REVIEW

Functional links between telomeres and proteins of the DNA-damage response

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In response to DNA damage, cells engage a complex set of events that together comprise the DNA-damage response (DDR). These events bring about the repair of the damage and also slow down or halt cell cycle progression until the damage has been removed. In stark contrast, the ends of linear chromosomes, telomeres, are generally not perceived as DNA damage by the cell even though they terminate the DNA double-helix. Nevertheless, it has become clear over the past few years that many proteins involved in the DDR, particularly those involved in responding to DNA double-strand breaks, also play key roles in telomere maintenance. In this review, we discuss the current knowledge of both the telomere and the DDR, and then propose an integrated model for the events associated with the metabolism of DNA ends in these two distinct physiological contexts.

All organisms respond to interruptions in the DNA double-helix by promptly launching the DNA-damage response (DDR). This involves the mobilization of DNA-repair factors and the activation of pathways, often termed checkpoint pathways, which temporarily or permanently delay cell cycle progression. Although the integrity of the DNA double-helix is perturbed by telomeres (the ends of linear chromosomes), these structures generally escape activating the DDR. Several explanations have been proposed to explain the exceptional nature of telomeres in this regard. Thus, it has been suggested that a telomere might not be recognized by components of the DDR because of its unique DNA sequence and structure, its specific localization within the cell nucleus, and/or because of the actions of specific proteins associated with it. Although this is partly correct, recent findings have revealed that, contrary to initial expectations, various proteins involved in the DDR physically associate with telomeres and actually play important roles in regulating normal telomeric functions. In this review, we focus on the role of DDR factors in regulating telomere length and stability, and also explain how dysfunctional telomeres can trigger the DDR. Before doing this, however, we first summarize the salient features of both telomeres and the DDR.

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Telomere structure and biology

The ends of linear chromosomes contain long stretches of DNA tandem repeats (TTAGGG in vertebrates) and terminate in a 3′ protruding single-stranded DNA overhang. Due to the inability of the standard lagging-strand DNA replication machinery to copy the most distal telomere sequences (i.e., those at the very end of the chromosome) and to the additional exonucleolytic processing needed to generate protruding overhangs at both ends, telomeric DNA progressively decreases in length as cells go through successive division cycles. Hence, in the absence of specialized telomere homeostatic mechanisms this would ultimately lead to the loss of all telomeric sequences and subsequently to the loss of more internal essential genetic information and ensuing cell death. To circumvent this, many cells maintain their telomeres by the action of telomerase, a specialized reverse transcriptase that uses its associated RNA component as a template to elongate the TG-rich telomeric DNA strand. Although in vitro telomerase activity is dependent on the activity of the reverse transcriptase catalytic subunit (Est2p in the budding yeast *Saccharomyces cerevisiae*; TERT in mammals) and the telomerase RNA template (Tlc1 in *S. cerevisiae* and hTR in humans), other factors are clearly needed for telomerase action in vivo (see Table 1). For instance, effective telomerase function in *S. cerevisiae* requires Est1p and Est3p, and the loss of either of these two proteins—like the loss of Tlc1 or Est2p—leads to progressive telomere shortening (for review, see Blackburn 2000). Furthermore, and as explained below, effective telomerase action in vivo also requires several proteins associated with the DDR.

The telomeric repeat sequences are essential for many of the key biological features of telomeres by virtue of them being recognized by a specific set of sequence- and structure-specific DNA-binding factors (Table 1; Fig. 1). Some of these bind to the double-stranded portion of the

Table 1. *Telomere-associated factors*

Mammals	*S. cerevisiae*	*S. pombe*	*C. elegans*
TRF1: telomere DNA binder and telomerase mediated-telomere length regulator	Rap1p: telomere length regulator	Taz1p: telomere length and structure regulator	
	Tbf1p: telomere binding factor		
TIN2: telomerase mediated telomere length regulator			
TANK1: TRF1 PARP modifier and telomere length regulator			
TANK2: TRF1 PARP modifier			
TRF2: telomere DNA binder with telomere end capping function and telomerase independent telomere length regulator			
RAP1: TRF2 interactor and telomere length regulator		Rap1p: Taz1p interactor and telomere length regulator	
ERCC1/XPF: TRF2 interacting endonuclease			
MRN complex: TRF2 interactor	MRX complex: telomere length and single stranded overhang regulator	MRN: in vivo component of the telomere and telomere length regulator	
Rif1: Trf2 interactor and in vivo component of mouse telomeres	Rif1/2p: Rap1p interactors and telomere length regulators	Rif1p: Taz1p interactor and telomere length regulator	
POT1: TRF1 interactor and single stranded telomeric DNA binder with telomere length regulation functions	Cdc13p: single stranded telomeric DNA binder with telomere capping and telomerase recruiting functions	Pot1p: single stranded telomeric DNA binder with telomere capping functions	
	Stn1p/Ten1p: Cdc13p interactors and mediators of its telomerase recruiting and capping functions		
Ku: in vivo component of the telomere and telomere length regulator (?)	Ku: in vivo component of the telomere, telomere length and single-stranded overhang regulator	Ku: in vivo component of the telomere and telomere length regulator	
DNA-PKcs: in vivo component of the telomere with telomere capping functions			
EST1A/B: telomere length regulator (?)	Est1p: in vivo cofactor of telomerase	Est1p: in vivo cofactor of telomerase	
TERT: catalytic component of the telomerase complex	Est2p: catalytic component of the telomerase complex	Trt1p: catalytic component of the telomerase complex	
TR: RNA component of the telomerase complex	Tlc1: RNA component of the telomerase complex		
PARP1: telomere length regulator			
	RPA: in vivo component of the telomere with Est1p-recruiting functions		
		9–1–1 complex: in vivo component of the telomere and telomere length regulator	MRT-2 and Hus1: regulators of telomere length and germline mortality
	Tel2p: telomere length regulator		Rad5: telomere length regulator (?)

telomeric DNA and are involved in telomere length regulation (e.g. *S. cerevisiae* Rap1p, *Schizosaccharomyces pombe* Taz1p, and mammalian TRF1 and TRF2), while others have important roles in capping the very end of the chromosome by virtue of their ability to recognize the telomeric 3′ overhang (e.g., *S. cerevisiae*

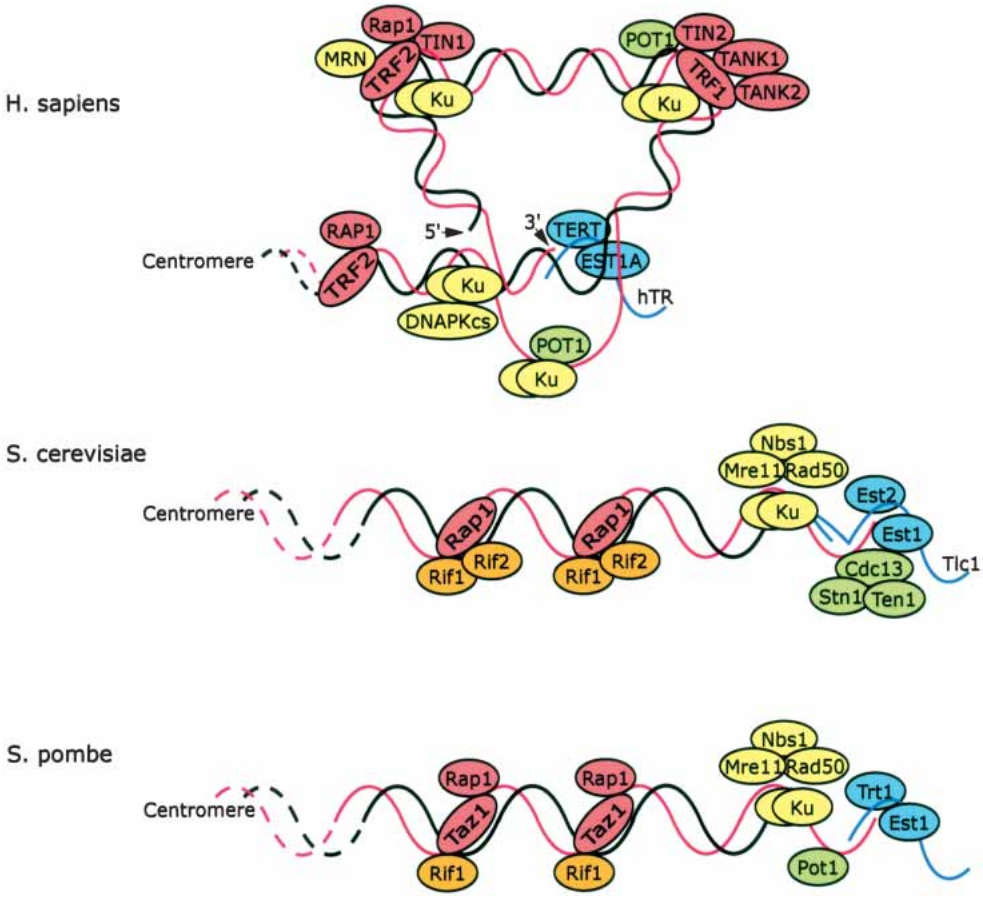

Figure 1. Schematic representation of telomere factors in different organisms.

Cdc13, *S. pombe* Pot1p, and possibly hPOT1). These latter factors bind single-stranded DNA through a conserved OB (oligonucleotide/oligosaccharide binding) fold domain (Mitton-Fry et al. 2002; Lei et al. 2003) and because a DDR ensues in their absence, are believed to play crucial roles in preventing the inappropriate triggering of the DDR by the telomere. Indeed, in *S. cerevisiae* lacking functional Cdc13p, the CA-rich telomeric strand complementary to that bound by Cdc13p is rapidly degraded, leading to *RAD9*-dependent cell-cycle arrest (Garvik et al. 1995; see below). Similarly, inactivation of *S. pombe* Pot1p leads to rapid and dramatic telomere shortening, leaving chromosome circularization as the preferred option to maintain cell viability (Baumann and Cech 2000).

In addition to it being bound by the proteins described above, there is evidence that telomeric DNA may adopt an unusual and specific structure, the so-called T loop. In this structure, the very end of the chromosome is folded back and the single-stranded telomeric 3′ overhang is tucked into a portion of the double-stranded telomeric DNA, resulting in a three-stranded structure (Griffith et al. 1999). This conformation has been suggested to prevent telomere ends from being recognized as DNA damage and triggering the DDR. Nevertheless, it is still unclear whether it is the structure per se or the factors associated with it, or a combination of both, that is crucial for evading the activation of a DDR. In vitro, the mammalian telomere repeat binding protein, TRF2, can promote T-loop formation (Stansel et al. 2001), and impairing the DNA-binding function of TRF2 in vivo leads to either ataxia telangiectasia mutated (ATM)- and p53-dependent cell death or to permanent cell cycle arrest, depending on the cell type (Karlseder et al. 1999). Although T loops have so far only been demonstrated in mammals and *Trypanosomes* (Munoz-Jordan et al. 2001), similar structures may exist in other organisms. In *S. cerevisiae*, evidence has been provided that telomeric DNA can loop back in a manner that requires Sir3p, a protein needed for the formation of transcriptionally silent chromatin flanking telomeres and at certain other genomic loci (de Bruin et al. 2001). However, it should be noted that inactivation of SIR proteins does not in itself trigger a DDR, indicating that if yeast does fold its telomeric termini by SIR-dependent mechanisms, it must also employ other systems to prevent telomeres from normally being recognized as DNA damage. These results also suggest that T loops and telomere chromatin looping-back in yeasts represent functionally different structures.

Another feature of telomeres in some eukaryotic cells is that at various cell cycle stages they appear to cluster

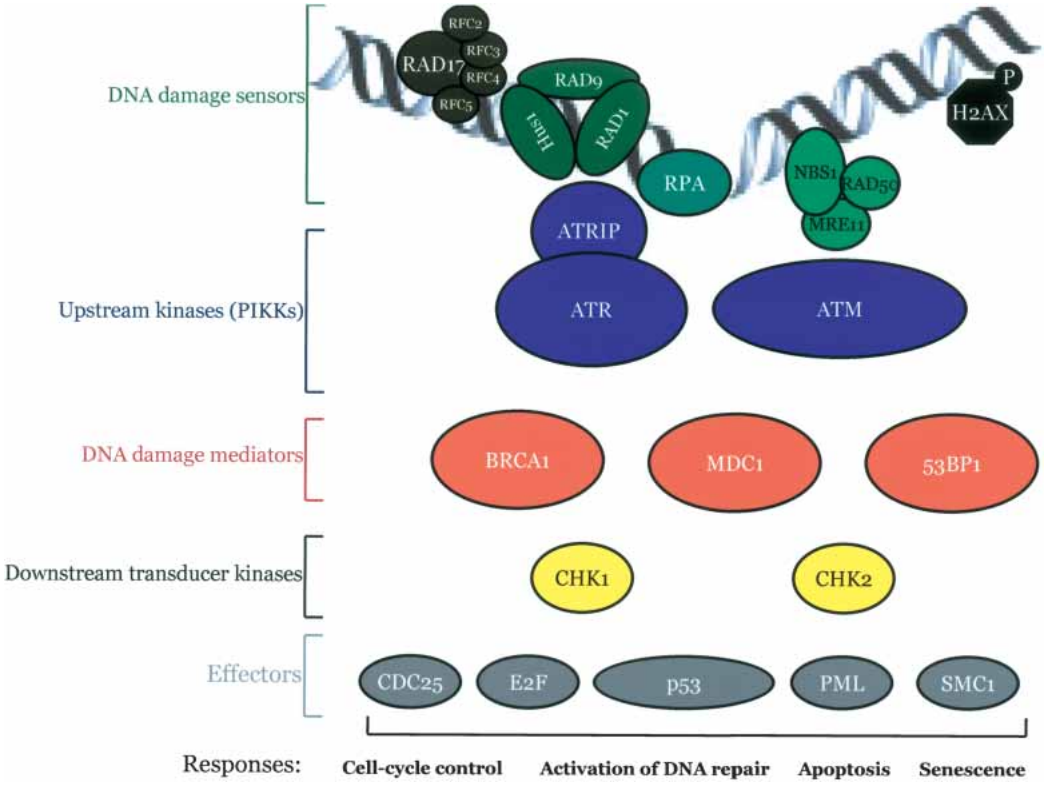

Figure 2. Schematic hierarchical representation of the factors activated following the generation of DNA damage.

and position preferentially at the nuclear periphery (Scherthan 2001). Although this is mostly associated with chromosomal separation during meiosis, at least in *S. cerevisiae* and *Plasmodium falciparum* it also occurs during interphase of the mitotic cell cycle (Gotta et al. 1996; Figueiredo et al. 2002). In such situations, the telomeres form clusters in perinuclear chromatin domains that constitute areas of transcriptional repression and modulate recombination between internal tracts of yeast telomeric DNA (Stavenhagen and Zakian 1998; Figueiredo et al. 2002). In *S. cerevisiae*, the telomeres appear to be tethered to such locations in part via their interaction with the DNA repair protein Ku (see below). To date, however, there is no firm evidence for analogous mechanisms operating in other eukaryotes.

The DNA-damage response

The DDR has evolved to optimize cell survival following DNA damage and control the proliferation of a damaged cell. Probably the best characterized—and most highly evolutionarily conserved—features of the DDR are the recruitment of DNA-repair proteins to sites of DNA damage and the "checkpoint" events that slow down or arrest cell-cycle progression, thus delaying key cell-cycle transitions until the damage has been removed (Zhou and Elledge 2000; Khanna and Jackson 2001). Once the DNA damage has been repaired, the blocks to cell-cycle progression are relieved and cell proliferation can resume. In multi-cellular organisms an inability to repair DNA damage and/or prolonged checkpoint activation can also lead to programmed cell death (apoptosis; Rich et al. 2000), or cause the cell to enter into permanent cell cycle arrest—a state known as senescence (Schmitt 2003). Other aspects of the DDR include changes in chromatin structure at sites of DNA damage (Fernandez-Capetillo and Nussenzweig 2004) and the transcriptional induction and posttranslational modification of DNA-repair and checkpoint proteins as well as other proteins that indirectly influence DNA repair, for example by modulating deoxyribonucleotide availability (Zhou and Elledge 2000; Rouse and Jackson 2002a).

Although it is often useful to study specific aspects of the DDR in isolation, recent findings have suggested that these distinctions are somewhat arbitrary. For example, in some situations "DNA-repair" factors are needed to process initial DNA lesions into structures that can trigger checkpoint activation, and "checkpoint proteins" can control the activity of DNA-repair factors and their recruitment to sites of DNA damage (Lydall and Weinert 1995; Rouse and Jackson 2002a). Consequently, it is probably best to consider the DDR as an integrated and highly coordinated set of events. These issues should therefore be borne in mind in the sections below where, for the sake of simplicity, we summarize the key features of DNA repair and DNA-damage checkpoint events separately and then discuss how each set of factors impacts on telomere biology.

DNA-damage checkpoint pathways

To the first approximation, DNA-damage checkpoint events can be likened to a classical intracellular signal-transduction pathway. Thus, the "stimulus" (DNA damage) is detected by a "sensor" (DNA-damage-binding protein) that then triggers the activation of a "transduction" system composed of upstream (proximal) and downstream (distal) protein kinases, together with a series of adaptor proteins (Fig. 2). This kinase cascade amplifies the initial DNA-damage signal and triggers a diverse set of outputs through targeting a range of "effector" proteins. Central to the DDR in all organisms studied are two large and highly conserved protein kinases of the PIKK (phosphatidyl inositol 3-kinase-like kinase) family. In humans these "checkpoint PIKK" proteins are termed ATM and ATR (ATM and RAD3-related), whereas in *S. cerevisiae* and *S. pombe* they are known as Tel1p and Mec1p, respectively, and Tel1p and Rad3p, respectively (see Table 2). The available evidence indicates that the two kinases have distinct but partially overlapping functions. Thus, mammalian ATM is involved primarily in sensing and responding to DNA double-strand breaks (DSBs) generated by agents such as ionizing radiation, although in the absence of ATM some of these functions are partly assumed by ATR (Shiloh 2003). By contrast, ATR responds to a wider range of lesions, probably after they have been processed to a common single-stranded DNA intermediate, and is particularly important in responding to DNA damage during S phase (Zou and Elledge 2003). Once activated, the checkpoint PIKK proteins phosphorylate a range of factors including the distal checkpoint kinases CHK1 and CHK2 (Chk1p and Rad53p in *S. cerevisiae*; Chk1p and Cds1p in *S. pombe*) that then target various effector proteins involved in modulating DNA repair, transcription, and cell-cycle progression (Bartek and Lukas 2003).

Precisely how the checkpoint-PIKKs are activated by DNA damage is still open to debate. One study suggested that several stresses that induce chromatin alterations in the absence of DSBs can lead to ATM autophosphorylation and activation (Bakkenist and Kastan 2003). Other reports have suggested that DSBs trigger efficient ATM activation after they have been bound and/or nucleolytically processed by the MRE11–RAD50–NBS1 (MRN) complex (D'Amours and Jackson 2002; Uziel et al. 2003; Weizman et al. 2003). Consistent with such a model, work in *S. cerevisiae* has shown that the analogous Mre11p–Rad50p–Xrs2p (MRX) complex promotes Tel1 activation (D'Amours and Jackson 2001; Usui et al. 2001). On the other hand, ATR activation requires its associated regulatory subunit ATR-interacting protein (ATRIP; Lcd1p/Ddc2p in *S. cerevisiae* and Rad26p in *S. pombe*; see Table 2). One substrate for such complexes is replication protein A (RPA)-coated single-stranded DNA (Zou and Elledge 2003), although evidence has also been provided for direct DNA binding by these complexes (Rouse and Jackson 2002b; Bomgarden et al. 2004; Unsal-Kacmaz and Sancar 2004). On their own, the above complexes appear to be sufficient for activation of the relevant PIKK and for this to phosphorylate a subset of its targets. One such target is the C terminus of the histone H2A variant H2AX (H2A in *S. cerevisiae*), which is phosphorylated extensively in the chromatin flanking sites of DNA damage (Nussenzweig 2004). The resulting phosphorylated species of H2AX, referred to as γ-H2AX, is then thought to facilitate the DDR by inducing changes in local chromatin structure and by facilitating the focal accumulation of DNA-repair and checkpoint proteins to the damaged regions.

[2]Notably, however, the phosphorylation of other checkpoint PIKK targets also requires additional factors, at least some of which may be classified as DNA-damage sensors as they are recruited to sites of DNA damage independently of the PIKK-containing complexes. In humans, these additional factors include the replication factor C (RF-C)-like complex containing hRAD17 in association with the small RF-C subunits, and the proliferating cell nuclear antigen (PCNA)-like hRAD9–hRAD1–hHUS1 (9–1–1) complex (Shiomi et al. 2002; for review, see Karnitz 2004; see Table 2 for yeast orthologs). Although other models for their actions exist, the PCNA- and RF-C-like checkpoint complexes might promote the DDR by enhancing the activity of the checkpoint PIKK proteins and/or by recruiting checkpoint PIKK substrates to the vicinity of DNA damage, thus facilitating their phosphorylation. Finally, efficient checkpoint activation also requires the recently characterized "mediator" proteins, which include mammalian BRCA1, 53BP1, MDC1/NFBD1, and Claspin, together with yeast counterparts such as *S. cerevisiae* Rad9p (Shiloh 2003; for review, see Stucki and Jackson 2004). One function of these proteins appears to be to facilitate the focal accumulation of checkpoint and DNA-repair factors in damaged regions, thus promoting their phosphorylation and leading to more efficient checkpoint activation and DNA repair (e.g., Gilbert et al. 2001; Goldberg et al. 2003).

DNA-repair pathways

Different DNA-damaging agents tend to yield chemically distinct classes of lesions and, generally speaking, each class of lesions is repaired by one or more distinct DNA-repair pathways (Friedberg et al. 1995). Of particular importance in regard to telomere functions are the two principal pathways of DNA DSB repair: homologous recombination (HR) and nonhomologous end-joining (NHEJ). Both of these systems have been highly conserved throughout eukaryotic evolution but, whereas NHEJ is a major pathway for DNA DSB repair in higher eukaryotes, single-celled organisms such as yeast rely most heavily on HR (Lieber et al. 2003; Sung et al. 2003). HR requires the *RAD52* epistasis group of genes and involves the damaged DNA entering into synapsis with an undamaged homologous partner. An early event in HR is the resection of the DNA DSB in the 5′-to-3′ direction by a nuclease, whose activity appears to be modulated by the MRN complex. The resulting 3′ single-stranded DNA tails are then bound by Rad51p (a process that is

Table 2. *Proteins involved in checkpoint–PIKK pathways are conserved from yeast to man*

Checkpoint factors characteristics	*S. cerevisiae*	*S. pombe*	Human
Upstream kinases (PIKKs)	Mec1p	Rad3p	ATR
	Tel1p	Tel1p	ATM
PIKK-interacting subunit	Lcd1p/Ddc2p	Rad26p	ATRIP
Signal-modifier	Mre11p–Rad50p–Xrs2p	Rad32p–Rad50p–Nbs1p	MRE11–RAD50–NBS1
RFC-like subunit (clamp-loader)	Rad24p	Rad17p	RAD17
PCNA-like subunit (sliding clamp)	Rad17p	Rad1p	RAD1
′	Ddc1p	Rad9p	RAD9
	Mec3p	Hus1p	HUS1
Mediators	Rad9p	Crb2p	53BP1, MDC1?
	Dpb11p	Cut5p	TOPBP1?
			BRCA1
	Mrc1p	Mrc1p	Claspin
Downstream transducer kinases	Chk1p	Chk1p	Chk1
	Rad53p and Dun1p	Cds1p	CHK2/CDS1

facilitated by a range of other HR factors), which catalyzes a strand-exchange reaction with a homologous undamaged DNA molecule. Subsequently, the 3′ terminus of the damaged molecule is extended by DNA polymerase, ligation takes place and the DNA crossovers (Holliday junctions) are resolved to yield two intact DNA molecules. By contrast, NHEJ does not require an undamaged partner molecule and essentially any two exposed double-stranded DNA ends can be re-ligated. In all eukaryotic species examined, NHEJ involves the heterodimeric DNA end-binding protein Ku together with DNA ligase IV in association with a regulatory subunit (XRCC4 in mammals). In vertebrates, efficient NHEJ also requires the DNA-dependent protein kinase catalytic subunit (DNA-PKcs; a member of the PIKK family), which is targeted to DNA DSBs by Ku (Smith and Jackson 1999; Downs and Jackson 2004). In many cases, NHEJ also involves additional proteins that help the processing of DNA ends prior to their ligation (Lieber et al. 2003). As discussed further below, proteins associated with certain other DNA-repair pathways have also been implicated in telomeric functions.

DNA-damage checkpoint proteins and telomeres

One of the most compelling indications for a central role of DNA-damage checkpoint factors at telomeres is the observation that inactivation of checkpoint PIKKs leads to major defects in telomere length control and telomeric stability in all organisms examined. For example, inactivation of Tel1p in *S. cerevisiae*, Rad3p in *S. pombe* or ATM in human cells causes telomere shortening (Lustig and Petes 1986; Greenwell et al. 1995; Metcalfe et al. 1996; Dahlen et al. 1998; Naito et al. 1998; Matsuura et al. 1999; Hande et al. 2001). In the above yeast mutants, the telomeres initially shorten rapidly but then stabilize at a new, shorter length. By contrast, the compound inactivation of both checkpoint PIKKs in *S. cerevisiae* or *S. pombe* leads to a total inability to maintain telomeric tracts by telomerase-dependent mechanisms, thus causing dramatic and progressive chromosome erosion and ensuing loss of proliferative capacity (Naito et al. 1998; Nakamura et al. 1998, 2002; Ritchie et al. 1999). An analogous analysis of mammalian cells defective in both ATM and ATR has not been possible because ATR is essential for cell viability (Brown and Baltimore 2000). Notably, it has recently been observed that Tel1p and Mec1p are alternatively associated with the telomere during the *S. cerevisiae* cell cycle—Mec1p peaking in S phase and Tel1p in the other phases—and that Mec1p kinase activity governs this association (Takata et al. 2004). Furthermore, in the absence of Tel1p, Mec1p associates with the telomere throughout the cell cycle. Taken together, these observations reveal a crucial role for the yeast checkpoint PIKKs in telomere maintenance, and suggest that these two kinases act in two distinct telomere maintenance pathways that can partially compensate for one another.

Mounting evidence suggests that the checkpoint PIKKs act in analogous ways at the telomere and in the DDR. For example, in all cases examined both roles require the integrity of the PIKK kinase catalytic domain (Greenwell et al. 1995; Mallory and Petes 2000). Furthermore, in line with functional interactions between *S. cerevisiae* Tel1p and the MRX complex in the DDR (D'Amours and Jackson 2001; Usui et al. 2001), Tel1p and MRX also work in the same pathway of telomere length maintenance (Boulton and Jackson 1998; Nugent et al. 1998; Ritchie and Petes 2000; Gallego and White 2001; Ranganathan et al. 2001). That is, the loss of any one of these proteins causes telomere shortening to a new, stable, length, but no further shortening is observed with compound mutants, at least as detectable with the available techniques. Moreover, as with inactivation of *TEL1*, disruption of *RAD50* in a *mec1* mutant background leads to dramatic telomere shortening and ensuing growth arrest (Ritchie and Petes 2000). In *S. pombe*, inactivation of either *RAD3* or *RAD26*—which encodes the regulatory subunit of Rad3p in the DDR—causes similar telomere shortening (Naito et al. 1998; Nakamura et al. 2002). Furthermore, despite the loss of *S. pombe* Tel1p or orthologs of the MRX complex having

modest, if any, effect on telomere length (Wilson et al. 1999; Hartsuiker et al. 2001; Manolis et al. 2001; Nakamura et al. 2002; Ueno et al. 2003), combining their loss with inactivation of *RAD3* leads, in each case, to an inability to maintain telomeres by telomerase-dependent mechanisms (Ritchie et al. 1999; Nakamura et al. 2002; Chahwan et al. 2003). This phenotype is also observed in *S. pombe* strains deleted for *TEL1* and *RAD26* (Nakamura et al. 2002). Finally in this regard, RPA—which facilitates the recruitment of mammalian ATR–ATRIP and *S. cerevisiae* Mec1p–Ldc1p/Ddc2p to single-stranded DNA (Zou and Elledge 2003)—has been implicated in telomere length control (Smith et al. 2000; Mallory et al. 2003) and in controlling the access of Est1p to the telomere (Schramke et al. 2004). Taken together, the available data therefore strongly suggest that triggering checkpoint PIKK activity is necessary for normal telomere homeostasis, and suggest that the mechanism by which this occurs is closely related to the events leading to PIKK activation in the DDR.

Other upstream components of the DDR, particularly potential sensors of DNA lesions, also impinge on telomere length regulation. Perhaps the most compelling evidence for this is the observation that *C. elegans* strains lacking MRT2—a functional ortholog of human RAD1 that forms part of the 9–1–1 complex—display progressive telomere shortening and loss of germ-line immortality (Ahmed and Hodgkin 2000). However, the deletion of components of the analogous complex in *S. cerevisiae* causes only mild telomere length changes, and some effects appear to be laboratory or strain specific (Corda et al. 1999; Longhese et al. 2000; Grandin et al. 2001a). There have also been contrasting reports on the potential role of the analogous *S. pombe* complex in telomere length regulation, although a recent extensive analysis concluded that these factors and Rad17p—a component of the RF-C-like checkpoint complex—do control telomere length and are associated with telomeric DNA in vivo (Nakamura et al. 2002 and references therein). Although the mechanism(s) by which these factors influence telomere length regulation is still unclear, one possibility is that they facilitate the phosphorylation of certain checkpoint PIKK targets involved in telomere maintenance. Alternatively, or in addition, the effects of these factors on telomere length might reflect them altering telomeric chromatin structure (Corda et al. 1999) or the maturation of telomeric lagging-strand DNA replication intermediates. It is noteworthy that *S. cerevisiae* cells lacking an alternative RF-C-like checkpoint complex containing Elg1p have long telomeres (Kanellis et al. 2003; Smolikov et al. 2004).

Significantly, combining the deletion of *TEL1* with deletion of components of the PCNA-like checkpoint complexes in *S. cerevisiae*, and the PCNA- and RF-C-like checkpoint complexes in *S. pombe*, do not result in the senescent phenotypes observed with deletion of *MEC1* and *TEL1* or *RAD3* and *TEL1*, respectively (Nakamura et al. 2002; Mieczkowski et al. 2003). However, telomere-to-telomere fusions do occur with increased frequency in *S. cerevisiae ddc1 tel1* and *mec3 tel1* mutants, a phenotype that is similar to that of *mec1 tel1* and *mec1 mre11* mutants (Mieczkowski et al. 2003). Similarly, components of the analogous *S. pombe* PCNA- and RF-C-like complexes influence telomeres via the *RAD3/RAD26* pathway but their loss does not fully recapitulate the phenotypes of *RAD3*- or *RAD26*-deficient strains (Nakamura et al. 2002). Some other less well-characterized DDR factors also regulate telomere functions. For example, *S. cerevisiae* Tel2p works in the same telomere maintenance pathway as Tel1p (Runge and Zakian 1996) and seems to bind to telomeric DNA (Kota and Runge 1998). Although a role of Tel2p in the DDR has not yet been described, its human counterpart controls sensitivity to DNA damaging agents whereas its *C. elegans* ortholog influences telomere length, the S-phase checkpoint, and controls life span and biological rhythms (Ahmed et al. 2001; Benard et al. 2001; Lim et al. 2001; Jiang et al. 2003).

It is interesting to note that the components of the DDR that tend to have most impact at the telomere are those that function in the upstream parts of the DDR signalling cascade. Thus, while the checkpoint PIKKs and factors involved in their regulation/activation have major roles in telomere homeostasis, proteins that play important but more downstream functions in the DDR—such *S. cerevisiae* Rad9p, Rad53p, Dun1p and Chk1p—do not. Furthermore, in instances where such downstream factors influence the telomere, this has generally been ascribed to an indirect effect. For example, the impact of *RAD53* or *DUN1* deletion on telomere length seems to at least in part reflect defective regulation of deoxyribonucleotide levels (Longhese et al. 2000; Mallory et al. 2003). Where analyzed, downstream components of the DDR in mammals, such as p53 and H2AX, have also not been found to have a major impact on telomere length regulation (Chin et al. 1999; Fernandez-Capetillo et al. 2003). Taken together, the available data are therefore consistent with a model in which telomere homeostasis involves (certain) sensor and upstream kinase components of the DDR that influence telomere structure and telomerase action by mechanisms that do not require the actions of more downstream transducers or effectors of the DDR.

Based on the above, it seems probable that checkpoint PIKKs and their regulatory factors respond to a specific DNA structure(s) arising at telomeres. One situation where such structures may occur is during S phase, when telomeres are replicated and their specialized functions might be temporarily disrupted. In this regard, it is noteworthy that Tel1p and the MRX complex function together in responding to DSBs during S phase (D'Amours and Jackson 2001; Grenon et al. 2001; Usui et al. 2001) and that replication of telomeres may transiently produce similar structures. At the telomere, leading- and lagging-strand DNA replication are expected to produce a blunt end and a recessed end with a 3′ overhang, respectively (Chakhparonian and Wellinger 2003). Although replication products bearing a 3′ overhang might directly serve as a template for telomerase with little or no processing needed, blunt-ended products would pre-

sumably require extensive processing to generate the 3′ overhang needed for the binding of telomeric single-stranded DNA-binding proteins such as Cdc13p. In line with this, differential processing of the two products has been revealed by studies with *S. cerevisiae* strains lacking the Rad27p nuclease, which functions in DNA-base excision repair and in the processing of Okazaki-fragment DNA-replication intermediates (Parenteau and Wellinger 2002).

The use of a de novo telomere addition assay employing a telomeric DNA substrate bearing a HO-endonuclease-induced 5′ overhang has revealed an involvement of MRX for telomerase action and Cdc13p binding to the de novo substrate (Diede and Gottschling 2001). Based on these results, it was proposed that the MRX complex helps to prepare telomeric DNA for the loading of Cdc13p, which then protects the chromosome from further degradation and recruits telomerase and other DNA replication components to synthesize telomeric DNA. However, in apparent opposition to this model, the association of Cdc13p with natural yeast telomeres was found to occur efficiently in the absence of Tel1p or MRX and moreover, mutations in the exonuclease domain of Mre11p did not affect telomere length (Moreau et al. 1999; Tsukamoto et al. 2001). The recent finding that the MRX complex does play a modest but detectable role in the generation of telomere overhangs outside S phase could reconcile the above observations (Larrivée et al. 2004). In addition, it is also possible that the MRX complex acts in a partially redundant manner with other proteins at the telomere; one such protein might be the conserved exonuclease Exo1p, which in *S. cerevisiae* regulates single-stranded telomeric DNA degradation in the absence of Ku (Maringele and Lydall 2002). Further evidence that the yeast MRX complex is involved in recruiting telomerase activity to telomeres is provided by the observation that robust telomere lengthening takes place in *mec1 mrx* and *mec1 tel1* mutant cells in situations where telomerase is targeted to telomeres by way of a protein fusion (Tsukamoto et al. 2001). Such a role may also exist in mammals, as NBS1 associates with telomeres during S phase when telomeres are elongated (Zhu et al. 2000), and is required for effective telomere elongation by telomerase (Ranganathan et al. 2001).

Although there are many ways in which the checkpoint PIKKs and associated components may influence telomere homeostasis, these can be reconciled with a model in which such factors regulate telomerase activity or telomerase access to the telomeric template. One possibility, discussed above, is that such factors are needed for the efficient processing of nascent telomeres into structures compatible with telomerase action. In addition, several lines of evidence suggest that they might also influence telomerase activity more directly. For example, ionizing radiation can influence hTERT nuclear localization (Wong et al. 2002), and telomerase activity was found to increase in extracts derived from rodent cells that had been treated with ionizing radiation or ultra-violet light (Hande et al. 1997, 1998). Conversely, DNA-damage-induced phosphorylation of hTERT by c-Abl (a protein implicated in DNA-PK- and ATM-dependent DDR events) has been found to inactivate telomerase activity (Kharbanda et al. 2000). Nevertheless, it seems unlikely that the checkpoint PIKK proteins control telomere length primarily by influencing intrinsic telomerase catalytic activity, since in vitro telomerase activity is largely unaffected by their loss and in *S. cerevisiae* the targeting of telomerase to telomeres by way of a protein fusion rescues the senescent phenotype of *mec1 tel1* mutant cells (Chan et al. 2001). Therefore, it seems most likely that checkpoint PIKK proteins such as Tel1p and Mec1p mainly control telomere length by regulating the access of telomerase to telomeres by targeting additional telomere-bound factors.

Some potential telomeric targets for the checkpoint PIKKs in yeast have arisen through the work of D. Shore and collaborators, who demonstrated that telomere elongation by telomerase is progressively inhibited in *cis* by telomere-bound Rap1p. In this elegant model of telomere length homeostasis (the so-called Rap1 counting mechanism; Marcand et al. 1997), progressive telomere shortening causes the gradual loss of telomere-bound Rap1p and, therefore, a progressive relief of its inhibitory function on telomerase activity, ultimately resulting in telomerase-mediated telomere elongation. Significantly, this Rap1 counting mechanism does not function in the absence of Tel1p and, furthermore, the deletion of the Rap1p-binding factors, Rif1p and Rif2p, leads to telomerase-dependent telomere elongation in wild-type but not in *tel1* mutant cells (Craven and Petes 1999; Ray and Runge 1999). Taken together, these results suggest a model in which Tel1p and the Rap1p/Rif1p/Rif2p complex promote telomere elongation by acting in the same genetic pathway. Notably, the human homolog of Rap1p, hRAP1, does not appear to bind DNA directly but instead acts together with the telomere-specific DNA-binding protein TRF2 to negatively regulate telomere length in a telomerase-dependent fashion (Li and de Lange 2003). The recent discovery of mammalian orthologues of Rif1p (Adams and McLaren 2004) and the surprising finding that human Rif1 plays important roles in the DDR but seemingly not in telomere homeostasis (Silverman et al. 2004) adds further potential layers of complexity to their functions.

DNA-repair proteins and telomeres

One of the first indications for an involvement of DNA-repair factors in normal telomeric functions was the discovery in 1996 that inactivation of either subunit of the NHEJ protein Ku leads to telomere shortening in *S. cerevisiae* (Boulton and Jackson 1996; Porter et al. 1996). It was subsequently shown that inactivation of Ku also triggers the rapid loss of telomeric repeats from chromosome termini in *S. pombe* (Baumann and Cech 2000). However, contrary to when telomerase components are deleted, these telomeres stabilize at a new, shorter, length and there is no progressive further telomere attrition leading to loss of cell proliferation (Boulton and Jackson 1998; Nugent et al. 1998; Polotnianka et al.

1998; Baumann and Cech 2000). Notably, whereas telomere shortening is also caused by the loss of *S. cerevisiae* Mre11p, Rad50p, or Xrs2p (which also function in NHEJ), this is not the case when *S. cerevisiae* DNA-ligase 4 or Lif1p (the XRCC4 homolog) are inactivated (e.g., Teo and Jackson 1997; Boulton and Jackson 1998; Herrmann et al. 1998; D'Amours and Jackson 2002). Consistent with these findings, the role of Ku at telomeres appears to be distinct from its roles in NHEJ, as Ku mutants have been identified that affect one function but not the other (Driller et al. 2000; Bertuch and Lundblad 2003; Stellwagen et al. 2003; Roy et al. 2004).

Chromatin immunoprecipitation and immunolocalization studies have shown that Ku is physically associated with telomeres in both *S. cerevisiae* and *S. pombe* (Gravel et al. 1998; Laroche et al. 1998; Nakamura et al. 2002), although it is not yet clear whether this reflects direct binding of Ku to telomeric DNA or it being tethered by protein–protein interactions, or both. One mechanism by which Ku functions at the telomere has been revealed by work showing that *S. cerevisiae* Ku regulates telomere length by interacting directly with TLC1 (Peterson et al. 2001; Stellwagen et al. 2003). Indeed, overexpression of a conserved stem loop of TLC1 leads to Ku-dependent telomere shortening, deletion of this stem loop causes telomere shortening, and a *YKU80* mutation that renders Ku unable to bind TLC1 results in short telomeres (Stellwagen et al. 2003). Taken together, these data suggest that the binding of Ku to the telomerase RNA and perhaps other telomere-specific proteins plays a key role in ensuring that telomerase is targeted appropriately to chromosomal ends (Fig. 1). Significantly, the deletion of Ku also impairs the synthesis and/or stability of chromosomal termini in *S. cerevisiae*. Thus, whereas the telomeric 3′ overhang is detectable only during S phase in wild-type cells, in Ku mutants these overhangs are observed in all cell-cycle phases (Gravel et al. 1998). This has lead to the suggestion that the lack of Ku leads to a defect in lagging-strand DNA replication of the telomere (Gravel and Wellinger 2002) and a lack of protection towards Exo1p and other exonucleases, resulting in the generation of the observed constitutive overhang (Maringele and Lydall 2002). In *S. cerevisiae*, Ku is also required for transcriptional silencing at telomeres (Tsukamoto et al. 1997; Boulton and Jackson 1998)—a function that may in part reflect interactions between Ku and SIR proteins (Tsukamoto et al. 1997; Roy et al. 2004)—and for tethering telomeres to the nuclear periphery (Laroche et al. 1998). Such tethering may limit HR between telomeres (Polotnianka et al. 1998) and ensure that telomeres are replicated in late S phase (Cosgrove et al. 2002).

Several lines of evidence indicate that Ku also functions in telomere maintenance in mammals. For example, it has been reported that human Ku interacts with both TRF1 and TRF2 (Hsu et al. 2000; Song et al. 2000; Peterson et al. 2001), suggesting that it may cooperate with these proteins to regulate telomere length and establish telomere end-protection, respectively. In line with this idea, chromatin immunoprecipitation studies have revealed that Ku physically associates with mammalian telomeres in vivo (Hsu et al. 1999; d'Adda di Fagagna et al. 2001). In addition, inactivation of one allele of the gene for Ku80 in human cells results in telomere shortening (Myung et al. 2004). Furthermore, inactivation of both alleles leads to cell death, although it is not clear whether this is due to further telomere shortening or an inability to cope with endogenous DNA damage (Li et al. 2002). Differently, Ku inactivation is not lethal in mice. Although the reason for this difference between humans and mice is not clear, it has been observed that the Ku80 locus expresses a primate-specific alternative form of the protein, known as KARP-1, that is absent in rodents (Myung et al. 1997). In mice, the analysis of the role of Ku at telomeres has generated some contrasting conclusions. One study showed that cells derived from transgenic mice lacking Ku80, and embryonic stem cells lacking Ku70, have shorter telomeres than their controls, while cells lacking Ligase IV or XRCC4 do not display marked telomere length alterations (d'Adda di Fagagna et al. 2001). This report also showed that Ku inactivation causes elevated chromosomal instability, leading to chromosomal fusions that generally lacked detectable telomeric repeats at the fusion sites. By contrast, a report from another group observed that inactivation of Ku80 did not lead to telomere shortening and that the chromosomal fusions retained telomeric DNA at the fusion points (Samper et al. 2000). Furthermore, an additional report from the same group suggested that Ku is a negative regulator of telomere access by telomerase (Espejel et al. 2002a). Since both groups analyzed mice with the same genetic deletion, the differences reported may originate from variations in the experimental procedures of telomere length measurement, or from differences in mouse or cell maintenance. Importantly, both analyses found that Ku inactivation does not lead to the dramatic changes in telomeric overhangs that are observed in yeast.

Perhaps surprisingly, inactivation of Ku in *Arabidopsis thaliana* was found to lead to telomerase-dependent telomere lengthening and inefficient C-strand maintenance (Bundock et al. 2002). However, the compound inactivation of Ku and telomerase in *A. thaliana* causes a faster rate of telomere shortening than telomerase inactivation alone (Riha et al. 2002; Riha and Shippen 2003). Significantly, the deletion of *MRE11* also caused telomere elongation in *A. thaliana* (Bundock and Hooykaas 2002). Although these findings were unexpected, it is noteworthy that while telomerase inactivation restricts life span in most organisms, it extends life span in *A. thaliana* (Riha et al. 2001). A unifying model for the telomeric functions of Ku in different species is further complicated by the observation that inactivation of Ku in chicken DT40 cells does not seem to affect telomere length (Wei et al. 2002).

In the mouse, inactivation of the Ku-associated NHEJ protein, DNA-PKcs, leads to telomere fusions in the absence of detectable telomere shortening, suggesting that it may be involved in telomere capping (Bailey et al. 2001; Gilley et al. 2001; Espejel et al. 2002b). Consistent

with this idea, DNA-PKcs is associated with telomeric DNA in human cells (d'Adda di Fagagna et al. 2001) and inhibition of DNA-PKcs catalytic activity by chemical inhibitors results in telomere fusions in human cells (Bailey et al. 2004). Finally, it has been shown that mice lacking DNA-PKcs and Terc display faster rates of telomere loss than mice lacking Terc alone (Espejel et al. 2002b).

Whether proteins associated with HR also have key functions at normal telomeres is still unclear. Thus, while loss of *RAD52* or *RAD51* does not affect telomere length in *S. cerevisiae*, *rad52 tlc1*, *rad51 tlc1*, or *rad52 est1* double mutant cells senesce at a faster rate than *tlc1* or *est1* single mutants (Lundblad and Blackburn 1993; Le et al. 1999) and *Rad54* knock-out mice have recently been shown to bear shorter telomeres than matched controls (Jaco et al. 2003). Furthermore, *Rad51* inactivation in chicken DT40 cells has been reported to increase the presence of the telomeric overhangs (Wei et al. 2002). Most recently, it was established that the RAD51-related protein RAD51D colocalizes with telomeres in human cells and that inactivation of this factor leads to cell death, possibly as a consequence of telomere uncapping (Tarsounas et al. 2004). In light of these findings, it is tempting to speculate that RAD51D, possibly in a complex with certain other HR factors, promotes telomere T-loop formation. In addition, and as discussed below, HR factors can play key roles in maintaining telomere length by telomerase-independent mechanisms.

Other DNA-repair proteins have also been implicated in telomere maintenance. For example, the mammalian DNA repair protein poly(ADP-ribose) polymerase (PARP-1)—which functions in DNA base-excision repair and single-strand break repair (D'Amours et al. 1999)—acts at the telomere. Indeed, a study of PARP-1 knock-out mice provided the first demonstration of a protein of the DDR functioning at the telomere in vertebrates (d'Adda di Fagagna et al. 1999). In this report, PARP-1 inactivation was shown to lead to stable, shortened, telomeres and genomic instability in two different mouse genetic backgrounds and in different tissues. Furthermore, the compound inactivation of PARP-1 and p53 lead to very long and heterogeneous telomeres (Tong et al. 2001), perhaps reflecting the ability of both PARP-1 and p53 to suppress HR (Mekeel et al. 1997; Schultz et al. 2003). However, a different group reported that PARP-1 inactivation does not affect telomere length (Samper et al. 2001). The use of two different genetic deletions in two different mouse strains may help to explain these apparently contradictory results. Finally, XPF/XRCC1—which interacts with ERCC1 to form a structure-specific endonuclease involved in nucleotide excision repair (de Laat et al. 1999)—was recently shown to regulate the stability of the telomeric 3′ overhang (Zhu et al. 2003).

DDR proteins at dysfunctional telomeres

Most human somatic cells do not express sufficient telomerase to cope with the inability of the DNA replication machinery to fully replicate chromosomal termini. Consequently, telomeres progressively shorten upon repeated cell divisions, ultimately becoming so short that their normal functions are perturbed. It is still unclear what is the minimal length below which a telomere triggers a DDR. Recently, it has been shown that a DDR at critically short telomeres is associated with the absence of TRF2, at least as detected by immunofluorescence experiments (Herbig et al. 2004), suggesting that the recruitment of this protein to a telomere could be the limiting factor. In some cell types telomere dysfunction lead to apoptosis whereas in others, such as human fibroblasts, it triggers a permanent growth arrest called senescence. Recent work has established that telomere-initiated senescence shares many features of a cell-cycle arrest induced by DNA-damaging agents that cause DSBs (d'Adda di Fagagna et al. 2003). These include the activation of upstream checkpoint PIKKs, mediators, and downstream kinases of the DDR, and the appearance of senescence-associated DNA damage foci (SDFs) containing DDR factors, as detected by immunofluorescence; one report, however, concluded that the detectability of such markers is only transient (Bakkenist et al. 2004). The appearance of DDR markers in senescent cells is triggered with the direct contribution of eroded telomeres, as revealed by the specific accumulation of γ-H2AX and other markers of the DDR at chromosome termini in senescent cells. Significantly, interfering with the actions of checkpoint kinases by siRNA or by dominant-negative constructs leads to a significant proportion of senescent cells resuming cell cycle progression into S phase, indicating that DNA-damage checkpoint activation is causally associated with the senescent state (d'Adda di Fagagna et al. 2003; Herbig et al. 2004).

Similarly, progressive telomere shortening caused by inactivation of telomerase in *S. cerevisiae* leads to the accumulation of cells that are unable to divide further and which display an activated DDR—as determined by the phosphorylation of Rad53p—and a morphology reminiscent of senescent mammalian cells (Enomoto et al. 2002; IJpma and Greider 2003). Moreover, inactivation of checkpoint factors such as Mec3p, Mec1p, Lcd1p/Ddc2p, or Rad24p allows a portion of such cells to bypass this senescence-like state and continue proliferating. Therefore, as in mammalian cells, severe telomere shortening in yeast leads to the activation of the DDR and concomitant cell-cycle arrest. These findings are consistent with biochemical experiments and micro-array expression analyses, which have shown that yeast cells with critically short telomeres have a global gene expression profile that overlaps with that of cells exposed to DNA-damaging agents (Nautiyal et al. 2002). Furthermore, the observation that mice with shortened telomeres are more sensitive to radiation (Goytisolo et al. 2000; Wong et al. 2000) is consistent with a model in which dysfunctional telomeres are perceived as DSBs and therefore cells bearing them are more sensitive to additional DNA damaging agents generating DSBs. Taken together, these results suggest that eroded telomeres and DNA damage

trigger very similar responses and ultimately produce similar outcomes.

Telomere shortening is not the only way the protective function of the telomere can be lost. In mammals, removal of TRF2 from the telomere leads to a DDR that results in cell-cycle arrest or apoptosis, depending on the cell type (van Steensel et al. 1998). Moreover, the DDRs in senescent and TRF2-inhibited cells appear to be strikingly similar (d'Adda di Fagagna et al. 2003; Takai et al. 2003). Taken together, these results suggest that the loss of telomeric DNA is not detrimental per se, but it is the loss of telomere-bound factors that results in telomere deprotection and concomitant activation of the DDR. This idea is further supported by the observation that cells senesce with a shorter mean telomere length if TRF2 is overexpressed; presumably the additional TRF2 helps to stabilize short telomeres (Karlseder et al. 2002). Analogously, inactivation of *S. cerevisiae CDC13*, *STN1*, or *TEN1*—which form a complex that binds to and protects the protruding telomeric 3′ overhang—leads to dramatic activation of the DDR (Garvik et al. 1995; Grandin et al. 1997, 2001b; Pennock et al. 2001). In addition, a DDR leading to rapid telomere degradation has been observed in *S. pombe* lacking Pot1p—a telomeric single-stranded protein similar to those found in ciliated protozoa (Baumann and Cech 2001). Whether human Pot1p has a similar protective role, however, is still unclear (Colgin et al. 2003; Loayza and de Lange 2003).

Perhaps unexpectedly, unregulated telomere lengthening can also induce a DDR, as has been observed in *S. cerevisiae* cells bearing short telomeres and overexpressing Tel1p (Viscardi et al. 2003), and also can cause genome instability and telomere fusions, as observed in *S. pombe* cells lacking Taz1p (Ferreira and Cooper 2001). Furthermore, in human cells the overexpression of a human ortholog of yeast Est1p—a factor necessary for telomerase mediated telomere elongation (Snow et al. 2003)—leads to telomere uncapping (Reichenbach et al. 2003). Although the mechanisms that trigger the DDR under these circumstances are still unclear, it is possible that the uncoupling of the synthesis of the two strands, caused by an overactive telomerase, might lead to generation of an excess of single-stranded DNA that triggers a DDR. Overall, these observations reveal that a variety of perturbations of telomere structure can trigger a DDR very similar to that caused by exogenous DNA-damaging agents.

Dysfunctional telomeres are not only substrates for the cell-cycle checkpoint machinery but are also targeted by the DNA-repair apparatus. Indeed, in both mammals and yeast, critically short telomeres are substrates for recombination and are prone to telomere–telomere fusions. This leads to frequent chromosomal circularization in *S. pombe* cells lacking telomerase (Nakamura et al. 1998), and chromosomal aberrations resulting from chromosome end fusions in human fibroblasts approaching replicative senescence and in late generation telomerase-deficient mice (Blasco 2002). Similarly, uncapped telomeres are substrates for end-joining events that involve well-known NHEJ factors (Baumann and Cech 2001; Ferreira and Cooper 2001; Smogorzewska et al. 2002; Mieczkowski et al. 2003). Notably, however, there are suggestions that differences exist between the mechanism of telomere end fusions and NHEJ of DNA DSBs caused by DNA-damaging agents. For example, although *S. cerevisiae* Nej1p—an essential NHEJ component—does not affect the stability of telomeres in wild-type cells, it suppresses telomere fusions mediated by NHEJ in yeasts maintaining their telomeres via HR (Liti and Louis 2003).

When telomeres become critically short in the absence of telomerase in *S. cerevisiae*, rare survivors emerge that maintain their telomeres through *RAD52*-dependent mechanisms of HR (Lundblad and Blackburn 1993; Le et al. 1999). These survivors employ either *RAD50*-dependent amplification of TG-repeats (type II recombination) or *RAD51*-dependent acquisition of subtelomeric elements (and their deletion derivatives) by a large number of telomeres (type I recombination; Lundblad and Blackburn 1993; Teng and Zakian 1999; Teng et al. 2000; Chen et al. 2001; for review, see Lundblad 2002). It is noteworthy that, although such events might occur most commonly on telomeres that either have lost telomerase activity or Ku (McEachern et al. 2000), recombination can also occur on long telomeres that have been uncapped by the loss of Cdc13p, suggesting that these factors protect chromosome ends from such reactions (Booth et al. 2001; Grandin et al. 2001a; DuBois et al. 2002; Tsai et al. 2002; Grandin and Charbonneau 2003). As mentioned previously, the loss of telomerase function in *S. pombe* leads to chromosomal circularization in surviving cells (Baumann and Cech 2000). However, when Taz1p is also deleted in such backgrounds, the ensuing survivors more frequently use recombinational modes for telomere maintenance (Nakamura et al. 1998). Thus, as in *S. cerevisiae*, telomere end-protection proteins actively inhibit HR among homologous telomeric sequences in *S. pombe*.

In mammals, a significant but relatively small portion of tumours (mostly sarcomas), and cell lines transformed by the SV40 virus, show a very heterogeneous telomeric pattern with some very long telomeres (Neumann and Reddel 2002). These cells do not express detectable telomerase and are believed to maintain their telomeres by HR, as demonstrated by their ability to amplify a tagged subtelomeric sequence in *trans* onto other chromosomal termini (Dunham et al. 2000; Niida et al. 2000; Varley et al. 2002). Significantly, a portion of cells maintaining telomeres by this "ALT" mechanism (for alternative lengthening of telomeres) display evidence of a DDR at some telomeres. In these cells, telomere-specific binding proteins and telomeric DNA—possibly including this in an extra-chromosomal form—colocalize in subnuclear structures known as PML bodies together with proteins usually associated with DNA damage checkpoint signalling and HR such as MRE11, NBS1, RAD50, RAD51, RAD52, RPA, BLM, and WRN (Henson et al. 2002). Although care should be used to interpret these colocalization data, as very long telomeres might render DDR proteins that are normally associated with

telomeres more detectable than when telomeres are shorter, it is tempting to speculate that such structures represent sites where telomeres are being maintained by HR-based mechanisms. How cells become able to maintain their telomeres in this manner is still open to conjecture. Cell-fusion experiments suggest that ALT cells generally carry a recessive mutation(s) (Neumann and Reddel 2002). Furthermore, circumstantial evidence suggests that p53 suppresses ALT, as cell lines derived from Li-Fraumeni syndrome patients—which bear inherited p53 mutations—are frequently ALT, as are SV40 transformed cell lines in which p53 activity has been essentially ablated. The observation that p53 negatively regulates HR (Mekeel et al. 1997), possibly by inhibiting RAD51 activity (Linke et al. 2003), lends further support to this idea. Finally, it is possible that changes in telomeric chromatin are associated with the assumption of ALT. For example, a change in telomeric chromatin that made it more open and accessible to HR proteins could render the cell more susceptible to the initiation of ALT. In this regard, it is noteworthy that inactivation of the *S. cerevisiae HHO1* gene, which encodes the linker histone Hho1p, makes it more easy for the yeast cell to enter into HR-dependent mechanisms of telomere maintenance (Downs et al. 2003). Perhaps inactivation or deregulation of linker histones, or possibly other chromatin changes, could lead to a similar situation in the mammalian system.

DDR proteins at functional and dysfunctional telomeres: what's the difference?

As discussed above, components of the DDR are necessary both for normal telomere homeostasis and for responding to dysfunctional telomeres. For example, the checkpoint PIKKs are necessary both for telomere homeostasis and to mount a DDR in reaction to the disruption of the telomere protective structure following telomere shortening and/or telomere deprotection. Similarly, Ku can protect normal telomeric ends from resection and ensuing end fusions, and yet the NHEJ apparatus actually mediates chromosomal end-to-end fusions caused by telomere dysfunction. A key challenge for the telomere field is to explain how the DDR apparatus distinguishes between functional and dysfunctional telomeres and produces two very different outcomes.

The most obvious difference between these two situations is the amount and constitution of telomere-associated proteins. It is therefore possible that the protein complexes associated in a sequence-specific manner with telomeric DNA have the ability to limit the DDR. Thus, when too few (or none) of such proteins are at a telomere, the DDR would become unrestrained, leading to chromosomal end fusions, cell-cycle arrest and/or apoptosis (Fig. 3). We envision several, not necessarily mutually-exclusive mechanisms by which telomeric proteins might inhibit a full DDR being elicited from a functional telomere. By direct steric hindrance and/or by facilitating the formation of higher-order telomeric DNA structures, or by confining the telomere to specific subnuclear regions these proteins might physically prevent DDR factors from gaining access to telomeric DNA. Examples of such activities might include single-stranded telomeric DNA-binding proteins with telomere protecting functions, factors promoting T-loop formation, the generally compact and repressive state of telomeric chromatin and the localization to the nuclear periphery of telomeres in some species.

Nevertheless, the observation that some DDR factors are associated with telomeres and are necessary for proper telomere homeostasis suggests a more complex regulatory mechanism than mere exclusion of access. Studies in *S. cerevisiae* have indicated that the induction of a full DDR is triggered by an unrepaired and most likely resected DSB (Pellicioli et al. 2001; Rouse and Jackson 2002a). Consistent with this idea, ATM activation in mammalian cells is compromised in cells impaired in the MRN nuclease complex (Uziel et al. 2003). Moreover, at least for mammalian ATR and *S. cerevisiae* Mec1, it appears that single-stranded DNA must be bound by RPA in order for efficient checkpoint activation to ensue (Zou and Elledge 2003). Therefore, a telomere might only activate the DDR if it becomes significantly resected (Maringele and Lydall 2002) and complexed with a sufficient number of RPA molecules. Indeed, short tracts of single-stranded telomeric DNA do not appear to normally activate the DDR, as such structures are present in cycling cells and in *S. cerevisiae* lacking Ku, where a large increase in these structures is generated in the absence of a detectable DDR (Gravel et al. 1998). In these situations the binding of Cdc13p (or Pot1p in *S. pombe*; Mitton-Fry et al. 2002) to single-stranded telomeric tracts presumably prohibits these being recognized by large amounts of RPA. Notably, work in yeast has shown that a DSB generated near a telomeric tract is not resected as efficiently as one located elsewhere in the genome, and that this difference depends on Cdc13p (Diede and Gottschling 1999). Thus, telomere bound factors such as Cdc13p can curtail the DDR by both binding to single-stranded telomeric tracts and therefore competing with RPA, and also by restricting further DNA resection into adjacent nontelomeric sequences (Fig. 3). In addition to potentially explaining why telomeres are not normally recognized as DNA damage, such a model could also help to explain why DNA damage generated at the telomere is generally less easily repaired than that at other chromosomal sites (von Zglinicki 2002).

One situation when a normal telomere might be particularly vulnerable to triggering the DDR is when it is replicated—a replication fork reaching the end of a chromosome end will face a situation very similar to that encountered by a fork replicating a chromosome carrying a DNA strand break. In addition to the above described mechanisms, it is possible that telomere-bound factors directly modulate the kinase activity of the upstream kinases of the DDR. Although this may be so, checkpoint PIKK activity is clearly needed for normal telomere homeostasis. One attractive possibility then is that the checkpoint PIKK proteins become only transiently acti-

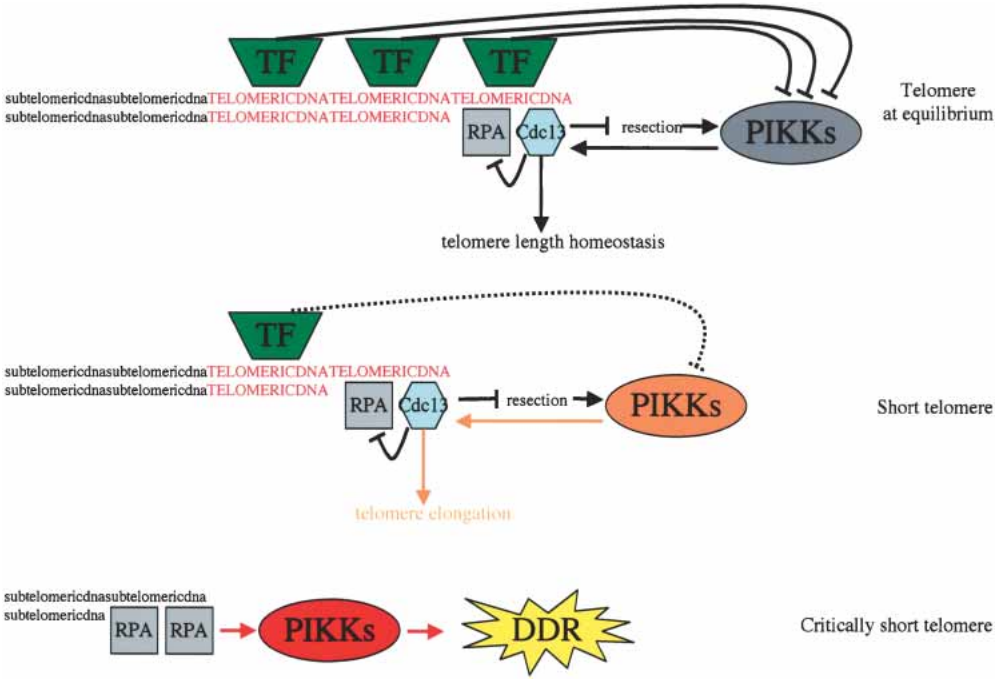

Figure 3. Schematic representation of the mechanisms controlling telomere length homeostasis, telomere elongation of a short telomere, and the generation of a DDR at a critically short telomere. At a telomere in equilibrium, telomere factors (TFs) inhibit the activation of upstream DNA damage kinases (PIKKs), preventing them from activating proteins (such as Cdc13p) that can trigger telomere elongation. PIKK activation is also inhibited by proteins (e.g., Cdc13p) that can restrain telomere resection and the consequent accumulation of RPA on telomeric DNA. Telomere shortening causes the loss of telomere bound TFs, resulting in diminished PIKK inhibition and unleashing telomere elongation mechanisms. In the absence of telomere maintenance mechanisms, further telomere shortening leads to the loss of factors such as Cdc13p that prevent single-stranded DNA erosion, leading to unrestrained resection; this may cause the generation of a single-stranded DNA/RPA complex of a sufficient length to trigger the generation of a robust DDR.

vated at telomeres at the end of S phase and that this activation is coupled to effective telomere end-maintenance. Indeed, regulating telomerase access by such a mechanism might provide opportunities for the cell to target this enzyme most effectively to the shorter telomeres in the population that are in most need of lengthening. Although such a control mechanism is hypothetical, we note that yeast Cdc13p has several conserved PIKK consensus phosphorylation sites, raising the possibility that such phosphorylations control the ability of the Cdc13p complex to recruit telomerase and/or cap telomeric ends (DuBois et al. 2002). A variation on the above models is one in which telomeric-binding factors modulate the activity of PIKKs by allowing the PIKKs to phosphorylate proteins involved in normal telomere homeostasis but preventing them from acting on downstream components and generating a full-blown DDR. One way this could be achieved is through differential use of the mediators of the pathway; indeed, mediators such as BRCA1 are only required for the phosphorylation of a subset of ATM and ATR substrates (Foray et al. 2003), suggesting that different components of the DDR pathway are required to differing extents depending on the initial signal and the final outcome.

In summary, we propose that the DDR apparatus in fact does not distinguish between functional and dysfunctional telomeres: it recognizes both structures and is active at both (Fig. 3). However, whereas its activity at functional telomeres is restrained by telomere-bound factors and thereby channelled towards telomere homeostasis, at dysfunctional telomeres the DDR is unrestrained and enforces a DNA damage checkpoint involving the entire cascade of DDR factors.

Future directions

Over the past decade, there has been much progress towards understanding the normal structure and functions of telomeres, how telomere homeostasis is maintained, and how telomeres are prevented from being recognized as DNA damage. Strikingly, this work has revealed that normal telomere maintenance requires many proteins associated with the DDR. Although we now have some knowledge of how these proteins function, there is still much to be learned. One of the most important issues facing the telomere field is to define precisely what it is about telomeres that prevent them from being recognized as DNA damage. Many insights into this will surely come from further defining the structure and functions of telomere-bound proteins and by establishing the range of proteins targeted by the checkpoint PIKKs, both at the telomere and in the DDR. Another major challenge will be to establish how the access of

telomerase to the telomere is tightly controlled. In addition, it will be interesting to ascertain whether additional DDR proteins act at normal and/or dysfunctional telomeres. Such work is likely to provide insights not only into telomere biology but also into responses to DNA damage in a wider context. In this regard, it will also be of great interest to see whether further factors that were initially identified through their functions at the telomere actually have more widespread roles in the DDR. Finally, it will be of key importance to establish how deregulation of pathways of telomere maintenance can lead to cancer and, perhaps, also contributes to a range of other age-related pathologies. Given the intense activity taking place in the telomere and DDR fields, it seems safe to predict that the answers to these and other questions will soon be upon us.

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