SUPPLEMENTAL DATA

METHODS

Cross-linking of Shelterin complexes to a radiolabeled photoactivatable telomeric DNA junction

In a final volume of 80 µl, reactions contained 16 µl of 5X binding buffer (20% glycerol, 5 mM $MgCl_2$, 5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl pH 7.5), 8 µg of sonicated *E. coli* DNA, and 16 µl of whole cell extracts made from HeLa cells transfected with no DNA (Mock), Flag-TRF2$^{\Delta B}$ or Flag-TRF2$^{\Delta B}$(R361P). After 5 minutes at room temperature, 1.4 x 10^5 cpm of [^{32}P]-labeled 2R-3'(BrdU) probe were added and the incubation performed for an additional 25 minutes. Next, samples were left in the dark or exposed to ultraviolet (UV) irradiation to photoactivate the BrdU base. UV treatment was performed at 4^{o}C for 20 minutes with the samples spotted on parafilm over a UV transilluminator (302 nm emission, FisherBiotech model FBTIV-88). After that, protein/DNA complexes containing the Flag-tagged proteins were captured using magnetic beads coated with the anti-Flag M2 antibody. Ten microliters of M2-coated beads were added and the suspension was rotated at room temperature for an hour. With the help of a magnet, beads were recovered and washed 3-times with 500 µl of ice-cold 1X binding buffer (as described in the EMSA section) containing 0.1% BSA. Next, the captured complexes were released by incubation of the beads with 40 µl of a 3XFLAG peptide solution (100 µg/ml in 1X binding buffer) for 10 minutes at room temperature. The eluted complexes were heat-denatured in Laemmli buffer and separated by electrophoresis on a SDS-PAGE gel. After electrophoresis, the gel was fixed, dried, and exposed to a PhosphoImager cassette to reveal the radiolabeled cross-linked proteins. [^{32}P]-labeled 2R-3'(BrdU) was made as described in the Materials and Methods Section of the parent article.

Expression vector for TPP1, RAP1 and TRF1

Plasmid pcDNA3.1-Flag-TPP1 is a derivative of pCMV-SPORT6-PTOP (Open Biosystem) encoding full length TPP1 (BC016904). Briefly, TPP1's open reading frame was PCR amplified with Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) using primer PTOP-F (5'-CCGGAATTCATGCCTGGCCGCTGT-CAGAGTGAC-3', EcoRI site underlined) and PTOP-R (5'-CGCGGATCCTCACATCGGAGTTGGCTC-AGACCCTG-3'). EcoRI-digested product was inserted in EcoRI/PmeI sites of plasmid pcDNA3.1-Flag, in frame with the Flag tag. pcDNA3.1-Flag is a pcDNA3.1(-) vector encoding a Flag epitope located downstream of a T7 promoter and followed by an EcoRI site. Plasmid pcDNA3.1-Flag-RAP1 is a derivative of pCMV-SPORT6-RAP1 (Open Biosystem) encoding full length RAP1 (BC078171). RAP1's open reading frame was PCR amplified using primer RAP1-F (5'-CCGGAATTCATGGCGGAGGC-GATGGATTTGG-3', EcoRI site underlined) and RAP1-R (5'-CGCGGATCCTTATTTCTTTCGAAATT-CAATCCTCCGAGC-3'), cut with EcoRI and clones into the EcoRI/PmeI sites of pcDNA3.1-Flag. Plasmid pcDNA3.1-Flag-TRF1 is a derivative of pTethTRF1 (a gift from Titia de Lange, Rockefeller University, NY)(1). TRF1's open reading frame was PCR amplified from pTethTRF1 using primer EcoRI-TRF1-F1 (5'-GCCGAATTCATGGCGGAGG-ATGTTTCC-3', EcoRI site underlined) and TRF1-BamHI-R (5'-GGCGGATCCTCAGTCTTCGCTGTCTGAG-3', BamHI site underlined), cut with EcoRI and BamHi and clones into the same sites of pcDNA3.1-Flag.

References

1. *van Steensel, B. and de Lange, T. (1997) Control of telomere length by the human telomeric protein TRF1. Nature, **385**, 740-743.*

FIGURE LEGENDS

Figure S1. Making telomeric DNA probes with distinct end structures. (A) Probes were made in a 2-step process. In the first step, a [^{32}P]-labeled M13 primer was annealed to a single-stranded template, and the primer was extended with Taq DNA polymerase. In the second step, these products were digested with an outside cutter and subsequently gel purified. Using cutters PpiI, HgaI or MlyI, probes harboring a 3'-overhang, 5'-extension, or blunt end were respectively made. Star denotes presence of a [^{32}P]-phosphate.

Figure S2. Cross-linking of Shelterin complexes to a radiolabeled photoactivatable telomeric DNA junction. A) Schematic description of the radiolabeled probe used. Probe 2R-3'(BrdU) carried two binding sites for the Myb domain of TRF2 (numbered 1 and 2) and a 3'-telomeric overhang of 5 bases. The overhang was designed to contain a photoactivatable BrdU nucleotide to allow cross-linking of the Shelterin component that establishes contacts with the overhang. [^{32}P]-phosphate was located at the 5'-end of the top strand. B) Experimental plan for cross-linking of the radiolabeled probe with components of the Shelterin Complexes. Extracts made from HeLa cells transfected with no plasmid (Mock), Flag-TRF2$^{\Delta B}$ or Flag-TRF2$^{\Delta B}$(R361P) were incubated with the [^{32}P]-labeled 2R-3'(BrdU) probe, after which binding reactions were exposed to UV light to activate the BrdU for cross-linking. After immunoprecipitation (IP) of the Flag-containing protein/DNA complexes, the isolated complexes were eluted with an excess of 3XFLAG peptide and then heat-denatured in Laemmli buffer. After separation by SDS-PAGE electrophoresis, the radiolabeled cross-linked proteins were revealed by exposure to a PhosphoImager cassette. C) Cross-linking of a Shelterin component with an estimated weight of 76 kDa. Cross-linking of the top strand of probe 2R-3'(BrdU) to this subunit required UV exposure (+). Recovery of the cross-linked product required Flag-TRF2$^{\Delta B}$ (Mock versus Flag-TRF2$^{\Delta B}$) and was blocked by the R361P mutation known to prevent assembly of the Shelterin complex (Flag-TRF2$^{\Delta B}$ versus Flag-TRF2$^{\Delta B}$(R361P)). Estimated molecular weight of the subunit cross-linked was calculated from the size of the observed product (85 kDa), after subtraction of the molecular weight of the probe's top strand (9 kDa).

Figure S3. Two Myb binding sites are required for binding of complex T2 to probes carrying a short 3'-telomeric overhang. (A) Graphical representations of the structures of all tested probes. Probes carrying a 3'-telomeric overhang of 5 bases were designed to contain different number of ds-TTAGGGTTA motifs (numbered 1 through 4). Shaded areas represent telomeric DNA. (B) Electrophoretic mobility shift assay of the probes described in A. Probes were incubated with no extracts (lane 1) or with extracts of HT1080-Vector (lane 2) or HT1080-TRF2 (lanes 3-11) cells, and the protein/DNA complexes formed were resolved by native electrophoresis on TAM-containing polyacrylamide gels. Five minutes prior to loading, some samples were incubated with M2 anti-Flag antibody (lane 10) or normal mouse IgG (lane 11). Short arrow indicates positions of the supershifted complex T2. NS, non-specific band.

Figure S4. Shelterin binding activity is increased after transfection of TRF1 but not TPP1 or RAP1. (A-C) Transfection of TPP1 or RAP1 produces no increases in Shelterin binding activity. Panel A: Whole cells extracts made from HeLa cells transfected with Flag-tagged RAP1 and TPP1 were probed with antibodies against the Flag tag, TPP1 and RAP1. Panel B: Probe used for EMSA shown in panel C. Shaded areas represent telomeric DNA. Panel C: EMSA analysis of whole cells extracts analyzed in panel A. As positive control, a whole cells extract from Hela cells transfected with Flag-TRF2$^{\Delta B}$ was used. Extracts were incubated with probe Ov-8m8, after which the protein/DNA complexes that formed were resolved by native electrophoresis on TAM-containing polyacrylamide gels. T2/DNA, complex T2 bound to DNA. NS, non-specific band. (D-E) Transfection of TRF1 leads to the formation of Shelterin complexes that contain TRF1 but lack TRF2. Panel D: Whole cells extracts made from HeLa cells transfected with Flag-tagged TRF1 were probed with antibodies against the Flag tag, TRF1 and TRF2. Panel E: EMSA analysis of the whole cells extracts analyzed in panel D. Extracts were incubated with probe C2 (Figure 3A), after which the protein/DNA complexes that formed were resolved by native electrophoresis on TBE-containing polyacrylamide gels. TBE gels were used in this experiment because Shelterin complexes that contain TRF1 but lack TRF2 (T1 Shelterin) are unstable in TAM gels. Upon transfection of Flag-TRF1, two new complexes were detected: a TRF1 dimer and T1 Shelterin. The dimer was supershifted by the TRF1 antibody but not by the antibody against TIN2. The T1 Shelterin was supershifted by both the TRF1 and

TIN2 antibodies but not by antibodies against TRF2 or RAP1. Short arrow indicates positions of the supershifted complexes. mIgG, normal mouse IgG. rIgG, normal rabbit IgG. NS, non-specific band.

Figure S5. DNA binding by Flag-TIN2 complexes is unaffected by the spacing between the ds/ss-junction and POT1 site. A) Graphical representations of the structures of all tested probes. Probes were designed to have ds/ss-junctions at different position (-1, -2, -4, -7) relative to the POT1 site (ss-TTAGGGTTAG). Shaded areas represent telomeric DNA. B) Affinity of the different probes for the Flag-TIN2 Complexes. Extracts of HeLa cells transfected with Flag-TIN2 were incubated with the [^{32}P]-labeled probes described in A, after which Flag-tagged protein/DNA complexes were captured with the M2 antibody and the amount of radioactivity recovered was counted. Results in triplicates show the percent of each probe recovered by the M2 antibody (mean +/- standard deviation; n=3).

Figure S6. Complex T2 binds ds/ss-telomeric junctions without constraints on either distance or orientation. (A) Graphical representations of the structures of all tested probes. Probes were designed to change the spatial relationship between the two ds-TTAGGGTTA motifs or between these motifs and the 3'-telomeric overhang. Shaded areas represent telomeric DNA, with the light and dark shadings utilized to distinguish the orientation of the repeats. (B) Electrophoretic mobility shift assay of probes described in A. Probes were incubated with extracts of TRF2$^{\Delta B}$-transfected HeLa cells and the protein/DNA complexes formed were resolved by native electrophoresis on TAM-containing polyacrylamide gels. NS, non-specific band.

Figure S1

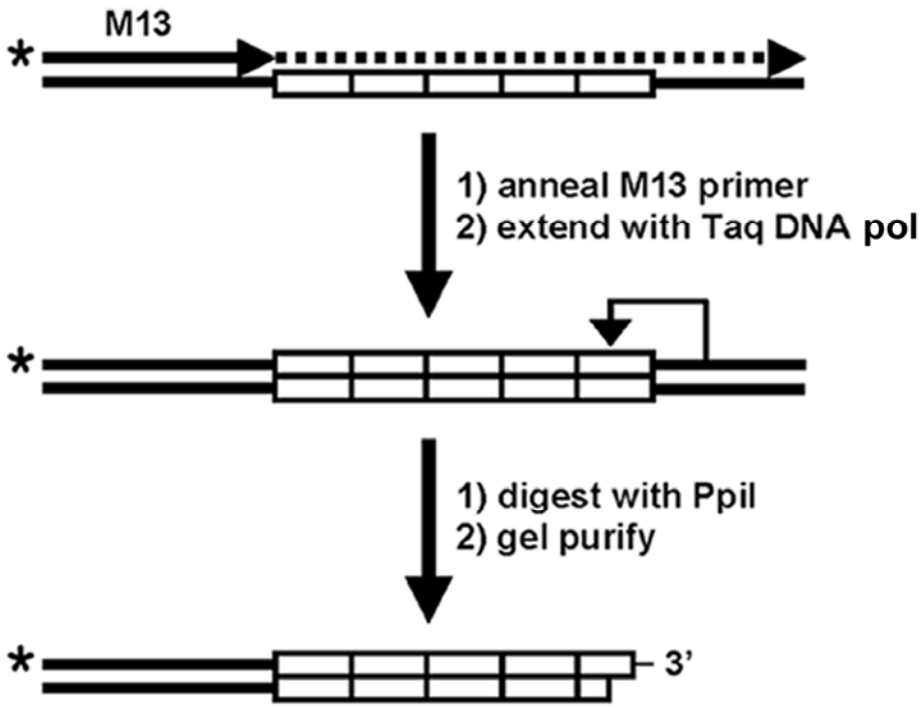
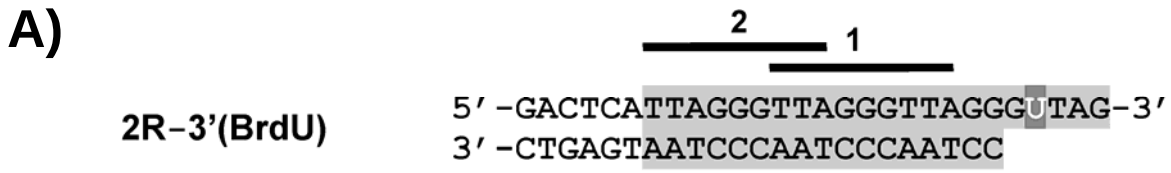
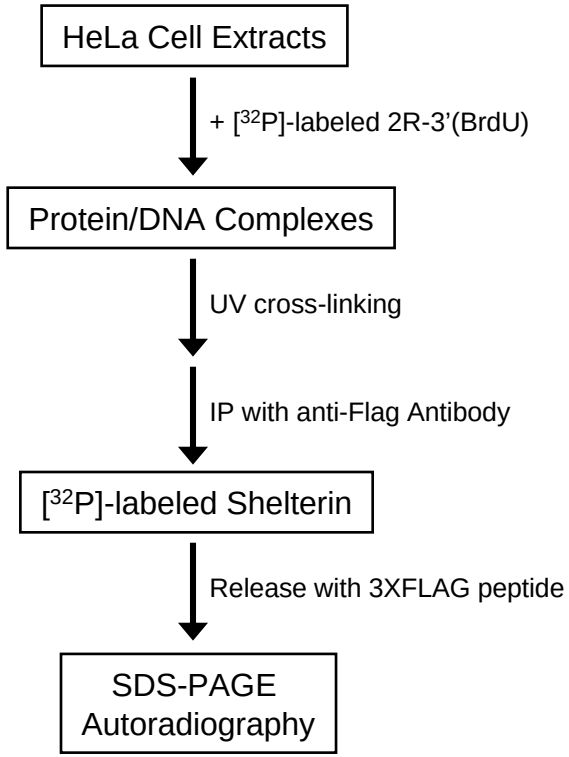

M13
1) anneal M13 primer
2) extend with Taq DNA pol
1) digest with PpiI
2) gel purify
3'

Figure S2A


A)
2
1
2R-3'(BrdU)
5'-GACTCATTAGGGTTAGGGTTAGGGUTAG-3'
3'-CTGAGTAATCCCAATCCCAATCC

Figures S2B-C

B)



C)

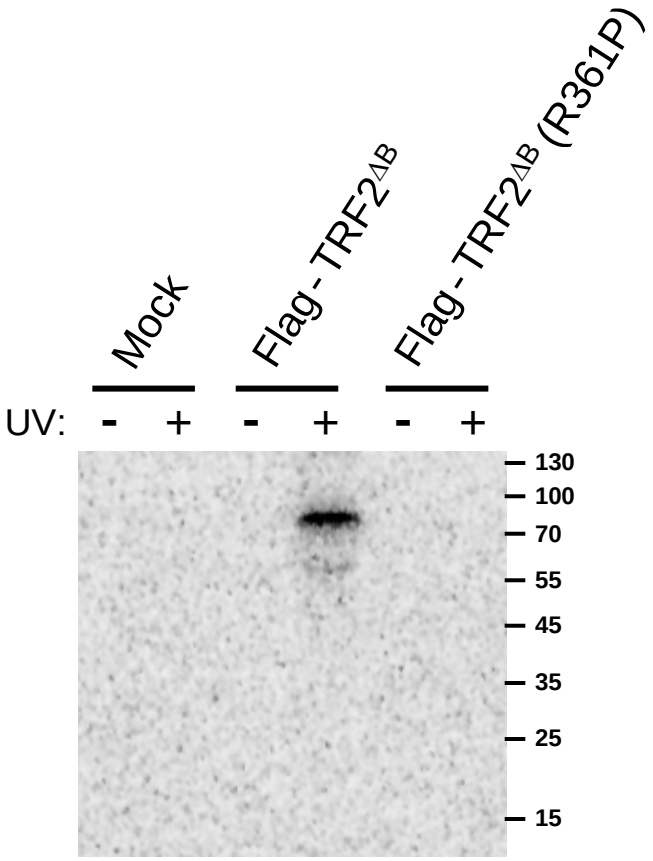


Figure S3A

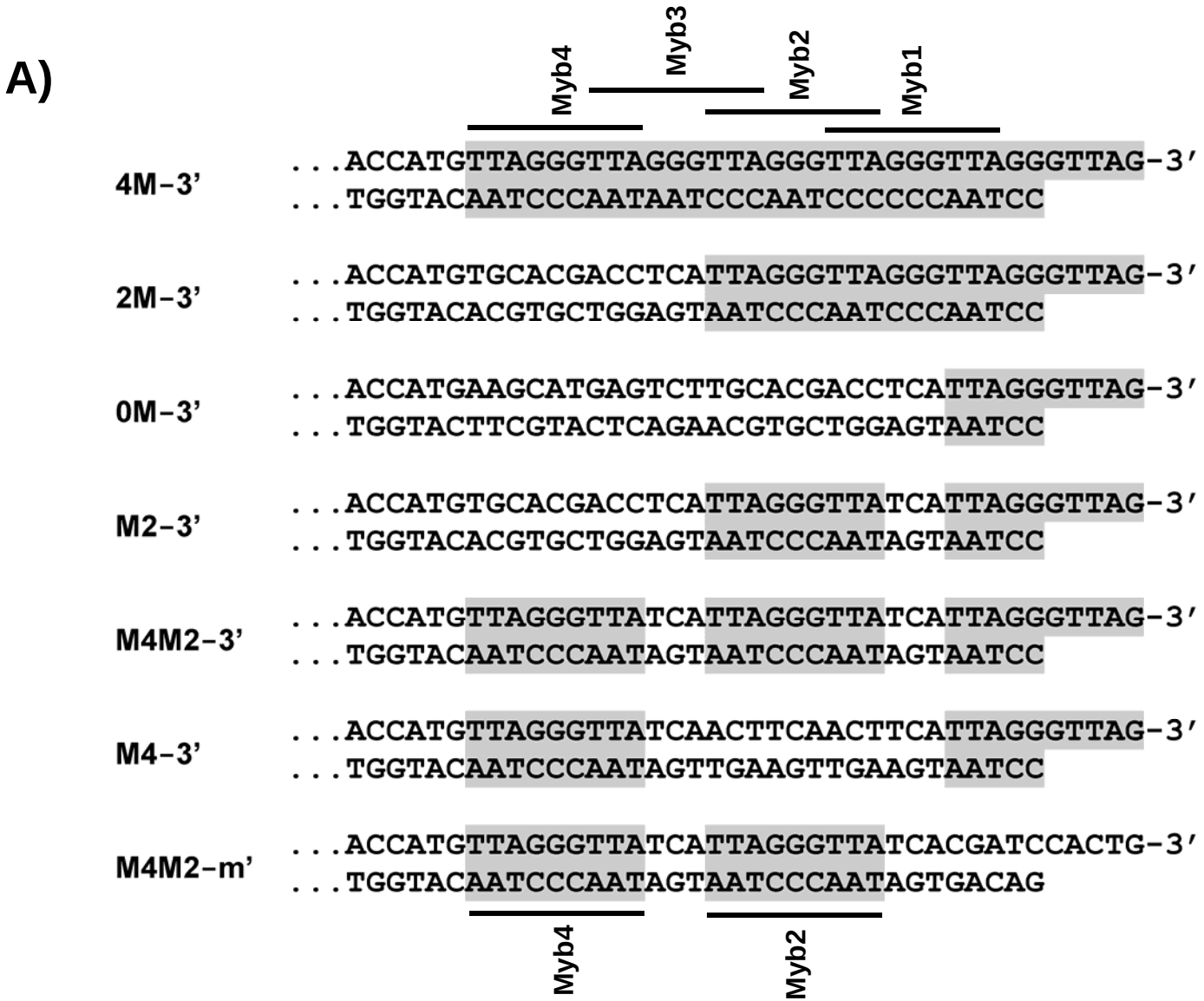

A)
Myb4
Myb3
Myb2
Myb1
4M-3'
...ACCATGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG-3'
...TGGTACAATCCCAATAATCCCAATCCCCCCAATCC
2M-3'
...ACCATGTGCACGACCTCATTAGGGTTAGGGTTAGGGTTAG-3'
...TGGTACACGTGCTGGAGTAATCCCAATCCCAATCC
0M-3'
...ACCATGAAGCATGAGTCTTGCACGACCTCATTAGGGTTAG-3'
...TGGTACTTCGTACTCAGAACGTGCTGGAGTAATCC
M2-3'
...ACCATGTGCACGACCTCATTAGGGTTATCATTAGGGTTAG-3'
...TGGTACACGTGCTGGAGTAATCCCAATAGTAATCC
M4M2-3'
...ACCATGTTAGGGTTATCATTAGGGTTATCATTAGGGTTAG-3'
...TGGTACAATCCCAATAGTAATCCCAATAGTAATCC
M4-3'
...ACCATGTTAGGGTTATCAACTTCAACTTCATTAGGGTTAG-3'
...TGGTACAATCCCAATAGTTGAAGTTGAAGTAATCC
M4M2-m'
...ACCATGTTAGGGTTATCATTAGGGTTATCACGATCCACTG-3'
...TGGTACAATCCCAATAGTAATCCCAATAGTGACAG
Myb4
Myb2

Figure S3B

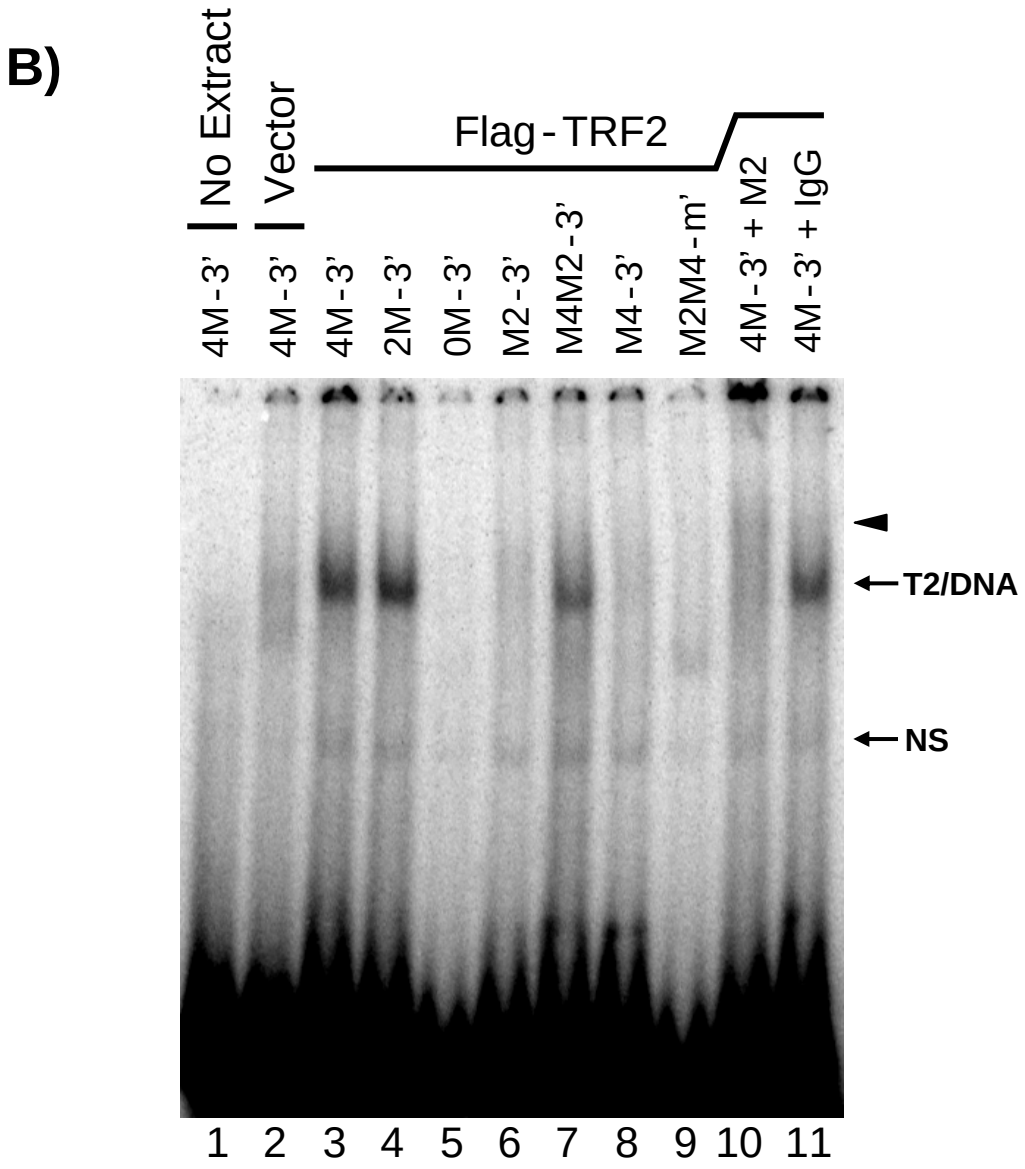
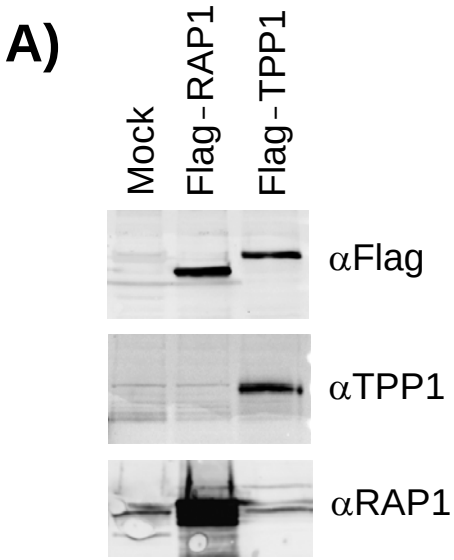
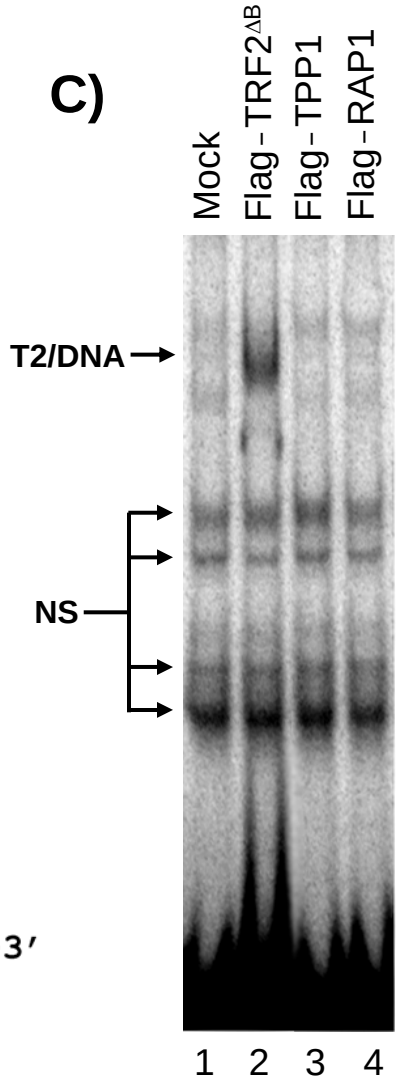

B)
No Extract
Vector
Flag-TRF2
4M-3'
4M-3'
4M-3'
2M-3'
0M-3'
M2-3'
M4M2-3'
M4-3'
M2M4-m'
4M-3' + M2
4M-3' + IgG
T2/DNA
NS
1 2 3 4 5 6 7 8 9 10 11

Figure S4A-C



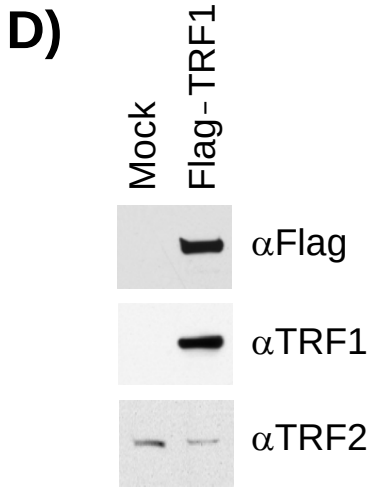


B)

Ov-8m8

5′-CGTACTTAGGGTTACACCAGCTATTAGGGTTAGGGTTAGGC-3′
3′-GCATGAATCCCAATGTGGTCGATAATCCCAATC

Figure S4D-E


D)
Mock
Flag-TRF1
αFlag
αTRF1
αTRF2

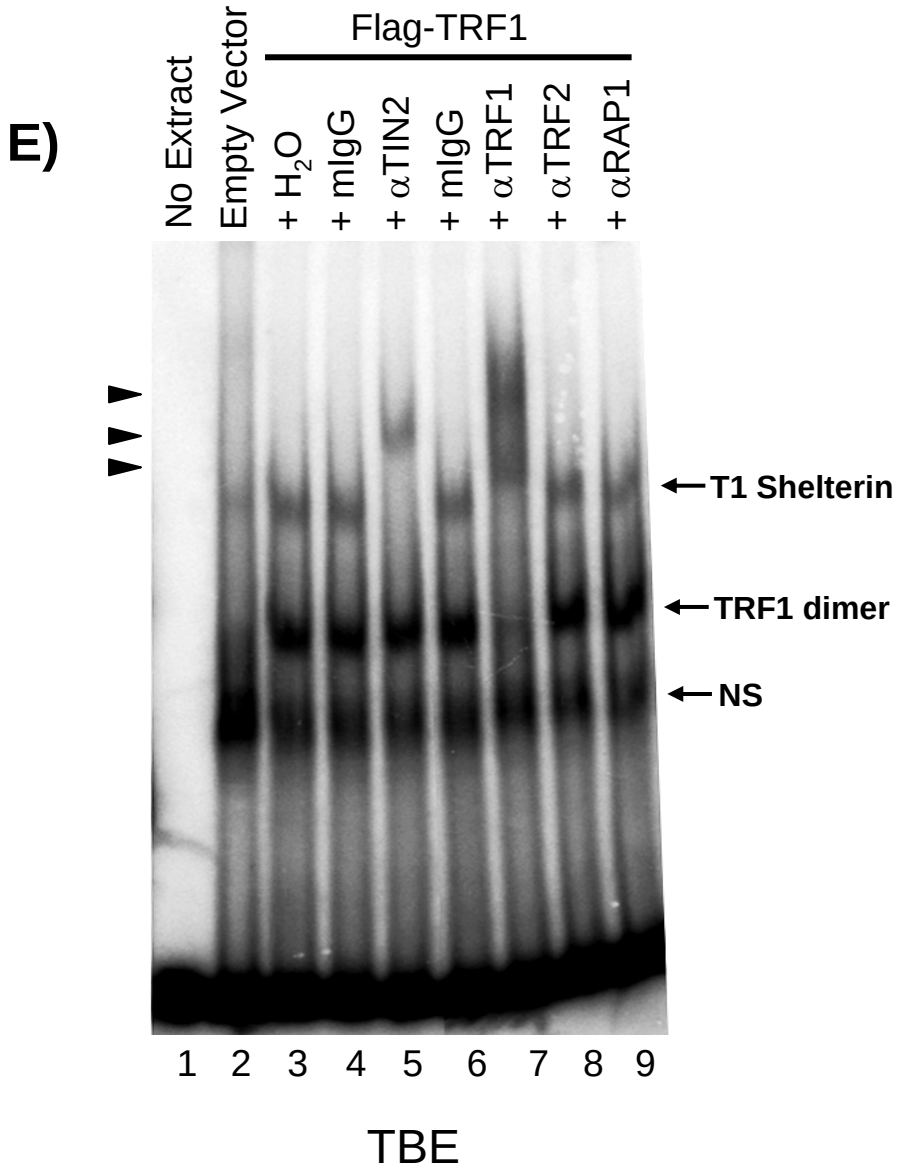

E)
No Extract
Empty Vector
Flag-TRF1
+ H2O
+ mIgG
+ αTIN2
+ mIgG
+ αTRF1
+ αTRF2
+ αRAP1
T1 Shelterin
TRF1 dimer
NS
1 2 3 4 5 6 7 8 9
TBE

Figure S5A

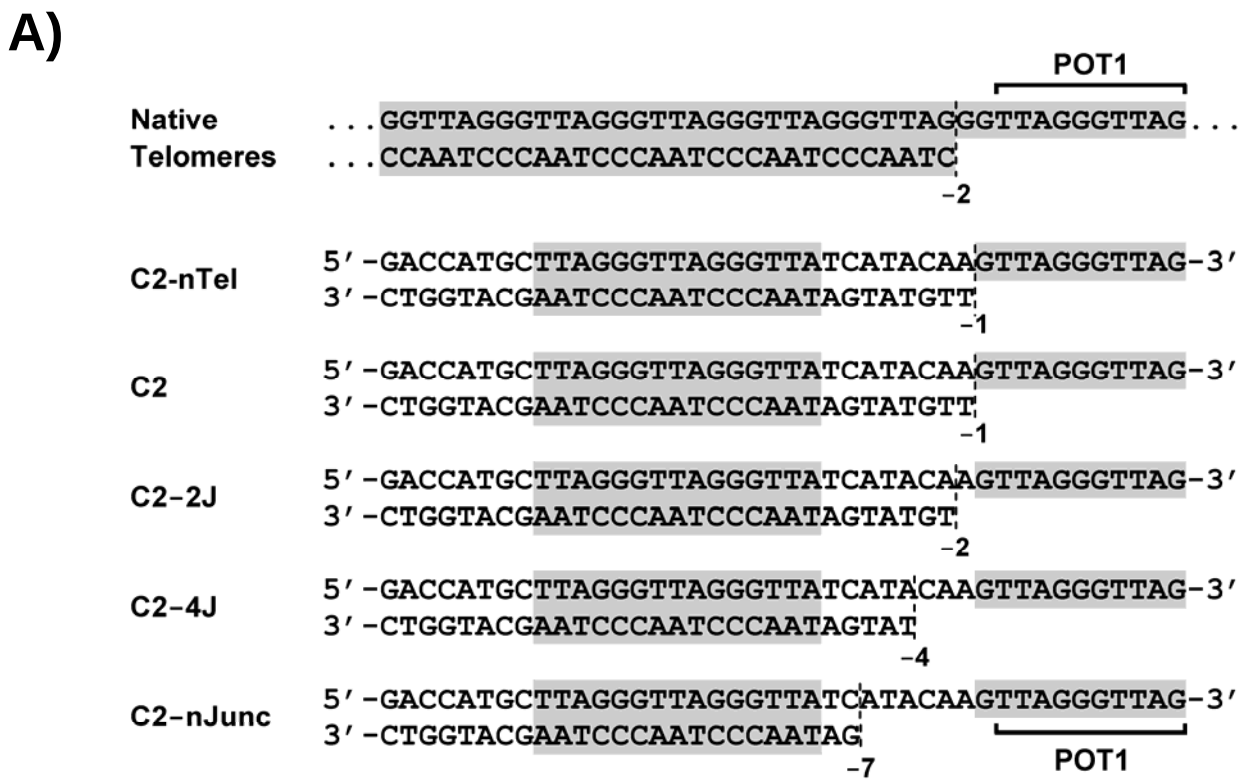

A)
POT1
Native Telomeres
...GGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG...
...CCAATCCCAATCCCAATCCCAATCCCAATC
-2
C2-nTel
5′-GACCATGCTTAGGGTTAGGGTTATCATACAAGTTAGGGTTAG-3′
3′-CTGGTACGAATCCCAATCCCAATAGTATGTT
-1
C2
5′-GACCATGCTTAGGGTTAGGGTTATCATACAAGTTAGGGTTAG-3′
3′-CTGGTACGAATCCCAATCCCAATAGTATGTT
-1
C2-2J
5′-GACCATGCTTAGGGTTAGGGTTATCATACAAGTTAGGGTTAG-3′
3′-CTGGTACGAATCCCAATCCCAATAGTATGT
-2
C2-4J
5′-GACCATGCTTAGGGTTAGGGTTATCATACAAGTTAGGGTTAG-3′
3′-CTGGTACGAATCCCAATCCCAATAGTAT
-4
C2-nJunc
5′-GACCATGCTTAGGGTTAGGGTTATCATACAAGTTAGGGTTAG-3′
3′-CTGGTACGAATCCCAATCCCAATAG
-7
POT1

Figure S5B

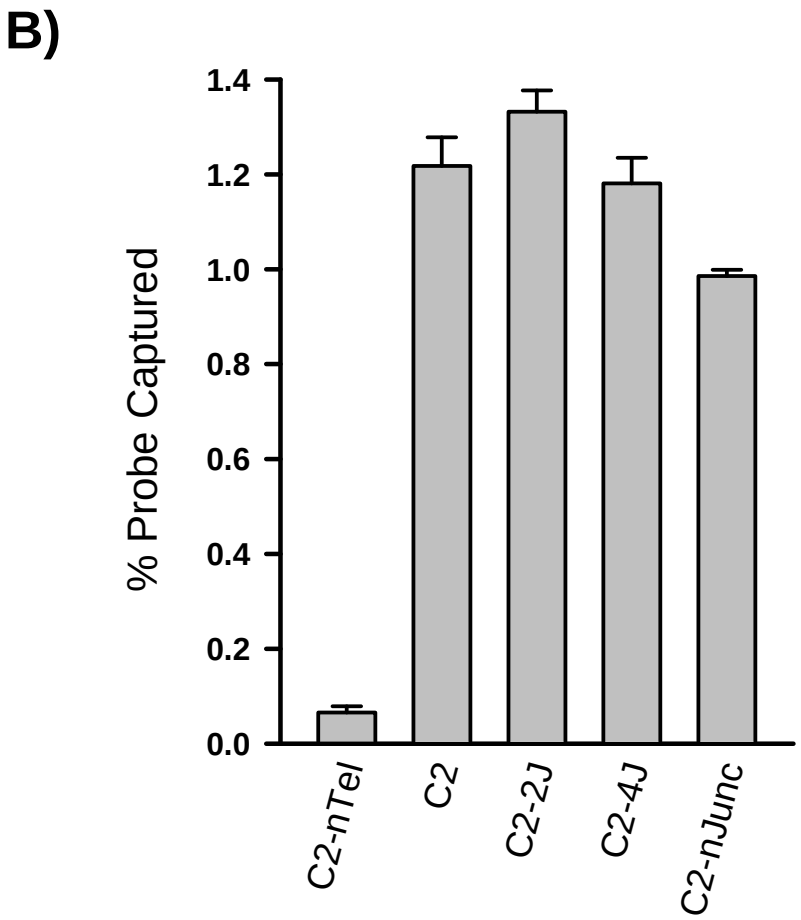

B)
% Probe Captured
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C2-nTel
C2
C2-2J
C2-4J
C2-nJunc

Figure S6A

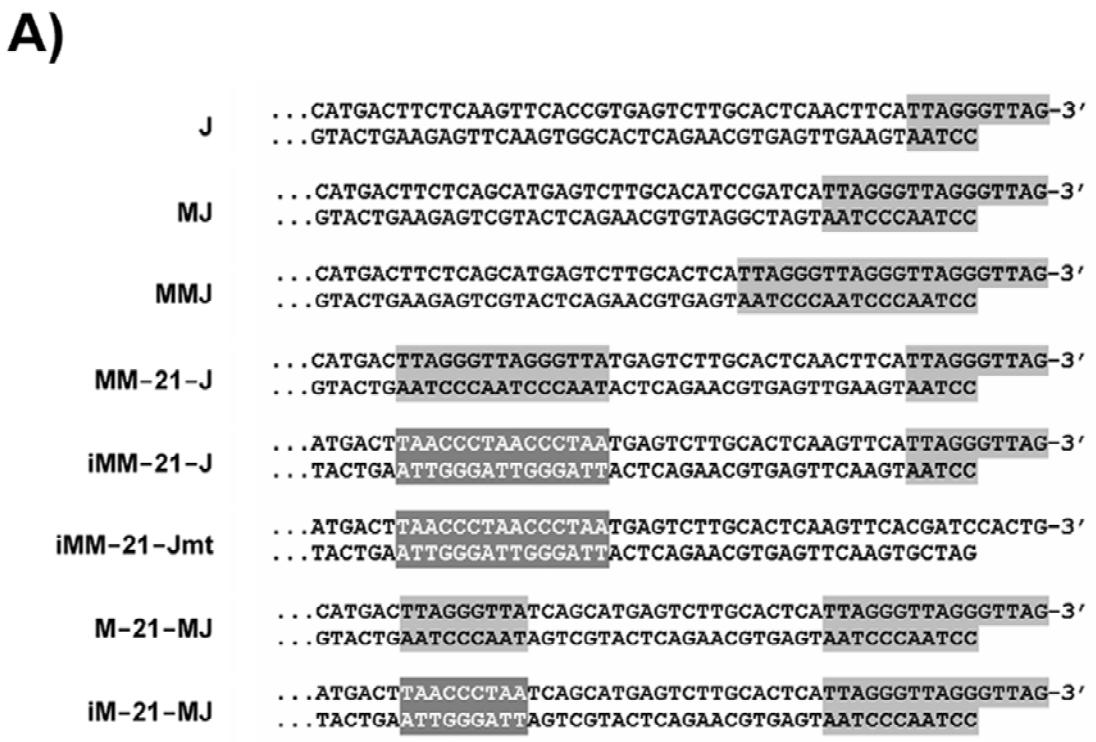
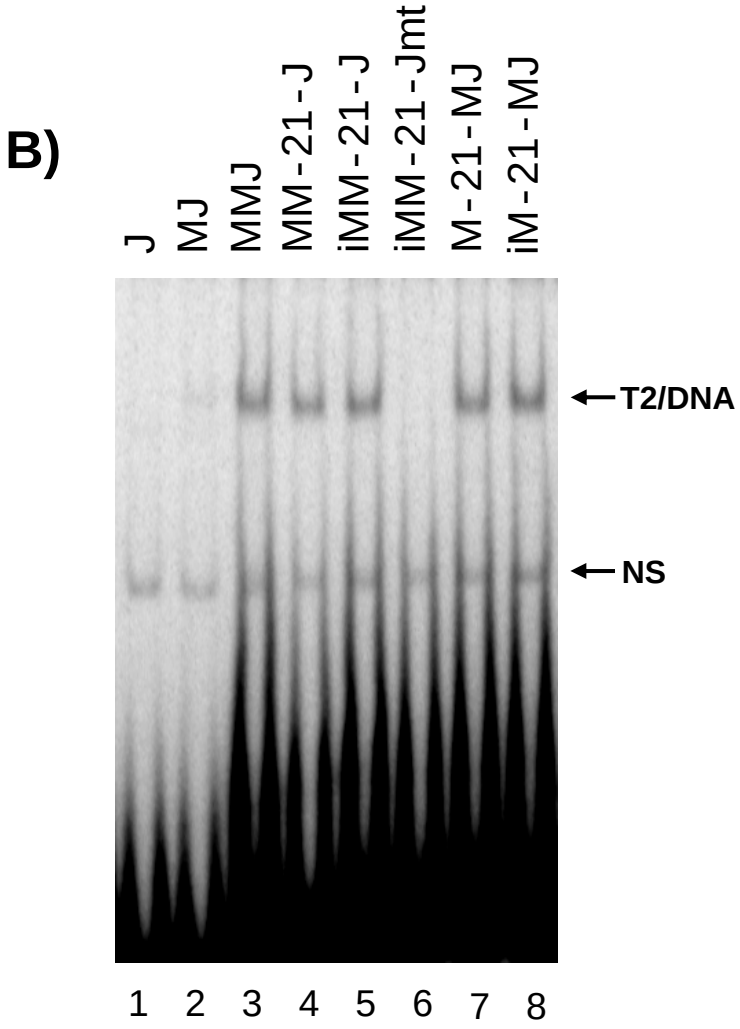

A)
J
...CATGACTTCTCAAGTTCACCGTGAGTCTTGCACTCAACTTCATTAGGGTTAG-3'
...GTACTGAAGAGTTCAAGTGGCACTCAGAACGTGAGTTGAAGTAATCC
MJ
...CATGACTTCTCAGCATGAGTCTTGCACATCCGATCATTAGGGTTAGGGTTAG-3'
...GTACTGAAGAGTCGTACTCAGAACGTGTAGGCTAGTAATCCCAATCC
MMJ
...CATGACTTCTCAGCATGAGTCTTGCACTCATTAGGGTTAGGGTTAGGGTTAG-3'
...GTACTGAAGAGTCGTACTCAGAACGTGAGTAATCCCAATCCCAATCC
MM-21-J
...CATGACTTAGGGTTAGGGTTATGAGTCTTGCACTCAACTTCATTAGGGTTAG-3'
...GTACTGAATCCCAATCCCAATACTCAGAACGTGAGTTGAAGTAATCC
iMM-21-J
...ATGACTTAACCCTAACCCTAATGAGTCTTGCACTCAAGTTCATTAGGGTTAG-3'
...TACTGAATTGGGATTGGGATTACTCAGAACGTGAGTTCAAGTAATCC
iMM-21-Jmt
...ATGACTTAACCCTAACCCTAATGAGTCTTGCACTCAAGTTCACGATCCACTG-3'
...TACTGAATTGGGATTGGGATTACTCAGAACGTGAGTTCAAGTGCTAG
M-21-MJ
...CATGACTTAGGGTTATCAGCATGAGTCTTGCACTCATTAGGGTTAGGGTTAG-3'
...GTACTGAATCCCAATAGTCGTACTCAGAACGTGAGTAATCCCAATCC
iM-21-MJ
...ATGACTTAACCCTAATCAGCATGAGTCTTGCACTCATTAGGGTTAGGGTTAG-3'
...TACTGAATTGGGATTAGTCGTACTCAGAACGTGAGTAATCCCAATCC

Figure S6B


B)
J
MJ
MMJ
MM-21-J
iMM-21-J
iMM-21-Jmt
M-21-MJ
iM-21-MJ
T2/DNA
NS
1 2 3 4 5 6 7 8