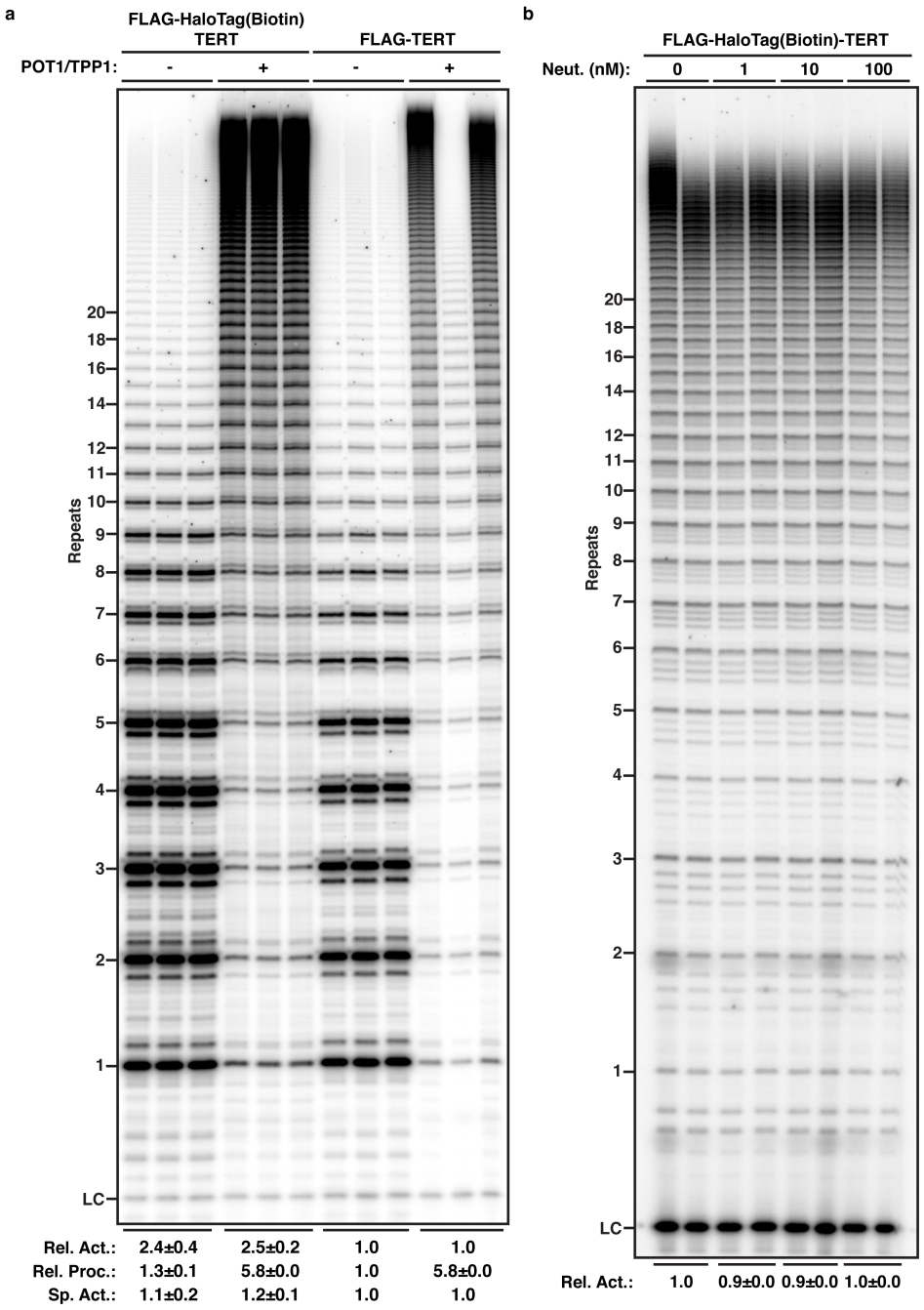
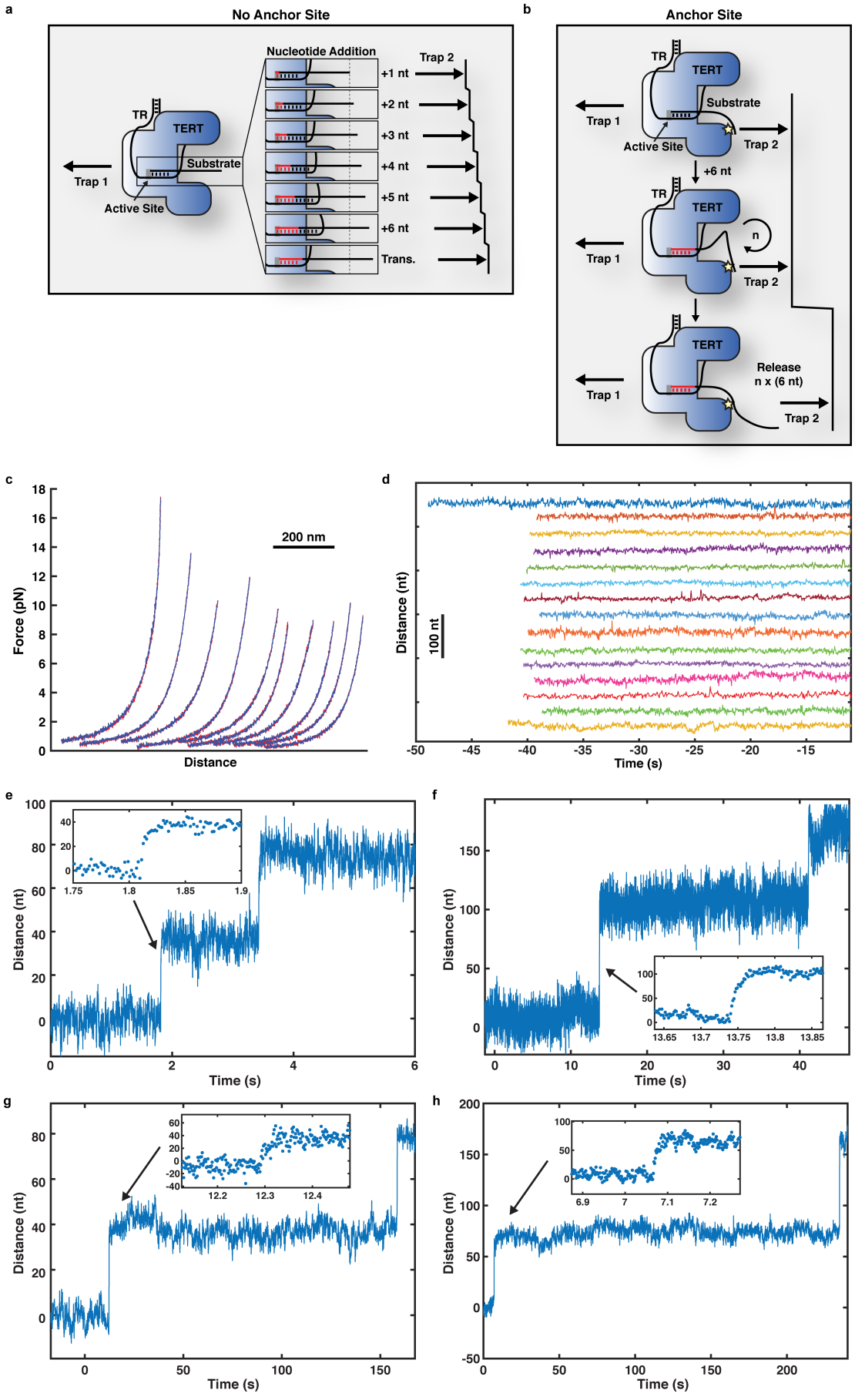
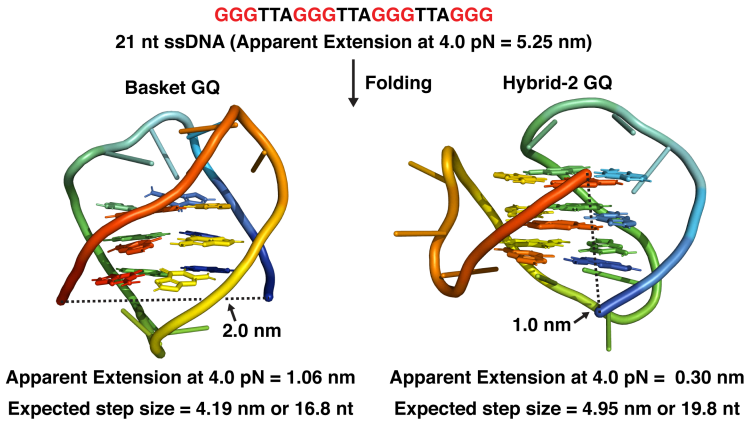


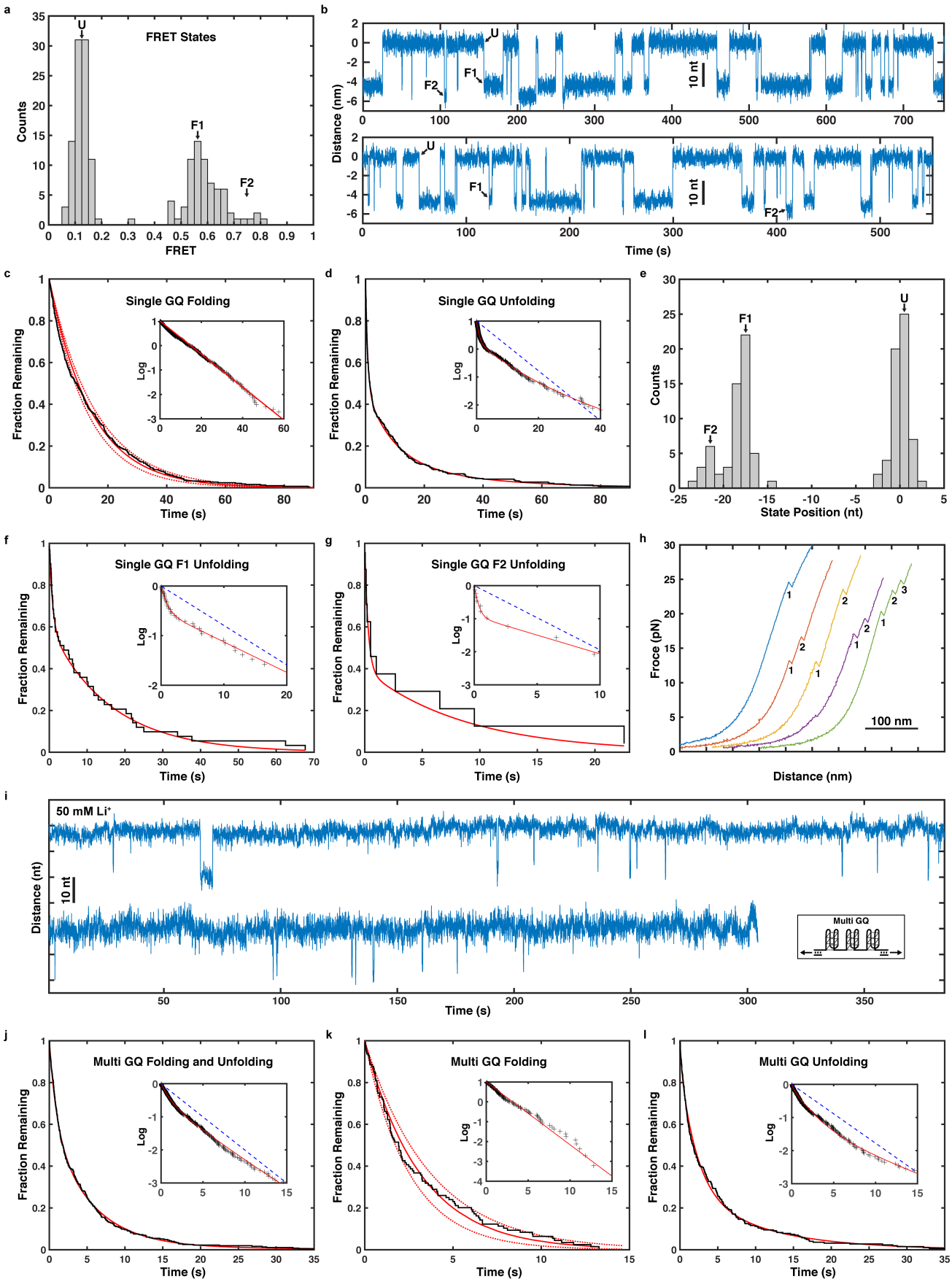
Supplementary Figure 1. Telomerase containing a 3xFLAG-HaloTag on TERT modified with biotin is stimulated by POT1/TPP1 and its activity is unaffected by neutravidin. a, Direct telomerase primer extension assay of 3xFLAG-HaloTag-TERT modified with biotin and 3xFLAG-TERT containing telomerase in the absence and presence of POT1/TPP1 in buffer containing 150 mM KCl. Relative activity is total lane intensity normalized to LC and the respective 3xFLAG-TERT telomerase activity. Specific activity is additionally normalized to the relative amount of TR (Fig. 1d). Processivity is calculated by dividing the intensity of products greater than 6 repeats by total lane intensity (n = 3, mean ± s.d.). **b,** Direct telomerase primer extension assay of 3xFLAG-HaloTag-TERT modified with biotin in reaction buffer containing 10 μM dNTPs, 50 mM KCl, and increasing concentrations of neutravidin. Relative activity is total lane intensity normalized to LC and activity in the absence of neutravidin (n = 2, mean ± s.d.).


a
No Anchor Site
Nucleotide Addition
Trap 2
+1 nt
+2 nt
+3 nt
+4 nt
+5 nt
+6 nt
Trans.
TR
TERT
Substrate
Trap 1
Active Site
b
Anchor Site
TR
TERT
Substrate
Trap 1
Active Site
Trap 2
+6 nt
n
Release
n x (6 nt)
c
200 nm
Force (pN)
Distance
d
Distance (nt)
100 nt
Time (s)
e
Distance (nt)
Time (s)
f
Distance (nt)
Time (s)
g
Distance (nt)
Time (s)
h
Distance (nt)
Time (s)

Supplementary Figure 2. Telomerase tethers are stable in the absence of nucleotides and extend in their presence. a, Expected elongation step pattern of telomerase without anchor site function. **b,** Expected elongation step pattern of telomerase with anchor site function. **c,** Representative force-extension curves of tethers formed by telomerase and substrate showing measurements (red) and polymer model (blue) (n = 30). **d,** Representative of 60 time trajectories (30 ms per data point) of telomerase catalysis prior to transfer into dNTP-containing buffer. **e-h**, Example traces showing consecutive anchor site release events (step sizes are much greater than expected step size for GQ unfolding) without intermitting reverse steps (**e,f** 1.5 ms per data point, **g,h** 75 ms per data point). Insets: zoom-in around the transition indicated by the arrow (1.5 ms per data point).

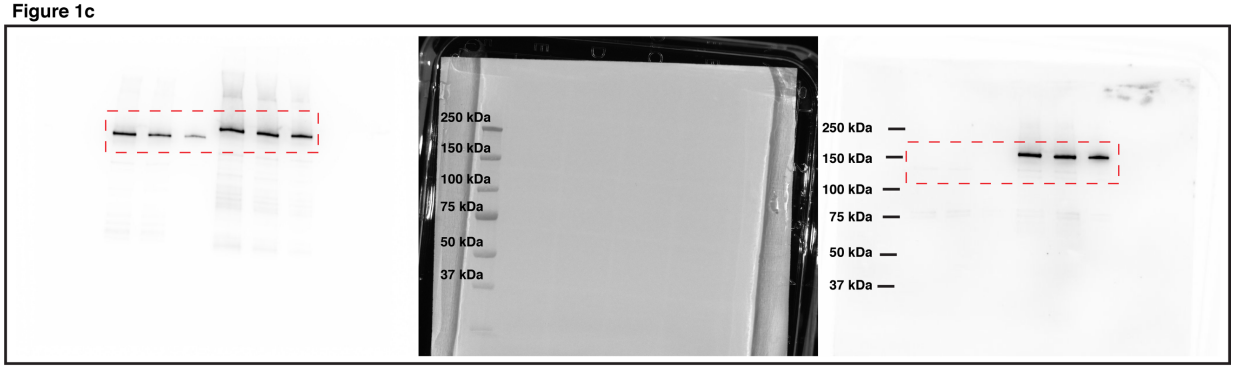


Supplementary Figure 3. Expected extension change for the formation of a G-quadruplex structures formed by four TTAGGG repeats. Models of basket (PDB: 143D) and hybrid-2 type GQs (PDB: 2JPZ). Apparent extensions and expected step sizes were derived from the freely jointed chain polymer model.


a
FRET States
U
F1
F2
Counts
FRET
b
U
F1
F2
10 nt
Distance (nm)
Time (s)
c
Single GQ Folding
Fraction Remaining
Log
Time (s)
d
Single GQ Unfolding
Fraction Remaining
Log
Time (s)
e
U
F1
F2
Counts
State Position (nt)
f
Single GQ F1 Unfolding
Fraction Remaining
Log
Time (s)
g
Single GQ F2 Unfolding
Fraction Remaining
Log
Time (s)
h
Froce (pN)
100 nm
Distance (nm)
i
50 mM Li+
10 nt
Distance (nt)
Multi GQ
Time (s)
j
Multi GQ Folding and Unfolding
Fraction Remaining
Log
Time (s)
k
Multi GQ Folding
Fraction Remaining
Log
Time (s)
l
Multi GQ Unfolding
Fraction Remaining
Log
Time (s)

Supplementary Figure 4. Analysis of G-quadruplex formation using high-resolution optical tweezers. a, Distribution of FRET states from single GQ tethers (n = 10 tethers, 163 states). **b,** Representative traces (n = 10) of two long-lasting tethers used to analyze unfolding rates of folded states F1 and F2 (72 ms per data point). **c,** Inverse cumulative distribution of dwell times for folding steps of single GQ tethers (black staircase, n = 22 tethers, 351 dwell times). Solid red line indicates a single exponential fit, dashed lines the 90% confidence intervals. Inset: Same distribution and fit on LOG scale. **d,** Inverse cumulative distribution of dwell times for unfolding steps of single GQ tethers (black staircase, n = 22 tethers, 350 dwell times). Solid red line indicates fit to the sum of three exponential decays ($\tau_{u1} = 0.73 \pm 0.12$, $\tau_{u2} = 9.0 \pm 3.2$, and $\tau_{u3} = 29 \pm 17$ s, weights of $52 \pm 5\%$, $32 \pm 9\%$ and $15 \pm 14\%$). Inset: Same distribution and fit on LOG scale, blue dashed line indicates single exponential decay fit. **e,** Distribution of trap position states for two long-lasting tethers used to analyze unfolding rates of folded states F1 and F2 (n = 2 tethers, 117 states). **f,** Inverse cumulative distribution of dwell times for unfolding steps from folded state F1 of single GQ tethers (black staircase, n = 2 tethers, 46 dwell times). Solid red line indicates fit to the sum of two exponential decays ($\tau_{u1} = 0.86 \pm 0.56$ and $\tau_{u2} = 16.0 \pm 5.2$, weights of $40 \pm 14\%$ and $60 \pm 13\%$). Inset: Same distribution and fit on LOG scale, blue dashed line indicates single exponential decay fit. **g,** Inverse cumulative distribution of dwell times for unfolding steps from folded state F2 of single GQ tethers (black staircase, n = 2 tethers, 12 dwell times). Solid red line indicates fit to the sum of two exponential decays ($\tau_{u1} = 0.29 \pm 0.4$ and $\tau_{u2} = 8.8 \pm 6.5$, weights of $60 \pm 21\%$ and $40 \pm 28\%$). Inset: Same distribution and fit on LOG scale, blue dashed line indicates single exponential decay fit. **h,** Force extension curves of $(TTAGGG)_{12}$ tethers. Numbers mark unfolding events. **i**, Representative traces of the folding dynamics of telomeric repeat ssDNA capable of forming up to three GQs in reaction buffer containing 50 mM LiCl (48 ms per data point, mean $\pm$ s.e.m. time in unfolded and folded state 15.4 $\pm$ 2.1 s and 0.3 $\pm$ 0.1 s, respectively, 6 tethers, n = 111 events). **j,** Inverse cumulative distribution of dwell times for folding and unfolding steps of multi GQ tethers (black staircase, n = 6 tethers, 339 dwell times). Solid red line indicates fit to the sum of three exponential decays ($\tau_{u1} = 1.0 \pm 0.5$, $\tau_{u2} = 5.0 \pm 1.5$, and $\tau_{u3} = 40 \pm 10$ s, weights of $40 \pm 15\%$, $60 \pm 10\%$ and 1%). Inset: Same distribution and fit on LOG scale, blue dashed line indicates single exponential decay fit. **k**, Inverse cumulative distribution of dwell times for folding steps from the completely unfolded state of multi GQ tethers (black staircase, n = 6 tethers, 102 dwell times). Solid red line indicates a single exponential fit, dashed lines the 90% confidence intervals. Inset: Same distribution and fit on LOG scale. **l,** Inverse cumulative distribution of dwell times for unfolding steps of multi GQ tethers (black staircase, n = 6 tethers, 237 dwell times). Solid red line indicates fit to the sum of three exponential decays. Inset: Same distribution and fit on LOG scale, blue dashed line indicates single exponential decay fit.

Figure 1c



TERT Western | Streptavidin HRP Blot | Streptavidin HRP Western

Figure 1d

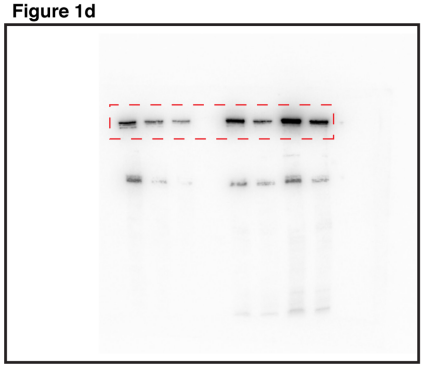
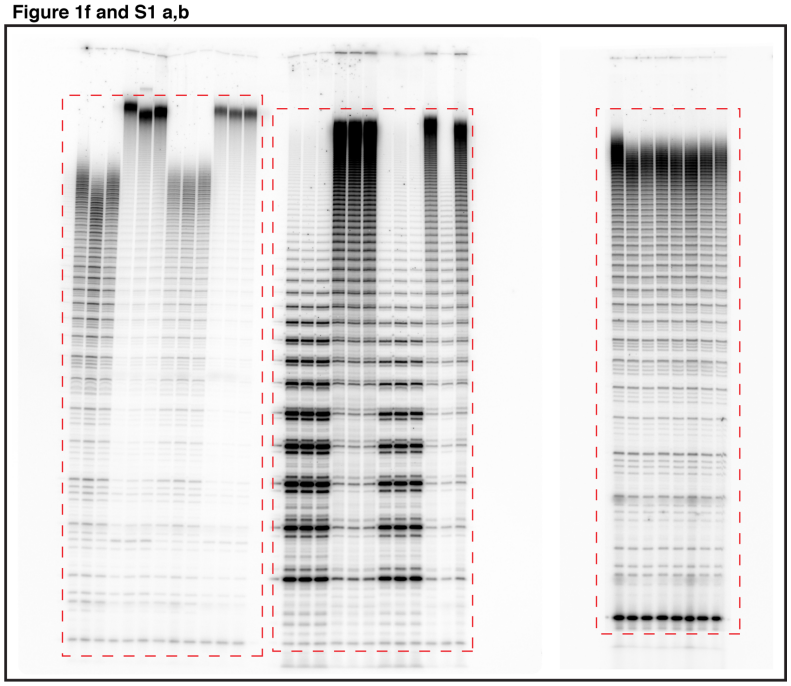
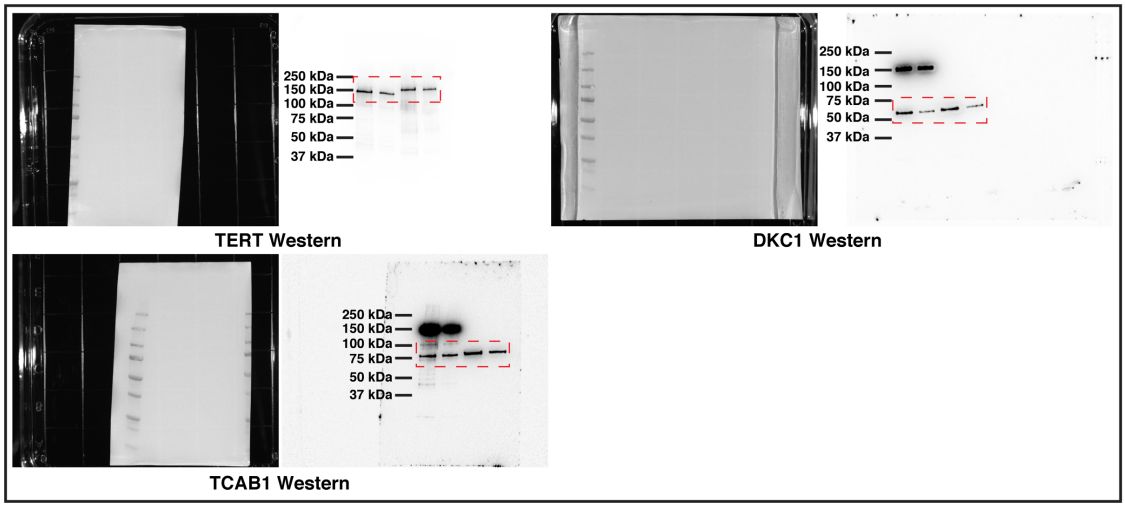


Figure 1f and S1 a,b



Direct Telomerase extension assay

Figure 1e



TERT Western | DKC1 Western

TCAB1 Western

Supplementary Figure 5. Unedited gel images for all data presented in this paper.

Supplementary Table 1. Oligonucleotides used in this study.

Name	Sequence
Substrate Handle Oligo 1	TGGCGACGGCAGCGAGGCTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTGGGTTAGGGTTAGGG
Substrate Handle Oligo 2	AAAAAAAAAAAAAAAAGCCTCGCTGCCGTCGCCA/3Dig_N/
Biotin-Dig Handle Oligo 1	/5Biosg/TGGTCTTCGGTTTCCGTGTT
Biotin-Dig Handle Oligo 2	/5DigN/CAACAACGTTGCGCAAACT
TR Probe 1	CTCCAGGCGGGGTTCGGGGGCTGGGCAG
TR Probe 2	CTTTTCCGCCCGCTGAAAGTCAGCGAG
TR Probe 3	CATGTGTGAGCCGAGTCCTGGGTGCACG
Primer A	TTAGGGTTAGGGTTAGGG
Primer A5	TTAGGGTTAGCGTTAGGG
Primer A-2	TTAGGGTTAGGGTTAG
GGG(TTAGGG)3	/5Phos/CCTGGAGGACTTGTTTTTAGGGTTAGGG TTAGGGTTAGGGTTCAGTATCGACCCACTGGC
FRET Donor	/5Cy3/ACAAGTCCT
FRET Acceptor	/5Phos/TCGATACTG/3Cy5Sp/
(TTAGGG)12	/5Phos/AGCTTTTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG TTAGGGTTAGGGTTAGGGTTAGGG TTAGGGTTAGGGTTAGGGTTCCCACTGGC
pBR322 for	/5Biosg/TGAAGTGGTGGCCTAACTACG
pBR322 rev	TTGCATGATAAAGAAGACAGTCAT
Lambda for	/5DigN/GGGCAAACCAAGACAGCTAA
Lambda rev	CCCGTCATACACTTGCTCCT