TRF1 and TRF2 Differentially Modulate Rad51-Mediated Telomeric and Non-Telomeric Displacement Loop Formation *In Vitro*†

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**Abbreviations Used**

Area under the curve, AUC; ataxia telangiectasia mutated, ATM; base pair, bp; bovine serum albumin, BSA; coding DNA, cDNA; cyanine 3, Cy3; deoxyribonucleic acid, DNA; displacement loop, D-loop; dithiothreitol, DTT; double-stranded DNA, dsDNA; electrophoretic mobility shift assay, EMSA; Fanconia anemia group D2 protein, FANCD2; Holliday junction, HJ; homologous recombination/repair, HR; kilobase pair, kbp; Mre11/Rad50/Nbs1 complex, MRN; non-homologous end joining, NHEJ; nucleotide, nt; polymerase chain reaction, PCR; post-translational modification, PTM; single-stranded DNA, ssDNA; single-stranded to double-stranded DNA, ss/dsDNA; sodium dodecyl sulfate, SDS; telomere loop, t-loop; telomere repeat-binding factor 1, TRF1; telomere repeat-binding factor 2, TRF2; TRF1 interacting nuclear protein 2, TIN2.

**ABSTRACT**: A growing body of literature suggests that the homologous recombination/repair (HR) pathway cooperates with components of the shelterin complex to promote both telomere maintenance and non-telomeric HR. This may be due to the ability of both HR and shelterin proteins to promote strand invasion, wherein a single-stranded DNA (ssDNA) substrate base pairs with a homologous double-stranded DNA (dsDNA) template displacing a loop of ssDNA (D-loop). Rad51 recombinase catalyzes D-loop formation during HR, and telomere repeat-binding factor 2 (TRF2) catalyzes the formation of a telomeric D-loop that stabilizes a looped structure in telomeric DNA (t-loop) that may facilitate telomere protection. We have characterized this functional interaction *in vitro* using a fluorescent D-loop assay measuring the incorporation of Cy3-labeled 90 nucleotide telomeric and non-telomeric substrates into telomeric and non-telomeric plasmid templates. We report that pre-incubation of a telomeric template with TRF2 inhibits the ability of Rad51 to promote telomeric D-loop formation when pre-incubated with a telomeric substrate. This suggests Rad51 does not facilitate t-loop formation, and suggests a mechanism whereby TRF2 can inhibit HR at telomeres. We also report a TRF2 mutant lacking the dsDNA binding domain promotes Rad51-mediated non-telomeric D-loop formation, possibly explaining how TRF2 promotes non-telomeric HR. Finally, we report telomere repeat binding factor 1 (TRF1) promotes Rad51-mediated telomeric D-loop formation, which may facilitate HR-mediated replication fork restart and explain why TRF1 is required for efficient telomere replication.

Mammalian telomeres consist of 5-15 kilobase pairs (kbp) of TTAGGG repeats that terminate in a 50-200 nucleotide (nt) single-stranded DNA (ssDNA) 3’ tail. The telomere repeats and the single-stranded-to-double-stranded DNA (ss/dsDNA) junction provide a binding site for telomere-specific proteins that shelter telomeres from being recognized as DNA damage. While these shelterin proteins may directly inhibit DNA damage signaling,[*1*](#_ENREF_1)*,* [*2*](#_ENREF_2) the presence of a DNA loop at the end of the telomeres (t-loop) may also mediate telomere protection. One shelterin component, telomere repeat-binding factor 2 (TRF2), is required for t-loop formation *in vivo*,[*3*](#_ENREF_3) and can promote t-loop formation *in vitro*[*4*](#_ENREF_4) by facilitating a strand invasion reaction between the ssDNA tail and upstream dsDNA in a telomere. However, telomere protection also requires components of the homologous recombination/repair (HR) pathway, which may facilitate telomere replication or promote t-loop formation.

*In vitro,* telomeric replication forks are prone to slipping,[*5*](#_ENREF_5) and replication of telomeric DNA is inefficient[*6*](#_ENREF_6) and prone to defects consistent with fork stalling.[*7*](#_ENREF_7) *In vivo* fork stalling can be mitigated by proteins involved in the HR pathway.[*8*](#_ENREF_8) Accordingly, replication of telomeric DNA *in vivo* is sensitive to disruption of that pathway. The BRCA2 tumor suppressor recruits the Rad51-recombinase to telomeres during replication, and disrupting the expression of either of these proteins results in telomere shortening and fragility. These phenotypes are attenuated in cells possessing short telomeres and are exacerbated by chemical inhibition of DNA replication.[*9*](#_ENREF_9) As such it’s likely that these defects are due in part to a telomere replication defect.

Disrupting the HR pathway in non-dividing cells results in aberrant telomere repair. Therefore, it is likely that the HR pathway also contributes to telomere protection in a replication-independent manner,[*9*](#_ENREF_9) possibly by promoting t-loop formation. Concordantly, both TRF2 and Rad51 are required for cell extracts to promote telomeric D-loop formation;[*10*](#_ENREF_10) a requisite step in t-loop formation. Interestingly, this relationship appears to be bi-directional. Overexpression of TRF2 promotes, while TRF2 knockdown inhibits HR *in vivo*.[*11*](#_ENREF_11) While these observations suggest that TRF2 and HR cooperate functionally *in vivo*, this hypothesis contradicts these proteins’ established *in vitro* activities. TRF2 induces positive supercoiling within telomeric dsDNA upon binding,[*12*](#_ENREF_12) but Rad51 most efficiently promotes D-loop formation when acting upon negatively supercoiled dsDNA templates.[*13*](#_ENREF_13)

To investigate functional interactions between shelterin proteins and the HR pathway, we undertook an *in vitro* characterization of the combined activities of purified proteins from these pathways. While the use of purified proteins permits an examination of their isolated functional interactions *in vitro*, such interactions may be affected by other proteins *in vivo*. The absence of such other proteins likely explains why the results of our assay contradict previous cell-extract based characterizations.[*10*](#_ENREF_10) We report that TRF2 inhibits the ability of Rad51 to promote telomeric D-loop formation, suggesting that Rad51 does not promote t-loop formation and elucidating a novel mechanism by which TRF2 inhibits aberrant DNA repair at the telomeres. In contrast, we report that TRF1 promotes Rad51-mediated telomeric D-loop formation, possibly explaining why TRF1 is required for efficient telomere replication. Finally, we report that a TRF2 mutant lacking the dsDNA binding domain was able to promote Rad51-mediated D-loop formation, suggesting that one or more TRF2 domains can positively modulate Rad51 activity and possibly explaining how TRF2 can facilitate HR.

**EXPERIMENTAL PROCEDURES**

**DNA Substrates, Templates and Competitors.** A pBluescript derived plasmid containing a 103 bp telomeric tract (pBB: TTAGGG)17T) was generated by conventional cloning via insertion of the BsmBI/BbsI fragment of pRST15[*4*](#_ENREF_4) into BsmBI cut pRST15. A pBluescript derived plasmid containing a non-telomeric insert (pGL GAP) was generated as previously described.[*14*](#_ENREF_14) All plasmids were cultured in DH10B *E. coli* and purified using Qiagen Maxiprep kits. HPLC purified 5’ Cy3 labeled G-rich telomeric 90 mer oligonucleotide (T90:[Cy3] (GGTTAG)15), D1 oligonucleotide ([Cy3]AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT) and T3 promoter primer ([Cy3]ATTAACCCTCACTAAAGGA) and HPSF purified unlabeled T7 promoter primer (TAATACGACTCACTATAGGG) were ordered from Eurofins MWG Operon. A 255 bp Cy3-labeled PCR product was amplified from pBB using the 5’ Cy3-labeled T3 and unlabeled T7 promoter primers and Q5 High Fidelity Polymerase (New England BioLabs) as per the manufacturer’s instructions and purified using a DNA Clean & Concentrator-25 column (Zymo Research).\

**Proteins.**Untagged Rad51 was expressed and purified as previous described[*15*](#_ENREF_15) from a pET-24 derived plasmid (EMD Millipore), which was generously provided by Dr. Richard Fishel (Ohio State University, USA). N-terminally hexahistidine tagged TRF2, TRF2∆B, TRF2∆M and TRF1 were purified from pTRC-HIS derived plasmids (Invitrogen) adapted from vectors generously provided by the laboratory of Dr. Eric Gilson[*12*](#_ENREF_12) (University of Nice, France) or modified from vectors previously described.[*16*](#_ENREF_16) All TRF2 cDNAs were modified to include the Ala434 codon that is absent in HeLa derived TRF2 clones.[*17*](#_ENREF_17) Briefly, a pTRC-HIS plasmid was transformed into BL21(DE3)PlysS *E. coli* and serially passaged to inoculate 1 L of Terrific Broth (Sigma-Aldrich) containing 50 μg/ml ampicillin. The culture was grown to an OD of 0.6 at 595 nm, and protein expression was then induced via addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (Promega) for 4 h at 37°C. The cells were then recovered via centrifugation, washed with phosphate buffered saline, resuspended in 100 ml of buffer containing 20 mM HEPES at pH 7.5, 300 mM NaCl, 10% Glycerol, 0.5 mM DTT and 50 mM Imidazole supplemented with protease inhibitors (Roche) and then flash frozen in liquid nitrogen. The cells were then thawed and lysed via sonication following addition of 1 mg/ml egg white lysozyme and 20 μL of RQ1 DNAse (Promega) and 20 μL of RNAse A (Sigma). The crude lysate was then centrifuged in an SW-41 Ti rotor at 41,000 RPM for 1.5 h. The supernatant was collected and serially purified over 1 ml HisTrap HP, HiTrap Heparin HP and HiTrap Q FF columns using an ÄKTApurifier FPLC (GE Bioscience). Rad51, TRF2, TRF2∆B and TRF2∆M protein were recovered in 20 mM HEPES at pH 7.5, 150 mM NaCl, 10% Glycerol and 0.5 mM DTT, while TRF1 was recovered in 20 mM HEPES at pH 7.5, 300 mM NaCl, 10% Glycerol, 0.5 mM DTT. These proteins were aliquoted, flash frozen with liquid nitrogen and stored at -80°C until use. Protein concentration was determined using a Biorad Protein Assay calibrated against a Bovine Gamma Globulin standard set (Biorad). For all proteins homogeneity was assessed as >90% by Coomassie staining of SDS-PAGE gels. Immediately prior to use in experiments TRF2, TRF2∆B, TRF2∆M and TRF1 were diluted to a final concentration of 4.25 μM in buffer containing 19 mM HEPES-KOH, 203.8 mM NaCl, 1 mM CaCl, 1 mM ATP, 7% glycerol, and 0.7 mM DTT. All protein concentrations are reported as monomeric protein. Rad51 was purified to a concentration of 27.5 μM and was used un-diluted in all experiments. Fraction V Bovine Serum Albumin (Fisher) was diluted to 10 mg/ml in 20 mM potassium phosphate at pH 7.0, 50 mM NaCl, 5% Glycerol and 0.1 mM EDTA.

**Displacement Loop Assay.**For the displacement loop assay 2.4 μM in nucleotides (nt) of the 5’ Cy3 labeled telomeric 90 mer (26.67 nM Oligo) was incubated with no protein or 1,000-1,500 nM Rad51 at 37 °C for 10 min in a reaction buffer containing 5 mM HEPES-KOH at pH 7.5, 1 mM CaCl, 1 mM ATP, 0.8 mM DTT and 100 μg/ml BSA. Simultaneously, the pBB plasmid, 35 μM in base pairs (bp) or 10 nM plasmid was incubated with no protein or 100-500 nM TRF2, TRF2∆B, TRF2∆M or TRF1 at 37 °C for 10 min in reaction buffer and 100 μg/ml BSA. Equal volumes of these reactions were then combined to give final Rad51 concentrations of 0 or 500-750 nM and a final concentration of 0-250 nM TRF2, TRF2∆B, TRF2∆M or TRF1. These reactions were incubated at 37 °C for 1 h then deproteinized via addition of 0.5% sodium dodecyl sulfate and 1 mg/ml Proteinase K (Ambion) and incubation at 37 °C for 15 min. Glycerol loading buffer (5% Glycerol, 1.67 mM Tris, 0.17 mM EDTA, 0.017% SDS) was then added to 1X and the samples were separated for 30 min in a small-format 1% 1/2X TBE agarose gel at 100 V (6.67 V/cm) in a light-protected box in a 4 °C cold room. All figures are labeled with the final respective protein concentrations.

**Electrophoretic Mobility Shift and Binding Competition Assay.** To demonstrate binding via an electrophoretic mobility shift assay, 2.55 μM in bp of the Cy3-labeled PCR product (10 nM product) was incubated with no protein or 100-500 nM TRF2, TRF2∆B, TRF2∆M or TRF1 at 37 °C for 10 min in reaction buffer supplemented with 100 μg/ml BSA. To demonstrate binding specificity via a competition assay, an additional set of 500 nM reactions were performed in buffer containing no competitor or between a 1:1 (2.55 μM bp) and 200:1 (510 μM bp) excess of pGL GAP and then incubated at 37 °C for 25 min. To demonstrate that the induced supershifts were protein-mediated a 500 nM reaction containing no competitor was incubated for 10 min then deproteinized with SDS and proteinase K for 15 min. Glycerol loading buffer containing no SDS was then added to 1X and the samples were separated for 30 min in a small-format 1/2X TBE agarose gel at 100 V (6.67 V/cm) in a light-protected box in a 4 °C cold room. All figures are labeled with the final respective protein concentrations.

**Imaging.**All Cy3 labeled gel products were imaged using a Biorad Typhoon Scanner equipped with a 532 nM green laser module and a 580 nM bypass filter. Gels were imaged with a photomultiplier setting of 600 and a pixel size of 100 microns. All gels were imaged with a +3 mm focal plane setting. Gel image intensity was then adjusted using ImageQuant software (GE Life Sciences) and quantified using ImageJ software (NIH).

**RESULTS**

**A Fluorescent TRF2 and Rad51-Mediated Displacement Loop Assay.**To investigate functional interactions between Rad51 and TRF2 we developed a fluorescent displacement loop (D-loop) assay (Figure 1A) adapted from previous TRF2 and Rad51 characterizations.[*12*](#_ENREF_12)*,* [*15*](#_ENREF_15) Untagged Rad51, and N-terminally hexahistidine tagged TRF1, TRF2 and TRF2 mutant proteins lacking either the N-terminal basic domain of TRF2 (TRF2∆B) or the C-terminal Myb domain of TRF2 (TRF2∆M) were purified from *E. coli* to >90% homogeneity (Supporting Information Figure S1). In this assay co-incubation of a Cy3-labeled telomeric ssDNA substrate (T90) with a dsDNA telomeric plasmid template (pBB) in the absence of any proteins resulted in low-to-undetectable levels (<0.5%) of spontaneous D-loop formation (Figure 1B, C: Lane 1). In contrast, pre-incubation of the substrate with purified Rad51 protein prior to its addition to the template promoted D-loop formation in a Rad51-concentration dependent manner (Figure 2A, B). Likewise, pre-incubation of the template with full length TRF2 protein prior to its addition to the substrate could promote D-loop formation across a discreet range of TRF2 concentrations (Figure 1B). TRF2∆B exhibited only 47% of the activity of full-length TRF2 (Table 1), but this residual activity was similarly optimal across a narrow range of concentrations (Figure 1B). In contrast, TRF2∆M and TRF1 respectively exhibited only 31% and 27% of the activity of full length TRF2 (Table 1), and were maximally active only at higher concentrations (Figure 1C).

Rad51-mediated D-loop formation was observed to be homology driven. Rad51 could promote D-loop formation between telomeric substrates and templates, and non-telomeric substrates and templates, but not between a telomeric substrate and a non-telomeric template (Supporting Information Figure S2A, B). Under identical conditions, Rad51-mediated telomeric D-loop formation was 6.4 fold higher (Table 1) than non-telomeric D-loop formation (Supporting Information Figure S2). This is consistent with previous characterizations showing that the activity of Rad51 is enhanced on repetitive and GT rich substrates.[*18*](#_ENREF_18)*,* [*19*](#_ENREF_19) In contrast to Rad51, TRF2-mediated D-loop formation was observed to be critically dependent upon telomeric homology. TRF2 could promote D-loop formation only between telomeric substrates and templates (Supporting Information Figure S2C, D).

**TRF2 Inhibits Rad51-Mediated Telomeric But Not Non-Telomeric D-Loop Formation.**To test for functional interactions between TRF2 and Rad51, D-loop assay reactions were prepared in which the template was pre-incubated with either a fixed concentration of TRF2 or no protein, while the substrate was pre-incubated with one of several concentrations of Rad51 or no protein prior to the combination of the substrate and template reactions. Pre-incubation of a telomeric template with TRF2 reduced the ability of Rad51 to promote D-loop formation between the template and a homologous telomeric substrate by 52±5.1% (Table 1; Figure 2A, B). In contrast, TRF2 did not significantly inhibit Rad51-mediated non-telomeric D-loop formation (Table 1; Figure 2C, D). Taken together, these data suggested that TRF2 differentially modulates Rad51-mediated telomeric and non-telomeric D-loop formation.

Rad51-mediated D-loop formation is a multi-step process initiated by Rad51 binding to ssDNA to form a nucleoprotein filament, which subsequently interrogates dsDNA for matching antisense sequence in a process known as ‘homology search’. In this process a Rad51-coated substrate initially forms a protein-mediated complex with a homologous template. Subsequently Rad51 promotes D-loop formation between the substrate and template.[*20*](#_ENREF_20) To determine what step or steps of this process might be inhibited by TRF2 we performed several order of addition experiments.

We observed that the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation was dependent upon addition of TRF2 early in the D-loop reaction (Supporting Information Figure S3). TRF2 could inhibit Rad51-mediated telomeric D-loop formation when pre-incubated with the telomeric template (T0) or when added to a combined reaction prior to D-loop formation (T0+10min). However, TRF2 could not inhibit Rad51-mediated D-loop formation if added after D-loop formation had already occurred (T0+3hrs). These observations suggested that TRF2 modulates Rad51-mediated D-loop formation via a passive mechanism, possibly by interfering with Rad51 filament formation, inhibiting homology search or by preventing subsequent D-loop formation.

We also observed that TRF2 could inhibit telomeric D-loop formation regardless of whether TRF2 was pre-incubated with the telomeric template or with the Rad51-coated substrate (Supporting Information Figure S4). However, the degree of this inhibition was reduced when TRF2 was incubated with the substrate compared with when it was incubated with the template. This suggests that TRF2 does not inhibit Rad51 at the level of filament formation. Instead, it appears that the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation is dependent upon the ability of TRF2 to bind to or modify the template.

To investigate whether the DNA binding activities of TRF2 mediate its ability to inhibit Rad51-mediated telomeric D-loop formation, we characterized the binding affinity and specificity of TRF2 using an electrophoretic mobility shift assay (EMSA) and a binding competition assay (Figure 2E). Incubating a Cy3-labeled template containing a 103 base pair bp telomere tract with increasing concentrations of TRF2 resulted in a supershift of the template, consistent with stable TRF2 binding (Figure 2F). The binding to TRF2 to the template was observed to be specific, and persisted even in the presence of high concentrations of non-telomeric competitor (Figure 2F: lanes 8-11). Nearly all low-mobility species generated by TRF2 binding became trapped in the wells. This supershift was protein mediated, and could be disrupted by incubation with SDS and proteinase K (Figure 2F: lane 12).

To further investigate possible mechanism by which TRF2 may inhibit Rad51-mediated telomeric D-loop formation we characterized the binding activity and the telomeric and non-telomeric Rad51-modulating activities of a variety of TRF2 mutant proteins and TRF1, a close homolog of TRF2.

**TRF2∆M Promotes Rad51-Mediated Telomeric But Not Non-Telomeric D-Loop Formation.** The dsDNA binding activity of TRF2 is primarily directed by its C-terminal Myb domain. Deletion of this Myb domain reduces telomeric dsDNA binding affinity by a factor of 2.9 (Table 1), eliminates telomeric binding specificity and grossly alters DNA binding properties compared to full-length TRF2 (Figure 3E).[*12*](#_ENREF_12) Interestingly and in contrast to TRF2, TRF2∆M does not inhibit Rad51-mediated telomeric D-loop formation (Table 1; Figure 3A, B). Also in contrast to TRF2, TRF2∆M was found to promote Rad51-mediated non-telomeric D-loop formation by 112±13.0% (Table 1; Figure 3C, D). Taken together these observations suggest that the Myb domain of TRF2 both contributes to the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation and suppresses the ability of TRF2 to promote Rad51-mediated non-telomeric D-loop formation.

**TRF2∆B Inhibits Telomeric But Not Non-Telomeric Rad51-Mediated D-Loop Formation.** In addition to its Myb domain, TRF2 possesses an N-terminal domain rich in basic residues that has been implicated in directing the binding of TRF2 to ss/dsDNA junctions and unusual DNA structures.[*4*](#_ENREF_4)*,* [*21*](#_ENREF_21) This domain also promotes the annealing and migration of DNA joints in a manner not unlike that required during D-loop formation.[*22*](#_ENREF_22) To investigate whether the basic domain of TRF2 contributes to the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation or the ability of TRF2∆M to promote Rad51-mediated non-telomeric D-loop formation we characterized the DNA binding affinity and specificity and Rad51-modulating activity of a TRF2 mutant protein lacking the basic domain of TRF2 (TRF2∆B).

Like TRF2 and in contrast to TRF2∆M, TRF2∆B was found to inhibit Rad51-mediated telomeric D-loop formation by 31±5.5% (Table 1; Figure 4A, B), suggesting that the joint-binding activity of TRF2 is not required for TRF2 to inhibit Rad51-mediated telomeric D-loop formation. In contrast, TRF2∆B was not observed to affect Rad51-mediated non-telomeric D-loop formation (Table 1; Figure 4C, D). Deletion of the basic domain resulted in an approximately 2.3-fold reduction in template binding affinity (Table 1) but did not reduce binding specificity (Figure 4E: lanes 8-11) compared to full length TRF2. Like TRF2, TRF2∆B binding resulted in the template becoming trapped in the wells.

**TRF1 Promotes Rad51-Mediated Telomeric But Not Non-Telomeric D-Loop Formation.** Our observation that TRF2 and TRF2∆B but not TRF2∆M could inhibit Rad51-mediated telomeric D-loop formation suggested that this inhibition could simply be due to Myb-domain directed dsDNA binding. To test this hypothesis we characterized the DNA binding and Rad51-modulating activity of TRF1, a TRF2 homolog with a highly similar Myb domain (Supporting Information Figure S1A).[*23*](#_ENREF_23) Interestingly and in contrast to TRF2, TRF1 was found to promote Rad51-mediated telomeric D-loop formation by 25±1.0% (Table 1; Figure 5A, B), suggesting that the ability of TRF2 to inhibit this process is not simply due to Myb domain binding. In contrast, TRF1 was found not to affect Rad51-mediated non-telomeric D-loop formation (Figure 5C, D). Comparisons between TRF1 and TRF2 must be made with caution, as despite possessing comparable DNA binding affinity and telomeric sequence specificity (Table 1) their binding behavior is otherwise grossly different when examined in an EMSA. Whereas TRF2 binding shifts a telomeric template into the wells (Figure 2F), TRF1 binding shifts the species into increasingly larger complexes as the TRF1 concentration is increased (Figure 5E). This behavior is perhaps consistent with previous observations that while TRF2 binds to telomeric dsDNA as a large oligomeric structure, TRF1 binds as a smaller complex.[*24*](#_ENREF_24)*,* [*25*](#_ENREF_25) Likewise, this property may be consistent with observations that TRF2 can promote the formation of unusual DNA structures and induce topological changes within telomeric DNA to a greater degree than TRF1.[*12*](#_ENREF_12)*,* [*26*](#_ENREF_26)

**DISCUSSION**

The results of this study suggest a model whereby TRF1 and TRF2 differentially regulate Rad51-mediated telomeric and non-telomeric D-loop formation. This would promote efficient telomeric DNA replication and non-telomeric HR while inhibiting aberrant HR at the telomeres. TRF1 promotes Rad51 mediated telomeric D-loop formation, which may facilitate replication fork restart and explain why TRF1 is required for efficient telomere replication. In contrast, TRF2 potently inhibits Rad51-mediated telomeric D-loop formation, providing yet another mechanism by which TRF2 can inhibit DNA repair at telomeres. Finally, TRF2∆M promotes Rad51-mediated D-loop formation, providing insight into how TRF2 may contribute to HR. Our findings are generally in good agreement with previous characterizations, and what contradictions exist are likely due to methodological differences.

Data from our *in vitro* characterization must be compared with other *in vivo* and genetic characterizations with caution. The activities of TRF1, TRF2, and Rad51 are modulated *in vivo* by a variety of other proteins, including other shelterin and HR proteins, which are absent from our assay. Additionally, the templates and substrates used in our assay are necessarily different from their *in vivo* analogs. The 3’ tails of telomeres are adjacent to a ss/dsDNA junction, which were absent from the substrates used in this work. Furthermore, telomeres are several kbp in length, whereas our template possesses only a 103 bp telomeric tract. Finally, while the topology of telomeric DNA *in vivo* is unknown, the templates used in our assay were negatively supercoiled.

While we observed that TRF2 inhibits Rad51, it has previously been reported that TRF2 and Rad51 appear to exhibit functional cooperation. Immunodepletion of TRF2 or Rad51 from cell extracts ablates the ability of those extracts to promote telomeric D-loop formation.[*10*](#_ENREF_10) Moreover, supplementation of such immunodepleted extracts with purified Rad51 or TRF2 can restore telomeric D-loop formation.[*10*](#_ENREF_10)*,* [*27*](#_ENREF_27) However, the presence of factors *in vivo* that are absent from our *in vitro* characterization may affect the activities of TRF2 and Rad51.

Although TRF1 and TRF2 can be found at telomeres throughout the cell cycle and TRF1 promotes efficient telomeric replication,[*7*](#_ENREF_7) TRF1 and TRF2 inhibit DNA replication *in vitro*.[*6*](#_ENREF_6) However, TRF1 and TRF2 binding are inhibited by post–translational modifications (PTMs), some of which are conferred by replication-complex associated proteins.[*28-34*](#_ENREF_28)

These proteins may facilitate replication by transiently removing TRF1 and TRF2 from telomeric DNA near the replication fork.[*30*](#_ENREF_30) Interestingly, PTMs that reduce TRF1 binding are inhibited *in vivo* by another shelterin protein, TIN2,[*28*](#_ENREF_28) and by FANCD2, a component of the Fanconi anemia pathway.[*31*](#_ENREF_31) Comparable PTMs of TRF2 are not likewise inhibited. However, these TRF2 PTMs likely disrupt TRF2 dimerization, which would be expected to abrogate Myb domain binding but that may not affect basic domain binding. As TRF1 can promote and TRF2 can inhibit Rad51-mediated telomeric D-loop formation, the depletion of TRF2 but not TRF1 from DNA near the replication fork may facilitate HR-mediated fork restart within the telomeres (Figure 6A). This process may also be facilitated by basic-domain mediated recruitment of TRF2 to regressed forks, where it can both protect the nascent Holliday junction (HJ) from HJ resolvases*[22](#_ENREF_22" \o "Poulet, 2009 #86)* and recruit RecQ helicases that can promote fork migration.[*35*](#_ENREF_35)*,* [*36*](#_ENREF_36) Likewise, the presence of TRF2 on telomeric dsDNA away from the fork may prevent HR-mediated strand invasion reactions and protect the telomeres from aberrant repair (Figure 6B).

The role of TRF2 in the HR pathway, especially in non-telomeric contexts, is not well understood. TRF2 is rapidly recruited to genomic dsDNA breaks (DSBs) and this recruitment requires the basic domain of TRF2 but not its Myb-domain[*37*](#_ENREF_37) and can occur in an ATM deficient background. Additionally, TRF2 is phosphorylated by ATM[*38*](#_ENREF_38)in response to DNA damage,[*39*](#_ENREF_39) and mutations that disrupt TRF2 phosphorylation inhibit DNA repair.[*40*](#_ENREF_40) While it has been suggested that this DNA repair defect may be due to impaired non-homologous end joining (NHEJ),[*39*](#_ENREF_39) the defect may also be due to impaired HR. Overexpression of TRF2 and TRF2∆M promote HR *in vivo*.[*11*](#_ENREF_11) Likewise, knockdown of TRF2 inhibits HR but not NHEJ *in vivo*.[*11*](#_ENREF_11) Our finding that TRF2∆M can promote Rad51-mediated D-loop formation suggests a novel mechanism by which TRF2 can promote HR (Figure 6C). Upon induction of a DSB, TRF2 may undergo basic-domain mediated recruitment to the site of damage. TRF2 may then help recruit proteins such as the Mre11/Rad50/Nbs1 (MRN) complex,[*41*](#_ENREF_41) which promotes end resection in preparation for HR. Following end resection, Rad51 binding and homology search, the basic domain of TRF2 may facilitate Rad51-mediated D-loop formation by promoting the opening of the template dsDNA in a manner similar to a Rad51 accessory protein, Rad54.[*13*](#_ENREF_13)

This model of the interaction of TRF1, TRF2 and Rad51 provides insight into both telomere biology and the HR pathway. Previous characterizations suggested that TRF2 and Rad51 cooperate to promote telomeric D-loop and possibly t-loop formation *in vivo*, despite apparent incompatibilities in the *in vitro* activities of these proteins. Our finding that TRF2 inhibits Rad51-mediated telomeric D-loop formation suggests that Rad51 does not contribute to t-loop formation, or that this inhibition must be alleviated by additional factors *in vivo*. While it has previously been reported that TRF1 is required for efficient telomere replication, this requirement seems at odds with other reports that TRF1 can inhibit telomere replication. Our finding that TRF1 promotes Rad51-mediated telomeric D-loop formation suggests that TRF1 may facilitate telomere replication by promoting HR-mediated replication fork restart. Finally, our observation that TRF2∆M can promote Rad51-mediated non-telomeric D-loop formation may explain how TRF2 can promote HR *in vivo*.

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| --- | --- | --- | --- | --- | --- | --- |
| **Table 1** Properties of TRF2, TRF2∆B, TRF2∆M and TRF1 | | | | | | |
| **Proteins** | **TRF-Induced**  **Telomeric**  **D-Loop Formation**  **AUC 0-250 nM**  *% of TRF2* | **Rad51-Mediated**  **Telomeric**  **D-loop Formation**  **AUC 500-750 nM**  *% Change From Buffer* | **Rad51-Mediated**  **Non-Telomeric**  **D-Loop Formation**  **AUC 500-750 nM**  *% Change From Buffer* | **Telomeric DNA Binding**  C1/2, nM | **Telomeric Binding Specificity** | **Migration in Agarose Gels** |
| **Buffer†** | N/A | **30.3 ± 0.4** | **4.7 ± 0.4** | N/A | **+** | - |
| *0 ± 0.7%* | *0 ± 7.6%* |
| **TRF2** | **6.0 ± 0.15** | **13.3 ± 0.8** \* | **4.7 ± 0.0** | 111 ± 8 | **+** | - |
| *100 ± 2.4%* | *- 52 ± 5.1% \** | *+ 4 ± 2.2%* |
| **TRF2∆B** | **2.8** | **22.1 ± 0.7** \* | **5.1 ± 0.7** | 257 | **+** | - |
| *47%* | *- 31 ± 5.5% \** | *+ 5 ± 7.2%* |
| **TRF2∆M** | **1.8** | **29.0 ± 1.4** | **9.6 ± 0.4** \* | 319 | - | **+ /** - |
| *31%* | *+ 4 ± 4.0%* | *+ 112 ± 13.0% \** |
| **TRF1** | **1.6** | **38.5 ± 1.9 \*** | **5.4 ± 0.9** | 152 | **+** | **+** |
| *27%* | *+ 25 ± 1.0% \** | *+ 9 ± 5.2%* |
| *†Buffer data are averaged. Proteins are statistically compared against matched buffer controls.* | | | | | | |
| *TRF-induced D-loop formation calculated as area under the curve (AUC: Complex%* × *[TRF] nM) from Figure 1.* | | | | | | |
| *Rad51-mediated D-loop formation calculated as AUC (Complex%* × *[Rad51] nM) from Figures 2-5.* | | | | | | |
| *AUC calculation examples in Supporting Information Figure S5.* | | | | | | |
| *C1/2 represents the concentration of TRF protein required to supershift 50% of template in EMSAs.* | | | | | | |
| *Errors shown are 95% confidence intervals from three independent experiments.* | | | | | | |
| *\* denotes significant difference (p<0.05) from buffer via two-tailed paired samples t-test.* | | | | | | |

**Figure 1** TRF-mediated telomeric D-loop formation. **(A)** Diagram of D-loop assay. **(B)** TRF2 and TRF2∆B promote telomeric D-loop formation with an activity peak when included at a final concentration of between 100 nM (lane 3) and 150 nM (Lane 4) of protein. **(C)** TRF2∆M and TRF1 promote telomeric D-loop formation only at higher concentrations.

**Figure 2** TRF2 inhibits Rad51-mediated telomeric but not non-telomeric D-loop formation. **(A)** Rad51 promotes telomeric D-loop formation in a concentration dependent manner that is inhibited by TRF2. **(B)** Quantification of data in (A).  **(C)** Rad51 promotes non-telomeric D-loop formation in a concentration dependent manner that is not affected by TRF2. **(D)** Quantification of data in (C). **(E)** Diagram of DNA binding and competition assay. **(F)** TRF2 binding supershifts the template into the wells. This binding is specific and persists in the presence of high concentrations of non-telomeric competitor and is protein-mediated. Error bars shown 95% confidence interval, significant difference between +Buffer and +TRF2 (**\***), paired samples t-test α=0.05 from three independent experiments.

**Figure 3** TRF2∆M promotes Rad51-mediated non-telomeric but not telomeric D-loop formation. **(A)** Rad51 promotes telomeric D-loop formation in a concentration dependent manner that is not affected by TRF2∆M. **(B)** Quantification of data in (A). **(C)** Rad51 promotes non-telomeric D-loop formation in a concentration dependent manner that is promoted by TRF2∆M. **(D)** Quantification of data in (C). **(E)** TRF2∆M binding supershifts the template into a lower mobility species and into the wells. This binding is non-specific and is disrupted by low concentrations of non-telomeric competitor and is protein-mediated. Error bars shown 95% confidence interval, significant difference between +Buffer and +TRF2∆M (**\***), paired samples t-test α=0.05 from three independent experiments.

**Figure 4** TRF2∆B promotes Rad51-mediated telomeric but not non-telomeric D-loop formation. **(A)** Rad51 promotes telomeric D-loop formation in a concentration dependent manner that is promoted by TRF2∆B. **(B)** Quantification of data in (A). **(C)** Rad51 promotes non-telomeric D-loop formation in a concentration dependent manner that is not affected by TRF2∆B. **(D)** Quantification of data in (C). **(E)** TRF2∆B binding supershifts the template into the wells. This binding is specific and persists in the presence of high concentrations of non-telomeric competitor and is protein-mediated. Error bars shown 95% confidence interval, significant difference between +Buffer and +TRF2∆B (**\***), paired samples t-test α=0.05 from three independent experiments.

**Figure 5** TRF1 promotes Rad51-mediated telomeric but not non-telomeric D-loop formation. **(A)** Rad51 promotes telomeric D-loop formation in a concentration dependent manner that is promoted by TRF1. **(B)** Quantification of data in (A).  **(C)** Rad51 promotes non-telomeric D-loop formation in a concentration dependent manner that is not affected by TRF1. **(D)** Quantification of data in (C). **(E)** TRF1 binding supershifts the template into several low-mobility species. This binding is specific and persists in the presence of high concentrations of non-telomeric competitor and is protein-mediated. Error bars shown 95% confidence interval, significant difference between +Buffer and +TRF1 (**\***), paired samples t-test α=0.05 from three independent experiments.

**Figure 6** TRF1 and TRF2 differentially modulate Rad51-mediated telomeric and non-telomeric D-loop formation. **(A)** Post translational modifications may deplete TRF2 but not TRF1 from telomeric DNA near a replication fork, possibly by inhibiting TRF2 Myb domain binding. Following fork collapse, basic domain directed TRF2 binding can protect regressed forks from cleavage and recruit factors that promote fork migration. Finally, TRF1 can promote D-loop formation away from the fork and thereby promote HR mediated fork restart. **(B)** TRF2 inhibits Rad51-mediated telomeric D-loop formation, which may prevent aberrant repair processes at the telomeres. **(C)** TRF2 is recruited to DSBs, where it may promote recruitment of enzymes that promote end resection. Afterwards, the basic domain of TRF2 may promote Rad51-mediated D-loop formation and thereby promote HR.

**Figure 1**

**C:\Users\Brian\Desktop\TRF1 and TRF2 differentially modulate telomeric and non-telomeric Rad51-mediated displacement loop formation in vitro\F1,DLoopTest1.tif**

**Figure 2**

**C:\Users\Brian\Desktop\TRF1 and TRF2 differentially modulate telomeric and non-telomeric Rad51-mediated displacement loop formation in vitro\F2,TRF2.tif**

**Figure 3**

**C:\Users\Brian\Desktop\TRF1 and TRF2 differentially modulate telomeric and non-telomeric Rad51-mediated displacement loop formation in vitro\F3,DeltaM.tif**

**Figure 4**

**C:\Users\Brian\Desktop\TRF1 and TRF2 differentially modulate telomeric and non-telomeric Rad51-mediated displacement loop formation in vitro\F4,DeltaB.tif**

**Figure 5**

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**Figure 6**

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**Supporting Information**

Supporting Information Figures S1-S5 are available free of charge via the Internet at http://pubs.acs.org.