Mre11–Rad50–Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity

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DNA double-strand breaks (DSBs) can be processed by the Mre11–Rad50–Nbs1 (MRN) complex, which is essential to promote ataxia telangiectasia-mutated (ATM) activation. However, the molecular mechanisms linking MRN activity to ATM are not fully understood. Here, using Xenopus laevis egg extract we show that MRN-dependent processing of DSBs leads to the accumulation of short single-stranded DNA oligonucleotides (ssDNA oligos). The MRN complex isolated from the extract containing DSBs is bound to ssDNA oligos and stimulates ATM activity. Elimination of ssDNA oligos results in rapid extinction of ATM activity. Significantly, ssDNA oligos can be isolated from human cells damaged with ionizing radiation and injection of small synthetic ssDNA oligos into undamaged cells also induces ATM activation. These results suggest that MRN-dependent generation of ssDNA oligos, which constitute a unique signal of ongoing DSB repair not encountered in normal DNA metabolism, stimulates ATM activity.

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Introduction

Chromosomal breakage induces a robust cellular response that leads to cell cycle arrest, DNA repair, or under some circumstances, apoptosis. The ataxia telangiectasia-mutated (ATM) kinase, a member of the phosphatidylinositol 3-kinase-like kinase (PIKK) family, is central to this response (Shiloh, 2006). Essential to full ATM activation, both in vivo and in vitro, is the heterotrimeric Mre11–Rad50–Nbs1 (MRN) complex (Uziel et al., 2003; Costanzo et al., 2004a; Falck et al., 2005; Lee and Paull, 2005). The effect of MRN on ATM and its activity is likely to happen on multiple levels, as well as recruitment of ATM by Nbs1 to the sites of DNA double-strand breaks (DSBs) (Falck et al., 2005), the MRN-dependent DSB unwinding and tethering activities are essential for efficient ATM activation (Costanzo et al., 2004a; Lee and Paull, 2005). MRN complex also possesses nucleolytic activity (Paull and Gellert, 1998), and interestingly, Mre11-deficient cells complemented with an Mre11 allele carrying a mutation in the nuclease catalytic site exhibit defective ATM activation (Uziel et al., 2003). These observations support the hypothesis that MRN nuclease activity is required for ATM activation and are consistent with the recent report showing that Mir1, an inhibitor of MRN nuclease activity, suppresses ATM activation (Dupre et al., 2008). The MRN complex has both exo- and endonuclease activities (Paull and Gellert, 1998). MRN endonucleolytic activity is important for DSB resection and is enhanced by Ctp1 (Sartori et al., 2007), which also has nuclease activity (Takeda et al., 2007). However, although MRN has a major function in DSB resection it is unclear whether this is linked to ATM activity. In budding yeast, continuous DNA resection, recruitment of DNA repair proteins and chromatin remodelling at the site of a DSB is required to maintain an active checkpoint response (Ira et al., 2004). This suggests that DSB processing is linked to the activation of the DNA damage response. One aspect of DSB processing that has not been investigated is that endonucleolytic processing of DSBs should lead to the generation of single-stranded DNA oligonucleotides (ssDNA oligos) as by-product. The role and the fate of these ssDNA oligos inside the nucleus are currently unknown. Recently, the presence of ssDNA oligos derived from a yet unidentified DNA-processing event has been linked to the chronic activation of the ATM-dependent DNA damage response in cells deficient for Trex1 (Yang et al., 2007), which is an exonuclease that degrades ssDNA to mononucleotides (Mazur and Perrino, 2001). In addition, ATM activation has been shown to require hSSB1, a novel ssDNA-binding protein (Richard et al., 2008). Overall, these findings suggest that ssDNA molecules have an important function in promoting and sustaining ATM activity. Here, using Xenopus laevis egg extract we have investigated how DNA ends are processed and how this processing influences the MRN- and ATM-dependent DNA damage response. The Xenopus system is ideal to study the rapid activation process of ATM following addition of DNA templates to the cell-free extract (Costanzo et al., 2004b). Using this approach, we found that double- and single-stranded DNA templates inducing ATM activation are extensively processed. Surprisingly, we discovered that DNA resection leads to the production of ssDNA oligos that associate with the MRN complex and influence ATM activity.

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Results

DNA end processing leads to formation of ssDNA oligos in Xenopus laevis egg extract

Synthetic DNA molecules such as annealed oligonucleotides consisting of 70 bases of random complementary sequences (rDSBs) or poly-dA70/poly-dT70 (pA70/pT70) induce ATM-dependent DNA damage response in Xenopus egg extract (Costanzo et al., 2000; Guo and Dunphy, 2000). We monitored the fate of different DNA molecules in the extract. Equal amounts of double-stranded rDSBs and pA70/pT70 or single-stranded pA70 and pT70 DNA molecules were labelled with 32P at the 5' or 3' ends and incubated in egg extracts. DNA was then isolated and ran on sequencing gels. All DNA molecules were resected very rapidly in the extract resulting in the accumulation of mononucleotides and of ssDNA oligos ranging from 4 to 12 nucleotides in size (Figure 1A). Native gel electrophoresis of DNA derived from the extract treated with DSB molecules confirmed that these ssDNA oligos were present as single-stranded DNA as there was no difference in their molecular weight compared with denaturing gels (Supplementary Figure 1). ssDNA oligos could also be detected from internally labelled DNA, indicating that they arise from unwinding of DSBs followed by the resection of single-stranded DNA throughout its entire length (Figure 1B). Although all DNA molecules underwent resection, there were significant differences in their stability. Double-stranded DNA molecules exhibited the highest stability, whereas single-stranded DNA molecules were completely degraded after 60 min (Figure 1C). It is notable that although resection of rDSBs and pA70/pT70 also led to the generation of ssDNA oligos, we did not observe a reduction in the molecular weight of these DNA molecules. This is probably due the fact that nucleolytic processing is balanced by ongoing DNA repair, as in contrast to single-stranded molecules, resected double-stranded DNA molecules could undergo fill-in DNA synthesis and end joining. Indeed, the appearance of DNA molecules with higher molecular weight than rDSBs and pA70/pT70 confirmed this prediction (Figure 1A).

Activation of ATM by different DNA structures

Activation of ATM can be monitored by detection of serine 1981 phosphorylation (Bakkenist and Kastan, 2003), and ATM kinase activity can be measured by phosphorylation of a histone H2AX carboxy-terminal peptide containing the serine 139 (Costanzo et al., 2004a). We measured ATM activity triggered by different DNA molecules at increasing concentrations. rDSBs and pA70/pT70 induced phosphorylation of histone H2AX and ATM serine 1981 (Figure 2A and B). Surprisingly, single-stranded poly-dT70 (pT70) and to a much lesser extent poly-dA70 (pA70) induced significant ATM activity. However, circular single-stranded M13 phage DNA could not induce ATM activation even at high doses, suggesting that DNA ends are necessary for the activation of ATM (Figure 2A). In these conditions, H2AX phosphorylation is mostly dependent upon ATM as shown by the suppression of H2AX phosphorylation induced by the ATM inhibitor KU55933 (Hickson et al., 2004) and, to a similar extent, by ATM depletion (Supplementary Figure 2A and B). The phosphorylation status of ATM serine 1981 was consistent with H2AX phosphorylation levels and was inhibited by the ATM inhibitor (Figure 2B). ATM activation can be induced by ATR (Stiff et al., 2006). However, depletion of ATR from the egg extract did not affect the induction of ATM activation by single- and double-stranded DNA molecules, indicating that ATM was directly activated by these DNA structures (Supplementary Figure 3A and B). pT70-induced ATM activation might be due to the conversion of single-stranded pT70 into double-stranded DNA in the extract. To rule out this possibility, we monitored pT70 replication at different concentrations. We observed efficient pT70 replication at concentrations higher than the ones already capable of inducing ATM activation (Supplementary Figure 4). This indicated that pT70 molecules were able to induce ATM activation in the single-stranded form. pT70 replication at high doses might be due to the saturation of the enzymes responsible for pT70 degradation resulting in subsequent stabilization of a fraction of pT70 molecules, which then become available to the single-

Figure 1 DNA end processing leads to generation of ssDNA oligos. (A) Different DNA structures such as rDSB and pA70/pT70 were stoichiometrically labelled at the 5' (532P). After incubation for the indicated times in the egg extract, the DNA was recovered and ran on a 22% denaturing acrylamide gel. In (B) rDSBs were internally labelled (internal 32P) at the thirty-fifth nucleotide from the 5' end and in (C) pT70 and pA70 were labelled at the 5' end (3'32P) before incubation.
strand replication machinery. Replicated pT70 molecules contribute to the induction of DNA damage response to DSBs in the egg extract as previously shown (Guo and Dunphy, 2000). We also measured ATM activity in response to low concentration of pT70 in the presence of high amounts of aphidicolin, which inhibits DNA polymerases and single-stranded DNA synthesis in the egg extract (Jenkins et al., 1992). As shown in Figure 2A, ATM activation by pT70 is refractory to aphidicolin treatment. Activation of ATM by pT70 was also observed after gel purification of pT70 oligos and was suppressed by pretreating pT70 oligos with DNAase, confirming that it was not due to contaminants of pT70 synthesis (data not shown). These data suggest that double as well as some single-stranded linear DNA molecules can stimulate ATM activity.

### ssDNA oligos and ATM activity

We then correlated the stability of DNA templates to the persistence of ATM activity. To this end, rDSBs, pA70, pT70 and single-stranded DNA molecules with random DNA sequence (R70) were incubated in the egg extract. ATM activity was monitored at various time points from DNA addition. Incubation of rDSBs, pT70, R70 and to a lesser extent of pA70 molecules in the extract resulted in stimulation of ATM activity (Figure 2C). Significantly, ATM activity reached its peak and was maintained at time points when most of the single-stranded DNA templates such as pT70 and pA70 were degraded into smaller ssDNA oligos (Figures 1C and 2C). Further degradation of ssDNA oligos to mononucleotides correlated instead with complete loss of ATM activity. In contrast, no significant loss of ATM activity was observed with rDSB molecules, whose stability was not affected over time (Figures 1C and 2C). ssDNA oligos derived from pA70, which, compared with pT70-derived ssDNA oligos were more rapidly degraded to mononucleotides, were less effective at promoting sustained ATM activity (Figure 2C). To further probe the role of ssDNA oligos, we measured H2AX phosphorylation after the removal of pA70/pT70 from the extract. To this end, biotinylated pA70/pT70 oligos were incubated in the extract and removed after 30 min with streptavidin beads (Figure 3A). To verify that ssDNA oligos had been generated from pA70/pT70 before its removal, we labelled pA70/pT70 on the 3′ end of the DNA complementary to the biotinylated strand. After 30 min of incubation in the extract, ssDNA oligos were generated from pA70/pT70 processing and could not be eliminated by the removal of biotinylated pA70/pT70 (Figure 3B). ATM activity was then measured in the pA70/pT70-depleted extracts. Consistent with previous observations (Dupre et al., 2006), removal of pA70/pT70 did not affect ATM activity, indicating that once the signal has been initiated the kinase is able to maintain its activity even in the absence of pA70/pT70. To demonstrate a role for ssDNA oligos in maintaining ATM activity, we tested different nucleases for their ability to eliminate ssDNA oligos. We found that phosphodiesterase I (PDEI), which is known to preferentially degrade single-stranded DNA to mononucleotides as terminal products (Razzell and Khorana, 1959a, b), was able to efficiently degrade small ssDNA oligos in the egg extract (Figure 3B) and to suppress ATM activity after removal of pA70/pT70 (Figure 3C). Although PDEI could also degrade RNA or poly-ADP ribose polymers (PARP), we found no effect of RNA degradation or PARP synthesis inhibition on ATM activity (Supplementary Figure 5). Importantly, we could demonstrate that PDEI in the conditions used for these experiments specifically degraded small ssDNA oligos and not larger double-stranded DNA molecules (Supplementary Figure 6). Taken together, these results indicate that the ssDNA oligos generated from linear DNA processing sustain ATM activity.

### The MRN complex mediates the effects of ssDNA oligos on ATM activity

As ATM activation requires the MRN complex in Xenopus egg extract at low doses of DSBs (Costanzo et al., 2004a; Dupre et al., 2006), we were intrigued to know whether the MRN complex was promoting ATM activity induced by ssDNA oligos. Biotinylated pA70/pT70 molecules were incubated in the extract for 30 min and then removed. The MRN complex
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Figure 3 ssDNA oligos sustain ATM activity. (A) Experimental procedure: biotinylated pA70/pT70 labelled with 32P on the 3’ end was incubated for 30 min in the extract and then removed with streptavidin beads. After pA70/pT70 removal, histone H2AX peptide phosphorylation was monitored. (B) Residual DNA left in the extract was isolated and run on a 15% TBE-urea denaturing gel. DNA was isolated from the extract incubated with biotinylated and labelled pA70/pT70 for 30 min (lane 1) and from extracts in which biotinylated pA70/pT70 was incubated for 30 min and then removed with streptavidin beads (Strep) (lanes 2 and 3). The extracts were untreated (lane 2) or supplemented with 0.001 U/μl PDEI (lane 3). (C) Histone H2AX peptide phosphorylation induced by 5 ng/μl biotinylated pA70/pT70 before and after pA70/pT70 removal in the absence (black bar) or in the presence (grey bar) of 0.001 U/μl PDEI. PDEI was added 30 min after DNA addition and was incubated for 10 min at 22°C. Activity is expressed as fold induction over the untreated extract (no DNA).

was subsequently immunoprecipitated from the extract with polyclonal antibodies raised against Mre11 (Costanzo et al., 2004a; Dupre et al., 2006). Depletion of the MRN complex led to the loss of ATM activity, suggesting that it was required to sustain ATM activity (Figure 4A). As the MRN complex tethers DNA fragments resulting in an increase in the local concentration of ATM and DNA ends (Costanzo et al., 2004a; Dupre et al., 2006), we postulated that the MRN complex might bind to ssDNA oligos. To test this possibility, biotinylated pA70/pT70 labelled with 32P at the 3’ end was incubated in the egg extract for 30 min and then removed. Following immunoprecipitation of Mre11, we could detect the presence of 32P-labelled ssDNA oligos in the immunoprecipitated samples (Figure 4B). Treatment of the immunoprecipitated Mre11 with PDEI led to degradation of labelled ssDNA oligos (Figure 4B). To verify whether the MRN–ssDNA oligos complexes were able to stimulate ATM activity, we incubated immunoprecipitated Mre11 derived from pA70/pT70-treated extracts in fresh extracts containing pA70/pT70 or rDSB at concentrations not sufficient to induce ATM activation. The presence of high molecular weight DNA facilitated the formation of DNA–protein complexes with increased local concentration of MRN and ATM molecules as previously shown (Costanzo et al., 2004a; Dupre et al., 2006). In this case, the MRN–ssDNA oligos complexes led to partial activation of ATM activity (Figure 4C). Significantly, MRN–ssDNA oligos complexes pretreated with PDEI, which was then washed away, were unable to induce ATM activation, suggesting that ssDNA oligos associated with MRN were active intermediates capable of promoting ATM activity (Figure 4C). To further confirm the role of ssDNA oligos in ATM activation, we incubated synthetic poly-dT5 (pT5) and poly-dT10 (pT10) oligonucleotides in the extract and measured ATM activity. However, these synthetic oligonucleotides failed to activate ATM in the extract (data not shown). This was likely due to the rapid degradation of exogenous pT5 and pT10 oligonucleotides in the egg cytoplasm (Supplementary Figure 7). ssDNA oligos generated from processed ends were instead stable for more than 60 min (Figure 2A), suggesting that ssDNA oligos are stabilized by the rapid association with protein complexes such as MRN immediately after their generation. Notably, ssDNA oligos did not interfere with the ability of the MRN complex to promote end-to-end bridging of linear double-stranded DNA molecules (Supplementary Figure 8), indicating that ssDNA oligos interact with parts of the MRN complex that are not involved in DNA tethering.

**ssDNA oligos formation from chromosomal DSBs is MRN dependent**

To show that ssDNA oligos generation is a physiologically relevant phenomenon, we sought to establish whether ssDNA oligos could be generated following induction of chromosomal breakage. Strikingly, EcoRI treatment of sperm nuclei induced the accumulation of ssDNA oligos that were efficiently degraded by PDEI (Figure 5A). ssDNA oligo formation over time was impaired by depletion of the MRN complex and restored by supplementing the egg extract with recombinant human MRN complex (Figure 5B). This indicates that ssDNA oligo generation is due to MRN-dependent processing of DNA ends produced by EcoRI. The fact that human MRN complex restored the formation of ssDNA oligos in the egg extract confirmed that the ability to promote DSB processing is a conserved feature of the MRN complex. EcoRI-dependent chromosomal breakage induced ATM activation in the egg extract as previously reported (Yoo et al., 2004). Consistent with the data obtained with synthetic DNA templates, the degradation of ssDNA oligos by PDEI also led to the suppression of ATM activation (Figure 5C) induced by chromosomal breakage. Importantly, ATM activation by EcoRI-induced breaks was MRN dependent as it was abolished in Mre11-depleted extract (Figure 5D).

**ssDNA oligos induce ATM activation in human cells**

To demonstrate that generation of ssDNA oligos at chromosomal breaks is a conserved phenomenon, we developed a protocol to isolate DNA oligos from human cells after
Figure 4 ssDNA oligos form DNA–protein complexes with MRN that are able to induce ATM activation. (A) Biotinylated labelled pA70/pT70 was incubated in the extract for 30 min and following DNA removal with streptavidin beads, Mre11 was depleted and ATM activity was monitored. Activity is expressed as fold induction over the untreated samples. (B) 32P-labelled DNA isolated from Mre11 or mock immunoprecipitations was run on a 15% TBE–urea denaturing gel. Immunoprecipitates were untreated or treated with 0.001 U/μl PDEI. (C) Histone H2AX peptide phosphorylation induced by Mre11 immunoprecipitated from an untreated or a pA70/pT70-treated extract and transferred to an extract supplemented with low concentrations of pA70/pT70 (0.2 ng/μl) (black bar), rDSB (0.2 ng/μl) (grey bar) or no DNA (red bar). Immunoprecipitates were also treated with 0.001 U/μl PDEI for 10 min at 22°C before transfer into the extract supplemented with low concentrations of DNA (white bar).

Figure 5 ssDNA oligos are generated from MRN-dependent processing of chromosomal DSBs. (A) Sperm nuclei were incubated in the extract for 30 min. The extract was then supplemented with 0.2 U/μl of EcoRI in the presence or absence of 0.001 U/μl of PDEI and the samples were incubated for a further 60 min. Sperm nuclei were permeabilized and processed to isolate soluble low molecular weight DNA. DNA was labelled with TdT in the presence of 32P-alpha-ddATP and ran on a 15% TBE–urea denaturing gel. (B) Upper panel: sperm nuclei were incubated in the extract for 30 min, after which the extract was supplemented with 0.2 U/μl EcoRI and immediately processed to isolate low molecular weight DNA (0 min) or incubated for a further 60 min. The extracts were untreated, mock depleted (mock) or Mre11-depleted (Mre11 dep) and supplemented with recombinant MRN complex (+ MRN) (250 nM). Lower panels: egg extract treated as indicated in the upper panel was probed with anti-Xenopus (xMre11) and anti-human Mre11 (hMre11) antibodies. (C) Sperm nuclei were incubated in the extract in the presence or absence of 0.2 U/μl of EcoRI or 0.001 U/μl of PDEI for 60 min. ATM was immunoprecipitated and immunoblotted as indicated. (D) Sperm nuclei were incubated in the extracts that were untreated (Un), mock depleted (mock) or Mre11 depleted (Mre11 dep) in the presence or absence of 0.2 U/μl of EcoRI for 60 min. ATM was immunoprecipitated and immunoblotted as indicated.

induction of DSBs. Using this protocol, we could isolate ssDNA oligos from human U2OS cells treated with ionizing radiation (IR) (Figure 6A). ssDNA oligos were also isolated from G1-arrested cells, suggesting that ssDNA oligo production as ATM activation is not restricted to S phase (Supplementary Figure 9). We then tested the effect of ssDNA oligos on ATM in mammalian cells. To this end, we microinjected pT10 oligonucleotides into human U2OS cells. This procedure allowed reaching a high nuclear concentration of ssDNA oligos overcoming ssDNA degradation that usually occurred with alternative methods based on liposome-mediated transfer (data not shown). To identify the injected cells, we used anti-goat IgG conjugated with Alexa Fluro 488 as injection marker. ATM activation in the injected cells was monitored by detecting ATM phospho-serine 1981 (pSerine 1981). The DNA–injection marker mixture was injected in proximity to the nuclear periphery. Microinjection of cells with pT10 led to activation of ATM in the injected cells (Figure 6B). This response was completely inhibited when cells were pre-incubated with the ATM.
inhibitor KU55933 (Figure 6B). Furthermore, microinjection of pA5 or a mixture of random 5-mers also led to ATM activation (Figure 6C). Injecting cells with the injection marker alone, dTTP or circular DNA did not lead to detectable ATM activation (Figure 6C). ATM activation by small ssDNA oligos was observed in the majority of the injected cells (Supplementary Figure 10A). Quantification of the immunofluorescence intensity obtained with anti-phospho-ATM also revealed a robust activation of ATM induced by ssDNA oligos in the injected cells (Supplementary Figure 10B). ATM activation was detected immediately after oligo injection to exclude the interference of ssDNA oligos with DNA metabolic processes such as DNA replication or transcription. ssDNA oligos-induced ATM activation resulted in small and diffuse foci (Figure 6B and C) that were different from the large DNA damage foci typical of chromosomal breakage induced by IR (Figure 6B). The absence of genomic DSBs in cells injected with various ssDNA oligos was confirmed by the absence of DNA damage foci containing phosphorylated histone H2AX (Supplementary Figure 11).

Discussion

**MRN-dependent ssDNA oligo generation at DSBs**

Here, we have shown that DNA molecules with free DNA ends triggering ATM activation are rapidly processed resulting in the generation of ssDNA oligos. These molecules are not simple by-products of DNA resection as they participate in the ATM-dependent DNA damage response. We have demonstrated that generation of ssDNA oligos from chromosome breaks requires the MRN complex. In addition, we have shown that the MRN complex bound to ssDNA oligos promotes ATM activity, indicating that ssDNA oligos function as allosteric cofactors activating the complex. It is known that the MRN complex containing nuclease-inactive Mre11 fails to promote ATM activation (Uziel et al., 2003). In addition, Mirin, a chemical that inhibits Mre11 nuclease activity without affecting MRN and ATM binding to DSBs, suppresses ATM activity (Dupre et al., 2008). Our findings are consistent with the requirement for the MRN complex nuclease activity in the ATM activation process. Once ssDNA oligos have been generated, they remain associated with the MRN complex and probably promote a stable conformation capable of inducing continuous stimulation of ATM molecules. This process, likely, requires the generation of a limited amount of ssDNA oligos bound to MRN complex and does not require extensive resection to activate a large number of ATM molecules. This model is compatible with the lack of extensive resection in G1-arrested cells in which ATM can be activated (Jazayeri et al., 2006; Sartori et al., 2007). In addition, we show that ssDNA oligo formation can take place also in G1-arrested cells after treatment with IR. This is consistent with the recently reported resection of ‘ragged’ DNA ends induced by IR in G1-arrested cells (Barlow et al., 2008). ssDNA oligos produced at DSBs could interact with one or more subunits of the MRN complex that have DNA-binding domains. The binding of dinucleoside polyphosphates to the MRN complex through the Rad50 subunit has recently been demonstrated.
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ssDNA oligos turnover is linked to ATM activation status

An important aspect of ssDNA oligo metabolism is their turnover as the persistence of ssDNA oligos is correlated with the activation status of ATM. We showed that in Xenopus egg extract, exogenous synthetic small ssDNA oligos are rapidly degraded, whereas ssDNA oligos derived from the resection of larger DNA templates are more stable. This is likely due to their association with factors involved in DNA resection such as the MRN complex or other ssDNA-binding proteins. Interestingly, the ssDNA-binding protein hSSB1, which is highly conserved in Xenopus, has a higher binding affinity for poly-pyrimidine than for poly-purine containing ssDNA molecules (Richard et al., 2008). The association of ssDNA oligos to proteins such as hSSB1, if proven, might explain the higher intrinsic stability and efficiency at stimulating ATM activity of poly-pyrimidine pT70 compared with poly-purine pA70 oligos in the egg extract.

Predictably, the elimination of ssDNA oligos would be required for efficient inactivation of ATM once DSBs have been repaired, whereas the persistence of ssDNA oligos would maintain ATM active. ssDNA oligos elimination could be mediated by an exonuclease capable of degrading ssDNA oligos. Among known nucleases, Trex1, which has an activity similar to PDEI, can degrade ssDNA oligos (Mazur and Perrino, 2001). Mutations in Trex1 are responsible for the Aicardi–Goutieres syndrome, a complex human disease that recapitulates the effects of an embryonic response to a viral DNA infection (Crow et al., 2006). The deficiency of Trex1 activity leads to the accumulation of free ssDNA oligos and this correlates with chronic stimulation of the ATM-dependent DNA damage response (Yang et al., 2007). Therefore, Trex1 is an ideal candidate for the regulation of ssDNA oligos stability and, indirectly, ATM activity (Figure 7). Interestingly, Trex1 is confined to endoplasmic vesicles and ssDNA oligos need to be exported from nuclei for degradation (Yang et al., 2007). In our experiments, direct injection of ssDNA oligos into cells bypasses this degradation pathway reaching a nuclear concentration able to stimulate ATM. Taken together, these findings indicate an important and unexpected role for the ssDNA oligos metabolism in the DNA damage response.

ssDNA oligos as an alarm signal

The creation of ssDNA oligos during the resection of DNA undergoing repair, either from 5' to 3' processing of DSBs or possibly from enlarging gaps in other forms of DNA repair is a unique signal of DNA damage. Whereas mononucleotides are produced by normal DNA metabolism, these ssDNA oligos are only present when DNA damage is being processed. ssDNA oligos could function as an alarm signal that promotes full activation of the DNA damage response. Thus, whereas DSB ends and ssDNA are necessary to establish a platform to assemble factors required for the localized activation of the checkpoint and for the repair of the damage, a widespread and efficient DNA damage response—which should be turned off when repair is complete—takes advantage of DNA species that are only produced while repair is ongoing. The fact that single-stranded circular DNA, which is not degraded, is unable to trigger sustained ATM activation is consistent with the hypothesis that ssDNA in the absence of DNA processing is not sufficient to activate ATM. In budding yeast, where repair can be carefully monitored,
resection continues at a rate of 4 kbp/h from DSB ends for as long as it takes the process of repair to be completed, and the checkpoint is turned off soon after repair is complete (Vaze et al., 2002; Keogh et al., 2006). In the absence of yeast Mre11, the DNA damage checkpoint is initiated, but not maintained as in wild-type cells (Lee et al., 1998; D’Amours and Jackson, 2001), indicating a role for Mre11 in sustaining the checkpoint. Importantly, ssDNA oligos can also be observed following induction of DSBs in human cells, indicating that this is a conserved physiological phenomenon. The introduction of ssDNA oligos in cancer cells could be therapeutically exploited to enhance DNA damage response without producing further damage to the genome.

Materials and methods

Xenopus egg extract

The egg extract was prepared as previously described (Costanzo et al., 2004a). Interphase extract was obtained by releasing CSF-arrested extract with 0.4 mM CaCl2.

DNA structures

All sequence corresponding to the first 20 nucleotides of rDSB oligos) was mixed with 10 μl of the egg extract and incubated at 22 °C for 0, 1, 30, 60 and 90 min. Reactions were stopped with 40 μl stop buffer (0.5% SDS, 80 mM Tris pH 8.0 and EDTA 8 mM). Here, 10 μl of the reaction was blotted on Hybond-N membrane (Amersham). The samples were loaded on 15% Tris–urea acrylamide sequencing gel using a Bio-Rad apparatus. Alternatively, 15% TBE–urea acrylamide or TBE–acrylamide pre-cast gels from Invitrogen were used. Oligonucleotide DNA marker (Amersham) was used as labelled with TTG as described above. Gels were washed in 1× TBE for 12 h, then dried and exposed to X-ray film. DNA markers were from Bethyl. Anti-human Mre11 antibodies were from Trenz et al., 2006). Detection of ATM pSerine 1981 by western blot was obtained with mouse anti-ATM pSerine 1981 (Rockland Immunochemicals) overnight in blocking solution using Advanced ECL (Amersham).

DNA-processing reaction

Poly-dAA/dT70, poly-dA10, poly-dT70 or rDSB labelled at the 5′ or 3′ end was mixed with 10 μl of the egg extract and incubated at 22 °C for 0, 1, 30, 60 and 90 min. Reactions were stopped with 40 μl stop buffer (0.5% SDS, 80 mM Tris pH 8.0 and EDTA 8 mM). Here, 10 μl of the reaction was blotted on Hybond-N membrane (Amersham). The samples were loaded on 15% Tris–urea acrylamide sequencing gel using a Bio-Rad apparatus. Alternatively, 15% TBE–urea acrylamide or TBE–acrylamide pre-cast gels from Invitrogen were used. Oligonucleotide DNA marker (Amersham) was used was labelled with TTG as described above. Gels were washed in 1× TBE for 12 h, then dried and exposed to X-ray film. DNA markers were from Bethyl. Anti-human Mre11 antibodies were from Trenz et al., 2006). Detection of ATM pSerine 1981 by western blot was obtained with mouse anti-ATM pSerine 1981 (Rockland Immunochemicals) overnight in blocking solution using Advanced ECL (Amersham).

Isolation of DNA oligonucleotides

DNA oligonucleotides associated with Mre11 immunoprecipitates were detected as follow: 100 ng poly-dAA/dT70 or dT70 was 32P-labelled at 3′ end of the poly-dT70 using TdT as described above. The reaction was scaled up by a factor of 10. 32P-labelled poly-dAA/dT70 (100 ng) was incubated in 100 μl egg extracts for 30 min. Protein A Sepharose beads (50 μl) coupled to 100 μl of anti-X-Mre11 serum were then added to the extracts. For mock depletions, protein A Sepharose beads washed in PBS were used. Reactions were incubated for 45 min at 22 °C. Sepharose beads were then isolated by 1 min centrifugation at 1000 t.p.m. at 4 °C and washed three times with PBS supplement with 0.4% NP40. The samples were then resuspended in TBE-urea loading buffer (Invitrogen), heated at 70 °C for 3 min and run on a 15% TBE-urea acrylamide pre-cast gels (Invitrogen).

DNA oligonucleotides accumulated in response to EcoRI treatment were isolated as follows. Sperm nuclei were incubated in uninduced, mock-depleted or Mre11-depleted extracts at 400 nuclei/μl for 30 min at 22 °C and the extract was supplemented with 0.2 U/μl of EcoRI and incubated for a further 60 min. The extract was then resuspended in 40 mM HEPES-KOH pH 7.5, 15 mM MgCl2, 100 mM KCl, 20 mM EDTA and 1% Triton X-100 and incubated on ice for 10 min. The samples were centrifuged at 6000 g for 5 min and the supernatant was collected and labelled with TdT. Briefly, 50 μl of TdT reaction mixture (30 U TdT, TdT reaction buffer and 50 μCi of alpha-32P-dATP 10 μCi/μl greater than 3000 Ci/mmole). Reactions were incubated at 37 °C overnight. Labelled DNA was purified through G25 gel filtration columns (Amersham). The samples were loaded on 15% Tris–urea acrylamide gels and run for 1 h at 2000V. This labelled DNA was transferred to Hybond-N membrane (Amersham) for 2 h at 300 V in 1 × TBE buffer. The membrane was then heated at 80 °C for 2 h and exposed (Figure 5A and B).
Isolation of DNA oligonucleotide from human cells was obtained as follows: 3 × 10^7 human U2OS cells arrested by confluence density or synchronized in G1 with 500 μM mimosine (Sigma-Genosys) were irradiated with 10 Gy using a caesium 137 source or mock treated. G1 arrest was monitored with standard protocols using a FACs sorter. The cells were washed with ice-cold PBS once, harvested with a cell scraper in PBS and collected by centrifugation. The cell pellets were then treated for 5 min with lysis buffer (2% sodium dodecyl sulphate, 20 mM EDTA, 20 mM EGTA, 50 mM Tris–HCl, pH 7.5). Tubes were incubated at room temperature for 10 min. Then 25 μl 5 M NaCl was added and tubes were gently inverted three times and stored for 24 h at 4°C. The genomic DNA was then pelleted following centrifugation for 30 min at 9000 g at 4°C. The supernatant was harvested and the small DNA species were extracted with one volume of phenol–chloroform followed by ethanol precipitation overnight at –20°C. DNA was recovered following centrifugation at 9000 g at 4°C for 30 min. DNA was washed once with 70% ethanol and after repeated centrifugation at 9000 g at 4°C for 30 min, the pelleted DNA was resuspended in 50 μl TdT labelling mix (30U TdT, TdT reaction buffer and 50μl of alpha-3P-ddATP 10μCi/μl greater than 3000 Ci/mmol) as per the Fermentas manufacturer’s protocol and incubated at 37°C for 30 min. RNase A was then added to the final concentration of 1 mg/ml. The labelling reaction was stopped using 5 μl 0.5 M EDTA. Formamide loading buffer (5 μl) was added to 5 μl of the labelling reaction. The samples were loaded on 15% Tris–urea acrylamide gels and run for 1 h at 200 V. Gels were washed in 35% methanol, 15% acetic acid, wrapped in saran-wrap and immediately exposed.

**DNA oligonucleotide injection and immunofluorescence**

Human U2OS cell derivatives were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were grown on poly-L lysine-treated cover slips for at least 48 h in DMED supplemented with 10% FCS prior to manipulations. For microinjections, injection mixtures (10 μl of Alexa Fluor 488 chicken anti-goat IgG (Molecular Probes) plus 5 μl of DNA at 5 μg/μl) were loaded onto Femtotip I (Eppendorf) and attached to InjectMan N2 System (Eppendorf) connected to Zeiss Axiomert 200 microscope. The cells were injected into the cytoplasm at ~100–150 hPa injection pressure. For each experiment, about 200 cells were injected and after 30 min cells were fixed in ice-cold 50% methanol–50% acetone mixtures for 10 min on ice. Following fixation, the cells were washed extensively with PBS and incubated for 30 min with blocking solution containing 5% (w/v) non-fat milk in TBST (TBS plus 0.1% Tween-20). The cells were then incubated with mouse anti-ATM pSerine 1981 (Rockland Immunochimicals) overnight in blocking solution. For secondary detection, we used Alexa Fluor 594 chicken anti-mouse IgG (Molecular Probes). The cells were visualized using a ZeissLSM 510 Confocal microscope.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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