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Mechanisms and regulation of DNA end resection

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DNA double-strand breaks (DSBs) are highly hazardous for genome integrity, because failure to repair these lesions can lead to genomic instability. DSBs can arise accidentally at unpredictable locations into the genome, but they are also normal intermediates in meiotic recombination. Moreover, the natural ends of linear chromosomes resemble DSBs. Although intrachromosomal DNA breaks are potent stimulators of the DNA damage response, the natural ends of linear chromosomes are packaged into protective structures called telomeres that suppress DNA repair/recombination activities. Although DSBs and telomeres are functionally different, they both undergo 5′–3′ nucleolytic degradation of DNA ends, a process known as resection. The resulting 3′-single-stranded DNA overhangs enable repair of DSBs by homologous recombination (HR), whereas they allow the action of telomerase at telomeres. The molecular activities required for DSB and telomere end resection are similar, indicating that the initial steps of HR and telomerase-mediated elongation are related. Resection of both DSBs and telomeres must be tightly regulated in time and space to ensure genome stability and cell survival.

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Introduction

DNA double-strand breaks (DSBs) can occur spontaneously during normal cell metabolism or can be induced by exposure to DNA-damaging agents, such as ionizing radiations (IR) and radiomimetic chemicals. They can also arise during DNA replication, such as when the DNA polymerase encounters a lesion in the template or a secondary DNA structure. DSBs pose a particularly dangerous threat to cell viability and genome integrity, because they can lead to mutagenic events when left unrepaired or inappropriately repaired. Though DSBs threaten genome stability, germ cells deliberately introduce them into their genome to initiate meiotic recombination. Moreover, eukaryotic cells contain natural DSBs that are represented by the ends of their linear chromosomes.

Cells have evolved different mechanisms to repair DSBs depending on the nature of the DSB and the cell cycle phase in which the damage is detected. Both accidental and meiosis-specific DSBs can be repaired by homologous recombination (HR), which involves the interaction between homologous DNA sequences. The primary function of HR in mitotic cells is to repair DSBs, whereas it ensures a correct segregation of the homologous chromosomes in meiosis by establishing physical connections between them (reviewed in San Filippo *et al*, 2008; Longhese *et al*, 2009). Furthermore, both accidental and meiosis-specific DSBs trigger activation of fine-tuned systems called DNA damage and recombination checkpoints, respectively, which regulate DNA repair/recombination pathways and coordinate progression through mitosis and meiosis with DNA repair capacity (reviewed in Harrison and Haber, 2006; Longhese *et al*, 2006; Putnam *et al*, 2009).

In contrast, the natural ends of linear chromosomes are protected from degradation, recombination, fusion and recognition by the checkpoint machinery (reviewed in Longhese, 2008; Lydall, 2009; Shore and Bianchi, 2009). This special feature depends on the organization of chromosomal ends into protective nucleoprotein complexes called telomeres. Failure to protect the natural chromosome ends leads to chromosomal rearrangements, general hallmarks for cancer cells in human beings, and cell death.

During the first step of HR, mitotic and meiotic DSBs undergo nucleolytic degradation of their 5′-ending strands. This process, known as resection, is a general feature of HR DSB repair during both mitosis and meiosis, because the resulting 3′-ended single-strand DNA (ssDNA) can invade a homologous template (White and Haber, 1990; Sun *et al*, 1991). Processing of the 5′ strand also occurs at telomeric ends, in which the resulting 3′-ended ssDNA is thought to allow telomerase action. Failure to execute and regulate ssDNA generation at both DSBs and telomeres threatens genome integrity and can contribute to human diseases. The mechanisms and regulation of DNA end processing are the focus of this review.

Resection of mitotic DSBs

One of the most important steps in DSB repair is deciding which specific repair pathway to use. One possible pathway is HR, which uses the genetic information stored in the sister chromatid or in the homologous chromosome to accurately restore lost genetic information at the break site (reviewed in San Filippo *et al*, 2008). On the other hand, non-homologous end joining (NHEJ) directly rejoins two chromosomal ends with no or minimal base pairing at the junction and can

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generate mutations at the end joining sites (reviewed in Daley *et al*, 2005). The commitment to a specific DNA repair pathway is tightly regulated and resection of the DSB ends represents an important regulatory step, as generation of 3′-ended ssDNA is needed for all HR pathways, while resected DNA decreases NHEJ efficiency.

Once a DSB occurs, the highly conserved Mre11, Rad50 and Xrs2 (MRX)/Mre11, Rad50 and Nbs1 (MRN) complex, composed of MRX subunits in budding yeast and MRN subunits in both fission yeast and mammals, is the first group of proteins recruited to DNA ends (Lisby *et al*, 2004). The Ku70/Ku80 heterodimer is also loaded onto DNA ends. If cells are in the G1 cell cycle phase, the presence of Ku prevents resection and, together with MRX, mediates recruitment of downstream NHEJ factors (Lee *et al*, 1998; Chen *et al*, 2001; Zhang *et al*, 2007; Clerici *et al*, 2008; Palmbos *et al*, 2008). DSB ends can then be religated by NHEJ, a process that requires the DNA ligase activity of the Dnl4-Lif1/XRCC4 heterodimer and the Nej1/XLF protein (reviewed in Daley *et al*, 2005).

If cells are in the S or G2 cell cycle phase when a DSB is detected, processing of the 5′ DSB ends generates 3′-ended ssDNA tails that inhibit NHEJ and target DSB repair to HR. It has been shown that the MRX complex functions together with the Sae2 protein in processing the DSB ends in a 5′–3′ direction. *Saccharomyces cerevisiae* mutants lacking Sae2 or any component of the MRX/MRN complex delay resection of an endonuclease-induced break by acting in the same epistasis group (Ivanov *et al*, 1994; Clerici *et al*, 2006). Furthermore, Sae2 and MRX are involved in mitotic DSB repair by single-strand annealing (Ivanov *et al*, 1996; Clerici *et al*, 2005) and they both have a unique function in processing hairpin-containing DNA structures (Lobachev *et al*, 2002; Yu *et al*, 2004; Rattray *et al*, 2005). Putative orthologues of *S. cerevisiae* Sae2 have been identified in other organisms such as *Schizosaccharomyces pombe* (Ctp1/Nip1), *Caenorhabditis elegans* (Com1/Sae2), *Arabidopsis thaliana* (Com1/Sae2) and *Homo sapiens* (CtIP). Studies in human and *S. pombe* cells have revealed that CtIP/Ctp1 has a critical function in promoting DNA end resection (Limbo *et al*, 2007; Sartori *et al*, 2007; Yun and Hiom, 2009), indicating that Sae2 involvement in DSB processing is conserved among eukaryotes.

Full understanding of how Sae2/CtIP and MRX/MRN function together to promote DSB end resection awaits further studies. The Mre11 subunit of the MRX complex has nuclease activities *in vitro*, including single-strand endonuclease and 3′–5′ double-strand exonuclease (Paull and Gellert, 1998; Williams *et al*, 2008). One possibility is that the Mre11 endonuclease activity initiates resection of the 5′ strand by catalysing the removal of an oligonucleotide to generate short 3′-ended ssDNA (Mimitou and Symington, 2008; Zhu *et al*, 2008) (Figure 1A). In turn, Sae2/CtIP may regulate MRX/MRN's nuclease activity, as human CtIP directly interacts with MRN and increases Mre11 nuclease activity *in vitro* (Sartori *et al*, 2007). Interestingly, budding yeast Sae2 itself exhibits ssDNA endonuclease activity (Lengsfeld *et al*, 2007), raising the possibility that Sae2 may act either as a regulator of Mre11 nuclease activity or as a nuclease. However, unlike in Mre11, there are no obvious nuclease motifs in Sae2 that could be mutated to assess whether Sae2 nuclease activity is important for DSB resection.

In the absence of either Mre11 or Sae2, the 5′–3′ double-strand-specific exonuclease Exo1 provides a compensatory activity to initiate end processing at endonuclease-induced breaks. In fact, *exo1*Δ *sae2*Δ and *exo1*Δ *mre11*Δ mutants show a synergistic decrease in DNA end resection and greater DNA damage sensitivity than each single mutant (Nakada *et al*, 2004; Clerici *et al*, 2006). Furthermore, overproduction of Exo1, but not of its nuclease-defective variant, partially suppresses the DSB repair and resection defects of *mrx* null mutant cells (Tsubouchi and Ogawa, 2000; Moreau *et al*, 2001; Lewis *et al*, 2002; Mantiero *et al*, 2007). However, residual resection still occurs in both *exo1*Δ *sae2*Δ and *exo1*Δ *mre11*Δ double mutants, suggesting further redundancy within the pathways.

In bacteria, the helicase RecQ acts together with the 5′–3′ exonuclease RecJ to resect DSB ends when RecBCD is absent (Amundsen and Smith, 2003). Simultaneous inactivation of *S. cerevisiae* Sgs1, the budding yeast RecQ orthologue, and Exo1 abolishes long-range DNA end resection of an endonuclease-induced break (Gravel *et al*, 2008; Mimitou and Symington, 2008; Zhu *et al*, 2008). Only some minimal processing, which depends on both Sae2 and Mre11, can be detected in *sgs1*Δ *exo1*Δ double mutants, suggesting that Sae2 and MRX initiate DSB processing that is then extended by Sgs1 and/or Exo1. Sgs1 helicase activity may unwind the double-stranded DNA to facilitate resection by a nuclease. The Sgs1-associated nuclease seems to be Dna2, a nuclease/helicase known to function in Okazaki fragment processing during DNA replication (Bae *et al*, 2001). Although Dna2 has both helicase and nuclease activities, only the nuclease activity is required for DSB resection (Zhu *et al*, 2008), consistent with the hypothesis that Sgs1 unwinds DNA ends and the Dna2 nuclease removes the 5′ strand. However, it remains possible that Sgs1 provides substrates also for Exo1, as it does in human beings (see below; Nimonkar *et al*, 2008). Helicase-nuclease coupling seems to be a general mechanism of the DSB resection machinery. Although the function of vertebrate Dna2 is unclear, the human counterparts of both Exo1 and Sgs1 are involved in DSB resection (Gravel *et al*, 2008; Nimonkar *et al*, 2008). As in yeast, the simultaneous downregulation of BLM, the human RecQ/Sgs1 orthologue, and Exo1 severely impairs ssDNA formation, suggesting that BLM is partially redundant with Exo1 (Gravel *et al*, 2008). However, BLM interacts with Exo1 and stimulates its activity (Nimonkar *et al*, 2008), suggesting that it can provide substrates for Exo1.

Altogether, the above observations led to the proposal that the MRX complex and Sae2 initiate together the resection of the 5′ strand possibly through an endonucleolytic cleavage. The resulting partially resected 5′ DNA end can be further processed by the action of either Exo1 or Sgs1 and Dna2 (Mimitou and Symington, 2008; Zhu *et al*, 2008) (Figure 1A). The initial endonucleolytic cleavage of the 5′ strand catalysed by MRX and Sae2 can be crucial for processing blocked ends, such as those formed by Spo11 or after exposure to IR, bleomycin and methylating agents. The *S. pombe* Ctp1 and Mre11 nuclease activities are involved in the removal of covalently bound topoisomerases from DNA (Hartsuiker *et al*, 2009b). Furthermore, both *S. cerevisiae sae2*Δ and *mre11* nuclease-defective mutants display a marked hypersensitivity to camptothecin, which traps covalent topoisomerase I-DNA complexes (Liu *et al*, 2002; Deng *et al*, 2005).

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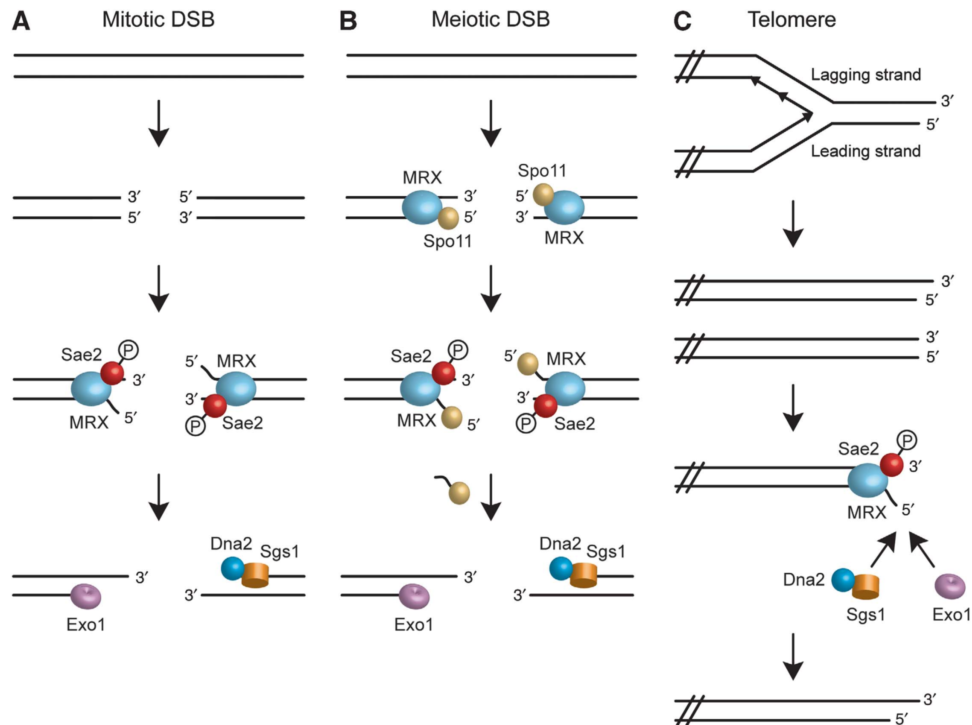

Figure 1 DNA end resection at DSBs and telomeres. (**A**) DSBs in mitotic cells are detected by both MRX and Sae2. Upon phosphorylation of Sae2 by Cdk1, MRX and Sae2 catalyse the initial processing of the 5′ strand, resulting in generation of short ssDNA stretches. The 5′ strand can be substrate for further nucleolytic resection by the concerted action of a helicase, Sgs1, and two nucleases, Exo1 and Dna2. (**B**) Spo11, MRX and other proteins catalyse the formation of a meiosis-specific DSB. Upon phosphorylation of Sae2 by Cdk1, MRX and Sae2 catalyse the removal of Spo11 by endonucleolytic cleavage. Spo11 removal allows the processing of the 5′ strand by either Exo1 or Dna2–Sgs1. (**C**) Telomere DNA replication is expected to leave a short 3′ overhang on the lagging strand (upon RNA primer removal) and a blunt end on the leading strand. End processing at the leading-strand telomere can then be initiated by Sae2/MRX, with Sae2 activity requiring Cdk1-mediated phosphorylation. Sgs1 and Exo1 can provide compensatory activities to resect the 5′ C-strand, with Sgs1 acting in conjunction with Dna2.

Finally, the same mutants are completely defective in resecting meiotic DSBs, where MRX and Sae2 catalyse the removal of Spo11 to allow processing of the 5′ strand (see below) (Figure 1B) (Keeney and Kleckner, 1995; Furuse *et al*, 1998; Tsubouchi and Ogawa, 1998; Usui *et al*, 1998; Moreau *et al*, 1999; Hartsuiker *et al*, 2009a). In contrast, the MRX-Sae2 initial endonucleolytic cleavage is not essential for processing DSBs generated by endonucleases (the so-called 'clean' DSBs), because mutants lacking Sae2 or the Mre11 nuclease activity impair only partially the processing of these DSB ends (Furuse *et al*, 1998; Tsubouchi and Ogawa, 1998; Moreau *et al*, 1999; Llorente and Symington, 2004; Clerici *et al*, 2005). This indicates that Exo1 and Sgs1–Dna2 can resect 'clean' DSBs even in the absence of the initial processing step. However, the resection defect of endonuclease-induced DSBs is more severe in *mre11*Δ than in *mre11* nuclease-defective mutants (Moreau *et al*, 1999; Llorente and Symington, 2004; Clerici *et al*, 2006), suggesting that Exo1, Sgs1 and/or Dna2 require the integrity of the MRX complex to fully exert their actions.

It is noteworthy that *mre11*Δ cells are defective in DSB resection when the break is present in G2 (Clerici *et al*, 2006), whereas they slow down resection only of two-fold when the break occurs when they are exponentially growing (Ivanov *et al*, 1994). This observation, together with the finding that a DSB is processed more efficiently during active DNA replication than in G2 (Zierhut and Diffley, 2008), suggests that the MRX requirement for initiating DSB resection can be partially bypassed when a replication fork encounters a DSB. The precise function of the replication forks remains to be determined, but it might be to promote recruitment of factors required for DSB processing, chromatin remodelling or histone modifications.

Formation and resection of meiotic DSBs

Meiotic recombination is initiated by the formation of self-inflicted DSBs made by the Spo11 protein in early meiotic prophase. An Spo11 dimer breaks both strands of a DNA molecule, creating a DSB in which the 5′ DNA ends are covalently linked to the catalytic tyrosine residue on each Spo11 monomer (reviewed in Keeney and Neale, 2006). In addition to Spo11, DSB formation in *S. cerevisiae* requires the presence of at least nine other proteins, among which are the three subunits of the MRX complex. Although *S. cerevisiae* mutants lacking any MRX complex subunit fail to generate meiotic DSBs, *mre11* mutations specifically impairing Mre11 nuclease activities allow Spo11-induced DSB formation (reviewed in Keeney and Neale, 2006). Thus, the integrity of the MRX complex, rather than its nuclease activity, is important for meiotic DSB formation.

Once Spo11 has catalysed DSB formation, it is removed from the DSB ends by endonucleolytic cleavage to allow further processing of the 5′ DNA ends to initiate HR (Neale *et al*, 2005). This cleavage requires Sae2 and the nuclease activity of the MRX complex (Figure 1B). In fact, both the lack of Sae2 and the Mre11 nuclease-defective variants allow Spo11-induced DSB formation, but prevent Spo11 removal

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and meiotic DSB end resection in *S. cerevisiae* and *S. pombe* cells (Keeney and Kleckner, 1995; Furuse *et al*, 1998; Tsubouchi and Ogawa, 1998; Usui *et al*, 1998; Moreau *et al*, 1999; Hartsuiker *et al*, 2009a). Moreover, *A. thaliana* and *C. elegans sae2* mutants accumulate unrepaired DSBs, but fail to form Rad51 foci, suggesting a defect in DSB resection (Penkner *et al*, 2007; Uanschou *et al*, 2007).

Noteworthy, the lengthening of ssDNA tracts at Spo11-induced DSBs depends on the same nucleases that resect mitotic DSBs (Figure 1B) (Manfrini *et al*, 2010). In fact, both Exo1 and Sgs1 are involved in 3′-ended ssDNA generation after the initial endonucleolytic removal of Spo11, with Exo1 having the major function. In contrast, Exo1 and Sgs1 are not required to remove Spo11 from 5′ ends (Manfrini *et al*, 2010), indicating that different sets of nucleases control the initiation and elongation steps of meiotic DSB resection. Generation of ssDNA at Spo11-induced DSBs depends also on the nuclease Dna2, which appears to contribute mainly to long-range resection (Manfrini *et al*, 2010). Thus, also in meiosis, the helicase activity of Sgs1 may unwind DSB ends to facilitate the access of nucleases. If these nucleases are Exo1 and/or Dna2 remains to be established. In any case, the finding that resection of Spo11-induced DSBs is reduced to a greater extent in cells crippled for both Exo1 and Sgs1 activities than in *exo1*Δ single mutant cells indicates that Sgs1 can act independently of Exo1 (Manfrini *et al*, 2010).

Resection of telomere ends

The structure of telomeric DNA is conserved in the majority of eukaryotes and is organized in short tandem DNA repeats, in which the 3′ strand contains clusters of guanines (3′ G-strand). Furthermore, telomeric DNA terminates with 3′ single-stranded overhangs (G-tails) because the 3′ G-strand extends beyond its complementary 5′ C-strand (Henderson and Blackburn, 1989; Wellinger *et al*, 1993). These single-stranded G-tails have a central function in modulating telomere homeostasis. In fact, they provide a substrate for telomerase, a specialized reverse transcriptase that needs a single-stranded 3′ overhang to anneal it to its associated RNA moiety for iterative reverse transcription of the RNA template. In *S. cerevisiae*, the single-stranded G-tails are bound by the ssDNA-binding protein Cdc13, which is necessary for the recruitment of telomerase through an interaction with the telomerase subunit Est1 (reviewed in Shore and Bianchi, 2009). *S. cerevisiae* telomerase action is negatively regulated by the Rap1 protein that, together with its interactors Rif1 and Rif2, binds telomeric double-stranded DNA repeats and inhibits both telomerase-dependent telomere elongation (Hardy *et al*, 1992; Marcand *et al*, 1997; Levy and Blackburn, 2004) and telomere fusions by NHEJ (Marcand *et al*, 2008). A complex called shelterin, which functionally resembles the Rap1–Rif1–Rif2 complex, has been found at telomeres also in other eukaryotes, such as fission yeast and mammals (reviewed in Palm and de Lange, 2008).

Single-stranded telomeric G-tails can be generated during lagging-strand replication after removal of the last RNA primer, whereas leading-strand polymerases are expected to fully replicate their template, thus generating blunt ends (Figure 1C). However, in the large majority of eukaryotes, 3′ single-stranded overhangs can be detected at both daughter telomeres (Wellinger *et al*, 1996; Makarov *et al*, 1997), implying that the 5′ strand of the leading-strand telomere must be resected to convert blunt ends into 3′ overhangs.

Notably, similar resection machineries create 3′ overhangs at both telomeres and DSBs (Diede and Gottschling, 2001; Larrivée *et al*, 2004; Bonetti *et al*, 2009) (Figure 1A and C). By using a telomere-healing assay in which an endonuclease-induced cleavage is adjacent to a short telomere seed sequence, the MRX complex and Sae2 have been shown to be important for 5′ C-strand resection, with MRX having the major function (Diede and Gottschling, 2001; Bonetti *et al*, 2009). It has been recently shown that the MRX complex is present only at the leading-strand telomere (Faure *et al*, 2010). In contrast, Cdc13 and telomerase are recruited at both leading- and lagging-strand telomeres, but only their binding at leading-strand telomeres requires MRX (Faure *et al*, 2010). As MRX is necessary to resect telomeric DNA, the above data suggests that leading-strand blunt ends are resected to generate ssDNA, whereas the 3′ ssDNA on lagging-strand telomeres could be generated by RNA primer removal and/or MRX-independent processing. How MRX is targeted only at leading-strand telomeres remains to be determined.

Sgs1 and Exo1 provide compensatory activities to initiate end processing in the absence of Sae2. In fact, 5′ C-strand degradation of an endonuclease-induced telomere is severely reduced in *sae2*Δ *exo1*Δ double mutant cells compared with *sae2*Δ single mutant cells, and it is almost completely abolished in *sae2*Δ *sgs1*Δ double mutant cells (Bonetti *et al*, 2009). As seen for DSB resection, Sae2 and MRX act in the same telomere resection pathway, whereas Sgs1 functions in conjunction with Dna2. The involvement of Dna2 in telomere resection has been observed also in *S. pombe* (Tomita *et al*, 2004). In any case, 3′-ended ssDNA at telomeres is less far reaching than that at DSBs. Moreover, the lack of Sgs1, Exo1 or Dna2 by itself does not affect the resection of an endonuclease-induced telomere (Bonetti *et al*, 2009). These observations suggest that the initial resection catalysed by MRX-Sae2 could be sufficient to generate 3′-ended ssDNA that can be long enough to allow telomerase action. In turn, Exo1 and Sgs1–Dna2 may provide a back-up mechanism for telomere resection when Sae2-MRX activity is compromised (Figure 1C). Alternatively, MRX and Sae2 could be specialized in resecting the 5′ strand on the leading-strand telomere, whereas Exo1, Sgs1 and Dna2 may extend the single-stranded overhangs generated by RNA primer removal at the lagging-strand telomere.

Sae2 and Sgs1 control two distinct, but partially complementary, pathways for telomere processing also at native telomeres (Bonetti *et al*, 2009). Consistent with the requirement of 3′-ended ssDNA to allow telomerase action, *sae2*Δ *sgs1*Δ double mutant cells show telomere shortening. However, the absence of both Sae2 and Sgs1 does not cause the complete telomere loss that is observed upon lack of telomerase activity, suggesting that other nuclease activities may resect telomeres even in the absence of Sae2 and Sgs1. Consistent with this hypothesis, telomere shortening in *sae2*Δ *sgs1*Δ double mutant cells can be partially suppressed by overexpressing Exo1, but not its nuclease-defective variant. Furthermore, this telomere shortening can be overcome after extensive subculturing, possibly through unknown changes that upregulated Exo1 and/or other regulators of end resection (Bonetti *et al*, 2009).

Positive regulators of DNA end resection

The choice of the pathway for DSB repair is highly regulated to ensure that cells use the most appropriate mechanism. Although NHEJ is used in G1, HR in haploid cells occurs during S and G2 cell cycle phases, when DNA has replicated and the sister chromatid is available as a repair template. However, the use of NHEJ and HR should be regulated as a function of the cell cycle phase at least in haploid cells, because 5′–3′ resection irreversibly channels a DSB to HR. Indeed, it has been shown that the choice between NHEJ and HR is governed by cyclin-dependent protein kinase (Cdk1 in budding yeast) activity, which promotes 5′–3′ nucleolytic degradation of DNA ends and generation of the 3′ ssDNA tails that are necessary for HR and concomitantly inhibit NHEJ (Aylon *et al*, 2004; Ira *et al*, 2004; Zhang *et al*, 2009). Low Cdk1 activity in *S. cerevisiae* cells, either G1 arrested or after inhibition of an analogue-sensitive variant of the Cdk1 catalytic subunit (Cdc28), does not allow resection and repair by HR of a single endonuclease-induced DSB (Aylon *et al*, 2004; Ira *et al*, 2004). This observation supports a model in which only NHEJ is allowed in haploid G1 cells, whereas Cdk1 activation in S and G2 phases leads to generation of 3′ ssDNA tails and subsequent HR. Cdk1 activity is also required to promote formation of 3′ overhangs at telomeres (Frank *et al*, 2006; Vodenicharov and Wellinger, 2006). As Cdk1 activity is low in G1, resection at telomeres can occur only during S/G2, coinciding with the time frame in which the length of the G-tails increases (Wellinger *et al*, 1993) and telomerase elongates telomeres (Marcand *et al*, 2000).

The Cdk1-mediated control of resection at both mitotic DSBs and telomeres involves phosphorylation of Sae2 Ser267 (Figure 1A and C) (Huertas *et al*, 2008; Bonetti *et al*, 2009), a mechanism that is conserved in the Sae2 vertebrate homologue CtIP (Huertas and Jackson, 2009). In *S. cerevisiae*, lack of Sae2 Ser267 phosphorylation because of the *sae2-S267A* allele impairs processing of both DSBs (Huertas *et al*, 2008) and telomeres (Bonetti *et al*, 2009). These defects are caused by the inability of Cdk1 to phosphorylate Sae2 Ser267, because the same processes take place quite efficiently in *sae2-S267E* cells, where Sae2 Ser267 is replaced by a glutamic residue mimicking constitutive phosphorylation. However, Sae2 Ser267 phosphorylation is necessary, but not sufficient for telomeric end resection in G1 (Bonetti *et al*, 2009). Furthermore, resection in *sae2-S267E* mutants is limited to a few kilobases flanking the break (Huertas *et al*, 2008), suggesting the existence of additional, as-yet-unidentified, Cdk1 substrates (see below).

Cdk1 activity is required to generate Spo11-induced DSBs during meiosis (Henderson *et al*, 2006; Wan *et al*, 2008), and, therefore, its involvement in allowing meiotic DSB processing has not been assessed. However, it has been shown that Cdk1-dependent phosphorylation of *S. cerevisiae* Sae2 Ser267 is required to initiate meiotic DSB resection (Figure 1B) (Manfrini *et al*, 2010). In fact, substitution of Sae2 Ser267 with a non-phosphorylatable alanine residue severely impairs both Spo11 removal and meiotic DSB processing, which instead take place when an aspartic residue mimicking constitutive phosphorylation replaces Ser267. This finding implies that Cdk1 activity is required not only for generation of meiotic DSBs, but also for their resection, thus coordinating this event with meiotic progression. However, spore viability is reduced to a lesser extent by the *sae2-S267A* allele compared with *sae2Δ*, although 3′-ended ssDNA is under the detection level in both *sae2Δ* and *sae2-S267A* cells (Manfrini *et al*, 2010). Thus, full Sae2 activity in meiosis may require Cdk1-dependent phosphorylation of additional residues. Besides Ser267, Sae2 contains two other potential Cdk1 target sites, Ser134 and Ser179, and a *sae2-S267A-S134A* double mutant allele causes a strong reduction in spore viability compared with *sae2-S267A* (Manfrini *et al*, 2010). This loss of viability is likely due to the lack of Ser134 phosphorylation, because the presence of either the *sae2-S267A-S134D* or *sae2-S267A* alleles causes similar spore viability (Manfrini *et al*, 2010). Thus, in addition to Ser267 phosphorylation, also Ser134 phosphorylation might be important for Sae2 meiotic functions.

How Cdk1-dependent phosphorylation modulates Sae2 activity at chromosome ends is still unknown. As Sae2 has been shown to be an endonuclease that acts cooperatively with the MRX complex *in vitro* (Lengsfeld *et al*, 2007), one possibility is that these phosphorylation events stimulate the nuclease activity of Sae2. However, Sae2 endonuclease activity is detected *in vitro* in the absence of phosphorylation events, indicating that they are not absolutely required for the observed Sae2 biochemical activity. These apparent differences between the *in vivo* and *in vitro* data suggest that unknown proteins inhibit Sae2 activity *in vivo* and Cdk1-mediated phosphorylation can relieve this inhibition. Alternatively, or in addition, phosphorylation of Sae2 may induce its interaction with positive regulators of DSB resection, thus enhancing its activity *in vivo*.

Negative regulators of DNA end resection

Inhibition of resection at DSBs

Processing of the DSB ends is inhibited during G1 by competition with NHEJ. In fact, deletion of *YKU70* or *YKU80*, as well as of the NHEJ genes *DNL4* or *LIF1*, increases DSB resection in *S. cerevisiae* cells with low CDK activity (Clerici *et al*, 2008; Zierhut and Diffley, 2008). Interestingly, up to three endonuclease-induced DSBs result in neither resection nor checkpoint activation in G1, but four DSBs are sufficient to initiate both DNA end resection and DNA damage checkpoint response in NHEJ-proficient G1 cells (Zierhut and Diffley, 2008). This suggests that NHEJ is rate limiting in the inhibition of DSB processing in this cell cycle phase. As NHEJ allows DSB ends to be religated, one possibility is that defective NHEJ may increase the time available to the resection machinery to initiate resection. In any case, religation of the DSB ends by NHEJ cannot be the only reason for reduced DSB processing during G1, as loss of Ku has a stronger effect in promoting 5′ DSB end degradation in G1 than loss of either Dnl4 or Lif1 (Clerici *et al*, 2008). The finding that the absence of Ku prevents the loading of Lig4, whereas Ku is still bound at DSBs in the absence of Lig4 (Wu *et al*, 2008), is consistent with an NHEJ-independent function of Ku in protecting DSBs from degradation.

Notably, resection of a single DSB in either NHEJ- or Ku-deficient G1 cells occurs independently of Cdk1 activity, suggesting that Cdk1 activity can relieve the inhibitory effect exerted by Ku and the NHEJ machinery. However, this resection in either NHEJ- or Ku-deficient cells is limited to the break-proximal sequence (Clerici *et al*, 2008; Zierhut and

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Diffley, 2008), suggesting that Cdk1 is still required to activate proteins involved in extensive DSB resection, such as Exo1, Sgs1 and/or Dna2. Alternatively, Cdk1 phosphorylation may prevent the action of proteins that inhibit extensive resection. One candidate for the latter is the checkpoint protein Rad9, as *RAD9* deletion increases DNA end resection even when Cdk1 is not active (Lazzaro *et al*, 2008). However, although Rad9 undergoes multiple Cdk1-dependent phosphorylation events (Ubersax *et al*, 2003), whether they can relieve the block for extensive DNA resection is presently unknown.

It has been shown that Ku and the MRX complex bind independently, and almost simultaneously, to DSB ends (Wu *et al*, 2008) and allow NHEJ to occur in G1 (Figure 2A). Ku and MRX appear to compete for the binding to DNA ends. In fact, in the absence of Ku, DSB resection depends primarily on MRX and the amount of Mre11 bound to the break is increased (Clerici *et al*, 2008). On the other hand, the lack of MRX increases the amount of DSB-bound Ku (Zhang *et al*, 2007; Wu *et al*, 2008), which acts as a block to resection by Exo1. In fact, the IR sensitivity of *mre11*Δ and *sae2*Δ budding and fission yeast mutants is partially suppressed by *KU70*

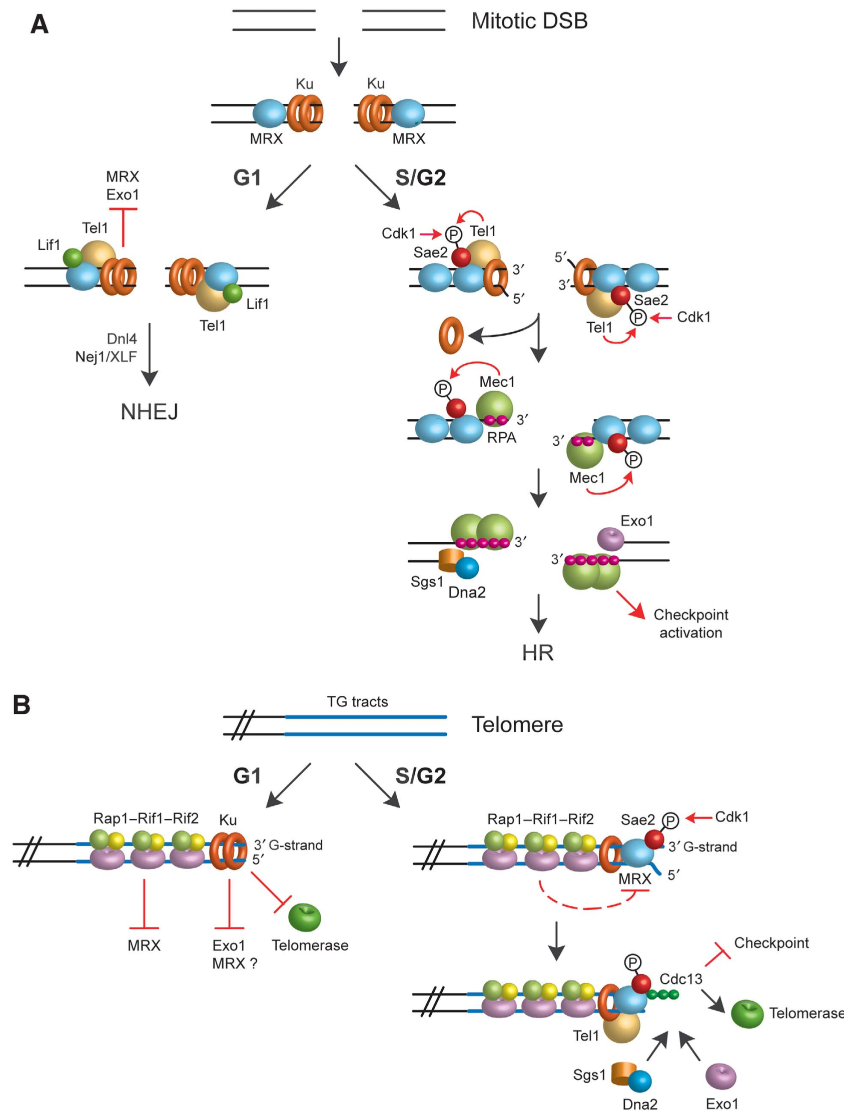

Figure 2 Regulation of 5′ resection at mitotic DSBs and telomeres. (**A**) The MRX complex and Ku almost simultaneously bind the DSB ends. In G1, Ku and MRX mediate recruitment of the NHEJ proteins (Lif1, Dnl4 and Nej1), which allow religation of the DSB ends. Recognition of the DSB by MRX also leads to Tel1 recruitment. Both Ku and the NHEJ proteins prevent initiation of resection. In the absence of Ku or NHEJ, the DSB undergoes MRX-dependent resection even in the absence of Cdk1. When the DSB ends are not bound by MRX, Ku also prevents Exo1-mediated resection. In S/G2, Sae2 is activated by Cdk1- and Tel1-dependent phosphorylation events. MRX and Sae2 then catalyse the initial processing of the 5′ strand possibly by endonucleolytic cleavage, which reduces the ability of Ku to bind the ends and promotes extensive 5′ strand resection by Sgs1, Exo1 and Dna2. The 3′-ended ssDNA tails coated by RPA allow recruitment of Mec1, which in turn phosphorylates Sae2, thus contributing to potentiate resection. Mec1 association to DSB ends also leads to DNA damage checkpoint activation. (**B**) In G1, Rap1, Rif1 and Rif2 mainly act by inhibiting MRX access, whereas Ku protects telomeres from Exo1. As Rap1, Rif1 and Rif2 still prevent MRX action in *yku70*Δ G1 cells, Ku may protect G1 telomeres also from MRX. The lack of telomeric ssDNA should prevent telomerase action. In S/G2, only Rap1, Rif2 and Rif1 still exert their inhibitory effects on telomere processing, but telomere resection can take place because Cdk1 activates Sae2-MRX, which in turn relieves the inhibitory effect of Ku. The resulting telomeric ssDNA is covered by Cdc13, which suppresses DNA damage checkpoint activation and allows telomerase action. If the shelterin-like proteins and/or Ku also regulate Sgs1 and Dna2 activities is still unknown.

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deletion in an Exo1-dependent manner (Tomita *et al*, 2003; Limbo *et al*, 2007; Wasko *et al*, 2009).

If the DSB is not repaired by NHEJ, progression of the cell cycle into S/G2 leads to Cdk1-dependent activation of Sae2, which initiates DSB resection together with the MRX complex. Noteworthy, the Ku dimer has high affinity for DSBs, whereas it binds poorly to ssDNA (Dynan and Yoo, 1998). Furthermore, Ku dissociation from DSB ends correlates with bulk resection (Wu *et al*, 2008). These findings suggest that the initial processing catalysed by Sae2 and MRX could generate a less suitable substrate for Ku binding, thus overriding the resection block imposed by Ku and committing DSB repair to HR (Figure 2A).

Interestingly, it has been shown that *S. pombe* Sae2/Ctp1 is retained at the break site through phosphorylation-dependent direct binding to the N-terminal FHA domain of Nbs1 (Lloyd *et al*, 2009; Williams *et al*, 2009). The Nbs1-binding sites in Ctp1 resemble a motif found in budding yeast Lif1 (Lloyd *et al*, 2009), suggesting that Lif1–Xrs2 interaction (Palmbos *et al*, 2008) may take place by a similar mechanism. Although the homology between Sae2 and Ctp1 is limited, this finding raises the possibility that Lif1 and Sae2 may compete for binding to Xrs2, thus regulating the choice between NHEJ and HR (Figure 2A).

Inhibition of resection at telomeres

The single-stranded G-tails of budding yeast telomeres are short (about 10–15 nucleotides) for most of the cell cycle, and their length increases transiently in late S phase (about 50–100 nucleotides) (Larrivée *et al*, 2004). Thus, 5′ resection of telomeric ends is less extensive than that of intrachromosomal DSB ends. As the nuclease requirements at DSB and telomere resection are similar (Bonetti *et al*, 2009), this finding suggests that telomeric ends are resistant to nuclease attack. Interestingly, the activity of Exo1 in generating ssDNA at uncapped telomeres is inhibited by the checkpoint machinery (Morin *et al*, 2008). However, it is unknown whether this negative feedback loop acts also at DSBs or it is specific to dysfunctional telomeres.

Indeed, the heterodimeric Ku complex has a function in inhibiting resection also at telomeres. In fact, its lack causes Exo1-dependent accumulation of telomeric ssDNA (Gravel *et al*, 1998; Polotnianka *et al*, 1998; Maringele and Lydall, 2002; Bertuch and Lundblad, 2004), as well as checkpoint-mediated cell cycle arrest at high temperatures (Barnes and Rio, 1997; Maringele and Lydall, 2002). Similar to what is observed at DSBs, Ku protects telomeres from degradation mainly in G1 (Figure 2B) (Bonetti *et al*, 2010). Interestingly, resection at an endonuclease-induced telomere in *yku70*Δ G1 cells is confined to the telomeric tips, indicating that either the rate or the processivity of resection is reduced in G1 compared with G2 even in the absence of Ku. Unlike at intrachromosomal DSBs (Clerici *et al*, 2008; Zierhut and Diffley, 2008), loss of Dnl4 does not allow ssDNA generation at the endonuclease-induced telomere in G1 (Bonetti *et al*, 2010), indicating that the Ku-mediated inhibition of telomeric processing is independent of Ku function in NHEJ. This finding is consistent with the observation that NHEJ is inhibited at telomeres (Pardo and Marcand, 2005), possibly because some of its components are not allowed to bind telomeric ends.

Besides Ku, protection from degradation of budding yeast telomeres depends on proteins that specifically bind single- or double-stranded telomeric DNA. In particular, inactivation of Cdc13 leads to accumulation of long ssDNA regions that extend into non-telomeric DNA sequences (Garvik *et al*, 1995; Nugent *et al*, 1996; Booth *et al*, 2001). Furthermore, the shelterin-like proteins Rif1, Rif2 and Rap1 have been recently shown to inhibit nucleolytic processing at telomeres during both G1 and G2 cell cycle phases (Figure 2B) (Bonetti *et al*, 2010), with Rif2 and Rap1 showing the strongest effect. Similarly, Rif2 and Rap1, but not Rif1, prevent telomeric fusions by NHEJ (Marcand *et al*, 2008). Telomeric ssDNA generation is increased to the same extent in the absence of Rif2 or Rap1 C-terminus, suggesting that the inhibitory effect exerted by Rap1 is likely mediated by Rif2, whose recruitment to telomeres depends on Rap1 C-terminal domain (Wotton and Shore, 1997). Interestingly, loss of mammalian Rap1 induces HR at telomeres without activation of the DNA damage checkpoint (Sfeir *et al*, 2010), raising the possibility that the shelterin complex can inhibit ssDNA generation also at mammalian telomeres.

Although resection of telomeres in *yku70*Δ G1 cells is confined to the telomeric tips, more resection events are initiated in *yku70*Δ than in *rif2*Δ G1 cells (Bonetti *et al*, 2010), arguing that Ku is mainly involved in inhibiting initiation of resection. On the other hand, the finding that the limited telomere processing in *yku70*Δ G1 cells is relieved upon loss of Rif2 suggests that Rif2 and Rap1 primarily limit extensive resection. Consistent with the different function of Ku and shelterin-like proteins in inhibiting telomere resection, the lack of both Ku and Rif2 causes a synergistic increase of ssDNA at an endonuclease-induced telomere (Bonetti *et al*, 2010).

Ku and the shelterin-like proteins appear to inhibit the action of different nucleases (Figure 2B). In fact, telomeric ssDNA generation in *yku70*Δ G1 cells requires Exo1 (Maringele and Lydall, 2002; Bertuch and Lundblad, 2004; Bonetti *et al*, 2010). In contrast, MRX is responsible for nucleolytic degradation of telomeres in both *rif2*Δ and *rap1*Δ*C* cells (Bonetti *et al*, 2010). MRX association at telomeres is enhanced in *rif2*Δ and *rap1*Δ*C* cells (Hirano *et al*, 2009; Bonetti *et al*, 2010), suggesting that Rap1 and Rif2 can prevent MRX action by inhibiting MRX recruitment onto telomeric ends. Interestingly, Ku prevents the action of Exo1 at telomeres, whereas it protects intrachromosomal DSBs mainly from MRX-dependent degradation (Clerici *et al*, 2008). However, the finding that Rif2 and Rap1 still inhibit MRX association at telomeres in *yku70*Δ cells can explain the apparent different involvement of nucleases in resecting DSBs versus telomeres in the absence of Ku. Finally, the inhibitory effect of the shelterin-like complex is not sufficient to block telomere resection in S/G2, because Cdk1 activates MRX/Sae2, which can relieve the inhibitory action exerted by Ku (Figure 2B).

DNA damage checkpoint activation by DNA ends

Checkpoint activation by mitotic DSBs

DSB formation triggers activation of the DNA damage checkpoint, whose important players are ATM and ATR in mammals and Tel1 and Mec1 in *S. cerevisiae* (Longhese *et al*, 2006). In both *S. cerevisiae* and human cells, the MRX/MRN complex recruits Tel1/ATM at blunt or minimally processed

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DNA ends (Nakada *et al*, 2003; Falck *et al*, 2005; Mantiero *et al*, 2007), arguing that MRX/MRN association at DSBs is the signalling event for checkpoint activation. Initiation of DSB processing and the subsequent generation of ssDNA coated by the replication protein A (RPA) complex leads to Mec1 recruitment and Mec1-dependent checkpoint activation (Zou and Elledge, 2003). The finding that Tel1 signalling activity at DSBs is compromised when the DSB ends are nucleolytically processed indicates that DSB resection regulates the transition not only from NHEJ to HR, but also from a Tel1/ATM- to a Mec1/ATR-controlled checkpoint (Jazayeri *et al*, 2006; Mantiero *et al*, 2007).

Indeed, the resection machinery is also a downstream target of Tel1/ATM and Mec1/ATR kinases. In fact, Mec1 and Tel1 phosphorylate Sae2 (Baroni *et al*, 2004; Cartagena-Lirola *et al*, 2006), whereas ATM phosphorylates CtIP (Li *et al*, 2000), and these phosphorylation events are important for Sae2/CtIP function in DSB metabolism. In particular, replacing with alanines the Sae2 serine and threonine residues belonging to the S/T-Q motifs preferred for phosphorylation by ATM/ATR kinases results in hypersensitivity to DNA-damaging agents, decreased rates of mitotic recombination between inverted *Alu* repeats (Baroni *et al*, 2004) and defective resection of mitotic DSBs (our unpublished observation) in *S. cerevisiae* cells. Furthermore, such replacements impair also Sae2 meiotic function, as it causes accumulation of unprocessed meiotic DSBs (Cartagena-Lirola *et al*, 2006).

In both *S. pombe* and human cells, Ctp1/CtIP recruitment to damaged DNA seems to be controlled by multiple kinases. In fact, ATM kinase activity is required for the recruitment of Ctp1/CtIP to damaged DNA (Limbo *et al*, 2007; Williams *et al*, 2009; You *et al*, 2009), although it is still unclear whether ATM exerts this function by phosphorylating Ctp1/CtIP. Furthermore, potential casein kinase 2 phosphorylation motifs in Ctp1 bind the FHA domain of the MRN subunit Nbs1, which then recruits Ctp1 to DSBs (Lloyd *et al*, 2009; Williams *et al*, 2009). In any case, Ctp1/CtIP targeting to sites of DNA damage is not a mechanism commonly used. In fact, impairment of Mec1- and Tel1-dependent Sae2 phosphorylation does not affect Sae2 localization at DSBs in *S. cerevisiae* (our unpublished observation), suggesting that Sae2 is not retained at the break site through direct phosphorylation-dependent binding to Xrs2. This is consistent with the finding that MRX is not required for the loading of Sae2 onto DSBs in *S. cerevisiae* (Lisby *et al*, 2004), whereas Ctp1/CtIP recruitment to DNA damage sites requires MRN in both *S. pombe* and mammals (Williams *et al*, 2009).

S. cerevisiae Sae2 is also involved in checkpoint deactivation, possibly by regulating MRX dissociation from damaged DNA. In fact, *sae2Δ* cells fail to turn off Tel1/ATM-dependent checkpoint (Usui *et al*, 2001; Clerici *et al*, 2006) and exhibit persistent MRX foci at DNA breaks (Lisby *et al*, 2004; Clerici *et al*, 2006). The observation that *mre11* nuclease-defective mutants display the same phenotypes (Lisby *et al*, 2004; Clerici *et al*, 2006) suggests that Sae2 promotes checkpoint switch off by stimulating MRX nuclease activity, which in turn promotes MRX release from DNA. Interestingly, the function of Sae2 in deactivating the checkpoint requires Mec1- and Tel1-dependent Sae2 phosphorylation (Clerici *et al*, 2006), suggesting that Mec1 and Tel1 may limit MRX ability to signal to the checkpoint machinery by phosphorylating and activating Sae2.

Checkpoint inhibition at telomeres

Functional telomeres are protected from checkpoints, as well as from HR and NHEJ that normally act at intrachromosomal DSBs (reviewed in Longhese, 2008; Lydall, 2009). One way to ensure that telomeres are not recognized as DSBs would be to exclude checkpoint/repair/recombination proteins from telomeres. However, many proteins involved in the DNA damage response bind telomeres and have critical functions in telomere metabolism, suggesting that the DNA damage response is attenuated, but not abolished at telomeres. The mechanism by which this is achieved is unclear, but it likely relies on different telomere features, such as the telomeric DNA sequence, the proteins localized at telomeres and the structure of telomeric DNA.

Mammalian telomeres have long single-stranded telomeric ends (Makarov *et al*, 1997). One solution to repress checkpoint activation in mammals is the remodelling of telomeric DNA into t-loops, which can hide the chromosome ends from being recognized by the DNA damage checkpoint. However, both *S. cerevisiae* and *S. pombe* telomeres are presumably too short to generate t-loops, and it is unclear whether all telomeres or only a subset of them are organized into t-loops in other organisms. Thus, alternative mechanisms should exist to prevent telomeric single-stranded overhangs from eliciting a DNA damage response. In mammals, inhibition of the shelterin component POT1 triggers an ATR-dependent checkpoint response (Lazzerini Denchi and de Lange, 2007), suggesting that POT1 inhibits ATR activation by blocking the recruitment of RPA to the single-stranded telomeric DNA (Lei *et al*, 2004; Kelleher *et al*, 2005). A similar mechanism may exist in yeast, in which the binding of Cdc13 to the single-stranded telomeric G-tails attenuates Mec1 association with these DNA ends (Hirano and Sugimoto, 2007). In any case, it is well known that ssDNA accumulation at DSBs invokes an ATR/Mec1-dependent DNA damage response when it exceeds a certain threshold (Pellicioli *et al*, 2001; Zierhut and Diffley, 2008). Thus, one way to ensure that telomeres do not activate the DNA damage response would be to reduce the amount of ssDNA by resisting to the nuclease attack.

The activity that protects telomeres from extensive nucleolytic degradation resides on proteins that bind telomeric DNA. Both lack of Ku and inactivation of Cdc13 cause a checkpoint-mediated cell cycle arrest at high temperatures in budding yeast (Garvik *et al*, 1995; Teo and Jackson, 2001; Maringele and Lydall, 2002; Zubko *et al*, 2004). Moreover, loss of the shelterin protein TRF2 leads to ATM-dependent DNA damage response in mammalian cells (Celli and de Lange, 2005; Lazzerini Denchi and de Lange, 2007). In contrast, the absence of Rif2 or Rap1 C-terminus does not elicit a DNA damage checkpoint in budding yeast, although it causes telomeric ssDNA accumulation (Bonetti *et al*, 2010). One possibility is that this ssDNA is still covered by Cdc13, which has been shown to inhibit Mec1 association to DNA ends by competing with RPA for binding to telomeric ssDNA (Hirano and Sugimoto, 2007). The above hypothesis is consistent with the finding that ATM activation induced by loss of human TRF2 does not require generation of ssDNA. As the ATM yeast orthologue, Tel1, has a very minor function in eliciting a DSB-induced checkpoint compared with Mec1 (Mantiero *et al*, 2007), Tel1 activation induced by loss of Rap1, Rif1 or Rif2 may be insufficient for inducing a detectable checkpoint response.

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Conclusions

DNA end resection is especially relevant both for DSB repair commitment to a specific pathway and for allowing telomerase-mediated telomere elongation. Thus, its regulation is very important to avoid aberrant DSB repair events, as well as extensive degradation and activation of a DNA damage response at telomeres. Owing to the critical function of both DNA repair and telomere homeostasis in maintaining genetic stability and in counteracting cancer development, increasing our knowledge of how resection is regulated is essential for the understanding of these defence mechanisms.

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Conflict of interest

The authors declare that they have no conflict of interest.

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