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Origin-dependent initiation of DNA replication within telomeric sequences

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ABSTRACT

Replication of telomeres requires the action of telomerase, the semi-conservative replication machinery and the stabilization of the replication fork during passage through telomeric DNA. Whether vertebrate telomeres support initiation of replication has not been experimentally addressed. Using *Xenopus* cell free extracts we established a system to study replication initiation within linear telomeric DNA substrates. We show binding of TRF2 to telomeric DNA, indicating that exogenous DNA exclusively composed of telomeric repeats is recognized by shelterin components. Interaction with telomere binding proteins is not sufficient to prevent a DNA damage response. Notably, we observe regulated assembly of the pre-replicative complex proteins ORC2, MCM6 and Cdc6 to telomeric DNA. Most importantly, we detect origin-dependent replication of telomeric substrates under conditions that inhibit checkpoint activation. These results indicate that pre-replicative complexes assemble within telomeric DNA and can be converted into functional origins.

INTRODUCTION

In vertebrates, telomeric DNA is composed of 5–50 kb of repetitive arrays of TTAGGG. These sequences are recognized by a protein complex called 'shelterin', which is essential for telomere end-protection and length regulation (1). Loss of telomeric proteins or shortening of telomeres beyond a critical length triggers a DNA damage response characterized by the recruitment of DNA damage response proteins to telomeric ends and the activation of checkpoints, which lead to senescence or apoptosis (1). Complete and faithful replication of telomeric DNA is essential to maintain chromosome stability and for cell cycle progression. However, little is known about the molecular mechanisms that underlie replication initiation and progression of the semi-conservative replication machinery through telomeric DNA. Telomeres are challenging structures to replicate due to their repetitive sequences and the structures they can adopt including G-quadruplexes and heterochromatin (2). In yeast and human cells, replication forks naturally stall at telomeric DNA (3,4), indicating that telomeric DNA is replicated slowly. TRF1 and TRF2 inhibit replication fork progression in an *in vitro* replication system of SV40 DNA, (5), whereas in fission yeast, absence of Taz1 induces replication fork stalling and entanglement of telomeres (4). In *Saccharomyces cerevisiae,* replication is initiated within autonomously replicating sequences (ARS), which are bound by the origin recognition complex (ORC) in a sequence specific manner (6). The origins used to replicate telomeric DNA lie in the subtelomeric region and origins are not fired within telomeric sequences (7,8). In contrast, initiation of DNA replication in humans, Drosophila and *Xenopus,* is mostly sequence independent and multiple factors including sequence bias, chromatin structure, DNA methylation patterns, transcriptional activities and protein chaperones participate in the selection of replication origins (9). Notably, binding of transcription factors increase site-specific origin firing, indicating that the local chromatin structure significantly affects origin selection (12). A number of DNA substrates containing random DNA sequences injected in *Xenopus leavis* eggs initiates replication efficiently at random locations (10,11). However, it has not been shown that DNA templates adopting a non-canonical chromatin structure including centromeric or telomeric DNA replicate in *Xenopus* eggs. Recent findings from studies in mammalian cells show that pre-replicative complex (pre-RC) proteins localize to telomeres through interaction to TRF2 (13,14). Whether these pre-RCs represent functional origins is not known.

Cell-free extracts from *X. laevis* unfertilized eggs contain nuclear and cytoplasmic proteins to support 12 cell divisions in the absence of transcription and have been instrumental to the study of DNA transactions

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including DNA damage response and DNA replication (15,16). When supplemented with sperm chromatin, cytosolic extracts support nuclear assembly followed by one round of cell cycle regulated, semi-conservative DNA replication (17). Origin assembly starts with binding of ORC proteins followed by Cdc6- and Cdt1-dependent loading of MCM helicase. Geminin, a protein that sequesters Cdt1 prevents origin assembly and origin-dependent DNA replication (18). Protein kinases activate this pre-RC to permit Cdc45, MCM10, GINS and polymerases to load.

Xenopus embryonic cells replicate their genome in less than 20 min and a replication fork should not travel more than 12 kb at a synthesis rate of 10 nt/s (19,20). *Xenopus* telomeres range from 10 kb to over 50 kb (21), making their replication originating uniquely from subtelomeric origins problematic. Given their length and inherent difficult replication, it would be beneficial to establish active origins within telomeric DNA.

To test this possibility, we used *X. laevis* cell-free extracts supplemented with exogenous linear DNA substrates containing exclusively telomeric repeats. We show that these substrates are specifically bound by TRF2, support the regulated assembly of pre-RC components and undergo origin-dependent DNA replication. Binding of shelterin components, however, is not sufficient to prevent a DNA damage response induced by the relatively short telomeric substrates. We establish that telomeric DNA supports the assembly and activation of functional origins.

MATERIALS AND METHODS

Cell free extracts

Cell-free extracts from unfertilized *Xenopus* eggs were prepared as described (28).

Cloning of non-telomeric substrate

A non-telomeric (NT) control plasmid pRST5_NT was generated by PCR amplification of positions 666–1254 of *Xenopus* XLX gene and cloned into the HindIII and BamHI restriction sites of pRST5.

Preparation of biotinylated substrates

One microgram of telomeric or NT DNA fragment (gel extracted from BsmBI and HindIII digested pRST5 or pRST5_NT, respectively) was end-labeled with 1 U T4 polymerase in the presence of 33 μM each of dATP, dGTP, dTTP and biotin-dCTP for 15 min at 12°C. Reactions were stopped by addition of 50 mM EDTA and incubated at 76°C. Labelled DNA was purified using PCR purification kit (Qiagen) and quantified by photospectrometry.

Pull-down experiments

Three hundred nanograms of end-labeled 0.6 kb linear NT or exclusively telomeric DNA was bound to 10 μl Streptavidin-bound magnetic beads according to the supplier (Dynal). Washed beads were resuspended in 11 μl dH_2O. One microliter was analyzed on gel electrophoresis using SYBR-gold to visualize bound DNA to quantify binding efficiency. In total, 5–10 μl beads were incubated with 90 μl egg cytosol (LSS) at a final concentration of $3x10^9$ double strand breaks (DSB)/μl for 20 min at 22°C. Beads were pelleted in a table top centrifuge for 10 sec at 1500 rpm prior to separation from the supernatant on a magnet. Beads were washed four times with 200 μl ELB, 0.2% Triton-X and resuspended in 10 μl Laemmli buffer for SDS-PAGE analysis. Pull-down experiments in the NPE system were performed by incubating 2 μl membrane-free egg cytosol (HSS) supplemented with energy mix (10 mM Creatin phosphate, 10 μg/ml Creatin kinase, 2 mM ATP, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.5, 1 mM DTT) for 30 min in the presence of 150 ng bead-bound 0.6 kb linear DNA and either buffer control or 100 ng/μl geminin. Two-fold volumes of NPE extract supplemented with energy mix was added and incubation continued for 25 min prior to processing as described above.

Western blot analysis

One microliter of extract was diluted in loading buffer, electrophoresed, transferred to nitrocellulose and probed with polyclonal antibodies specific for *Xenopus* TRF2 (a generous gift from Dr Ishikawa), Cdc6, Orc2, MCM6 (48), Mre11 (25), ATM (26), Nbs1 (a generous gift of Dr H. Lindsay) and human Ku70 (MMS-263R, Covance) and P-Chk1 (Ser345 polyclonal, Cell Signaling). Bead bound substrates were directly dissolved in 10 μl loading buffer.

Preparation of NHEJ substrates

In total, 0.6 kb of telomeric substrate was excised from pRST5 by DdeI and BsmBI digestion. NT fragment was excised by HindIII and BsmBI. Digested products were gel extracted.

NHEJ assays

A typical NHEJ reaction consisted of 9 μl egg cytosol (LSS) and 1 μl DNA substrate. Samples were incubated at 17°C for 2.5 h. Reactions were stopped by the addition of 200 μl stop solution (10 mM Tris, pH 7.5, 5 mM EDTA and 1% SDS) and digested with 1 mg/ml proteinase K (Roche) at 50°C for 1 h. DNA was Phenol-Chloroform extracted and Ethanol precipitated. DNA pellets were resuspended in 10 μl TE, pH 7.5. 2–5 μl of extracted DNA was separated on a 0.7% native Agarose gel.

Southern blot analysis

Agarose gels were depurinated in 0.2 M HCl for 10 min and washed briefly with dH_2O. DNA was denatured by incubating the gel in 100 ml 1.5 M NaCl, 0.5 M NaOH for twice 15 min and neutralized in 1.5 M NaCl, 1 M Tris, pH 7.4 for twice for 15 min. DNA was transferred on Nylon membranes (Hybond-XL, Amersham) by capillary blotting in 10× SSC and membranes were UV-crosslinked. NT probes were prepared by random labeling

of 10 ng of pRST5_NT in the presence of 50 μCi (α-^{32}P)dCTP and hybridized in Hybridisation buffer at 60°C overnight. Telomeric probes were generated by 5′-end-labelling of 100 pmol of a $(C_3TA_2)_6$ oligonucleotide with 5U of PNK in the presence of 50 μCi of (γ-^{32}P)dATP. Hybridisation was for 3 h at 50°C. Membranes were rinsed with 2× SSC, 0.1% SDS and washed twice with 0.5× SSC, 0.1% SDS for 20 min and once with 0.1× SSC, 0.1% SDS. Membranes were exposed to a phosphorimager and signals quantified using ImageQuant. For quantification, the mean value from three independent experiments of the ratio between LD and LM was calculated from dilutions 3×10^9, 10^9 and 3×10^9 DSB/μl.

Replication assays

Caffeine (Sigma) was dissolved in 10 mM PIPES. Five nanograms of pRST5_NT linearized with BsmBI or 5 ng of a 0.6 kb NT or telomeric fragment excised with HindIII and BsmBI from pRST5 or pRST5_NT, respectively, was incubated with membrane-free egg cytosol (HSS) supplemented with energy mix for 30 min in the presence of either buffer control, 5 mM caffeine, 100 ng/μl geminin or caffeine and geminin together at 22°C. Two-fold volumes of NPE extract supplemented with energy mix and 0.1 μl (α-^{32}P)dCTP was added and incubation continued for 1 h. Reactions were stopped by addition of 200 μl stop solution (10 mM Tris, pH 7.5, 5 mM EDTA and 1% SDS) and incubated with 1 mg/ml proteinaseK at 50°C for 1 h. Samples were phenol-chloroform extracted and ammonium acetate precipitated in the presence of 5 μg Glycogen. Precipitated DNA was resuspended in 10 μl TE and half of the reaction was separated on a 1% native Agarose gel and fixed in 50% TCA for 30 min before squeezing the gel and drying under vacuum. Gels were exposed to a phosphorimager screen and signals were quantified using a phosphorimager and ImageQuant software.

RESULTS

Generation and characterization of telomeric substrates

Because telomeres only represent a minor part of chromosomal DNA and are composed of the same repeated sequence elements, mapping telomeric sites of DNA replication initiation using current technologies has been challenging. To circumvent this limitation, we established a system to monitor replication of linear DNA substrates with defined sequence compositions and to characterize the proteins they are associated with. To prepare telomeric DNA substrates, the 96 telomeric repeats present in pRST5 were excised by restriction digest with HindIII/BsmBI (Figure 1A). This generates 0.6 kb long linear substrates, which contain exclusively telomeric DNA. To characterize proteins bound to DNA, the 5′ overhangs were filled-in with T4 DNA polymerase, dNTP's and biotinylated dCTP, which labels the 3′-end of the C-rich strand and leaves the opposite end blunt and unlabeled. Biotinylated substrates were immobilized on streptavidin beads before addition to extracts. A control plasmid (pRST5_NT) yields a NT DNA sequence of similar length upon restriction digest.

Using these short biotinylated NT or telomeric (T) DNA substrates immobilized on beads, we first monitored binding of associated proteins. The telomere binding protein TRF2 was specifically enriched on the telomeric substrate (Figure 1B, lane 5), indicating that these templates are recognized as telomeric DNA. The conservation of a stable multiprotein telomeric complex throughout different species (1,22) and the high homology between *Xenopus* and human TRF1 and TRF2 (23) supports the idea that a stable multisubunit telomeric complex also exist in *Xenopus* and that other components of the TRF2 complex were bound to telomeric sequences. Notably, incubation of bead-bound DNA did not deplete TRF2 from the supernatant (Figure 1B, lane 2), indicating that the amount of TRF2 present in extracts is not rate-limiting for the amount of DNA used. Our observations are consistent with the reported binding of ^{35}S-labeled xTRF1 protein to telomeric DNA in cell-free extracts (23).

Linear DNA substrates incubated in cytosolic extracts are recognized as DNA double-strand breaks (DSBs) and induce a rapid DNA damage response, which is characterized by recruitment of the Mre11/Rad50/Nbs1 (MRN) complex to DNA ends followed by activation of ATM at sites of DSBs, cell cycle checkpoint activation and repair of broken DNA through NHEJ (24–26). Interestingly, a number of DNA damage response proteins including ATM, MRN, Ku70/80 heterodimer, DNA-PKcs, ERCC1 and RAD51 associate with telomeres during a normal cell cycle and are important for telomere protection and telomere length homeostasis (1,27). We monitored binding of DNA damage proteins to the 0.6 kb linear telomeric substrates compared to NT control DNA. As expected, we observed ATM, Mre11 and Ku70 assembly on NT DNA (Figure 1B) and consistent with previous observations, the binding of repair proteins to telomeric DNA, which occurred with similar efficiency.

Origin-dependent assembly of pre-RC components on telomeric DNA

Next, we wanted to determine whether components of the pre-replicative complex assemble onto telomeric DNA. We incubated the immobilized 0.6 kb biotinylated telomeric and NT DNA substrates in cytosolic extracts for 20 min and monitored binding of pre-RC components. ORC2, Cdc6 and MCM6 proteins loaded on NT and telomeric DNA with similar efficiency (Figure 2A, lanes 4 and 5), indicating that neither the structure and the sequence of the DNA, nor the presence of the shelterin component TRF2 influence significantly pre-RC assembly.

Next, we wanted to determine whether the assembled pre-RC proteins could be converted into pre-initiation complexes that support origin firing and DNA replication. Cytosolic extracts faithfully recapitulate DNA damage signaling and pre-RC assembly but fail to support origin-dependent replication of small DNA templates.

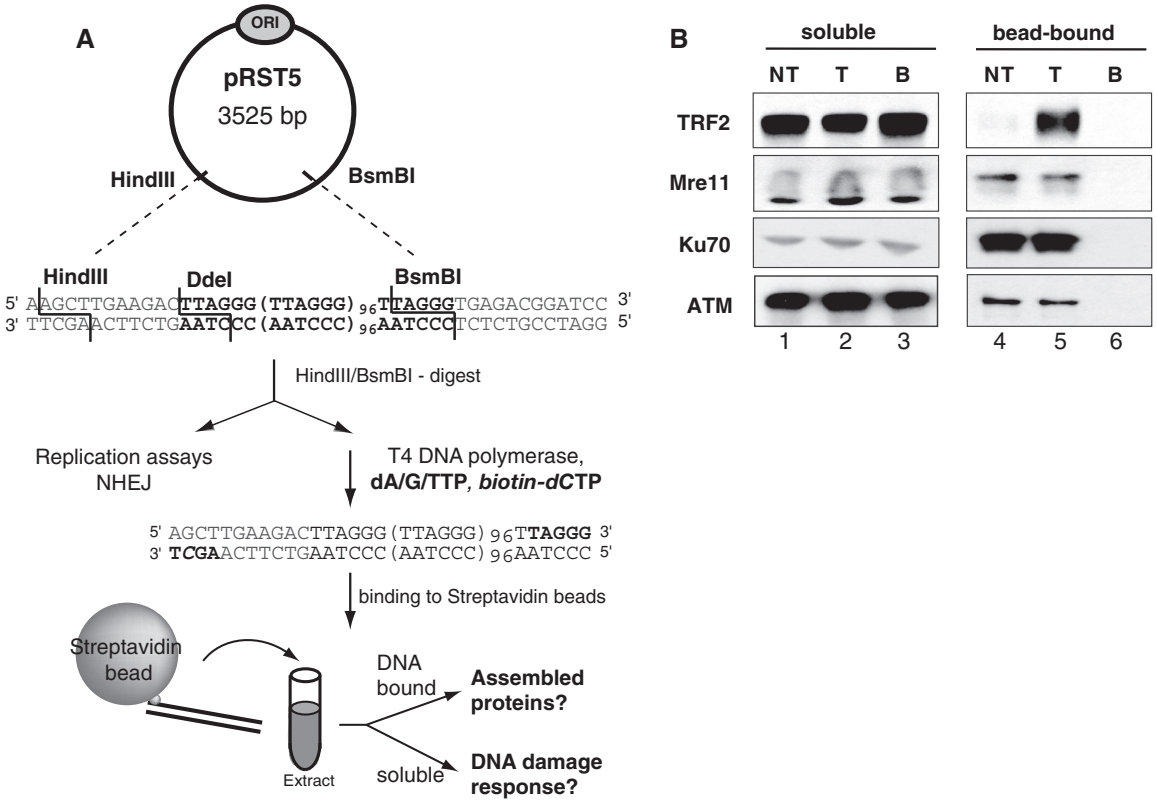

Figure 1. Generation and characterization of telomeric DNA substrates. (**A**) pRST5 contains an insert of 98 telomeric repeats (bold), which is excised by BsmBI/HindIII to generate 0.6 kb linear substrates containing exclusively telomeric DNA. Overhangs are filled by T4 DNA polymerase, dA/G/TTP and biotinylated dCTP. After incubation of immobilized substrates for 20 min in cytosolic extracts, proteins in the soluble fraction or bound to DNA are monitored by western blotting. Non-biotinylated DNA is used for replication and NHEJ assays. A NT control plasmid pRST5_NT harbors a random sequence of similar length. (**B**) Bead-bound NT or telomeric (T) DNA was incubated with cytosolic extracts at 2×10^9 ends/µl. Soluble fractions were separated (lanes 1–3), beads washed and bead-bound fractions (lanes 4–6) analyzed by western blotting. Lanes 3 and 6, beads only.

We therefore turned to a two-step cell-free extract system that uses concentrated nuclear extract (NPE), which contains high kinase activities to support conversion of a pre-RC into a functional initiation complex on plasmid DNA templates (28). Previous studies showed that MCM3 and ORC2 load on linear dsDNA templates as short as 100 bp in length and addition of NPE converts DNA templates into functional pre-initiation complexes as seen by binding of Cdc45 (29). This suggests that the relatively short length of our substrates (600 bp) should allow us to assess DNA replication initiation. We monitored the binding of pre-RC proteins following incubation of immobilized DNA substrates in NPE (Figure 2B). Consistent with the results obtained with cytosolic extracts (Figures 1B and 2A), TRF2 was enriched on telomeric substrates and similar levels of ORC2 and MCM6 assembled on both templates (Figure 2B, lanes 4 and 5) A non specific protein signal from the Ponceau stain was used to normalize the signals for quantification (Figure 2C). Importantly, treatment with geminin, which prevents the assembly of MCM proteins through sequestering Cdt1 (18), abolished MCM but not ORC loading, as anticipated (Figure 2B, lanes 7 and 8). Our observations establish that the presence of TRF2 on DNA does not interfere with assembly of a pre-RC and suggest that cell cycle regulated loading of MCMs to telomeric DNA and to *bona fide* origins are regulated by similar mechanisms. It further indicates that pre-RC assembly to telomeric DNA is not due to non-specific binding to telomeric chromatin. We consistently observe increased ORC2 loading in geminin treated extracts, with a more pronounced ORC2 association on NT DNA substrates (Figure 2C). It is possible that TRF2 or other shelterin components are modulating some of the steps during pre-RC assembly. Overall, our data are consistent with *in vivo* studies showing cell cycle regulated assembly of pre-RC proteins to telomeres (13) and with the recent report of active origins within telomeres in mouse embryo fibroblasts (30).

A number of components of the DNA damage response including ATM, Nbs1/Mre11/Rad50 or PARP1 harbor a conserved FxLxP motif and bind TRF2 via its TRFH protein docking site (31). To assess a potential role for these DNA damage response proteins in pre-RC assembly, we immunodepleted Mre11 and ATM from cytosolic extracts and monitored binding of MCM6, ORC2 and Cdc6 to immobilized substrates. ATM or Mre11 depletion did not affect the assembly of pre-RC components (Supplementary Figure S1). Due to the lack of antibodies to quantitatively deplete TRF2 from extracts, we were not able to test whether TRF2 is involved in assembly of a pre-RC.

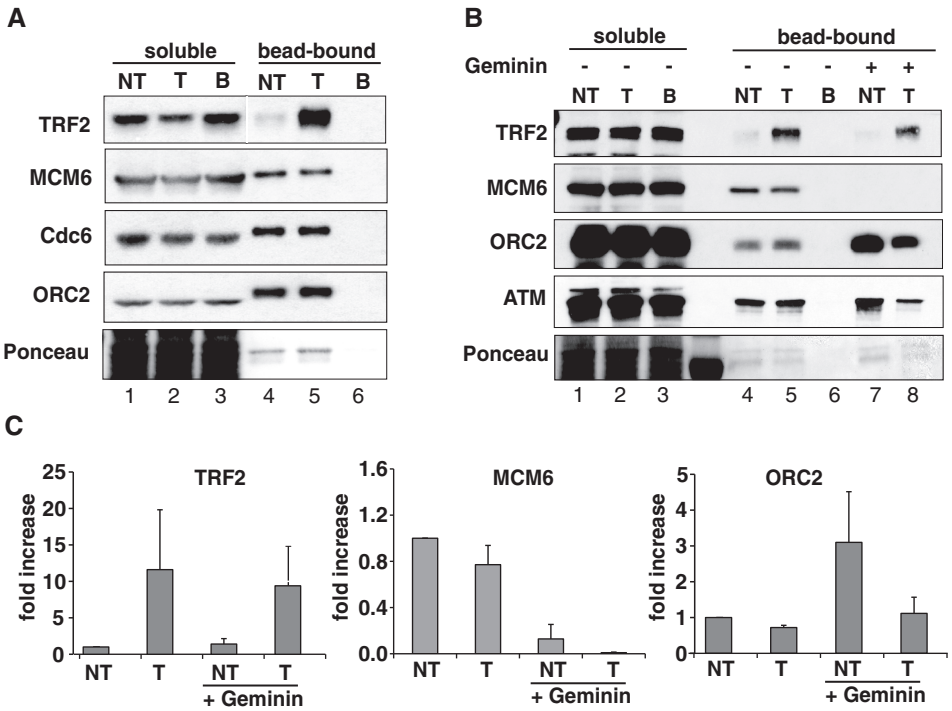

Figure 2. Assembly of pre-RC components on telomeric DNA. (**A**) Assembly of pre-RC proteins on immobilized 0.6 kb telomeric or NT DNA in cytosolic extracts as described in Figure 1B. A part of the membrane stained with Ponceau was used as an internal loading control. (**B**) Immobilized 0.6 kb NT or telomeric substrates were incubated with egg cytosol at 30 ng/μl for 30 min before addition of 2-fold volumes of NPE. Reactions were processed after 25 min as in Figure 1B. 100 ng/μl Geminin was added to the egg cytosol in lanes 7 and 8. (**C**) Quantification of the signals for TRF2, ORC2 and MCM6 from (B) for three independent experiments. Signals were normalized to the signal of the Ponceau stain. Values for untreated NT conditions are set to 1.

Linear DNA substrates can replicate in *Xenopus* extracts

To assess the functional significance of pre-RC assembly we sought to establish conditions that support initiation-dependent DNA replication of linear DNA substrates in *Xenopus* extracts. Small circular plasmids can be replicated in the two-step cell-free NPE extract system but it has not been reported whether this system also supports replication of linear DNA substrates. We anticipated that the ends of linear DNA templates would be sensed as DSBs. Broken DNA triggers a DNA damage response that interferes with origin firing due to activation of ATM/ATR checkpoint kinases, which inhibit the S-phase kinase Cdk2 (32). When a 3.5 kb linear NT DNA substrate was incubated in NPE in the presence of ^{32}P-dCTP, we detected incorporation of radiolabelled nucleotides (Figure 3A, lane 1). Treatment with caffeine, an inhibitor of the ATM/ATR protein kinases, significantly enhanced the signal as predicted (Figure 3A, lane 2 and Figure 3B). This strongly suggests that nucleotide incorporation was restricted by checkpoint activation and that the caffeine-sensitive signal is the result of origin-dependent DNA replication. Geminin, which specifically inhibits origin assembly and firing but does not affect DNA repair, abolished the caffeine-sensitive DNA synthesis (Figure 3A, lanes 3 and 4 and Figure 3B). The high molecular weight molecules that appeared in addition to the linear plasmid suggest that DNA ligation, most likely through NHEJ, was taking place in extracts (25). Electrophoresis of the replication products on denaturing gels partially resolves the labeled products (Figure 3A, bracket on the left) into discrete bands, similar to products detected in NHEJ reactions (see below and data not shown), indicating that repair through end-joining contributes to formation of the high molecular weight products. Quantification of replicated DNA substrates revealed that 23% of input linear DNA substrate was replicated, compared to 50% of input circular plasmids (data not shown). Nucleotide incorporation was inhibited by treatment with the Cdk2 inhibitor Roscovitine, confirming that nucleotide synthesis resulted from origin-dependent DNA replication (Supplementary Figure S2, lanes 3 and 4). DNA replication was strictly dependent on exogenous DNA templates (Supplementary Figure S2, lane 5). Taken together, our results show that linear DNA substrates can replicate in this *Xenopus* extract system.

Origin-dependent replication of telomeric DNA

Next, we performed replication assays using untagged linear 0.6 kb NT or telomeric DNA templates. Both substrates incorporated radiolabelled nucleotides (Figure 4A, lanes 1 and 5) and caffeine treatment reproducibly enhanced the signal significantly (Figure 4A, lanes 2 and 6; 4B), suggesting that DNA synthesis was restricted by checkpoint activation, as observed in Figure 3. Next, we wanted to confirm that a caffeine-sensitive checkpoint

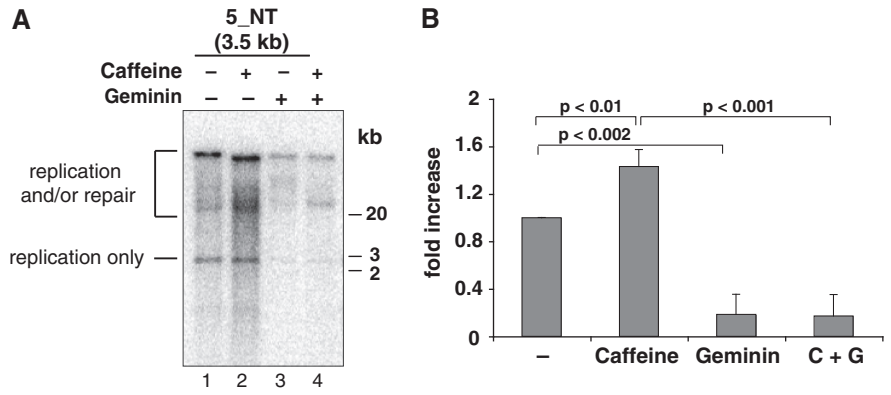

Figure 3. Replication of linear substrates. (**A**) BsmBI linearized 3.5 kb NT pRST5_NT (5_NT) was incubated with egg cytosol at 1.5 ng/μl in the presence of either buffer control, 5 mM Caffeine, 100 ng/μl Geminin or Caffeine and Geminin for 30 min before addition of 2-fold volumes of NPE and (α-^{32}P)dCTP. Reactions were processed after 60 min for native agarose gel electrophoresis. The radioactive signal of three independent experiments were quantified by Phosphorimager (**B**). Values for untreated conditions are set to 1. *P*-values were calculated using a *t*-test.

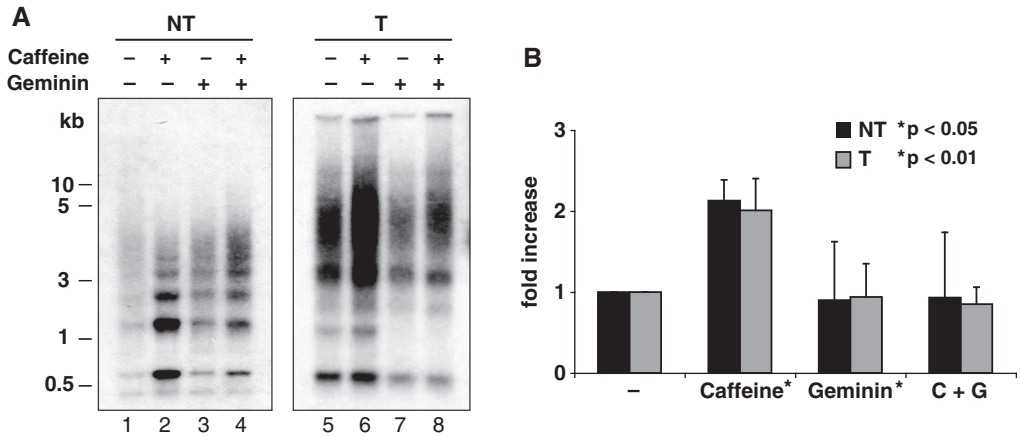

Figure 4. Telomeric DNA is replicated. (**A**) Replication assays with 3 ng/μl of 0.6 kb NT (lanes 1–4) or telomeric (lanes 5–8) DNA were performed as in Figure 3A. Reactions were processed after 40 min. (**B**) Quantification of replication assays from three (NT) or 4 (T) independent experiments. Values for untreated conditions are set to 1. *P*-values were calculated using a *t*-test from Caffeine versus Geminin treated extracts, as indicated by asterisks.

affecting origin-dependent DNA replication was limiting DNA synthesis. Indeed, addition of geminin largely abolishes incorporation of ^{32}P-dCTP (Figure 4A, lanes 4 and 8; Figure 4B). This strongly suggests that active origins assemble and fire within DNA composed exclusively of telomeric repeats and that, under our experimental conditions, their activity is limited by a caffeine sensitive DNA damage checkpoint. We observed that the caffeine-insensitive nucleotide incorporation (Figure 4A, lanes 1 and 5) was not decreased by geminin treatment (Figure 4A, lanes 3 and 7). We propose that this origin-independent DNA synthesis could be due to processing events at DNA termini. Quantification of the replication efficiency reveals that 5% (NT) and 7% (T) of the 0.6 kb substrates replicated, compared to 23% for the linear 3.5 kb substrates. Thus, origin-driven DNA replication is less efficient for small than for long linear templates and the geminin-resistant signal represents a larger fraction of nucleotide incorporation for short templates. End processing and ligation of the linear substrates by NHEJ could also account for the more complex pattern of nucleotide incorporation of short substrates (compare Figures 3A and 4A and also see Figure 5A). We also observe ~1.4 fold higher efficiency of replication of telomeric versus NT short substrates. The reason for this difference is not entirely clear but could indicate a role of shelterin components in stabilization of the replication fork on passage through telomeric DNA.

Short telomeric DNA substrates are repaired by NHEJ and induce a DNA damage response

To test the possibility that initiation-independent DNA synthesis was coupled to repair by non-homologous end-joing (NHEJ), we monitored the formation of repair products by Southern blot analysis. Linear DNA templates are efficiently repaired by NHEJ in *Xenopus* extracts (33). End-joining is very robust and independent of the nature of the end termini (25). Upon incubation of

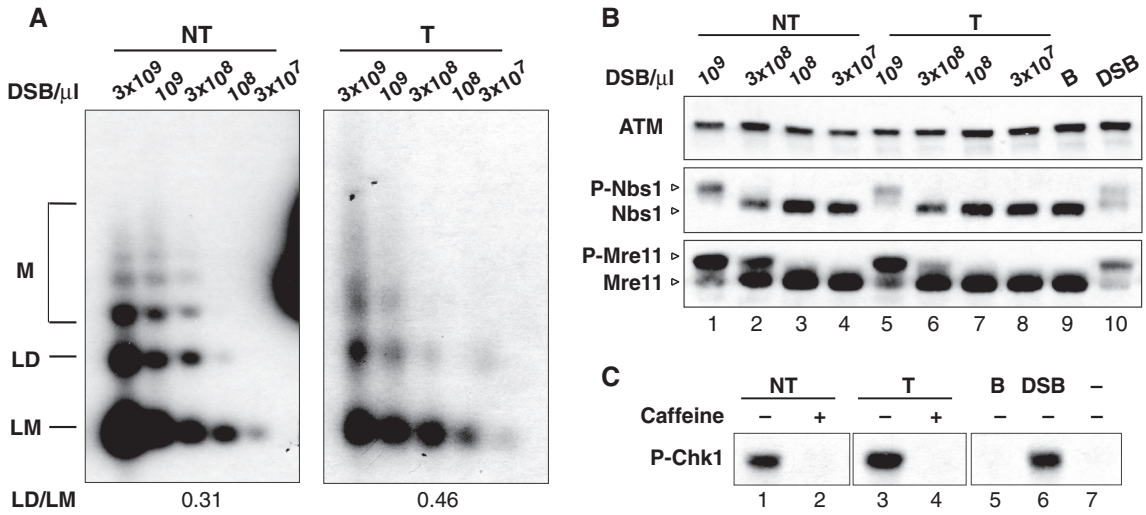

Figure 5. Short telomeric substrates activate a DSB response and are repaired by NHEJ. (**A**) Southern blot analysis of NHEJ-mediated repair products. In total, 0.6 kb of untagged NT or telomeric DNA was added to cytosolic extracts for 150 min. Reactions were processed for native agarose gel electrophoresis before transfer onto a Nitrocellulose membrane. Telomeric substrates are hybridized with a 5′ ^{32}P-labeled $(CCCTAA)_6$ oligonucleotide and NT substrates with random labeled pRST5_NT. LM linear monomer, LD linear dimer, M multimers. Signals from three independent experiments were quantified by phosphorimager analysis and expressed as ratios of the LD to LM. (**B**) Immobilized NT or telomeric DNA was incubated with cytosolic extracts at indicated concentrations and activation of DNA damage response proteins in the soluble fraction was monitored by western blotting. Lane 9, buffer control, lane 10, 2 ng/μl HaeII-digested pBS was added. (**C**) Immobilized NT or telomeric DNA was incubated with cytosolic extracts at 2×10^9 DSB/μl and checkpoint activation in the soluble fraction was monitored by western blotting using an antibody against phosphorylated Chk1. Beads only, lane 5, 2 ng/μl HaeII digested pBS, lane 6, buffer control, lane 7.

the linear 0.6 kb unlabelled DNA substrates in cytosolic extracts, we observed formation of linear dimer (LD) and multimer (M) products with similar efficiency for NT and telomeric DNA (Figure 5A). Dilution of the DNA did not affect the repair patterns, suggesting that depletion of telomere binding proteins is not the explanation for the failure to protect telomeres. Quantification of the ratios between linear dimers (LD) and linear monomers (LM) indicated that ligation was at least as efficient for telomeric as for NT substrates (Figure 5A). These findings differ from recent experiments in mammalian cell extracts where stretches of 12 telomeric repeats at the ends of linear plasmid DNA were sufficient to protect linear substrates from NHEJ (34). Next, we performed NHEJ assays with 3.5 kb DNA substrates that harbor a stretch of 75 TTAGGG repeats at one end only (Supplementary Figure S3). We observed efficient intra- and intermolecular end-joining, regardless of the sequence of the substrate. The difference between our observations and previously described results could reflect the fact that *Xenopus* cytosolic extracts support efficient NHEJ regardless of end termini unlike mammalian cell-free extracts that repair primarily compatible ends with lower efficiency (35). In addition, it has been shown that intra-molecular end-joining in *Xenopus* extracts is dependent on Ku70 (25,36), whereas inter-molecular end-joining in *Xenopus* is not (25) and might reflect non-canonical (i.e. alternative) NHEJ pathways.

Telomeric substrates generated less sharp repair products possibly due to processing events occurring specifically at telomeric DNA and is consistent with the appearance of higher molecular bands appearing in the replication gels in Figure 4A. These events could include microhomology-directed end-joining (MMEJ) favored by the high degree of homology along the telomeric DNA templates (37). Priming events on a single stranded overhang generated by telomerase (23) could also contribute to the extension of telomeric substrates (Figure 5A) and geminin-insensitive nucleotide incorporation (Figure 4).

Our observation that telomeric DNA could be repaired by NHEJ prompted us to determine whether these telomeric substrates could trigger a DNA damage response. As predicted, NT DNA substrates induced phosphorylation of Nbs1 and Mre11 at concentrations higher than 10^8 ends/μl (Figure 5B, lanes 1–4). Notably, we observed a similar response induced from telomeric ends (Figure 5B, lanes 5–8). We also observed caffeine sensitive phosphorylation of Chk1 (Figure 5C), suggesting that ATM/ATR kinases are involved in the signal transduction. Overall, our data indicate that assembly of telomeric proteins on a 0.6 kb telomeric DNA-only fragment is not sufficient to establish telomere protection in *Xenopus* cell extracts. This suggests that other processing events such as the generation of a single stranded overhang are required to establish a protective end-structure. We propose that the short substrates used here might reflect a deprotected state as it is often linked to critically short telomeres (38,39). The induction of a DNA damage response however, does not interfere with the assembly of a pre-RC (Figure 2).

DISCUSSION

Xenopus cell-free extracts can support assembly and replication of at least 10 000 nuclei/μl. Given a telomere length of 50 kb, this represents 4×10^7 kb telomeric sequences/μl and would correspond to 8×10^7 DNA molecules/μl for a

600 bp long DNA substrate. Here, we have developed a system to study proteins assembled on telomeric DNA substrates independent from other sources of DNA and at concentrations close to these physiological levels of telomeric DNA (Figure 5). Exogenous telomeric DNA fragments can be added to *X. laevis* cell-free extracts and are specifically bound by TRF2 (Figures 1 and 2), an aspect of telomere biology that is fully recapitulated. Furthermore, *Xenopus* extracts support origin-dependent DNA replication of linear DNA substrates (Figures 3 and 4). Therefore, this system represents a powerful tool to study various aspects of telomere biology by allowing to use physiological amounts of telomeric DNA substrates in a soluble environment that contains nucleoplasmic proteins.

Using this approach we investigated whether telomeric DNA supports replication initiation. Our data show that DNA templates containing uniquely telomeric sequences and bound by TRF2 support origin-dependent initiation of DNA replication in *Xenopus* cell-free extracts under conditions close to physiological levels of natural telomeres. We also show that ATM and Mre11, two shelterin accessory factors, are not involved in the regulation of pre-RC assembly. These novel observations suggest that in *Xenopus* and possibly in other organisms harboring long telomeres, origins of DNA replication can assemble and could fire within telomeric DNA (Figures 2 and 4). Indeed, origin firing within telomeres was recently reported in mouse embryo fibroblasts by single molecule analysis of replicated telomeres. The fraction of telomeres that displayed active origins was small (3%), suggesting that in mouse embryo fibroblasts the majority of replication forks travel from subtelomeric origins into telomeric DNA (30).

It has been proposed that the localization of a pre-RC to telomeres is cell cycle regulated and could be influenced by telomere structure (14). We find that localization of pre-RC components to telomeric DNA does not require a native telomere structure, e.g. the presence of a single stranded overhang or the formation of a T loop. This would permit assembly of pre-RCs away from the ends of natural telomeres that represent only a minor fraction of total telomeric DNA. Indeed, within a native 5–50 kb long vertebrate telomere, the 3′ overhang and/or the formation of a D-loop only accounts for a small portion of the total telomeric complex.

The exact functional relationships between TRF2 or other shelterin components and pre-RC assembly and activation are not fully understood. TRF2 is associated to telomeres throughout the cell cycle (23,40). Here, we show that pre-RC assembly on telomeric DNA is inhibited by geminin and therefore regulated in a cell cycle dependent manner, as in human cells (13). Human ORC1 physically interacts with the amino terminal domain of TRF2 and down-regulation of either TRF2 or ORC1 results in lower levels of pre-RC assembly within telomeric DNA (13,14). The former suggests that pre-RC assembly is influenced by the formation of proper telomeric chromatin. Conversely, ORC2 depletion by siRNA or hypomorphic cell lines for ORC2 display loss of telomere repeat DNA (14), indicating that pre-RC components are involved in telomere length homeostasis. Telomere instability in the absence of ORC could also be explained by collapsed replication forks within telomeres, which cannot be rescued by a neighboring origin. While our results demonstrate cell cycle regulated assembly of a pre-RC, we cannot exclude that shelterin components are involved in some of the steps of pre-RC assembly or the stabilization of a replication fork that travels through telomeric DNA. In fact, recent data in mammalian cells demonstrate that TRF1 is required for efficient replication through telomeric DNA and prevents replication fork arrest (30).

The genome in *Xenopus* eggs replicates within 20 min, which requires a very efficient and fast duplication of the 5–50 kb long *Xenopus* telomeres. Mammalian telomeres, in contrast, replicate their telomeres throughout S phase in an average of 6–8 h (41). Therefore, *Xenopus* chromosomes must have the ability to complete faithful replication of their telomeres in a short amount of time. This task is complicated by the fact that the repetitive G-rich regions within telomeric DNA are difficult stretches to replicate and result in higher frequencies of stalled replication forks (3,4). Consequently, additional factors including DNA helicases that stabilize passage of a replication fork and prevent secondary structure formation are required for telomere replication (4,42). *In vivo* studies in other eukaryotes showed that one single short telomere induces cell cycle arrest (43), which indicates a tight requirement for complete replication of telomeric DNA. Stalled replication forks within chromosomal DNA can be repaired by several pathways including break-induced replication or homologous recombination. However, the recombinogenic potential within the repetitive sequence of telomeres is repressed by the action of shelterin components (44) in order to prevent recombination events that could yield short telomeres, which can affect the proliferative life span of cells. Furthermore, unlike other chromosomal regions, where DNA synthesis from a collapsed polymerase can be completed by adjacent replication forks, a stalled polymerase traveling from subtelomeric regions towards the end of the telomeres could not. Therefore, restoring of stalled replication forks is likely to occur through a different mechanism. In a NT DNA context, replication fork stalling triggers the activation of surrounding dormant origins (45,46), a mechanism that could apply to telomeres to solve the 'random completion' problem there (47). Our data demonstrate that the levels of pre-RC components recruited to telomeric DNA and the efficiency of replication is comparable to substrates containing a random DNA sequence. Thus, in *Xenopus*, origin firing within telomeric DNA may occur at least as efficient as in NT DNA. The presence of multiple origins within telomeric DNA should provide a mechanism to replicate telomeres more rapidly and/or to compensate for failing replication forks originating in sub-telomeric regions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Palm,W. and de Lange,T. (2008) How shelterin protects mammalian telomeres. *Annu. Rev. Genet.*, **42**, 301–334.
2. Williamson,J.R., Raghuraman,M.K. and Cech,T.R. (1989) Monovalent cation-induced structure of telomeric DNA: the G-quartet model. *Cell*, **59**, 871–880.
3. Makovets,S., Herskowitz,I. and Blackburn,E.H. (2004) Anatomy and dynamics of DNA replication fork movement in yeast telomeric regions. *Mol. Cell Biol.*, **24**, 4019–4031.
4. Miller,K.M., Rog,O. and Cooper,J.P. (2006) Semi-conservative DNA replication through telomeres requires Taz1. *Nature*, **440**, 824–828.
5. Ohki,R. and Ishikawa,F. (2004) Telomere-bound TRF1 and TRF2 stall the replication fork at telomeric repeats. *Nucleic Acids Res.*, **32**, 1627–1637.
6. Bell,S.P. and Stillman,B. (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature*, **357**, 128–134.
7. Wellinger,R.J., Wolf,A.J. and Zakian,V.A. (1993) Structural and temporal analysis of telomere replication in yeast. *Cold Spring Harb. Symp. Quant. Biol.*, **58**, 725–732.
8. Gilson,E. and Geli,V. (2007) How telomeres are replicated. *Nat. Rev. Mol. Cell Biol.*, **8**, 825–838.
9. Gilbert,D.M. (2004) In search of the holy replicator. *Nat. Rev. Mol. Cell Biol.*, **5**, 848–855.
10. Harland,R.M. and Laskey,R.A. (1980) Regulated replication of DNA microinjected into eggs of Xenopus laevis. *Cell*, **21**, 761–771.
11. Mechali,M. and Kearsey,S. (1984) Lack of specific sequence requirement for DNA replication in Xenopus eggs compared with high sequence specificity in yeast. *Cell*, **38**, 55–64.
12. Danis,E., Brodolin,K., Menut,S., Maiorano,D., Girard-Reydet,C. and Mechali,M. (2004) Specification of a DNA replication origin by a transcription complex. *Nat. Cell Biol.*, **6**, 721–730.
13. Tatsumi,Y., Ezura,K., Yoshida,K., Yugawa,T., Narisawa-Saito,M., Kiyono,T., Ohta,S., Obuse,C. and Fujita,M. (2008) Involvement of human ORC and TRF2 in pre-replication complex assembly at telomeres. *Genes Cells*, **13**, 1045–59.
14. Deng,Z., Dheekollu,J., Broccoli,D., Dutta,A. and Lieberman,P.M. (2007) The origin recognition complex localizes to telomere repeats and prevents telomere-circle formation. *Curr. Biol.*, **17**, 1989–1995.
15. Costanzo,V., Robertson,K. and Gautier,J. (2004) Xenopus cell-free extracts to study the DNA damage response. *Methods Mol. Biol.*, **280**, 213–227.
16. Tutter,A.V. and Walter,J.C. (2006) Chromosomal DNA replication in a soluble cell-free system derived from Xenopus eggs. *Methods Mol. Biol.*, **322**, 121–137.
17. Blow,J.J. and Laskey,R.A. (1986) Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. *Cell*, **47**, 577–587.
18. Wohlschlegel,J.A., Dwyer,B.T., Dhar,S.K., Cvetic,C., Walter,J.C. and Dutta,A. (2000) Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science*, **290**, 2309–2312.
19. Mahbubani,H.M., Paull,T., Elder,J.K. and Blow,J.J. (1992) DNA replication initiates at multiple sites on plasmid DNA in Xenopus egg extracts. *Nucleic Acids Res.*, **20**, 1457–1462.
20. Callan,H.G. (1972) Replication of DNA in the chromosomes of eukaryotes. *Proc. R. Soc. Lond. B. Biol. Sci.*, **181**, 19–41.
21. Bassham,S., Beam,A. and Shampay,J. (1998) Telomere variation in Xenopus laevis. *Mol. Cell Biol.*, **18**, 269–275.
22. O'Connor,M.S., Safari,A., Xin,H., Liu,D. and Songyang,Z. (2006) A critical role for TPP1 and TIN2 interaction in high-order telomeric complex assembly. *Proc. Natl Acad. Sci. USA*, **103**, 11874–11879.
23. Nishiyama,A., Muraki,K., Saito,M., Ohsumi,K., Kishimoto,T. and Ishikawa,F. (2006) Cell-cycle-dependent Xenopus TRF1 recruitment to telomere chromatin regulated by Polo-like kinase. *EMBO J.*, **25**, 575–584.
24. Costanzo,V., Robertson,K., Ying,C.Y., Kim,E., Avvedimento,E., Gottesman,M., Grieco,D. and Gautier,J. (2000) Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage. *Mol. Cell*, **6**, 649–659.
25. Di Virgilio,M. and Gautier,J. (2005) Repair of double-strand breaks by nonhomologous end joining in the absence of Mre11. *J. Cell Biol.*, **171**, 765–771.
26. Dupre,A., Boyer-Chatenet,L. and Gautier,J. (2006) Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex. *Nat. Struct. Mol. Biol.*, **13**, 451–457.
27. 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.
28. Walter,J., Sun,L. and Newport,J. (1998) Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell*, **1**, 519–529.
29. Edwards,M.C., Tutter,A.V., Cvetic,C., Gilbert,C.H., Prokhorova,T.A. and Walter,J.C. (2002) MCM2-7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in Xenopus egg extracts. *J. Biol. Chem.*, **277**, 33049–33057.
30. Sfeir,A., Kosiyatrakul,S.T., Hockemeyer,D., MacRae,S.L., Karlseder,J., Schildkraut,C.L. and de Lange,T. (2009) Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. *Cell*, **138**, 90–103.
31. Chen,Y., Yang,Y., van Overbeek,M., Donigian,J.R., Baciu,P., de Lange,T. and Lei,M. (2008) A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins. *Science*, **319**, 1092–1096.
32. Shechter,D., Costanzo,V. and Gautier,J. (2004) ATR and ATM regulate the timing of DNA replication origin firing. *Nat. Cell Biol.*, **6**, 648–655.
33. Pfeiffer,P. and Vielmetter,W. (1988) Joining of nonhomologous DNA double strand breaks in vitro. *Nucleic Acids Res.*, **16**, 907–924.
34. Bae,N.S. and Baumann,P. (2007) A RAP1/TRF2 complex inhibits nonhomologous end-joining at human telomeric DNA ends. *Mol. Cell*, **26**, 323–334.
35. Baumann,P. and West,S.C. (1998) DNA end-joining catalyzed by human cell-free extracts. *Proc. Natl Acad. Sci. USA*, **95**, 14066–14070.
36. Labhart,P. (1999) Ku-dependent nonhomologous DNA end joining in Xenopus egg extracts. *Mol. Cell Biol.*, **19**, 2585–2593.
37. Verkaik,N.S., Esveldt-van Lange,R.E., van Heemst,D., Bruggenwirth,H.T., Hoeijmakers,J.H., Zdzienicka,M.Z. and van Gent,D.C. (2002) Different types of V(D)J recombination and end-joining defects in DNA double-strand break repair mutant mammalian cells. *Eur. J. Immunol.*, **32**, 701–709.
38. d'Adda di Fagagna,F., Reaper,P.M., Clay-Farrace,L., Fiegler,H., Carr,P., Von Zglinicki,T., Saretzki,G., Carter,N.P. and Jackson,S.P. (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature*, **426**, 194–198.

39. IJpma,A.S. and Greider,C.W. (2003) Short telomeres induce a DNA damage response in Saccharomyces cerevisiae. *Mol. Biol. Cell*, **14**, 987–1001.
40. Konishi,A. and de Lange,T. (2008) Cell cycle control of telomere protection and NHEJ revealed by a ts mutation in the DNA-binding domain of TRF2. *Genes Dev.*, **22**, 1221–1230.
41. Wright,W.E., Tesmer,V.M., Liao,M.L. and Shay,J.W. (1999) Normal human telomeres are not late replicating. *Exp. Cell Res.*, **251**, 492–499.
42. Crabbe,L., Verdun,R.E., Haggblom,C.I. and Karlseder,J. (2004) Defective telomere lagging strand synthesis in cells lacking WRN helicase activity. *Science*, **306**, 1951–1953.
43. Hemann,M.T., Strong,M.A., Hao,L.Y. and Greider,C.W. (2001) The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell*, **107**, 67–77.
44. Celli,G.B., Denchi,E.L. and de Lange,T. (2006) Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination. *Nat. Cell Biol.*, **8**, 885–890.
45. Ge,X.Q., Jackson,D.A. and Blow,J.J. (2007) Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. *Genes Dev.*, **21**, 3331–3341.
46. Courbet,S., Gay,S., Arnoult,N., Wronka,G., Anglana,M., Brison,O. and Debatisse,M. (2008) Replication fork movement sets chromatin loop size and origin choice in mammalian cells. *Nature*, **455**, 557–560.
47. Hyrien,O., Marheineke,K. and Goldar,A. (2003) Paradoxes of eukaryotic DNA replication: MCM proteins and the random completion problem. *Bioessays*, **25**, 116–125.
48. Ying,C.Y. and Gautier,J. (2005) The ATPase activity of MCM2-7 is dispensable for pre-RC assembly but is required for DNA unwinding. *EMBO J.*, **24**, 4334–4344.