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Protection of mammalian telomeres

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Telomeres allow cells to distinguish natural chromosome ends from damaged DNA. When telomere function is disrupted, a potentially lethal DNA damage response can ensue, DNA repair activities threaten the integrity of chromosome ends, and extensive genome instability can arise. It is not clear exactly how the structure of telomere ends differs from sites of DNA damage and how telomeres protect chromosome ends from DNA repair activities. What are the defining structural features of telomeres and through which mechanisms do they ensure chromosome end protection? What is the molecular basis of the telomeric cap and how does it act to sequester the chromosome end? Here I discuss data gathered in the last few years, suggesting that the protection of human chromosome ends primarily depends on the telomeric protein TRF2 and that telomere capping involves the formation of a higher order structure, the telomeric loop or t-loop.
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The key factor in telomere protection: TRF2

TRF2 coats the length of all human telomeres at all stages of the cell cycle (Figure 1a) (Bilaud *et al*., 1997; Broccoli *et al*., 1997). This small, ubiquitously expressed protein, is estimated to be present at more than 100 copies per chromosome end, binding directly to the tandem array of duplex TTAGGG repeats. The protection of human telomeres crucially depends on this factor and it is reasonable to assume that the requirement for TTAGGG repeats at chromosome ends reflects the need for TRF2 binding. Indeed, expression of a mutant telomerase that adds sequences to chromosome ends lacking TRF2 binding sites, results in a deleterious phenotype similar to that of TRF2 inhibition (Guiducci *et al*., 2001; Kim *et al*., 2001).

In the experiments reviewed below, inhibition of TRF2 is achieved a using dominant negative allele that binds the endogenous TRF2 and forms an inactive heterodimer, unable to bind DNA (Figure 1b) (van Steensel *et al*., 1998). This allele, $TRF2^{\Delta B\Delta M}$, blocks the accumulation of TRF2 on chromosome ends and effectively strips TRF2 and its interacting factors off the telomeres (Li *et al*., 2000; van Steensel *et al*., 1998; Zhu *et al*., 2000). $TRF2^{\Delta B\Delta M}$ does not affect other telomeric DNA binding factors, such as TRF1. Inhibition of TRF2 in cultured human cells has been very informative with regard to the exact consequences of telomere dysfunction at the cellular, chromosomal, and molecular level. From the analysis of what goes wrong with telomeres in the absence of TRF2, a view has emerged of how telomeres normally protect chromosome ends.

Cellular consequences of telomere dysfunction: apoptosis and senescence

The cellular outcomes of TRF2 inhibition suggest that TRF2-depleted telomeres are perceived as if they represent sites of DNA damage (Karlseder *et al*., 1999; van Steensel *et al*., 1998). In many cell types, including primary lymphocytes, TRF2 inhibition leads to immediate induction of apoptosis (Figure 1c) (Karlseder *et al*., 1999). Apoptosis is accompanied by the stabilization and activation of p53, resulting in expression of its downstream targets p21 and bax. The p53 response is required to initiate apoptosis which does not occur in human and mouse cells lacking a functional p53 pathway. In addition to p53, the ATM PI3 kinase is required for the initiation of this program. Two B-cell lines from ataxia telangiectasia patients lacking this kinase and cells from mice lacking a functional ATM gene failed to show apoptosis after TRF2 inhbition (Karlseder *et al*., 1999; Karlseder and de Lange, unpublished). Consistent with a role for the ATM kinase in its activation, p53 is phosphorylated on serine 15 (Karlseder and de Lange, unpublished). The involvement of the ATM kinase in this response pathway suggests that telomeres lacking TRF2 are perceived as damaged DNA. ATM has been previously implicated in the response to γ-irradiation, generally believed to cause double strand breaks (DSBs) (Canman *et al*., 1994; Khanna and Lavin, 1993). Therefore, the loss of TRF2 from telomeres may create a structure that resembles this type of damage.

When TRF2 is inhibited in G1 cells, apoptosis can occur before the onset of DNA replication, indicating that neither telomere replication nor chromosome

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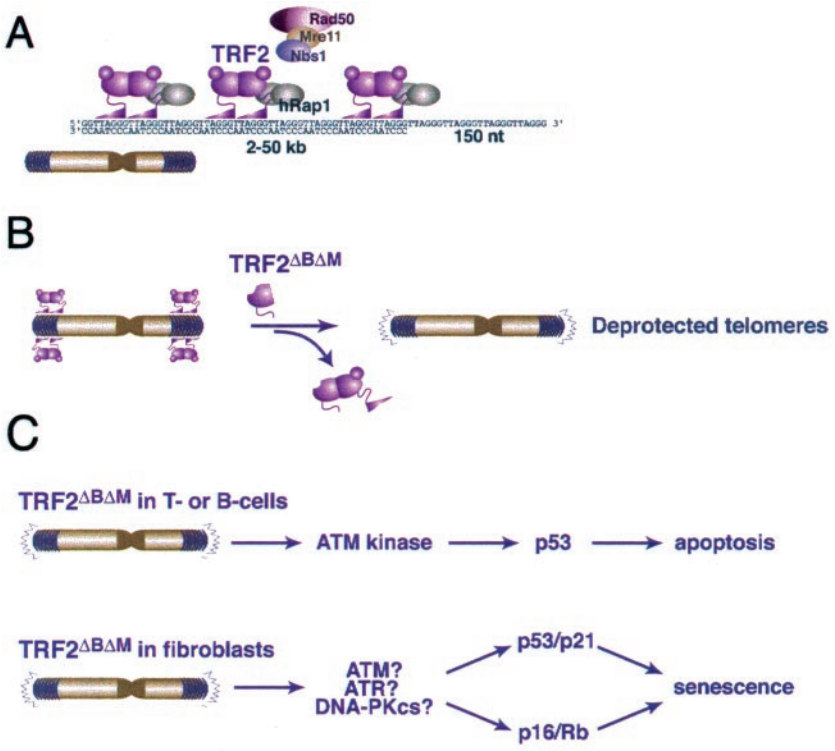

Figure 1 TRF2 and the cellular consequences of its inhibition. (**a**) Schematic of mammalian telomeres containing the TRF2 complex. (**b**) Inhibition of TRF2 using a dominant negative allele, lacking the N-terminal basic domain and the C-terminal Myb DNA binding domain, $TRF2^{\Delta B\Delta M}$. (**c**) Cellular consequences of telomere deprotection due to inhibition of TRF2

segregation are required to generate the apoptotic signal. Apparently the chromosome end is immediately altered when TRF2 is removed and this alteration leads to activation of the ATM/p53 response pathway.

Apoptosis also occurs when telomere dysfunction is enforced by different methods. For instance, human cells expressing a mutant telomerase generating telomeric repeats without TRF2 binding sites undergo apoptosis (Guiducci *et al*., 2001; Kim *et al*., 2001) and apoptosis occurs in telomerase-deficient mouse cells once the telomeres have reached a critically short length (Chin *et al*., 1999). As is the case for TRF2 inhibition, apoptosis in the telomerase deficient mice can be bypassed when the mice also lack p53. However, there also appears to be a p53-independent apoptosis response to shortened telomeres. Both in the telomerase-deficient mice and in tumor cell lines expressing a dominant negative allele of the telomerase reverse transcriptase eventually undergo apoptosis even when p53 is absent (Chin *et al*., 1999; Hahn *et al*., 1999; Zhang *et al*., 1999). Interestingly, one of the tumor cell lines that undergoes p53-independent apoptosis after prolonged telomerase inhibition (SW613 (Hahn *et al*., 1999)), does not show an apoptotic response to $TRF2^{\Delta B\Delta M}$ (Karlseder and de Lange, unpublished). Perhaps this differential response indicates that cells experiencing TRF2 inhibition sustain a different type of lesion or have less extensive damage than when their telomeres are shortened.

In a subset of human cell types, most prominently primary fibroblasts, TRF2 inhibition results in senescence rather than apoptosis (Figure 1c) (Karlseder *et al*., 1999; Smogorzewska and de Lange, in preparation). These cells display all the morphological and molecular signs of senescence, including a large and flat cell shape, frequent occurrence of multiple nuclei, a 2n or 4n DNA content, and staining with the senescence associated marker SA-β-gal (Smogorzewska and de Lange, in preparation). The pathway leading to this response resembles the induction of senescence by shortened telomeres, involving upregulation of p53 (and its target p21) as well as effects on the Rb pathway, including induction of the Cdk inhibitor p16 and concomitant lack of Rb phosphorylation.

The inhibitory effects of SV40 large T and HPV16 E6 and E7 were used in order to determine the contribution of the p53 and p16/Rb pathways to the senescent signal (Smogorzewska and de Lange, in preparation; for reviews on HPV and SV40 oncoproteins, see Pipas and Levine, 2001; Tommasino and Crawford, 1995, respectively). When p53 was inactivated with the HPV16 E6 oncoprotein or a dominant negative allele of p53, $TRF2^{\Delta B\Delta M}$ could still induce a cell cycle arrest. Similarly, inhibition of pRb with E7 failed to abrogate the cell cycle arrest. Abrogation of the $TRF2^{\Delta B\Delta M}$ induced arrest was only observed in cells expressing both E6 and E7 or SV40 large T antigen. Such cells presumably lack both the p53 and the Rb pathway. Apparently both tumor suppressors are capable of preventing entry into S phase when telomeres are damaged. These findings closely parallel

the signaling of senescence in cells undergoing replicative aging (reviewed in Lundberg *et al*., 2000; Sherr and DePinho, 2000), suggesting that telomeres lacking TRF2 resemble critically short telomeres.

An important question concerns the signaling components upstream of p53 and p16/Rb that alert cells to telomere dysfunction. The ATM kinase is likely to be involved in the induction of senescence as well as in apoptosis. However, fibroblasts from A-T patients will still execute $TRF2^{\Delta B\Delta M}$-induced senescence indicating that this kinase is not alone in activating the response pathways (Smogorzewska and de Lange, in preparation). In fact, p53 is still upregulated in TRF2-depleted A-T fibroblasts, implicating perhaps other PI3 kinases such as the ATR or DNA-PKcs in this process. Although DNA-PKcs is not involved in the activation of p53 after γ- or UV-induced damage (Jimenez *et al*., 1999; Rathmell *et al*., 1997) it is not excluded that this kinase phosphorylates p53 at dysfunctional telomeres. Particularly suggestive in this regard is the fact that DNA-PKcs appears to be located at telomeres (d'Adda di Fagagna *et al*., 2001). An additional important challenge is to determine the upstream component(s) involved in the upregulation of p16 in response to dysfunctional telomeres. So far there is no suggestion on how the expression level of p16 is increased in cells experiencing telomere damage or other forms of DNA damage.

In general, the induction of senescence by loss of TRF2 or telomere shortening resembles the cellular response to extensive DNA damage. When allowed sufficient time to express their phenotype, human fibroblasts treated with a large dose of γ-irradiation are virtually indistinguishable from aged human cells or $TRF2^{\Delta B\Delta M}$ expressing fibroblasts in terms of morphology, senescence markers, and alterations in the p53 and p16/Rb pathways (Di Leonardo *et al*., 1994; Robles and Adami, 1998; Smogorzewska and de Lange, in preparation). In each case, p53 and p21 are induced and high levels of p16 accompany a reduction in pRb phosphorylation. Perhaps the irreparable damage caused by the uncapping of multiple telomeres mimics by the ultimate consequences of extensive genome-wide radiation damage. Telomere-directed senescence may thus reflect the results of persistent damage, irrespective of its original proximate cause. The use of sudden telomere deprotection as effected by $TRF2^{\Delta B\Delta M}$ will be a useful tool to further dissect the genetic requirements for this pathway.

Molecular consequences: loss of the 3′ telomeric overhang and NHEJ

When TRF2 is inhibited, a substantial fraction of the telomeres (~15%) become fused to each other (van Steensel *et al*., 1998) (Figure 2). These fused telomeres can be detected in genomic blots as newly-formed telomeric DNA fragments with a molecular weight twice that of the original telomeres. Molecular analysis indicates that the fusions are formed by ligation of the

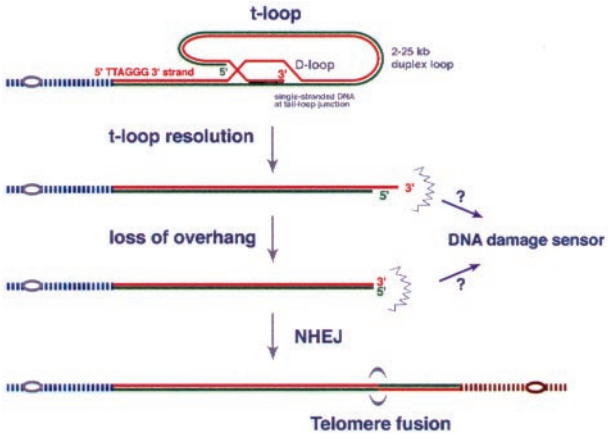

Figure 2 Molecular consequences of TRF2 inhibiton. The figure shows a speculative model for the formation of telomere fusions after inhibition of TRF2. Inhibition of TRF2 is proposed to result in the resolution of t-loops or failure to reform t-loops after DNA replication. The exposed 3′ overhang or its processing product activates DNA damage checkpoints. Loss of the 3′ overhang can occur in absence of DNA replication. Alternatively, DNA replication can create a blunt ended product through leading strand DNA synthesis. Due to lack of TRF2 function, the blunt-ended telomere is not processed and becomes a substrate for ligation by DNA ligase IV (NHEJ). The result is the covalent fusion of two telomeres, either before or after DNA replication and a dicentric chromosome is formed. Note that the duplex telomeric repeat array is maintained

TTAGGG repeat strand of one telomere to the CCCTAA strand of another (Smogorzewska and de Lange, in preparation). The fused telomeric sequences are no longer at the physical end of a chromosome as demonstrated by their resistance to the exonuclease Bal31, a standard method for distinguishing terminal and interstitial loci in eukaryotic genomes. Furthermore, the telomere fusions remain stable in NaOH, arguing against base-pairing as their mode of association, and suggesting that they are formed by DNA ligation. Given the nature of the sequences that are fused together, non-homologous end joining (NHEJ) is the most likely mechanism by which the telomeres are joined. Indeed, recent experiments using mouse cells lacking DNA ligase IV, the main ligase involved in NHEJ, showed a >30-fold drop in fusion frequency in response to TRF2 inhibition (Smogorzewska and de Lange, in preparation). Collectively, these data are most consistent with a simple end-to-end ligation of the telomeres, executed by the NHEJ pathway once the protective role of TRF2 is impeded. The data also demonstrate that one of the functions of telomeres is to protect chromosome ends from the NHEJ pathway.

All human telomeres carry a 3′ overhang of several hundred nt of single-stranded TTAGGG repeats (Figure 1A) (Huffman *et al*., 2000; Makarov *et al*., 1997; McElligott and Wellinger, 1997; Wright *et al*., 1997). It seems unlikely that telomeres with such a 3′ protrusion would be a substrate for NHEJ. Indeed, there is a precipitous loss of the 3′ overhang after expression of $TRF2^{\Delta B\Delta M}$. Up to 50% of the G-strand signal disappears within a few cell divisions (van

Steensel *et al*., 1998). This loss can be demonstrated on those telomeres that have not (yet) been fused, suggesting that G overhang loss precedes ligation.

It is not clear by what mechanism the 3′ overhang is lost. Perhaps the overhangs are simply not regenerated after the telomeres are replicated. It is generally believed that the maintenance of 3′ protrusions in dividing cells requires an active process of exonucleolytic attack on the C-strand. C-strand processing is certainly required at the ends generated by leading strand DNA synthesis, which are predicted to be blunt. But even the ends resulting from lagging strand synthesis may require additional modification since the overhang left by removal of the last RNA primer or by unfinished DNA replication may not have the correct length. If TRF2 is involved in the recruitment or activation of the nuclease assigned to this task, inhibition of TRF2 is expected to result in a gradual drop of the overall overhang signal as the culture expands. Consistent with passive loss of the overhangs, the G-strand signal slowly disappears after TRF2 inhibition, taking several cell divisions to reach a 50% drop (van Steensel *et al*., 1998). However, inhibition of TRF2 can also result in a reduction of overhang signal in absence of DNA replication (Karlseder and de Lange, unpublished), suggesting that active degradation of the overhangs can occur and that blunt-ended chromosomes might also be generated in G1.

Knowledge of the exact molecular status of the telomere termini after inhibition of TRF2 is relevant to the question which structural feature of the uncapped chromosome ends activates the DNA damage response in these cells (Figure 2). It is possible that simple exposure of the 3′ overhang is sufficient for the signal. This would be consistent with the idea that single-stranded DNA rather than duplex breaks are detected by the general DNA damage response machinery. There is good evidence for this view in budding yeast where dysfunctional telomeres are rendered single-stranded by degradation of their 5′ ended strands and signal to the RAD9/MEC1 checkpoint (Garvik *et al*., 1995; Lydall and Weinert, 1995). However, in mammalian cells, there is no evidence for exonucleolytic attack on the 5′ (C rich) strand of uncapped human telomeres. The C-strand remains intact both in TRF2$^{\Delta B\Delta M}$ expressing cells (van Steensel *et al*., 1998) and in cells undergoing replicative senescence (Zhu and de Lange, unpublished data). Hence, either the exposure of the resident telomeric overhang is sufficient for the signal, or the signal does not require single-stranded DNA. Indeed, recent evidence from *S. cerevisiae* suggests that DSBs can signal directly to a DNA damage checkpoint that includes the Tel1p kinase and the Mre11 complex (Usui *et al*., 2001). Interestingly, the mammalian Tel1p ortholog, ATM, is involved in telomere signaling (Karlseder *et al*., 1999), and the Mre11 complex is associated with telomeres (Zhu *et al*., 2000), raising the possibility that this pathway may be capable of detecting telomere dysfunction without further generation of single-stranded DNA.

Chromosomal consequences: chromosome end fusions and associated aberrations

The telomeres in cells expressing TRF2$^{\Delta B\Delta M}$ become fusogenic and generate end-to-end fused chromosomes that can be detected as dicentric and multicentric chromosomes in metaphase spreads (van Steensel *et al*., 1998). Telomere fusions can occur before and after DNA synthesis (Figure 3). If the telomere fusion takes place prior to chromosome duplication, both chromatids of a metaphase chromosome will be joined to another chromosome resulting in a so-called chromosome-type dicentric. Chromatid-type dicentrics are formed when a fusion takes place after DNA synthesis and only one of the two chromatids becomes joined to another telomere. It is clear that only a fraction of the actual fusions are detectable by inspection of metaphase chromosomes because many cells with dysfunctional telomeres arrest before mitosis. Indeed, the molecular data from genomic blots indicates that up to 20% of the telomeres fuse after removal of TRF2, whereas only ~1% of the chromosome ends observed in metaphase cells show a fusion (van Steensel *et al*., 1998).

A striking feature of the TRF2$^{\Delta B\Delta M}$-induced chromatid type dicentrics is that they usually involve telomeres created by leading strand DNA synthesis (Bailey *et al*., 2001). Bailey and co-workers used chromatid orientation fluorescent *in situ* hybridization (CO-FISH) to infer which of the two strands in a fused telomere was synthesized in the preceding round of replication. CO-FISH uses BrdU/BrdC incorporation during a single S phase, followed by destruction of the substituted strand in metaphase chromosomes by treatment with UV and exonucleaseIII. The remaining DNA strand, which is detected with a strand-specific fluorescent probe, represents the parental DNA strand. Thus, if a telomere can be detected with a TTAGGG probe, its CCCTAA-strand must have been the parental strand. Such a telomere is inferred to be the product of leading strand synthesis in which the G-strand is synthesized *de novo* in the 5′→3′ direction. The preferential fusion of the leading strand ends is further confirmed by the observation that sister telomeres do not fuse, even though they must be much more close to each other than to any other chromosome end. Why is there preferential fusion of the leading strand ends? One explanation is that leading strand DNA synthesis creates a blunt end, a good substrate for NHEJ. By contrast, the lagging strand end is predicted to contain a short overhang representing the site where the last RNA primer was synthesized. Perhaps the presence of this overhang is sufficient to prevent any NHEJ involving the lagging strand ends.

Collectively, the data on the chromosome end fusions after TRF2 inhibition suggest the following scenario. In absence of TRF2, cells fail to regenerate the 3′ overhang and the ends created by leading strand synthesis remain blunt ended after DNA replication. Therefore, these ends remain unprotected, for instance because they can not be processed into t-loops. A

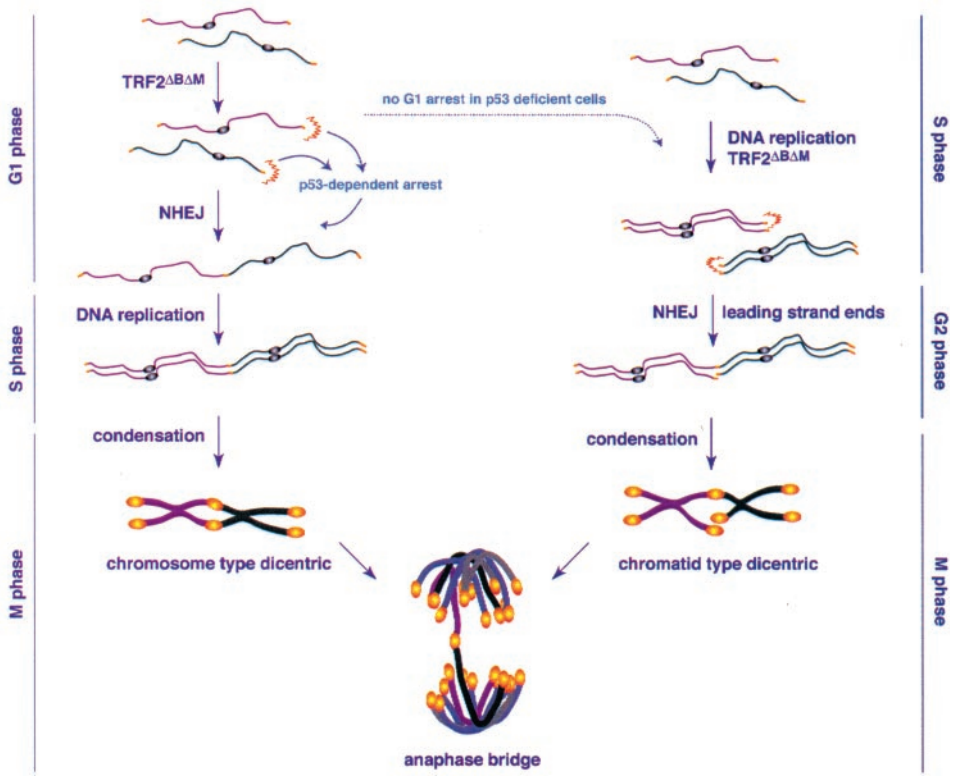

Figure 3 Chromosomal consequences of TRF2 inhibition. See text for details

DNA damage response ensues in G2 that ultimately results in ligation of the leading strand ends by NHEJ. When the defective telomeres have been processed the cells proceed into mitosis and dicentric chromosomes are observed in metaphase spreads. If such a dicentric survives anaphase intact, it will be present as a chromosome type fusion in the next mitosis. In addition, chromosome type fusions can result from end-to-end fusions in G1. Inhibition of TRF2 function in G1 results in reduction in the G overhang signal (Karlseder and de Lange, unpublished), possibly generating the blunt substrate for NHEJ.

In interpreting cytogenetic data, it is important to consider the checkpoint status of the cells and how the arrest might affect different types of fusions. For instance, in checkpoint-proficient primary fibroblasts, chromosome type fusions are prominent (Smogorzewska and de Lange, unpublished) suggesting frequent G1 events. By contrast, chromatid type fusions dominate in the p53-deficient tumor cell line HT1080 (Bailey *et al*., 2001; van Steensel *et al*., 1998). Because these cells lack the p53 pathway, telomere dysfunction will not provoke a prolonged G1 arrest. Thus, the period available for pre-replicative DNA repair will be limited and cells have the opportunity to proceed through S phase generating two sister ends before the deprotected telomeres become fused (see Figure 3).

Dicentric chromosomes are generally unstable in mitosis. If two centromeres in a end-to-end fused chromosome attach to opposite poles, an anaphase bridge is formed (Figure 3) and either a break in the chromosome or a rupture in connecting microtubules is required for cell division to proceed. McClintock observed that dicentric corn chromosomes can initiate multiple bridge-breakage-fusion cycles leading to continual generation of abnormal broken products until the breaks are healed (McClintock, 1941). Similarly, in human cells, general genome instability ensues from the inhibition of TRF2 presumably as a direct result of the end-to-end fusions. The consequences include translocations and other rearrangements as well as anaphase bridges which likely result in non-disjunction and changes in chromosome number (Smogorzewska, Jauch, de Lange, in preparation). The same spectrum of chromosome aberrations is observed when human cells near the end of their replicative life-span (Benn, 1976) suggesting that the chromosomal consequences of TRF2 inhibition mimic those of telomere shortening. As discussed in detail several years ago (de Lange, 1995), the consequences of chromosome end fusions can explain the majority of karyotypic changes seen in human cancer suggesting that telomere dysfunction is a driving force in tumor associated genome instability.

T-loops as a way to protect chromosome ends

When TRF2 is inhibited the telomeric DNA remains largely intact, yet there is a precipitous loss of telomere function. Why does the removal of TRF2 lead to immediate deprotection of chromosome ends? TRF2 is a duplex TTAGGG repeat binding protein and does

not interact with single-stranded DNA, making it unlikely that TRF2 directly protects the 3′ or 5′ end of the chromosome. A solution to this conundrum was provided by the observation that TRF2 can remodel telomeric DNA into t-loops (Griffith *et al*., 1999; Stansel *et al*., 2001).

T-loops are large duplex telomeric loops that appear to be formed by the invasion of the 3′ overhang into the duplex part of the telomeric repeat array (Figure 2) (Griffith *et al*., 1999). Loop sizes range from as small as 0.3 kb in trypanosomes to up to 30 kb in mouse cells and this variability suggests that the loop itself does not form a functional unit (Griffith *et al*., 1999; Munoz-Jordan *et al*., 2001; Murti and Prescott, 1999). Instead, the crucial feature of t-loops appears to be the invasion of the telomere terminus into the double-stranded telomeric DNA. The exact structure at the base of the t-loop is not known but it is clear that there is a short segment of single-stranded DNA, likely representing a D-loop of TTAGGG repeats that are displaced by the invading 3′ overhang. It is not excluded that a short segment of the C-strand is also invaded resulting in a Holliday junction. As discussed previously, the t-loop structure resembles a DNA recombination intermediate as it occurs during reinitiation of a stalled replication fork or during homologous recombination (de Lange and Petrini, 2000; Griffith *et al*., 1999).

T-loops could provide cells with an architectural solution to the telomere protection problem (Figure 2). The invasion of the 3′ overhang may simply sequester the telomere terminus from being mistaken for a site of damage or additional factors could help to cover up the single-stranded DNA and other non-duplex structures. One of the pleasing aspects of the t-loop solution to telomere capping is that it relies on the most conserved feature of eukaryotic telomeres, the presence of tandem telomeric repeats ending in a single stranded overhang.

The idea that t-loops could protect chromosome ends is consistent with the finding that TRF2, when given the right substrate, can form t-loop like structures *in vitro* (Griffith *et al*., 1999; Stansel *et al*., 2001). TRF2 can form looped structures out of a model telomeric DNA substrate containing a stretch of 0.5 kb duplex TTAGGG repeats and a short (<54 nt) 3′ overhang. The loops are ~ 0.5 kb in size and contain a large amount of TRF2 at their base. When the DNA is treated with psoralen and UV light to generate interstrand cross-links, the loops are stable after removal of TRF2, indicating that a strand-invasion has occurred. It will be important to determine how TRF2, which lacks helicase motifs, is able to induce a strand invasion of this type. Furthermore, it is of immediate interest to determine the effect of the $TRF2^{\Delta B \Delta M}$ allele on the relative frequency of t-loops *in vivo*.

An additional way by which TRF2 might facilitate the protection of chromosome ends is by recruiting a protein that can bind the telomere terminus. A candidate telomere end binding factor, Pot1, was recently identified in the database based on homology to a similar protein from the ciliate Oxytricha nova (Baumann and Cech, 2001). Although little is known about this factor in human cells, fission yeast Pot1 is essential for telomere protection (see below). It will be interesting to determine to what extend Pot1 and TRF2 act in the same protection pathway and to determine whether Pot1 interacts with TRF2.

The role of TRF2 interacting factors

Although TRF2 can form t-loops *in vitro* by itself, it is likely that its role at telomeres *in vivo* requires interacting factors. TRF2 has several known binding partners (Figure 1a). Human Rap1, like its ortholog in *S. cerevisiae*, contributes to the regulation of telomere length (Li *et al*., 2000). So far, tests using a set of potential dominant negative alleles of hRap1 have failed to show a role in telomere protection (Li and de Lange, unpublished), but a conclusion in this regard will have to await more definitive data on cells lacking Rap1 function. A second interacting partner of TRF2 is the Mre11 complex, composed of Mre11, Rad50, and Nbs1 (Zhu *et al*., 2000). As discussed in detail elsewhere (de Lange and Petrini, 2000), the presence of this complex at telomeres is particularly interesting with regard to the generation of 3′ overhangs and t-loops. The Mre11 complex has been suggested to be the nuclease responsible for overhang formation (de Lange and Petrini, 2000; Diede and Gottschling, 2001) although its nuclease activity has the opposite ($3'-5'$) polarity *in vitro* (Paull and Gellert, 1998). The involvement of this complex in homologous recombination and the role of its prokaryotic orthologs in replication restarts are suggestive of a possible role for this TRF2 tethered complex in the generation, maintenance, and processing of t-loops (reviewed in de Lange and Petrini, 2000). A further hint that the Mre11 complex contributes to structural alterations and other processing of telomeres comes from the finding that its regulatory subunit, Nbs1, joins TRF2 at telomeres only in S phase (Zhu *et al*., 2000), a likely moment for the resolution and re-establishment of t-loops and a time when overhangs need to be regenerated. Since mammalian cells lacking this complex are not viable (Luo *et al*., 1999), conditional ablation may be required to determine the contribution of the Mre11 complex to telomere protection.

Telomere protection by DNA-PKcs and Ku70/80

The DNA dependent protein kinase (DNA-PK) is a heterotrimeric complex that first attracted attention because of its involvement in VDJ recombination and in NHEJ after extensive radiation damage (reviewed in Critchlow and Jackson, 1998). Ironically, it now turns out that all three subunits of the complex, Ku70, Ku80, and the catalytic subunit (DNA-PKcs) contribute to the protection of telomeres (Bailey *et al*.,

1999; d'Adda di Fagagna *et al*., 2001; Goytisolo *et al*., 2001; Hsu *et al*., 2000; Samper *et al*., 2000). Mouse embryo fibroblasts (MEFs) from DNA-PKcs$^{-/-}$ mice or cells containing the scid mutation in this locus have a significant increase in end-to-end fused chromosomes. These fusions take place in cells with adequate telomere length (Goytisolo *et al*., 2001) and there is telomeric DNA at the fusion sites (Bailey *et al*., 1999; Goytisolo *et al*., 2001), indicating that the function of the DNA-PKcs is important for the protection of chromosome ends by telomeric DNA. The fusions can give rise to chromatin bridges in anaphase (Goytisolo *et al*., 2001), and like the TRF2$^{\Delta B\Delta M}$ fusions, they never occur between sister chromatids (Bailey *et al*., 2001). The fusions can be both of chromosome type and chromatid type, but when single chromatids are involved in the fusions, the fused ends are always created by leading strand synthesis (Bailey *et al*., 2001).

Similar to the DNA-PKcs null cells, MEFs from Ku70−/− and Ku80−/− mice also show an increase in chromosome end fusions in metaphase (Bailey *et al*., 1999; Hsu *et al*., 2000; Samper *et al*., 2000). Since NHEJ is severely impaired in these mutants, it will be interesting to determine which pathway is responsible for the fusions. Overall, the telomere insult in this context is probably relatively minor since DNA-PKcs and Ku deficient mice survive to adulthood and there is no indication that a DNA damage checkpoint is activated. This result contrasts with TRF2 inhibition in cultured human and mouse cells, which blocks cell proliferation.

Based on chromatin immunoprecipitation experiments, each of the three subunits of DNA-PK may be present at telomeres (d'Adda di Fagagna *et al*., 2001; Hsu *et al*., 1999; Loayza and de Lange, unpublished) as well as elsewhere in the genome. The binding of this complex has been suggested to involve an interaction with TRF1 (Hsu *et al*., 2000) or TRF2 (Song *et al*., 2000) but it is also possible that it loads directly onto the chromosome end, since Ku is an end-loading ring (Walker *et al*., 2001) and TTAGGG repeat termini are an adequate substrate for this protein (Bianchi and de Lange, 1999). A crucial question is which factor is phosphorylated by DNA-PKcs and what the effects is of this phosphorylation.

Comparison of telomere deprotection in mammals and yeast

Fission yeast has a TRF2 ortholog, Taz1, with a very similar telomere protection function (Cooper *et al*., 1997; Fairall *et al*., 2001; Godhino Ferreira and Promisel Cooper, 2001; Li *et al*., 2000). Under conditions that prevent homologous recombination, loss of Taz1 results in loss of viability and frequent chromosome end fusions (Godhino Ferreira and Promisel Cooper, 2001). Fusions occur on ends that have maintained the telomeric DNA and give rise to anaphase bridges, demonstrating the formation of dicentric chromosomes. In cells lacking Ku or DNA ligase IV, no fusions occur after Taz1 loss, demonstrating that like telomeres lacking TRF2, Taz1 deficient telomeres are processed by NHEJ. The status of the telomere termini in taz1 deficient cells is not yet known.

A second telomere protection factor in *S. pombe* is Pot1, a protein distantly related to the alpha subunit of the telomere end binding complex of Oxytricha and other hypotrichous ciliates (Baumann and Cech, 2001). Pot1 deficiency also has a deprotection phenotype. However, in this case, the telomeric DNA is lost and occasional circularization of all three fission yeast chromosomes allows the infrequent survival of cells in a telomere-free state. Whether the human ortholog of Pot1 has the same protective function remains to be established. Like their ciliate counterparts the Pot1 proteins are single-stranded DNA binding proteins suited to bind either the 3′ overhang or the D-loop at the t-loop base (Baumann and Cech, 2001; reviewed in de Lange, 2001).

The telomeres of budding yeast appear not to require a duplex telomeric DNA binding protein for their protection. Indeed, no ortholog of TRF2 or Taz1 is present in the budding yeast genome and it is likely that this factor was lost during evolution (Li *et al*., 2000). Unlike its mammalian and fission yeast orthologs, budding yeast Rap1 can bind to double stranded telomeric DNA but there is no data to suggest that Rap1 or its interacting factors contribute to telomere protection (Marcand *et al*., 1997; Shore, 1994). However, deletion of Rap1 is lethal and while this phenotype is generally ascribed to the function of Rap1 in transcriptional activation, it is not excluded that Rap1 has a role in the capping of telomeres as well. Similarly, the budding yeast Mre11 complex, while involved in telomere maintenance (Boulton and Jackson, 1998; Nugent *et al*., 1998; Ritchie and Petes, 2000), appears to be dispensable for telomere protection. Without the Mre11 complex, cells continue to divide suggesting that they continue to have the ability to distinguish DNA damage and natural chromosome ends. However, the Mre11 complex itself could also be required for the recognition of dysfunctional telomeres (for instance, by creating single-stranded DNA or as part of the TEL1 dependent checkpoint (Usui *et al*., 2001)), making the experiments difficult to interpret.

The best understood factor acting to protect chromosome ends in budding yeast is Cdc13 (Garvik *et al*., 1995), which may be a distant ortholog of Pot1 and the ciliate end binding protein (D Wuttke and V Lundblad, personal communication). This protein binds to single-stranded telomeric DNA *in vitro* (Lin and Zakian, 1996; Nugent *et al*., 1996) and protects telomeres by recruiting Stn1p and Ten1p (Grandin *et al*., 1997, 2001; Pennock *et al*., 2001; reviewed in Lustig, 2001). Without this complex, yeast telomeres are rapidly degraded from their 5′ ends and the resulting single-stranded DNA activates the RAD9-dependent DNA damage checkpoint and leads to cell cycle arrest (Garvik *et al*., 1995; Lydall and Weinert, 1995). In absence of RAD9, cells continue to divide but eventually perish due to chromosome instability (Grandin *et al*., 2001).

In budding yeast, the Ku heterodimer is associated with telomeric DNA and maintains telomere integrity (Gravel *et al*., 1998). Ku deficiency results in an altered telomere structure which appears to activate a DNA damage pathway at elevated temperature resulting in an inherent ts phenotype of Ku null strains (Barnes and Rio, 1997; Teo and Jackson, 2001). Thus, Ku acts as a telomere protecting factor both in mammals and in yeast.

Early on, the question was raised whether telomerase could be a component of the protective complex at telomeres (Broccoli *et al*., 1995; de Lange, 1995). In some cases telomere protecting factors directly bind telomerase components. For instance, Cdc13 binds to the telomerase component Est1 and genetic analysis implies that Ku can bind the yeast telomerase RNA, TLC1 (Pennock *et al*., 2001; Peterson *et al*., 2001; Qi and Zakian, 2000). However, since many telomeric proteins play a dual role in protection as well as in telomerase recruitment, such interactions may only be relevant to the latter function. The fact that telomerase deficient yeasts and mice have no obvious phenotype until their telomeric DNA has run out (Blasco *et al*., 1997; Lundblad and Szostak, 1989), argues against an important capping function for the enzyme. It has been proposed that human telomerase may help to protect partially disabled (very short) telomeres in transformed cells (Zhu *et al*., 1999), but in that setting it is difficult to distinguish between the ability of telomerase to restore the telomeres to functional length as suggested by Sabatier and colleagues (Ducray *et al*., 1999) or a true capping function that does not require synthesis of telomeric DNA.

Now that substantial progress has been made on the proteins involved in telomere protection in three very different systems, it is clear that some players are encoded by highly conserved genes and likely to represent conserved functions (Ku, the Mre11 complex); some components are hard to recognize at the amino acid sequence level yet are present in all systems (human and fission yeast Pot1 and budding yeast Cdc13); and others are completely missing in one of the organisms (no TRF2/Taz1 gene in the budding yeast genome). In addition, the phenotypes resulting from telomere deprotection have certain constant features (for instance, the activation of a DNA damage checkpoint by loss of Cdc13 in budding yeast or loss of TRF2 in mammals); while other downstream effects are quite variable (NHEJ of intact telomeres occurs in fission yeast and mammals but has not been seen in budding yeast). Some of these differences should caution against extrapolation from one eukaryote to another. Yet important hints on how things might work can emerge from the diversity of solutions to the telomere problem in different systems. As is the case for other aspects of chromosome biology, including centromere function, replication, DNA damage response, and cell cycle control, a deep understanding of telomere function will require knowledge of both the unifying principles and the instructive exceptions in a wide variety of eukaryotes. In this regard, the recent strengthening of Arabidopsis (Riha *et al*., 2001), *C. elegans* (Ahmed and Hodgkin, 2000), and Trypanosomes (Aline and Stuart, 1989; Munoz-Jordan *et al*., 2001) as systems to analyse telomere function represents a welcome expansion of the number of genetically tractable model organisms for telomere biology.

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