Published online 10 February 2010 *Nucleic Acids Research, 2010, Vol. 38, No. 9* ***2955–2963***
doi:10.1093/nar/gkp1248

ATR contributes to telomere maintenance in human cells

Gaëlle Pennarun[1], Françoise Hoffschir[1], Deborah Revaud[1], Christine Granotier[1], Laurent R. Gauthier[1], Patrick Mailliet[2], Denis S. Biard[3] and François D. Boussin[1,*]

[1]CEA/DSV/iRCM/SCSR, Laboratoire de Radiopathologie, INSERM-Université Paris VII U967, 92265 Fontenay-aux-Roses, [2]Sanofi-Aventis, centre de recherche de Paris, 94403 Vitry-sur-Seine France and [3]CEA/DSV/iRCM/SCSR, INSERM U935, Institut A. Lwoff, 94801 Villejuif Cedex, France

Received April 20, 2009; Revised December 18, 2009; Accepted December 27, 2009

ABSTRACT

Telomere maintenance is essential to preserve genomic stability and involves several telomere-specific proteins as well as DNA replication and repair proteins. The kinase ATR, which has a crucial function in maintaining genome integrity from yeast to human, has been shown to be involved in telomere maintenance in several eukaryotic organisms, including yeast, *Arabidopsis* and Drosophila. However, its role in telomere maintenance in mammals remains poorly explored. Here, we report by using telomere-fluorescence *in situ* hybridization (Telo-FISH) on metaphase chromosomes that ATR deficiency causes telomere instability both in primary human fibroblasts from Seckel syndrome patients and in HeLa cells. The telomere aberrations resulting from ATR deficiency (i.e. sister telomere fusions and chromatid-type telomere aberrations) are mainly generated during and/or after telomere replication, and involve both leading and lagging strand telomeres as shown by chromosome orientation-FISH (CO-FISH). Moreover, we show that ATR deficiency strongly sensitizes cells to the G-quadruplex ligand 360A, enhancing sister telomere fusions and chromatid-type telomere aberrations involving specifically the lagging strand telomeres. Altogether, these data reveal that ATR plays a critical role in telomere maintenance during and/or after telomere replication in human cells.

INTRODUCTION

Telomeres are specialized nucleoprotein structures that protect the natural ends of eukaryotic chromosomes (1). Mammalian telomeres contain a duplex array of telomeric repeats (5′-TTAGGG-3′) ending by a 3′ single-strand overhang and capped by a specialized telomere protein complex referred to as shelterin (2,3). Telomeres are thought to adopt a protected conformation, known as T-loop, through invasion of the duplex telomeric region by the terminal overhang, hiding the ends of the telomeres from DNA repair mechanisms (4). Deprotection of telomeres induces DNA damage response pathways leading to cell cycle arrest, genomic instability, apoptosis or senescence (3,5). Besides, several DNA damage response factors are involved in telomere replication and in the formation of T-loop in S and G2 phase (6–10).

Ataxia-telangiectasia-mutated and Rad3-related (ATR), a key protein in DNA damage response, is essential for maintenance of genomic stability from yeasts to humans (11). ATR belongs to a family of phosphatidylinositide 3-kinase-like kinases, which includes ATM (ataxia telangiectasia mutated). ATM is primarily activated by DNA double-strand breaks, while ATR responds to a broad spectrum of DNA damages, in particular those interfering with DNA replication, and phosphorylates many substrates involved in replication checkpoint and repair (11).

Defective ATR-dependent DNA damage signaling pathway, related to an hypomorphic mutation of ATR in a few patients, is associated with the Seckel syndrome (MIM 210600), which is characterized by growth retardation, microcephaly, mental retardation and craniofacial abnormalities (12–14).

ATR plays a key role during S-phase by monitoring the progress of DNA replication forks (15). ATR pathway stabilizes stalled replication forks arrested by a lesion or an unusual DNA structure, thereby enabling removal or repair of blocking events (16). The ATR pathway is thought to prevent illegitimate recombination at stalled forks and promote helicase activity that could reverse stalled fork structures (17–19).

*To whom correspondence should be addressed. Tel: +33 1 46 54 97 91; Fax: +33 1 46 54 97 02; Email: boussin@cea.fr

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

ATR or its functional homologs are also required for telomere maintenance in *Arabidopsis* (20), *Saccharomyces cerevisiae* (Mec1) (21), S*chizosaccharomyces pombe* (Rad3) (22–24) and Drosophila (Mei-41) (25). In both mice and humans, ATR has been reported to signal dysfunctional telomeres leading to the formation of telomere dysfunction induced foci (TIF) and cell cycle arrest (26–28). However, a more direct role of ATR in mammalian telomere maintenance is not yet well demonstrated, although ATR has been shown to localize at human telomeres in a cell cycle-dependent manner, mainly in late S until G2 phase, and to be required for *in vitro* generation of telomeric D loops (8).

The aim of this study was to investigate the importance of ATR for telomere maintenance in human cells. We therefore investigated the occurrence of telomere aberrations in primary Seckel fibroblasts and in HeLa cells knocked down for ATR (ATR^{KD} HeLa cells) by using telomere-fluorescence *in situ* hybridization (Telo-FISH) on metaphase chromosomes. We found a significant induction of specific types of telomere aberrations (i.e. sister telomere fusions and chromatid type-telomere aberrations) in both Seckel fibroblasts and ATR^{KD} HeLa cells, suggesting that ATR contributes to human telomere stability during and/or after replication. Moreover, we showed that ATR knockdown increased telomere aberrations induced by the G-quadruplex (G4) ligand 360A that destabilizes telomeres during S-G2 phases (29–31). Hence, we provide new evidence that ATR contributes to the reconstitution of stable and correct telomere state during the replication process and thus plays an important role in human telomere maintenance.

MATERIALS AND METHODS

Cell cultures

Normal primary fibroblasts from normal donors of various ages (ranging from 3 to 56 years, Table 1) were kindly given by Dr M. Martin (HFP537, HFP536, HFP4196, HFPM23, HFP14) and Dr J.P. Puget (NHF). Primary fibroblasts GM18366 and GM09812 (Coriell Cell Repositories) were isolated from Seckel patients (a 6-year-old male and a 15-year-old female, respectively) with defective ATR signaling pathway and reduced ATR protein levels (13,14,32). This has been linked to a mutation in ATR (2101 A > G) leading to an alternative splicing in GM18366 cells (13). Fibroblasts were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (ATGC), 2 mM glutamine (Sigma), and antibiotics (penicillin, 100 U/ml and streptomycin, 100 μg/ml, Sigma).

Table 1. Telomere instability in ATR-deficient Seckel primary fibroblasts

	Age of donors (years)	Number of passages	Means of chromosomes with telomere aberration/cell ± SEM (%)
Normal fibroblast			
HFP537	3	5	6.1 ± 0.6
HFP536	3	6	3.1 ± 0.4
NHF	30	30	9.2 ± 0.6
HFP4196	31	7	7.7 ± 0.5
HFPM23	41	7	8.6 ± 0.7
HFP14	56	7	8.6 ± 1
Seckel fibroblasts			
GM18366	6	18	19.6 ± 1.0
GM09812	15	16	19.7 ± 1.1

Means of chromosomes with telomere aberration/cell ± SEM (%) were determined by Telo-FISH on at least 47 metaphases per sample. The different types of telomere aberrations observed are listed in Figure 1 and Supplementary Figure S1. The table also gives age of donors and number of passages at which Telo-FISH has been done.

ATR knockdown (ATR^{KD}) HeLa cells stably transfected with an Epstein–Barr virus (EBV) vector expressing small interfering RNA (siRNA) targeting ATR and control (Ct^{KD}) HeLa cells stably transfected with a control EBV vector (vector pBD650) expressing an inactive siRNA have been previously described elsewhere (33). Cells were cultured at 10^6 cells/75 cm^2 flasks in DMEM medium supplemented with 10% FBS, 2 mM glutamine and antibiotics (penicillin, 100 U/ml and streptomycin, 100 μg/ml), 1 mM Hepes (Invitrogen), hygromycin B at 250 μg/ml (Invitrogen) and 0.05% dimethyl sulfoxide (DMSO).

Treatment with the G4 ligand 360A

The pyridine derivative, G4 ligand 360A was provided by Sanofi-Aventis (Vitry/seine, France) and has been previously described elsewhere (30). The 360A stock solution was prepared at 10 mM in DMSO. Cells were cultured as described above in medium supplemented with 5 μM 360A or 0.05% DMSO as controls. Every 3–4 days, cells were trypsinized, counted by trypan blue exclusion, and then reseeded at the initial density in fresh medium.

Telomere-fluorescent *in situ* hybridization

Telo-FISH using a Cy-3-labeled $(CCCTAA)_3$ PNA probe (Applied Biosystems) specific to the G-rich telomeric strand was carried out on metaphase spreads as described elsewhere (31) using standard procedures (34). The chromosome preparations were counterstained with DAPI and observed under a fluorescence microscope (Olympus IX81). Digital images were recorded using a coolsnap HQ camera (Princetown instrument) and analyzed with Metamorph software. Telomere aberrations scored were essentially: sister telomere losses, sister telomere fusions, telomere doublets, terminal deletions, telomeric–DNA containing double-minute chromosomes and dicentric chromosomes with or without telomere sequences at the fused points, as previously described (31). Statistical analyses were done with StatView software (Abacus Concepts).

Chromosome orientation-FISH

CO-FISH has been performed as previously published (35). Briefly, cells were cultured in complete medium supplemented with 10 μM BrdU (Sigma) for one cell cycle. Metaphase spreads obtained as described above were stained with Hoechst 33258 (Sigma), then exposed to

ultraviolet (UV) light at 365 nm (Fisher Bioblock Scientific) and digested with exonuclease III (Promega) to remove newly synthesized DNA strands. Successive hybridizations with a FITC-labeled $(TTAGGG)_3$ PNA probe (Applied Biosystems), then with a Cy-3-labeled $(C CCTAA)_3$ PNA probe allowed to detect by fluorescence microscopy parental telomere C and G strands, respectively.

RESULTS

Telomere instability in Seckel primary fibroblasts

To investigate whether ATR plays a role in human telomere maintenance, we performed fluorescent *in situ* hybridization with a telomere probe (Telo-FISH) on metaphase spreads from primary fibroblasts isolated from Seckel syndrome patients (GM18366 and GM09812) with defective ATR signaling pathway and reduced ATR protein levels (13,14,32) and normal fibroblasts from six healthy donors of various ages (ranging from 3 to 56 years) as controls.

The levels of telomere instability in control fibroblasts were in relatively similar range (3.1 ± 0.4% to 9.2 ± 0.6% of chromosomes with telomere aberrations per cell, Table 1). However, the lowest levels of telomere instability were found in the fibroblasts from the two younger donors, whereas the highest telomere instability was found in the sample with the highest number of *in vitro* passages, consistently with the increase of telomere instability with age and number of *in vitro* passages. As shown in Table 1, despite the fact that 4/6 of the control fibroblasts were obtained from older donors and one had a higher number of *in vitro* passages than the Seckel fibroblasts, telomere instability was increased by 2.1 to 6.3 times in Seckel fibroblasts (19.6 ± 1.0% of chromosomes with telomere aberrations in GM18366 and 19.7 ± 1.1% in GM09812) compared to each of the controls (P-values < 0.0001). Strikingly, this increase relied exclusively on only four different types of telomere aberrations (Figure 1A): sister telomere fusions (36–38), sister telomere losses [i.e. the loss of the telomere signal at only one chromatid end (39–41)], telomere doublets [characterized by the presence of an extra telomeric signal at one chromatid end (42–44)] and terminal deletions (i.e. lack of telomere signals at both sister chromatids).

Sister telomere fusions were detected on 2.3 ± 0.3% and 0.8 ± 0.2% of the chromosomes in GM18366 and GM09812 cells, respectively, but rarely in the six normal fibroblast controls (Figure 1B and Supplementary Figure S1, $P < 0.002$). Sister telomere losses were the most frequent telomere aberrations (9.4 ± 0.7% chromosomes/cell in GM18366 and 13.1 ± 0.9% in GM09812 versus 2.3 ± 0.4% to 6.5 ± 0.6% in the controls; P-values < 0.001; Figure 1B and Supplementary Figures S1 and S2). The percentages of chromosomes with a telomere doublet increased by a ratio ranging from 3.7 to 18.8 in GM18366 (4.8 ± 0.4%) and 2.6 to 13.3 in GM09812 (3.4 ± 0.4%) compared to controls (ranging from 0.256 ± 0.1% to 1.3 ± 0.2%, $P < 0.0001$; Figure 1B and Supplementary Figures S1 and S2). Terminal deletions were found on 3.1 ± 0.4% and 2.5 ± 0.4% of chromosomes in GM18366 and GM09812, respectively, versus 0.5 ± 0.1% to 1.3 ± 0.2% in normal fibroblasts, $P < 0.05$; Figure 1B and Supplementary Figures S1 and S2).

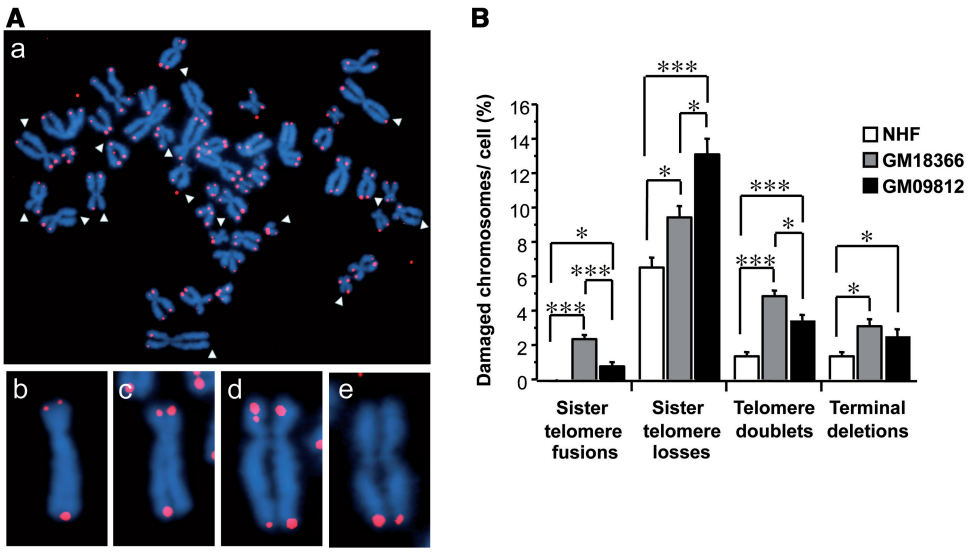

Figure 1. Increase in sister telomere fusions and chromatid-type telomere aberrations in ATR-deficient Seckel fibroblasts. (**A**) Representative metaphase spread from ATR-deficient fibroblasts GM18366 (a) hybridized with a telomeric PNA probe (red) in Telo-FISH experiments. Examples of telomere aberrations found in metaphases from Seckel fibroblasts (b-e): sister telomere fusion (b); sister telomere loss (c); telomere doublet (d) and terminal deletion (e). (**B**) Histograms showing the mean percentages of chromosomes with the indicated telomere aberrations per cell from Seckel fibroblasts (GM18366 and GM09812) and NHF, one of the six normal fibroblasts used as controls, showing the highest levels of telomere aberrations—results obtained with the five others are given in Supplementary Figure S1. Mean percentages ± standard errors of the mean (SEM) were calculated from at least 47 metaphases for each cell lines (*t-test P-value ≤ 0.05; **$P < 0.001$; ***$P < 0.0001$). Box graph showing the distributions of the percentages of the telomere aberrations per cell is given in Supplementary Figure S2.

Terminal deletions may be the result of telomere dysfunctions (39), but could also arise from double-strand breaks at interstitial sites. However, this seems unlikely to occur in Seckel fibroblasts since we did not detect chromosome or chromatid breaks in these cells (data not shown) in accordance with a previous study (45).

Most of the telomere aberrations increased in Seckel fibroblasts (i.e. sister chromatid fusions, sister telomere losses and telomere doublets) result from a defect occurring during and/or after telomere replication (31). We did not observe any dicentric chromosomes—the most frequent telomere aberrations detected in other models of telomere destabilization—resulting from fusion of two chromosomes during G1 or early S-phase because of the loss or the destabilization of their telomeres (36). Altogether, these data showed that telomere instability is increased during and/or after telomere replication in Seckel fibroblasts suggesting the importance of ATR signaling pathway for telomere maintenance in normal human cells.

ATR knockdown leads to telomere instability in HeLa cells

In order to further investigate the role of ATR in human telomere maintenance, we studied consequences of changing the ATR status on telomere stability in a same genetic background by comparing stable HeLa cell line knocked down for ATR (ATR^{KD}), by means of long-term RNA interference, with a control HeLa cell line (Ct^{KD}) expressing an inactive siRNA (33). ATR^{KD} cells showed little residual levels of ATR protein (5%) compared to Ct^{KD} cells (Supplementary Figure S3A). ATR^{KD} cells also showed a slower growth rate than Ct^{KD} cells (Supplementary Figure S3B), consistently with previous studies reporting slow growth in cells deficient for ATR (46,47). This was not related to apoptosis induction or associated with an accumulation in a particular phase of the cell cycle (Supplementary Figure S3C and D).

Telo-FISH revealed a nearly 2-fold increase in telomere aberrations per cell in ATR^{KD} ($13.2 \pm 0.4\%$) compared to Ct^{KD} cells ($7.3 \pm 0.4\%$; P-value < 0.0001; Figure 2A). We also found a moderate but significant induction of spontaneous chromatid breaks in ATR^{KD} cells ($1.6 \pm 0.2\%$ chromosomes/cells) that were not detected in Ct^{KD} cells (Figure 2A). However, chromatid breaks were 10-times less frequent than telomere aberrations in ATR^{KD} cells. As for Seckel fibroblasts, the main telomere aberrations in ATR^{KD} HeLa cells were sister telomere fusions ($3.2 \pm 0.3\%$ chromosomes/cell in ATR^{KD} versus $1.8 \pm 0.1\%$ in Ct^{KD} cells, $P < 0.0001$), sister telomere losses ($3.3 \pm 0.3\%$ chromosomes/cell in ATR^{KD} versus $2.1 \pm 0.2\%$ in Ct^{KD} cells, $P < 0.001$) and telomere doublets ($5.9 \pm 0.3\%$ chromosomes/cell in ATR^{KD} versus $3.3 \pm 0.3\%$ in Ct^{KD} cells, $P < 0.0001$; Figure 2B and Supplementary Figure S4). At a lower level, ATR inhibition also resulted in a 3-fold increase in terminal deletions ($0.57 \pm 0.11\%$ chromosomes/cell in ATR^{KD} compared to $0.17 \pm 0.07\%$ in Ct^{KD} cells, $P < 0.01$,

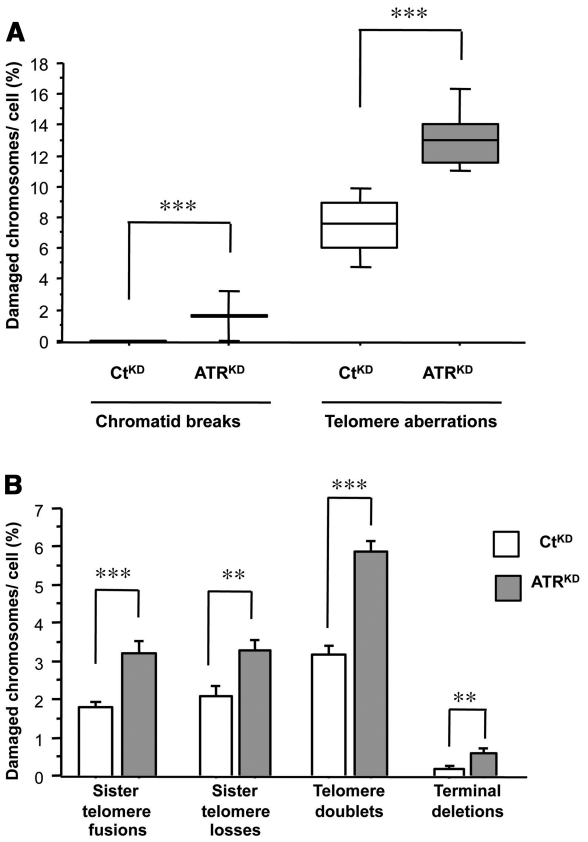

Figure 2. ATR inhibition leads to telomere instability in HeLa cells. (**A**) Increase in telomere aberrations in ATR^{KD} compared to Ct^{KD} HeLa cells. Box graph shows distributions of percentages of chromosomes with chromatid breaks or telomere aberrations per cell evidenced by Telo-FISH. Boxes include 50% of the values centered on the median (the horizontal line through the box). The vertical lines begin at the 10th percentile and end at the 90th percentile (***t-test P-value < 0.0001; at least $n = 30$ metaphases per condition). Similar levels of spontaneous telomere aberrations were found in Ct^{KD} HeLa cells and untransfected HeLa cells (data not shown). (**B**) Histograms showing the percentages of chromosomes with the indicated telomere aberrations per cells. Percentages ($\pm$SEM) were calculated from at least $n = 30$ metaphases (*t-test P-value ≤ 0.05; **$P < 0.001$; ***$P < 0.0001$). Box graph showing the distributions of the percentages of the telomere aberrations per cell is given in Supplementary Figure S3.

Figure 2B). Furthermore, we did not observe a significant increase in dicentric chromosomes in ATR^{KD} HeLa cells confirming the previous results in Seckel fibroblasts (Supplementary Figure S5).

In order to better characterize telomere instability induced by ATR deficiency, we performed CO-FISH, which enables to identify parental telomeric C and G strands on metaphase chromosomes after degradation of newly synthesized strands (35) (Figure 3). This allows to distinguish telomeres replicated by either leading or lagging strand synthesis. Results showed that almost all fused sister chromatids in ATR^{KD} cells had both parental telomeric C and G strand sequences at the fusion points (Supplementary Figure S6), confirming the involvement of both sister telomeres in sister chromatid fusions and thus that sister chromatid fusions resulted directly from telomere destabilization in ATR^{KD} cells.

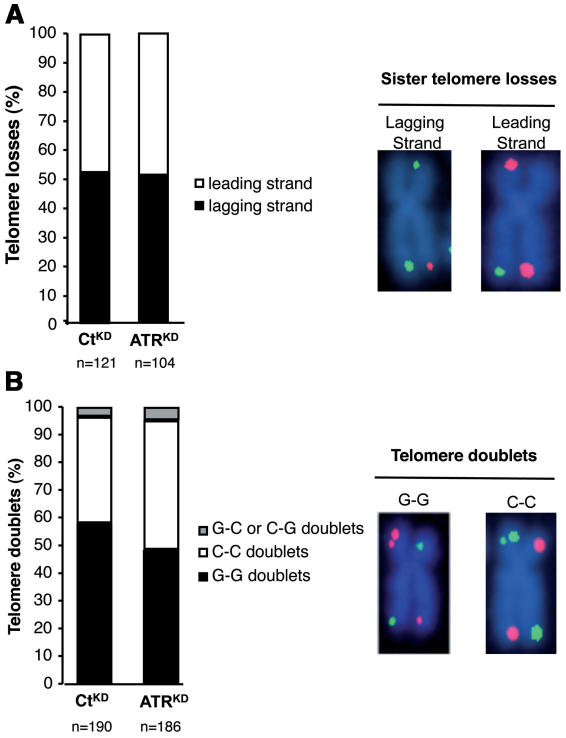

Figure 3. ATR inhibition destabilizes both lagging and leading strand telomeres. (**A**) Histograms show the percentages of sister telomere losses affecting the lagging or the leading strand in ATR^{KD} and Ct^{KD} cells detected by CO-FISH on metaphase spreads. 'n' represents the total number of telomere losses analyzed. No significant difference in the repartition of missing lagging or leading strand telomeres between the two cell lines was detected by chi-square analysis. Representative images of chromosomes missing lagging or leading strand telomeres are shown on the right. Lagging strand telomeres are labeled in red by hybridization of the parental G strands with Cy3-PNA probe and the leading telomeres in green by hybridization of the parental C strand with FITC-PNA probe. (**B**) Histograms show the respective percentages of telomere doublets containing two parental C-strand telomeres (C–C doublets) or two parental G-strand telomeres (G–G doublets) or both parental strand telomeres (C–G or G–C doublets) in ATR^{KD} and Ct^{KD} cells. 'n' represents the total number of telomere doublets analyzed. No significant difference in the repartition of the three classes of telomere doublets between the two cell lines was detected by chi-square analysis. Representative images of telomere doublets detected by CO-FISH are shown on the right.

CO-FISH also showed that sister telomere losses concerned almost equally parental telomere C or G strands in ATR^{KD} cells and thus that deficiency in ATR led indifferently to the loss of the leading or lagging strand telomeres (Figure 3A).

Finally, CO-FISH revealed that 95% of the telomere doublets hybridized with only one of the probes (i.e. telomere signals on a single chromatid were either both leading or both lagging strand telomeres, Figure 3B). This suggests that telomere doublets in ATR^{KD} cells were not due to amplification of telomeric sequences, but rather to recombination between parental telomeric sequences and interstitial sequences in *cis*. Interestingly, ATR deficiency led to a similar increase in doublets of parental telomeric C strands than of parental telomeric G strands, indicating that ATR deficiency induced telomere doublets indifferently at leading and lagging strand telomeres.

Altogether, these data support the hypothesis that ATR contributes to telomere maintenance during and/or after replication, conferring stability to both leading and lagging strand telomeres.

ATR prevents telomere destabilization induced by the G4 ligand 360A

To better understand the contribution of ATR to telomere maintenance, we used the G4 ligand, 360A, which preferentially binds telomeric G-quadruplex DNA structures (29,30) and destabilizes telomeres during and/or after replication (31).

Treatment with 5 μM 360A led to a progressive decrease in the rate of population doublings until complete growth arrest and apoptosis induction in Ct^{KD} cells as well as in ATR^{KD} cells (Supplementary Figure S3B and C), as previously observed in various cell lines treated by the G4 ligand (30,31). However, clonogenic survival assay showed that ATR deficiency significantly enhanced the sensitivity of HeLa cells to 360A (Supplementary Figure S3E).

Telo-FISH analysis after 8 days of treatment—this treatment duration was chosen because of the lack of detectable perturbation of the cell cycle and apoptosis that could bias the observation and which occur at later times (Supplementary Figure S3C and D)—showed that 360A induced a strong telomere instability in Ct^{KD} HeLa characterized by an increase in sister telomere fusions, sister telomere losses and telomere doublets as previously described (31) (Figure 4).

ATR knockdown led to a dramatic increase in telomere aberrations induced by the G4 ligand ($21.8 \pm 0.9\%$ chromosomes/cell in ATR^{KD} cells compared to $9.3 \pm 1\%$ in Ct^{KD} cells; $P < 0.0001$; Figure 4A). Some telomere aberrations occurring in G1 or early S-phase (i.e. dicentric chromosomes and telomeric DNA containing double-minute chromosomes (TDM) (39) were increased in 360A-treated ATR^{KD} compared to 360A-treated Ct^{KD} cells (Supplementary Figure S5). However, major consequences of ATR deficiency were observed on induction of sister telomere fusions, sister telomere losses and telomere doublets, which were dramatically increased in 360A-treated ATR^{KD} cells compared to 360A-treated controls (Figure 4B).

Terminal deletions induced by the G4 ligand were also increased in ATR^{KD} cells compared to Ct^{KD} cells ($1.8 \pm 0.3\%$ versus $0.5 \pm 0.1\%$, $P < 0.001$; Figure 4B), but remained at a significantly lower level than each of the main telomere aberrations listed above. The percentage of chromosomes with chromatid breaks induced by 360A was also enhanced in ATR^{KD} compared to Ct^{KD} cells ($1.3 \pm 0.3\%$ versus $0.1 \pm 0.07\%$, $P < 0.0001$; Figure 4A). However, chromatid breaks were 16.7 times less abundant than telomere aberrations.

CO-FISH showed that most of 360A-induced sister telomere fusions contained both leading and lagging strand telomere sequences at the fusion points in

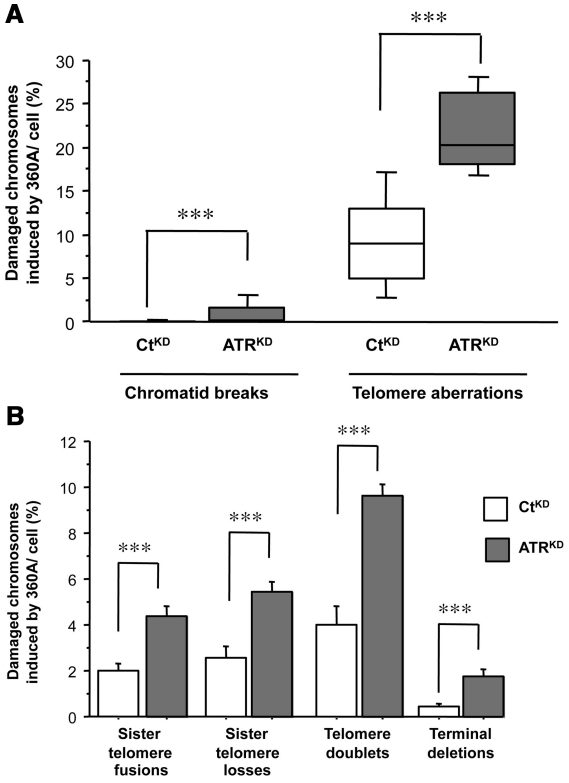

Figure 4. ATR knockdown increases telomere instability induced by the G4 ligand 360A. (**A**) Increase in telomere aberrations in ATR^{KD} compared to Ct^{KD} HeLa cells after 8 days of treatment with 5 μM of the G4-ligand 360A found by Telo-FISH experiments. Box graphs show distributions of percentages of chromosomes with chromatid breaks or telomere aberrations per cell induced by the G4 ligand. Values are given minus the mean percentage of chromosomes with telomere aberrations found in basal conditions (DMSO 0.05%, 8 days). Boxes include 50% of the values centered on the median (the horizontal line through the box). The vertical lines begin at the 10th percentile and end at the 90th percentile (***t-test P-value < 0.0001; at least $n = 30$ metaphases per condition). (**B**) Histograms show the percentages of chromosomes with the indicated telomeres aberrations induced by the ligand per metaphase from ATR^{KD} and Ct^{KD} HeLa cells as described in (A). Percentages (±SEM) were calculated from at least n = 30 metaphases per cell lines minus the mean percentage of chromosomes with telomere aberrations found in basal conditions (DMSO 0.05%, 8 days) (*t-test P-value ≤ 0.05; **$P < 0.001$; ***$P < 0.0001$). Box graph showing the distributions of the percentages of the telomere aberrations per cell is given in Supplementary Figure S5.

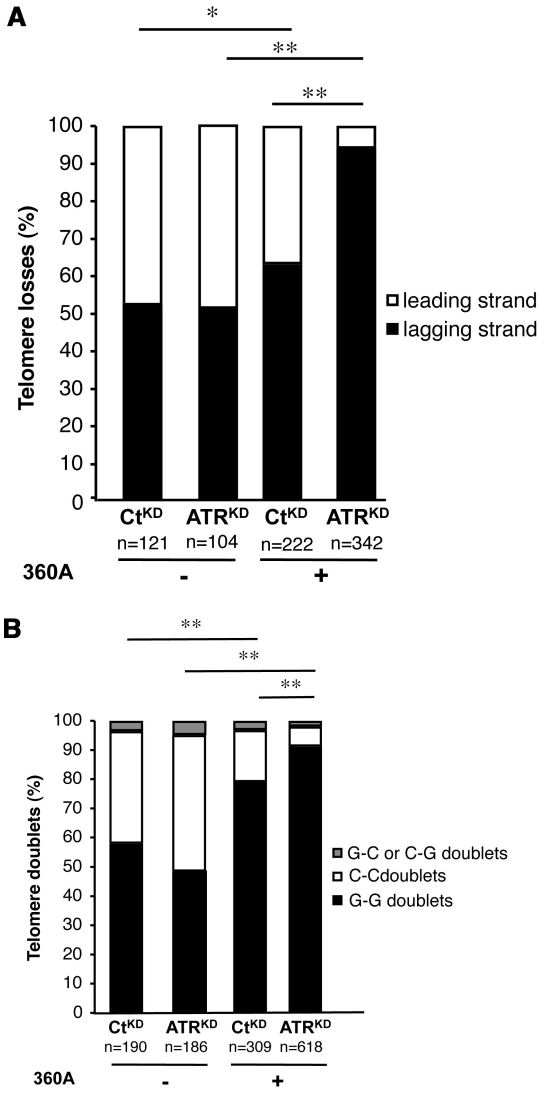

Figure 5. ATR knockdown increases lagging strand telomeres destabilization after G4 stabilization in HeLa cells. CO-FISH were performed on metaphases of ATR^{KD} and Ct^{KD} HeLa cells treated with (+) or without (–) 5 μM 360A for 8 days. (**A**) Histograms show the percentages of sister telomere losses affecting lagging or leading strand in ATR^{KD} and Ct^{KD} cells. 'n' represents the total number of telomere losses analyzed. Chi-square analysis was performed to detect differences in the repartition of missing lagging or leading strand telomeres between the different conditions (*$P < 0.05$; **$P < 0.001$). (**B**) Histograms show the respective percentages of telomere doublets containing two parental C-strand telomeres (C–C doublets) or two parental G-strand telomeres (G–G doublets) or both parental strand telomeres (C–G or G–C doublets) in ATR^{KD} and Ct^{KD} HeLa cells. 'n' represents the total number of telomere doublets analyzed (**, Chi-square $P < 0.001$).

ATR^{KD} cells (Supplementary Figure S6) confirming that ATR deficiency specifically increased telomere destabilization induced by the G4 ligand. Whereas telomere doublets and sister telomere losses concerned similar proportions of leading and lagging strand telomeres in untreated cells, CO-FISH showed that the proportions of chromatid-type aberrations involving the lagging strand telomeres were significantly increased in 360A-treated cells (Figure 5A and B). These data indicate that chromatid-type aberrations induced by the G4 ligand primarily concerned the lagging strand telomeres in accordance with the specific targeting of parental G-strand by 360A that we reported before (31). Interestingly, these proportions were dramatically increased in 360A-treated ATR^{KD} cells (in which the lagging-strand telomeres concerned 91% of telomere doublets and 94% of telomere losses) compared to 360A-treated Ct^{KD} cells (80 and 62%, respectively). These data confirm thus the specific induction by 360A of chromatid-type aberrations involving the lagging strand telomeres and that ATR deficiency specifically increased these types of telomere aberrations in response to 360A (Figure 5A and B).

Altogether, these data confirm that ATR has an important role in telomere maintenance during and/or after

telomere replication, both in basal conditions and in response to the G4 ligand.

DISCUSSION

We have shown here an increase in telomere instability occurring during and/or after telomere replication (sister telomere fusions and chromatid-type aberrations) in Seckel fibroblasts displaying defective ATR signaling pathway. A similar pattern of telomere instability was observed in HeLa cells knocked down for ATR. Altogether, these data unveil an important role for ATR in human telomere maintenance during and/or after replication in S and G2 phases, which is consistent with its localization at functional telomeres in human cells during these phases of the cell cycle (8).

Telomere instability in human cells related to ATR deficiency has not been reported before but this observation is in accordance with a recent report showing that embryonic fibroblasts from a mouse model for ATR-mutated Seckel syndrome have increased multi-telomeric signals (reminiscent of telomere doublets) (48).

The exact mechanisms leading to the main telomere aberrations found in ATR-deficient cells remained to be determined but they likely result from defective telomere replication and/or defective formation of the T-loop. Incomplete telomere replication could thus generate dysfunctional telomeres that cannot form a T-loop leading to sister telomere fusions mediated by NHEJ (36). Alternatively, sister telomere associations due to inefficient replication could also occur independently of NHEJ as recently shown in mouse TRF1 null cells (49). We have previously proposed that telomere doublets result from improper T-loop formation leading to recombination between telomeres and interstitial sequences in *cis* (31). Another possibility is that telomere doublets result from incomplete replication or processing of stalled forks leading to altered packaging and/or condensation of the chromatin due to extended areas of single-stranded DNA as recently suggested for fragile telomeres, which are reminiscent of telomere doublets in mouse cells (49). Sister telomere losses could be also due to incomplete replication (40) or result from unstable T-loop leading to the loss of telomere sequences by a process known as T-loop HR (31,50).

Importantly, CO-FISH showed that sister telomere losses and telomere doublets occurred in similar proportions at both leading and lagging strand telomeres and that sister chromatid fusions involved both leading and lagging strand telomeres in ATR knockdown cells. ATR appears thus equally important for stability of both telomeres generated by leading and lagging strand synthesis. This differs from DNA-PKcs, which is required for post-replicative processing of only telomeres replicated by the leading strand DNA synthesis. DNA-PKcs inhibition has been thus shown to induce chromatid-type fusions involving exclusively the leading strand telomeres of distinct chromosomes (35). We did not detect any chromatid-type telomeres fusion, but sister telomere fusion in Seckel fibroblasts and ATR^{KD} HeLa cells, supporting the hypothesis that, contrary to DNA-PKcs, ATR contributes to maintenance of both leading and lagging strand telomeres.

Although normal fibroblasts express only low levels of telomerase activity in S-phase (51) and Hela cells express largely higher levels (data not shown) (52), we observed the same pattern of telomere instability in both cell types. This suggests that telomerase status does not interfere with telomere instability induced by ATR deficiency, and thus that the role of ATR at telomeres is independent of telomerase activity.

ATR is needed to stabilize and rescue stalled replication forks, thereby preventing fork cleavage and subsequent illegitimate recombination (16). The data presented here support thus the hypothesis that efficient and complete telomere replication requires the ATR-dependent machinery (8).

The ability of telomere chromatin to form unusual structures can also lead to stalled replication forks during S-phase (53). ATR may preserve telomere stability by resolving structural DNA blocks, such as G-quadruplexes, within the telomere sequences, allowing efficient and complete telomere replication and T-loop formation. This possible role of ATR is well supported by the increase in telomere aberrations induced by the G-quadruplex ligand 360A in ATR knockdown cells compared to controls. Particularly, we showed a significant increase in chromatid-type aberrations involving specifically the lagging strand telomeres in 360A-treated ATR^{KD} cells, consistently with the specific targeting of the parental G-strand by the G-quadruplex ligand (31) and the importance of the ATR signaling pathway for their prevention. Upon stalled replication, ATR has been reported to phosphorylate and co-localize with the RecQ helicases WRN and BLM (17,18,54), which both could unwind G-quadruplex structures (55,56). Moreover sister telomere losses, almost exclusively concerning the lagging strand telomeres, have been reported in cells lacking WRN helicase (40,57). Hence, ATR could recruit WRN and BLM helicases to resolve G-quadruplex structures stabilized by the ligand.

To conclude, our data suggest that ATR plays an important role in telomere maintenance during and/or after replication. ATR may prevent telomere instability by allowing the completion of telomere replication and reformation of T-loop. ATR could act through initiation of the resolution of unusual DNA structures at telomeres such as G-quadruplexes as suggested by our results obtained with the G4 ligand. However, further experiments are needed to fully address this role in normal human cells, such as either knocking down ATR in normal fibroblasts or rescuing ATR-mutant Seckel cells using a wild type ATR gene. Finally, our study reveals a novel link between telomere instability and the Seckel syndrome. The importance of telomere instability for the pathogenesis of this disease should be further investigated.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We would like to thank Dr M. Martin (CEA, France), Drs C. Laurent and J.P. Puget (IRSN, France) for normal primary fibroblasts and Dr P. Bertrand (CEA, France) for GM09812 Seckel fibroblasts. We also would like to thank all members of the LRP lab and Drs J.P. Radicella and V. Pennaneach for advice and support.

FUNDING

Funding for open access charge: CEA.

Conflict of interest statement. None declared.

REFERENCES

1. Gilson,E. and Geli,V. (2007) How telomeres are replicated. *Nat. Rev. Mol. Cell. Biol.*, **8**, 825–838.
2. Makarov,V.L., Hirose,Y. and Langmore,J.P. (1997) Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell*, **88**, 657–666.
3. de Lange,T. (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.*, **19**, 2100–2110.
4. Griffith,J.D., Comeau,L., Rosenfield,S., Stansel,R.M., Bianchi,A., Moss,H. and de Lange,T. (1999) Mammalian telomeres end in a large duplex loop. *Cell*, **97**, 503–514.
5. Karlseder,J., Broccoli,D., Dai,Y., Hardy,S. and de Lange,T. (1999) p53-and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science*, **283**, 1321–1325.
6. d'Adda di Fagagna,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.
7. 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.
8. 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.
9. Longhese,M.P. (2008) DNA damage response at functional and dysfunctional telomeres. *Genes Dev.*, **22**, 125–140.
10. Attwooll,C.L., Akpinar,M. and Petrini,J.H. (2009) The Mre11 complex and the response to dysfunctional telomeres. *Mol. Cell. Biol.*, **29**, 5540–5551.
11. Cimprich,K.A. and Cortez,D. (2008) ATR: an essential regulator of genome integrity. *Nat. Rev. Mol. Cell. Biol.*, **9**, 616–627.
12. Seckel,H.P.G. (1960) Bird-*Headed* Dwarfs: Studies in Developmental Anthropology Including Human Proportions. Illinois, Springfield.
13. O'Driscoll,M., Ruiz-Perez,V.L., Woods,C.G., Jeggo,P.A. and Goodship,J.A. (2003) A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. *Nat. Genet.*, **33**, 497–501.
14. Alderton,G.K., Joenje,H., Varon,R., Borglum,A.D., Jeggo,P.A. and O'Driscoll,M. (2004) Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway. *Hum. Mol. Genet.*, **13**, 3127–3138.
15. Dart,D.A., Adams,K.E., Akerman,I. and Lakin,N.D. (2004) Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase. *J. Biol. Chem.*, **279**, 16433–16440.
16. Paulsen,R.D. and Cimprich,K.A. (2007) The ATR pathway: fine-tuning the fork. *DNA Repair (Amst)*, **6**, 953–966.
17. Pichierri,P., Rosselli,F. and Franchitto,A. (2003) Werner's syndrome protein is phosphorylated in an ATR/ATM-dependent manner following replication arrest and DNA damage induced during the S phase of the cell cycle. *Oncogene*, **22**, 1491–1500.
18. Davies,S.L., North,P.S., Dart,A., Lakin,N.D. and Hickson,I.D. (2004) Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest. *Mol. Cell. Biol.*, **24**, 1279–1291.
19. Tripathi,V., Kaur,S. and Sengupta,S. (2008) Phosphorylation-dependent interactions of BLM and 53BP1 are required for their anti-recombinogenic roles during homologous recombination. *Carcinogenesis*, **29**, 52–61.
20. Vespa,L., Couvillion,M., Spangler,E. and Shippen,D.E. (2005) ATM and ATR make distinct contributions to chromosome end protection and the maintenance of telomeric DNA in Arabidopsis. *Genes Dev.*, **19**, 2111–2115.
21. 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.
22. Matsuura,A., Naito,T. and Ishikawa,F. (1999) Genetic control of telomere integrity in Schizosaccharomyces pombe: rad3(+) and tel1(+) are parts of two regulatory networks independent of the downstream protein kinases chk1(+) and cds1(+). *Genetics*, **152**, 1501–1512.
23. Naito,T., Matsuura,A. and Ishikawa,F. (1998) Circular chromosome formation in a fission yeast mutant defective in two ATM homologues. *Nat. Genet.*, **20**, 203–206.
24. Nakamura,T.M., Moser,B.A. and Russell,P. (2002) Telomere binding of checkpoint sensor and DNA repair proteins contributes to maintenance of functional fission yeast telomeres. *Genetics*, **161**, 1437–1452.
25. Bi,X., Srikanta,D., Fanti,L., Pimpinelli,S., Badugu,R., Kellum,R. and Rong,Y.S. (2005) Drosophila ATM and ATR checkpoint kinases control partially redundant pathways for telomere maintenance. *Proc. Natl Acad. Sci. USA*, **102**, 15167–15172.
26. Herbig,U., Jobling,W.A., Chen,B.P., Chen,D.J. and Sedivy,J.M. (2004) Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol. Cell*, **14**, 501–513.
27. Denchi,E.L. and de Lange,T. (2007) Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. *Nature*, **448**, 1068–1071.
28. Guo,X., Deng,Y., Lin,Y., Cosme-Blanco,W., Chan,S., He,H., Yuan,G., Brown,E.J. and Chang,S. (2007) Dysfunctional telomeres activate an ATM-ATR-dependent DNA damage response to suppress tumorigenesis. *EMBO J.*, **26**, 4709–4719.
29. Granotier,C., Pennarun,G., Riou,L., Hoffschir,F., Gauthier,L.R., De Cian,A., Gomez,D., Mandine,E., Riou,J.F., Mergny,J.L. *et al.* (2005) Preferential binding of a G-quadruplex ligand to human chromosome ends. *Nucleic Acids Res.*, **33**, 4182–4190.
30. Pennarun,G., Granotier,C., Gauthier,L.R., Gomez,D., Hoffschir,F., Mandine,E., Riou,J.F., Mergny,J.L., Mailliet,P. and Boussin,F.D. (2005) Apoptosis related to telomere instability and cell cycle alterations in human glioma cells treated by new highly selective G-quadruplex ligands. *Oncogene*, **24**, 2917–2928.
31. Pennarun,G., Granotier,C., Hoffschir,F., Mandine,E., Biard,D., Gauthier,L.R. and Boussin,F.D. (2008) Role of ATM in the telomere response to the G-quadruplex ligand 360A. *Nucleic Acids Res.*, **36**, 1741–1754.
32. Liu,X., Matsuda,A. and Plunkett,W. (2008) Ataxia-telangiectasia and Rad3-related and DNA-dependent protein kinase cooperate in G2 checkpoint activation by the DNA strand-breaking nucleoside analogue 2′-C-cyano-2′-deoxy-1-beta-D-arabino-pentofuranosylcytosine. *Mol. Cancer Ther.*, **7**, 133–142.
33. Biard,D.S. (2007) Untangling the relationships between DNA repair pathways by silencing more than 20 DNA repair genes in human stable clones. *Nucleic Acids Res.*, **35**, 3535–3550.
34. Lansdorp,P.M., Verwoerd,N.P., van de Rijke,F.M., Dragowska,V., Little,M.T., Dirks,R.W., Raap,A.K. and Tanke,H.J. (1996) Heterogeneity in telomere length of human chromosomes. *Hum. Mol. Genet.*, **5**, 685–691.
35. Bailey,S.M., Cornforth,M.N., Kurimasa,A., Chen,D.J. and Goodwin,E.H. (2001) Strand-specific postreplicative processing of mammalian telomeres. *Science*, **293**, 2462–2465.
36. Smogorzewska,A., Karlseder,J., Holtgreve-Grez,H., Jauch,A. and de Lange,T. (2002) DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. *Curr. Biol.*, **12**, 1635–1644.

37. Dynek,J.N. and Smith,S. (2004) Resolution of sister telomere association is required for progression through mitosis. *Science*, **304**, 97–100.
38. Dimitrova,N. and de Lange,T. (2009) Cell cycle-dependent role of MRN at dysfunctional telomeres: ATM signaling-dependent induction of nonhomologous end joining (NHEJ) in G1 and resection-mediated inhibition of NHEJ in G2. *Mol. Cell. Biol.*, **29**, 5552–5563.
39. Zhu,X.D., Niedernhofer,L., Kuster,B., Mann,M., Hoeijmakers,J.H. and de Lange,T. (2003) ERCC1/XPF removes the 3′ overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. *Mol. Cell.*, **12**, 1489–1498.
40. 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.
41. Saharia,A., Guittat,L., Crocker,S., Lim,A., Steffen,M., Kulkarni,S. and Stewart,S.A. (2008) Flap endonuclease 1 contributes to telomere stability. *Curr. Biol.*, **18**, 496–500.
42. Philippe,C., Coullin,P. and Bernheim,A. (1999) Double hybridization signals on the chromatid revealed by telomeric probes. *Chromosome Res.*, **7**, 497–499.
43. Undarmaa,B., Kodama,S., Suzuki,K., Niwa,O. and Watanabe,M. (2004) X-ray-induced telomeric instability in Atm-deficient mouse cells. *Biochem. Biophys. Res. Commun*, **315**, 51–58.
44. van Overbeek,M. and de Lange,T. (2006) Apollo, an Artemis-related nuclease, interacts with TRF2 and protects human telomeres in S phase. *Curr. Biol.*, **16**, 1295–1302.
45. Casper,A.M., Durkin,S.G., Arlt,M.F. and Glover,T.W. (2004) Chromosomal instability at common fragile sites in Seckel syndrome. *Am. J. Hum. Genet.*, **75**, 654–660.
46. Brown,E.J. and Baltimore,D. (2000) ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev.*, **14**, 397–402.
47. Wang,H., Powell,S.N., Iliakis,G. and Wang,Y. (2004) ATR affecting cell radiosensitivity is dependent on homologous recombination repair but independent of nonhomologous end joining. *Cancer Res.*, **64**, 7139–7143.
48. Martinez,P., Thanasoula,M., Munoz,P., Liao,C., Tejera,A., McNees,C., Flores,J.M., Fernandez-Capetillo,O., Tarsounas,M. and Blasco,M.A. (2009) Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice. *Genes Dev.*, **23**, 2060–2075.
49. 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.
50. Wang,R.C., Smogorzewska,A. and de Lange,T. (2004) Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell*, **119**, 355–368.
51. Masutomi,K., Yu,E.Y., Khurts,S., Ben-Porath,I., Currier,J.L., Metz,G.B., Brooks,M.W., Kaneko,S., Murakami,S., DeCaprio,J.A. *et al.* (2003) Telomerase maintains telomere structure in normal human cells. *Cell*, **114**, 241–253.
52. Morin,G.B. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*, **59**, 521–529.
53. Maizels,N. (2006) Dynamic roles for G4 DNA in the biology of eukaryotic cells. *Nat. Struct. Mol. Biol.*, **13**, 1055–1059.
54. Pichierri,P. and Franchitto,A. (2004) Werner syndrome protein, the MRE11 complex and ATR: menage-a-trois in guarding genome stability during DNA replication? *Bioessays*, **26**, 306–313.
55. Sun,H., Karow,J.K., Hickson,I.D. and Maizels,N. (1998) The Bloom's syndrome helicase unwinds G4 DNA. *J. Biol. Chem.*, **273**, 27587–27592.
56. Mohaghegh,P., Karow,J.K., Brosh,R.M. Jr, Bohr,V.A. Jr and Hickson,I.D. (2001) The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases. *Nucleic Acids Res.*, **29**, 2843–2849.
57. Laud,P.R., Multani,A.S., Bailey,S.M., Wu,L., Ma,J., Kingsley,C., Lebel,M., Pathak,S., DePinho,R.A. and Chang,S. (2005) Elevated telomere-telomere recombination in WRN-deficient, telomere dysfunctional cells promotes escape from senescence and engagement of the ALT pathway. *Genes Dev.*, **19**, 2560–2570.