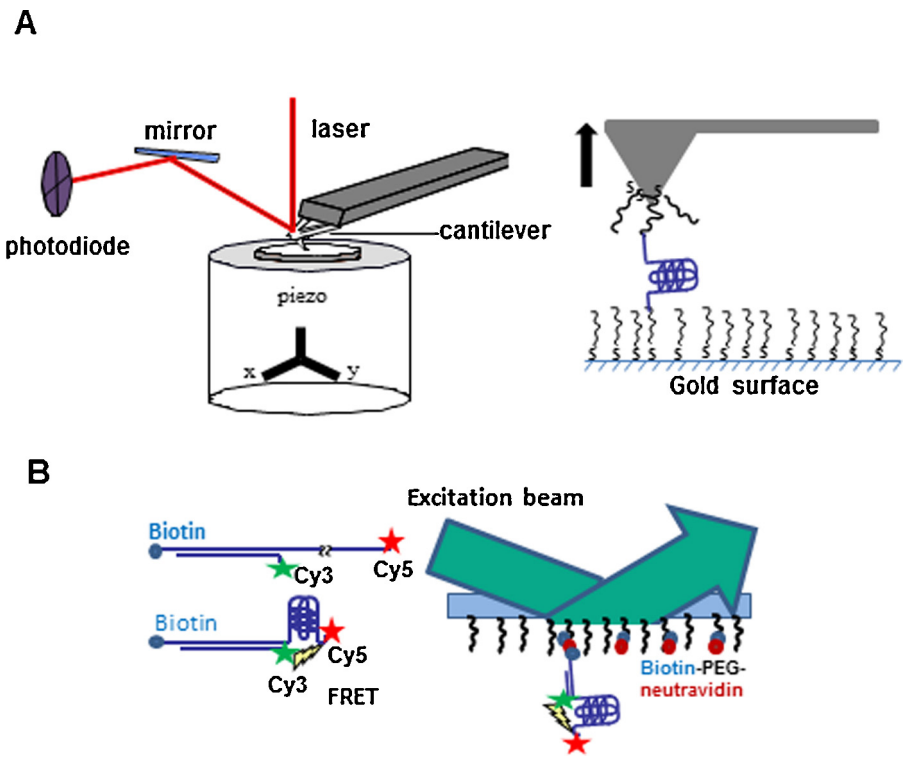

Fig. 2. Single-molecule imaging techniques used in the studies of telomere structure and function. (A) Atomic force microscopy (AFM) (left) and force spectroscopy (right) setup [71]. (C) Single-molecule FRET setup [92].

measured and translated into an interaction force F (Fig. 2A, right). EM and AFM imaging carried out in different laboratories have illuminated the mechanisms of G-quadruplex formation (Section 3.1), and the functions of shelterin proteins including POT1, TRF1, TRF2, and RAP1 (Sections 3.3 and 3.4) [49–52].

2.2. Single-molecule fluorescence imaging

Since 1996, when the first Förster (fluorescence) resonance energy transfer (smFRET) study demonstrated single-molecule sensitivity [53,54], smFRET has established itself as a powerful technique for studying conformational changes and dynamics of biological molecules (Fig. 2C) [55]. In FRET, the efficiency of non-radiative energy transfer reports the distance between two fluorescent dye molecules, donor and acceptor. The efficiency of energy transfer (E) is described by $E = 1/(1 + (R/R_0)^6)$, where R is the inter-dye distance, and R_0 is the Förster radius at which $E = 0.5$. FRET can detect distance changes within ~2–8 nm inter-dye distance range for $R_0 = 5$ nm, and follow single-molecule reactions on a time scale of ~1 ms to minutes. For detecting FRET signal at the single-molecule level, reduction of the background signal is essential. This is achieved through using total internal reflection fluorescence (TIRF) microscopy. TIRF creates an evanescent field of only ~100–200 nm depth, exciting only a thin layer of molecules in proximity of the substrate surface (Fig. 2C). To suppress nonspecific binding, these (typically quartz slide) surfaces are coated with PEG or a lipid layer, or biomolecules are entrapped inside lipid vesicles [56]. DNA and protein molecules can be immobilized onto the slide surface through biotin/streptavidin interactions, or to a PEG-coated surface engineered to carry Ni^{2+} or Cu^{2+} chelated on iminodiacetic acid (or nitrilotriacetic acid) groups via 6x histidine (His_6) tags on proteins [56].

Different from FRET, two-color coincident detection (TCCD) requires two-color excitation and two-color detection to follow coincident fluorescence bursts in a small, subfemtoliter confocal volume defined by a tightly focused laser beam [57,58]. Ratiometric analysis of two fluorescence intensities can be used to determine the relative and absolute composition of the complexes, and the kinetic stability of the specific complexes.

Several groups have studied the folding of G-quadruplex structures and telomerase RNA by taking advantage of the sensitivity of smFRET to conformational changes of biomolecules (Sections 3.1 and 3.2) [35,59]. TCCD also provided new insights into telomerase processivity and stoichiometry (Section 3.2) [57,58].

3. Results from single-molecule studies

3.1. G-quadruplex structures

G-quadruplex structures consist of a network of Hoogsteen hydrogen bonds, in which each of the four guanine residues serves as both a donor and an acceptor of two hydrogen bonds (PDB: 2KF8 and 2GKU) [60]. G-quadruplexes have been implicated in regulating DNA transcription, replication, and recombination processes [61,62]. G-quadruplex structures formed with telomeric sequences inhibit telomerase activity, supporting the idea that the G-quadruplex formed at 3′ overhang serves to negatively control telomeric extension by telomerase [63]. With reactivated telomerase being detected in more than 90% of malignant tumors, understanding the structure and dynamics of the G-quadruplex structures is essential for developing cancer therapeutics that target G-quadruplex DNA stability. We will highlight single-molecule studies of the structure and dynamics of G-quadruplexes formed by human telomeric sequences, and mechanisms of G-quadruplex stabilizing ligands [64].

AFM imaging was able to demonstrate, based on quantitative comparison of DNA molecular heights, that single-stranded human telomeric DNA forms higher-order structures [65]. Interestingly, another AFM study also showed that in the presence of crowding agent PEG, four TTAGGG repeats at the end of

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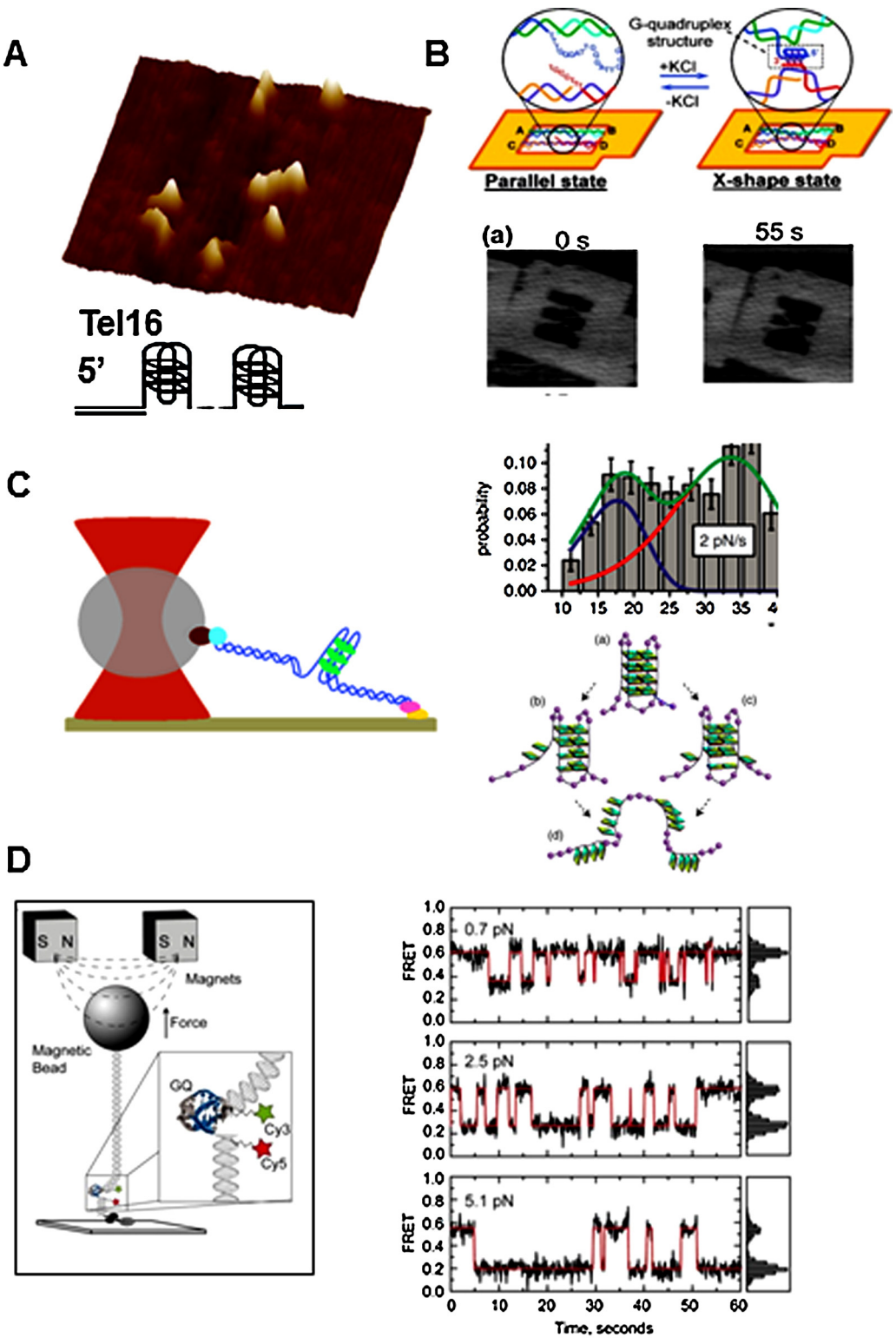

Fig. 3. Single-molecule studies of G-quadruplex structures. (A) An AFM image of beads-on-a-string structures formed by $(TTAGGG)_{16}$ [52]. (B) High-speed AFM imaging of interstrand G-quadruplex structure (X-shape) formation in real-time using nanoscaffold [69]. Reprinted with permission from [69], 2010 American Chemical Society. (C) Laser tweezers. Left: a schematic representation of laser tweezers set up. Right: Dynamic force spectroscopy study of G-quadruplexes using laser tweezers. Top right: Force distributions are consistent with two quadruplex conformations, parallel (blue) and antiparallel (red). Bottom right: proposed unfolding model: 2–3 bp disruption of the G-quadruplex under force leads to the transition state and disruption of G-quadruplex structures [72]. Reprinted with permission from [72], 2012 American Physical Society. (D) Magnetic tweezers and smFRET integration [73] for studying G-quadruplexes. Reprinted with permission from [73], 2013 Oxford University Press.

blunt-ended double-stranded DNA can form G-quadruplex structures as judged by their molecular height of ~2 nm [66]. This result suggests that G-quadruplex structure may form *in vivo* at blunt telomeric ends, providing an alternative protective structure at telomeres. In addition, G-rich telomeric RNA was observed as round particles or short, thick rods in EM micrographs supporting the idea that G-rich RNA in TERRA folds into a string of G-quadruplex beads [67]. Telomeric RNA can also directly interact

with telomeric DNA to form hybrid intermolecular G-quadruplexes, adding another layer of complexity to the telomere regulation [68]. Prior to single-molecule studies, the arrangements and numbers of G4-quadruplex units that can form on biologically relevant longer telomeric tails were not well understood. Our AFM study showed that the majority of molecules with 16 telomeric DNA repeats (TTAGGG) formed two G-quadruplex structures with a beads-on-a-string conformation (Fig. 3A), instead of the maximum of four that would occur if all the TTAGGG repeats folded into quadruplexes [52]. These results have important implications for the mechanism of POT1 interaction with 3′ overhangs (Section 3.3). Furthermore the high-speed AFM enabled a direct observation of G-quadruplex formation in real-time and at nanometer resolution [69]. In this study, a special DNA nanoscaffold based on DNA origami was employed (Fig. 3B). The DNA substrates used to create this scaffold contained complementary ssDNA connection sites on the DNA frame as well as single-stranded G-rich overhangs for the formation of interstrand G-quadruplexes. On introducing K^+ ions, interstrand G-quadruplex formation led to the appearance of an X shape within the nanoscaffold. This study has provided the groundwork for future studies of G-quadruplex binding ligands and proteins in real-time.

The folding of the single-stranded telomeric DNA into compact G-quadruplex structures has been probed using smFRET. For these studies, the DNA substrates were labeled with a donor dye on the complementary stem region and an acceptor dye on the G-quadruplex strand (Fig. 2B). Single-molecule FRET data demonstrated that single-stranded telomeric DNA with 4 repeats containing GGGs exhibits extreme conformational diversity with at least six different conformational states [70].

Both AFM and optical tweezers force spectroscopy showed that, when applying a linearly increasing force over time, G-quadruplex structures are disrupted when the force reaches ~50 pN [71,72]. In one AFM study, the gold-coated Si_3N_4 probe as well as the gold surface were functionalized with a self-assembled monolayer of DNA containing G-rich domains [71]. When the tip approached the surface, intermolecular G-quadruplexes were formed between the tip and surface. Optical tweezers based dynamic force spectroscopy, in which the distribution of rupture forces was measured for different loading rates, was used to characterize the transition state barrier for unfolding of the G-quadruplex structure [72]. The high precision of the optical tweezers allowed de Messieres et al. to resolve two distinct force distributions for parallel and antiparallel quadruplexes at different loading rates (Fig. 3C) [72]. While optical tweezers can achieve sub-nanometer resolution of G-quadruplex DNA when relatively large stretching forces are applied (>10 pN), Long et al. addressed the conformational change of G-quadruplexes under smaller biologically relevant forces. They applied a recently developed novel method, which integrates smFRET and magnetic tweezers to directly probe the conformational change of G-quadruplexes formed by human telomeric sequences under force (Fig. 3D) [73]. These data showed that G-quadruplex structures were very sensitive to force between ~1 and 8 pN (Fig. 3D). Higher forces biased the G-quadruplex folding/unfolding equilibrium to unfolding. The distance between the folded quadruplex and transition state was determined to be ~0.6 nm, with the transition state barrier closer to the unfolded state than the folded state. Consistent with this result, optical tweezers experiments also suggested that the formation of G-quadruplex is a highly cooperative process requiring a relatively small perturbation to the structure (2–3 bp) to reach the transition state [72].

Telomestain, a natural product, and a macrocyclic hexaoxazole telomestatin derivative (6OTD), have been shown to bind strongly to G-quadruplex structures. Using AFM force spectroscopy, Nakamura et al. demonstrated that larger unbinding forces were observed with G-quadruplex DNA in the presence of 6OTD dimer in comparison with 6OTD monomer. This finding suggested that the dimer binds to the G-quadruplex DNA simultaneously, possibly by sandwiching the G-quadruplex [74]. In addition, smFRET showed that binding of quinolinecarboxamide macrocycle 1, a potent quadruplex stabilizing ligand, does not prevent the intrinsic dynamics of G-quadruplex structures, but does selectively stabilize the conformation that is not normally favored under physiological conditions [75].

In summary, the observations from single-molecule imaging and manipulation studies have provided a foundation for further investigations of mechanisms of protein binding to the 3′ overhang, and for design of drugs that target G-quadruplexes for telomerase inhibition.

3.2. Telomerase

Telomerase is an essential ribonucleoprotein (RNP) that compensates for DNA replication-mediated telomere shortening by adding multiple tandem telomeric repeats to the DNA 3′ end (Fig. 1) [1,13,76]. Multisubunit telomerase is a complex structure comprising telomerase reverse transcriptase (TERT), a template containing RNA component (TR), and additional protein cofactors involved in the regulation of enzyme assembly and activity (PDB: 4LMO, 3KYL, 3DU6, and 3DU5). The core structure of TRs contains an RNA pseudoknot (PK), template, template boundary elements (TBE), and a stem terminal element (STE). The telomerase catalytic cycle is dynamic with three general steps: DNA primer alignment, telomere repeat addition, and translocation [77]. Due to the low natural abundance, poor expression, and inefficient *in vitro* assembly of telomerase, stoichiometry and dynamics of telomerase assembly and action had been elusive before the application of single-molecule techniques. Next, we will briefly highlight several exciting results from single-molecule studies of telomerase. For more detail, readers are encouraged to consult a comprehensive review on this topic [35].

The Klenerman and Balasubramanian groups were the first to apply TCCD to study human telomerase [57]. TCCD was used to detect the coincident fluorescence bursts of a reference fluorophore (Alexa 488) on the DNA primer and individual Cy5-dATP incorporated into the DNA substrate by telomerase [57]. The ratios of Cy5-dATP to the reference fluorophore fluorescence were used to determine the DNA length extended by telomerase and hence enzyme processivity. The authors reported a mean processivity of ~0.32, in good agreement with the value of 0.37 derived from a radioactivity based assay. They also demonstrated a stable 1:1:1 stoichiometry for the catalytically active hTERT:hTR:substrate complex [58] and the first direct evidence of a physical interaction between hTR and dyskerin [78]. Dyskerin is a highly conserved essential protein required for telomerase RNA (TR) stability, telomerase activity, and telomere maintenance.

The presence of PK, in which the single-stranded terminal loop of an RNA stem-loop is base-paired with a single-stranded region elsewhere in the RNA molecule, has been proposed as a universal feature of telomerase RNAs from all species [79]. Conservation of this motif underlies its important role in telomerase activity. Labeling of PK with donor and acceptor fluorescence dyes allowed direct investigation of PK folding using smFRET. The Zhuang group reported that an isolated *Tetrahymena* PK sequence formed a stable PK structure with high FRET values (Fig. 4A) [80]. In contrast, due to interferences from other parts of the RNA, the full-length telomerase RNA was not able to form the stable PK structure in the absence of protein cofactors, as indicated by substantially lower FRET values (Fig. 4A). Interestingly, the PK structure was formed stably in the telomerase RNP [80]. The stem IV dependent interaction of p65 (a telomerase specific RNA folding protein) with telomerase RNA induced a marked structural rearrangement of telomerase RNA

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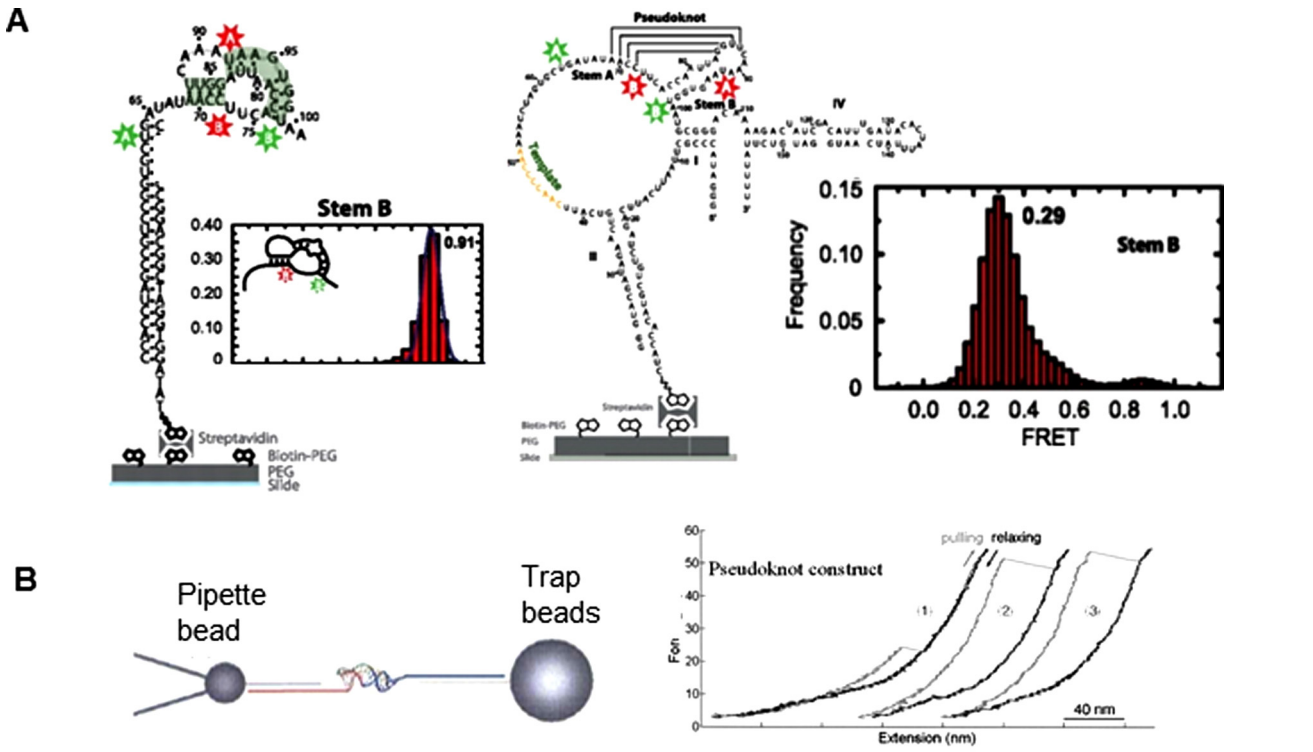

Fig. 4. Single-molecule studies of telomerase. (A) smFRET study of the structure of isolated PK (left) and full-length RNA sequence (right) [80]. The isolated PK sequence form stable PK structure indicated by high FRET. In contrast, the full-length sequences do not form stable PK structure indicated by low FRET. Reprinted with permission from [80], 2011 National Academy of Sciences. (B) Studying the dynamics of PK folding and unfolding using laser tweezers [83]. Top: the experimental setup, bottom: representative force–extension curves. Reprinted with permission from [83], 2007 RNA Society.

(indicated by shifting of FRET values). This conformational change in turn directed the binding of TERT to form the functional ternary complex. In addition, the binding of TERT further altered the RNA conformation, resulting in a compact RNA tertiary fold (indicated by high FRET values) within the functional telomerase RNP. Taking these results together, a hierarchical assembly mechanism for telomerase RNP was proposed [81]. Hengesbach et al. employed smFRET to study the effect of Mg^{2+} on the folding dynamics of human telomerase RNA PK [82]. This work revealed that the hTR PK domain has intrinsic ability to form alternative conformations with higher FRET efficiency distinct from the native PK fold. The nearly full length hTR PK had no detectable folding in the absence of Mg^{2+}. While WT hTR PK folded into a conformation with high FRET values in near physiological buffer containing Mg^{2+}, PK with a GC(107–108)AG mutation, which is genetically linked to dyskeratosis congenital patients, displayed no domain folding. This study highlights the importance of Mg^{2+} ions in stabilizing the native PK fold and the link between a pathogenic phenotype and telomerase RNA folding defect.

Chen et al. used optical tweezers to study the mechanical unfolding and folding of the isolated hairpin-type PK in human telomerase RNA (Fig. 4B) [83]. In the force ramp experiments, unfolding/folding transitions were characterized by sudden increase/decrease in extension and decrease/increase in force. Fig. 4B shows the rips (abrupt unfolding) at an unfolding force of ~24 pN (curve 1) and ~50 pN (curves 2 and 3), corresponding to the unfolding force of an intermediate state and complete PK, respectively. Interestingly, high-force (~50 pN) unfolding of PK in comparison with a RNA hairpin construct (~24 pN) is consistent with a shearing mechanism, but is inconsistent with breaking secondary structure interactions one base pair at a time. The return traces revealed re-folding of PK at relatively low forces (<10 pN) (Fig. 4B).

While telomerase has a single active site for nucleotide addition, it also has the unique ability to synthesize multiple copies of the template following a single binding event, known as repeat addition processivity (RAP). The mechanism of template positioning for achieving RAP is still not well understood. Biochemical assays suggested that direct connectivity of TBE and the template recognition element (TRE) on both sides of the template sequence is important for RAP [84]. Using smFRET on the *Tetraphymena thermophile* telomerase system, Berman et al. directly probed the RNA conformational changes within individual telomerase–DNA complexes by labeling telomeric DNA primers with a donor dye and TRE or TBE with an acceptor dye. In their experimental designs, high and low FRET values indicated compressed and extended RNA, respectively. Reciprocal trends were observed for the FRET values as a function of the 3′-extension of the primer when the acceptor dye was on TBE versus TRE. This finding is consistent with what is predicted by an accordion model: the single-stranded RNA elements flanking the DNA template act as a molecular accordion, undergoing reciprocal extension and compaction during telomerase translocation.

In summary, single-molecule techniques have revealed mechanisms of telomerase RNA folding, ribonucleoprotein assembly and composition, processivity, as well as contributed to new techniques to detect telomerase activity [35].

3.3. Structure and function studies of POT1–TPP1

The 3′ DNA overhang in telomeres is the substrate for telomeric ssDNA-binding proteins. POT1 binds telomeric single-stranded DNA with exceptionally high sequence specificity (PDB: 1XJV and 3KJO) [85,86]. Highly specific telomeric DNA binding by POT1 is promoted by base stacking and unusual G-T base pairing interactions that compact DNA. Besides binding to the 3′ overhang, POT1 can be loaded onto duplex telomeric DNA through protein–protein

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interactions (Fig. 1) [87,88]. TPP1 can recruit POT1 and bridge POT1 to the TRF1–TIN2 complex. It was proposed that this unique function enables POT1 to relay information about telomere length, measured by TRF1, to the telomere terminus, where telomerase is regulated [89,90]. In addition, it was suggested that POT1 and TPP1 form a complex with telomeric DNA that can switch from inhibiting telomerase, to serving as a processivity factor for telomerase during telomere extension [91]. Furthermore, POT1 was shown to trap unfolded telomere repeats in an extended state to prevent G-quadruplex folding [63].

Recent AFM and smFRET studies provided detailed mechanisms regarding how POT1–TPP1 regulates telomeric overhang structural dynamics [52,92]. Firstly, AFM imaging revealed that the underfolding (i.e. formation of less than the maximum potential number of G4 units) of long telomeric ssDNA provides a previously unrecognized route for POT1 binding [52]. POT1 can coexist with G-quadruplex on the same telomeric 3′ overhang (compare Figs. 3A and 5A). However, POT1 loading shifts the population distribution toward molecules that have fewer G4 units or that are completely unfolded. These studies support a model whereby POT1 acts as a "steric driver" to promote unfolding of adjacent G4 structures upon binding to a long telomeric tail. Applying the smFRET system to examine telomeric G-quadruplex dynamics (Fig. 5B) revealed that POT1 promotes G-quadruplex unfolding in a step-wise manner as indicated by four steps of monotonic FRET decrease upon POT1 addition [92]. Strategic mutation of nucleotides within the telomeric overhang supported a model whereby two POT1 monomers bind the $(TTAGGG)_4$ sequence one OB fold at a time (Fig. 5B). Crystal structure data revealed that POT1 harbors two OB folds that make distinct interactions with nucleotides in the TTAGGGTTAG sequence [93]. Further smFRET analyses revealed that POT1 bound to its partner TPP1 induced highly dynamic exchanges between folded and unfolded states of the $(TTAGGG)_4$ overhang. Labeling TPP1 with a dye that could FRET with the Cy3-labeled telomeric overhang uncovered a sliding action of the TPP1–POT1 complex along DNA, offering a mechanistic explanation for the observed dynamics of G-quadruplex folding. In line with this, bulk biochemical studies showed that POT1–TPP1 greatly enhances telomerase processivity *in vitro* by promoting translocation [94]. Thus, single-molecule techniques revealed novel mechanisms by which POT1 and the POT1–TPP1 complex modulate telomeric overhang conformations and supported a model for the POT1–TPP1 complex as a sliding clamp to promote telomerase processivity.

3.4. Structure and function studies of TRF1, TRF2, and RAP1

TRF1 and TRF2 share 30% homology, bind to duplex telomeric DNA with high affinity and are essential for the maintenance of functional telomeres (Fig. 1 and Fig. 6A) [95]. Together with tankyrase, TRF1 functions as a negative regulator of telomere length: overexpression of TRF1 leads to telomere shortening [96]. TRF1 promotes efficient replication of TTAGGG repeats and prevents replication fork stalling at telomeres [97]. TRF1 interacts with the cohesin subunit SA1 and specifically targets SA1 instead of SA2 to telomeres [98]. TRF2 is also involved in telomere length regulation [99], and is crucial in capping and protecting chromosome ends [3,100]. Removal of TRF2 from the telomeres leads to loss of the 3′ overhang, covalent fusion of telomeres, and induction of ATM and p53 dependent apoptosis [101,102]. Overexpression of TRF2 in telomerase-negative cells prevents short telomeres from fusing and delays the onset of senescence [101]. Furthermore, TRF2 plays important roles in the assembly of telomeric chromatin [103]. Importantly, post-translational modifications of TRF1 and TRF2 regulate their functions, including DNA binding, dimerization, localization, degradation, and interactions with other proteins [104]. In addition, several groups have reported DNA binding and gene regulation functions for both TRF1 and TRF2 proteins outside of telomeres [105–111].

Both TRF1 and TRF2 contain a TRFH domain that mediates homodimerization and a C-terminal Myb type domain that mediates sequence-specific binding to telomeric DNA (PDB: 1H6P, 1VFC, and 3SJM) (Fig. 6A) [95]. In both proteins, the DNA binding domain and the dimerization domain are joined together by long linkers (~100 amino acids in TRF1 and 150 amino acids in TRF2). The dimerization domains from human TRF1 and TRF2 have the same α-helical architecture [112]. However, TRF1 and TRF2 dimerization interfaces feature unique interactions that prevent heterodimerization. Solution structures of Myb domains of TRF1 and TRF2 bound to DNA with the sequence GTTAGGGTTAGGG show that both proteins recognize the central AGGGTT sequence through both hydrophilic and hydrophobic interactions between the protein and DNA [113]. However, the DNA binding domain of TRF2 has ~four-fold weaker DNA binding activity than that of TRF1 (equilibrium dissociation constants K_d: 750 nM vs. 200 nM, respectively). Interestingly, a single amino acid change (lysine on TRF2 to arginine on TRF1) is the main contributor to this difference in binding affinity.

The binding of TRF1 and TRF2 to telomeric DNA has been studied using EM and AFM [25,50,51,114–116]. TRF1 was observed as a dimer or a tetramer, while TRF2 was observed as a dimer on DNA in the EM micrographs [25,51,114]. TRF1 has extreme spatial flexibility and can induce DNA looping by binding to two distant half-sites (Fig. 6B) [114]. In addition, TRF1 forms protein filaments on longer telomeric repeats (≥27 repeats) and promotes parallel pairing of telomeric tracts (Fig. 6C) [51]. This activity may play important roles in promoting T-loop formation (in conjunction with TRF2) and sister telomere associations (in conjunction with SA1) [117]. *In vitro*, TRF2 can remodel linear telomeric DNA into T-loops (Fig. 6D) [50,115], and displays domain B dependent binding to DNA junctions at replication forks and the center of Holliday junctions [110]. Recent evidence suggested that TRF2 condenses DNA and generates positive supercoiling on DNA, thereby favoring strand invasion (Fig. 6E) [116]. The DNA condensation activity is from the TRFH domain. Unlike TRF2, TRF1 alone lacks the ability to condense DNA due to the repression by its acidic domain (Fig. 6E) [118]. TRF2 can simultaneously bind to TERRA and telomeric G-quadruplex DNA [68], and a TERRA-like RNA molecule greatly reduced its ability to condense DNA [118].

TRF1 and TRF2 are the only shelterin proteins that bind to duplex telomeric DNA with high affinity. Dynamic movements on DNA, such as 1-dimensional (1-D) sliding (translocation while maintaining continuous DNA contact), jumping and hopping (microscopic dissociation and rebinding events), are essential for a protein to achieve its function inside cells where nonspecific DNA is in vast excess and bound by histones and other proteins.[119–122]. How TRF1 and TRF2 find their cognate sites and protein partners to form the shelterin complex, and to regulate the functions of proteins involved in DNA repair and cell-cycle progression are not fully understood. [123]. Recently, we used DNA tightrope assay based single-molecule fluorescence imaging to directly probe how TRF1 and TRF2 locate their target DNA sequences and protein partners (Fig. 7) [124]. Fluorescent labeling of TRF1 and TRF2 was achieved by conjugating 6x histidine (His_6) tagged TRF1 and TRF2 to streptavidin-conjugated quantum dots (QDs) using the biotinylated multivalent chelator tris-nitrilotriacetic acid (^{BT}tris-NTA) (Fig. 7A) [125]. The measured diffusion constants for TRF1- QDs ($3.8–7.5 \times 10^{-2}$ $\mu m^2/s$) and TRF2-QDs ($8.4–9.5 \times 10^{-2}$ $\mu m^2/s$) obtained using fluorescence imaging of QD-labeled proteins on λ DNA tightropes were consistent with rotational tracking of the DNA helix. However, the attached lifetime of TRF1 at non-telomeric sequences was 10-fold shorter than that of TRF2 (0.3 s vs. 3.4 s at 225 mM from molecules with both on and off events observed). While the majority (>89%) of TRF2 molecules

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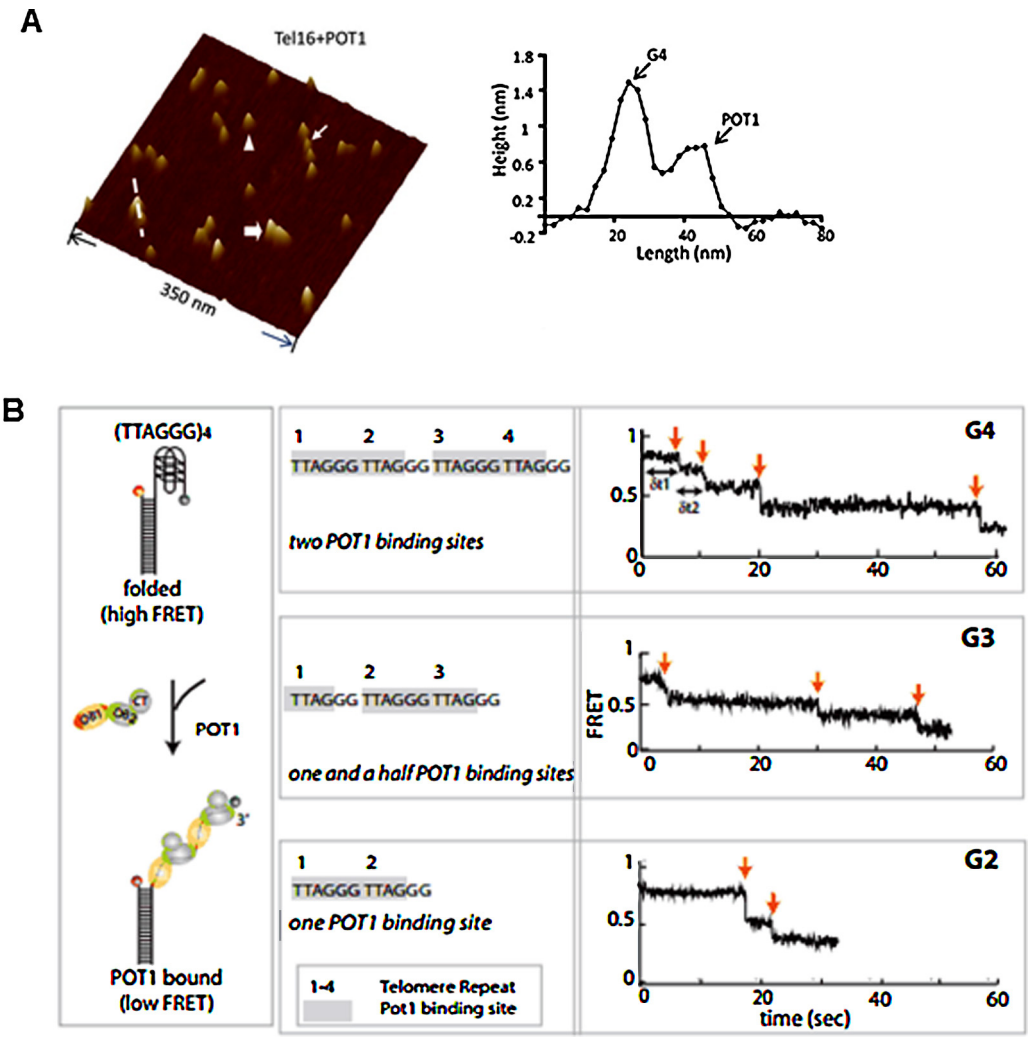

Fig. 5. Mechanisms of G-quadruplex unfolding mediated by POT1. (A) POT1 disrupts G-quadruplexes by acting as a "steric drive" to promote unfolding [52]. Left panel: a representative AFM surface plot of Tel16 DNA containing 16 TTAGGG repeats in the presence of POT1. The thick arrow points to a structure with folded G4. The triangle points to an individual POT1 molecule. The thin arrow points to a structure with multiple POT1 proteins. Right panel: cross-section of the molecule highlighted in the left panel by the dotted line demonstrating that G-quadruplex (left peak) and POT1 (right peak) coexist on the same molecule. (B) POT1 binds to single-stranded telomeric DNA resulted in a stepwise FRET decrease [92]. Left: Schematic of the experimental design. Middle: POT1 binding sites marked in gray. Right: the numbers of steps shown in the single-molecule FRET traces are consistent with two POT1 monomers bind the $(TTAGGG)_4$ sequence one OB fold at a time.
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were motile on λ DNA across all ionic strengths (75–225 mM), the fraction of motile TRF1 proteins ranged only from 15% to 33%. This difference between TRF1 and TRF2 is partly due to the sequences at the N-termini of TRF proteins. For TRF2 this region contains a basic domain, the deletion of which (TRF2ΔB) led to a clear reduction in the percentage of motile protein complexes on λ DNA. We concluded while both TRF1 and TRF2 can bind non-telomeric sequences, TRF2 uses 1-D search more efficiently to locate telomeric DNA. Furthermore, on telomeric DNA, the dynamics of TRF1 and TRF2 converge: they both slide, albeit with ~17- to 37-fold slower diffusion constants at telomeric regions in comparison with non-telomeric DNA (Supplementary Material Movie S1). These differences correspond to an increase of ~3.4 (for TRF1) and 1.1 $\kappa_B T$ (for TRF2) in relative binding energy at the telomeric sequences. The metastable and dynamic nature of the TRF protein binding to telomeric sequences is in stark contrast to stable binding at specific sites characteristic of mismatch repair protein MutSα and Type III restriction enzyme EcoP15I [126,127]. We proposed that slower 1-D sliding allows TRF1 and TRF2 to strike a balance between searching for protein partners and achieving specificity on long repetitive telomeric DNA sequences. We suggested that upon encountering other shelterin subunits, shelterin complex formation is stabilized by combined protein–DNA interaction energies. 1-D sliding on DNA and subsequent stabilization through protein–protein interactions provide an external energy-independent means to proofread their interactions. We called this process "tag-team proofreading". In cells, the intrinsic dynamics of TRF1 and TRF2 could potentially serve as an important switch for regulating the assembly and disassembly of shelterin complexes and different telomere structures (capped and uncapped states).

RAP1 is the most conserved telomere binding protein from yeast to humans (Fig. 1). Human RAP1 has an N-terminal BRCT domain followed by one Myb type DNA binding motif, and a highly conserved protein–protein interaction domain (RCT) at the C-terminus (PDB: 3K6G). RAP1 interacts with telomeric repeats through its interaction with TRF2. These multiple protein–protein and protein–DNA interaction domains make RAP1 a versatile adaptor protein with both telomeric and non-telomeric functions [128–131]. Human RAP1 is a repressor of non-homologous end joining and recombination at telomeres, while it is not essential for

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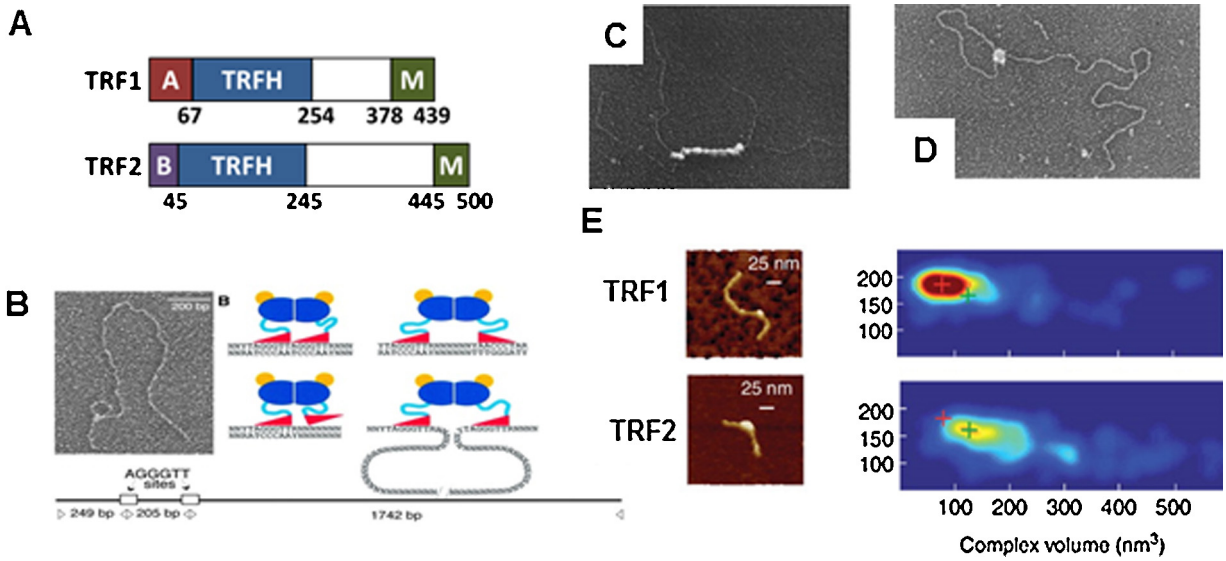

Fig. 6. TRF1 and TRF2 play important architectural roles at telomeres. (A) Schematic representations of the domain structures of TRF1 and TRF2. A: Acidic domain, B: Basic domain. M: Myb type domain. (B) An EM micrograph showing that TRF1 induces DNA looping between two AGGGTT sites separated by 205 bp (left). Right: models for how TRF1 uses one or two Myb domains to contact specific DNA sites [114]. (C) An EM micrograph showing that TRF1 promotes parallel pairing of telomeric tracts [51]. (D) An EM micrograph showing that TRF2 promotes T-loop formation *in vitro* [25]. (E) AFM imaging showing higher oligomers of TRF2 with larger volumes condensed DNA leading to shortening of DNA length [118]. Reprinted with permissions from [114] (1999 Nature Publishing Group), [51] (1998 Elsevier), [25] (1999 Elsevier), and [118] (2012 Oxford University Press).

telomere capping [132,133]. Previously, bulk biochemical assays showed that RAP1 does not bind to ds$(TTAGGG)_{12}$ or single-stranded telomeric DNA [134]. While recent EM analysis confirmed that RAP1 binds weakly to duplex DNA, it also showed that RAP1 has surprisingly strong preference for ds/ss DNA junctions, Holliday junctions, and replication forks [49]. However, unlike TRF2, RAP1 binding to ds-ssDNA junctions is not sequence dependent. Compared to either protein alone, the TRF2/RAP1 complex displayed a 2× higher affinity for dsDNA with telomeric sequence and more than 10-fold higher affinity for the telomeric 3′ end [49]. It was also proposed that TRF2-RAP1 can slide and search for the ds-ssDNA junction to regulate DNA end-joining events at telomeres. Taken together, single-molecule studies established that TRF1, TRF2, and RAP1 play important architectural roles and demonstrated unique dynamics of protein–DNA interactions along repetitive sequences at telomeres.

4. Summary and perspectives

Single-molecule imaging and manipulation techniques have recently become an integral part of telomere research. Many biomolecular mysteries remain to be solved. For example, we still do not fully understand mechanisms driving the assembly and disassembly of capped and uncapped structures at telomeres.

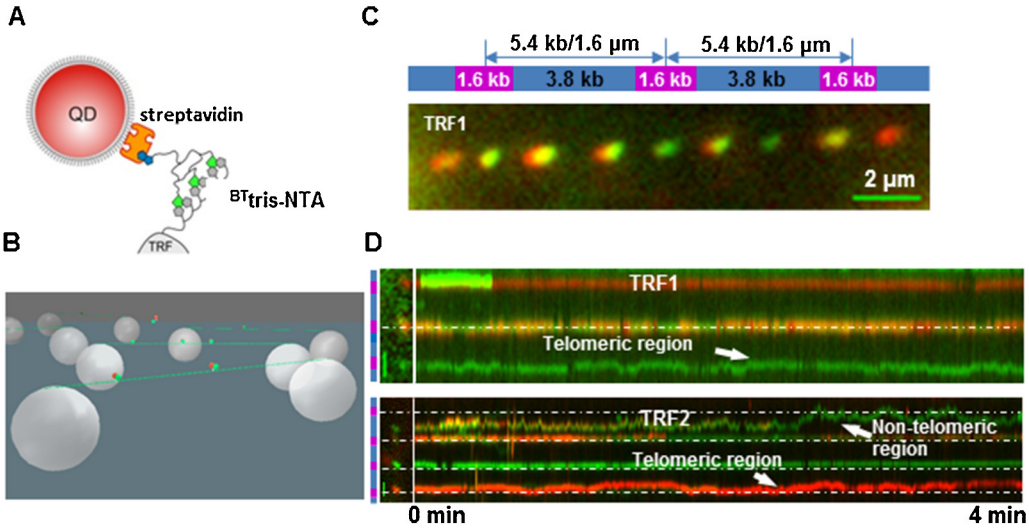

Fig. 7. DNA tightrope assay based oblique-angle fluorescence imaging of TRF1- and TRF2-QDs on λ DNA tightropes [124]. (A) Schematic representations of TRF1- and TRF2-QD conjugates. (B) A schematic drawing of the DNA tightropes between silica beads; red ball: QD; proteins: green balls. The drawing is not to scale. (C) A schematic drawing of the ligated T270 DNA substrate (top, purple: $(TTAGGG)_{270}$ telomeric DNA, blue: non telomeric DNA) and a representative fluorescence image of dual color (655 and 565 nm) labeled TRF1-QDs on the ligated T270 DNA substrate. (D) TRF1 and TRF2 show different diffusional properties over telomeric region versus non-telomeric regions. Kymographical analysis of dual color (655 and 565 nm) labeled TRF1 (top) and TRF2 (bottom) on the T270 DNA with alternating telomeric (purple) and non-telomeric regions (blue). The scale bar is 1 μm.
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Complex relationships between the shelterin complex and DNA repair proteins need to be further studied to fully understand mechanisms of telomere maintenance. Advancements in real-time single-molecule imaging and manipulation techniques will continue to meet the challenge of solving these mysteries. Future real-time single-molecule fluorescence microscopy studies will help us further understand the dynamics of telomere maintenance by following the dynamics of DNA binding and protein assembly in real-time using defined *in vitro* systems. Exciting new techniques such as time-correlated detection counting PALM (tcPALM) using photoconvertible fluorescent proteins hold the promise to reveal the dynamics of telomere complexes with sizes smaller than the diffraction limit in live cells [135]. There is also a great need to develop a high-throughput real-time single-molecule telomerase activity assay, similar to what has been achieved for DNA sequencing using zero-mode waveguides [136]. Collective efforts from telomere and single-molecule imaging research communities are needed to train a new generation of scientists to establish new frontiers of single-molecule imaging and address most important biological questions regarding telomeres.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2014.01.012.

References

[1] E.H. Blackburn, Telomeres and telomerase: their mechanisms of action and the effects of altering their functions, FEBS Letters 579 (2005) 859–862.
[2] T. de Lange, Shelterin: the protein complex that shapes and safeguards human telomeres, Genes & Development 19 (2005) 2100–2110.
[3] T. de Lange, Protection of mammalian telomeres, Oncogene 21 (2002) 532–540.
[4] T.R. Cech, Beginning to understand the end of the chromosome, Cell 116 (2004) 273–279.
[5] A. Sfeir, Telomeres at a glance, Journal of Cell Science 125 (2012) 4173–4178.
[6] A. Smogorzewska, T. de Lange, Regulation of telomerase by telomeric proteins, Annual Review of Biochemistry 73 (2004) 177–208.
[7] R.S. Maser, R.A. DePinho, Connecting chromosomes, crisis, and cancer, Science 297 (2002) 565–569.
[8] W.E. Wright, J.W. Shay, Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology, Nature Medicine 6 (2000) 849–851.
[9] R.J. Wellinger, V.A. Zakian, Everything you ever wanted to know about *Saccharomyces cerevisiae* telomeres: beginning to end, Genetics 191 (2012) 1073–1105.
[10] M.J. Giraud-Panis, S. Pisano, A. Poulet, M.H. Le Du, E. Gilson, Structural identity of telomeric complexes, FEBS Letters 584 (2010) 3785–3799.
[11] K.A. Lewis, D.S. Wuttke, Telomerase and telomere-associated proteins: structural insights into mechanism and evolution, Structure 20 (2012) 28–39.
[12] V.L. Makarov, Y. Hirose, J.P. Langmore, Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening, Cell 88 (1997) 657–666.
[13] J. Nandakumar, T.R. Cech, Finding the end: recruitment of telomerase to telomeres, Nature Reviews: Molecular Cell Biology 14 (2013) 69–82.
[14] R.E. Verdun, J. Karlseder, Replication and protection of telomeres, Nature 447 (2007) 924–931.
[15] Z. Songyang, D. Liu, Inside the mammalian telomere interactome: regulation and regulatory activities of telomeres, Critical Reviews in Eukaryotic Gene Expression 16 (2006) 103–118.
[16] Y. Miyake, M. Nakamura, A. Nabetani, S. Shimamura, M. Tamura, S. Yonehara, M. Saito, F. Ishikawa, RPA-like mammalian Ctc1-Stn1-Ten1 complex binds to single-stranded DNA and protects telomeres independently of the Pot1 pathway, Molecular Cell 36 (2009) 193–206.
[17] L.Y. Chen, S. Redon, J. Lingner, The human CST complex is a terminator of telomerase activity, Nature 488 (2012) 540–544.
[18] X.D. Zhu, L. Niedernhofer, B. Kuster, M. Mann, J.H. Hoeijmakers, T. de Lange, ERCC1/XPF removes the 3′ overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes, Molecular Cell 12 (2003) 1489–1498.
[19] P.L. Opresko, M. Otterlei, J. Graakjaer, P. Bruheim, L. Dawut, S. Kolvraa, A. May, M.M. Seidman, V.A. Bohr, The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2, Molecular Cell 14 (2004) 763–774.
[20] O. Bombarde, C. Boby, D. Gomez, P. Frit, M.J. Giraud-Panis, E. Gilson, B. Salles, P. Calsou, TRF2/RAP1 and DNA-PK mediate a double protection against joining at telomeric ends, EMBO Journal 29 (2010) 1573–1584.
[21] B. Luke, J. Lingner, TERRA: telomeric repeat-containing RNA, EMBO Journal 28 (2009) 2503–2510.
[22] S. Feuerhahn, N. Iglesias, A. Panza, A. Porro, J. Lingner, TERRA biogenesis, turnover and implications for function, FEBS Letters 584 (2010) 3812–3818.
[23] Z. Deng, J. Norseen, A. Wiedmer, H. Riethman, P.M. Lieberman, TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres, Molecular Cell 35 (2009) 403–413.
[24] I. Lopez de Silanes, M. Stagno d'Alcontres, M.A. Blasco, TERRA transcripts are bound by a complex array of RNA-binding proteins, Nature Communications 1 (2010) 33.
[25] J.D. Griffith, L. Comeau, S. Rosenfield, R.M. Stansel, A. Bianchi, H. Moss, T. de Lange, Mammalian telomeres end in a large duplex loop, Cell 97 (1999) 503–514.
[26] K.G. Murti, D.M. Prescott, Telomeres of polytene chromosomes in a ciliated protozoan terminate in duplex DNA loops, Proceedings of the National Academy of Sciences of the United States of America 96 (1999) 14436–14439.
[27] J.L. Munoz-Jordan, G.A. Cross, T. de Lange, J.D. Griffith, t-loops at trypanosome telomeres, EMBO Journal 20 (2001) 579–588.
[28] A.J. Cesare, N. Quinney, S. Willcox, D. Subramanian, J.D. Griffith, Telomere looping in *P. sativum* (common garden pea), Plant Journal 36 (2003) 271–279.
[29] S. Luke-Glaser, H. Poschke, B. Luke, Getting in (and out of) the loop: regulating higher order telomere structures, Frontiers in Oncology 2 (2012) 180.
[30] L. Tomaska, J. Nosek, J. Kramara, J.D. Griffith, Telomeric circles: universal players in telomere maintenance? Nature Structural & Molecular Biology 16 (2009) 1010–1015.
[31] M. Fry, Tetraplex DNA and its interacting proteins, Frontiers in Bioscience 12 (2007) 4336–4351.
[32] N. Maizels, Dynamic roles for G4 DNA in the biology of eukaryotic cells, Nature Structural & Molecular Biology 13 (2006) 1055–1059.
[33] E.Y. Lam, D. Beraldi, D. Tannahill, S. Balasubramanian, G-quadruplex structures are stable and detectable in human genomic DNA, Nature Communications 4 (2013) 1796.
[34] G. Biffi, D. Tannahill, J. McCafferty, S. Balasubramanian, Quantitative visualization of DNA G-quadruplex structures in human cells, Nature Chemistry 5 (2013) 182–186.
[35] M. Hengesbach, B.M. Akiyama, M.D. Stone, Single-molecule analysis of telomerase structure and function, Current Opinion in Chemical Biology 15 (2011) 845–852.
[36] C. Bustamante, J.C. Macosko, G.J. Wuite, Grabbing the cat by the tail: manipulating molecules one by one, Nature Reviews: Molecular Cell Biology 1 (2000) 130–136.
[37] K.C. Neuman, T. Lionnet, J.F. Allemand, Single-molecule micromanipulation techniques, Annual Review of Materials Research 37 (2007) 33–67.
[38] H. Wang, Y. Yang, D.A. Erie, Characterization of protein–protein interactions using atomic force microscopy, in: P. Schuck (Ed.), Protein Interactions Biophysical Approaches for the Study of Complex Reversible Systems, Springer Science + Business Media, LLC, 2007, pp. 39–78.
[39] J.F. Allemand, D. Bensimon, G. Charvin, V. Croquette, G. Lia, T. Lionnet, K.C. Neuman, O.A. Saleh, H. Yokota, Studies of DNA–protein interactions at the single molecule level with magnetic tweezers, Lecture Notes in Physics 711 (2007) 123–140.
[40] I. De Vlaminck, C. Dekker, Recent advances in magnetic tweezers, Annual Review of Biophysics 41 (2012) 453–472.
[41] M. Ritzefeld, V. Walhorn, D. Anselmetti, N. Sewald, Analysis of DNA interactions using single-molecule force spectroscopy, Amino Acids 44 (2013) 1457–1475.
[42] J.D. Griffith, G. Christiansen, Electron microscope visualization of chromatin and other DNA–protein complexes, Annual Review of Biophysics and Bioengineering 7 (1978) 19–35.
[43] G. Binning, H. Rohrer, C. Gerber, E. Weibel, Surface studies by scanning tunneling microscopy, Physical Review Letters 49 (1982) 57–61.
[44] C. Bustamante, C. Rivetti, Visualizing protein–nucleic acid interactions on a large scale with the scanning force microscope, Annual Review of Biophysics and Biomolecular Structure 25 (1996) 395–429.

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[45] A. Janicijevic, D. Ristic, C. Wyman, The molecular machines of DNA repair: scanning force microscopy analysis of their architecture, Journal of Microscopy 212 (2003) 264–272.
[46] Y.L. Lyubchenko, A.A. Gall, L.S. Shlyakhtenko, Atomic force microscopy of DNA and protein–DNA complexes using functionalized mica substrates, Methods in Molecular Biology 148 (2001) 569–578.
[47] T. Ando, High-speed atomic force microscopy coming of age, Nanotechnology 23 (2012) 062001.
[48] H. Wang, M.J. DellaVecchia, M. Skorvaga, D.L. Croteau, D.A. Erie, B. Van Houten, UvrB domain 4, an autoinhibitory gate for regulation of DNA binding and ATPase activity, Journal of Biological Chemistry 281 (2006) 15227–15237.
[49] N.O. Arat, J.D. Griffith, Human Rap1 interacts directly with telomeric DNA and regulates TRF2 localization at the telomere, Journal of Biological Chemistry 287 (2012) 41583–41594.
[50] R.M. Stansel, T. de Lange, J.D. Griffith, T-loop assembly in vitro involves binding of TRF2 near the 3′ telomeric overhang, EMBO Journal 20 (2001) 5532–5540.
[51] J. Griffith, A. Bianchi, T. de Lange, TRF1 promotes parallel pairing of telomeric tracts in vitro, Journal of Molecular Biology 278 (1998) 79–88.
[52] H. Wang, G.J. Nora, H. Ghodke, P.L. Opresko, Single molecule studies of physiologically relevant telomeric tails reveal POT1 mechanism for promoting G-quadruplex unfolding, Journal of Biological Chemistry 286 (2011) 7479–7489.
[53] T. Ha, T. Enderle, D.F. Ogletree, D.S. Chemla, P.R. Selvin, S. Weiss, Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor, Proceedings of the National Academy of Sciences of the United States of America 93 (1996) 6264–6268.
[54] C. Joo, H. Balci, Y. Ishitsuka, C. Buranachai, T. Ha, Advances in single-molecule fluorescence methods for molecular biology, Annual Review of Biochemistry 77 (2008) 51–76.
[55] T. Ha, Single-molecule fluorescence resonance energy transfer, Methods 25 (2001) 78–86.
[56] R. Roy, S. Hohng, T. Ha, A practical guide to single-molecule FRET, Nature Methods 5 (2008) 507–516.
[57] X. Ren, H. Li, R.W. Clarke, D.A. Alves, L. Ying, D. Klenerman, S. Balasubramanian, Analysis of human telomerase activity and function by two color single molecule coincidence fluorescence spectroscopy, Journal of the American Chemical Society 128 (2006) 4992–5000.
[58] D. Alves, H. Li, R. Codrington, A. Orte, X. Ren, D. Klenerman, S. Balasubramanian, Single-molecule analysis of human telomerase monomer, Nature Chemical Biology 4 (2008) 287–289.
[59] B. Okumus, T. Ha, Real-time observation of G-quadruplex dynamics using single-molecule FRET microscopy, Methods in Molecular Biology 608 (2010) 81–96.
[60] S. Burge, G.N. Parkinson, P. Hazel, A.K. Todd, S. Neidle, Quadruplex DNA: sequence, topology and structure, Nucleic Acids Research 34 (2006) 5402–5415.
[61] S. Balasubramanian, L.H. Hurley, S. Neidle, Targeting G-quadruplexes in gene promoters: a novel anticancer strategy? Nature Reviews: Drug Discovery 10 (2011) 261–275.
[62] A. Verma, V.K. Yadav, R. Basundra, A. Kumar, S. Chowdhury, Evidence of genome-wide G4 DNA-mediated gene expression in human cancer cells, Nucleic Acids Research 37 (2009) 4194–4204.
[63] A.J. Zaug, E.R. Podell, T.R. Cech, Human POT1 disrupts telomeric G-quadruplexes allowing telomerase extension in vitro, Proceedings of the National Academy of Sciences of the United States of America 102 (2005) 10864–10869.
[64] P.S. Shirude, S. Balasubramanian, Single molecule conformational analysis of DNA G-quadruplexes, Biochimie 90 (2008) 1197–1206.
[65] Y. Xu, M. Komiyama, The structural studies of human telomeric DNA using AFM, Nucleic acids Symposium Series (2007) 241–242.
[66] Z.Y. Kan, Y. Lin, F. Wang, X.Y. Zhuang, Y. Zhao, D.W. Pang, Y.H. Hao, Z. Tan, G-quadruplex formation in human telomeric (TTAGGG)4 sequence with complementary strand in close vicinity under molecularly crowded condition, Nucleic Acids Research 35 (2007) 3646–3653.
[67] A. Randall, J.D. Griffith, Structure of long telomeric RNA transcripts: the G-rich RNA forms a compact repeating structure containing G-quartets, Journal of Biological Chemistry 284 (2009) 13980–13986.
[68] G. Biffi, D. Tannahill, S. Balasubramanian, An intramolecular G-quadruplex structure is required for binding of telomeric repeat-containing RNA to the telomeric protein TRF2, Journal of the American Chemical Society 134 (2012) 11974–11976.
[69] Y. Sannohe, M. Endo, Y. Katsuda, K. Hidaka, H. Sugiyama, Visualization of dynamic conformational switching of the G-quadruplex in a DNA nanostructure, Journal of the American Chemical Society 132 (2010) 16311–16313.
[70] J.Y. Lee, B. Okumus, D.S. Kim, T. Ha, Extreme conformational diversity in human telomeric DNA, Proceedings of the National Academy of Sciences of the United States of America 102 (2005) 18938–18943.
[71] S. Lynch, H. Baker, S.G. Byker, D. Zhou, K. Sinniah, Single molecule force spectroscopy on G-quadruplex DNA, Chemistry 15 (2009) 8113–8116.
[72] M. de Messieres, J.C. Chang, B. Brawn-Cinani, A. La Porta, Single-molecule study of G-quadruplex disruption using dynamic force spectroscopy, Physical Review Letters 109 (2012) 058101.
[73] X. Long, J.W. Parks, C.R. Bagshaw, M.D. Stone, Mechanical unfolding of human telomere G-quadruplex DNA probed by integrated fluorescence and magnetic tweezers spectroscopy, Nucleic Acids Research 41 (2013) 2746–2755.
[74] Y. Amemiya, Y. Furunaga, K. Iida, M. Tera, K. Nagasawa, K. Ikebukuro, C. Nakamura, Analysis of the unbinding force between telomestatin derivatives and human telomeric G-quadruplex by atomic force microscopy, Chemical Communications 47 (2011) 7485–7487.
[75] P.V. Jena, P.S. Shirude, B. Okumus, K. Laxmi-Reddy, F. Godde, I. Huc, S. Balasubramanian, T. Ha, G-quadruplex DNA bound by a synthetic ligand is highly dynamic, Journal of the American Chemical Society 131 (2009) 12522–12523.
[76] C.W. Greider, E.H. Blackburn, The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity, Cell 51 (1987) 887–898.
[77] J.Y. Wu, M.D. Stone, X. Zhuang, A single-molecule assay for telomerase structure–function analysis, Nucleic Acids Research 38 (2010) e16.
[78] B. Ashbridge, A. Orte, J.A. Yeoman, M. Kirwan, T. Vulliamy, I. Dokal, D. Klenerman, S. Balasubramanian, Single-molecule analysis of the human telomerase RNA–dyskerin interaction and the effect of dyskeratosis congenita mutations, Biochemistry 48 (2009) 10858–10865.
[79] J.A. Yeoman, A. Orte, B. Ashbridge, D. Klenerman, S. Balasubramanian, RNA conformation in catalytically active human telomerase, Journal of the American Chemical Society 132 (2010) 2852–2853.
[80] M. Mihalusova, J.Y. Wu, X. Zhuang, Functional importance of telomerase pseudoknot revealed by single-molecule analysis, Proceedings of the National Academy of Sciences of the United States of America 108 (2011) 20339–20344.
[81] M.D. Stone, M. Mihalusova, M.C. O'Connor, R. Prathapam, K. Collins, X. Zhuang, Stepwise protein-mediated RNA folding directs assembly of telomerase ribonucleoprotein, Nature 446 (2007) 458–461.
[82] M. Hengesbach, N.K. Kim, J. Feigon, M.D. Stone, Single-molecule FRET reveals the folding dynamics of the human telomerase RNA pseudoknot domain, Angewandte Chemie 51 (2012) 5876–5879.
[83] G. Chen, J.D. Wen, I. Tinoco Jr., Single-molecule mechanical unfolding and folding of a pseudoknot in human telomerase RNA, RNA 13 (2007) 2175–2188.
[84] A.J. Berman, B.M. Akiyama, M.D. Stone, T.R. Cech, The RNA accordion model for template positioning by telomerase RNA during telomeric DNA synthesis, Nature Structural & Molecular Biology 18 (2011) 1371–1375.
[85] M. Lei, P. Baumann, T.R. Cech, Cooperative binding of single-stranded telomeric DNA by the Pot1 protein of *Schizosaccharomyces pombe*, Biochemistry 41 (2002) 14560–14568.
[86] D. Loayza, H. Parsons, J. Donigian, K. Hoke, T. de Lange, DNA binding features of human POT1: a nonamer 5′-TAGGGTTAG-3′ minimal binding site, sequence specificity, and internal binding to multimeric sites, Journal of Biological Chemistry 279 (2004) 13241–13248.
[87] J.Z. Ye, D. Hockemeyer, A.N. Krutchinsky, D. Loayza, S.M. Hooper, B.T. Chait, T. de Lange, POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex, Genes & Development 18 (2004) 1649–1654.
[88] D. Liu, A. Safari, M.S. O'Connor, D.W. Chan, A. Laegeler, J. Qin, Z. Songyang, PTOP interacts with POT1 and regulates its localization to telomeres, Nature Cell Biology 6 (2004) 673–680.
[89] D. Loayza, T. de Lange, POT1 as a terminal transducer of TRF1 telomere length control, Nature 423 (2003) 1013–1018.
[90] V. Lundblad, Telomeres: taking the measure, Nature 423 (2003) 926–927.
[91] F. Wang, E.R. Podell, A.J. Zaug, Y. Yang, P. Baciu, T.R. Cech, M. Lei, The POT1–TPP1 telomere complex is a telomerase processivity factor, Nature 445 (2007) 506–510.
[92] H. Hwang, N. Buncher, P.L. Opresko, S. Myong, POT1–TPP1 regulates telomeric overhang structural dynamics, Structure 20 (2012) 1872–1880.
[93] M. Lei, E.R. Podell, T.R. Cech, Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection, Nature Structural & Molecular Biology 11 (2004) 1223–1229.
[94] C.M. Latrick, T.R. Cech, POT1–TPP1 enhances telomerase processivity by slowing primer dissociation and aiding translocation, EMBO Journal 29 (2010) 924–933.
[95] R. Court, L. Chapman, L. Fairall, D. Rhodes, How the human telomeric proteins TRF1 and TRF2 recognize telomeric DNA: a view from high-resolution crystal structures, EMBO Reports 6 (2005) 39–45.
[96] B. van Steensel, T. de Lange, Control of telomere length by the human telomeric protein TRF1, Nature 385 (1997) 740–743.
[97] A. Sfeir, S.T. Kosiyatrakul, D. Hockemeyer, S.L. MacRae, J. Karlseder, C.L. Schildkraut, T. de Lange, Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication, Cell 138 (2009) 90–103.
[98] S. Canudas, B.R. Houghtaling, J.Y. Kim, J.N. Dynek, W.G. Chang, S. Smith, Protein requirements for sister telomere association in human cells, EMBO Journal 26 (2007) 4867–4878.
[99] A. Smogorzewska, B. van Steensel, A. Bianchi, S. Oelmann, M.R. Schaefer, G. Schnapp, T. de Lange, Control of human telomere length by TRF1 and TRF2, Molecular and Cellular Biology 20 (2000) 1659–1668.
[100] K. Ancelin, C. Brun, E. Gilson, Role of the telomeric DNA-binding protein TRF2 in the stability of human chromosome ends, Bioessays 20 (1998) 879–883.
[101] B. van Steensel, A. Smogorzewska, T. de Lange, TRF2 protects human telomeres from end-to-end fusions, Cell 92 (1998) 401–413.
[102] J. Karlseder, D. Broccoli, Y. Dai, S. Hardy, T. de Lange, p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2, Science 283 (1999) 1321–1325.

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[103] R. Benetti, S. Schoeftner, P. Munoz, M.A. Blasco, Role of TRF2 in the assembly of telomeric chromatin, Cell Cycle 7 (2008) 3461–3468.
[104] J.R. Walker, X.D. Zhu, Post-translational modifications of TRF1 and TRF2 and their roles in telomere maintenance, Mechanisms of Ageing and Development 133 (2012) 421–434.
[105] P.S. Bradshaw, D.J. Stavropoulos, M.S. Meyn, Human telomeric protein TRF2 associates with genomic double-strand breaks as an early response to DNA damage, Nature Genetics 37 (2005) 193–197.
[106] Z. Mao, A. Seluanov, Y. Jiang, V. Gorbunova, TRF2 is required for repair of nontelomeric DNA double-strand breaks by homologous recombination, Proceedings of the National Academy of Sciences of the United States of America 104 (2007) 13068–13073.
[107] P. Zhang, M.J. Pazin, C.M. Schwartz, K.G. Becker, R.P. Wersto, C.M. Dilley, M.P. Mattson, Nontelomeric TRF2-REST interaction modulates neuronal gene silencing and fate of tumor and stem cells, Current Biology: CB 18 (2008) 1489–1494.
[108] T. Simonet, L.E. Zaragosi, C. Philippe, K. Lebrigand, C. Schouteden, A. Augereau, S. Bauwens, J. Ye, M. Santagostino, E. Giulotto, F. Magdinier, B. Horard, P. Barbry, R. Waldmann, E. Gilson, The human TTAGGG repeat factors 1 and 2 bind to a subset of interstitial telomeric sequences and satellite repeats, Cell Research 21 (2011) 1028–1038.
[109] D. Yang, Y. Xiong, H. Kim, Q. He, Y. Li, R. Chen, Z. Songyang, Human telomeric proteins occupy selective interstitial sites, Cell Research 21 (2011) 1013–1027.
[110] N. Fouche, A.J. Cesare, S. Willcox, S. Ozgur, S.A. Compton, J.D. Griffith, The basic domain of TRF2 directs binding to DNA junctions irrespective of the presence of TTAGGG repeats, Journal of Biological Chemistry 281 (2006) 37486–37495.
[111] N. Bosco, T. de Lange, A TRF1-controlled common fragile site containing interstitial telomeric sequences, Chromosoma 121 (2012) 465–474.
[112] L. Fairall, L. Chapman, H. Moss, T. de Lange, D. Rhodes, Structure of the TRFH dimerization domain of the human telomeric proteins TRF1 and TRF2, Molecular Cell 8 (2001) 351–361.
[113] S. Hanaoka, A. Nagadoi, Y. Nishimura, Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA-binding activities, Protein Science 14 (2005) 119–130.
[114] A. Bianchi, R.M. Stansel, L. Fairall, J.D. Griffith, D. Rhodes, T. de Lange, TRF1 binds a bipartite telomeric site with extreme spatial flexibility, EMBO Journal 18 (1999) 5735–5744.
[115] S.H. Yoshimura, H. Maruyama, F. Ishikawa, R. Ohki, K. Takeyasu, Molecular mechanisms of DNA end-loop formation by TRF2, Genes to Cells 9 (2004) 205–218.
[116] S. Amiard, M. Doudeau, S. Pinte, A. Poulet, C. Lenain, C. Faivre-Moskalenko, D. Angelov, N. Hug, A. Vindigni, P. Bouvet, J. Paoletti, E. Gilson, M.J. Giraud-Panis, A topological mechanism for TRF2-enhanced strand invasion, Nature Structural & Molecular Biology 14 (2007) 147–154.
[117] K.K. Bisht, Z. Daniloski, S. Smith, SA1 binds directly to DNA through its unique AT-hook to promote sister chromatid cohesion at telomeres, Journal of Cell Science 126 (2013) 3493–3503.
[118] A. Poulet, S. Pisano, C. Faivre-Moskalenko, B. Pei, Y. Tauran, Z. Haftek-Terreau, F. Brunet, Y.V. Le Bihan, M.H. Ledu, F. Montel, N. Hugo, S. Amiard, F. Argoul, A. Chaboud, E. Gilson, M.J. Giraud-Panis, The N-terminal domains of TRF1 and TRF2 regulate their ability to condense telomeric DNA, Nucleic Acids Research 40 (2012) 2566–2576.
[119] O.G. Berg, R.B. Winter, P.H. von Hippel, Diffusion-driven mechanisms of protein translocation on nucleic acids. 1. Models and theory, Biochemistry 20 (1981) 6929–6948.
[120] P.H. von Hippel, O.G. Berg, Facilitated target location in biological systems, Journal of Biological Chemistry 264 (1989) 675–678.
[121] J. Gorman, E.C. Greene, Visualizing one-dimensional diffusion of proteins along DNA, Nature Structural & Molecular Biology 15 (2008) 768–774.
[122] A. Tafvizi, L.A. Mirny, A.M. van Oijen, Dancing on DNA: kinetic aspects of search processes on DNA, Chemphyschem 12 (2011) 1481–1489.
[123] T. de Lange, Telomere biology and DNA repair: enemies with benefits, FEBS Letters 584 (2010) 3673–3674.
[124] J. Lin, P. Countryman, N. Buncher, P. Kaur, L.E.Y. Zhang, G. Gibson, C. You, S.C. Watkins, J. Piehler, P.L. Opresko, N.M. Kad, H. Wang, TRF1 and TRF2 use different mechanisms to find telomeric DNA but share a novel mechanism to search for protein partners at telomeres, Nucleic Acids Research (2013).
[125] A. Reichel, D. Schaible, N. Al Furoukh, M. Cohen, G. Schreiber, J. Piehler, Noncovalent, site-specific biotinylation of histidine-tagged proteins, Analytical Chemistry 79 (2007) 8590–8600.
[126] J. Gorman, F. Wang, S. Redding, A.J. Plys, T. Fazio, S. Wind, E.E. Alani, E.C. Greene, Single-molecule imaging reveals target-search mechanisms during DNA mismatch repair, Proceedings of the National Academy of Sciences of the United States of America 109 (2012) E3074–E3083.
[127] F.W. Schwarz, J. Toth, K. van Aelst, G. Cui, S. Clausing, M.D. Szczelkun, R. Seidel, The helicase-like domains of type III restriction enzymes trigger long-range diffusion along DNA, Science 340 (2013) 353–356.
[128] S. Kabir, A. Sfeir, T. de Lange, Taking apart Rap1: an adaptor protein with telomeric and non-telomeric functions, Cell Cycle 9 (2010) 4061–4067.
[129] F. Yeung, C.M. Ramirez, P.A. Mateos-Gomez, A. Pinzaru, G. Ceccarini, S. Kabir, C. Fernandez-Hernando, A. Sfeir, Nontelomeric role for Rap1 in regulating metabolism and protecting against obesity, Cell Reports 3 (2013) 1847–1856.
[130] P. Martinez, G. Gomez-Lopez, F. Garcia, E. Mercken, S. Mitchell, J.M. Flores, R. de Cabo, M.A. Blasco, RAP1 protects from obesity through its extratelomeric role regulating gene expression, Cell Reports 3 (2013) 2059–2074.
[131] J.M. Platt, P. Ryvkin, J.J. Wanat, G. Donahue, M.D. Ricketts, S.P. Barrett, H.J. Waters, S. Song, A. Chavez, K.O. Abdallah, S.R. Master, L.S. Wang, F.B. Johnson, Rap1 relocalization contributes to the chromatin-mediated gene expression profile and pace of cell senescence, Genes & Development 27 (2013) 1406–1420.
[132] J. Sarthy, N.S. Bae, J. Scrafford, P. Baumann, Human RAP1 inhibits non-homologous end joining at telomeres, EMBO Journal 28 (2009) 3390–3399.
[133] A. Sfeir, S. Kabir, M. van Overbeek, G.B. Celli, T. de Lange, Loss of Rap1 induces telomere recombination in the absence of NHEJ or a DNA damage signal, Science 327 (2010) 1657–1661.
[134] B. Li, S. Oestreich, T. de Lange, Identification of human Rap1: implications for telomere evolution, Cell 101 (2000) 471–483.
[135] I.I. Cisse, I. Izeddin, S.Z. Causse, L. Boudarene, A. Senecal, L. Muresan, C. Dugast-Darzacq, B. Hajj, M. Dahan, X. Darzacq, Real-time dynamics of RNA polymerase II clustering in live human cells, Science (2013).
[136] P. Zhu, H.G. Craighead, Zero-mode waveguides for single-molecule analysis, Annual Review of Biophysics 41 (2012) 269–293.
[137] A.J. Cesare, J.D. Griffith, Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops, Molecular and Cellular Biology 24 (2004) 9948–9957.

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