Published in final edited form as:

Nat Struct Mol Biol. 2008 January ; 15(1): 79–84.

Pot1 and cell cycle progression cooperate in telomere length regulation

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Abstract

Removal of the vertebrate telomere protein Pot1 results in a DNA damage response and cell cycle arrest. Here we show that loss of chicken Pot1 causes Chk1 activation, and inhibition of Chk1 signaling prevents the cell cycle arrest. However, arrest still occurs after disruption of *ATM*, which encodes another DNA damage response protein. These results indicate that Pot1 is required to prevent a telomere checkpoint mediated by another such protein, ATR, that is most likely triggered by the G-overhang. We also show that removal of Pot1 causes exceptionally rapid telomere growth upon arrest in late S/G2 of the cell cycle. However, release of the arrest slows both telomere growth and G-overhang elongation. Thus, Pot1 seems to regulate telomere length and G-overhang processing both through direct interaction with the telomere and by preventing a late S/G2 delay in the cell cycle. Our results reveal that cell cycle progression is an important component of telomere length regulation.

Telomeres are essential for genome stability, because they prevent chromosome ends from being ligated together by the repair pathways that act on DNA double-strand breaks^{1,2}. The telomeric DNA is shielded from double-strand break repair activities by specialized telomere proteins that bind along the duplex region of the telomere and to the overhang on the 3' G-rich strand. In vertebrate cells the telomeric duplex is packaged by a complex of six proteins (Trf1, Trf2, Rap1, Tin2, Tpp1 and Pot1) termed shelterin¹. Two of the proteins, Pot1 and Tpp1, also bind to the G-strand overhang. Pot1 is a specialized G-strand binding protein, whereas Tpp1 interacts with both telomerase and other components of the shelterin complex^{3,4}.

Although telomere proteins shield the chromosome end from unwanted repair activities, a wide range of DNA damage response proteins are also found at telomeres (for example, ATM, ATR, MRN and Ku)^{1,2,5}. The presence of these proteins is not merely a hallmark of a dysfunctional telomere. Instead, damage response proteins are recruited to functional telomeres during replication, where they cooperate with telomere proteins to ensure correct processing of the G-overhang and appropriate telomere length regulation⁶⁻¹⁰. Notably, the recruitment of ATM and ATR in late S/G2 does not activate a full DNA damage response^{11,12}, indicating that downstream signaling must be quenched during telomere replication. However, if telomeres are critically short or otherwise defective, ATM-ATR signaling is not quenched and a full DNA damage response ensues^{5,13,14}. Although the telomeric defects leading to ATM signaling have been explored in some detail, little is known about the stimuli leading to ATR activation. Our results now point to a role for Pot1 in quenching ATR-Chk1 signaling.

In *Schizosaccharomyces pombe*, Pot1 depletion leads to rapid loss of telomeric and subtelomeric DNA and widespread cell death¹⁵. This observation suggested that Pot1 proteins

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AUTHOR CONTRIBUTIONS

D.C. and C.M.P. designed the research; D.C. performed the experiments; C.M.P. wrote the article.

function by protecting the G-strand overhang from degradation, thus preventing telomere loss and chromosome fusions. However, gene knockouts in mice and chicken cells indicate that this is not the case in all eukaryotes, because vertebrate cells lacking Pot1 retain telomeric G-overhangs and show only a slight increase in chromosome fusions¹⁶⁻¹⁸. We have studied Pot1 function using a conditional chicken DT40 cell line in which one *POT1* allele is disrupted and the other is replaced by a cDNA encoding an estrogen receptor-Pot1 fusion protein (Er-Pot1)¹⁷. The Er-Pot1 cells grow normally in tamoxifen, but drug removal results in loss of Pot1 from telomeres, a telomeric DNA damage response and cell cycle arrest in late S/G2. Pot1 loss also leads to an increase in G-overhang length and, as described below, to rapid telomere growth. The absence of G-overhang degradation indicates that other proteins must substitute for Pot1 in overhang protection. However, they are unable to prevent the telomere from being detected as DNA damage. Our findings indicate that a major function of Pot1 is to prevent the G-overhang from activating a DNA damage response. A second function is to regulate telomere length.

Although ATM is involved in damage signaling at critically short or Trf2-depleted telomeres^{5,13,14}, several observations suggested that the checkpoint activated after Pot1 loss might instead be mediated by ATR. First, a telomere with its 3' G-strand overhang provides the classic structure (a 5' double-stranded/single-stranded DNA (ds/ssDNA) junction) for ATR activation¹⁹. Second, Rpa (replication protein A), a key protein in ATR recruitment, is known to bind to telomeres during S phase¹² and hence is likely to protect the overhangs in late S/G2 in cells that lack functional Pot1. We have used the Er-Pot1 cell line to explore the role of ATM and ATR in the checkpoint response to Pot1 removal. We show that the checkpoint is ATM independent but requires Chk1 signaling, a hallmark of ATR activation. We also show that Pot1 removal leads to unusually rapid telomere growth but that the rate of growth is decreased if Chk1 signaling is inhibited and the Er-Pot1 cells can cycle. Our results indicate that cell cycle progression is an important component of telomere length regulation.

RESULTS

Loss of Pot1 leads to Chk1 signaling

Initial studies of the Er-Pot1 cell line revealed that addition of caffeine but not wortmannin could prevent cell cycle arrest after tamoxifen removal¹⁷ (Supplementary Fig. 1 online). Although caffeine and wortmannin both inhibit ATM, wortmannin is a less effective inhibitor of ATR²⁰. Thus, it seemed that Pot1 removal might lead to signaling by ATR rather than ATM. To explore this possibility, we looked for phosphorylation of the checkpoint kinases Chk2 and Chk1 (Fig. 1a). Phosphorylation of Chk2 indicates ATM signaling, whereas Chk1 phosphorylation is a hallmark of ATR signaling²¹. Western blot analysis of γ -irradiated cells revealed a shift in Chk2 migration, indicating Chk2 phosphorylation. However, we detected little if any shift after tamoxifen removal, and Chk2 levels gradually dropped at later time points. Thus, Chk2 did not seem to be activated in response to Pot1 loss. In contrast, Chk1 levels remained unchanged, and antibody to Chk1 phosphorylated on Ser345 revealed many phosphorylated isoforms. The phosphorylation began to increase soon after tamoxifen removal, reached a peak at 12-16 h and then gradually declined. Although the degree of Chk1 phosphorylation was quite modest and was hard to detect with antibody to total Chk1, fluorescence-activated cell sorting (FACS) analysis indicated that the peak in phosphorylation correlated with maximal cell cycle arrest (data not shown). Likewise, the decline in phosphorylation reflected the later escape from the arrest¹⁷. These results again pointed to a role for ATR-Chk1 in checkpoint activation with a subsequent reduction in signaling leading to escape from the checkpoint.

We next tested whether the Chk1 inhibitor Gö6976 could prevent cell cycle arrest after Pot1 loss. Gö6976 is a staurosporine derivative that has an IC₅₀ of 50-100 nM for Chk1 and of 10

μM for Chk2 (ref. 22). When Gö6976 was added to Er-Pot1 cells at the time of tamoxifen removal, a concentration of 1 μM completely prevented the G2 arrest, thus allowing the cells to cycle (Fig. 1b and Supplementary Fig. 2a online). Initially 100 nM Gö6976 was less effective, but ultimately the G2 arrest was again prevented and the cells were able to cycle at a similar rate to the cells treated with 1 μM Gö6976. This abrogation of the G2 arrest with 0.1-1 μM Gö6976 again indicated a role for Chk1 but not Chk2 in the response to Pot1 loss.

Cells treated with Gö6976 continued to divide and showed a normal cell cycle distribution for multiple days (Supplementary Fig. 2b). However, cell mortality increased with time, so that the cell number only increased two- to sixfold before declining (Supplementary Figure 2c and Fig. 1b). DAPI staining did not reveal defects in chromosome segregation (data not shown)—the cause of cell death when Er-Pot1 cells escape the G2 checkpoint in the absence of Gö6976 (ref. 17). Thus, the high mortality may result from the additive effect of telomere dysfunction and nontelomeric DNA damage due to Chk1 inhibition. Chk1 is not essential in DT40 cells, but Chk1-deficient cells accumulate DNA damage as a result of increased stalling of replication forks²³.

We also examined whether cell cycle arrest could be prevented by depletion of Chk1 with RNA-mediated interference (Supplementary Fig. 3 and **Supplementary Methods** online). We were able to deplete Chk1 protein levels by 80-90% using two different short interfering RNAs (siRNAs). This reduction in Chk1 caused a decrease in the population of late S/G2 Er-Pot1 cells after tamoxifen removal and a 70% increase in cell number relative to a nonspecific siRNA control. Although Chk1 depletion was less effective than Gö6976 in preventing cell cycle arrest, our results again supported a role for Chk1 signaling in the response to Pot1 removal. The residual arrest in the siRNA-depleted cells may have been due to the remaining Chk1 protein activating a partial checkpoint response. An alternative possibility was that both Chk1 and the p38MAPK-MK2 stress response pathway are activated by Pot1 removal and also inhibited by Gö6976 (refs. 24-25). Because such a dual response to Pot1 removal could also explain why Chk1 phosphorylation was quite modest, we looked for p38MAPK-MK2 signaling by monitoring MK2 phosphorylation. However, we did not detect a change in phosphorylation that correlated with Pot1 removal and onset of the cell cycle arrest (data not shown).

ATM is not required for checkpoint activation

Although our results pointed to a role for ATR rather than ATM in the DNA damage response to Pot1 removal, cross-talk is known to occur between the two signaling pathways²⁶. Thus, to further assess the contribution of ATM to the response, we generated conditional Er-Pot1 cells in which the *ATM* gene was disrupted. The two *ATM* alleles were disrupted sequentially using previously described gene-targeting constructs that remove a conserved region of the kinase domain (Supplementary Fig. 4 online)^{27,28}.

Not surprisingly, the *ATM*^{-/-} Er-Pot1 cells resembled *ATM*^{-/-} single knockout cells in that they grew more slowly in tamoxifen than the *ATM*^{+/+} Er-Pot1 cells (population doubling (PD) of ~24 h versus 10 h) and showed an increased mortality (25% versus ≤5%) (Fig. 2 and data not shown). Notably, after tamoxifen removal the *ATM*^{-/-} Er-Pot1 cells underwent a cell cycle arrest in late S/G2 and eventually died. Thus, the response of the *ATM*^{+/+} and *ATM*^{-/-} Er-Pot1 cells to Pot1 loss was very similar. We therefore conclude that ATM is not required for checkpoint activation.

The main differences in the response of the two cell lines were that the cell cycle arrest was delayed by 6-12 h in the *ATM*^{-/-} cells and that those cells arrested less synchronously. However, examination of H2AX and Chk1 phosphorylation patterns suggested that these differences stem from the slower growth rate of the *ATM*^{-/-} cells rather than from a requirement for ATM.

The *ATM*^{-/-} cells had an elevated basal level of γ H2AX phosphorylation (Fig. 2c), suggesting a constant low-level DNA damage response. Nonetheless, the *ATM*^{+/+} and *ATM*^{-/-} cell lines showed a similar gradual increase in H2AX and Chk1 phosphorylation after tamoxifen removal. Although the accumulation of γ H2AX and phospho-Chk1 was delayed in the *ATM*^{-/-} cells, the peak in Chk1 phosphorylation still correlated with the time of maximum G2 arrest. This similarity in phosphorylation pattern would not be expected if ATM were required to initiate checkpoint activation. The less synchronous nature of the arrest can be explained by the slower growth rate of the *ATM*^{-/-} cells, because cells entering G2 soon after drug removal later would escape the arrest before other cells completed S phase. Our results therefore indicate that ATM plays little if any role in the response to Pot1 removal. Because Chk1 activation is a highly conserved hallmark of ATR signaling²⁹, our results suggest that ATR alone is sufficient to activate the resulting cell cycle checkpoint. Moreover, because ATR signaling occurs in response to ssDNA, our results also suggest that this checkpoint is triggered by the G-strand overhang.

Pot1 loss promotes rapid telomere growth

During our initial analysis of the Er-Pot1 cell line, we showed that the amount of G-overhang increases two- to threefold within 24 h of tamoxifen removal¹⁷. To determine whether the telomeres also elongate, we performed a telomere length analysis on DNA from Er-Pot1 cells harvested at various times after tamoxifen removal (Fig. 3a). Because chicken chromosomes harbor large regions of interstitial telomeric sequence, the telomeric restriction fragments were detected by nondenaturing in-gel hybridization of a probe to the G-strand overhang. Analysis of mean telomere length³⁰ revealed that after a short time lag the telomeres did indeed elongate. When we calculated the rate of increase between 12 and 24 h (the period corresponding to maximum cell cycle arrest and cell viability), we found that it averaged 317 base pairs (bp) in 12 h. This telomere growth is most unusual because it occurs in the absence of cell division and it is exceptionally fast. The most rapid growth observed previously in response to a change in a vertebrate telomere protein (overexpression of mutant human TIN2 or POT1 (refs. 31, 32)) is 200-230 bp/PD (that is, per 24 h). Abnormal expression of other telomere proteins leads to a length increase of 30-80 bp/PD³³⁻³⁷.

The rapid telomere growth after removal of chicken Pot1 was reminiscent of what we had previously observed in Pot1-deficient *Tetrahymena thermophila* cells³⁸, and it was striking that in both organisms much of the growth occurred in the absence of cell division. This observation led us to ask whether the telomere growth and increase in G-strand overhang was caused by arrest of the Er-Pot1 cells in late S/G2, a period of the cell cycle when telomeres may be more accessible to telomere lengthening and C-strand processing activities. To address this question, we first examined the timing of both telomere growth and the increase in G-overhang signal in synchronized cultures (Fig. 3b-d). Cells were synchronized in mitosis with nocodazole; the nocodazole and tamoxifen were removed simultaneously and cells harvested at 2- to 6-h intervals. Telomere length was determined by nondenaturing in-gel hybridization as just described. To detect changes in amount of G-overhang, the total hybridization signal was quantified for each lane and normalized for the amount of DNA loaded. Analysis of Exo1- or mung bean nuclease-treated DNA confirmed that the probe hybridized only to the G-overhang, in that the signal was lost after nuclease treatment (data not shown). Both the telomere length and G-overhang analysis revealed the same striking result, namely that there was little change in telomere or G-overhang length during G1 and early to mid S phase, but both increased rapidly after the cells entered late S/G2 (Fig. 3c,d). Thus, the telomere does not seem to become accessible to the G-overhang processing and telomere elongation activities until late S/G2 of the cell cycle.

To separate the consequence of the prolonged cell cycle arrest from the physical effect of removing Pot1 from the G-overhang, we next examined the change in telomere and G-overhang length in cells that lacked Pot1 at their telomeres but that could continue to divide because of addition of 1 μ M Gö6976. Gö6976 prevents cell cycle arrest by inhibiting Chk1 phosphorylation of Cdc25 but does not affect ATR activity^{21,22,39}. Thus, addition of Gö6976 should not affect telomere length by preventing ATR phosphorylation of telomere proteins. When Gö6976 was added to Er-Pot1 cells at the time of tamoxifen removal, we found that telomere length and amount of G-overhang still increased with time. However, the rate of increase was markedly lower than in G2-arrested cells (Fig. 4 and Supplementary Fig. 2). The telomere growth dropped from an average of ~320 bp per 12 h to 50-150 bp per 12 h (50-150 bp/PD), a rate that is more similar to that observed in mammalian cells with defects in Trf1, Rap1 and Tpp1 expression³⁴⁻³⁷. The rate of increase in G-overhang signal dropped by about 50%, and although the amount of overhang signal continued to increase for many days, it never reached the level observed in G2-arrested cells. These results indicate that both telomere growth and G-overhang processing are limited by the amount of time a cell spends in late S or G2 of the cell cycle. We therefore conclude that timely progression through the cell cycle is an important element of telomere length regulation.

Although telomere growth was slowed by addition of the Chk1 inhibitor, it was not prevented. This finding fits with previous studies indicating that Pot1 may limit telomere growth by sequestering the DNA terminus from telomerase^{40,41}. Likewise, the more limited increase in amount of G-overhang in cycling cells supports previous reports that Pot1 restricts degradation of the telomeric C strand^{16,42}. Overall our results indicate that the telomere and G-overhang growth observed after Pot1 loss has two separate causes. First, removal of Pot1 from the telomere increases access to telomerase and processing nucleases. Second, the G2 arrest greatly extends the time the telomere remains in an open chromatin conformation and thus is accessible to telomerase and processing nucleases.

DISCUSSION

In previous work, we showed that chicken Pot1 is required to prevent the telomere from activating a catastrophic DNA damage response¹⁷. We have now shown that this damage response is ATM independent and involves Chk1 signaling. Because Chk1 signaling depends on ATR activation, our results imply that the damage response is ATR mediated. This same conclusion has been reached by Denchi and de Lange, who recently showed that Pot1 depletion leads to an ATR-mediated damage response in mammals⁴³. Thus, Pot1 loss elicits a different response from critically short telomeres or telomeres that lack components of the shelterin complex, where the predominant response is ATM mediated^{5,9,13,14,43}. Because Pot1 binds specifically to telomeric G-strand DNA and loss of Pot1 does not cause dissociation of other shelterin components^{17,44}, our results imply that Pot1 prevents the G-overhang from eliciting a DNA damage response and specific 'G-overhang' checkpoint. Given that ATR requires Rpa-coated ssDNA for recruitment and activation^{19,45}, our results further suggest that Pot1 prevents the G-overhang checkpoint by limiting Rpa binding.

Because Pot1 loss leads to cell cycle arrest via Chk1 signaling, we were able to use a Chk1 inhibitor to abrogate the arrest and thus study the effect of Pot1 loss on G-overhang processing and telomere length regulation in cycling cells. We have shown that both G-overhang and telomere length increase in the absence of Pot1 in late S/G2-arrested cells, and that the rate of telomere growth is exceptionally fast. However, the increase in G-overhang and rate of telomere elongation is slowed when the cells are able to cycle. Our findings support previous studies indicating that Pot1 regulates G-overhang and/or telomere length by binding to the telomere and thus limiting access to telomerase and/or nuclease activities^{40,41}. However, our studies have also revealed a previously unknown aspect of telomere length regulation—

namely, the importance of cell cycle progression. Prolonging the G2 phase seems to allow continued telomere processing and elongation leading to deregulation of the terminal DNA structure. Thus, Pot1 plays a hitherto unexpected role in telomere length regulation by preventing a G2 delay due to checkpoint activation.

Restraining telomere checkpoints

Although the current studies were performed with cells that lack active Pot1, our results have important implications for the mechanism by which Pot1 may limit DNA damage signaling during telomere replication in wild-type cells. Both ATM and ATR are recruited to telomeres during the late stages of telomere replication when the overhang is generated by nuclease processing and then extended by telomerase⁹. Although this recruitment of ATM, ATR or both and the subsequent phosphorylation of telomere proteins seems to be an integral step in telomere replication^{7-9,11}, the activated ATM and ATR do not cause a full DNA damage response. For example, ATM does not phosphorylate Chk2 or cause stabilization of p53 (ref. 11). Full amplification of the ATM signaling pathway in humans seems to be prevented by binding of TRF2 (ref. 46). The interaction of TRF2 with ATM prevents autophosphorylation on Ser1981, a key step in ATM activation. Our results suggest that Pot1 binding to the G-overhang may provide a parallel pathway to prevent amplification of ATR signaling. By displacing Rpa or limiting Rpa association with the overhang, Pot1 binding would reduce or prevent recruitment of Atrp-ATR and the Rad9-Rad1-Hus1 checkpoint clamp and hence ATR activation.

Role of cell cycle progression in telomere length regulation

Regulation of telomere length is a complex process that balances elongation and shortening of the telomere by telomerase and nuclease activities. The amount of DNA extension or degradation seems to be regulated both through the direct action of telomere proteins on telomerase and by the opening of the telomeric chromatin structure to increase access to the DNA terminus during telomere replication^{1,4,40,41,47,48}. Components of shelterin (Trf1, Tin2, Rap1, Trf2) seem to act by regulating opening of the telomeric chromatin, whereas the Pot1-Tpp1 heterodimer directly affects telomerase activity. *In vitro* assays indicate that the two subunits of the POT1 heterodimer provide opposing functions: POT1 restricts telomere elongation by decreasing access to the DNA terminus^{40,41}, whereas TPP1 promotes telomere elongation by recruiting telomerase, increasing telomerase processivity or both^{4,48}. Our results showing telomere elongation in cycling Er-Pot1 cells fully support this direct role for Pot1 in telomere length regulation. However, the unusually rapid elongation in G2-arrested cells most likely occurs because the telomeres are being held in an open conformation for an extended period of time. This would allow continued telomere elongation.

Although we have not directly investigated the mechanism of telomere elongation, we suspect it is caused by telomerase instead of by a recombination-based mechanism, because the telomeres show a steady increase in length rather than the sudden jumps in length characteristic of alternative lengthening of telomeres. If this supposition is true, our results indicate that association of Tpp1 with the G-overhang is not needed for telomerase-mediated telomere growth in Pot1-deficient cells, because TPP1 only binds G-strand DNA as a POT1-TPP1 heterodimer^{4,48}. However, the situation in wild-type cells is likely to be different, because stimulation or recruitment of telomerase by Tpp1 may be essential to offset the inhibitory effect of Pot1 binding to the chromosome terminus.

Opening of the telomeric chromatin is presumed to occur in late S/G2 in conjunction with passage of the replication machinery. However, our understanding of how telomere replication is linked to cell cycle regulation is in its infancy. Recent studies in *Saccharomyces cerevisiae* have shown that processing of the G-overhang and addition of telomeric repeats by

telomerase are regulated by Cdk1 and hence occur only in late S/G2 when Cdk1 levels are high^{49,50}. Our studies now indicate that exit from late S/G2 is also a key event in telomere replication, because a prolonged G2 phase can lead to deregulation of G-strand processing and telomere elongation.

METHODS

Cell culture, irradiation and flow cytometry

Culture of DT40 cells and FACS analysis were performed as described¹⁷. Where applicable, tamoxifen was added to 100 nM, nocodazole to 0.5 $\mu\text{g ml}^{-1}$ and Gö6976 to 10 nM–1 μM . Cells were exposed to 254-nm UVC light while suspended in $1 \times$ phosphate-buffered saline–1% (v/v) fetal bovine serum at 6×10^5 cells/ml. Cells were γ -irradiated in complete medium with a ¹³⁷Cs γ -ray source (Gammacel 40; Atomic Energy of Canada) at a dose rate of 1.7 Gy min^{-1} . The cells were harvested 1–2 h after exposure to either UV light or γ -irradiation. Cells were synchronized by treatment with nocodazole and tamoxifen for 10 h, the nocodazole and tamoxifen were removed and cells were harvested for FACS and DNA isolation as they progressed into G1, S and G2.

Disruption of the *ATM* gene locus

Generation of the conditional Er-Pot1 cell line was described elsewhere¹⁷. *ATM*^{-/-} Er-Pot1 cells were generated from Er-Pot1 cells using previously developed gene-targeting vectors (pATM-hygro and pATM-puro) that replaced the BamHI-XhoI fragment of the ATM kinase region with a drug resistance cassette²⁷. Transfection was with a Nucleofector Device (Amaxa) using 5×10^6 log phase cells and 5 μg DNA in 100 μl Nucleofector Solution L (Amaxa). Cells transfected with the pATM-hygro vector were selected in medium containing 0.9 mg ml^{-1} hygromycin and 100 nM tamoxifen. *ATM*^{+/-} Er-Pot1 clones were identified by Southern hybridization²⁷; one clone was then transfected with the pATM-puro vector, and the cells were selected in medium containing 0.5 $\mu\text{g ml}^{-1}$ puromycin and 100 nM tamoxifen. *ATM*^{-/-} Er-Pot1 cells were identified by Southern hybridization.

Immunoblotting and antibodies

Whole-cell lysates were prepared by resuspending 5×10^6 cells in 50 μl phosphate-buffered saline and adding an equal volume of $2 \times$ SDS gel sample buffer containing 4% (w/v) SDS. Phosphorylated and unphosphorylated forms of Chk2 and Chk1 were resolved by SDS-PAGE in 10% or 12% (w/v) gels with a 120:1 ratio of acrylamide to bisacrylamide. Total protein corresponding to $\sim 5 \times 10^5$ cells was loaded in each lane. Western blot analysis followed standard procedures, except that membranes were blocked with 5% (w/v) bovine serum albumin in TBST (10 mM Tris pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween-20) for detection of phosphoproteins and 5% (w/v) milk in TBST for detection of other proteins. Proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibody sources were as follows: α -tubulin, DSHB, 12G10; γ H2AX, Upstate, 05-636; actin, Oncogene, CP01; Chk1, Sigma, C9358; phospho-Chk1 (Ser345), Cell Signaling, 2341. Chicken Chk2 antibody was a gift (see Acknowledgments)²³.

Telomere length and G-overhang analysis

Telomeric restriction fragments were detected by in-gel nondenaturing hybridization of ³²P-labeled (C₃TA₂)₄ oligonucleotide to the G-overhang, and the signal was detected with a PhosphorImager and quantified with ImageQuant software (Molecular Dynamics) as earlier described³⁰. To compare the relative amount of G-overhang in different lanes, the gel was denatured and rehybridized with the same (C₃TA₂)₄ probe. A block of bands corresponding to interstitial telomeric DNA was quantified and used to normalize for differences in loading.

Digestion with Exo1 and mung bean nuclease were performed before restriction digestion. DNA was digested with 10 U Exo1 per microgram of DNA for 24 h at 37 °C and with 0.5 U mung bean nuclease per microgram of DNA for 15-60 min at 30 °C. To determine telomere length, each lane was divided into 50 separate boxes, and the signal in each box was determined along with its size in base pairs. The weighted mean telomere length was determined using the formula $L_{wm} = \Sigma(L_i \times Sig_i) / \Sigma Sig_i$, where L_{wm} is the mean length (in base pairs) of the telomere signal, L_i is the length in base pairs of an individual box and Sig_i is the signal intensity of that box³⁰.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank C. Morrison (National University of Ireland) for helpful comments and for providing the ATM gene targeting constructs, D. Gillespie (Beatson Institute for Cancer Research) for the chicken Chk2 antibody and Y. Sanchez (Dartmouth Medical School) for helpful discussions. This work was supported by US National Institutes of Health grant GM041803 to C.M.P.

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Figure 1.

Activation of Chk1 signaling after Pot1 removal. **(a)** Western blots showing Chk2 and Chk1 phosphorylation state in Er-Pot1 cell lysates after 4-24 h growth without tamoxifen (Tam). Control cultures grown with Tam were treated with UV light or γ -irradiated to induce Chk1 or Chk2 phosphorylation. Blots were reprobbed with actin antibody to visualize loading. **(b)** Growth of Er-Pot1 cells after Chk1 inhibition. Cells were grown with or without Tam with 0, 10 nM, 100 nM or 1 μ M Gö6976 (Go). DMSO was added as solvent control. The results are representative of four independent experiments with two different Er-Pot1 clones.

Figure 2.

ATM is dispensable for checkpoint activation in response to Pot1 loss. **(a)** FACS analysis of DNA content showing development of the cell cycle arrest in ATM^{-/-} Er-Pot1 cells after tamoxifen (Tam) removal. **(b)** Growth of ATM^{+/+} and ATM^{-/-} Er-Pot1 cells with or without Tam. Mean values are plotted for two independent experiments. Error bars indicate s.d. **(c)** Western blot analyses showing the time course of H2AX and Chk1 phosphorylation after tamoxifen removal from ATM^{+/+} and ATM^{-/-} Er-Pot1 cells. Cultures treated with UV light were used as a positive control. Blots were reprobbed with α -tubulin antibody to visualize loading.

Figure 3.

Increase in telomere length and G-overhang signal in Er-Pot1 cells. **(a)** Increase in telomere length in asynchronous cultures. The plot shows the mean length of telomeric restriction fragments from Er-Pot1 cells isolated at various times after tamoxifen removal. The data are the mean of five independent experiments. Error bars indicate s.d. **(b-d)** The increase in telomere length and G-overhang occurs after the late S/G2 arrest. **(b)** FACS analysis showing DNA content of cells synchronized with nocodazole (Noc) and released into medium with or without tamoxifen for the indicated times. **(c)** Change in telomere length for synchronized cells shown in **b**. **(d)** G-overhang signal from cells shown in **b**. Data shown in **b-d** are from one representative experiment.

Figure 4.

Inhibition of the late S/G2 checkpoint reduces the rate of telomere and G-overhang elongation. Mean telomere restriction fragment length and telomeric G-overhang signal were measured for control (DMSO treated) and G6976-treated Er-Pot1 cells grown with or without tamoxifen. **(a)** Change in telomere length with time. The in-gel hybridization for this experiment is shown in Supplementary Figure 2d online. Data are shown for one representative experiment. **(b)** Change in G-overhang signal. The plot shows the mean values for two independent experiments; the error bars indicate the range.