MRX-dependent DNA Damage Response to Short Telomeres

Valeria Viscardi,* Diego Bonetti,* Hugo Cartagena-Lirola,*[†] Giovanna Lucchini,* and Maria Pia Longhese*

*Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, 20126 Milan, Italy; and [†]Division of Hepatology and Gene Therapy, Centro de Investigación Médica Aplicada, Universidad de Navarra, 31008 Pamplona, Spain

Submitted March 28, 2007; Revised May 4, 2007; Accepted May 17, 2007
Monitoring Editor: Orna Cohen-Fix

Telomere structure allows cells to distinguish the natural chromosome ends from double-strand breaks (DSBs). However, DNA damage response proteins are intimately involved in telomere metabolism, suggesting that functional telomeres may be recognized as DNA damage during a time window. Here we show by two different systems that short telomeres are recognized as DSBs during the time of their replication, because they induce a transient MRX-dependent DNA damage checkpoint response during their prolonged elongation. The MRX complex, which is recruited at telomeres under these conditions, dissociates from telomeres concomitantly with checkpoint switch off when telomeres reach a new equilibrium length. We also show that MRX recruitment to telomeres is sufficient to activate the checkpoint independently of telomere elongation. We propose that MRX can signal checkpoint activation by binding to short telomeres only when they become competent for elongation. Because full-length telomeres are refractory to MRX binding and the shortest telomeres are elongated of only a few base pairs per generation, this limitation may prevent unscheduled checkpoint activation during an unperturbed S phase.

INTRODUCTION

Telomeres, the physical ends of linear eukaryotic chromosomes, are composed of G/C-rich repeated sequences and the G-rich strand terminates with a 3' single-stranded DNA (ssDNA) overhang known as G-tail. Similarly, interruptions in duplex DNA molecules are resected by 5'-3' exonucleases to generate 3'-ended ssDNA tails, which elicit a DNA damage response, resulting in checkpoint-mediated cell cycle delay and DNA repair (reviewed in Longhese *et al.*, 2006). Despite their resemblance to double-strand breaks (DSBs), telomeres are not subjected to homologous recombination, end-to-end fusions or other events that normally promote repair of DNA breaks (reviewed in Viscardi *et al.*, 2005). Moreover, they do not activate the DNA damage checkpoint, whose key players belong to a protein kinase family, including mammalian ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad3-related) and *Saccharomyces cerevisiae* Tel1 and Mec1 (reviewed in Longhese *et al.*, 2006; Shiloh, 2006). This implies that cells must be able to differentiate natural chromosome ends from intrachromosomal DSBs, the latter serving as potent stimuli of the DNA damage response and resulting in checkpoint-mediated cell cycle arrest until repaired. This differentiation is thought to be the consequence of a unique organization of chromosomal ends into specialized nucleoprotein complexes (reviewed in Chakraborty and Wellinger, 2003; Smogorzewska and de

Lange, 2004). In humans, attenuation of checkpoint signaling at telomeres may be achieved through the action of TRF2 that binds directly to telomere repeat sequences and inhibits the ATM protein kinase (Karlseder *et al.*, 2004). Conversely, budding yeast telomeres possess a yet unidentified telomeric "antichkpoint" activity that might inhibit Mec1 signaling (Michelson *et al.*, 2005). In any case, telomeric double-stranded DNA is bound by specialized DNA-binding proteins that protect the chromosomal ends from being sensed as DSBs (reviewed in d'Adda di Fagagna *et al.*, 2004; de Lange, 2005; Viscardi *et al.*, 2005) and ensure their complete replication via a specialized reverse transcriptase called telomerase (Hug and Lingner, 2006). Telomeric-capping function is dependent on proper DNA end structure as well as on telomere-binding proteins. In *S. cerevisiae*, the G-tail binding protein Cdc13 and its interacting factors Stn1 and Ten1 are essential components of the telomeric cap (Garvik *et al.*, 1995; Grandin *et al.*, 1997, 2001). Moreover, the double-stranded telomeric repeat binding protein Rap1 is required to avoid telomere fusions (Pardo and Marcand, 2005).

Telomere length alterations by loss of telomere-associated proteins or by inhibition of telomere replication signal a DNA damage response. Extensive telomere shortening in yeast cells lacking telomerase triggers a Mec1-dependent checkpoint (Ijima and Greider, 2003), as does inactivation of the duplex TG-repeat-binding protein Rap1 (Pardo and Marcand, 2005). Moreover, degradation of the C-rich strand caused by inactivation of the telomere end-binding protein Cdc13 leads to accumulation of ssDNA and subsequent DNA damage checkpoint activation (Garvik *et al.*, 1995; Lydall and Weinert, 1995).

Although telomeres are apparently shielded from being recognized as DSBs, many DNA damage response proteins

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-03-0285>) on May 30, 2007.

Address correspondence to: Maria Pia Longhese (mariapia.longhese@unimib.it).

are associated with chromosomal termini and contribute to several aspects of telomere metabolism. For instance, budding yeast Tel1 and Mec1 exhibit cell cycle-dependent association to telomeres (Takata *et al.*, 2004; Verdun *et al.*, 2005). Paradoxically, chromatin immunoprecipitation experiments suggest that Tel1 and Mre11 may be at telomeres in different stages of the cell cycle (Takata *et al.*, 2004). This contrasts with recent data showing that ATM and Nbs1 both colocalize to telomeres during G2 in human cells (Verdun *et al.*, 2005). Tel1/ATM appears to be particularly important for telomere length maintenance in yeast, *Drosophila*, and mammalian cells, and its inactivation results in shortened telomeres and increased chromosomal end-to-end fusions by nonhomologous end joining (Metcalf *et al.*, 1996; Ritchie *et al.*, 1999; Bi *et al.*, 2004; Silva *et al.*, 2004). Epistasis analysis shows that Tel1 acts at telomeres together with the MRX (Mre11-Rad50-Xrs2)/MRN (Mre11-Rad50-Nbs1) complex (Ritchie and Petes, 2000; Tsukamoto *et al.*, 2001), which is required for the generation of 3' ssDNA tails in a telomere formation assay (Diede and Gottschling, 2001) and for the proper establishment of the constitutive G-tail end structure (Larrivee *et al.*, 2004). Moreover, MRX/MRN appears to protect telomeres from nuclease and DNA repair activities in both yeast and *Drosophila* (Bi *et al.*, 2004; Ciapponi *et al.*, 2004; Foster *et al.*, 2006). MRX and Tel1 are thought to contribute to yeast telomere length maintenance by promoting telomerase association to telomeres. In fact, recruitment of both the telomerase catalytic subunit Est2 and the telomerase accessory protein Est1 is severely reduced in Mre11 or Tel1 lacking cells (Goudsouzian *et al.*, 2006).

In any case, the finding that several factors known to be directly involved in DNA repair and DNA damage checkpoints localize at telomeres and contribute to telomere metabolism suggests that there may be a time window where functional telomeres are recognized as DNA breaks. Consistent with this hypothesis, we have previously shown that rapid telomere expansion caused by turning on Tel1 overexpression in cells with short but otherwise stable telomeres causes the activation of a Rad53-dependent checkpoint. This suggests that telomeres can be recognized as DSBs in a

window of time during their replication (Viscardi *et al.*, 2003). In this study, we try to assess the signals activating the checkpoint during telomere elongation by analyzing the checkpoint response during prolonged expansion of either a single or multiple shortened telomeres.

MATERIALS AND METHODS

Yeast Strains and Media

All yeast strains were derivatives of W303 (*MATa* or *MAT α* , *ade2-1*, *can1-100*, *his3-11,15*, *leu2-3,112*, *trp1-1*, *ura3*) and are listed in Table 1. Strain *GAL-TEL1*, carrying one copy of the *GAL1-TEL1* fusion disrupting the *TEL1* chromosomal gene, was obtained as previously described (Viscardi *et al.*, 2003). Strains expressing the fully functional *MRE11-MYC18*-tagged allele at the *MRE11* chromosomal locus were constructed as previously described (Clerici *et al.*, 2006). The *GAL-TEL1* strain was used to disrupt the *SAE2* and the *MRE11* genes, to replace *RAD50* with the *rad50s-K811* allele (Alani *et al.*, 1990) or to integrate the *GAL-SAE2* fusion at the *LEU2* locus. To generate *rad50s* mutants, *GAL-TEL1* cells were transformed with *MscI*-digested plasmid pML533, carrying the first 666 base pairs (bp) of the *RAD50* coding region with the K811 mutation. Strains Lev187, Lev189, and Lev220 were kindly provided by S. Marcand (Centre National de la Recherche Scientifique [CNRS], Fontenay-aux-Roses, France). Lev220 was used to disrupt the *RAD50*, *MEC1*, *SMI1*, or *TEL1* genes. *GAL1-TEL1* strains carrying either the 2 μ vector or 2 μ *RIF2* plasmid were constructed by transforming the *GAL-TEL1* strain with plasmids YEplac195 (2 μ *URA3*) and pML415 (2 μ *RIF2 URA3*), respectively. *cdc15-2* strains expressing the methionine-regulatable *MET-CDC6* fusion at the *CDC6* locus were kindly provided by S. Piatti (University of Milano-Bicocca, Milan, Italy) and were crossed to *GAL-TEL1 MRE11-MYC18* strain to obtain strains DMP4702/2C, DMP4701/26C, DMP4699/26B, and DMP4700/34D.

The accuracy of all gene replacements and integrations was verified by Southern blot or PCR analysis. Standard yeast genetic techniques and media were according to Rose *et al.* (1990). Cells were grown in YEP medium (1% yeast extract, 2% bactopectone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP+raf) or 2% raffinose and 1% galactose (YEP+raf+gal). Transformants carrying the *KANMX4* cassette were selected on YEPD plates containing 400 μ g/ml G418 (US Biological, Swampscott, MA).

Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation (ChIP) analysis was performed as in http://www.molbio.princeton.edu/labs/zakian/2004_methods.htm. Multiplex PCR reactions for Mre11 immunoprecipitates were carried out for 30 cycles. Gel quantitation was determined by using the NIH image program. The relative fold enrichments of telomere-bound Mre11 were calculated as follows: $[\text{TEL}_{\text{IP}}/\text{ARO}_{\text{IP}}]/[\text{TEL}_{\text{input}}/\text{ARO}_{\text{input}}]$, where IP and input represent the

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Reference/source
YLL1139/32	<i>MATa MRE11-MYC18::TRP1</i>	This study
YLL1679/1C	<i>MATa tel1::GAL1-TEL1::LEU2 MRE11-MYC18::TRP1</i>	This study
DMP3619/5A	<i>MATa tel1::GAL1-TEL1::LEU2 MRE11-MYC18::TRP1 mre11Δ::HIS3</i>	This study
DMP4617/4A	<i>MATa tel1::GAL1-TEL1::LEU2 MRE11-MYC18::TRP1 rad50s::URA3</i>	This study
DMP4629/2A	<i>MATa tel1::GAL1-TEL1::LEU2 MRE11-MYC18::TRP1 sae2Δ::KANMX4</i>	This study
DMP4595/1B	<i>MATa tel1::GAL1-TEL1::LEU2 MRE11-MYC18::TRP1 GAL1-SAE2::LEU2</i>	This study
YLL1686	<i>MATa tel1::GAL1-TEL1::LEU2 MRE11-MYC18::TRP1 [YEplac195 2 μ <i>URA3</i>]</i>	This study
YLL1798	<i>MATa tel1::GAL1-TEL1::LEU2 MRE11-MYC18::TRP1 [pML415 2 μ <i>RIF2 URA3</i>]</i>	This study
DMP4702/2C	<i>MATa cdc15-2 MRE11-MYC18::TRP1</i>	This study
DMP4701/26C	<i>MATa cdc15-2 MRE11-MYC18::TRP1 cdc6Δ::hisG-URA3-hisG trp1-1::TRP1-MET3-CDC6</i>	This study
DMP4699/26B	<i>MATa cdc15-2 MRE11-MYC18::TRP1 tel1::GAL1-TEL1::LEU2</i>	This study
DMP4700/34D	<i>MATa cdc15-2 MRE11-MYC18::TRP1 cdc6Δ::hisG-URA3-hisG trp1-1::TRP1-MET3-CDC6 tel1::GAL1-TEL1::LEU2</i>	This study
Lev187	<i>MATa leu2::LEU2-GAL10-FLP1[ciro] adh4::FRT-URA3-FRT-TEL</i>	Marcand <i>et al.</i> (1999)
Lev189	<i>MATa leu2::LEU2-GAL10-FLP1[ciro] adh4::FRT-URA3-TEL270-FRT-TEL</i>	Marcand <i>et al.</i> (1999)
Lev220	<i>MATa leu2::LEU2-GAL10-FLP1[ciro] adh4::FRT-URA3-TEL270-FRT-TEL</i>	Marcand <i>et al.</i> (1999)
DMP4744/1A	<i>MATa leu2::LEU2-GAL10-FLP1[ciro] adh4::FRT-URA3-TEL270-TEL270-FRT-TEL MRE11-MYC18::TRP1</i>	This study
YLL2017	<i>MATa leu2::LEU2-GAL10-FLP1[ciro] adh4::FRT-URA3-TEL270-TEL270-FRT-TEL tel1Δ::HPH</i>	This study
YLL2034	<i>MATa leu2::LEU2-GAL10-FLP1[ciro] adh4::FRT-URA3-TEL270-TEL270-FRT-TEL mec1Δ::TRP1 smi1Δ::KANMX4</i>	This study
YLL2011	<i>MATa leu2::LEU2-GAL10-FLP1[ciro] adh4::FRT-URA3-TEL270-TEL270-FRT-TEL rad50Δ::HIS3</i>	This study

amount of PCR product in the immunoprecipitates and input samples before immunoprecipitation, respectively. Sequences for all primers are available upon request.

Southern Blot Analysis of Telomere Length

Yeast DNA was prepared according to standard methods and digested with *Xho*I or *Stu*I enzyme. The resulting DNA fragments were separated by gel electrophoresis in 0.8% agarose gel and transferred to a GeneScreen nylon membrane (New England Nuclear, Boston, MA), followed by hybridization with a ³²P-labeled poly(GT) probe or a ³²P-labeled probe corresponding of an 840-bp *Hind*III-*Stu*I *ADH4* fragment and exposure to x-ray-sensitive films. Standard hybridization conditions were used.

Other Techniques

Visualization of the single-strand overhang at telomeres was done as described in Dionne and Wellinger (1996). Total protein extract preparation and Western blot analysis were performed as described in Clerici *et al.* (2001). Rad53 was detected using anti-Rad53 polyclonal antibodies kindly provided by J. Diffley (Clare Hall Laboratories, South Mimms, United Kingdom) and C. Santocanale (Nerviano, Milano, Italy). Secondary antibodies were purchased from Amersham (Piscataway, NJ), and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer.

RESULTS

MRX Requirement and Recruitment during the Checkpoint Response to Elongation of Multiple Short Telomeres

We previously showed that prolonged telomere elongation caused by turning on *Tel1* overexpression in cells with short telomeres (due to the lack of *Tel1* or *Ku*) triggers transient activation of a Rad53-dependent checkpoint that inhibits the G2/M transition (Viscardi *et al.*, 2003). Prolonged telomere elongation may disrupt specialized capping functions, thus generating signals, such as blunt ends and/or 3' single-stranded overhangs, which persist long enough to be detected as DNA lesions by the checkpoint machinery. The MRX complex is implicated together with *Mec1* and *Tel1* in triggering checkpoint activation after generation of intrachromosomal DSBs (Ira *et al.*, 2004; Nakada *et al.*, 2004; Clerici *et al.*, 2006) and associates at telomeres during S phase (Zhu *et al.*, 2000; Takata *et al.*, 2005). We therefore evaluated the possible MRX involvement in the above telomere elongation and checkpoint activation. It is worth mentioning that a galactose-inducible *GAL-TEL1* construct is the only *Tel1* source in all the *GAL-TEL1* strains used in this work (*tel1::GAL-TEL1*; see Table 1), which therefore have stable short telomeres when grown in the absence of galactose (Viscardi *et al.*, 2003). G1-arrested cell cultures in raffinose-containing medium were released from α -factor in the presence of galactose. As shown in Figure 1, *GAL-TEL1* cells transiently arrested, as expected, with 2C DNA content (Figure 1A) and underwent phosphorylation of the effector checkpoint kinase Rad53 (Figure 1C), while gradually elongating telomeres to a new equilibrium length (Figure 1B). Conversely, galactose-induced *GAL-TEL1* cells lacking the *Mre11* subunit of MRX did not undergo either telomere elongation (Figure 1B) or cell cycle arrest and Rad53 phosphorylation (Figure 1, A and C). Thus, the MRX complex is necessary for telomere elongation and checkpoint activation caused by ectopic *Tel1* expression.

To assess whether transient telomere elongation and checkpoint activation in *GAL-TEL1* cells involved MRX telomere association, we performed ChIP analysis in *GAL-TEL1* cells carrying a fully functional *MRE11-MYC*-tagged allele. Sheared chromatin prepared at different time points after galactose addition from formaldehyde cross-linked cell samples was immunoprecipitated with anti-myc antibodies. Quantitative multiplex PCR was then used to monitor co-immunoprecipitation of the nontelomeric *ARO1* fragment of chromosome IV, of the subtelomeric *ADH4* fragment located

5.3 kilobase pairs (kbp) from telomere VII-L and of the unique *TEL* fragment, located 52 bp away from telomere VI-R (Figure 1D). We also monitored the efficiency of *Mre11-myc* immunoprecipitation (ppt in Figure 1D) to ensure that differences in telomere association were not due to different protein accessibility. As shown in Figure 1, D and E, *Mre11* association to telomeres, which was enriched about three-fold over background in G1-arrested uninduced *GAL-TEL1* cells (α), strongly increased (35-fold) in these cells within 2 h after release into the cell cycle in the presence of galactose, and it remained high during the subsequent 10 h. *Mre11* telomere association under these conditions paralleled with telomere elongation, cell cycle arrest, and checkpoint activation. In fact, the amount of telomere-bound *Mre11* gradually declined (Figure 1, D and E) at the same time telomere length reached a new equilibrium (Figure 1B) and the checkpoint was switched off (Figure 1, A and C).

MRX is required for the generation of 3' ssDNA tails in a telomere formation assay (Diede and Gottschling, 2001) and for the proper establishment of the constitutive G-tail end structure (Larrivee *et al.*, 2004). We therefore asked whether MRX prolonged binding at elongating telomeres may lead to accumulation of long single-stranded 3' overhangs that are strong signals for checkpoint activation (Zou and Elledge, 2003). However, nondenaturing in-gel hybridization with a C-rich radiolabeled oligonucleotide (Dionne and Wellinger, 1996) of genomic DNA derived from either galactose-induced *GAL-TEL1* or wild-type cells yielded faint signals compared with those detected in cells lacking *Ku70*, which are known to accumulate telomeric single-stranded G-tails (Figure 2; Gravel *et al.*, 1998). This indicates that accumulation of ssDNA at telomeres is not likely responsible for checkpoint activation in galactose-induced *GAL-TEL1* cells. This is in agreement with recent data showing that a DSB adjacent to the telomeric sequence causes checkpoint activation before ssDNA generation (Michelson *et al.*, 2005).

MRX Association and Persistence at Telomeres Correlate with Checkpoint Activation

If *Mre11* telomere association were the signal for checkpoint activation in *GAL-TEL1* cells, its increased persistence should lead to prolonged checkpoint activation. To evaluate this possibility, we used *GAL-TEL1* cells lacking or overexpressing the *Sae2* protein, which works in concert with MRX in DSB processing and in subsets of recombination pathways (Rattray *et al.*, 2001; Lobachev *et al.*, 2002; Clerici *et al.*, 2005). *Sae2* appears to regulate MRX persistence at DSBs. In fact, both the lack of *Sae2* and the *rad50s* mutation, which is thought to alter MRX ability to interact with *Sae2*, increase MRX persistence at DNA breaks and prevent DNA damage checkpoint switch off in the presence of a single irreparable DSB (Lisby *et al.*, 2004; Clerici *et al.*, 2006). Furthermore, *SAE2* overexpression impairs both MRX foci formation and checkpoint activation in response to unrepaired DSBs (Clerici *et al.*, 2006). When G1-arrested cell cultures in raffinose containing medium were released from α -factor in the presence of galactose, Rad53 phosphorylation (Figure 1C), *Mre11* association to *TEL* (Figure 1, D and E) and cell cycle arrest (Figure 1A) persisted longer in both *GAL-TEL1 sae2Δ* and *GAL-TEL1 rad50s* cells than in *GAL-TEL1* cells. On the contrary, when G1-arrested *GAL-TEL1 GAL-SAE2* cell cultures in raffinose-containing medium were released from G1 arrest in the presence of galactose, both *TEL* association of *Mre11* (Figure 3, A and B) and Rad53 phosphorylation (Figure 3C, bottom) were strongly reduced compared with *GAL-TEL1* cells under the same conditions. Thus, the lack of *Sae2* and the *rad50s* mutation prevent both checkpoint switch off

Figure 1. Checkpoint activation by ectopic Tel1 expression correlates with Mre11 telomere association. Wild type (wt), *GAL-TEL1*, *GAL-TEL1 mre11Δ*, *GAL-TEL1 sae2Δ*, and *GAL-TEL1 rad50s* cells, carrying *GAL-TEL1* as the only Tel1 source and the *MRE11-MYC18* allele at *MRE11* chromosomal locus, exponentially growing in YEP+raf (raf), were arrested in G1 (αf) and then released from G1 arrest in the presence of galactose. (A) FACS analysis at the indicated times after galactose addition. (B) Genomic DNA was digested with XhoI and hybridized with a poly(GT) telomere-specific probe to detect telomere length. (C) Western blot analysis using anti-Rad53 antibodies at the indicated times after galactose addition. (D) Representative ChIP time course analysis. Soluble chromatin prepared from formaldehyde-treated cells collected at the indicated time points after galactose addition were subjected to immunoprecipitation with anti-Myc antibodies followed by multiplex

Figure 2. Single-strand overhangs in galactose-induced *GAL-TEL1*. *GAL-TEL1* cell cultures exponentially growing in YEP+raf (raf) were shifted to YEP+raf+gal. Genomic DNA prepared at the indicated times after galactose addition was digested with *Xho*I and the single-strand telomere overhangs were visualized by in-gel native hybridization using an end-labeled C-rich oligonucleotide (top). Before hybridization total DNA in the same gel was visualized by ethidium bromide staining to serve as internal loading control (bottom).

and Mre11 dissociation from telomeres in galactose-induced *GAL-TEL1* cells. On the contrary, Sae2 high levels impair both Tel1-induced checkpoint activation and Mre11 telomere association under the same conditions. These data strongly suggest that checkpoint activation under these conditions depends on MRX recruitment at telomeres.

To further support this hypothesis, we asked whether MRX telomere association and checkpoint activation may be limited by high levels of Rif2, a negative regulator of telomere elongation (Hardy *et al.*, 1992; Wotton and Shore, 1997) that we identified as multicopy suppressor of *GAL-TEL1* checkpoint response (Viscardi *et al.*, 2003). Indeed, Mre11 telomere association (Figure 3, D and E), Rad53 phosphorylation and telomere elongation (Figure 3F) were severely impaired in galactose-induced *GAL-TEL1* cells carrying the *RIF2* gene on a 2 μ plasmid compared with otherwise isogenic cells containing the empty vector.

MRX Recruitment at Short Telomeres Activates the Checkpoint Independently of Telomere Elongation

Consistent with defective MRX association at telomeres due to Sae2 excess and with impaired MRX functions caused by

the *sae2* Δ or *rad50s* mutations (Clerici *et al.*, 2005, 2006), telomere elongation was not detectable in galactose-induced *GAL-TEL1 sae2* Δ , *GAL-TEL1 rad50s*, and *GAL-TEL1 GAL-SAE2* cells (Figures 1B and 3C). On the other hand, *GAL-TEL1 sae2* Δ and *GAL-TEL1 rad50s* cells exhibited persistent DNA damage checkpoint activation (Figure 1, A and C). This finding suggests that MRX recruitment at telomeres may signal checkpoint activation independently of telomere elongation. We then asked whether inhibition of telomere elongation without altering MRX recruitment at telomeres could still lead to checkpoint activation after *GAL-TEL1* induction. We could not verify this possibility by deleting the telomerase encoding genes in order to inhibit telomere elongation, because extensive telomere shortening and degradation in *est* Δ cells (Lendvay *et al.*, 1996) caused MRX dissociation from telomeres (data not shown).

We therefore used a system based on the observation that defective resection of an intrachromosomal DSB caused by CDK1 inactivation does not impair MRX association at DSB ends (Ira *et al.*, 2004). Because generation of 3'-ended single-stranded G telomeric tails, which is necessary to elongate telomeres, is dependent on the passage of replication forks (Dionne and Wellinger, 1998; Vodenicharov and Wellinger, 2006), inhibition of replication origin firing may impair telomere elongation without altering MRX recruitment at telomeres. We blocked initiation of DNA replication by preventing prereplicative complex (pre-RC) assembly through depletion of the Cdc6 protein (Piatti *et al.*, 1995; Cocker *et al.*, 1996) in a *GAL-TEL1* strain where the only functional Cdc6 was expressed from the methionine-repressible *MET3* promoter. A *cdc15* mutation in the same strain allowed to uniformly arrest cells in late anaphase in the absence of galactose and methionine. Cells were then released into cell cycle at permissive temperature in the presence of methionine and galactose to fully repress *MET-CDC6* and induce *GAL-TEL1*, respectively. Under these circumstances, cells exited from their late anaphase arrest in the absence of de novo Cdc6 synthesis and, as a consequence, both *MET-CDC6 cdc15* and *MET-CDC6 GAL-TEL1 cdc15* cells failed to replicate DNA (Figure 4A). Moreover, the appearance of cells with less than 1C DNA content ~210–240 min after the shift to 25°C indicated that they eventually underwent a reductional anaphase in the absence of DNA replication, as previously described (Piatti *et al.*, 1995; Figure 4A). Conversely, both *cdc15* and *GAL-TEL1 cdc15* cells expressing *CDC6* from its own promoter entered S phase after the shift to 25°C in the presence of methionine and galactose. *GAL-TEL1 cdc15* cells then eventually arrested with 2C DNA content because of the activation of the DNA damage checkpoint (Figure 4A). As shown in Figure 4B, galactose-induced *GAL-TEL1 cdc15* cells that could enter S phase underwent telomere elongation. On the contrary, preventing Cdc6 synthesis and replication origin firing in similarly treated *MET-CDC6 GAL-TEL1 cdc15* cells did not allow these cells to elongate telomeres (Figure 4B). Strikingly, inhibition of telomere elongation did not prevent either checkpoint activation or MRX association to telomeres. In fact, Rad53 phosphorylation (Figure 4C) and Mre11 association to TEL (Figure 4, D and E) were induced in both *GAL-TEL1 cdc15* and *MET-CDC6 GAL-TEL1 cdc15* cells after methionine and galactose addition. Conversely, Rad53 phosphorylation was below the detection level in similarly treated *cdc15* and *MET-CDC6 cdc15* cells (Figure 4C), which did not show any increase in MRX telomere association (Figure 4, D and E) and did not elongate their already full-length telomeres (Figure 4B and data not shown). Altogether, these data further support the notion that DNA damage checkpoint activation induced by

Figure 1 (cont). PCR amplification. After DNA purification, PCR was carried out with telomeric (TEL), subtelomeric (ADH), and nontelomeric (ARO) primers on DNA from immunoprecipitates (Mre11-IP) or whole cell lysates (Input). Twofold serial dilutions of the input DNA establish the linear range of PCR. Western blot analysis with anti-MYC antibodies of the proteins in the immunoprecipitate (ppt) is also shown. (E) Quantitative analysis of Mre11-telomere association. Data from D are expressed as the relative fold enrichment of TEL over ARO signal independently for each time point and have been normalized to input DNA samples background. In each case bars indicate SE observed in two or more experiments.

Figure 3. SAE2 and RIF2 overexpression prevent both checkpoint activation and Mre11 association to short telomeres after Tel1 induction. (A and B) GAL-TEL1 and GAL-TEL1 GAL-SAE2 cell cultures, carrying the MRE11-MYC18 allele at MRE11 chromosomal locus, exponentially growing in YEP+raf (raf), were arrested in G1 with α -factor (α f) and then released from G1 arrest in the presence of galactose. (A) Representative ChIP time course analysis of the indicated DNA fragments as described in Figure 1D. Mre11 protein levels at each time point were identical to each other (data not shown). (B) Data from A are expressed as Myc tag-dependent fold enrichment of TEL over ARO signal independently for each time point. Bars, SE observed in two or more experiments. (C) Genomic DNA from cells in A was digested with XhoI and hybridized with a poly(GT) telomere-specific probe to detect telomere length (top), and protein extracts from the same cultures were subjected to Western blot analysis with anti-Rad53 antibodies at the indicated times after galactose addition (bottom). (D-F) GAL1-TEL1 [2 μ] and GAL1-TEL1 [2 μ RIF2] cell cultures exponentially growing in SC-ura+raf (raf) were arrested in G1 with α -factor (α f) and then released from G1 arrest in YEP+raf+gal. (D) Representative ChIP time-course analysis of the indicated DNA fragments as described in Figure 1D. Mre11 protein levels at each time point were identical to each other (data not shown). (E) Data from D are expressed as the relative fold enrichment of TEL over ARO signal independently for each time point and have been normalized to input DNA samples background. In each case bars indicate SE observed in two or more experiments. (F) Genomic DNA from cells in D was digested with XhoI and hybridized with a poly(GT) telomere-specific probe to detect telomere length (top), and protein extracts from the same cultures were subjected to Western blot analysis with anti-Rad53 antibodies (bottom).

ectopic Tel1 expression does not require telomere elongation, but correlates with increased MRX association at short telomeres.

MRX Recruitment at a Single Shortened Telomere Is Sufficient To Activate the Checkpoint

Because MRX association to intrachromosomal DSBs is responsible for the subsequent checkpoint activation (Ira *et al.*, 2004; Nakada *et al.*, 2004; Clerici *et al.*, 2006), we asked whether MRX association to a single telomere could trigger checkpoint activation also in the presence of physiological Tel1 levels. To address this issue, we took advantage of a system developed by S. Marcand (CNRS) that allows to reduce the size of a single telomere without affecting the length of the other telomeres in the cell and to follow its subsequent elongation until returning to equilibrium (Marcand *et al.*, 1999). In this system (Figure 5A), generation of one short telomere can be achieved using the yeast strain Lev187, where the left part of chromosome VII beyond the ADH4 gene was replaced by a short stretch of TG₁₋₃ repeats and a URA3 gene, which is in turn flanked by two recognition target sites (FRT) for the recombinase Flp1. The latter can be expressed from the inducible GAL1 promoter.

Because the probability of telomere elongation increases dramatically as distal TG-tract length decreases below 200 bp (Teixeira *et al.*, 2004; Negrini *et al.*, 2007), we used also strains Lev189 and Lev220, which differ from each other and from Lev187 for the length of the distal TG₁₋₃ telomeric repeats, whose mean size is 265 bp in Lev187, 140 bp in Lev189, and 90 bp in Lev220 (Figure 5A; Marcand *et al.*, 1999). This difference is due to the fact that the length regulation mechanism takes into account also the additional 270 bp blocks of TG₁₋₃ telomeric repeats, which are present in single (TEL270) or double copy (2 TEL270) between the URA3 gene and the telomere-proximal Flp1 site in strains Lev189 and Lev220, respectively (Marcand *et al.*, 1997; Figure 5A).

As depicted in Figure 5A, galactose addition leads to the deletion of the internal telomeric tract containing URA3 by an in vivo recombination event triggered by GAL1-FLP1 induction, leaving in the three strains a short telomere, which can be detected 5 h after galactose addition by Southern blot analysis of StuI-digested DNA using an ADH4 probe. The circular DNA molecule that is concomitantly generated cannot be sensed by the checkpoint machinery. When Lev187, Lev189, and Lev220 cells, exponentially

Figure 4. Cdc6 depletion prevents telomere elongation but not checkpoint activation after Tel1 induction. Strains were as follows: *cdc15*, *cdc6Δ MET-CDC6 cdc15*, *GAL-TEL1 cdc15*, and *cdc6Δ MET-CDC6 GAL-TEL1 cdc15* cells, carrying the *MRE11-MYC18* allele at *MRE11* chromosomal locus, growing in synthetic complete medium in the presence of raffinose and without methionine at 25°C (raf 25°C), were arrested in late anaphase at 37°C, the *cdc15* nonpermissive temperature (37°C raf). Cells were then transferred to YEP with 1% galactose and 2 mM methionine at 25°C (+gal +met). (A) FACS analysis of DNA contents at the indicated times in the presence of methionine and galactose. (B) DNA was digested with *XhoI* and hybridized with a poly(GT) telomere-specific probe to detect telomere length. (C) Western blot analysis with anti-Rad53 antibodies at the indicated times in the presence of methionine and galactose. (D) Representative ChIP time-course analysis of the indicated DNA fragments. PCR was carried out as described in Figure 1D with telomeric (TEL) and nontelomeric (ARO) primers on DNA from immunoprecipitates (Mre11-IP) or whole cell lysates (Input). (E) Data from D are expressed as the relative fold enrichment of TEL over ARO signal independently for each time point and have been normalized to input DNA sample backgrounds. In each case results without error bars were observed in two or more experiments. Mre11 protein levels at each time point were identical (data not shown).

Figure 5. The return to equilibrium length of single shortened telomere triggers DNA damage checkpoint activation. Strains Lev187, Lev189, and Lev220, exponentially growing in raffinose (raf), were transferred to 2% galactose to induce *FLP1* expression. (A) Schematic representation of the experimental system used to generate shortened telomeres with different length. The positions of the *StuI* restriction site is indicated and other details are given in the text. (B) Genomic DNA was digested with *StuI* and hybridized with an 840-bp *ADH4* fragment to detect telomere length (probe in A). The DNA molecules detected by this probe are indicated in A with arrows. (C) Western blots of protein extracts prepared at the indicated times after galactose addition were probed with anti-Rad53 antibodies. (D) The percentage of mononucleate large budded cells at the indicated times after galactose addition was determined. (E) Lev220, Lev220 *tel1Δ*, and Lev220 *mec1Δ sml1Δ* cells, exponentially growing in raffinose (raf), were transferred to galactose to induce *FLP1* expression. Western blots of protein extracts prepared at the indicated times after galactose addition were probed with anti-Rad53 antibodies.

growing in raffinose, were shifted to galactose, Flp1 induction led to an artificially shortened telomere of different length in the three strains (Figure 5B). In agreement with the

observation that the probability of telomere elongation increases dramatically as TG-tract length decreases below 200 bp (Teixera *et al.*, 2004; Negri *et al.*, 2007), we found that the

Flp1-induced Lev187 telomere, whose length is essentially equivalent to that of a native telomere, did not show a significant increase in length even 33 h after Flp1 induction (Figure 5B). Conversely, generation of 140- and 90-bp tracts of telomeric TG-repeats in Lev189 and Lev220 cells, respectively, resulted in their subsequent elongation, although to different extents, until they reached a new equilibrium length (Figure 5B). Strikingly, the increase in size of the shortened telomeres correlated with DNA damage checkpoint activation. In fact, Rad53 phosphorylated forms were below the detection level in all uninduced cell cultures and in galactose-induced Lev187 cells, while they became detectable 5 h after galactose addition in both Lev189 and Lev220 cells, and gradually disappeared concomitantly with the decline of telomere elongation rate (Figure 5, B and C). Furthermore, the amount and persistence of phosphorylated Rad53 were sufficient to cause transient accumulation of large budded cells with a single nucleus in Lev220 cells (Figure 5D). The lower amount and persistence of phosphorylated Rad53 in Lev189 cells did not seem sufficient to significantly affect cell cycle progression.

Rad53 phosphorylation in galactose-induced Lev220 cells requires both Mec1 and Tel1. In fact, it was below the

detection level in galactose-induced Lev220 *mec1*Δ cells, and its amount was reduced in Lev220 *tel1*Δ cells compared with wild type under the same conditions (Figure 5E).

Similar to what was observed for checkpoint activation in GAL-TEL1 cells, both telomere elongation and checkpoint activation during the return to equilibrium of the Flp1-shortened telomere depend on MRX and correlate with MRX telomere association. In fact, when Lev220 *rad50*Δ cells exponentially growing in raffinose were shifted to galactose, neither the length of the Flp1-shortened telomere increased, nor Rad53 was phosphorylated (Figure 6, A and B). Moreover, ChIP analysis showed that the association of Mre11 to a unique TEL fragment 80 bp away from telomere VII-L was strongly induced (35-fold) in Lev220 cells 5 h after galactose addition and gradually decreased as telomere elongation rate declined (Figure 6, A, C, and D).

Both Mre11 association to TEL VII-L (Figure 6E) and Rad53 phosphorylation (Figure 6F) were prevented when Flp1 was induced in Lev220 cells that were kept arrested in the G2 phase by nocodazole treatment during galactose addition. Similarly to what was previously observed (Marcand *et al.*, 2000) and consistent with defective MRX-telomere association, the Flp1-induced shortened telo-

Figure 6. Checkpoint activation during elongation of a Flp1-induced shortened telomere requires MRX and correlates with its telomeric association. (A and B) Lev220 and Lev220 *rad50*Δ cells, exponentially growing in raffinose (raf), were transferred to galactose to induce *FLP1* expression. (A) Genomic DNA was digested with *StuI* and hybridized with an 840-bp *ADH* fragment to detect telomere length. (B) Western blots of protein extracts prepared at the indicated times after galactose addition were probed with anti-Rad53 antibodies. (C) Representative ChIP time course analysis. Lev187 and Lev220 cells, carrying the *MRE11-MYC18* allele at *MRE11* chromosomal locus, exponentially growing in raffinose (raf), were transferred to galactose to induce *FLP1* expression and soluble chromatin prepared from formaldehyde-treated cells collected at the indicated time points after galactose addition was subjected to immunoprecipitation with anti-Myc antibodies followed by multiplex PCR amplification with telomeric (TEL) and nontelomeric (ARO) primers on DNA from immunoprecipitates (Mre11-IP) or whole cell lysates (Input). An anti-MYC Western blot of the proteins in the immunoprecipitate (ppt) is also shown. (D) Quantitative analysis of Mre11-telomere association. Data from C are expressed as the relative fold enrichment of TEL over ARO signal independently for each time point and have been normalized to input DNA samples background. In each case bars indicate SE observed in two or more experiments. (E–G) Galactose was added to nocodazole-arrested Lev220 cells that were kept arrested in G2 with nocodazole in the presence of galactose (+galactose +nocodazole) or that were released from the G2 arrest in the presence of galactose (+galactose). (E) Representative ChIP time-course analysis. Soluble chromatin prepared from formaldehyde-treated cells collected at the indicated time points after galactose addition was subjected to immunoprecipitation with anti-Myc antibodies followed by multiplex PCR amplification as described in C. (F) Protein extracts from cells in E that were kept arrested in the presence of nocodazole during galactose addition were subjected to Western blot analysis with anti-Rad53 antibodies. (G) Genomic DNA from cells in E that were kept arrested in G2 in the presence of nocodazole during galactose addition was digested with *StuI* and hybridized with an 840-bp *ADH4* fragment to detect telomere length.

mere did not elongate in these cells (Figure 6G). This result contrasts with the observation that an HO-induced short telomere can be elongated to some extent in nocodazole-arrested cells (Diede and Gottschling, 2001). We believe this discrepancy might be due to the different systems used to generate the short telomere. In agreement with previous findings indicating that MRX binds to telomeres only during S phase (Takata *et al.*, 2005), our data suggest that only S phase short telomeres are suitable to recognition by MRX and checkpoint signaling in the presence of physiological Tel1 levels.

DISCUSSION

Telomeres differ from DSBs because they do not induce a DNA damage response. However, proteins required for the latter are intimately involved in the regulation of telomere function, suggesting a time window where functional telomeres can be recognized as DNA breaks. By using two different systems, here we provide evidence that telomeres elicit an MRX-dependent DNA damage response when they become competent for elongation. Specifically, ectopic Tel1 induction in cells with otherwise stable short telomeres results in telomere elongation and checkpoint activation. Similarly, conversion into a full-length telomere of a single Flp1-induced telomere with short TG tracts parallels DNA damage checkpoint activation. In both cases, checkpoint activation correlates with telomere elongation, strongly suggesting that telomeres are dealt with in a similar manner as DSBs during the time of their replication.

We also show that the MRX complex, which is required in both the systems above for checkpoint activation and telomere elongation, is recruited to short telomeric ends concomitantly with their elongation and checkpoint activation. Furthermore, both the lack of Sae2 and the *rad50s* mutation increase MRX persistence at short telomeres and prevent checkpoint switch off after Tel1 induction. On the contrary, Sae2 high levels reduce MRX telomere association and impair checkpoint activation in *GAL-TEL1* cells. This strongly suggests that checkpoint activation during telomere elongation can be ascribed to MRX recruitment at telomeres.

Interestingly, the MRX complex is not required to activate the checkpoint when telomeres undergo extensive shortening due to the lack of telomerase or when they are exposed to exonuclease degradation leading to ssDNA accumulation due to the lack of Cdc13 (Ijima and Greider, 2003; Foster *et al.*, 2006). This indicates that elongating telomeres generate

checkpoint signals that are different from those of uncapped telomeres. Consistent with this hypothesis, we found that accumulation of ssDNA at telomeres is not likely responsible for the MRX-dependent checkpoint activation at elongating telomeres.

The specific roles of Mec1 and Tel1 in activating the MRX-dependent checkpoint during elongation of multiple short telomeres is difficult to assess, because high Tel1 levels can bypass Mec1 requirement (Clerici *et al.*, 2001). On the other hand, we found that both Mec1 and Tel1 contribute to checkpoint activation in response to elongation of a single short telomere. This situation is reminiscent of the checkpoint activated by a single DSB, where MRX mediates the recruitment of Tel1 at the DSB ends (Nakada *et al.*, 2003) and is necessary to activate the Mec1-dependent pathway, possibly by allowing generation of RPA-coated ssDNA (Zou and Elledge, 2003; Nakada *et al.*, 2004; Mantiero *et al.*, 2007). Once MRX is recruited at telomeres, checkpoint activation does not require telomere elongation. In fact, the *sae2Δ* and *rad50s* alleles, which cause MRX persistence at short telomeres and do not allow DNA damage checkpoint switch off in galactose-induced *GAL-TEL1* cells, also prevent telomere elongation in the same cells, possibly by altering MRX nuclease activity (Clerici *et al.*, 2005, 2006). Furthermore, we found that inhibition of replication origin firing impairs telomere elongation in galactose-induced *GAL-TEL1* cells without affecting either MRX association at short telomeres or checkpoint activation. These data indicate that telomere-bound MRX is sufficient to activate the checkpoint independently of telomere elongation, suggesting that MRX binding at short telomeres is the signaling event for checkpoint activation. They also imply that only telomeres that become susceptible to be bound by MRX and, therefore, suitable for elongation, can be recognized as DSBs by the checkpoint machinery. Indeed, MRX was shown to be enriched at telomeres during S phase (Zhu *et al.*, 2000; Takata *et al.*, 2005), and only telomeres with short TG tracts are avidly bound by MRX, as well as by the telomerase enzyme (Negrini *et al.*, 2007; this study).

In this context, our results indicate that, under unperturbed conditions, only S phase telomeres are potentially detectable as DSBs by the checkpoint machinery (Figure 7). However, the yeast telomerase enzyme only acts on short telomeres within one cell cycle (Teixera *et al.*, 2004), and the rate of telomere elongation appears limited to a few base pairs per generation (Marcand *et al.*, 1999). This limitation may prevent unscheduled checkpoint activation during an

Figure 7. A model for DNA damage checkpoint activation by short telomeres. During the time of telomere replication (S phase), only short telomeres are suitable to MRX binding and elongation. Telomere-bound MRX, besides triggering telomere elongation, can activate a Mec1- and Tel1-dependent checkpoint. Checkpoint activation, which is independent of telomere elongation and ssDNA generation, takes place only when MRX remains bound at telomeres long enough to be sensed by the checkpoint machinery. MRX access, telomere elongation, and checkpoint activation are instead prevented at full-length telomeres.

unperturbed S phase, because the checkpoint signals do not persist long enough to be detected by the checkpoint machinery.

MRX access, telomere elongation, and checkpoint activation are instead prevented at long or full-length telomeres, suggesting that the latter have acquired a structure that may physically hide the telomeric ends from MRX and telomerase. This protective cap can be formed immediately after DNA replication and maintained until the next S phase. Although the precise molecular nature of this capped structure is unknown, we have found that high levels of Rif2 inhibit both telomere elongation and checkpoint activation in galactose-induced *GAL-TEL1* cells. Because it has been shown that Rap1 is necessary to suppress both Cdc13 and MRX recruitment at long TG tracts (Negrini *et al.*, 2007), this suggests that Rap1, Rif1, and Rif2 might favor the formation of a t-loop structure similar to the one observed in many other organisms (de Lange, 2005).

In conclusion, we have shown that telomeres behave similarly to intrachromosomal DSBs when they are suitable for elongation. This biological response appears to be conserved throughout evolution, because functional human telomeres have been shown to undergo a structural change that elicits a DNA damage response during or after DNA replication (Verdun *et al.*, 2005; Verdun and Karlseder, 2006).

ACKNOWLEDGMENTS

We thank J. Diffley, S. Marcand, and C. Santocanale for providing antibodies and yeast strains; S. Piatti for providing yeast strains and for critical reading of the manuscript; and all the members of our laboratory for useful discussions and criticisms. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro to M.P.L. and Cofinanziamento 2005 MIUR/Università di Milano-Bicocca to G.L. V.V. and H.C. were supported by the fellowship Ninni Gattuso from Fondazione Italiana per la Ricerca sul Cancro and by an EC Research Training Network Grant (HPRN-CT-2002-00238), respectively. M.P.L. dedicates this work to Giorgio Terragni, who was born on 1 November 2006.

REFERENCES

- Alani, E., Padmore, R., and Kleckner, N. (1990). Analysis of wild-type and *rad50* mutants of yeast suggests an intimate relationship between meiotic chromosome synapsis and recombination. *Cell* 61, 419–436.
- Bi, X., Wei, S. C., and Rong, Y. S. (2004). Telomere protection without a telomerase; the role of ATM and Mre11 in *Drosophila* telomere maintenance. *Curr. Biol.* 14, 1348–1353.
- Chakhpourian, M., and Wellinger, R. J. (2003). Telomere maintenance and DNA replication: how closely are these two connected? *Trends Genet.* 19, 439–446.
- Ciapponi, L., Cenci, G., Ducau, J., Flores, C., Johnson-Schlitz, D., Gorski, M. M., Engels, W. R., and Gatti, M. (2004). The *Drosophila* Mre11/Rad50 complex is required to prevent both telomeric fusions and chromosome breakage. *Curr. Biol.* 14, 1360–1366.
- Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P. (2005). The *Saccharomyces cerevisiae* Sae2 protein promotes resection and bridging of double strand break ends. *J. Biol. Chem.* 280, 38631–38638.
- Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P. (2006). The *Saccharomyces cerevisiae* Sae2 protein negatively regulates DNA damage checkpoint signalling. *EMBO Rep.* 7, 212–218.
- Clerici, M., Paciotti, V., Baldo, V., Romano, M., Lucchini, G., and Longhese, M. P. (2001). Hyperactivation of the yeast DNA damage checkpoint by *TEL1* and *DDC2* overexpression. *EMBO J.* 20, 6485–6498.
- Cocker, J. H., Piatti, S., Santocanale, C., Nasmyth, K., and Diffley, J. F. (1996). An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature* 379, 180–182.
- d'Adda di Fagnana, F., Teo, S. H., and Jackson, S. P. (2004). Functional links between telomeres and proteins of the DNA damage response. *Genes Dev.* 18, 1781–1799.
- de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* 19, 2100–2110.
- Diede, S. J., and Gottschling, D. E. (2001). Exonuclease activity is required for sequence addition and Cdc13p loading at a de novo telomere. *Curr. Biol.* 11, 1336–1340.
- Dionne, I., and Wellinger, R. J. (1996). Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. *Proc. Natl. Acad. Sci. USA* 93, 13902–13907.
- Dionne, I., and Wellinger, R. J. (1998). Processing of telomeric DNA ends requires the passage of a replication fork. *Nucleic Acids Res.* 26, 5365–5371.
- Foster, S. S., Zubko, M. K., Guillard, S., and Lydall, D. (2006). MRX protects telomeric DNA at uncapped telomeres of budding yeast *cdc13-1* mutants. *DNA Repair* 5, 840–851.
- Garvik, B., Carson, M., and Hartwell, L. L. (1995). Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the *RAD9* checkpoint. *Mol. Cell Biol.* 15, 6128–6138.
- Goudsouzian, L. K., Tuzon, C. T., Zakian, V. A. (2006). *S. cerevisiae* Tel1p and Mre11p are required for normal levels of Est1p and Est2p telomere association. *Mol. Cell* 24, 603–610.
- Grandin, N., Damon, C., and Charbonneau, M. (2001). Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13. *EMBO J.* 20, 1173–1183.
- Grandin, N., Reed, S. I., and Charbonneau, M. (1997). Stn1, a new *Saccharomyces cerevisiae* protein, is implicated in telomere size regulation in association with Cdc13. *Genes Dev.* 11, 512–527.
- Gravel, S., Larrievée, M., Labrecque, P., and Wellinger, R. J. (1998). Yeast Ku as a regulator of chromosomal DNA end structure. *Science* 280, 741–744.
- Hardy, C. F., Sussel, L., and Shore, D. (1992). A Rap1-interacting protein involved in silencing and telomere length regulation. *Genes Dev.* 6, 801–814.
- Hug, N., and Lingner, J. (2006). Telomere length homeostasis. *Telomere length homeostasis. Chromosoma* 115, 413–425.
- Ijima, A. S., and Greider, C. W. (2003). Short telomeres induce a DNA damage response in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 14, 987–1001.
- Ira, G. *et al.* (2004). DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature* 431, 1011–1017.
- Karlseder, J., Hoke, K., Mirzoeva, O. K., Bakkenist, C., Kastan, M. B., Petrini, J. H., and de Lange, T. (2004). The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. *PLoS Biol.* 2, E240, 1150–1156.
- Larrievée, M., LeBel, C., and Wellinger, R. J. (2004). The generation of proper constitutive G-tails on yeast telomeres is dependent on the MRX complex. *Genes Dev.* 18, 1391–1396.
- Lendvay, T. S., Morris, D. K., Sah, J., Balasubramanian, B., and Lundblad, V. (1996). Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional EST genes. *Genetics* 144, 1399–1412.
- Lisby, M., Barlow, J. H., Burgess, R. C., and Rothstein, R. (2004). Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. *Cell* 118, 699–713.
- Lobachev, K. S., Gordenin, D. A., and Resnick, M. A. (2002). The Mre11 complex is required for repair of hairpin-capped double-strand breaks and prevention of chromosome rearrangements. *Cell* 108, 183–193.
- Longhese, M. P., Mantiero, D., and Clerici, M. (2006). The cellular response to chromosome breakage. *Mol. Microbiol.* 60, 1099–1108.
- Lydall, D., and Weinert, T. (1995). Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. *Science* 270, 1488–1491.
- Mantiero, D., Clerici, M., Lucchini, G., and Longhese, M. P. (2007). Dual role for *S. cerevisiae* Tel1 in the checkpoint response to double-strand breaks. *EMBO Rep.* 8, 380–387.
- Marcand, S., Brevet, V., and Gilson, E. (1999). Progressive cis-inhibition of telomerase upon telomere elongation. *EMBO J.* 18, 3509–3519.
- Marcand, S., Brevet, V., Mann, C., and Gilson, E. (2000). Cell cycle restriction of telomere elongation. *Curr. Biol.* 10, 487–490.
- Marcand, S., Gilson, E., and Shore, D. (1997). A protein-counting mechanism for telomere length regulation in yeast. *Science* 275, 986–990.
- Metcalfe, J. A., Parkhill, J., Campbell, L., Stacey, M., Biggs, P., Byrd, P. J., and Taylor, A. M. (1996). Accelerated telomere shortening in ataxia telangiectasia. *Nat. Genet.* 13, 350–353.
- Michelson, R. J., Rosenstein, S., and Weinert, T. (2005). A telomeric repeat sequence adjacent to a DNA double-stranded break produces an antieckpoint. *Genes Dev.* 19, 2546–2559.

- Nakada, D., Hirano, Y., and Sugimoto, K. (2004). Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway. *Mol. Cell. Biol.* 24, 10016–10025.
- Nakada, D., Matsumoto, K., and Sugimoto, K. (2003). ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism. *Genes Dev.* 16, 1957–1962.
- Negrini, S., Ribaud, V., Bianchi, A., and Shore, D. (2007). DNA breaks are masked by multiple Rap1 binding in yeast: implications for telomere capping and telomerase regulation. *Genes Dev.* 21, 292–302.
- Pardo, B., and Marcand, S. (2005). Rap1 prevents telomere fusions by nonhomologous end joining. *EMBO J.* 24, 3117–3127.
- Piatti, S., Lengauer, C., and Nasmyth, K. (1995). Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae*. *EMBO J.* 14, 3788–3799.
- Rattray, A. J., McGill, C. B., Shafer, B. K., and Strathern, J. N. (2001). Fidelity of mitotic double-strand-break repair in *Saccharomyces cerevisiae*: a role for *SAE2/COM1*. *Genetics* 158, 109–122.
- Ritchie, K. B., and Petes, T. D. (2000). The Mre11/Rad50/Xrs2 complex and the Tel1 function in a single pathway for telomere maintenance in yeast. *Genetics* 155, 475–479.
- Ritchie, K. B., Mallory, J. C., and Petes, T. D. (1999). Interactions of *TLC1* (which encodes the RNA subunit of telomerase), *TEL1*, and *MEC1* in regulating telomere length in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 19, 6065–6075.
- Rose, M. D., Winston, F., and Hieter, P. (1990). *Methods in Yeast Genetics*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Shiloh, Y. (2006). The ATM-mediated DNA-damage response: taking shape. *Trends Biochem. Sci.* 31, 402–410.
- Silva, E., Tiong, S., Pedersen, M., Homola, E., Royou, A., Fasulo, B., Siriaco, G., and Campbell, S. D. (2004). ATM is required for telomere maintenance and chromosome stability during *Drosophila* development. *Curr. Biol.* 14, 1341–1347.
- Smogorzewska, A., and de Lange, T. (2004). Regulation of telomerase by telomeric proteins. *Annu. Rev. Biochem.* 73, 177–208.
- Takata, H., Kanoh, Y., Gunge, N., Shirahige, K., and Matsuura, A. (2004). Reciprocal association of the budding yeast ATM-related proteins Tel1 and Mec1 with telomeres in vivo. *Mol. Cell* 14, 515–522.
- Takata, H., Tanaka, Y., and Matsuura, A. (2005). Late S phase-specific recruitment of Mre11 complex triggers hierarchical assembly of telomere replication proteins in *Saccharomyces cerevisiae*. *Mol. Cell* 17, 573–583.
- Teixeira, M. T., Arneric, M., Sperisen, P., and Lingner, J. (2004). Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states. *Cell* 117, 323–335.
- Tsukamoto, Y., Taggart, A.K.P., and Zakian, V. A. (2001). The role of the Mre11-Rad50-Xrs2 complex in telomerase-mediated lengthening of *Saccharomyces cerevisiae* telomeres. *Curr. Biol.* 11, 1328–1335.
- Verdun, R. E., and Karlseder, J. (2006). The DNA damage machinery and homologous recombination pathway act consecutively to protect human telomeres. *Cell* 127, 709–720.
- Verdun, R. E., Crabbe, L., Haggblom, C., and Karlseder, J. (2005). Functional human telomeres are recognized as DNA damage in G2 of the cell cycle. *Mol. Cell* 20, 551–561.
- Viscardi, V., Baroni, E., Romano, M., Lucchini, G., and Longhese, M. P. (2003). Sudden telomere lengthening triggers a Rad53-dependent checkpoint in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 14, 3126–3143.
- Viscardi, V., Clerici, M., Cartagena-Lirola, H., and Longhese, M. P. (2005). Telomeres and DNA damage checkpoints. *Biochimie* 87, 613–624.
- Vodenicharov, M. D., and Wellinger, R. J. (2006). DNA degradation at unprotected telomeres in yeast is regulated by the CDK1 (Cdc28/Clb) cell-cycle kinase. *Mol. Cell* 24, 127–137.
- Wotton, D., and Shore, D. (1997). A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in *Saccharomyces cerevisiae*. *Genes Dev.* 11, 748–760.
- Zhu, X. D., Kuster, B., Mann, M., Petrini, J. H., and de Lange, T. (2000). Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat. Genet.* 25, 347–352.
- Zou, L., and Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 300, 1542–1548.