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## p53 Binds Telomeric Single Strand Overhangs and t-Loop Junctions *in Vitro*\*

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**The interaction of p53 with a human model telomere *in vitro* was examined by electron microscopy. p53 demonstrated a sequence-independent affinity for telomeric DNA *in vitro*, localizing to the 3' single strand overhang and the t-loop junction both in the presence and absence of associated TRF2. Binding was not observed above background along the duplex telomeric repeats. However, the efficiency of TRF2-catalyzed t-loop formation on the model DNA was increased 2-fold in the presence of p53 although a variety of single strand or Holliday junction-binding proteins did not facilitate t-loop formation. These results suggest that p53 has an active role in telomere maintenance and structure through association with the t-loop junction.**

Recent studies have implicated several proteins required for DNA damage recognition and repair in telomere maintenance. The mammalian protein Ku, which binds double strand (ds)<sup>1</sup> ends and is central to ds break repair has been shown to bind telomeric DNA directly (1, 2) and to associate with the two duplex telomere repeat binding factors TRF1 and TRF2 (3, 4). Further, in *Saccharomyces cerevisiae*, the yeast homolog yKu is required for proper telomere function (5). Recent studies by Tong *et al.* (6) revealed that the tumor suppressor protein p53 is involved in the maintenance of telomeric tract length in mice. They observed that although the level of telomere-associated fluorescence in p53<sup>-/-</sup> mouse embryo fibroblasts was equivalent to that of wild type cells, there was an increased heterogeneity of telomere lengths with some telomeres being longer than those seen in wild type cells whereas others were very short or lost entirely. These shortened telomeres resulted in a high frequency of telomere-telomere fusions.

The telomere-specific protein TRF2 plays a central role in concealing telomere ends from ds break recognition and repair

factors (7, 8). Expression of a dominant-negative allele of TRF2 in cultured human cells triggers changes typical of those induced by ds breaks: loss of the 3' single strand (ss) overhang, induction of end-to-end chromosome fusions (8), and induction of apoptosis through the p53/ATM-dependent DNA damage checkpoint pathway (7). The induction of p53 is not dependent on DNA replication, suggesting that inhibition of TRF2 function at the telomeres signals p53 directly.

Our recent studies of telomere architecture provide a possible structural solution to how telomere ends are hidden from DNA break repair/recognition factors. These studies showed that mammalian telomeres are arranged into large duplex loops *in vivo* (t-loops) (9). The formation of t-loops *in vitro* requires TRF2 and a telomeric junction, which consists of a 3' ss overhang of at least one TTAGGG repeat adjacent to the ds portion of the telomere (10). The termini of the micronuclear chromosomes of *Oxytricha nova* (11), the telomeres of *Trypanosoma brucei* minichromosomes (12), and the telomeres of *Pisum sativum*<sup>2</sup> have been shown to form looped structures *in vivo* whereas *S. cerevisiae* telomeres appear to form fold-back structures (13–15). It is plausible that telomeric looping is a common mechanism for protecting the termini of linear chromosomes.

It has been proposed that the t-loop structure is formed by strand invasion of the G-rich ss overhang into the preceding duplex TTAGGG tract (9, 10). This invasion would generate a D-loop, which would effectively hide the natural end of the DNA to protect it from the machinery that scans DNA for broken ends. In addition, recent data support the possibility that some portion of the C-rich strand of the ss/ds telomeric junction may also invade the duplex, resulting in the formation of a Holliday junction-like structure at the base of the t-loop (Fig. 1A) (10). p53 tightly binds Holliday junctions *in vitro* and enhances their resolution by junction-cleaving enzymes (16). Further, p53 has a strong affinity for ss DNA (17) and would presumably bind the ss telomeric overhang of the telomere. The affinity of p53 for these structures points to the importance of investigating p53 binding to both the telomeric overhang and the t-loop junction as well as examining the influence of p53 on t-loop assembly by TRF2 *in vitro*.

Using electron microscopy (EM), we found that p53 was present at the t-loop junction both in the presence and absence of associated TRF2. In addition, localization of p53 to the 3' ss overhang was observed. TRF2-mediated t-loop formation was increased 2-fold on a model telomere DNA by the addition of p53. These studies suggest that p53 may be involved in the maintenance of telomere structure.

### EXPERIMENTAL PROCEDURES

**DNA Substrates and Proteins**—The model telomere contains 3 kb of plasmid sequences followed by 576 bp of duplex telomeric DNA, which terminates in a 54-nt telomeric overhang (5'-(TTAGGG)<sub>9</sub>-3') (10). The overhang was removed by treatment with mung bean nuclease to create a telomeric tract that terminates in a blunt end (10). Linear DNA substrates containing the 576-bp tract located internally were generated by cleavage of pRST5 plasmid with *Afl*III (New England Biolabs Inc., Beverly, MA), placing it 334 bp from the 5' end and 2597 bp from the 3' end.

Human p53 protein was overexpressed in SF9 cells using a vector provided by Dr. Arnold Levine and purified as described previously (18).

<sup>2</sup> J. D. Griffith, unpublished studies.

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<sup>1</sup> The abbreviations used are: ds, double strand; ss, single strand; EM, electron microscopy; nt, nucleotide; AMT, 4'-aminomethyltrioxsalen; RPA, replication protein A.

His-tagged TRF2 protein was overexpressed in SF9 cells using a vector provided by Dr. Titia de Lange and purified as described previously (19). *Escherichia coli* SSB was purified as described previously (20). T4 gene 32 protein was a gift of Dr. Nancy Nossal. Human RPA was a gift of Dr. Louise T. Chow. Human MSH 2/6 protein was overexpressed in SF9 cells using a vector provided by Dr. Richard Fishel and purified as described previously (21). The HMG(IY) bacterial plasmid was provided by Dr. Beverly Emerson, and the protein was purified as described previously (22).

**Binding of p53 to Telomeric DNA**—To examine the binding of p53 to telomeric DNA, 25 ng of DNA was incubated with purified p53 at a ratio of 2–8 tetramers of p53 per DNA molecule in a buffer containing 20 mM Hepes (pH 7.5) and 50 mM KCl for 20 min at room temperature. The complexes were fixed by adding glutaraldehyde to a final concentration of 0.6% for 5 min at room temperature followed by chromatography over a 2-ml column of Bio-Gel A-5m (Bio-Rad) equilibrated with 10 mM Tris (pH 7.5) and 1 mM EDTA. The samples were mixed with a buffer containing spermidine, adsorbed to glow-charged thin carbon foils, dehydrated through a series of water/ethanol washes, and rotary shadow cast with tungsten as described previously (9).

**Generation of t-Loops and Electron Microscopy**—t-Loops were formed on the model DNA by TRF2 as previously described (10). Protein-free t-loops were generated by cross-linking the DNA with 4'-aminomethyl-trioxsalen (AMT; Sigma) and UV light followed by treatment with 0.5% SDS and 0.5 mg/ml proteinase K for 15 min at 37 °C. The DNA was purified by chromatography over a 2-ml column of Bio-Gel A-5m equilibrated with 10 mM Tris (pH 7.5) and 1 mM EDTA. The absence of any residual TRF2 was verified by EM.

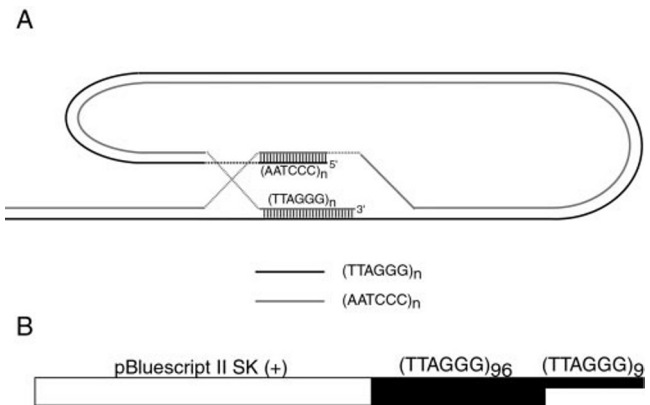
All samples were examined in a Philips EM400 or CM12 instrument. Micrographs were scanned from negatives using a Nikon 4500AF multifomat film scanner. The contrast was optimized and panels were arranged using Adobe Photoshop. The location of p53 binding to the DNA molecules was measured using a Phillips CM-12 and Digital Micrograph software (Gatan Inc., Pleasanton, CA).

**Effect of p53 and Single Strand and Holliday Junction-binding Proteins on t-Loop Formation**—DNA (100 ng) was incubated with TRF2 (3 dimers per TTAGGG repeat) and either p53 (2–8 tetramers per DNA), *E. coli* SSB (6 tetramers per DNA), T4 gene 32 protein (2 heterotrimers per DNA), human RPA (2 monomers per DNA), human MSH2/6 (2–4 heterodimers per DNA), or HMG(IY) (5–10 monomers per DNA) for 20 min at room temperature in a buffer containing 20 mM Hepes (pH 7.5) and 50 mM KCl. The complexes were then cross-linked with AMT/UV, deproteinized, and purified as previously described (9, 10).

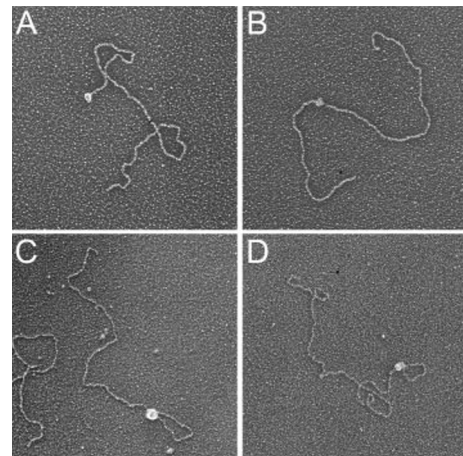
**Immunological Electron Microscopy**—To examine the binding of p53 to t-loops in the presence of TRF2, both proteins were incubated with the model telomere DNA in 20 mM Hepes (pH 7.5) and 50 mM KCl for 20 min at room temperature. The complexes were then incubated with polyclonal rabbit IgG raised against p53 (0.5  $\mu$ l; Cell Signaling Technology, Beverly, MA) for 30 min at room temperature. Gold-conjugated protein A (10-nm particles; 0.5  $\mu$ l; Amersham Biosciences) was added for an additional 30 min at room temperature followed by fixation and preparation for EM as described above.

## RESULTS

**p53 Binds to t-Loop Junction with High Affinity**—Recent data support a model of the t-loop junction in which both strands can insert to form a Holliday junction-like structure (Fig. 1A) (10). It was of interest to examine the ability of p53 to bind t-loop structures. t-Loops were generated by incubation of the model telomere with TRF2, stabilization by AMT/UV photocross-linking, and protein removal (see "Experimental Procedures") (9). p53 was incubated with the purified DNA, and the resulting complexes were examined by EM. The purified DNA contained both looped ( $10 \pm 3\%$ ,  $n = 100$ /sample, three experiments) and unlooped ( $90 \pm 3\%$ ,  $n = 100$ /sample, three experiments) molecules. Overall,  $59\% (\pm 21\%, n = 100$ /sample, three experiments) of the input DNA was bound by p53. The p53 binding occurred at one end of the DNA, internally along the duplex repeats, or at the t-loop junction. The p53 bound to the non-looped species showed a strong preference for the DNA ends (Fig. 2, A and B; see below). Of the t-loops observed, 88% ( $\pm 11\%$ ,  $n = 100$ /sample, three experiments) showed p53 bound exclusively at the t-loop junction (the junction of the loop and the linear tail) (Fig. 2, C and D). The mass of the p53 complex



**FIG. 1. t-Loop models.** A, diagram of a t-loop form in which both the G-rich strand overhang and a portion of the C-rich strand have invaded the duplex repeat region to form a Holliday junction-like structure. B, the model telomere DNA used in this study (10) consists of a ~3-kb plasmid segment followed by 576 bp of duplex TTAGGG repeats and terminates in a 54-nt 3' overhang.

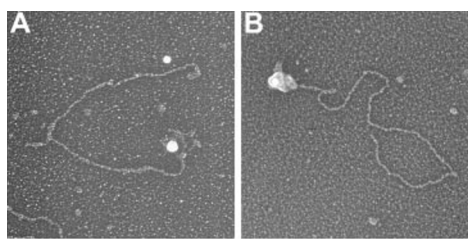


**FIG. 2. Visualization of the binding of p53 to the model telomere DNA and t-loops.** DNA was assembled into t-loops by TRF2, stabilized by cross-linking, deproteinized, and incubated with p53. Samples were prepared for EM by fixation, air-drying, and rotary shadow casting with tungsten (see "Experimental Procedures"). p53 associated with the DNA end (A) with a higher frequency than along the duplex DNA tract (B). p53 localized with high specificity to the t-loop junction (C and D). Reverse contrast is used; bar is equivalent to 1 kb.

at the t-loop junction appeared consistent with the presence of one or two tetramers with a small portion of the molecules containing larger complexes. Similarly, when TRF2 was incubated with the deproteinized, cross-linked DNA, binding was observed exclusively at the t-loop junction ( $100 \pm <1\%$ ,  $n = 100$ /sample, three experiments). TRF1, however, rarely bound the junction, binding instead along the telomeric tract (data not shown). These results demonstrate that p53 has a high affinity for the t-loop junction.

**p53 Does Not Bind Telomeric DNA in a Sequence-dependent Manner**—Based on the observation that p53 binds to the t-loop junction, it was of interest to determine whether the association of p53 with the loop is sequence-specific. It has been shown that p53 is capable of associating with DNA in both a sequence-dependent (reviewed in Refs. 23–25) and sequence-independent manner (26–27). Electrophoretic mobility shift assays and EM were used to examine the affinity of p53 for duplex TTAGGG repeat tracts as well as for a model telomere containing a duplex tract adjacent to a ss 3' overhang containing TTAGGG repeats. Electrophoretic mobility shift assays were performed with p53 and a 200-bp DNA fragment consisting of tandem TTAGGG repeats. Even with an input ratio of 30





**FIG. 3. p53 localizes to the t-loop junction in the presence of TRF2.** Complexes of p53 and TRF2 were formed on the model telomere DNA and detected by the addition of an anti-p53 polyclonal rabbit IgG followed by incubation with 10-nm gold particles conjugated with protein A. Samples were prepared for EM as described in Fig. 2. Gold labeling was observed at the t-loop junction (A) and at DNA ends (B). Reverse contrast is used; bar is equivalent to 1 kb.

tetramers per DNA molecule, p53 showed very low binding, and this was easily competed with nonspecific DNA (data not shown).

The association of p53 with telomeric repeats was examined by EM. p53 was incubated with a 96-repeat TTAGGG tract located in the center of a 3-kb DNA molecule. At a ratio of 20 p53 tetramers per DNA molecule, 60% of the input template was bound by p53 with no molecule containing more than one p53 particle. The location of p53 molecules along the DNA was measured from the micrographs, and the results revealed that of the bound DNA, 12% had p53 located within the telomeric tract, and the remaining 88% contained p53 randomly distributed along the length of the DNA ( $n = 200$ ). From these observations we conclude that p53 does not bind the TTAGGG repeat tract in a sequence-dependent manner.

The binding of p53 to the natural telomeric overhang was examined by EM using a model telomere containing a terminal 576-bp duplex tract and an adjacent 54-nt 3' ss TTAGGG overhang (Fig. 1B; see "Experimental Procedures") (10). Because p53 has been shown to bind ss DNA with high affinity, the non-telomeric end of the DNA terminated in a blunt end. Conditions were optimized so that each DNA showed no more than one p53 particle bound (20 tetramers per DNA) (see "Experimental Procedures"). Of the bound molecules,  $68 \pm 9\%$  ( $n = 100$ /sample, three experiments) contained p53 localized to the end of the DNA whereas the remainder ( $32 \pm 9\%$  of the bound molecules,  $n = 100$ /sample, three experiments) showed p53 scattered at random along the length of the DNA (Fig. 2, C and D). Removal of the 3' overhang decreased the fraction of end-bound p53 to 6%, demonstrating that the end localization of p53 is most likely because of its association with the ss overhang. Based on these data, we conclude that p53 binds to the ss telomeric overhang with strong affinity.

**TRF2-mediated t-Loop Formation Is Enhanced by p53**—It was of interest to examine the effect of p53 on the ability of TRF2 to form t-loops *in vitro*. It is possible that p53 binding to the ss DNA overhang would inhibit loop formation by preventing TRF2 from localizing to the ss/ds junction, a critical step in t-loop assembly (10). Alternately, binding to the ss overhang might enhance t-loop formation by facilitating the strand invasion event. Studies from others have suggested that p53 has some strand transfer activity (28), which might either assist in TRF2-mediated loop formation or catalyze loop formation in the absence of TRF2. Finally, p53 may stabilize loops once they are formed by binding to the t-loop junction either at the Holliday junction-like structure or the displaced ss portion of the D-loop. To investigate these possibilities, p53 and TRF2 were incubated together with the model telomere DNA. The number of t-loops assembled was monitored by EM.

First, the possible interaction between p53 and TRF2 in the absence of DNA was examined. To our knowledge, no evidence

of such an association has been reported. When p53 and TRF2 were coexpressed in SF9 cells and immunoprecipitated using standard techniques, no association was detected. Similarly, when purified TRF2 was incubated with purified p53, the proteins failed to coimmunoprecipitate (data not shown).

When TRF2 and p53 were incubated together with the model telomere DNA, smaller loops could have been obscured because of the presence of a large protein mass at the junction. Thus, an alternative approach for scoring looped molecules was utilized. Following assembly of the complexes, the DNA molecules were photocross-linked, deproteinized, and prepared for EM examination using a classic surface-spreading method with cytochrome *c* (see "Experimental Procedures"). Looped structures were rarely seen on the DNA in the absence of TRF2 ( $3 \pm 1\%$ ,  $n = 100$ /sample, three experiments) or when treated with p53 alone ( $4 \pm 1\%$ ,  $n = 100$ /sample, three experiments). When the incubations contained TRF2 and no p53,  $13\% (\pm 3\%, n = 100$ /sample, three experiments) of the input DNA was assembled into t-loops. This number is slightly reduced from the number of t-loops usually observed (10), most likely because of the use of a buffer optimized for the binding of both proteins. When both p53 and TRF2 were added to the reaction (four p53 tetramers per DNA and three TRF2 dimers per TTAGGG repeat) the number of t-loops rose significantly ( $24 \pm 3\%$ ,  $n = 100$ /sample, three experiments;  $p = 0.005$ ). Reduction of the level of p53 (two p53 tetramers per DNA) eliminated this increase in t-loop assembly ( $14 \pm <1\%$ ,  $n = 100$ /sample, two experiments). No further rise in efficiency was detected with either increased levels of p53 (six or eight p53 tetramers per DNA;  $23 \pm 1\%$ ,  $n = 100$ /sample, two experiments) or time of incubation (data not shown).

One possible explanation for the increase in the number of t-loops observed in the presence of TRF2 and p53 is that the t-loops may be stabilized by p53. *In vitro*, t-loops may form and dissociate throughout the incubation. The association of p53 with the displaced ss or the Holliday junction-like portion of the t-loop junction might stabilize the looped form, thus increasing the fraction of loops present at any one time. In this case, any ss or Holliday junction binding protein would be expected to produce the same effect. Three ss binding proteins, *E. coli* SSB, T4 gene 32 protein, and human RPA, were separately incubated with the model DNA and TRF2, and the loops were stabilized by AMT/UV cross-linking, deproteinized, and prepared for EM. No increase in the number of t-loops was observed with the addition of SSB ( $14 \pm 1\%$ ,  $n = 100$ /sample, three experiments), T4 gene 32 protein ( $14 \pm 4\%$ ;  $n = 100$ /sample, three experiments), or human RPA ( $14 \pm 2\%$ ;  $n = 100$ /sample, three experiments) to the reaction. Similarly two Holliday junction-binding proteins, human MSH2/6 and HMG(IY), were separately incubated with the model DNA and TRF2, the DNA prepared for EM. Neither MSH2/6 nor HMG(IY) showed any ability to enhance TRF2-mediated t-loop assembly *in vitro* ( $13 \pm 2$  and  $15 \pm 1\%$ , respectively;  $n = 100$ /sample, three experiments). These results suggest a more specific role for p53 in t-loop formation/stabilization.

p53 may increase the numbers of t-loops by facilitating loop formation by TRF2. Once bound to the overhang, p53 might assist in the strand invasion process. Alternately, p53 might stabilize the assembled t-loops by binding directly to the t-loop junction (as contrasted to the displaced ss). If binding to the overhang is responsible for the increase in t-loops, preincubation with p53 might be expected to further increase the numbers of t-loops formed as compared with preincubation with TRF2. If, instead, p53 stabilizes the t-loop junction, preincubation with p53 would not be expected to further enhance t-loop formation. To test these possibilities, p53 or TRF2 were first

incubated individually with the model telomere DNA for 5 min followed by addition of the second protein for 15 min. TRF2 assembled 13% ( $\pm <1\%$ ,  $n = 100/\text{sample}$ , two experiments) of the DNA into t-loops in the absence of p53. When the DNA was preincubated with p53 followed by addition of TRF2, the number of t-loops rose to 20% ( $\pm 7\%$ ,  $n = 100/\text{sample}$ , two experiments). Similarly, 19% ( $\pm 2\%$ ,  $n = 100/\text{sample}$ , two experiments) of the DNA molecules were looped when preincubated with TRF2 followed by addition of p53. Thus the order of addition of p53 and TRF2 with the model telomere DNA does not affect the ability of p53 to enhance loop frequency. Together, these data suggest that the p53-dependent increase in t-loop observed by EM is not due simply to binding of p53 to the ss overhang but instead may involve a more direct role of p53 on loop formation or stabilization.

**TRF2 Does Not Exclude p53 from Binding the t-Loop Junction**—The larger mass of protein present at the base of the t-loop when both p53 and TRF2 were present in the reaction suggests that the proteins are capable of binding to the junction simultaneously. This possibility was examined using immunoelectron microscopy. TRF2 and p53 were incubated with the model telomere DNA. p53 was then detected by addition of an anti-p53 polyclonal rabbit IgG followed by incubation with 10-nm gold particles conjugated to protein A. There was no cross-reactivity of the antibody with TRF2 as seen by EM and Western blot; hence all labeled molecules must contain p53 (data not shown). Because p53 is unable to assemble t-loops in the absence of TRF2 and we have previously shown that TRF2 binds all t-loop junctions, the looped molecules must also contain TRF2 (10). All t-loop junctions that are labeled with gold particles, therefore, are presumed to contain both p53 and TRF2.

By this approach, 100 individual molecules were placed into five classes: protein-free DNA (38%), DNA with a single protein complex bound at the end (23%), DNA with a single protein complex bound along the DNA not at the end (7%), DNA with protein bound at the base of a t-loop (14%), and DNA aggregates held together by a large protein mass (17%). Each class was further divided into molecules that were gold-labeled (20%) and molecules that remained unlabeled (80%). In these studies, gold particles were only observed on end-bound molecules, t-loops, and aggregates. Of the t-loops detected, 86% were labeled with gold particles at the t-loop junction, demonstrating the association of both TRF2 and p53 (Fig. 3A). In addition, 43% of the end-bound molecules were tagged with gold labels (Fig. 3B). The labeled end bound complexes may contain both proteins or only p53. These results provide direct evidence for the presence of p53 at TRF2-bound t-loop junctions. The location of p53 binding within the junction, either to the displaced strand or the Holliday junction-like structure, could not be resolved using these methods.

#### DISCUSSION

In this study we have used purified TRF2, p53, and a model telomere DNA to examine the binding of p53 to telomeric structures including the duplex repeats, the ss overhang, and a t-loop junction. No evidence for preferential binding of p53 to duplex telomeric DNA was observed. p53 does bind strongly to the ss overhang, as expected based on previous studies showing that p53 has a high affinity for ss DNA (17). p53 also associates with the t-loop junction with high affinity and was found to cooperate with TRF2 in formation of t-loops on the model telomere template. This produced a 2-fold increase in the fre-

quency of t-loops as monitored by EM. p53 was detected at the t-loop junction in the presence of TRF2 binding, suggesting that both proteins are present in a complex at the t-loop junction.

It remains possible that the enhancement of TRF2-mediated t-loop formation by p53 results from its interactions with the overhang or junction. Interactions of p53 with the junction may add stability and prevent t-loop loss *in vitro*. t-Loops could be lost because of migration of the junction along the duplex repeat tract or dissociation of TRF2. Association with the ss overhang may facilitate localization of TRF2 to the ss/ds overhang, which would result in an increase in loop assembly.

Several biologically significant roles for p53 localization to the t-loop junction can be envisioned. Normally, p53 translocates from the cytoplasm to the nucleus at the G<sub>1</sub>/S transition and is shuttled back to cytoplasm shortly thereafter. The presence of p53 at the t-loop junction just prior to DNA replication may promote resolution of the junction to facilitate telomere replication. It is also possible that p53 is sequestered at the t-loop junction to allow immediate recognition of any loss of end protection by p53. Cell cycle arrest or apoptosis then can be triggered. Additionally, other protein factors may be recruited to the chromosome end through interactions with p53. These factors may be essential for telomere structure and function.

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