ATR suppresses telomere fragility and recombination but is dispensable for elongation of short telomeres by telomerase

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Telomere shortening caused by incomplete DNA replication is balanced by telomerase-mediated telomere extension, with evidence indicating that the shortest telomeres are preferred substrates in primary cells. Critically short telomeres are detected by the cellular DNA damage response (DDR) system. In budding yeast, the important DDR kinase Tel1 (homologue of ATM [ataxia telangiectasia mutated]) is vital for telomerase recruitment to short telomeres, but mammalian ATM is dispensable for this function. We asked whether closely related ATR (ATM and Rad3 related) kinase, which is important for preventing replicative stress and chromosomal breakage at common fragile sites, might instead fulfill this role. The newly created ATR-deficient Seckel mouse strain was used to examine the function of ATR in telomerase recruitment and telomere function. Telomeres were recently found to resemble fragile sites, and we show in this study that ATR has an important role in the suppression of telomere fragility and recombination. We also find that wild-type ATR levels are important to protect short telomeres from chromosomal fusions but do not appear essential for telomerase recruitment to short telomeres in primary mouse embryonic fibroblasts from the ATR-deficient Seckel mouse model. These results reveal a previously unnoticed role for mammalian ATR in telomere protection and stability.

Introduction

Telomeres are nucleoprotein structures that protect the ends of eukaryotic chromosomes from DNA repair and degradation activities. Telomeres comprise complexes of tandem DNA repeats (TTAGGG in vertebrates) bound by a specialized multiprotein complex known as shelterin (Palm and de Lange, 2008). Shelterin contributes to the protective function of telomeres by cancelling DNA damage response (DDR) activation at telomeres and preventing potential telomere fragility (Martínez et al., 2009b; Sfeir et al., 2009). In particular, recent studies demonstrate that the shelterin protein TRF1 is essential to prevent telomere breakage associated with replication fork stalling at telomeres (Martínez et al., 2009b; Sfeir et al., 2009). TRF1 deletion leads to uncapping and fusion of telomeres and to increased numbers of telomeres with multiple telomeric signals (MTSs), an aberrant structure that is enhanced by conditions known to cause replication fork stalling, such as aphidicolin treatments (Martínez et al., 2009b; Sfeir et al., 2009). Chromosomal defects associated with TRF1 deficiency are also accompanied by the activation of an ATM (ataxia telangiectasia mutated)/ATR (ATM and Rad3 related)-dependent DDR (Martínez et al., 2009b; Sfeir et al., 2009). Interestingly, the important DDR kinase ATR functions protecting and stabilizing stalled replication forks during DNA synthesis and is essential for preventing chromosomal gaps and breaks at common

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Abbreviations used in this paper: ALT, alternative lengthening of telomeres; ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3 related; CO-FISH, chromosome orientation FISH; CT, computed tomography; DDR, DNA damage response; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; MEF, mouse embryonic fibroblast; MTS, multiple telomeric signal; PIKK, phosphoinositide-3-kinase–related kinase; Q-FISH, quantitative FISH; RLF, Robertsonian-like fusion; SCE, sister chromatid exchange; SFE, signal-free end; SKY, spectral karyotyping; TRAP, telomerase repeat amplification protocol; T-SCE, telomere SCE.

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genomic fragile sites (Casper et al., 2002, 2004; Murga et al., 2009). The recent description of telomeres resembling fragile sites (Sfeir et al., 2009) suggests that ATR may have an important function in the maintenance of telomere stability. A role for ATR at mammalian telomeres is further supported by evidence showing that ATR can be found at mammalian telomeres during late replication (Verdun and Karlseder, 2006) as well as by the known role of *Drosophila melanogaster* ATR in telomere function (Bi et al., 2005).

Telomere length can be modulated by telomerase, a telomere-specific reverse transcriptase that uses its intrinsic RNA subunit as a template to add telomere repeats to chromosome ends (Chan and Blackburn, 2002). Telomerase is expressed in stem cell compartments and cancer cells, but adult tissues do not express sufficient telomerase for continuous telomere maintenance (Blasco, 2005). Because of incomplete replication of linear DNA ends, telomeres shorten with progressive rounds of cell division until critically short, leading to increased genomic instability and eventually to cell cycle arrest and apoptosis, which in turn contribute to organismal ageing (Blasco, 2005). Under conditions of telomere shortening, the shortest telomeres are thought to be the preferred substrate for telomere lengthening by telomerase in yeast, primary mouse cells, and some human cell lines (Hemann et al., 2001; Perrem et al., 2001; Samper et al., 2001a; Liu et al., 2002; Teixeira et al., 2004; Zhao et al., 2009), although this is not the case for some human cancer cell lines (Zhao et al., 2009). However, the mechanism enabling telomerase to recognize short telomeres is not clearly defined. A paradigm exists in budding yeast *Saccharomyces cerevisiae* in which the Tel1 checkpoint kinase (homologue of mammalian ATM) is a critical telomere length regulator; Tel1 deletion results in dramatic telomere shortening, telomere fusion, and elevated telomere recombination (Greenwell et al., 1995; DuBois et al., 2002). Telomere length in *tel1Δ* cells eventually stabilizes at a minimum length of 50 bp (about sevenfold shorter than wild-type telomeres; Lustig and Petes, 1986), with residual telomere length being maintained by the activity of the closely related Mec1 kinase (homologue of mammalian ATR). Deletion of Mec1 on its own has little effect on telomere length, but *mec1Δ tel1Δ* double mutants exhibit progressive telomere attrition and eventual complete loss of telomeres (Ritchie et al., 1999), a phenotype similar to cells lacking telomerase components (Singer and Gottschling, 1994; Lendvay et al., 1996). Telomerase activity is normal in these cells, and the short telomere phenotype of the *tel1Δ* and *mec1Δ tel1Δ* strains can be overcome by fusing telomerase to the telomere end–binding protein Cdc13 (Chan et al., 2001; Tsukamoto et al., 2001), suggesting that these cells are defective in the recruitment of functional telomerase to short telomeres (Pennock et al., 2001; Bianchi et al., 2004; Tseng et al., 2006). Furthermore, budding yeast Mec1 has been proposed to have roles facilitating fork progression at specific regions in the genome (Cha and Kleckner, 2002), which are characterized by slow replication, as well as in regulating dNTP (deoxyribonucleotide triphosphate) pools (Zhao et al., 1998), suggesting essential roles for Mec1 in replication. Although Tel1 is the major regulator of telomerase recruitment in budding yeast, an opposite scenario exists in fission yeast *Schizosaccharomyces pombe*, in which deletion of the ATR homologue Rad3 causes severe telomere shortening, but *tel1Δ* cells have normal telomeres (Dahlen et al., 1998; Nakamura et al., 2002). Simultaneous deletion of *rad3Δ tel1Δ* is synergistic and causes the complete loss of telomere signal, defective telomerase recruitment, elevated telomere recombination, and reduced binding of the shelterin components Tpz1 and Ccq1 (Nakamura et al., 2002; Moser et al., 2009). Thus, in yeast cells, the phosphoinositide-3-kinase–related kinase (PIKK) family members ATM and ATR make important contributions to telomerase recruitment and telomere length maintenance.

Given their resemblance to DNA double-strand breaks, it is not surprising that short dysfunctional telomeres activate DDRs mediated by the ATM and ATR checkpoint kinases (Guo et al., 2007; Longhese, 2008). In the presence of DNA damage, ATM and ATR phosphorylate a variety of downstream target proteins, variously affecting checkpoint-mediated cell cycle arrest, DNA repair, apoptosis, and senescence (Matsuoka et al., 2007). A short telomere–induced DDR is manifested through the accumulation at critically short telomeres of phosphorylated ATM, 53BP1, γ-H2AX, the MRN (Mre11–Rad50–Nbs1) complex, and Rif, forming so-called telomere dysfunction–induced foci (d'Adda di Fagagna et al., 2003; Takai et al., 2003; Verdun and Karlseder, 2006; Longhese, 2008). Paradoxically, several important DDR proteins also localize to telomeres in the absence of DNA damage, some of which have a role in telomere protection from DDRs, telomere length maintenance, and telomere replication, including ATM, ATR, MRN, Ku86, ERCC1, PARP2, BLM (Bloom syndrome protein), and WRN (Werner syndrome protein; Blasco 2005; Verdun and Karlseder 2006), demonstrating that there is critical interplay between DDR proteins and telomere metabolism in eukaryotic cells.

Because it was first observed that *ATM*-deficient human cells have shorter telomeres than normal cells (Metcalfe et al., 1996; Pandita, 2002) and this was later confirmed in *ATM*-null mice (Hande et al., 2001), a role for ATM in telomere length regulation in mammals has been sought. However, simultaneous deletion of *ATM* and *Terc* in mice does not lead to accelerated telomere shortening with respect to the single $Terc^{-/-}$ controls, although it causes increased genomic instability and increased apoptosis relative to mice lacking either gene alone, implying that ATM depletion increases dysfunction of short telomeres but is not required for inducing cell death or for telomere maintenance (Qi et al., 2003; Wong et al., 2003). In line with this, ATM was demonstrated to be dispensable for telomerase-mediated elongation of short telomeres in mammalian cells (Feldser et al., 2006). These findings prompted us to ask whether ATR might be a critical regulator of telomerase recruitment, as well as of the recently described resemblance between telomeres and common fragile sites (Sfeir et al., 2009).

Results

Generation of mice and cells doubly deficient in *ATR* and *Terc*

In contrast to ATM, analyzing the role of ATR in mammals has been hampered by the essential nature of the kinase

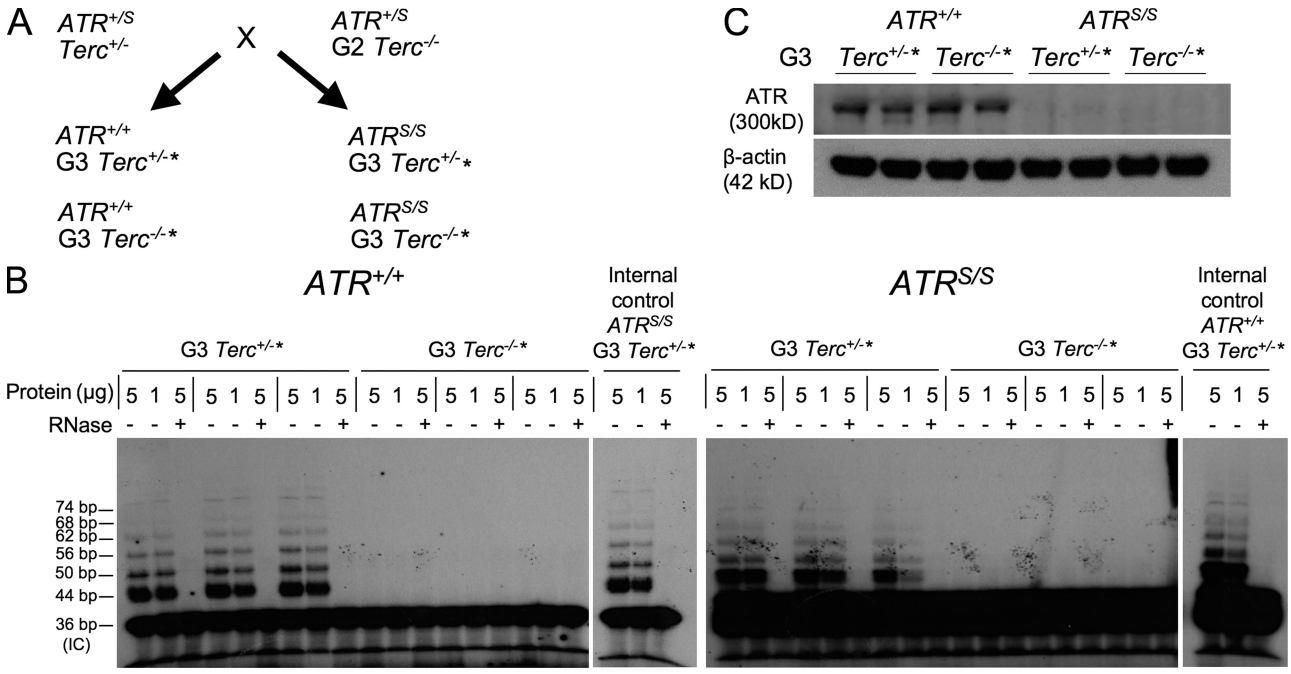

Figure 1. **Generation and characterization of Seckel Terc compound mice.** (A) Outline of the $ATR^{+/S}/Terc^{+/-}$ × $ATR^{+/S}$/G2 $Terc^{-/-}$ cross. G3 progeny inherit one set of short telomeres (from the G2 $Terc^{-/-}$ parent) and one set of long telomeres (from the $Terc^{+/-}$ parent), but only G3 $Terc^{+/-}$* progeny will be telomerase proficient. (B) The Seckel mutation does not affect telomerase activity. The TRAP assay shows successful telomerase reconstitution in G3 $Terc^{+/-}$* MEFs compared with their G3 $Terc^{-/-}$* littermates. Protein concentrations used are indicated above each lane. As a negative control, extracts were treated with RNase (+) or left untreated (−). IC, internal control. (C) Reduced ATR expression in $ATR^{S/S}$ cells. Western blot showing ATR expression levels in $ATR^{+/+}$ and $ATR^{S/S}$ MEFs. Actin loading control is indicated below.

(Brown and Baltimore, 2000; de Klein et al., 2000). Thus, the effects of ATR depletion in adult mice and cells have been tested only in the context of in vitro cell culture models or conditional *ATR* deletion in already adult animals (Ruzankina et al., 2007). To address the role of ATR in the regulation of telomerase recruitment, we used the Seckel syndrome mouse model recently generated by us (Murga et al., 2009). In a subset of human patients with Seckel syndrome, a single point mutation in *ATR* exon (E) 9 leads to aberrant splicing and severely decreased ATR protein expression and is the cause of growth retardation, microcephaly, brain and skeletal abnormalities, and highly unstable cellular genomes (O'Driscoll et al., 2003; Casper et al., 2004). The Seckel mouse model used in this study harbors a humanized ATR allele in which the region encompassing murine E8, E9, and E10 has been swapped by the human counterpart containing the Seckel mutation in E9 (Murga et al., 2009). This leads to a splicing defect in the E8–E10 boundary, which severely reduces ATR levels and recapitulates the human Seckel syndrome phenotypes in mice (Murga et al., 2009).

Mice lacking the RNA component of telomerase, *Terc*, show progressive telomere shortening from the first generation until the third or fourth generation (when in a pure C57BL6 background), by which stage they present critically short telomeres, defective stem cell proliferative capacity, infertility as the result of germ cell apoptosis, and increased genomic instability (Blasco et al., 1997; Herrera et al., 1999; Flores et al., 2005). We and others have shown that the restoration of telomerase activity in these mice, by reintroduction of one copy of the *Terc* gene, rescues critically short telomeres and reverses chromosomal instability and cell and tissue defects associated with late-generation telomerase deficiency (Hemann et al., 2001; Samper et al., 2001a; Siegl-Cachedenier et al., 2007).

To directly test whether ATR proficiency is necessary for telomerase action specifically on short telomeres in primary mouse embryonic fibroblasts (MEFs), we crossed $ATR^{+/S}/Terc^{+/-}$ females with G2 $ATR^{+/S}/Terc^{-/-}$ males to generate littermate populations of $ATR^{S/S}$ and $ATR^{+/+}$ mice that were also either G3 $Terc^{-/-}$ or G3 $Terc^{+/-}$. The G3 progeny of these crosses inherited a set of chromosomes with short telomeres from the male G2 $ATR^{+/S}/Terc^{-/-}$ parent and a set of chromosomes with normal telomeres from the female $ATR^{+/S}/Terc^{+/-}$ parent (and are hereafter referred to as G3 $Terc^{-/-}$* or G3 $Terc^{+/-}$*; Fig. 1 A), but only the G3 $Terc^{+/-}$* progeny would inherit a copy of the *Terc* gene and be telomerase proficient (Fig. 1 B). To confirm ATR abrogation in MEFs derived from the $ATR^{S/S}$ progeny, we first demonstrated that ATR expression is undetectable in $ATR^{S/S}$ compared with $ATR^{+/+}$ MEFs, as determined by Western blotting experiments (Fig. 1 C). As expected, *Terc* deficiency did not affect ATR expression levels (Fig. 1 C, compare the first and second lanes with the third and fourth lanes). We also confirmed that reintroduction of the *Terc* allele successfully reconstituted telomerase activity in both $ATR^{+/+}$/G3 $Terc^{+/-}$* and $ATR^{S/S}$/G3 $Terc^{+/-}$* MEFs (Fig. 1 B), demonstrating that telomerase processivity and activity is not affected by ATR depletion, which is similar to what has been previously reported for cells lacking ATM (Sprung et al., 1997).

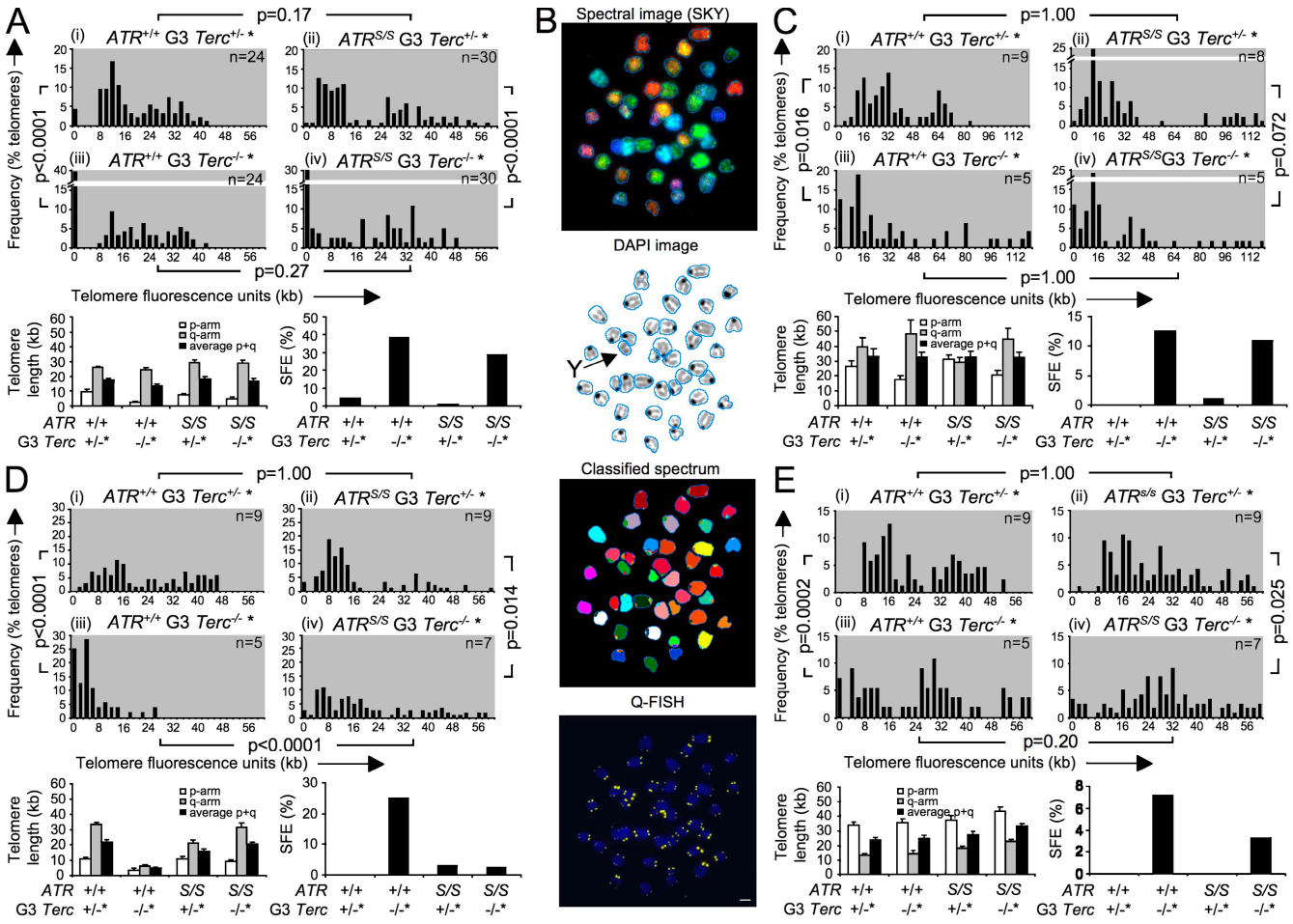

Figure 2. **ATR proficiency is dispensable for elongation of short telomeres.** (A) Telomere length histograms for each genotype (top, i–iv); mean lengths of p arm, q arm, and total telomeres (bottom left); and proportion of SFEs (bottom right) from Y chromosome as determined by Q-FISH are shown. Statistical comparison of proportion of chromosomes <1 kb using Fisher's exact test is shown. (B) Representative image of combined Q-FISH and SKY. (C–E) Telomere length distribution histograms for each genotype (top, i–iv); mean lengths of p arm, q arm, and total telomeres (bottom left); and proportion of SFEs (bottom right) from chromosome 1 (C), chromosome 2 (D), and chromosome 11 (E), as determined by Q-FISH, are shown. Statistical comparison of proportion of chromosomes <1 kb (C) and <4 kb (D and E) using Fisher's exact test is shown. (A and C–E) Error bars represent SEM. n, number of metaphases analyzed. Bar, 1 µm.

Severely decreased ATR levels in primary Seckel MEFs do not significantly impair elongation of short telomeres by telomerase

Next, we sought to directly test whether short telomeres inherited from the G2 $Terc^{-/-}$ father are elongated properly in telomerase-reconstituted $ATR^{S/S}$ cells. For this, we determined the telomere length of the Y chromosome using quantitative FISH (Q-FISH; Poon et al., 1999) in male littermate MEFs of each genotype under study: $ATR^{+/+}$/G3 $Terc^{+/-}$*, $ATR^{+/+}$/G3 $Terc^{-/-}$*, $ATR^{S/S}$/G3 $Terc^{+/-}$*, and $ATR^{S/S}$/G3 $Terc^{-/-}$*. Fig. 2 A shows the telomere length distribution frequency for Y chromosomes from littermate MEFs. As expected, cells receiving the *Terc*-null allele had a large peak of chromosome ends with undetectable telomeres (bottom panel, signal-free ends [SFEs]), which were completely rescued in cells inheriting the wild-type *Terc* allele ($P < 0.0001$; compare i with iii), which is in agreement with telomerase preferentially elongating critically short telomeres in MEFs (Samper et al., 2001a). Importantly, when a similar comparison is made for the ATR-deficient $ATR^{S/S}$ littermates (Fig. 2 A, compare ii with iv and see SFE in bottom panel), we found that telomerase-reconstituted $ATR^{S/S}$/G3 $Terc^{+/-}$* were also able to properly elongate critically short telomeres in a similar manner to their $ATR^{+/+}$ MEF siblings ($P = 0.17$ for frequency of SFEs; Fig. 2 A, compare i with ii), indicating that wild-type ATR proficiency is dispensable for the rescue of critically short Y chromosome telomeres and that Seckel cells are not compromised in telomerase recruitment to chromosome ends. Fig. 2 A (bottom left) also shows the mean Y chromosome telomere lengths for each genotype. As expected, $ATR^{+/+}$/G3 $Terc^{+/-}$* cells show longer mean telomeres (17.87 kb) than their $ATR^{+/+}$/G3 $Terc^{-/-}$* littermates (13.45 kb), but this lengthening is observed most dramatically for Yp telomeres, which were 7.14 kb longer; Yq telomeres were only moderately shorter in *Terc*-null cells (1.69 kb shorter). A similar trend is observed when comparing $ATR^{S/S}$/G3 $Terc^{+/-}$* and $ATR^{S/S}$/G3 $Terc^{-/-}$* cells, in which very short Yp telomeres were lengthened to a greater extent than Yq telomeres (2.74 kb longer and 0.42 kb longer, respectively). This pattern likely reflects preferential telomerase activity at short telomeres in mouse primary cells, where it appears that only

those telomeres that fall below a critical threshold are lengthened and not all telomeres in the cell (Hemann et al., 2001; Samper et al., 2001a).

To further confirm our observation of rescue of short telomeres in *ATR*-deficient mouse cells, we extended this analysis to additional chromosomes. We used spectral karyotyping (SKY) analysis in combination with Q-FISH (Fig. 2 B) to identify and measure the telomere lengths of specific chromosomes. Fig. 2 C shows that, as we observed for telomeres from the Y chromosome (Fig. 2 A), a population of critically short telomeres identified in *Terc*-null MEFs on chromosome 1 was effectively rescued by telomerase reconstitution in *ATR*-deficient $ATR^{S/S}$/G3 $Terc^{+/-}$* MEFs to a similar same extent as in $ATR^{+/+}$/G3 $Terc^{+/-}$* MEFs (P = 1.00 for SFE; comparison of i with iii and of ii with iv shows rescue of critically short telomeres), again indicating that murine *ATR*-deficient cells are not compromised in their ability to rescue short telomeres. This pattern is also reflected in the mean telomere lengths observed for chromosome 1 for the different genotypes in Fig. 2 C (bottom left). Although the overall mean telomere length did not change in each of the genotypes, the shorter chromosome 1p telomeres observed in *Terc*-null mice were increased to an equal length in both $ATR^{+/+}$/G3 $Terc^{+/-}$* and $ATR^{S/S}$/G3 $Terc^{+/-}$* cells (by 10.72 kb and 10.79 kb, respectively).

Using combined Q-FISH and SKY, we also examined the specific lengths of telomeres from chromosomes 2 and 11 in the different littermate MEFs. Chromosome 2 was examined because it has been found to have a shorter mean telomere length than other mouse chromosomes in several independent mouse strains (Zijlmans et al., 1997; Hande et al., 1999; Samper et al., 2001a), whereas chromosome 11 was chosen because it is homologous to human chromosome 17, which was also reported to have relatively short telomeres (Martens et al., 1998). In the telomere length histograms depicted in Fig. 2 (D [chromosome 2] and E [chromosome 11]), we see that telomerase deficiency in the $ATR^{+/+}$/G3 $Terc^{-/-}$* MEFs causes the accumulation of a population of critically short telomeres that were rescued in their $ATR^{+/+}$/G3 $Terc^{+/-}$* littermates. In the $ATR^{S/S}$/G3 $Terc^{-/-}$* MEFs, this critically short telomere population was less apparent than it was for $ATR^{+/+}$/G3 $Terc^{-/-}$*; however, the proportion of telomeres <4 kb was reduced by a statistically significant amount in $ATR^{S/S}$/G3 $Terc^{+/-}$* MEFs (P = 0.014 for chromosome 2; P = 0.025 for chromosome 11), indicating that telomerase is able to rescue short telomeres in MEFs devoid of detectable ATR, as previously demonstrated for telomeres on chromosome 1 and the Y chromosome (Fig. 2, A and C). In most cases, the absence of *Terc* in the $ATR^{+/+}$/G3 $Terc^{-/-}$* $ATR^{S/S}$/G3 $Terc^{-/-}$* caused increased frequency of SFEs in comparison with their $Terc^{+/-}$* counterparts, except for the telomeres of chromosome 2 in the $ATR^{S/S}$ MEFs. The reason for this anomaly is not clear but may be related to the presence of shorter telomeres in this chromosome and consequent telomerase-independent lengthening by recombination in the $ATR^{S/S}$/G3 $Terc^{-/-}$* population or $ATR^{S/S}$/G2 $Terc^{-/-}$* parent, leading to fewer SFEs because $Terc^{-/-}$ cells with short telomeres undergo more frequent telomere recombination than wild-type cells (Benetti et al., 2007b). Curiously, we noticed that the mean length of chromosome 11q telomeres was ~50% shorter than that of 11p telomeres in this MEF population, which is in contrast to the usual trend for p-arm telomeres to be shorter than q-arm telomeres (Fig. 2 E, bottom left; Zijlmans et al., 1997).

Further to our analysis of individual chromosomes, we also examined the telomere length distributions for all chromosomes for each genotype, incorporating three independent MEFs for each. As shown in Fig. 3 A, comparison of $ATR^{+/+}$/G3 $Terc^{+/-}$* with $ATR^{+/+}$/G3 $Terc^{-/-}$* and of $ATR^{S/S}$/G3 $Terc^{+/-}$* with $ATR^{S/S}$/G3 $Terc^{-/-}$* shows significantly (P < 0.0001) longer telomeres in the respective G3 $Terc^{+/-}$* populations, demonstrating telomere elongation upon telomerase restoration in both $ATR^{+/+}$ and $ATR^{S/S}$ lines. Note especially the dramatic reduction in critically short undetectable telomeres in these cell populations, as shown by arrows in Fig. 3 A. We found that the median telomere length in telomerase-competent $ATR^{+/+}$/G3 $Terc^{+/-}$* and $ATR^{S/S}$/G3 $Terc^{+/-}$* cells was not significantly different (P = 0.2173) but, surprisingly, the median telomere length of ATR-deficient $ATR^{S/S}$/G3 $Terc^{-/-}$* MEFs was significantly longer than $ATR^{+/+}$/G3 $Terc^{-/-}$* MEFs, even though the frequency of critically short telomeres was similar in both populations (P < 0.0001).

Severely decreased ATR levels augment telomere recombination in Seckel MEFs

The overall longer telomeres in *ATR*-deficient $ATR^{S/S}$/G3 $Terc^{-/-}$* MEFs are suggestive of either telomere lengthening caused by increased usage of recombination-based telomere lengthening mechanisms such as ALT (alternative lengthening of telomeres) in *ATR*-deficient MEFs in the absence of telomerase (Bailey et al., 2004; Gonzalo et al., 2006) or of reduced telomere shortening caused by compromised cell division of *ATR*-deficient cells (Brown and Baltimore, 2003; Murga et al., 2009). To explore the first possibility, that ATR might suppress telomeric recombination in *Terc*-deficient cells, we used chromosome orientation FISH (CO-FISH; Fig. 3 B) to directly visualize telomeric sister chromatid exchange (SCE [T-SCE]). A positive T-SCE was scored when chromatid exchange was observed between both leading and lagging strand telomeres (Fig. 3, B and C). Similar to previous observations, we found that $ATR^{+/+}$ cells lacking telomerase showed increased T-SCE (Fig. 3 D), likely reflecting increased recombination stimulated by short telomeres (Benetti et al., 2007a; Morrish and Greider, 2009). Interestingly, we also observed a significant increase in T-SCE in $ATR^{S/S}$/G3 $Terc^{+/-}$* cells in comparison with $ATR^{+/+}$/G3 $Terc^{+/-}$* cells, suggesting that ATR may negatively regulate telomeric recombination (Fig. 3 D). In $ATR^{S/S}$/G3 $Terc^{-/-}$* cells, T-SCEs were not significantly different to $ATR^{S/S}$/G3 $Terc^{+/-}$* or $ATR^{+/+}$/G3 $Terc^{-/-}$* cells, implying that the combined deficiency of *ATR* and *Terc* does not further increase telomeric recombination (Fig. 3 D). Importantly, global recombination frequencies (SCE) along whole chromosomes were not significantly influenced by either *Terc* or *ATR* deficiencies, suggesting a preferential impact of these deficiencies on telomeric repeats (Fig. 4, A and B). Collectively, these data indicate that ATR may negatively regulate telomeric recombination independently of the presence of functional telomerase. These results further indicate that increased telomeric

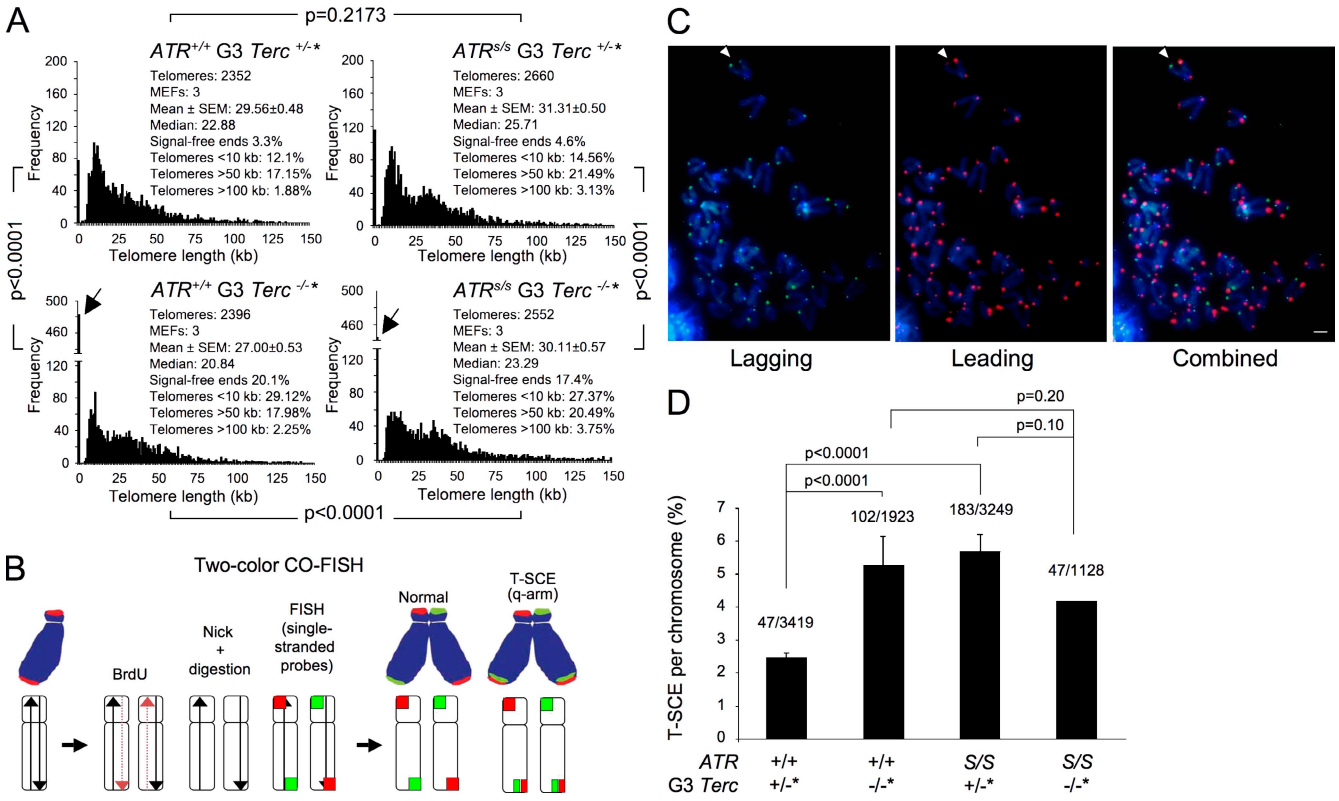

Figure 3. **Analysis of overall telomere length and telomeric recombination.** (A) Histogram of telomere lengths from all chromosomes in G3 $ATR^{+/+}$ and $ATR^{S/S}$ MEFs. Arrows indicate critically short telomeres in G3 $Terc^{-/-}$* MEFs that are rescued in G3 $Terc^{+/-}$* MEFs. Data from three independent MEF cultures per genotype are shown. (B) Schematic of CO-FISH telomere labeling resulting from leading-strand (red) and lagging-strand (green) DNA synthesis. T-SCE results in combined green and red fluorescence. (C) Representative CO-FISH images of full metaphases hybridized with probes against the leading (red) and lagging (green) telomeres. Arrowheads depict chromosomes where signals are interchanged at both sister telomeres. (D) Quantification of T-SCE. Only T-SCE events with exchanges between both leading- and lagging-strand probes simultaneously were considered positive. The number of T-SCE events scored out of total number of chromosomes is indicated. Data represent at least two independent MEF cultures per genotype. Statistical comparison using Fisher's exact test is shown. Error bars represent SEM. Bar, 1 µm.

recombination typically associated with ALT is unlikely to contribute to the significant increase in telomere length in $ATR^{S/S}$/G3 $Terc^{-/-}$* cells over their $ATR^{+/+}$/G3 $Terc^{-/-}$* counterparts, as both cells have similarly elevated telomere recombination frequencies. Although similarities in T-SCE frequencies alone are not sufficient to exclude the use of ALT, the increase in telomere length is more likely the result of delayed cell cycle kinetics in utero caused by limiting amounts of ATR (Murga et al., 2009) or may be the result of the existence of alternative recombination-independent, telomerase-independent mechanisms of telomere elongation.

Severely reduced ATR levels do not augment end-to-end fusions caused by short telomeres

Because of its essential role in DNA replication, *ATR*-deficient cells exhibit massive genome instability, particularly at fragile sites, that are sensitive to the formation of breaks and gaps after replication stress and are hotspots for SCEs, translocations, and deletions (Glover and Stein, 1987, 1988; Casper et al., 2004; Murga et al., 2009). Short dysfunctional telomeres also provoke genome instability, most often in the form of chromosome end-to-end fusions. In mouse primary cells, these fusions mostly involve p:p Robertsonian-like fusions (RLFs) and, to a lesser extent, q:q dicentrics and p:q fusions (Blasco et al., 1997; Herrera et al., 1999). Therefore, we were interested in determining the effects of combined *ATR* and *Terc* depletion on chromosomal instability. Short telomeres caused significantly increased end-to-end chromosome fusions in $ATR^{+/+}$/G3 $Terc^{-/-}$* cells compared with $ATR^{+/+}$/G3 $Terc^{+/-}$* (P = 0.02), a majority of which were RLFs (Fig. 5 A, black bars). Interestingly, ATR depletion in $ATR^{S/S}$/G3 $Terc^{+/-}$* cells did not increase RLFs over $ATR^{+/+}$/G3 $Terc^{+/-}$*, but other types of fusions were significantly increased (rings, complex aberrations, dicentrics, p:q arm fusions, etc.), likely reflecting stochastic joining of broken chromosomes and chromatids resulting from genome-wide instability in $ATR^{S/S}$ cells (Murga et al., 2009). The fact that the frequency of RLFs is similarly low in $ATR^{+/+}$/G3 $Terc^{+/-}$* and $ATR^{S/S}$/G3 $Terc^{+/-}$* (Fig. 5 A) indicates that severe ATR dysfunction does not, per se, lead to loss of telomere capping. Instead, the higher variety of chromosomal fusions in $ATR^{S/S}$/G3 $Terc^{-/-}$* double deficient cells in comparison with either of the single mutations (P = 0.004 in both cases) suggests that ATR depletion augments the dysfunctionality of short telomeres, as has been previously reported for ATM (Qi et al., 2003; Wong et al., 2003), and likely reflects opportunistic fusion of uncapped or fragile telomeres to chromosome breaks and fragments rather than a direct role for *ATR* in telomere capping, as suggested by the absence of any

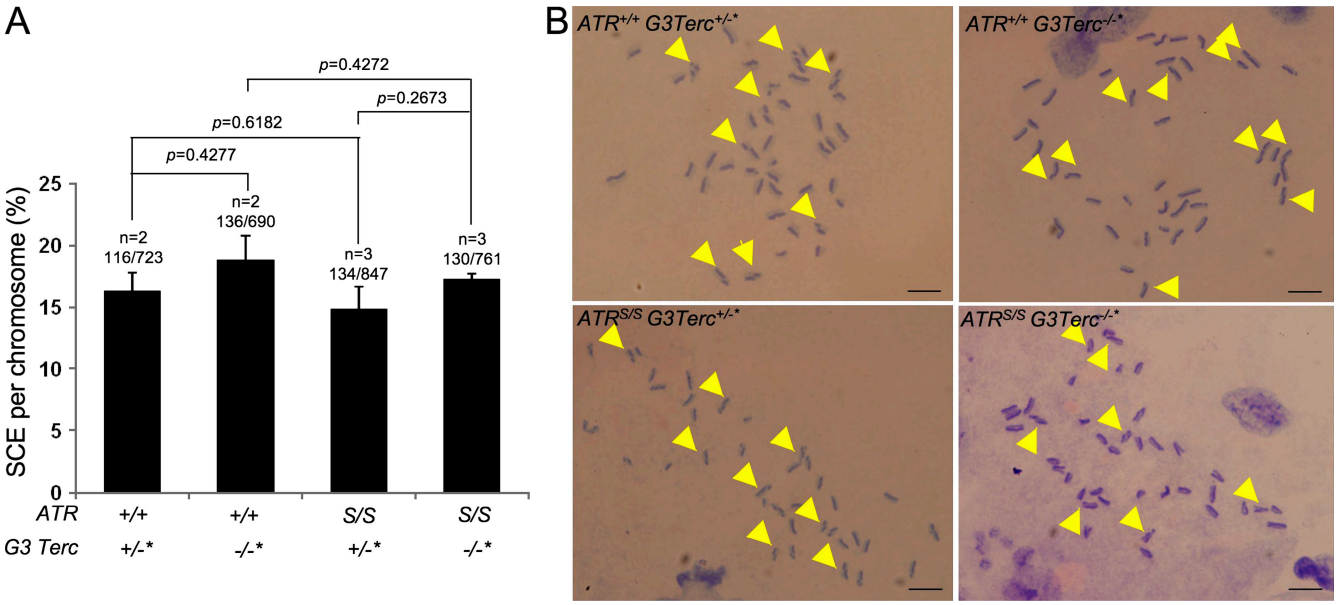

Figure 4. **Determination of global recombination frequencies (SCE).** (A) Quantification of global SCE frequencies. The number of SCE events scored out of the total number of chromosomes is indicated. Data represent at least two independent MEF cultures (n) per genotype. Statistical comparison using Student's *t* test is shown. Error bars represent SEM. (B) Representative global SCE images of full metaphases. Arrowheads depict chromosomes where there are SCE events. Bars, 5 µm.

telomere length anomalies (Fig. 2) and by similar frequencies of RLFs in $ATR^{+/+}$ and $ATR^{S/S}$ cells (Fig. 5 A). Finally, as previously reported by us, there was significant elevation in the number of chromatid breaks and fragments in the $ATR^{S/S}$ cells compared with their $ATR^{+/+}$ littermates (Fig. 5 B; Murga et al., 2009), but these were not affected by the *Terc* status of the cells because these aberrations do not typically result from critical telomere shortening.

ATR prevents telomeres from becoming fragile sites

Consistent with very recent findings, we observed a significant ATR-dependent increase in chromosome ends possessing MTSs, which is also increased in $ATR^{+/+}$ cells upon aphidicolin treatment (Fig. 5 C; Martínez et al., 2009b). Previously, we have demonstrated that MTSs are increased under conditions of replication stress, such as in *ATR*-deficient Seckel MEFs as well as in wild-type cells after treatment with the DNA polymerase α inhibitor aphidicolin, and thus, telomeres exhibit hallmark characteristics of common genomic fragile sites (Martínez et al., 2009b). MTSs are also increased in cells lacking TRF1, which is required for efficient telomere replication, and therefore are a direct indicator of telomere fragility resulting from replication fork stalling (Martínez et al., 2009b; Sfeir et al., 2009). In this study, we further demonstrate that *ATR*-dependent MTSs occur independently of the *Terc* status of the cell, indicating that telomere fragility is unrelated to telomere length (Fig. 5 C), thus suggesting that short telomeres are not more prone to replication fork stalling. Importantly, we further extended these findings to cells derived from human Seckel patients (O'Driscoll et al., 2003), which also present significantly increased MTSs, as well as increased breaks and fragments, which are further aggravated upon aphidicolin treatment (Fig. S1 A). Of note, we also observed slightly shorter telomeres in human Seckel cells compared with ATR wild-type controls (Fig. S1 B), although this modest telomere shortening was not accompanied by detectable end-to-end chromosome fusions (Fig. S1 A) and therefore is unlikely to cause telomere uncapping.

Notably, in $Terc^{+/-}$* cells with long telomeres, the ATR-dependent increase in MTSs was also paralleled by an ATR-dependent increase in T-SCE (Fig. 3 D). In addition to breaks and gaps, increased chromosomal instability means that fragile sites are also hotspots for increased recombination by SCE; 70% of all gaps and breaks at a common fragile site also have SCEs at the same site (Glover and Stein, 1987; Durkin and Glover, 2007). Therefore, we suggest that increased telomere recombination observed in $ATR^{S/S}$ MEFs is a direct consequence of increased telomere fragility and that our results indicate that we have revealed an important role for ATR in suppressing two outcomes of fragile telomeres: MTSs and recombination. Interestingly, we also observed a marked elevation in aneuploidy (primarily tetraploidy) in $ATR^{S/S}$ cells in comparison with $ATR^{+/+}$ (Fig. 5 D), which was not caused by the presence of short telomeres and may instead reflect defective G2/M checkpoint activation in *ATR*-deficient cells leading to an accumulation of cells with a 4N DNA content (Lin and Dutta, 2007), but the exact molecular mechanism of this remains to be confirmed.

Severe developmental defects in Seckel mice simultaneously abrogated for *Terc*

The *ATR* mutation linked to Seckel syndrome causes an array of characteristic phenotypes in both human patients and Seckel mice, including intrauterine and postnatal growth retardation, microcephaly, mental retardation, progeria, and facial and osteoskeletal abnormalities (O'Driscoll et al., 2003; Murga et al., 2009). Because *Terc* deficiency has been shown to suppress the

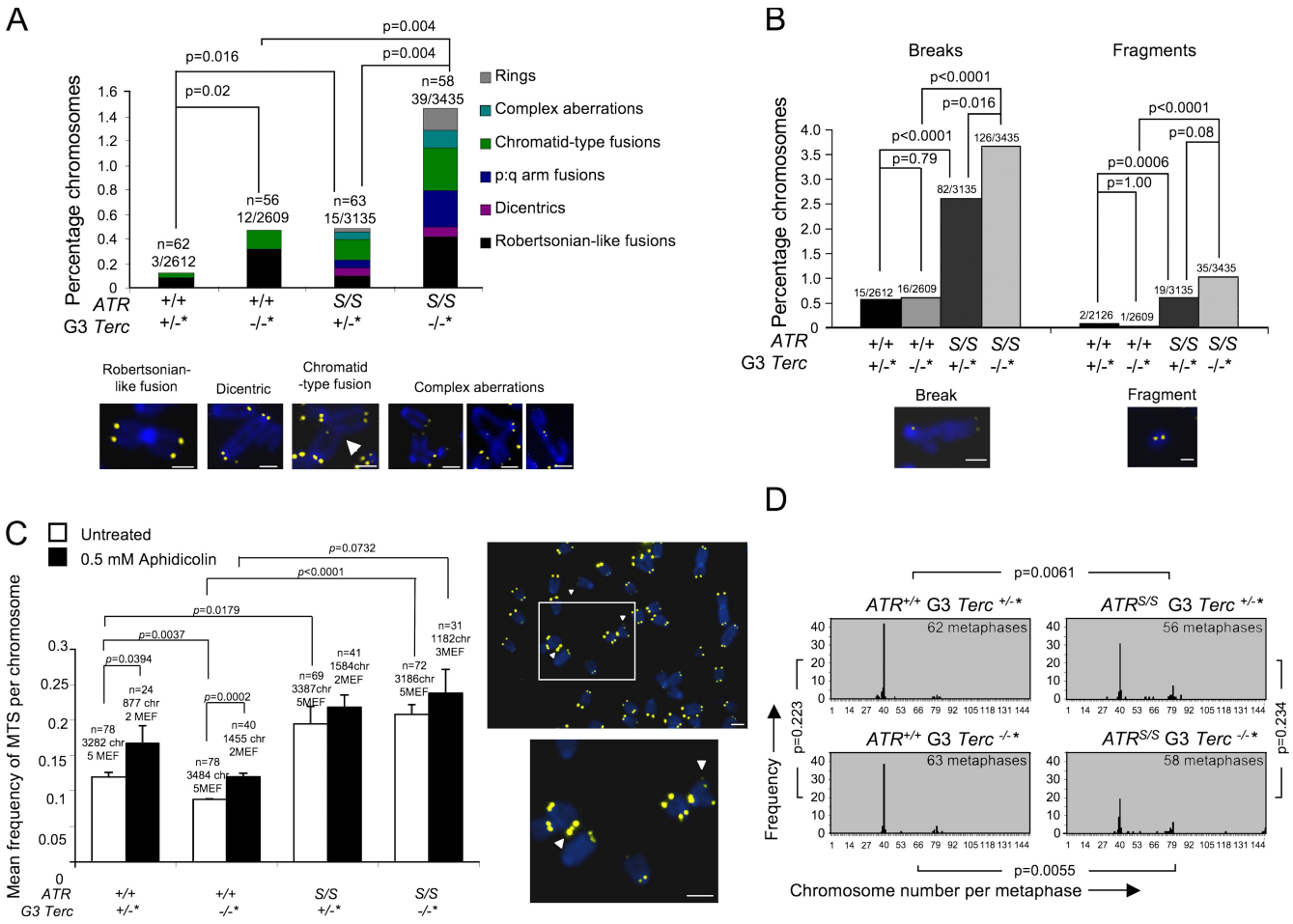

Figure 5. **Chromosomal aberrations in *ATR/Terc* MEFs.** (A–C) Quantification of chromosome fusions (A), chromatid breaks and fragments (B), and MTSs (C) in G3 $ATR^{+/+}$ and $ATR^{S/S}$ MEFs before (nontreated) or after treatment with aphidicolin. n, number of metaphases from two to five independent MEFs per indicated genotype. The number of events out of the total number of telomeres or chromosomes scored is indicated above the bars. Statistical comparisons using Fisher's exact test are shown. Error bars represent SEM. Photos show representative images of a metaphase and of individual aberrations. Arrowheads show the chromatid-type fusion (A) and MTSs (C). The boxed area in the larger micrograph in C shows examples of MTSs in detail below. (D) Increased aneuploidy in $ATR^{S/S}$ MEFs. Histograms showing the number of chromosomes per metaphase for each genotype are shown. Aneuploidy is defined as metaphases having other than 40 chromosomes. Statistical comparisons using Fisher's exact test are shown. Bars, 1 µm.

severity of phenotypes associated with ATM deletion (median lifespan and tumorigenesis; Qi et al., 2003; Wong et al., 2003), we were similarly interested in determining the interaction between *ATR* and *Terc* at the whole organism level.

To study the effect of *Terc* deficiency on Seckel mouse phenotypes, we first examined the impact on reduced lifespan. Critical telomere shortening leads to premature ageing and shortened lifespan in G3 $Terc^{-/-}$ mice, which we confirmed with the observation of significantly reduced survival of $ATR^{+/+}$/G3 $Terc^{-/-}$ mice compared with $ATR^{+/+}$/G3 $Terc^{+/-}$ mice (Fig. 6 A). Interestingly, we observed a tendency toward a reduced longevity in $ATR^{S/S}$/G3 $Terc^{-/-}$ mice compared with $ATR^{S/S}$/G3 $Terc^{+/-}$ (Fig. 6 A, P = 0.081; almost significant), most likely reflecting the increase in genome instability owed to combined deficiencies of *ATR* and *Terc* (Blasco et al., 1997; Murga et al., 2009). However, *Terc* deficiency did not contribute to the severe dwarfism of Seckel mice, which is already noticeable at birth, here indicated from the measurement of mean body weight at 7 wk of age (Fig. 6 B). Because of severe intrauterine growth defects and developmental abnormalities, Seckel mutant mice were born at reduced non-Mendelian frequencies in comparison with their wild-type and heterozygote littermates, and this was not further aggravated by simultaneous *Terc* deficiency (Table I; Murga et al., 2009). In line with this, G3 $Terc^{-/-}$* mice were born at the same frequency as G3 $Terc^{+/-}$* for all of the *ATR* genotypes under study (Table I).

Some of the most prominent phenotypes attributable to the $ATR^{S/S}$ mutation are severe microcephaly and osteoskeletal and cranial defects. Because telomere dysfunction has previously been associated with osteoporosis (Hofer et al., 2005; Pignolo et al., 2008), we were interested in analyzing the severity of osteoporosis and cranial abnormalities in G3 $Terc^{+/-}$* and G3 $Terc^{-/-}$* mice by computed tomography (CT) of 10-wk-old mice. However, the already severe osteoporosis associated with the $ATR^{S/S}$ mutation was not worsened in $ATR^{S/S}$/G3 $Terc^{-/-}$* mice compared with their $ATR^{S/S}$/G3 $Terc^{+/-}$* siblings (Fig. 6 C). Even though telomere defects have previously been linked to osteoporosis in mice, the severity of this is not apparent until old age (14 mo old) in comparison with the early appearance of osteoporosis in much younger (2 mo old) Seckel mice (Murga

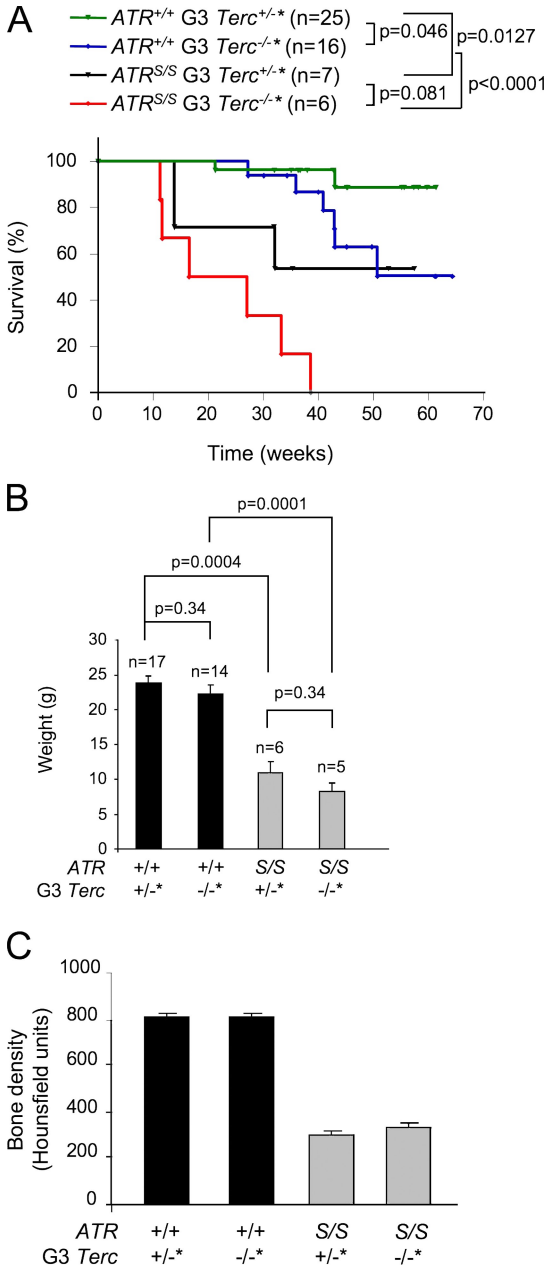

Figure 6. **Telomerase deficiency aggravates lifespan attenuation in Seckel mutant mice.** (A) Kaplan-Meier survival curve of mice of the indicated genotype. n, number of mice per genotype. Statistical comparisons using log-rank test are shown. (B) Reduced body weight of Seckel mice at 7 wk of age is not worsened by telomerase deficiency. Statistical comparisons using Student's *t* test (unpaired, two sided) are shown. n, number of mice. (C) Severe osteoporosis associated with ATR Seckel mutation is not increased by telomerase deficiency (two to three mice per genotype). (B and C) Error bars represent SEM.

et al., 2009). Similarly, the severe microcephaly of Seckel mice was not worsened by *Terc* deficiency, and there were no measurable differences in the dimensions of the cranial cavity, the extent of kyphosis, the length and angle of the nasal bone, mandibular lengths, or the cross-sectional dimensions of the thorax (Fig. S2, A–H) between $ATR^{S/S}$/G3 $Terc^{+/-}$* and $ATR^{S/S}$/G3 $Terc^{-/-}$* mice. Owing to the fact that Seckel mice have a greatly reduced lifespan, these mice never develop tumors (Murga et al., 2009),

Table I. **Telomerase deficiency does not further reduce the low birth rates of $ATR^{S/S}$ mice**

Genotype	Number of mice (percentage)
$ATR^{+/+}$/G3 $Terc^{+/-}$*	18 (15.5)
$ATR^{+/+}$/G3 $Terc^{-/-}$*	23 (19.8)
$ATR^{+/S}$/G3 $Terc^{+/-}$*	30 (25.9)
$ATR^{+/S}$/G3 $Terc^{-/-}$*	32 (27.6)
$ATR^{S/S}$/G3 $Terc^{+/-}$*	7 (6.0)
$ATR^{S/S}$/G3 $Terc^{-/-}$*	6 (5.2)

Table indicates the number of mice born for each genotype from a total of 19 $ATR^{+/S}/Terc^{+/-}$ × $ATR^{+/S}$/G2 $Terc^{-/-}$ crosses. Statistical analysis using χ^2 test is shown for comparison of G3 $Terc^{+/-}$* versus G3 $Terc^{-/-}$* for each ATR genotype. Parents were $ATR^{+/S}/Terc^{+/-}$ × $ATR^{+/S}$/G2 $Terc^{-/-}$. Mean litter size was 4.8 pups. The total number of mice studied is 116. P = 0.435 comparing $ATR^{+/+}$/G3 $Terc^{+/-}$* with $ATR^{+/+}$/G3 $Terc^{-/-}$*; P = 0.799 comparing $ATR^{+/S}$/G3 $Terc^{+/-}$* with $ATR^{+/S}$/G3 $Terc^{-/-}$*; and P = 0.593 comparing $ATR^{S/S}$/G3 $Terc^{+/-}$* with $ATR^{S/S}$/G3 $Terc^{-/-}$*.

which prevented addressing the impact of combined *Terc* and *ATR* deficiencies in carcinogenesis. Collectively, these results suggest that combined telomerase and *ATR* deficiencies seem to have a negative impact of longevity but do not worsen characteristic phenotypes of $ATR^{S/S}$ mice, most likely because of the early onset of Seckel phenotypes.

Discussion

Given that the yeast Tel1 and Mec1/Rad3 checkpoint kinases are critical for telomere length maintenance, a similar role was expected for their mammalian counterparts, and because murine ATM was proven dispensable for the lengthening of short telomeres, ATR was suspected to fulfill this role (Feldser et al., 2006; Sabourin and Zakian, 2008). We demonstrate in this study for the first time that a severe reduction in ATR levels does not impair telomerase-mediated elongation of short telomeres in mouse primary fibroblasts but has an important role in the prevention of telomere fragility and recombination.

ATR primarily functions to prevent replicative stress and genomic instability. In particular, ATR depletion leads to increased chromosome breakage, especially at fragile sites, both in human and mouse ATR-deficient Seckel cells (Casper et al., 2004; Murga et al., 2009). Consistent with this, we observed an ATR-dependent, Terc-independent increase in MTSs at chromosome ends, an aberration diagnostic of telomere fragility resulting from stalled DNA replication (Martínez et al., 2009b; Sfeir et al., 2009), and we further confirmed these results in cells derived from human Seckel patients. Furthermore, we also observed increased T-SCE in both $ATR^{S/S}$/G3 $Terc^{+/-}$* and $ATR^{S/S}$/G3 $Terc^{-/-}$* MEFs, indicating a role for ATR in the negative regulation of recombination at telomeres. Common fragile sites are genomic regions prone to replication fork stalling that exhibit increased formation of chromosome gaps and breaks and are also hotspots for increased recombination by SCE (Durkin and Glover, 2007). Given that 70% of all gaps and breaks at a common fragile site also have SCEs at these sites, our observation of ATR-dependent increase in MTSs paralleled by ATR-dependent increase in T-SCE underscores the notion that telomeres are bona fide fragile sites

whose stability is protected by ATR (Glover and Stein, 1987). Telomeres present inherent difficulties for the replication machinery, including topological interference by the t-loop and the tendency for secondary structure formation (such as G-quadruplexes) within repetitive sequences (Gilson and Géli, 2007). Replication forks stall naturally at mammalian telomeres and require an ATR-dependent restart for replication to complete (Verdun and Karlseder, 2006) and depend on TRF1 for efficiency of completion (Sfeir et al., 2009). Similarly, the fission yeast homologue of TRF1, Taz1, is crucial for efficient replication of yeast telomeres (Miller et al., 2006). Thus, similarly to the case for TRF1, increased telomere fragility in the absence of ATR most likely results from replication fork stalling at telomeres. Interestingly, budding yeast Mec1 has been proposed to have roles facilitating fork progression at specific regions in the genome (Cha and Kleckner, 2002), which are characterized by slow replication, as well as in regulating dNTP pools (Zhao et al., 1998), suggesting essential roles for Mec1 in replication.

We know from a study in yeast that ATM and ATR homologues have crucial roles in telomerase recruitment and telomere length maintenance (Moser et al., 2009), but the same does not appear to be true for higher eukaryotes, suggesting a degree of evolutionary divergence and that other mechanisms exist to enable telomerase recruitment to critically short telomeres in mice. Compared with yeast telomeres (300 bp), human (8–15 kb) and murine (30–50 kb) telomeres are extremely long, which may account for the divergence in function of the checkpoint proteins in telomere length regulation, even though other elements of telomere biology are evolutionarily conserved. Factors regulating the recruitment and action of telomerase may vary according to species and cell type or context and multiple factors may be required. For example, recent work by Zhao et al. (2009) suggests that in the case of some immortalized human cell lines under telomere equilibrium conditions, telomerase acts to lengthen most telomeres each cell cycle, whereas in yeasts, primary murine cells, and other human cell lines, telomerase has been shown by numerous groups to act preferentially on the shortest telomeres under conditions of telomere shortening similar to those used in the current study (Hemann et al., 2001; Perrem et al., 2001; Samper et al., 2001a; Liu et al., 2002; Teixeira et al., 2004; Zhao et al., 2009). Whether these very different findings reflect experimental artifacts caused by the usage of immortalized versus nonimmortalized cell lines, differences between higher eukaryote species, or differential regulation of telomerase under conditions of telomere disequilibrium remains undetermined, and careful future work is required to resolve this important discrepancy. Although we and others have shown that neither ATM or ATR alone are required for telomerase-mediated elongation of short telomeres (Feldser et al., 2006; Sabourin and Zakian, 2008; this study), it is possible that because of their functional redundancy, both kinases together might function in this capacity in mice, as recently reported for fission yeast (Moser et al., 2009). However, because of their combined essential functions in cell cycle checkpoints, codepletion of ATM and ATR results in severe embryonic lethality when $ATR^{S/S}$ mice are crossed with $ATM^{-/-}$ to generate doubly depleted offspring (Murga et al., 2009). The combined deficiency is severe enough to prevent even the generation of viable MEFs (Murga et al., 2009), meaning that this hypothesis cannot be properly tested with the currently available mouse models. ATM and ATR are both members of the PIKK family. Intriguingly, a third PIKK family member, DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs), also has important functions at the telomere. DNA-PKcs (along with its heterodimeric regulatory subunit Ku) functions primarily in DNA double-strand break repair by nonhomologous end joining but is also required for effective telomere capping (Bailey et al., 1999; Gilley et al., 2001; Goytisolo et al., 2001). The localization of DNA-PK at telomeres and the observation of progressive telomere shortening with increasing generations of DNA-PKcs deficiency raise the intriguing mechanistic possibility that DNA-PKcs may function in telomerase recruitment in mammalian cells (Espejel et al., 2004). Further studies are required to determine whether this dramatic shortening results from degradation of telomeres related to uncapping or the fascinating possibility of a telomerase recruitment defect.

In murine cells, ATM functions to protect short telomeres (Qi et al., 2003; Wong et al., 2003). In late generation $ATM^{-/-}/Terc^{-/-}$ mice, ATM loss results in a synergistic increase in telomere dysfunction and chromosome fusion. Similarly, in double mutant $ATR^{S/S}$/G3 $Terc^{-/-}$* cells, we also observe dramatic genomic instability and increased chromosome end-to-end fusions compared with either mutant alone, suggesting that, like ATM, ATR also functions to protect the stability of telomeres in murine cells. We hypothesize that the synergistic increase in fusions in $ATR^{S/S}$/G3 $Terc^{-/-}$* cells likely results from a combination of fusion of short telomeres as a protective strategy with an increased availability of chromosome ends at breaks at fragile sites, including replicating telomeres, caused by ATR deficiency.

A role for ATM and ATR in telomere length maintenance was recently studied in *Arabidopsis thaliana* (Vespa et al., 2005). In contrast to mammalian cells lacking ATR, *A. thaliana* lacking either ATM or ATR are viable, and the authors demonstrated that ATM- and ATR-null cells showed wild-type telomere length homeostasis for several generations, which is consistent with our findings in Seckel mice and previous data from ATM-null mice (Feldser et al., 2006). Collectively, these results further demonstrate that ATM and ATR have distinct functions in the DDR and telomerase regulation in higher and lower eukaryotes. In both mice and *A. thaliana*, cells doubly null for ATM and telomerase exhibit increased telomere dysfunction without gross telomere shortening (Qi et al., 2003; Vespa et al., 2005), but *A. thaliana* ATR/telomerase double mutants show profound genomic instability in concert with dramatic telomere loss. This differs to our observations of moderate telomere lengthening in ATR-deficient $ATR^{S/S}$/G3 $Terc^{-/-}$* MEFs and implies that in plants, ATR functions to protect telomeres from deletion or degradation but that in mammalian cells, the shelterin complex is sufficient to protect mammalian telomeres from drastic shortening in the absence of ATR and Terc.

Use of the Seckel mouse model has allowed us to monitor the effects of ATR depletion on telomere length maintenance. It is important to emphasize that ATR expression in MEFs is undetectable by Western blotting and causes cell cycle arrest defects and extreme genomic instability (Murga et al., 2009). In particular,

Seckel MEFs show rapid reduction in cell proliferation and the onset of senescence by the second passage after isolation (Murga et al., 2009). Because differences in apoptosis, senescence, and proliferation rates may affect telomere length measurements, we were careful to always use only cells in the first passage after harvest. Moreover, by restricting our analysis to metaphase cells derived by mitotic arrest of actively dividing MEF cultures, we exclude apoptotic and senescent cells from analysis.

In conclusion, we show in this study that severe reduction in ATR levels does not impair telomerase recruitment to short telomeres but increases telomere fragility and telomere recombination, indicating that the important DDR checkpoint kinases ATM and ATR function differently in telomere maintenance in mammals and yeast. Given the important role of ATR in preventing telomere fragility and the occurrence of chromosomes with multiple telomere signals, our results suggest that telomere dysfunction associated with ATR deficiency may in part be responsible for some of the pathologies associated with Seckel syndrome.

Materials and methods

Generation, genotyping, and handling of mice
To generate $ATR^{S/S}/Terc^{-/-}$ mice, $ATR^{S/S}$ mice (Murga et al., 2009) were crossed with $Terc^{-/-}$ animals (Blasco et al., 1997), and resulting double heterozygous breeding pairs were used to generate G1 of single $Terc^{-/-}$ and double $ATR^{+/S}/Terc^{-/-}$ animals. G2 mouse colonies were generated by intercrossing G1 $ATR^{S/+}/Terc^{-/-}$ pairs. To generate $ATR^{+/+}$/G3 $Terc^{+/-}$*, $ATR^{+/+}$/G3 $Terc^{-/-}$*, $ATR^{S/S}$/G3 $Terc^{+/-}$*, and $ATR^{S/S}$/G3 $Terc^{-/-}$* mice, we crossed $ATR^{+/S}$/G2 $Terc^{-/-}$ males with $ATR^{+/S}/Terc^{+/-}$ females. All mice analyzed were from a mixed 129Sv/C57BL6 background. Terc and Seckel mice were genotyped as described previously (Blasco et al., 1997; Murga et al., 2009). Mice were maintained at the Spanish National Cancer Centre (CNIO) under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations. CT analysis of 10-wk-old mice was performed in the Molecular Imaging Unit at the CNIO using a CT scanner (VISTA; GE Healthcare). Bone density was determined for both proximal and distal femurs and expressed as Hounsfield units.

Isolation of MEFs
MEFs were isolated from day 13.5 embryos as described previously (Blasco et al., 1997), maintained in DME supplemented with 10% (vol/vol) fetal bovine serum and antibiotics/antimycotics, and grown in a 5% CO_2 humidified incubator at 37°C. All MEF experiments used cells from the first passage after isolation.

Human Seckel cell lines
ATR wild-type and ATR Seckel human cell lines were obtained from the Coriell Cell Repositories.

Western blotting
Whole cell extracts were prepared from primary MEFs, and Western blots were performed as described previously (Martínez et al., 2009a). After transfer, membranes were incubated with an anti-ATR antibody (1:500; AbD Serotec) and anti–β-actin monoclonal (1:10,000; Sigma-Aldrich). Antibody binding was detected using enhanced chemiluminescence after incubation with horseradish peroxidase–coupled secondary antibody.

Telomerase repeat amplification protocol (TRAP)
TRAP was performed as described previously (Siegl-Cachedenier et al., 2007). In brief, primary MEFs were trypsinized and washed in PBS, and S-100 extracts were prepared in lysis buffer with 10% CHAPS. Protein concentration was determined by the Bio-Rad Laboratories protein assay, and two protein concentrations were used for each sample (5 and 1 µg). Extension and amplification by PCR reactions and electrophoresis were performed as described previously (Blasco et al., 1997). A negative control was included by preincubating each MEF extract with RNase for 10 min at 30°C before the extension reaction. An internal control for PCR efficiency was included.

Telomere length analysis by Q-FISH
Q-FISH was performed on metaphases harvested by incubating MEFs or *ATR*-deficient human cells with 0.1 µg/ml colcemid (Invitrogen) for 4 h at 37°C followed by fixation in methanol/acetic acid (3:1). Q-FISH on chromosome metaphase spreads was performed as described previously (Herrera et al., 1999; Samper et al., 2000); metaphase spreads were stained with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories). Telomere fluorescence values were extrapolated from the telomere fluorescence of lymphoma cell lines LY-R (R cells) and LY-S (S cells) with known telomere lengths of 79.9 kb and 9.7 kb, respectively. Images were recorded at room temperature using a camera (CCK; Cohu, Inc.) on a fluorescence microscope (DMRB; Leica) with a mercury vapor lamp source (CS 100 W-2; Philips) with a 100× oil objective to get a 1,000 magnification. They were captured using Q-FISH software (Leica) in linear acquisition mode. TFL-Telo software (a gift from P. Lansdorp, BC Cancer Agency, Vancouver, British Columbia, Canada) was used to quantify fluorescence intensity of telomeres (Zijlmans et al., 1997). Images from littermate $ATR^{+/+}$/G3 $Terc^{+/-}$*, $ATR^{+/+}$/G3 $Terc^{-/-}$*, $ATR^{S/S}$/G3 $Terc^{+/-}$*, and $ATR^{S/S}$/G3 $Terc^{-/-}$* metaphases as well as from human *ATR* wild-type and *ATR* Seckel cells were captured on the same day, in parallel, and blindly.

Combined Q-FISH and SKY
After Q-FISH image capture, coverslips were removed, and slides were washed in PBS for 10 min. Painting probes for each mouse chromosome were generated, labeled, hybridized, and washed according to the manufacturer's protocol (Applied Spectral Imaging) as described previously (García-Cao et al., 2004). The same metaphases previously captured for Q-FISH were relocated, and SKY images captured and processed according to Samper et al. (2001b). Images were recorded at room temperature with a Spectra Cube (SD300; Applied Spectral Imaging) mounted on an imaging microscope (Axioplan 2; Carl Zeiss, Inc.) with a 100× oil Iris objective to get a 1,000 magnification. They were captured using the SkyView 1.6 software (Applied Spectral Imaging) and processed with the Case Data Manager 5.5 software (Applied Spectral Imaging). Y chromosomes were identified by reverse DAPI banding in the original Q-FISH images.

Cytogenetic analyses
At least 56 metaphases per genotype were analyzed for chromosomal aberrations by superimposing the Q-FISH telomere image on the DAPI image using TFL-Telo. Analysis was performed blindly. Metaphases were scored for RLFs (p:p arm fusions), dicentrics (q:q arm fusions), p:q arm fusions, rings (chromosomes with two fused chromatids without telomere signal at the fusion point), chromatid fusions (fusions between chromosomes by one chromatid), complex aberrations, chromatid breaks (gaps in chromatids with an identifiable corresponding chromosome), and fragments (with two telomere signals or less without an identifiable corresponding chromosome). For MTS analysis, primary MEFs and human *ATR* wild-type and *ATR* Seckel cells were treated with aphidicolin at 0.5 µM for 24 h when indicated, as described previously (Martínez et al., 2009b).

CO-FISH
MEFs were grown at 37°C in DME containing 10 µM 5′-bromo-2′-deoxyuridine (Sigma-Aldrich) and allowed to replicate their DNA once. 1 µg/ml colcemid was added for the last 4 h. Cells were recovered, and metaphases were prepared as described previously (Samper et al., 2000). CO-FISH was performed as described previously (Bailey et al., 2004; Gonzalo et al., 2006) using a Cy3-labeled $(TTAGGG)_7$ probe followed by a Rhodamine green–labeled $(CCCTAA)_7$ probe (Applied Biosystems). Images of metaphase spreads were captured at room temperature using a CCK camera on a DMRB fluorescence microscope with a mercury vapor lamp source (CS 100 W-2) with a 100× oil objective to get a 1,000 magnification. They were recorded using the Q-FISH software.

Global SCE determination
MEFs were grown at 37°C in DME containing 3 µg/µl 5′-bromo-2′-deoxyuridine for two rounds of replication. Colcemid was added at a concentration of 1 µg/ml for the last 4 h of incubation. Cells were then recovered, and metaphase chromosome spreads were prepared as described for the Q-FISH (Samper et al., 2000), and genomic SCEs were visualized using an adapted fluorescence plus Giemsa protocol as described previously (Jaco et al., 2008). Metaphases on slides were immersed in 5 µg/ml Hoechst 333258 solution (Invitrogen) and washed with abundant water. The slides were exposed to UV light for 15 min in the presence of 2× SSC and washed with water. Then, they were stained with Giemsa solution (Merck) for 2 min

and washed with abundant water. Once they were dried, images were recorded using a camera (DP70; Olympus) on a research microscope (Provis AX70; Olympus) with photomicrography controller (Olympus). They were captured with immersion oil at room temperature using DP Controller and DP Manager softwares (Olympus) with a Plan-Apochromat 100× NA 1.40 oil objective to get a 1,000 magnification. Metaphases were analyzed for harlequin staining. Each color switch was scored as one SCE.

Statistical analysis and p:q ratio determination

Fisher's exact test (two sided) was used to calculate statistical significance of proportions of short telomeres in G3 littermates, T-SCE, SFEs, MTSs, chromosome fusions, fragments, breaks, and aneuploidy. The Wilcoxon-Mann-Whitney rank sum test was used for statistical comparison of median telomere length in MEFs. Statistical comparison of survival data was performed using the log rank (Mantel-Cox test). p:q telomere ratios were calculated using telomere length values from TFL-Telo for p and q arms of individual chromosomes. Ratios were calculated by dividing q/p, and proportions of chromosomes with p:q ratios greater than fivefold ($q/p \geq 5$ or ≤ 0.2) were calculated. Chromosome arms with SFEs were deemed to have p:q ratios ≥5 (Morrish and Greider, 2009). A two-sided Student's *t* test was used to calculate the statistical significance of p:q ratios and SCE. Unless otherwise indicated in the figure legend, all data with error bars represent mean ± SEM.

Online supplemental material

Fig. S1 shows that cells derived from human Seckel patients show increased telomere fragility. Fig. S2 shows that telomerase deficiency does not worsen cranial phenotypes of Seckel mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200908136/DC1.

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