Focus Review

Taming the tiger by the tail: modulation of DNA damage responses by telomeres

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Telomeres are by definition stable and inert chromosome ends, whereas internal chromosome breaks are potent stimuli of the DNA damage response (DDR). Telomeres do not, as might be expected, exclude DDR proteins from chromosome ends but instead engage with many DDR proteins. However, the most powerful DDRs, those that might induce chromosome fusion or cell-cycle arrest, are inhibited at telomeres. In budding yeast, many DDR proteins that accumulate most rapidly at double strand breaks (DSBs), have important functions in physiological telomere maintenance, whereas DDR proteins that arrive later tend to have less important functions. Considerable diversity in telomere structure has evolved in different organisms and, perhaps reflecting this diversity, different DDR proteins seem to have distinct roles in telomere physiology in different organisms. Drawing principally on studies in simple model organisms such as budding yeast, in which many fundamental aspects of the DDR and telomere biology have been established; current views on how telomeres harness aspects of DDR pathways to maintain telomere stability and permit cell-cycle division are discussed.

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Telomeres, the chromosome ends

Telomeres are the natural ends of linear chromosomes and are found on most eukaryotic nuclear chromosomes. When the concept of telomeres was proposed it was suggested that ‘the terminal gene must have a special function, that of sealing the end of the chromosome’ (Muller, 1938). Of course, at this time, the nature of a gene was not clear and therefore there could have been little idea about the special function that would allow the terminal gene to cap (seal) the chromosome end. Since then much has been discovered about the nucleic acid and protein constituents of chromosome ends and about the mechanisms by which these structures cap chromosome ends to ensure that telomeres are much more stable and inert than internal chromosome breaks (Blackburn et al., 2006). It has also become clear that telomere capping is not only important for maintaining genetic stability and protecting against cancer, but also for permitting cell division and protecting against ageing (Stewart and Weinberg, 2006; Aubert and Lansdorp, 2008; Deng et al., 2008; Jeyapalan and Sedivy, 2008). Before describing how telomeres cap chromosome ends it is worth considering how cells respond to chromosome breaks elsewhere.

DNA damage responses to double strand breaks

A double strand break (DSB) is the DNA lesion that most resembles a telomere. All cell types from bacteria with circular chromosomes to human cells with linear chromosomes have evolved a powerful DNA damage response (DDR) to DSBs and other types of DNA damage. DSBs are perhaps the most potent inducers of DDRs because a single un-repaired DSB can lead to loss of DNA distal to the break when chromosomes are segregated to daughter cells. The importance of the DDR is illustrated by the fact that many DDR protein functions are conserved through evolution, in bacteria, archaea and eukaryotes.

In essence, the DDR comprises three coordinated responses: DNA repair pathways reverse lesions in DNA; checkpoint pathways inhibit cell-cycle progression while repair occurs; and apoptosis ensures that cells with high levels of DNA damage are killed rather than permitted to divide and pass on damaged genomes. Two major DNA repair pathways engage with DSBs: non-homologous end joining (NHEJ) fuses broken chromosome ends together, whereas homologous recombination (HR) uses sequence homology to repair DSBs (Frank-Vaillant and Marcand, 2002; Kim et al., 2005).

Scores of proteins contribute to cellular responses to DSBs and some of these are listed in Table I. Some proteins engage early with DSBs, others engage later, and with the final repair outcome depending on competition between different repair pathways (Lisby et al., 2004; Symington and Heyer, 2006; Kanaar et al., 2008). The left part of Figure 1 shows some of the budding yeast proteins binding to a DSB as it undergoes HR repair and the right part shows some of the same proteins and telomere-specific proteins playing roles in telomere maintenance. Interestingly, in budding yeast many of the ‘early’ DDR proteins at DSBs are involved in physiological telomere maintenance, whereas ‘late’ DDR proteins seem, generally, to have less of a role in telomere maintenance. Late DDR proteins do have important functions in the case of...
telomere failure, either in back up mechanisms of telomere maintenance, such as alternative lengthening of telomeres (ALTs), or inhibiting cell-cycle progression if telomeres are uncapped (Lydall and Weinert, 1995; Enomoto et al., 2002; Lundblad, 2002; IJpma and Greider, 2003).

The simplest way to repair a DSB is to fuse the ends back together, and this is essentially what the NHEJ repair pathway does. NHEJ depends on little or no homology between the ends being joined, and therefore carries the risk the DNA ends that were not adjacent previously may be joined, causing a chromosomal translocation. Different eukaryotic organisms use variations on a core set of proteins to perform NHEJ. DSBs are recognised by the Ku heterodimer, which forms a ring and binds DSB ends with high affinity and is an early recruit to DSBs (Figure 1C). In many eukaryotes, Ku interacts with accessory factors, such as DNA PK (DNA-dependent protein kinase), a PI3-kinase-like protein kinase (PIKK), XRCC4 and polymerases and nucleases that fill in or trim DNA ends before ligation. In all eukaryotes DNA ligase IV is required to fuse (ligate) the ends. In budding yeast, the Mre11 complex contributes to NHEJ, and recent evidence suggests that the Mre11 complex also contributes to NHEJ in mammals (D’Amours and Jackson, 2002; Deriano et al., 2009).

The other major route to repair DSBs is HR. HR has the advantage that homology between the DSBs site and homologous DNA sequences, mean that repair by HR can occur without error, with complete fidelity. Recently, there has been much progress in dissecting some of the early steps of HR (Gravel et al., 2008; Mimitou and Symington, 2008; Raynard et al., 2008; Zhu et al., 2008). Some early aspects of HR are indicated in Figure 1A, C, E and G. The Mre11 complex, comprising Mre11, Rad50 and Xrs2 in budding yeast, is recruited rapidly to DSB ends, at about the same time as the Ku complex. The Mre11 complex does two things at the lesion, it recruits the PIKK, Tel1 (orthologue of ATM), which signals cell-cycle arrest through its kinase activity. Tel1 as the name suggests contributes to telomere homeostasis. Second, and in combination with Sae2/CTIP, the Mre11 complex is important for initiating resection to generate ssDNA, an important intermediate in HR. Resection further from the break appears to be due to several complementary nuclease and helicase activities, including Dna2, Exo1 and Sgs1. Dna2 and Exo1 possess nuclease activity and Dna2 and Sgs1 helicase activity. ssDNA generated at DSBs is bound by the hetero-trimeric ssDNA-binding complex, replication protein A (RPA). RPA is important for regulating recruitment of HR proteins such as Rad52 and Rad51, and checkpoint proteins. During HR repair, checkpoint pathways recognise that repair is incomplete and signal cell-cycle arrest. Several checkpoint proteins bind close to DSBs. The Mre11/Tel1 complex contributes to a weak checkpoint pathway, termed the TM pathway (Usui et al., 2001). A complementary and more potent checkpoint pathway depends on a different PIKK, Mec1 (ATR), which binds with its partner Ddc2 to RPA-coated ssDNA. Full checkpoint kinase activation and cell-cycle arrest after DSBs also depend on the loading of the 911 complex, comprising Rad17, Mec3 and Ddc1 in budding yeast, which is loaded onto to DNA by the Rad24/RFC complex, comprising Rad24 and the four small Replication Factor C subunits (Figure 1G) (Majka et al., 2006). Rad9, another checkpoint protein, binds in the vicinity of DSBs, in part, through its interaction with chromatin

Table 1 DNA Damage response proteins and telomeres

| Budding yeast | Human cells | DSB arrival | Telomere length | Biochemical function |
|---------------|-------------|-------------|-----------------|---------------------|
| Rad50         | Rad50       | Early       | Short           | Mre11, Rad50 and Xrs2 complex functions in meiotic recombination, checkpoint signalling |
| Mre11         | Mre11       | Early       | Short           | PIKK recruited by Mre11 complex to DSBs |
| Xrs2          | Nbs1        | Early       | Short           | Nuclease recruited to DSBs |
| Tel1          | ATM         | Early       | Short           | Ku70/Ku86 heterodimer, which functions in NHEJ |
| Sae2          | CTIP        | Normal      | Normal          | DNA ligase for NHEJ |
| Yku70         | Yku70       | Early       | Short           | Yku70/Ku86 heterodimer, which functions in NHEJ |
| Yku80         | Yku86       | Early       | Short           | DNA ligase for NHEJ |
| Lig4          | Lig4        | Late        | Normal          | Ligase, a PIKK and Ddc2, recruited to RPA-coated ssDNA |
| Mec1          | ATR         | Late        | Normal          | Downstream checkpoint kinase |
| Ddc2          | ATRIP       | Late        | Normal          | Downstream checkpoint kinase |
| Chk1          | Chk1        | Normal      | Normal          | 5’-3’ exonuclease, DSB and mismatch repair |
| Rad53         | Chk2        | Normal      | Normal          | Helicase |
| Exo1          | Exo1        | Late        | Normal          | Helicase/nuclease required for DNA replication |
| Sgs1          | BLM/WRN     | Late        | Normal          | RecA orthologue, HR strand exchange |
| Dna2          | Dna2        | Late        | Normal          | Required with Rad51 during HR |
| Rad51         | RAD51       | Late        | Normal          | Checkpoint mediator, binds near DSBs |
| Rad52         | RAD52       | Late        | Normal          | Checkpoint, components of 911 complex loaded at DSBs |
| Rad9          | 53BP1       | Late        | Normal          | Checkpoint, Replication Factor C type subunit, works with small RFC subunits to load 911 complex |
| Rad17         | Rad1        | Late        | Normal          | Checkpoint, Replication Factor C type subunit, works with small RFC subunits to load 911 complex |
The DDR, similar to many biological defence mechanisms, is potentially harmful. For example, DNA repair enzymes often transiently amplify DNA damage during the process of DNA repair, for example, during resection of DSBs undergoing HR repair. Similarly, if the DDR responded to telomeres as it does to DSBs elsewhere in the genome, chromosome ends might be ‘repaired’ by NHEJ leading to chromosome fusions and/or activate checkpoint pathways, leading to perpetual cell-cycle arrest. For these reasons it is critical that functional telomeres do not stimulate the most powerful DDRs.

It is possible to make a crude calculation of the comparative checkpoint stimulating activities of telomeres versus DSBs. A single un-repaired DSB in the budding yeast genome causes cell-cycle arrest before entry into anaphase for many generation times (Sandell and Zakian, 1993; Michelson et al., 2005). Budding yeast has 16 chromosomes and if a DSB were made in G1 and replicated without being repaired, this could lead to a maximum of four internal ends in G2 but the same yeast cell would contain 64 inert telomeric DNA ends, which do not induce arrest. Therefore, telomeres in yeast must be at least 16 times less potent than DSB-induced ends in activating cell-cycle arrest. Furthermore, if native telomeres induced even a 1% cell-cycle delay such growth-inhibited cells would be out competed by cells that better hid chromosome ends from the DDR. An induced DSB induces arrest for several generation times (Michelson et al., 2005), which can be conservatively calculated as a four-fold or 400% cell-cycle delay. Therefore, it seems reasonable to estimate that a budding yeast DSB is 64/4 × 400 or 6400-fold more potent stimulator of checkpoint pathways than a telomere. A conservative estimate is therefore that a budding yeast telomere is at least a 1000-fold less potent than an internal chromosome end at inducing cell-cycle arrest (Figure 2).

**Harnessing DNA damage responses for telomere maintenance**

One way to ensure that telomeres do not activate DDRs would be to exclude DDR proteins from telomeres. Powerful biological damage defence responses are sometimes excluded from specific locations as a mechanism of attenuating responses that might do more harm than good. For example, the potentially harmful effects of the immune response are limited in organs such as the eye and brain (Ferguson et al., 2002; Streilein, 2003; Caspi, 2006). Although DDRs are clearly attenuated at telomeres, it is evident that rather than being excluded from telomeres many DDR proteins bind telomeres and indeed have critical functions in telomere maintenance and physiology, as well as in cellular responses to telomere uncapping.

Why do DDR proteins bind and have such important functions at telomeres rather than being excluded from the ends of chromosomes? Perhaps the major reason is simply that it would be too difficult to exclude DDR proteins from telomeres given the clear requirement that DDR proteins have methylated histone, H3K79, and phosphorylated H2A(X), and is also critical for cell-cycle arrest in response to DSBs.

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high-affinity for DNA damage elsewhere in the genome. In addition, passage of the replication fork, which is associated with many DDR proteins, through telomeres would make it difficult to exclude DDR proteins from telomeric ends.

In principal, telomere-capping proteins and DDR proteins have opposing goals when interacting with chromosome ends. The goal of telomere-capping proteins is to stably maintain the DNA end at telomeres, whereas the goal of DDR proteins is to 'repair' these ends. However, the evidence suggests that telomere-capping proteins and DDR proteins have evolved to work together, interdependently, to maintain telomere and genetic stability. Thus, one view is that telomeres have evolved to harness the power of the DDR pathways at chromosome ends. If so, then perhaps telomeres, similar to a skilled judo practitioner, use their opponent's weight, strength and momentum for their own benefit or, as suggested by the title of this review, the most powerful, tiger-like aspects of the DDR are tamed at telomeres.

NHEJ, HR and checkpoint proteins all play roles in physiological telomere maintenance. DDRs occur in the context of chromatin, and vary through the cell cycle, as does the function of telomere-capping proteins. However, for simplicity the effects of the cell cycle and chromatin on DDRs at the telomeres will only be superficially discussed in this review. A number of complementary reviews on the interplay between DDRs and telomere function have been published recently and are excellent sources for alternative and more detailed views on these and other relevant areas of telomere biology (Verdun and Karlseder, 2007; Longhese, 2008; Rog and Cooper, 2008; Sabourin and Zakian, 2008).

Diversity in telomere structures

To understand the roles of DDR proteins at telomeres it is necessary to understand telomere structure. Many eukaryotic telomeres are similar in structure, including human and yeast telomeres (Blackburn et al, 2006). However, some radically different approaches to telomere capping are used by organisms like Drosophila, which does not use telomerase (Louis and Vershinin, 2005). In human and yeast cells, telomeric DNA is composed of G-rich repetitive sequences with the G-rich strand ending in a 3' DNA is composed of G-rich repetitive sequences with the G-rich strand ending in a 3' strand overhang (Figure 3A). The chromosomal 'end replication problem', which conventional DNA replication enzymes cannot replicate the end of linear DNA molecules, is solved in yeast and human cells using telomerase, a reverse transcriptase-like enzyme, which synthesises G-rich DNA without the need for an DNA template (Greider and Blackburn, 1985). The 3' overhang structure at telomeres provides not only a suitable substrate for telomerase (Lingner and Cech, 1996) but also resembles one half of a resected DSB, an intermediate in HR repair (Figure 3B). In many eukaryotes, including human cells, the 3' overhang can invade the double stranded DNA, to form a t loop, which is similar to the D loop, an intermediate in HR (Griffith et al, 1999)(Figure 3C). The t loop structure probably contributes to protecting the end of the chromsome from the DDR. Other structures, such as G quartets, which form in G-rich DNA, are likely to form at telomeres and may also contribute to telomere function.

Recent experiments show that 3' overhangs are not found in all organisms that use telomerase to maintain telomere length. Caenorhabditis elegans, which expresses telomerase, contains 5' and 3' overhangs at telomeres (Figure 3D) (Raices et al, 2008). Furthermore, the fruit fly Drosophila does not express telomerase and instead large transposon element arrays are found at its chromosome ends (Figure 3E) (George et al, 2006). It is not yet established whether single strand overhangs or t loops are found at telomeres in Drosophila but it seems that there needs be no special DNA sequence at Drosophila telomeres and that therefore the telomeres are epigenetic in nature (Rong, 2008). Overall, when the spectrum of functional telomeric DNA structures is considered, it is perhaps to be expected that the role of DDR proteins at telomeres will be numerous and diverse.

Gain and loss of telomeres

Chromosome and telomere structure are very stable over evolutionary time periods. For example, the chromosomal structure of apes and humans is very similar, with evidence for just a single chromosome fusion event occurring during evolution (Hartl and Jones, 2009). However, chromosome stability is most likely a product of the stabilising influence of meiosis and sexual cycles, which act to limit chromosomal changes, rather than because of the innate stability of chromosome and/or telomere structures. In some organisms, such as Tetrahymena and in the largely non-sexual yeast Candida, in tumours and in the laboratory, chromosomal structure and hence telomere position can be remarkably
flexible (Maser and DePinho, 2002; Maringele and Lydall, 2004b; Blackburn et al, 2006; Murnane, 2006; Titen and Golic, 2008; Polakova et al, 2009). Indeed, the enzyme telomerase was first purified from Tetrahymena, a protozoan organism that as part of its developmental programme divides its large chromosomes into thousands of smaller segments each of which needs telomeres to be added de novo (Blackburn et al, 2006). Therefore, it is clear that eukaryotic cells can readily gain or lose telomeres.

Most eukaryotic genomes are linear and most bacterial genomes circular but this is not universally the case, for example the bacteria that causes Lyme disease, Borrelia contains linear chromosomes (Tourand et al, 2007). Furthermore, in the laboratory, linear chromosomes can become circular and vice versa. For example, when fission yeast Schizosaccharomyces pombe, which contains three linear chromosomes, is cultured in the absence of telomere maintenance mechanisms, such as the telomere-capping protein, Pot1, chromosomes can become circularised (Baumann and Cech, 2001). In the opposite direction, the bacterium Escherichia coli, which contains a single circular chromosome, can be manipulated to grow with a linear chromosome by the insertion of N15 phage telomeres (Cui et al, 2007). Thus, it seems that in principal both bacteria and eukaryotes could live with either linear or circular chromosomes, and that all cell types are very flexible with respect to chromosomal structure.

Telomere length homeostasis

In all organisms telomeres need to be resistant to DNA repair activities, such as nucleases and ligases that are active at DSBs. In addition, in dividing cells, the end replication problem needs to be solved and telomeres should not stimulate checkpoint pathways. Many organisms, including human, yeast and Tetrahymena use telomerase to solve the end replication problem (Blackburn et al, 2006). Telomerase adds telomeric DNA to chromosome ends without the need of a chromosomal DNA template, and therefore telomerase activity needs to be properly regulated to ensure that telomeres do not extend indefinitely because, if for no other reason, generating lots of unnecessary telomeric DNA this would be extremely wasteful. Telomerase does not act on every telomere in every cell cycle, rather telomerase is activated stochastically with short, rather than long, telomeres being the most likely substrates for telomere extension (Teixeira et al, 2004; Chang et al, 2007). A balance between telomerase inhibitory activities, regulated in part by telomeric length, and telomerase recruiting/activating activities regulate telomere length (Bianchi and Shore, 2008). DNA repair activities also contribute to telomere length homeostasis, for example telomere rapid deletion can reduce the size of very long telomeres (Li and Lustig, 1996; Bucholc et al, 2001; Pickett et al, 2009). Therefore, telomere length measurement is a simple readout of all the competing activities that contribute to telomere maintenance. Mutant cells with a phenotype of short telomeres could, for example, be less efficient at recruiting telomerase to short telomeres or less efficient at inhibiting the DNA repair activities that act to reduce telomere length. Mutant cells with a phenotype of unusually long telomeres might promiscuously engage telomerase activity or fail to engage telomere rapid deletion pathways.

How telomeres inhibit DNA damage responses

When telomeres fail and become uncapped telomeric DNA ends can induce all three major consequences of the DDR at DSBs—HR, NHEJ and checkpoint activation. The mechanisms by which functional telomeres inhibit downstream DDRs are unclear but most likely depend on telomeric DNA sequences, the proteins localised at or near telomeres, location (e.g. nuclear periphery) and structures of telomeric DNA. Elegant experiments in yeast clearly show that a DSB placed adjacent to a telomere is a much less potent inducer of cell-cycle arrest than a DSB placed internally in the chromosome (Michelson et al, 2005).

In mammalian cells a stable complex of six proteins, TRF2, TRF1, TIN2, Rap1, TPP1 and POT1, collectively termed Shelterin, binds telomeric DNA (de Lange, 2005). As the name suggests, Shelterin protects the ends of chromosomes from being recognised as DSBs, but in addition components of Shelterin both inhibit and activate telomerase activity (de Lange, 2005; Wang et al, 2007). Analogous proteins bind telomeric DNA in all organisms, these include Taz1 and Pot1 in fission yeast (Cooper et al, 1997; Baumann and Cech, 2001) and Rap1 and Cdc13 in budding yeast. In Drosophila, HOAP and HP1 do not bind specific telomeric sequences, but bind the ends of chromosomes to mark them epigenetically as ‘telomeres’ (Cenci, 2009). In the absence of many of these proteins, NHEJ, HR and checkpoint-dependent cell-cycle arrest can be induced.

Cdc13, Stn1, Ten1 are three essential budding yeast proteins, which bind telomeric ssDNA and behave like a telomere-specific RPA complex (Gao et al, 2007)(Figure 1). Cdc13 has at least two roles at telomeres—recruiting telomerase to chromosome ends and protecting chromosome ends from numerous DDR activities (Nugent et al, 1996; Jia et al, 2004; Zubko et al, 2004). One way that Cdc13, Stn1 and Ten1 protect against DDR pathways is that they outcompete the RPA complex for binding to telomeric ssDNA (Figure 1). Presumably, the lower levels of RPA near telomeres reduce the recruitment of DNA repair proteins such as Rad52 and Rad51 and also reduce the likelihood that RPA-bound ssDNA recruits and activates the Mecl/Ddc2-dependent checkpoint kinase cascade. Interestingly, in certain circumstances when aspects of the DDR are disabled or telomeres are being maintained by ALT-like mechanisms, cells can grow in the absence of these normally essential telomere-specific ssDNA-binding proteins (Larrivee and Wellinger, 2006; Petreaca et al, 2006; Zubko and Lydall, 2006).

In budding yeast, the essential protein, Rap1, binds telomeric dsDNA, as well as other genomic locations, for example at mating type loci (Shore and Nasmyth, 1987). Rap1 in turn interacts with two other non-essential proteins, Rif1 and Rif2, and these proteins seem to inhibit telomerase activity because rif1Δ and rif2Δ mutants contain very long telomeres (Wotton and Shore, 1997). Part of the mechanism by which Rif1 and Rif2 inhibit telomere lengthening is by inhibiting the Tel1 protein from binding telomeres (Hirano et al, 2009). Rap1 binding at telomeres inhibits NHEJ (Pardo and
In budding yeast, systematic genome-wide screens have identified approximately 300 gene products that affect telomere length homeostasis, showing that telomere length control is complex and regulated by numerous processes and pathways (Askree et al., 2004; Gatbonton et al., 2006; Shachar et al., 2008). Interestingly, among the many proteins that affect telomere lengths are two critically important DDR protein complexes, the Ku complex and the Mre11 complex, which are discussed below in more detail.

**Mre11 complexes and telomere homeostasis**

In budding yeast, the Mre11/Rad50/Xrs2 complex (Mre11, Rad50 and Nbs1 in mammals) is recruited early to DSBs and is involved in HR, NHEJ and checkpoint activation (D’Amours and Jackson, 2002; Lisby and Jackson, 2002; Askree et al., 2004; Sabourin et al., 2004). The Mre11 complex interacts with many other DDR proteins to contribute to these three different responses to DSBs. In all organisms examined, the Mre11 complex has roles in telomere maintenance. For example, loss of Mre11 results in a short telomere phenotype in budding yeast and human cells (Ritchie and Petes, 2000; Ranganathan et al., 2001) and high levels of telomere fusions in Drosophila (Bi et al., 2004; Ciapponi et al., 2004).

To activate checkpoint pathways, the Mre11 complex component, Xrs2 (Nbs1) protein, interacts through its C terminus with the PIKK, Tel1 (ATM) (Nakada et al., 2003). Tel1 and the Mre11 complex seem to act in a single pathway for telomere length maintenance in budding yeast because single and double mutants have similarly short telomere lengths (Ritchie and Petes, 2000). However, in other contexts, for example DSB repair and meiosis, the Mre11 complex functions independently of Tel1 (Ritchie and Petes, 2000; Carballo et al., 2008). Sae2, which works with the Mre11 complex to process meiotic DSBs and to regulate resection at meiotic and mitotic DSBs, seems to have little role at telomeres because sae2Δ mutants show, in contrast to mre11Δ and tell1Δ mutants, a minor telomere length phenotype in yeast (Kim et al., 2008).

There are at least two ways in which the Mre11 complex could contribute to maintain telomere length homeostasis. The Mre11 complex could increase telomerase activity at chromosome ends, contribute to telomere capping or both. There is evidence for both. In budding yeast, genetic experiments show that Mre11 and telomerase work in the same pathway for telomerase activation at telomeres (Nugent et al., 1998). Interestingly, in Drosophila, which caps telomeres in the absence of any telomerase activity, loss of Mre11 or ATM leads to a failure of telomere capping and telomere fusion (Bi et al., 2004; Ciapponi et al., 2004). These observations in Drosophila clearly show that Mre11 and ATM have roles in telomere capping that are completely independent of telomerase activity. In budding yeast, there is also evidence for the Mre11 complex having a role capping telomeres in cdc13-1 mutants, defective in an essential telomere-capping protein (Foster et al., 2006). In Drosophila, the Mre11 complex and ATM contribute to capping by providing a substrate for binding of the heterochromatin factors HP1 and HOAP binding (Cenci, 2009).

In yeast and mammalian cells, the Mre11 complex is involved in making the 3' overhang at the end of telomeres (Larriée et al., 2004; Chai et al., 2006). Mre11-independent mechanisms to generate 3' overhangs also exist and different mechanisms for 3' overhang generation most likely occur on leading and lagging strand telomeres (Lydall, 2003). The 3' overhang may help chromosome capping in at least three ways: first a 3' overhang, rather than a blunt end, is a necessary substrate for telomerase (Lingner and Cech, 1996); second, as mentioned earlier, essential telomere-capping proteins, such as Cdc13/Stn1/Ten1, in budding yeast bind to the ssDNA telomeric overhang (Gao et al., 2007); third, a 3' overhang is necessary to form a t loop. In yeast, Mre11 is necessary for recruiting Tel1 specifically to short telomeres and for telomere extension at these short telomeres (Hector et al., 2007; Sabourin et al., 2007). The Tel1 pathway inhibits NHEJ at telomeres (Chan and Blackburn, 2003). In mammals, the Mre11 complex binds mammalian telomeres every cell cycle and contributes to telomere length control (Verdun et al., 2005; Verdun and Karlseder, 2006; Wu et al., 2007). In summary, the Mre11 complex is likely to have numerous roles at telomeres and seems to have evolved to have different roles in different organisms.

**Tel1/ATM and Mec1/ATR redundancy at telomeres**

In budding yeast, fission yeast and Drosophila there is evidence that the Tel1 (ATM) and Mec1/(Rad3, ATR) PIKKs have redundant roles in telomere maintenance. In vitro the two PIKKs are capable of phosphorylating a very similar range of substrates but the kinases have different roles in vivo (Baldó et al., 2008). In budding yeast, mec1 tel1 double mutants are incapable of maintaining telomeres and, similar to telomerase deficient cells, use recombination-dependent mechanisms of telomere elongation (Ritchie et al., 1999). Similarly, in fission yeast, rad3 tel1 double mutants are incapable of maintaining telomeres and generate survivors by creating circular chromosomes (Naito et al., 1998). In Drosophila, ATM and ATR function together to help recruit the heterochromatic factors HOAP and HP1 to telomeres (Okemus et al., 2006; Rong, 2008).

Interestingly, in budding yeast, mec1 mutants have a very minor effect on telomere length, whereas tel1 mutants have profound effects on telomere length (Longhese et al., 2000). This is in contrast to the relative importance of the two proteins in signalling checkpoint-dependent cell-cycle arrest, in which Mec1 has a much more important function (Usui et al., 2001; Mantiero et al., 2007). The telomere-length phenotype of the double mutants could perhaps best be explained if Mre11/Tel1 complex bind and become activated at blunt telomeres or telomeres with short telomeric ssDNA regions, when Cdc13, Stn1 and Ten1 bind the G-rich ssDNA (Figure 1D, F, H). The preferential binding of Cdc13, Stn1 and Ten1 to G-rich ssDNA at telomeres would help inhibit Mec1/Ddc2 binding, because Mec1/Ddc2 has high affinity for RPA-coated ssDNA. However, if telomeres shorten further, because for example the Mre11 complex is non-functional, there is a higher probability that RPA binds ssDNA near telomeres (intriguingly, Rpa2 and Rpa3 have higher affinity for telomeric ssDNA than random DNA (Gao et al., 2007)). At such telomeres, Mec1/Ddc2 will bind near telomeres and
can, assuming resection is not excessive, contribute as a back up mechanism for telomere protection that only engages if Tel1 fails to recruit telomerase activity to short telomeres (dashed arrow between Figure 1G and H). If the two PIKKs, Mec1/Rad3 and Tel1/ATM are missing, chromosomes circu- larise (fission yeast), or engage recombination-dependent, telomerase-independent, pathways of telomere maintenance (budding yeast) (McEachern and Haber, 2006).

The KU dimer and telomere homeostasis

Ku is an abundant heterodimeric ring protein, comprising Ku70 and Ku80, with high affinity for DSBs. Ku, similar to the Mre11 complex discussed above, is an early recruit to DSBs (Frank-Vaillant and Marcand, 2002; Kim et al, 2005; Wu et al, 2008) (Figure 1). Ku contributes to NHEJ but seems to inhibit HR, in part, by inhibiting resection of DSBs to create the 3’ ssDNA that is an intermediate in HR (Lee et al, 1998; Barlow et al, 2008). Ku has a role in telomere maintenance in budding yeast, mice, human cells, Drosophila and indeed all organisms examined (Myung et al, 2004; Fisher and Zakian, 2005; Celli et al, 2006). Ligase IV, which ligates DNA molecules during NHEJ, does not seem to have a role at telomeres because unlike yku70A and mre11A mutants, lig4A mutants show no telomere length phenotype (Teo and Jackson, 1997). This shows that it is not NHEJ per se that contributes to telomere length homeostasis.

Interestingly, the effect of Ku on telomere length in different organisms can vary dramatically. In budding and fission yeast, human cells and trypanosomes, the effect of deleting Ku is reduction in telomere length, often dramatically. Although in Arabidopsis and Drosophila, the effect of deleting Ku is to lengthen telomeres (reviewed in (Fisher and Zakian, 2005)). Therefore, it can be inferred that Ku performs different functions at telomeres in different organisms.

In budding yeast, Ku performs at least four functions at telomeres. (1) Ku binds telomerase TLC1 RNA and is important for recruiting telomerase to telomeres (Stellwagen et al, 2003). (2) Ku protects telomeres from nuclease activities and this helps stop telomeres undergoing recombination or stimulating checkpoint-dependent cell-cycle arrest (Fellerhoff et al, 2000; Maringele and Lydall, 2002). (3) Ku is required for the silencing of telomeres, helping recruit the Sir2/3/4 complex (Boulton and Jackson, 1998). (4) Ku is required to localise some telomeres to the nuclear periphery (Laroche et al, 1998).

Given the numerous roles for Ku at budding yeast telomeres it is not surprising that not all of the roles of Ku are conserved and that the effects of deleting Ku in different organisms vary. For example, if in plants and Drosophila, Ku ensures that telomerase is inhibited or transposons move less readily to chromosome ends, this would explain telomere the length increases observed in these organisms when Ku is inactivated (Riha and Shippen, 2003; Melnikova et al, 2005).

Interplay between Ku and Mre11 complexes

The Ku and MRX complexes, which are early and independent recruits to DSBs (Figure 1), have redundant roles in telomere capping in budding yeast. When the Ku complex and the MRX complex are simultaneously deleted from budding yeast, cells no longer maintain telomere length using telomerase and instead use HR-dependent mechanisms to maintain telomere length (analogous to meclA tel1A mutants above) (DuBois et al, 2002; Maringele and Lydall, 2004a). Furthermore, both Ku and MRX seem to have independent roles to the essential Cdc13, Snt1 and Ten1 complex in telomere capping because they contribute to the robust growth of temperature-sensitive cdc13-1 mutants at permissive temperatures (Polotnianka et al, 1998; Addinall et al, 2008).

HR proteins and telomere homeostasis

In budding yeast, proteins recruited late to DSBs, such as Rad51 and Rad52, tend to have a lesser or non-detectable role in telomere length homeostasis. Two genome-wide screens in budding yeast showed that about 300 to 4500 non-esssential budding yeast genes have important functions in telomere length maintenance. The Ku and MRX complexes were identified as important for telomere length homeostasis but interestingly very few other DNA repair genes involved at DSBs were identified by these systematic screens (Askree et al, 2004; Gabtonet et al, 2006; Shachar et al, 2008). However, in mammals there is a role for HR proteins in telomere homeostasis (Tarsounas et al, 2004).

Checkpoint proteins and telomere homeostasis

In budding yeast, many of the DNA damage checkpoint proteins including the 911 complex (Rad17, Mec3 and Ddc1), the clamp loader (Rad24, RFC) and Rad9 have comparatively small effects on telomere length in comparison with Ku or Mre11 proteins and are late recruits to DSBs (Longhese et al, 2000) (see Figure 1). However, all of these checkpoint proteins have critical roles in responding to uncapped telomeres as discussed below (see cdc13-1 below). However, in C. elegans, genes encoding components of the 911 complex and its clamp loader have critical roles maintaining telomere length (Boerckel et al, 2007). In C elegans, telomere loss rates in telomerase mutants, 911 mutants and telomerase 911 double mutants, suggest that telomerase and the 911 complex belong in the same pathway of maintaining telomere length (Boerckel et al, 2007). Indeed, MRT2, encoding the Rad1 component of the 911 complex, was first identified on its telomere loss phenotype (Ahmed and Hodgkin, 2000). In mouse and human cells, the 911 complex also has critical roles in telomere maintenance (Francia et al, 2006).

Telomerase limitation

Understanding DNA repair and checkpoint activation at uncapped telomeres in human cells is critically important for understanding carcinogenesis and ageing. In most human somatic cells insufficient telomerase is expressed to counteract the end replication problem. Consequently, in these cells telomeres shorten with each cell division until telomeres reach a critically short length, are no longer functional and are perceived like DSBs (Takai et al, 2003; d’Adda di Fagagna et al, 2003). This leads to p53- and p21-dependent cell-cycle arrest in G1 (Artandi and Attardi, 2005). This checkpoint-dependent cell-cycle arrest induced by short telomeres is thought to act as a tumour suppressor mechanism because it acts to limit indefinite cell division. In the absence of
proficient checkpoint controls (e.g. in p53 defective cells) cells continue to divide with ever shorter telomeres and high levels of genetic instability are induced, presumably as chromosome ends become less and less ‘telomeric’ and more DNA repair events, such as NHEJ, lead to chromosome fusions and subsequent breakage fusion bridge cycles (Artandi and Attardi, 2005). Short telomeres seem to be the substrates for DNA repair events that lead to telomere fusion events (Capper et al., 2007). After a period of ‘crisis’ characterised by high levels of genetic instability, comparative genetic stability is often restored in cancer cells when telomerase is reactivated or ALT mechanisms are engaged (Stewart and Weinberg, 2006; Cesare and Reddel, 2008).

Experiments in yeast may shed light on the variety of plausible ALT mechanisms because yeast can be genetically manipulated to grow in the absence of telomerase. In such yeast cells telomeres shorten and cells enter a period of crisis, when growth is slow and checkpoint pathways are activated by the dysfunctional telomeres (Enomoto et al., 2002; Uppma and Greider, 2003). However, after this period of slow growth, yeast cells often regain the ability to extend telomeres by using HR (RAD52)-dependent mechanisms (Lundblad, 2002; McEachern and Haber, 2006). Very recent experiments show that the requirement for HR in contributing to ALT can vary between different strain backgrounds (Grandin and Charbonneau, 2009; LeBel et al., 2009). Further experiments in budding yeast have also shown that when telomerase, HR and other aspects of the DDR are inactivated (rad52Δ, exo1Δ, mre11Δ, sgs1Δ), budding yeast cells are often able to divide without any discernable mechanisms for capping chromosome ends (Maringele and Lydall, 2004b, 2005; Lee et al., 2008). Such cells cannot divide indefinitely, losing DNA at chromosome ends because of the end replication problem and nuclear activities, and therefore to counteract loss of DNA from chromosome ends, large palindromes often form at chromosome ends.

In budding yeast, Exo1, which is a major nuclease involved in generating ssDNA at DSBs and uncapped telomeres, also accelerates the rate at which telomerase deficient budding yeast cells enter telomere-initiated senescence and also contributes to the recombination-dependent recovery from tcl1A-initiated senescence (Bertuch and Lundblad, 2004; Mariingele and Lydall, 2004a). Interestingly, this role of Exo1 is conserved in mammals because in telomerase knockout mice, Exo1 contributes to ssDNA generation and early death of telomerase deficient mice (Schaetzel et al., 2007).

**Experimental uncapping of telomeres**

Even in yeast, when telomerase genes can be entirely deleted, telomere attrition caused by insufficient telomerase expression takes a long time to induce somewhat asynchronous and therefore difficult to study DDRs (Enomoto et al., 2002; Nautiyal et al., 2002; Uppma and Greider, 2003). Therefore, it is largely through artificial but experimentally tractable manipulations that much has been learnt about the role of DDR proteins at uncapped telomeres. Telomere uncapping has been induced by gene deletion, siRNA and use of dominant negative or temperature-sensitive alleles. The results of a number of different experiments, in a number of different model systems, show that there is no universal response to telomere-capping defects and uncapped telomeres can be subject to NHEJ, HR and induce different types of checkpoint pathways.

**cdc13-1**

The budding yeast temperature-sensitive cdc13-1 mutation affects the essential Cdc13 protein that binds telomeric ssDNA in budding yeast (Hartwell et al., 1973)(Figure 1F). Under restrictive conditions cdc13-1 mutants rapidly and efficiently activate checkpoint-dependent cell-cycle arrest before anaphase, with greater than 90% of cells arrested at this point within 2 h. While arrested, cdc13-1 mutants accumulate large regions of ssDNA at telomeres but not at other locations in the genome (Garvik et al., 1995). Cdc13-1 seems to be completely non-functional at 36 °C (Garvik et al., 1995) but passage of the replication fork through the telomere is necessary for ssDNA to be generated at telomeres (Vodenicharov and Wellinger, 2006). The ssDNA at telomeres in cdc13-1 mutants resembles a DSB-end, initiating 5‘-3‘ resection to generate a 3’ overhang that is an intermediate of HR, and ssDNA can be detected up to 30 kb from chromosome ends (Booth et al., 2001; Zubko et al., 2004). All the DNA damage checkpoint proteins that play roles in inducing cell-cycle arrest after DSBs also play a role in inducing cell-cycle arrest after telomere uncapping. In fact, cdc13-1 was the tool first used to identify or show that Mec1, Mec3, Rad17, Rad24 and Rad53 checkpoint proteins played a role in checkpoint pathways (Weinert and Hartwell, 1993; Weinert et al., 1994). In addition to its role in telomere capping, Cdc13 has a separate function of recruiting telomerase to telomeres. Cells containing the cdc13-2 allele, which is defective in interacting with the Est1 subunit of telomerase, enter telomere-initiated senescence at the same rate (i.e. over many generation times) as cells deleted of telomerase activity (Nugent et al., 1996).

Although telomeres of cdc13-1 mutants resemble DSBs undergoing HR there are some differences. A number of recent papers have shown that the 5‘-3‘ dsDNA exonuclease, Exo1, the Mre11 complex and Sae2, the Sgs1 helicase and the Dna2 helicase/nuclease all contribute to DSB resection (Gravel et al., 2008; Mimiotou and Symington, 2008; Raynard et al., 2008; Zhu et al., 2008). At DSBs, it seems that the Mre11/ Sae2 complex is important for initiating resection, and that Exo1 and Sgs1/Dna2 are important for resection further from the site of the initial break. Interestingly, at uncapped cdc13-1 telomeres the Mre11 complex is not required to initiate resection and instead the Mre11 complex contributes to capping (Foster et al., 2006). The difference between cdc13-1 telomeres and DSBs is probably for a combination of reasons, first some ssDNA must already exist at telomeres for Cdc13-1 to bind telomeric DNA at permissive temperatures (Figure 1F), and second the Mre11 complex contributes to telomere capping through its interaction with Tel1. The Mre11 complex is not involved in signalling cell-cycle arrest of cdc13-1 mutants (Foster et al., 2006).

Just as found at DSBs, Exo1 is not the only nuclease generating ssDNA at cdc13-1 telomeres (Zubko et al., 2004). The roles of Sgs1 and Dna2, recently reported to play roles in resection at DSBs, at cdc13-1 telomeres have not so far been reported. However, interestingly there is a Rad24-, Rad17-, Mec3- and Ddc1 (checkpoint sliding clamp and checkpoint
clamp loader)-dependent nuclease activity (ExoX) that is active at cdc13-1 telomeres (Lydall and Weinert, 1995; Booth et al, 2001; Zubko et al, 2004). There are at least two reports that a similar activity is functional at DSBs (Aylon and Kupiec, 2003; Dubrana et al, 2007). It will be interesting to see how or if the Rad24-dependent nuclease activity relates to the Sgs1-, Dna2- or Mre11-dependent activities.

Resection at DSBs and uncapped telomeres needs to be properly regulated. Studies in cdc13-1 mutants have shown that Rad9-dependent and checkpoint kinase-dependent mechanisms inhibit resection at uncapped telomeres (Lydall and Weinert, 1995; Jia et al, 2004; Lazzaro et al, 2008). It seems there are at least two routes to inhibit resection at cdc13-1 telomeres and that these mechanisms are also probably active at DSBs. First, Rad9 binds histone H3 methylated on the K79 residue close to uncapped telomeres. H3K79 methylation is Dot1-dependent and is found in about 90% of H3 molecules (van Leeuwen et al, 2002). Dot1 contributes to the Rad9-dependent inhibition of resection, as well as Rad9-dependent cell-cycle arrest (Lazzaro et al, 2008). Second, a Rad9-, Rad24-, Rad53- and Mec1-dependent kinase cascade phosphorylates Exo1 and inhibits its nuclease activity (Morin et al, 2008). However, other mechanisms affecting the mobility of telomeric DNA are suggested by the finding that a Rad9 orthologue, 53BP1, contributes to telomere mobility and NHEJ in mammalian cells with capping defects (Dimitrova et al, 2008). In addition, Rad9, which contains a BRC1 domain, and/or other proteins may interact with phosphorylated H2AX near DSBs and uncapped telomeres to inhibit resection.

Extensive studies of cdc13-1 mutant cells have generated much insight into cellular responses to telomere uncapping and DSBs. The cdc13-1 defect is useful in part because it is tunable, in the sense that by simply increasing temperature by small increments (e.g. 0.5°C), telomeres can become slightly less telomeric and slightly more DSB-like, and subtle effects of DDR pathways on growth of cells with telomere-capping defects can be distinguished (Addinall et al, 2008; Morin et al, 2008). Furthermore, cdc13-1-induced DNA damage is better tolerated than DNA damage induced elsewhere because growth of cdc13-1 mutants is improved by inactivation of checkpoint pathways, whereas growth of cells with less localised chromosomal damage, for example caused by defects in DNA-ligase (cdc9), is exacerbated by inactivation of the same checkpoint pathways (Weinert and Hartwell, 1993). Systematic genetic, transcriptomic and mathematical modelling of cdc13-1-induced responses suggest that this allele, first identified nearly 40 years ago, still has much to teach about the interplay between telomeres and the DDR (Proctor et al, 2007; Addinall et al, 2008; Greenall et al, 2008).

Lack of Ku

In budding yeast, loss of Ku causes a short telomere phenotype, as discussed above, but also a temperature-sensitive telomere-capping defect. At DSBs, Ku seems to inhibit resection and HR, whereas favouring the NHEJ pathway of repair (Frank-Vaillant and Marcand, 2002; Barlow et al, 2008). At high temperature, such as 37°C, yku70Δ mutants accumulate excessive levels of Exo1-dependent ssDNA in sub-telomeric regions and thus telomeres in Ku mutants look like intermediates in HR (Maringele and Lydall, 2002).

Interestingly, cell-cycle arrest of yku70Δ mutants at high temperature depends on only some of the checkpoint genes that respond to the cdc13-1 defect, such as Rad9, Chk1 and Mec1 but arrest is independent of Rad17, Rad24, Mec3, Ddc1 (the checkpoint sliding clamp and clamp loader) and Rad53. Therefore, although in most contexts, for example at DSBs and in cdc13-1 mutants, the 911 complex has a critical role in signalling cell-cycle arrest, its role is unnecessary for signalling cell-cycle arrest of Ku mutants with uncapped telomeres. Interestingly, there is also a role for spindle checkpoint genes in responding to the Ku defect, as there is in Drosophila mutants with telomere-capping defects, and S. pombe taz1Δ defective cells (see next section) (Maringele and Lydall, 2002; Miller and Cooper, 2003; Cenci, 2009).

Lack of Taz1

In S. pombe Taz1, an orthologue of human Trf1 and Trf2, binds the telomeric double stranded DNA. Interestingly, lack of Taz1 leads to uncontrolled telomere elongation, a cold-sensitive phenotype, and a failure of DNA replication through telomeric DNA (Ferreira et al, 2004). It seems that Dna2, rather than Exo1, is important for generating the high level of ssDNA observed in taz1Δ cells (Tomita et al, 2003, 2004). Interestingly, the RecQ helicase, Rqh1, analogous to Sgs1 in budding yeast, also contributes to the defects in taz1Δ cells, inducing telomere breakage (Rog et al, 2009).

Lack of Pot1

Pot1 genes encode ssDNA-binding proteins that bind the ssDNA at telomeres of S. pombe and mammalian cells (Lei et al, 2003). In many senses Pot1 is analogous to Cdc13 in Saccharomyces cerevisiae. Pot1 is essential for telomere function because in S. pombe loss of Pot1 leads to chromosome circularisation (Baumann and Cech, 2001). In mice there are two Pot1 genes, Pot1a and Pot1b. Loss of Pot1a leads to p53-dependent cell-cycle arrest, increased levels of 3' ssDNA overhang length, elevated levels of HR and chromosome instability (Wu et al, 2006). Loss of Pot1b also increases the amount of ssDNA found at mouse telomeres (Hockemeyer et al, 2006, 2008). Loss of Pot1 from chicken cells, also increases levels of ssDNA at telomeres and induces ATR and Chk1-dependent cell-cycle arrest (Churikov and Price, 2008). However, in human cells siRNA depletion of Pot1 resulted in a loss of the G-rich 3’ overhang at telomeres (Hockemeyer et al, 2005). It is clear that in all organisms Pot1 contributes to telomere capping (Denchi and de Lange, 2007). Interestingly, the Pot1 complex does more than simply cap telomeres because it increases telomerase processivity and activity (Wang et al, 2007).

Lack of TRF2

TRF2 is one component of the mammalian shelterin complex proteins and it directly binds telomeric DNA. Human fibroblasts expressing a dominant negative version of TRF2 (TH2ΔBAM) induce rapid cell-cycle arrest in G1, and telomeres look like DSBs because, for example, there is the accumulation of phosphorylated H2AX, Mre11 and ATM, and telomere fusions are detected (van Steensel et al, 1998; d’Adda di Fagagna et al, 2003). Mouse cells, in which TRF2 is deleted by Cre-dependent
between telomere repeats can also to unequal exchanges resulting in increases or decreases in the number of telomere repeats on individual chromosome ends. The 3′ overhang found at telomeres inhibits the NHEJ pathway of repair and makes it difficult to fuse telomeres (Figure 4B). The repetitive nature of telomeric DNA ensures that the 3′ ssDNA overhang cannot fold back on itself and base pair to generate a 3′ end that points internally towards the centromere (Figure 4C) instead the base paring ensures that a t loop pointing towards the telomere is formed (Figure 4C). A 3′ end pointing towards the centromere would be dangerous because it could initiate break-induced replication and perhaps lead to the formation of large palindromes at chromosome ends that have lost telomeric DNA repeats, as proposed in cells that have lost telomerase-dependent repeats found in most eukaryotic cells mean that should NHEJ occur between telomeres then a palindromic sequence will be formed. Palindromes, although more stable in eukaryotes than prokaryotes, can form cruciform structures and be susceptible to cleavage through resolvase activities, and therefore the fusion could be reversed. When human telomere fusions have been sequenced they invariably have lost all the telomeric DNA from at least one side of the fusion (Capper et al., 2007).

**Conclusions**

Telomeres and DDRs have evolved side-by-side. In all organisms, including those that maintain telomeres in the absence of telomerase, it seems that DDR proteins have critically important functions in telomere physiology as well as when telomeres fail. Just as different solutions to telomere capping have evolved in different organisms so have the roles of individual DDR proteins at telomeres in different organisms. Understanding the diverse roles of DDR proteins at telomeres of model organisms, as well as in human cells, will have important implications for the development and treatment of cancer and for understanding ageing.

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