Emerging Accessibility Patterns in Long Telomeric Overhangs

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ABSTRACT: We present single molecule experimental and computational modeling studies investigating the accessibility of human telomeric overhangs of physiologically relevant lengths. We studied 25 different overhangs that contain 4-28 repeats of GGGTTA (G-Tract) sequence and accommodate 1-7 tandem G-quadruplex (GQ) structures. Using FRET-PAINT, we probed the distribution of accessible sites via a short imager strand, which is complementary to a G-Tract and transiently binds to available sites. We report accessibility patterns that periodically change with overhang length and interpret these patterns in terms of the underlying folding landscape and folding frustration. Overhangs that have [4n]G-Tracts, (12, 16, 20…), demonstrate the broadest accessibility patterns where the PNA probe accesses G-Tracts throughout the overhang. On the other hand, constructs with [4n+2]G-Tracts, (14, 18, 22…), have narrower patterns where the neighborhood of the junction between single and double stranded telomere is most accessible. We interpret these results as the folding frustration being higher in [4n]G-Tract constructs compared to [4n+2] G-Tract constructs. We also developed a computational model that tests the consistency of different folding stabilities and cooperativities between neighboring GQs with the observed accessibility patterns. Our experimental and computational studies suggest the neighborhood of duplex junction is least stable and most accessible, which has implications for Shelterin complex formation that require access to this site.

SIGNIFICANCE STATEMENT: We report single molecule FRET-PAINT measurements and computational modeling studies probing the accessibility of 25 different human telomeric overhangs which, to our knowledge, is the most comprehensive range studied to date and covers a significant portion of the physiologically relevant length scale. Our measurements demonstrate novel repeating accessibility patterns with a well-defined periodicity, with certain regions having significantly higher accessibility than others. These accessibility patterns also suggest the underlying folding frustration depends on telomere length and constructs with a certain number of telomeric repeats demonstrate elevated levels of frustration compared to others. These patterns have significant implications for telomere organization, protection of free 3'-end against exonuclease activity and telomerase-catalyzed extension, and folding cooperativity between neighboring G-quadruplex structures.

INTRODUCTION

Telomeres are specific DNA-protein complexes, located at the ends of linear chromosomes and play a vital role in promoting their integrity. They are involved in safeguarding the chromosome ends by differentiating them from DNA double-strand breaks, which would otherwise trigger unwanted DNA damage response (1, 2). Human telomeric DNA is composed of tandem repeats of hexanucleotide d(GGGTTA) arranged into a long double-stranded region followed by a 50-300 nucleotide (nt) long 3′ single-stranded overhang (3). These unique telomeric repeats are conserved across vertebrates and beyond (4). The length of telomeres is important for their protective functions (5, 6); however, the inability of DNA replication machinery to complete the replication and processing of chromosome ends causes progressive telomere shortening during successive cell divisions (7). When a critical telomere length is reached, apoptosis or cell senescence is activated in somatic cells (8). Certain oncogenic cells continue to proliferate by overexpressing telomerase ribonucleoprotein complex which elongates telomeres, eventually leading to development of malignant tumors (6, 9). Telomerase includes a reverse transcriptase enzyme that synthesizes telomeric DNA repeats d(GGTTAG) using an internal RNA template (3, 10).

The G-rich overhang allows human telomeric DNA to fold into compact non-canonical structures called G-quadruplex (GQ). GQ is formed when four repeats of d(GGGTTA) are arranged into planar quartet geometry, stabilized by Hoogsteen hydrogen bonding and centrally located monovalent cation. For brevity, the d(GGGTTA) sequence will be referred to as a "G-Tract". Potentially GQ forming sequences are concentrated in certain regions of the human genome, including telomeres and promoters, and GQs have been confirmed to form in vitro (11, 12) and in vivo (13–15). GQs are implicated in crucial biological processes, such as transcription, recombination, and replication (16, 17). Telomeric GQs can inhibit telomerase activity, preventing telomere elongation (18).

Most studies on GQs have typically focused on understanding the structure and function of "single" DNA or RNA GQ molecules (19–21) and their interactions with DNA binding proteins (22–24) and helicases (25–28). However, the presence of many G-Tracts in human telomeric overhangs allows formation of 5-10 tandem GQs with varying separations. How these tandem GQs interact with each other, and their folding characteristics would clearly impact telomere accessibility. Prior studies on DNA constructs that form multiple telomeric GQs have typically investigated the thermodynamic, kinetic, or structural characteristics of isolated DNA molecules but have not directly studied telomere accessibility, which is the primary goal of this study. The studies on isolated tandem GQs have not

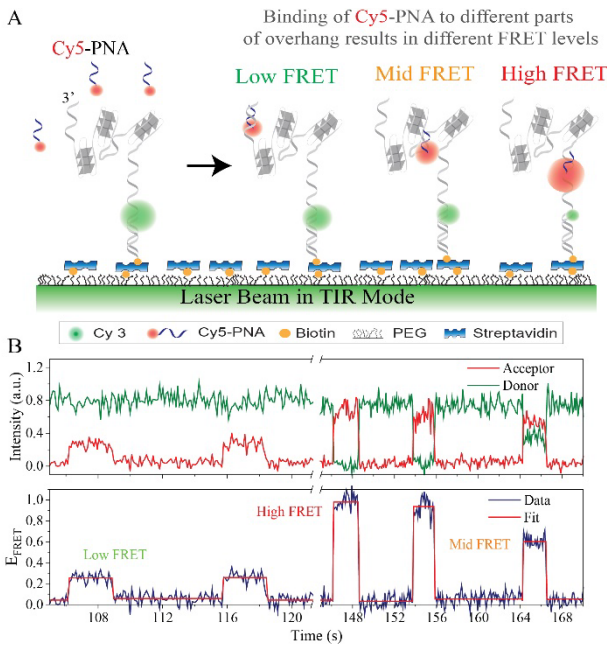

Figure 1: Schematic of FRET-PAINT assay. (A) Partial duplex DNA (pdDNA) construct with donor fluorophore (Cy3) is immobilized on the PEGylated surface via biotin-streptavidin attachment. The pdDNA construct has a telomeric overhang with multiple GGGTTA (G-Tract) repeats, which can form GQs separated from each other with unfolded regions of varying length. FRET signals with varying levels are observed when the imager strand (Cy5-PNA), which is complementary to a G-Tract, transiently binds to different segments of the overhang. (B) An example smFRET time trace showing five binding events with low, mid, and high FRET levels. The red line is a fit to the FRET trace to indicate the corresponding FRET levels.

reached a consensus and have either concluded negligible interactions, stabilizing (positive cooperativity) stacking interactions, or destabilizing interactions (negative cooperativity) take place between neighboring GQs (29–34). Variations in ionic conditions, temperatures, sensitivity of probes to different thermodynamics parameters, or the design of the DNA constructs might have played a role in these different conclusions.

In this study, we investigated the accessibility patterns of telomeric single-stranded DNA (ssDNA) molecules that contain 4-28 G-Tracts, a total of 25 constructs, using the single molecule FRET-PAINT (Förster Resonance Energy Transfer-Point Accumulation for Imaging in Nanoscale Topography) method (34). The telomeric ssDNA was part of a partial-duplex DNA (pdDNA) construct that contained a double-stranded DNA (dsDNA) stem and single stranded telomeric overhang. This also mimics the physiological structure where the single stranded

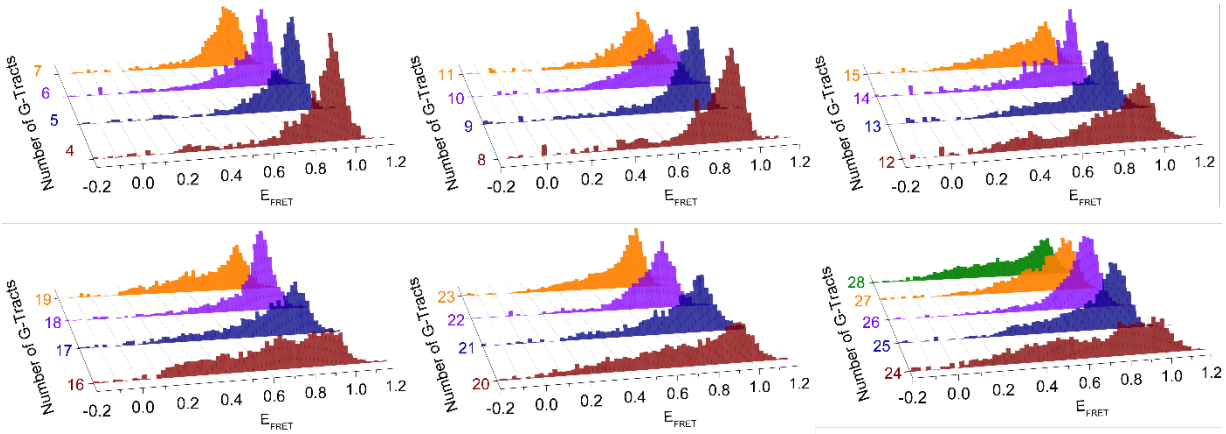

Figure 2: Normalized FRET histograms for telomeric overhangs with 4-28 G-Tracts. The histograms are grouped in sets of 4 with [4n]G-Tracts in brown, [4n+1]G-Tracts in blue, [4n+2]G-Tracts in violet and [4n+3]G-Tracts in orange. The 28G-Tract construct is added to the last histogram in green. The FRET histograms, which represent accessibility patterns, are broadest in [4n]G-Tract constructs and narrowest in [4n+2]G-Tract constructs.

telomeric overhang is connected to the double stranded telomeric region. The sequences we studied can form 1-7 tandem GQs or could have multiple unfolded G-Tracts at different segments of the overhang. Binding of a Cy5-labeled short complementary Peptide Nucleic Acid (PNA) strand (TAACCCTT-Cy5 in which the underlined nucleotides are complementary to telomeric sequence) to available G-Tract results in a FRET signal, which is used as an indicator for telomere accessibility. This FRET signal depends on the position of the binding site within the overhang and the folding pattern between the binding site and the ssDNA/dsDNA junction, where Cy3 is located. The resulting FRET distributions represent the accessibility patterns of the overhang.

As accessibility patterns depend on the underlying GQ folding landscape, we also studied their implications for folding characteristics. For this, we developed a computational model that calculates the resulting accessibility patterns when folding stabilities for different segments of the overhang and nature of interactions between neighboring GQs are modulated. With this approach, we mapped the resulting patterns for a broad range of stability constants and investigated the resulting patterns for positive, negative and negligible cooperativity between neighboring GQs.

A two state telomere model with nearest neighbor interactions (a kind of Ising model) was introduced by Mittermaier *et al.* who noticed that combinatorial factors associated with the location of the folded GQs along the telomere and its finite length conspire to produce non-trivial accessibility patterns (33). The authors introduced the term frustration to describe the diversity of folding patterns in states with similar energy. This entropic and topological frustration has thermodynamic and kinetic consequences in G-tract accessibility of telomeres. In the present paper, we find that this simple model supports a surprising diversity of thermodynamic accessibility patterns which are sensitive to GQ interaction strength, stability, and telomere length.

More broadly, frustration in biomolecules (35) is perhaps more familiar as a component of the funneled energy landscape of proteins (36). While natural protein sequences are minimally frustrated, landscape ruggedness and frustration is evident in patterns of frustration in many proteins (37), with locally frustrated regions often associated with allosteric conformation change (38) and protein-ligand binding sites (37). The frustration associated with folding of repeat proteins with coupled neighboring repeat units is perhaps most closely related to the frustration of GQ folding in telomeres. In repeat proteins, the balance between the energy of folding each element and the interactions is subtle, leading to a rich variety of behaviors. Proteins poised at a particular balance may be susceptible to local perturbations causing large structural effects (39). One-dimensional Ising models similar to the GQ model employed in this paper have been used to understand the folding thermodynamics and kinetics of repeat proteins (39, 40).

While the energy landscape of DNA GQ folding has been studied much less than in proteins, it appears that the folding landscape of human telomeric GQ is not funneled (41) and has a high degree of frustration as evidenced by stable folding intermediates (42), alternative conformers (43), and sensitivity of the GQ topology to perturbations such as small changes in loop length (44). In this paper, the frustration does not refer to the to the frustration in folding of a single GQ (which we take as two state in the model) but rather to the frustration associated with the folding patterns along the telomere in the sense of the term introduced by Mittermaier *et al.*

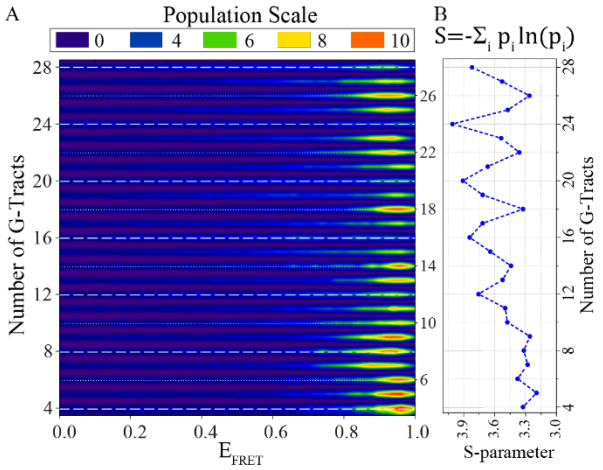

Figure 3: Contour plot of accessibility patterns and variation of S-parameter with overhang length. (A) The histograms in Figure 2 are combined in a contour plot that demonstrates broad histograms for [4n]G-Tract constructs (for 12G-Tract and longer) and narrower histograms for other constructs, especially [4n+2]G-Tract constructs. The overhangs with [4n]G-Tracts are marked with dotted white lines. (B) To quantify the broadness of the histograms in Figure 2, an S-parameter (defined on top) is introduced. S-parameter is analogous to Shannon entropy and is a measure of folding frustration: the larger the S-parameter the greater the folding frustration. At lengths longer than 12G-Tract, a pattern is established where the maxima of S-parameter occur at [4n]G-Tract constructs (maximum frustration) while minima are at [4n+2]G-Tract (minimum frustration).

RESULTS AND DISCUSSION

We investigated the accessibility patterns of 4-28 G-Tract long telomeric overhangs using a FRET-PAINT assay (Figure 1A). FRET-PAINT combines attributes of the super-resolution microscopy technique DNA-PAINT (45) with those of single-molecule FRET (smFRET) (46). This methodology takes advantage of transient binding of a labeled probe (called imager strand) to a target strand. As the imager strand, we used an acceptor (Cy5) labeled PNA that is complementary to a 7-nt segment of telomeric sequence. The imager strand is introduced to a microfluidic chamber that contains surface-immobilized, donor (Cy3) labeled partial duplex DNA (pdDNA) constructs that have a single stranded telomeric overhang (Figure 1A). The surface immobilized DNA is annealed and slowly cooled to room temperature to ensure the folding pattern reaches a thermodynamic steady state. A FRET signal that depends on the position of the binding site is observed when Cy5-PNA transiently binds to an accessible G-Tract (Figure 1B).

When fully folded, the longest telomeric overhang we studied (28G-Tract) can form up to seven tandem GQs. However, the number of folded GQs and how the unfolded G-Tracts are distributed would vary depending on the folding pattern. This would also influence the observed FRET levels. Figure 2 shows the normalized FRET distributions obtained from transient bindings of Cy5-PNA to unfolded G-Tracts in telomeric overhangs. The histograms are normalized such that each binding event (regardless of how long it lasts) contributes by the same amount and the cumulative area under each histogram equals 100. In accordance with this constant normalization factor of 100, these distributions represent probability distributions of binding events. The data are grouped in sets of four histograms, with the shortest construct in each set having an integer multiple of 4 G-Tracts, [4n], while the longest construct has an additional three G-Tracts, [4n+3]. The constructs that do not have any unfolded G-Tracts or those whose unfolded sites are not accessible to the probe do not contribute to these histograms.

These FRET distributions reveal surprising accessibility patterns that emerge when the telomeric overhang reaches a length of about 10 G-Tracts. The patterns have a periodicity of 4 G-Tracts and persist in the 10-28 G-Tract range. While the [4n]G-Tract constructs have the broadest distributions, the [4n+2]G-Tract constructs are typically the narrowest. These narrower distributions are concentrated at high-FRET levels, which represent binding to sites closer to the ssDNA/dsDNA junction (5´-side). The broad distributions of [4n]G-Tract constructs suggest the binding sites, i.e., unfolded G-Tracts, are distributed throughout the overhang. Figure 3A shows a contour plot of the combined data for all 25 constructs that illustrates alternating broad (marked with white dashed lines) and narrow (marked with white dotted lines) distributions. The same bin-size was used in this contour plot as that used in the histograms in Figure 2. To quantify the broadness of FRET distributions, we defined an S-parameter which is analogous to Shannon entropy. The S-parameter describes the uncertainty associated with determining where on the telomeric overhang the imager strand would bind (Figure 3B). This uncertainty is greater for broad distributions since the imager strand has similar probabilities to bind to different sites. Therefore, the maxima of S-parameter should occur at [4n]G-Tract constructs and the minima at [4n+2]G-Tract constructs.

The accessibility patterns presented in Figure 2 have implications about the folding landscape. Broad distributions suggest Cy5-PNA binding sites are distributed throughout the overhang and show significant variations for different molecules. On the other hand, narrow distributions suggest binding sites are concentrated in a particular region and show a higher level of consistency among different molecules.

Even though not identical to the way it is defined in some studies (33), these patterns could be interpreted in terms of folding frustration. We would expect the binding sites to be distributed over broader regions and show greater variability between different molecules for systems with higher folding frustration. With this identification in mind, our data suggest [4n]G-Tract constructs have significantly higher frustration compared to other constructs. Adding 1-3 G-Tracts resolves this frustration to different extents, resulting in a concentration of unfolded sites in the vicinity of the ssDNA/dsDNA junction, on the 5´-side. This implies the G-Tracts at the 3´-end or intermediate regions have higher folding stability compared to those at the 5´-side. The proximity of these sites to a duplex DNA, which mimics the way telomeric DNA is in a physiological setting, could be the reason for their reduced stability.

The [4n]G-Tract constructs have an exact number of G-Tracts that can accommodate n GQs. Therefore, attaining complete folding requires a perfect progression of the folding throughout the overhang. For these constructs, nucleation of GQ folding from multiple sites will likely result in leaving unfolded G-Tracts between neighboring GQs. Also, skipping of even one G-Tract during progression of the folding, in other words two neighboring GQs separated by a single unfolded G-Tract, would result in three more unfolded G-Tracts at different regions of the overhang. We assume GQs with long loops that incorporate one or more G-Tracts (9 nt or longer loops instead of the canonical 3 nt long loops) are highly unlikely due to entropic considerations (33). On the other hand, constructs with additional G-Tracts ([4n+1], [4n+2] and [4n+3]) are more tolerant for shifts in the G-Tract register during folding or initiation of folding from different sites. Assuming lower folding stability at the 5´-side, nucleation of folding from the 3´-end or intermediate regions would more likely result in unfolded G-Tracts at the 5´-side. Once a particular folding landscape is established, it persists for long periods of time due to the high stability of associated GQ structures, which prevents resolving the frustration in folding (33). The observed patterns also imply relatively efficient progression of folding towards the 5´-side without leaving many unfolded G-Tracts between neighboring GQs. These observations have physiologically significant implications as the free 3´-ends of telomeres are more prone to degradation by exonuclease activity and folding into GQ helps in protecting these ends (47). In addition, both telomerase and alternative lengthening of telomere (ALT) mechanisms require unfolded 3´-ends for the extension of telomeres (48) and presence of such secondary structures could potentially render telomeres inaccessible. The ssDNA/dsDNA junction that is more likely to be unfolded is the region where POT1-TPP1 connect to members of Shelterin complex that are localized on duplex DNA (49). Therefore, having unfolded G-Tracts at this region would facilitate binding of POT1 and establishing this connection. This would also reduce accessibility of these regions to other agents. Interestingly, the observed accessibility pattern onsets when telomeric overhang reaches ~10G-Tract, which corresponds to ~60 nt. This is similar to the minimum telomere length (~50 nt) that is required before the cells undergo senescence or apoptosis. Further studies are required to establish whether these repeating accessibility and folding patterns persist in the presence of the Shelterin complex or play any role in telomere maintenance or organization.

There are several important aspects regarding the experimental approach employed in this study that need to be discussed and clarified in order establish the validity and significance of our approach. As the main goal of our study is to create accessibility maps of telomeric overhangs, a probe that can access telomeres needs to be employed. This aspect is different from most prior studies where folding patterns and interactions between GQs were studied for telomeric DNA in isolation. These studies did not directly probe accessibility; therefore, did not require a probe. Our study is more akin to what telomerase would experience, for example, as its RNA template needs to access and hybridize with the telomeric overhang. Naturally, every agent that accesses telomeric DNA would impact the underlying folding pattern to a certain degree. There are many physiologically relevant interactions that impact the folding patterns in the telomeres and our study should be considered as a case where such a scenario is mimicked *in vitro*. Nevertheless, by choosing a very small PNA probe that is adequate for studying experimentally detectable binding events (which last shorter than a few seconds), we sought to minimize the impact of the probe on the underlying folding pattern. The Cy5-PNA only base-pairs with 7 nt telomeric overhang and is smaller than the minimum unfolded site, which would be 9 nt long (TTAGGGTTA). To determine the thermodynamic stability of the PNA/DNA duplex that would form upon binding of Cy5-PNA to a telomeric sequence, we performed FRET melting measurements under identical ionic conditions to those of smFRET measurements and obtained T_m=12.7±0.3 °C (Supplementary Figure S2). This is consistent with the estimate of a model that predicts T_m of a PNA/DNA duplex based on the melting point of a DNA/DNA duplex of the same sequence. According to this model, T_m=13.7 °C (with a typical deviation of a few °C from experimental results) under the ionic conditions and oligo concentrations of our assay (50). Even such a weak disturbance will impact the stability of the telomeric GQs to a certain extent; however, the impact would be minimal (at least at an experimentally detectable scale). This is also evident in the low

frequency of the binding events we observe and the fact that 80% or more of all the DNA molecules we observe do not show any binding events during the experimental observation time (2-3 minutes). To provide a quantitative comparison, the frequency of binding events to a single G-Tract (an exposed G-Tract in the absence of GQ) is over an order of magnitude greater than the frequency of binding events we observe for the 4G-Tract construct (Supplementary Figure S3). In this context, one might expect that there should not be any binding events for a 4G-Tract construct since all G-Tracts would be protected within a GQ. However, this assumes all the DNA molecules are perfectly folded and remain so throughout the few minutes observation time. Even though this is true for most of molecules which do not show any binding events, it is not valid for all molecules that might be misfolded or unfolded for a brief period when the probe gains access to telomeres. Another piece of evidence for the Cy5-PNA probe not destabilizing the GQs in KCl comes from the observation that the frequency of binding events are much higher in LiCl compared to KCl, as we demonstrated in an earlier study (34).

The impact of the 3D structure of the telomeric overhang on the observed FRET levels is another aspect that needs to be considered. Potential interactions between neighboring GQs and the flexible segments (for instance the junction region or the TTA sequences that link consecutive GQs) in the telomeric overhang could impact the relationship between the observed FRET and telomere length. However, our data show that the correlation between the observed FRET efficiencies and telomere length are maintained. This is particularly evident in the histograms for [4n]G-Tract constructs where the binding sites are distributed throughout the overhang and hence provide more information about the 3D structure compared to constructs where binding sites are concentrated in a particular region. In the [4n]G-Tract constructs, the FRET distributions systematically shift to lower FRET values as the overhang length is increased, suggesting the correlation between FRET efficiency and location of binding site with respect to the junction (where the donor fluorophore is located) is not eliminated. If for example, the telomeric overhang were to fold onto itself beyond a certain length (e.g. the 3'-end were to position in the vicinity of the ssDNA/dsDNA junction), we would have expected a shift towards higher FRET values beyond this telomere length. However, this is not what is observed. We quantified this by studying the fraction of FRET population for $E_{FRET}<0.50$ and demonstrate that this population increases as telomere length is increased (Supplementary Figure S4) (34). Clearly, there are degeneracies and ambiguities in mapping a particular FRET value to a specific binding site: Depending on the folding pattern, binding to different sites might result in similar FRET efficiencies or binding to a particular site could result in different FRET values. The approach employed in this study does not claim to resolve these issues and our conclusions are based on the patterns and are limited to only distinguishing three regions in the overhang: the junction region, the broad intermediate region, and the vicinity of the 3'-end. Similarly, in the modeling efforts that will be described next, we based our conclusions on the patterns observed in the FRET distributions and their periodicity (Fig. 3), which have persisted despite such ambiguities and degeneracies. If 3D structure of the overhang were to eliminate the correlation between the measured FRET efficiency and binding site position, such patterns would not have persisted. Therefore, we believe the data are not overinterpreted.

Another structural issue that needs discussion is whether the FRET-PAINT approach remains sensitive to binding events in the vicinity of the 3'-end of long telomeric overhangs. For unstructured ssDNA, it would not have been possible to detect binding events 100 nt away from a reference site (location of donor fluorophore) using Cy3/Cy5 as FRET pairs. However, the telomeric overhangs are highly folded and stable (most DNA molecules do not show any binding events during the 2-3 min observation time) under our assay conditions and contain flexible linkers between consecutive GQs that reduce end-to-end separation. Therefore, the overhangs are much more compact than unstructured DNA. If our approach did not have sensitivity to binding events at the distal regions beyond a certain overhang length, the FRET distributions for [4n]G-Tract constructs would broaden until a certain length and remain unchanged beyond that. However, this is not what we observe as the distributions continue to broaden as the overhang length increases. This is quantified by comparing the population for $E_{FRET}<0.5$ with that for $E_{FRET}>0.5$ as a function overhang length, where the lower FRET population continues to increase throughout the range of constructs we study. We performed additional measurements to test sensitivity of the method by designing DNA constructs that have two binding sites, one on either side of 1-4 consecutive GQs with a modified sequence (Supplementary Figure S5). In another set of measurements, we designed constructs that have a single binding site after 1-5 consecutive modified GQs (Supplementary Figure S6). In these measurements, we replaced the telomeric GQ formed by $(GGGTTA)_4$ sequence with a GQ formed by $(GGGT)_4$, which is much more stable and attains only the parallel conformation (22). To enable Cy5-PNA binding to specific sites, we placed a TTAGGGTTA sequence at these locations. In both sets of measurements (Fig. S5-S6), we demonstrate that while the FRET levels for distal binding site gradually decrease as overhang becomes longer, they remain well within detectable range even for the longest construct we studied.

Another piece of evidence for sensitivity of the method is the fact that the FRET distributions alternate between broad and narrow distributions even though the overhang length is systematically increased. If the method did not have sensitivity to these distal binding events, the histograms would not have switched from narrow at 26 G-Tract to broad at 28G-Tract and 24 G-Tract.

Whether accessibility of a particular open site is impacted by its neighborhood is another relevant issue. To illustrate, an open site (a G-Tract that is not part of a GQ) could have folded GQs on both sides of it, a folded GQ on one side and another open site on the other, or open sites on both sides of it. Possible arrangements multiply if beyond nearest neighbors are considered. Since we do not have any control on which of these arrangements prevails for a given open site, we did not quantify the impact of the neighborhood structures on accessibility. However, since over a hundred binding events are used to create each of the histograms in Fig. 2, these effects are expected to average out. Attaining more precise information would require performing systematic measurements on many constructs where certain configurations are biased by introducing mutations in the sequence, which will be the focus of future studies.

The impact of the fluorescent probe (Cy5) on GQ stability is another issue that needs to be discussed. The Cy5 is attached to PNA via a flexible linker and an additional thymine (TAACCCTT-Cy5, the T that is not underlined does not hybridize with the DNA) separates the fluorophore from PNA/DNA hybrid duplex. Even if Cy5-PNA binds in the immediate vicinity of a GQ, there will be a TT overhang (the A in TTA loop will hybridize with the last underlined T in Cy5-PNA) between the GQ and the PNA/DNA duplex. So overall, there will be 3 nucleotides that are not part of the GQ or the PNA/DNA duplex, the width of the PNA/DNA duplex, and the 6-carbon long flexible linker connecting the Cy5 to PNA that separate the Cy5 from the nearest GQ. Similarly, the Cy3 fluorophore at the junction (placed on the short strand that does not contain the telomeric overhang) is separated by the nearest GQ by at least three nucleotides (TTA in the junction, in addition to six carbon long flexible linker). Earlier studies have shown that Cy3/Cy5 fluorophores placed at the terminal nucleotide tend to stack on the dsDNA (51) (similar to an additional base), and therefore these fluorophores are not expected to have a significant impact on the nearest GQ that is separated by at least three nucleotides. In most studies that employ fluorescent dyes (including bulk FRET melting assays), the fluorophores are separated from the GQ by 0-2 nt overhangs and a flexible linker. Due to their small size, brightness, and photostability, Cy3/Cy5 are among the most used fluorophores in these studies. Even though these would not warrant zero interaction between the fluorophore and the GQ (52), the impact should be small and should be considered as an overall impact of the probe on the telomeric overhang.

The observed accessibility patterns have implications about the underlying folding topography. We sought to explore this connection by a computational model that estimates unfolding probability (P_i) of a G-Tract based on folding cooperativity (K_c) between neighboring GQs and the position of the G-Tract, which influences both combinatorial considerations and the statistical weight of being in the folded state, i.e. being one of the four G-Tracts that form a GQ. For a telomeric overhang with N G-Tracts, the stabilities of a GQ in the interior ($K_f = 60$), a GQ ending at the 3´-end ($K_{f,N} = 229$), and a GQ ending at the neighboring G-Tract ($K_{f,N-1} = 80$), were kept similar to those reported in a recent study by Mittermaier *et al.* due to similarity of our pdDNA construct to their ssDNA construct in the middle and 3´-ends (33). Varying these parameters from these values resulted in similar results as long as $K_{f,N-1}$ was greater than K_f and $K_{f,N}$ was significantly greater than $K_{f,N-1}$. On the 5´ side, our constructs have a ssDNA/dsDNA junction rather than a free end as in ssDNA, which resulted in significantly lower stability for G-Tracts in this region. A similar destabilization due to flanking duplex regions was recently reported for the GQs that form in KIT promoter (53). We estimated the folding parameters associated with the G-Tracts in the vicinity of ssDNA/dsDNA junction ($K_{f,1}$ and $K_{f,2}$) and the cooperativity parameter (K_c) by comparing the patterns observed for the experimentally measured and computationally calculated S-parameters, as described below.

The FRET signal measured in the experiment probes the location of unfolded G-Tracts. To compare the outcomes of the model with the experimental distribution measured as a function of telomere length, we calculate how delocalized the distribution of unfolded G-Tracts is for a given length via the S-parameter, as defined in the Methods section. As discussed earlier, this approach does not distinguish between different accessibilities for open sites that have different neighborhoods (folded GQs or open sites in immediate vicinity). We find that the model accommodates a variety of patterns of the calculated S-parameter as a function of telomere length depending on folding and cooperativity parameters in the model. Figure 4 shows a phase diagram as a function of the stability for a GQ starting with the 5´ G-Tract, $K_{f,1}$, and the cooperativity K_c. In this figure, we choose the weight for a GQ starting at the second G-Tract from the junction to be twice that of starting at the first G-Tract, i.e. $K_{f,2} = 2K_{f,1}$. Similar patterns are observed as long as $K_{f,2}$ is greater than $K_{f,1}$ and significantly smaller than K_f (Figure S7). For simplicity, we focus on a window composed of N=20 to N=24 G-Tracts to characterize the

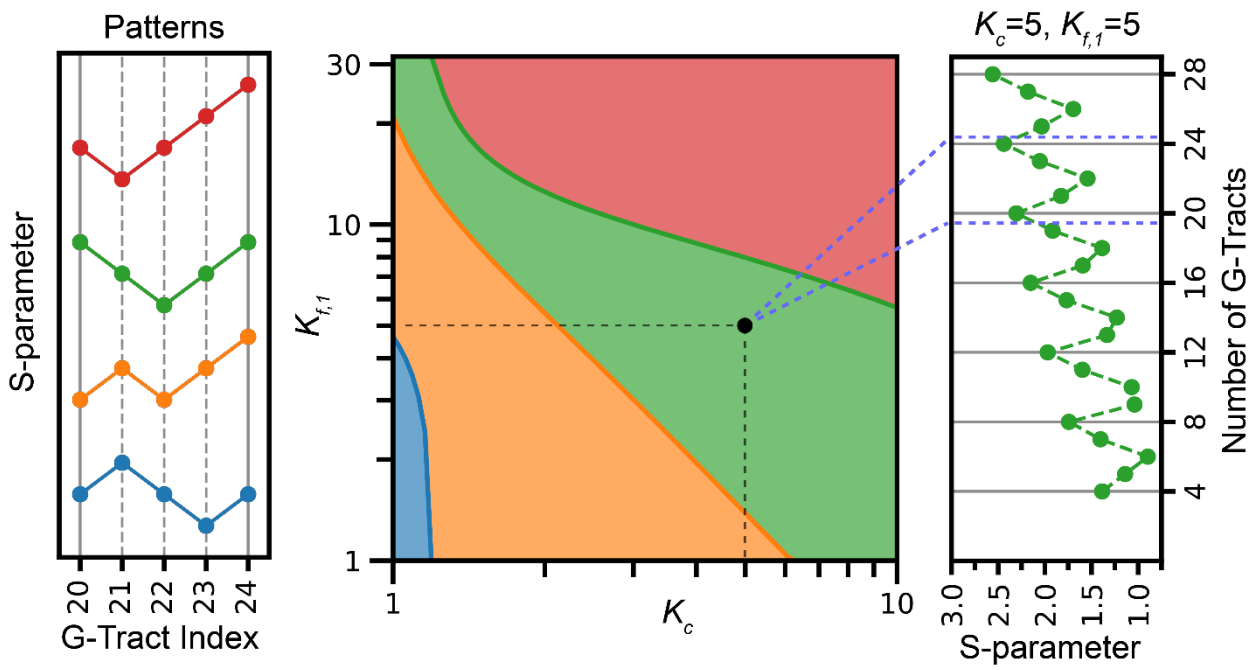

Figure 4: Computational patterns observed for the S-parameter for N=20-24 G-Tracts for varying $K_{f,1}$ and K_c parameters. In these calculations, $K_{f,1}$ and K_c are varied while $K_{f,2}$=2$K_{f,1}$, K_f=60, $K_{f,N-1}$=80, and $K_{f,N}$=229. The left panel shows the four patterns observed depending on whether the S-parameter increases or decreases as the length of the overhang is increased by one G-Tract. The phase diagram in the middle shows the parameter range where different patterns are observed in colors that match the patterns on the left panel. The green range corresponds to the pattern observed in the experiment. The right panel shows the calculated S-parameters for K_c=5 and $K_{f,1}$=5, which can be compared to the experimentally observed pattern in Figure 3B.

pattern of the S-parameter and monitor if the S-parameter increases or decreases as *N* increases within this range. With positive cooperativity, four patterns are observed for the S-parameters. The pattern observed in the FRET efficiency (colored green in Figure 4) occurs within a specific (though rather broad) range for parameters $K_{f,1}$, $K_{f,2}$, and K_c. For K_c >6, the stabilities of GQ starting near the ssDNA/dsDNA junction must be below an upper threshold to observe the appropriate pattern, and for smaller cooperativity, these stabilities must lie between a lower and upper bound. The S-parameter as a function of *N*, shown in Figure 4, is similar to what was experimentally observed. Because of this similarity to the experimentally measured S-parameter, we will use parameters ($K_{f,1}$ = 5, $K_{f,2}$ = 10, and K_c = 5) as an illustration in what follows.

To get a feel for the stabilities suggested by this model, at T = 25 C, the stability of an interior GQ relative to an unfolded G-Tract is given by ΔG = -2.43 kcal/mol and a GQ involving the first G-Tract at the ssDNA/dsDNA junction is ΔG = -0.95 kcal/mol. The free energy of two adjacent GQ has free energy ΔG = -5.81 kcal/mol, of which -0.95 kcal/mol is associated with cooperative stabilizing interactions between the GQ's. Destabilization of GQs at the ssDNA/dsDNA junction and positive cooperativity are consistent with the high FRET peak observed in many constructs. The free energies for GQ stability calculated from our model are consistent with those reported in literature(54); although, there is no consensus about whether the interaction between neighboring GQs is stabilizing or destabilizing. Several studies have investigated the interactions between neighboring GQs and possible higher-order structures that might form in telomeric overhangs using different constructs and experimental methods. However, these studies have not reached a consensus with conclusions varying from negligible stacking interactions (beads on a string type arrangement) (29) and destabilizing interactions (negative cooperativity) (33, 55) to higher order structures with multiple GQs condensing into compact structures (30, 34, 56, 57), which would suggest positive cooperativity. Our results are more consistent with these latter studies that propose positive cooperativity.

Figure 5 shows that the parameters identified by comparison of the computational and experimental S-parameters correspond to very stabilized GQs. Although the strong stability weakens as the number of G-Tracts increases, the average number of folded GQs (<n_{GQ}>) is nearly maximal for G-Tracts ranging from *N* = 4 to 28 (Figure 5A). Nevertheless, constructs with 4n

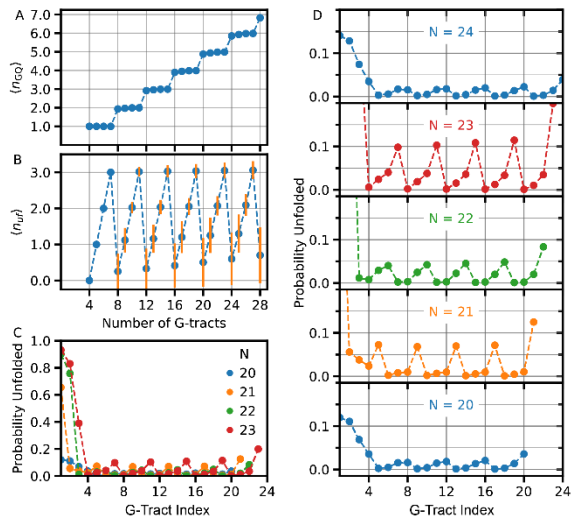

Figure 5. Folding levels for the set of parameters identified in Figure 4: $K_{f,1}$=5, $K_{f,2}$=10, K_f =60, $K_{f,N-1}$ =80, $K_{f,N}$=229 and K_c=5. These parameters result in high levels of folding for all constructs investigated although periodic patterns were observed for probability of being unfolded depending on the position of the G-Tract and length of overhang. (A) For the set of parameters used in these studies, the average number of GQ (<n_{GQ}>) is close to the maximum possible throughout 4-28 G-Tract overhang length. (B) Average number of unfolded G-Tracts (<n_{uf}>) as a function of overhang length. As expected, constructs with 4n+1, 4n+2, and 4n+3 G-Tracts have at least 1, 2, and 3 unfolded G-Tracts, respectively. As can be most clearly seen for 4n constructs, <n_{uf}> increases as the overhang length increases. The orange vertical lines are standard deviation in <n_{uf}>. (C) Probability of ith G-Tract being unfolded as a function its position for overhangs with N=20-24 G-Tracts. In this diagram i=1 for the G-Tract at ssDNA/dsDNA junction and i=*N* for the G-Tract at 3´-end. (D) The plots that are overlaid in (C) are separated for each N, and the y-axis scale is limited to unfolding probability in 0.0-0.2 range (0.0<P_i<0.2) to better illustrate the oscillations in the unfolding probability. The amplitude of oscillations in P_i vary depending overhang length, which can be related to the patterns observed for S-parameter.

and 4n+1 G-Tracts have somewhat higher average number of unfolded G-Tracts (<n_{uf}>) than the minimum associated with fully folded GQs (Figure 5B). This is consistent with the experiment where most of telomeric constructs do not show any binding events during experimental observation time (~3 min). Also, constructs with 4n and 4n+1 G-Tracts have large relative fluctuations so that <n_{uf}> is not a sharp value for these constructs, suggesting that the number of sites available to PNA binding can be significantly larger than indicated by <n_{uf}>.

Even though the unfolded G-Tracts are only a small fraction of the telomeric overhang, they are nevertheless physiologically very significant as they might serve as the nucleation sites for triggering DNA damage response, nuclease activity, or telomerase-mediated telomere extension. Therefore, understanding how they are distributed, and their relative concentration is important.

Patterns in the S-parameter can be understood as balancing the population of unfolded G-Tracts within the destabilized junction region, and the rest of the telomeric G-Tracts given the underlying frustration of the landscape and the minimum number of unfolded G-Tracts when the length of the construct differs from 4n. Because the first two repeats near the junction at the 5´ side are destabilized compared to others, the unfolded distribution is shifted towards the first two repeats. As shown in Figure 5C-D, the G-Tracts are mostly folded for *N* = 4n, with a weak pattern in unfolding propensity along the chain. For *N* = 4n+1, which has at least one unfolded G-Tract, the first G-Tract is much more likely unfolded, and the pattern for the unfolded G-Tract along the chain is more prominent. This localization of the unfolded G-Tracts is reflected in a decrease in the S-parameter. For *N* = 4n+2, the first two G-Tracts are unfolded with high probability and the remaining unfolding propensity decreases along the chain. Thus, the distribution of P_i is more localized and results in a local minimum of the S-parameter. For *N* = 4n + 3, unfolding of the first three G-Tracts are more likely, though the propensity of unfolding for other G-Tracts increases, giving rise to a more delocalized distribution along the chain, and hence an increase in the S-parameter.

A similar analysis of the patterns of the S-parameters shows that the pattern observed in the experiment does not appear for negative cooperativity (Supplement Figure S8). Also, looking at a broader window *N* = 12 – 24, we see that the pattern can shift from one type to another as a function of *N* near the boundaries of phases of the S-parameter (Supplementary Figure S9). Furthermore, these kinds of patterns occur not only in the S-parameter which explicitly depends on the distribution of unfolded G-Tracts, but also in the entropy as a function of *N* (Supplementary Figure S10).

As mentioned earlier, a consensus has not yet been established about the nature of interactions and cooperativity between neighboring GQs. While there are electron microscopy images showing several telomeric GQs stacking together to form compact structures (31) (which would suggest positive cooperativity), there are also thermodynamic studies that suggest negligible or destabilizing interactions (suggesting negative cooperativity) (30, 33). This is clearly a very important question that will continue to be investigated with different approaches and needs

more commentary about exactly what different techniques measure and what they are sensitive to. Whether the nature of interactions vary with temperature, asymmetry between the ends of the DNA construct (e.g., two free ends *vs.* presence of a destabilizing duplex at one end), proximity to a surface, ionic conditions, or employment of a probe will all need to be considered. This study differs in several significant aspects to most studies in literature: (i) We employ DNA constructs that contain a duplex DNA on side (similar to the physiological telomeric DNA) rather than two free ends as done in most studies; (ii) We directly probe accessibility of the telomeric overhang to a complementary probe (as would be the case for telomerase for example) and interpret the results in terms of their implications for relative stability of different segments of the overhang and interactions between neighboring GQs. Most studies in literature investigate telomeric DNA in isolation, establish stabilities of different segments and interpret the implications of these studies in terms of the accessibility of the overhang. To our knowledge, the accessibility maps demonstrated in Fig. 3 are the first demonstration of this kind of a direct approach. (iii) We employ a PNA probe in our studies as the main goal is to establish accessibility maps. Even though we argued the impact of this probe on the underlying folding topography is small, it is nevertheless not zero and differs in this respect from most studies in literature that study telomeric DNA in isolation. We do not consider this as a flaw in the approach but a required component to investigate accessibility. This approach clearly has physiological relevance as is evident from the cases of telomerase or TERRA, which contain nucleic acids sequences that interact with telomeres and impact underlying GQ structures. To summarize, our results should be evaluated in this broader perspective and should be considered as another piece of evidence that will help forming a coherent and more complete understanding of telomeric overhangs.

CONCLUSIONS

Our results demonstrate repeating accessibility and folding frustration patterns for telomeric overhangs of physiological lengths. Overhangs with [4n]G-Tracts demonstrate elevated levels of frustration where the PNA probe is able to access sites throughout the overhang. On the other hand, overhangs with two additional G-Tracts, [4n+2]G-Tracts, show minimal frustration where most accessible sites are concentrated in the vicinity of ssDNA/dsDNA junction. These accessibility patterns would help with protecting the free 3´-end against exonuclease activity and inhibit telomerase-mediated elongation while facilitating binding of POT1/TPP1 in the vicinity of ssDNA/dsDNA junction and connecting with the rest of the Shelterin complex. Both observations are consistent with the role of terminal GQs protecting telomeres against exonuclease activity. Our computational studies also capture the requirement for lower stability at the ssDNA/dsDNA junction to attain patterns consistent with those observed in the experiment.

METHODS

Nucleic Acid Constructs: DNA strands were purchased either from Eurofins Genomics or IDT DNA and their sequences are given in Table S1 and Table S2. The sequence of the Cy5-PNA probe was as follows: TAACCCTT-Cy5, the underlined nucleotides being complementary to telomeric repeats. The oligonucleotides were purified in-house using denaturing polyacrylamide gel electrophoresis (PAGE). The corresponding gel images are shown in Supplementary Figure S1. HPLC purified Cy5-PNA strand was purchased from PNA-Bio Inc. The pdDNA constructs (Figure 1A) were formed by annealing a long strand that contains the telomeric overhang (4-28 G-Tracts) with a short strand (18, 24, or 30nt) that has a biotin at the 3´-end and Cy3 at the 5´-end (Table S2). The two strands were annealed in a thermal cycler in 150 mM KCl and 10 mM $MgCl_2$ at 95°C for 3 minutes followed by a slow cooling to 30 °C (5°C decrease every 3 minutes). This slow cooling process ensures attaining a thermodynamic steady state for the folding pattern. It is important to note that 10 mM $MgCl_2$ was used only during the annealing process (to improve hybridization), while all measurements were performed at 2 mM $MgCl_2$. During annealing, excess long strand (500 nM for 100 nM of short strand) was used to ensure all biotinylated and Cy3-labeled short strands have a matching long strand. The unpaired long strands are washed out of channel after the DNA is immobilized on surface via biotin-streptavidin linker.

The smFRET assay and Imaging Setup: A home-built prism-type total internal reflection fluorescence (TIRF) microscope was used for these measurements following protocols described in earlier work (58). The slides and coverslips were initially cleaned with 1M potassium hydroxide (KOH) and acetone, followed by piranha etching, surface functionalization by amino silane and surface passivation by polyethylene glycol (PEG). To reduce nonspecific binding, the surfaces were treated with a mixture of m-PEG-5kDa and biotin-PEG-5kDa in the ratio 40:1 followed by another round of passivation with 333 Da PEG to increase the density of the PEG brush. The microfluidic chamber was created by sandwiching a PEGylated slide and a coverslip with double sided tape, followed by sealing the chamber with epoxy. The chamber was treated with 2% (v/v) Tween-20 to further reduce non-specific binding.

After washing the excess detergent from the chamber, 0.01 mg/mL streptavidin was incubated in the chamber for 2 minutes. The pdDNA samples were diluted to 10 pM in 150 mM KCl and 2 mM $MgCl_2$ and

added to the chamber for 2-5 minutes resulting in surface density of ~300 molecules per imaging area (~50 μm × 100 μm). The excess or unbound DNA was removed from the chamber with 150 mM KCl + 2 mM $MgCl_2$. Unless otherwise specified, the imaging buffer contained 50 mM Tris-HCl (pH 7.5), 2 mM Trolox, 0.8 mg/mL glucose, 0.1 mg/mL glucose oxidase, 0.1 mg/mL bovine serum albumin (BSA), 2 mM $MgCl_2$, 150 mM KCl and 40 nM Cy5-PNA. The Cy5-PNA strand was heated to 85 °C for 10 minutes prior to adding it to the imaging buffer. 1500-2000 frame long movies with 100 ms frame integration time were recorded using an Andor Ixon EMCCD camera. A green laser beam (532 nm) was used to excite the donor fluorophore and the fluorescence signal was collected by an Olympus water objective (60x, 1.20 NA).

Data Analysis: A custom written C++ software was used to record and analyze the movies, and to generate single molecule time traces of donor and acceptor intensities. The time traces of each molecule were further analyzed by a custom MATLAB code to select the molecules that passed a single molecule screening test. The background was subtracted for each of these molecules based on the remnant donor and acceptor intensities after donor photobleaches. The FRET efficiency (E_{FRET}) was calculated using E_{FRET} = Acceptor Intensity/(Acceptor Intensity + Donor Intensity). The molecules that did not show any binding event formed a donor-only (DO) peak at FRET efficiency E_{FRET}=0.06, which was used as a reference to rescale the FRET distribution such that DO peak corresponds to E_{FRET}=0.00. After this rescaling, the DO peak was subtracted from the histograms. Therefore, the FRET histograms presented in Figure 2 were generated from single molecules that show at least one Cy5-PNA binding event. The numbers of molecules that contributed to each histogram in Figure 2 are presented in Table S3, but typically each histogram was constructed from binding events to 100-150 DNA molecules, resulting in several hundred binding events in each histogram.

The FRET distributions were normalized such that each molecule contributes equally to the histogram and the total population across the entire FRET range was normalized to 100%, i.e., the population of a particular FRET bin represents its percent population. Because of this normalization, dividing the FRET distribution by 100 gives a distribution of probabilities for different FRET levels where the total probability is normalized to 1.0. Therefore, the probability p_i of a particular FRET level i (a particular FRET bin of width 0.02 FRET) is obtained by dividing population at that FRET level by 100. To quantify the spread in the FRET histograms, we define an S-parameter: $S = -\sum_i p_i \ln p_i$ where the summation is carried over the entire FRET range and p_i is the unfolded probability, normalized so that $\sum_{i=1}^{N} p_i = 1$. (Figure 3). To calculate the S-parameter predicted by the computational model, p_i is taken as the unfolded probability of ith G-Tract and the total probability is normalized so that $\sum_{i=1}^{N} p_i = 1$. The S-parameter is analogous to Shannon entropy of a particular FRET distribution, with broader distributions resulting in a larger S-parameter which represents greater uncertainty in determining the position of the binding site. These calculations were performed in Origin 2015.

Computational Model: We consider a simple model introduced by Mittermaier and co-workers consisting of N G-Tracts, each of which can either be unfolded or folded with three neighboring G-Tracts in a GQ (33). The statistical weight for a state α with N_f^{α} folded GQs and N_{int}^{α} interfaces between nearest neighbor GQs is given by $\omega_\alpha = K_f^{N_f^{\alpha}} K_c^{N_{int}^{\alpha}}$, where K_f is the weight associated with a folded GQ, and K_c models interactions between adjacent GQs. Folding cooperativity between neighboring GQs can be positive ($K_c > 1$), negative ($K_c < 1$), or neutral ($K_c = 1$). In Mittermaier's study, the DNA constructs were in the form of single stranded DNA with symmetrical 3′ and 5′ ends. This study demonstrated higher folding stability for GQs beginning or ending on the two terminal G-Tracts on each end: initiating on the 1st and 2nd G-tract at the 5'-end, or ending on the (N-1)th and Nth G-Tract at the 3'-end. Compared to the internal G-Tracts: $K_{f,1}, K_{f,2}, K_{f,N-1}$ *and* $K_{f,N}$ were greater than K_f for internal G-Tracts. In our study, we used a partial duplex DNA, which is a better representative of physiological telomeric DNA that includes a dsDNA/ssDNA junction at the 5′ side. Since the free 3′-end and interior regions of our constructs are identical to those of the Mittermaier study, we used similar statistical weights for $K_f, K_{f,N-1}$ *and* $K_{f,N}$ as those estimated in that study. However, the vicinity of the dsDNA/ssDNA junction was significantly different from the rest in our constructs, which required using much lower folding parameters. These considerations are implemented as $K_{f,N} > K_{f,N-1} > K_f > K_{f,2} > K_{f,1}$ in our study.

The probability for a state α is given by $p_\alpha = \omega_\alpha / Z$ where $Z = \sum_\alpha \omega_\alpha$ is the partition function. The total number of states can be written as $\Omega(N) = \sum_{N_f=0}^{\lfloor N/4 \rfloor} \frac{(N_f+N_{uf})!}{N_f!\, N_{uf}!}$, where the combinatorial factor is the number of states with N_f folded GQs and $N_{uf} = N - 4N_f$ unfolded G-Tracts. For telomeric overhangs of interest, $\Omega(N)$ is relatively small. For example, a telomere of 32 G-Tracts has only 16493 possible states, assuming long loops that contain one or more G-Tracts (such loops would be 9, 15, or 21 nt long compared to the canonical 3 nt loops) are not allowed due to their lower stability (33). This allows the partition sum to be computed explicitly by generating all states and evaluating the statistical weight of each state in the sum. The probability that the ith G-Tract is unfolded is

given by $P_i = \sum_\alpha \delta_{i,\alpha} p_\alpha$, where $\delta_{i,\alpha} = 1$ if the ith G-Tract is unfolded and $\delta_{i,\alpha} = 0$ if the G-Tract is in a folded GQ. Overall, average properties of the telomere can be computed, such as the average number of folded GQs, $\langle n_{GQ} \rangle = \sum_\alpha N_f^\alpha p_\alpha$, the average number of unfolded G-Tracts, $\langle n_{uf} \rangle = N - 4\langle n_{GQ} \rangle$, and the fluctuations of the unfolded G-Tracts, $\langle \Delta^2 n_{uf} \rangle = \langle n_{uf}^2 \rangle - \langle n_{uf} \rangle^2$.

For short chains, this model predicts a non-uniform unfolding probability along the sequence of G-Tracts under conditions strongly favoring folding (33). Here, combinatorial factors associated different ways in which specific G-Tracts can participate in GQ formation, as well as competing states with similar free energy conspire to produce patterns in P_i as a function of G-Tract index i; that is, some G-Tracts are more likely to be unfolded than others depending on their position. The experimental FRET distribution reflects binding of PNA to unfolded G-Tracts modeled by the distribution P_i which depends on the location of the G-Tract along the chain and the total number of repeats. This degree of localization of the unfolded G-Tracts is characterized by computing the S-parameter: $S = -\sum_{i=1}^{N} \tilde{P}_i \ln \tilde{P}_i$, where $\tilde{P}_i$ is the unfolded probability normalized to $\sum_{i=1}^{N} \tilde{P}_i = 1$.

SUPPORTING INFORMATION

Supplementary figures and tables. This material is available free of charge at https://www. pnas.org.

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Author Contributions

S.S., J.J.P. and H.B. designed research. S.S., G.M. and S.G.K. performed FRET-PAINT experiments and analyzed the data. M.E.H. purified the oligonucleotides. E.Y. wrote a Matlab code for data analysis. J.J.P. performed computational research and analyzed data. S.S., J.J.P, and H.B. wrote the manuscript.

Notes

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