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Differences in the recruitment of DNA repair proteins at subtelomeric and interstitial I-SceI endonuclease-induced DNA double-strand breaks

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Abstract

Telomeres are nucleoprotein structures that are required to protect chromosome ends. Dysfunctional telomeres are recognized as DNA double-strand breaks (DSBs), and elicit the activation of a DNA damage response (DDR). We have previously reported that DSBs near telomeres are poorly repaired, resulting in a high frequency of large deletions and gross chromosome rearrangements (GCRs). Our previous genetic studies have demonstrated that this sensitivity of telomeric regions to DSBs is a result of excessive processing. In the current study, we have further investigated the sensitivity of telomeric regions to DSBs through the analysis of repair proteins associated with DSBs at interstitial and telomeric sites. Following the inducible expression of I-SceI endonuclease, chromatin immunoprecipitation (ChIP) and real-time quantitative PCR were used to compare the recruitment of repair proteins at I-SceI-induced DSBs at interstitial and subtelomeric sites. We observed that proteins that are specifically associated with processing of DSBs during homologous recombination repair, RAD51, BRCA1, and CtIP, are present at a much greater abundance at subtelomeric DSBs. In contrast, Ku70, which is specifically involved in classical nonhomologous end joining, showed no difference at interstitial and subtelomeric DSBs. Importantly, ATM was lower in abundance at subtelomeric DSBs, while ATR was in greater abundance at subtelomeric DSBs, consistent with the accumulation of processed DSBs near telomeres, since processing is accompanied by a transition from ATM to ATR binding. Combined, our results suggest that excessive processing is responsible for the increased frequency of large deletions and GCRs at DSBs near telomeres.

Keywords

DNA repair; double-strand breaks; telomere

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Conflict of Interest Statement

None declared

1. Introduction

Cellular DNA is susceptible to DNA lesions in the form of DNA double-strand breaks (DSBs) or single strand breaks (SSBs), which can be generated by many processes, such as the collapse of replication forks, ionizing radiation, and oxidative damage [1, 2]. DSBs are one of the most deleterious types of DNA damage and must be repaired in a timely manner to maintain genomic stability. Failure to properly repair DSBs leads to chromosome fusions, chromosome translocations, and eventually tumorigenesis. DSBs activate a well-established mechanism known as the DNA damage response (DDR) pathway, which is responsible for recognizing damaged DNA and recruiting repair proteins [2].

The recognition of the ends of chromosomes as DSBs is prevented by a nucleoprotein structure known as a telomere, which is composed of the TTAGGG repeat sequence and a complex composed of six proteins called shelterin [3]. Telomeres are essential for protecting chromosome ends from degradation, recombination processes, and chromosome fusion [3, 4]. Uncapping of telomeres elicits the DDR that leads to the activation of either the ataxia telangiectasia mutated (ATM) or ataxia-telangiectasia-and-Rad3-related (ATR) kinases, which phosphorylate downstream proteins leading to cell cycle arrest and ultimately chromosome fusions and genomic instability [3].

There are two primary pathways that repair mammalian genomic DSBs, classical nonhomologous end joining (C-NHEJ) and homologous recombination repair (HRR) [5, 6]. C-NHEJ utilizes a protein complex composed of Ku70, Ku86, and DNA-PKcs, which promotes the ligation of the broken ends by DNA ligase IV [7]. C-NHEJ can repair DSBs in all parts of the cell cycle except for mitosis. C-NHEJ is inhibited by the processing and resection of the 5' ends of DSBs, which generates single-stranded 3' overhangs [7]. In contrast, HRR requires the processing and resection of the 5' ends of DSBs and the presence of single-stranded 3' overhangs [8, 9]. HRR in mammalian cells utilizes the sister chromatid as a template for repair, and therefore can only repair DSBs during the late S/early G2 phases of the cell cycle when a sister chromatid is available [10]. HRR utilizes a large number of proteins, including MRE11, CtIP, and BRCA1, which are involved in generating the single-stranded 3' overhang, while other proteins, including RAD51, are involved in strand transfer between the sister chromatids [11, 12]. Some proteins, such as phosphorylated H2AX (γ H2AX) and p53 binding protein 1 (53BP1), are found at DSBs during repair in both C-NHEJ and HRR, since they can be phosphorylated by either DNA-PKcs or ATM [13]. 53BP1 is a large multi-domain protein with key roles in DSB repair pathway choice, and promotes C-NHEJ by suppressing 5' processing and resection of DSBs [14–17]. In the case of dysfunctional telomeres, the binding of 53BP1 is followed by processing and resection of DNA ends, which is mediated by CtIP, BLM and EXO1 proteins [18].

DSB repair processes are highly influenced by chromatin structure. Most DSBs are repaired within one to two hours [19]. However, 15–20% of DSBs are repaired with slow kinetics [19, 20], which includes DSBs that are difficult to repair, such as those in heterochromatin that require ATM for repair by C-NHEJ [21, 22]. Although ATM is involved in the initial response to DSBs, the extensive resection of DSBs promotes a switch from ATM to ATR

[23, 24], which is activated in response to single-stranded DNA. ATR also plays a major role in DNA damage encountered during DNA replication, in that it binds to the single stranded (SS) DNA resulting from stalled replication forks [25].

The processing and resection of DSBs in stages of the cell cycle other than late S/early G2 phase can result in chromosome rearrangements, because these processed DSBs cannot be repaired by either HRR (no sister chromatids are available) or C-NHEJ (single-stranded overhangs cannot be repaired by C-NHEJ) [26, 27]. Instead, these inappropriately processed DSBs are repaired by the alternative-NHEJ (A-NHEJ) repair pathway, which is involved in the formation of the DNA deletions, translocations and gross chromosome rearrangements (GCRs) [27–32]. Similar to HRR, A-NHEJ also requires the processing of DSBs to generate a single-stranded 3' overhang [27], which involves the MRE11/RAD50/NBS1 complex (MRN) and CtIP protein, along with the nuclease activity of EXO1 [26, 29, 33, 34]. However, unlike HRR, extensive resection is not required for A-NHEJ [27].

Our laboratory has previously investigated DSB repair using a plasmid containing *I-SceI* endonuclease recognition sites to introduce DSBs at subtelomeric or interstitial sites [31]. Using various reporter assays, we have previously shown that DSBs near telomeres are much more likely to result in GCRs and chromosome instability than DSBs occurring at interstitial locations [30–32, 35]. Recent studies from other laboratories have also shown a persistent DDR and deficient DSB repair at telomeric regions using ionizing radiation, genotoxic drugs, or H₂O₂ [36, 37]. This persistent DNA damage at telomeric regions has been proposed to be due to the suppression of DNA repair proteins by the telomere-binding protein TRF2 [35, 36]

Our earlier genetic studies concluded that the sensitivity of telomeric regions to DSBs is not a result of a direct defect in HRR or C-NHEJ. Instead, we concluded that the sensitivity of telomeric regions to DSBs is a result of excessive processing by MRE11 and other nucleases [32]. However, the reason for the abnormal processing of DSBs near telomeres is not yet known. To complement our genetic studies, in our current study, we have analyzed the differences in repair proteins associated with interstitial and subtelomeric DSBs. Our results are consistent with the presence of persistent extensively-processed DSBs near telomeres, as shown by the increased abundance of repair proteins RAD51, BRCA1, and CtIP at subtelomeric DSBs. Importantly, we also found that ATM is decreased in abundance at subtelomeric DSBs, while ATR is greater in abundance at subtelomeric DSBs, consistent with an accumulation of processed DSBs near telomeres. Finally, our current results show no difference in the amount of Ku70 at interstitial and subtelomeric DSBs, consistent with our previous conclusion that C-NHEJ functions normally near telomeres. Therefore, as with our earlier genetic studies, we conclude from our analysis of proteins associated with DSBs that the deficiency in DSB repair near telomeres is the result of extensive processing, which indirectly interferes with efficient repair, leading to an increased frequency of large deletions and GCRs.

2. Materials and Methods

2.1 Cell lines and Cell Culture

The cell lines used in this study were derived from the EJ-30 human cell bladder cell carcinoma cell line (also known as MGH-U1), as previously described [31, 32, 35]. Cells were grown in α -MEM culture medium (UCSF Cell Culture Facility) supplemented with 5% fetal calf serum (Invitrogen-Gibco), 5% newborn calf serum (Invitrogen-Gibco), and 1 mM *L*-glutamine (Invitrogen-Gibco) at 37 °C with 5% CO₂.

The GFP-7F1 and GFP-6D1 cell clones containing the pGFP-*I-SceI* plasmid integrated at interstitial and telomeric sites respectively, were previously used for the detection of large deletions and chromosome rearrangements [30–32]. The induction of a single DSB at interstitial and subtelomeric sites was accomplished using the inducible *I-SceI* system, ddSceGR, which uses an *I-SceI* endonuclease that contains a destabilization domain (dd) on one end and a glucocorticoid receptor (GR) on the other end. The addition of Shield1 (Clontech) to block the degradation of the *I-SceI*, and Triamcinolone acetonide (TA), a glucocorticoid analog, to transport the *I-SceI* endonuclease into the nucleus, results in nearly immediate induction of DSBs at *I-SceI* recognition sites in human cells [38, 39]. Clones GFP-7F1 and GFP-6D1 were transfected with the ddSceGR plasmid and cells containing the stably integrated plasmid were selected with 400 μ g/ml G418. Clones that express the *I-SceI* gene were then identified by PCR. RNA was isolated using RNeasy (Qiagen) and converted to cDNA with iScript (Bio-Rad) using protocols provided by the manufacturers. PCR was then performed using primers *I-SceI*-F2 5'-GGGTGATGCTTACATCCGTTC and *I-SceI*-R3 5'-ATCATCTGCGGGATCAGGTAC (Supplemental Figure 1A).

2.2 Inducible *I-SceI* endonuclease and analysis of small and large deletions

I-SceI endonuclease-induced DNA DSBs were generated by the addition of the Shield1 (0.5 μ M) and TA (100 nM) ligands to cultures of clones GFP-6D1 and GFP-7F1 that contain the integrated ddSceGR plasmid (Supplemental Figure 1A). For ChIP analysis, triplicate cultures were incubated with the ligands for 2 days, the medium containing the ligands replaced, and the cultures incubated for a total of 4 days. For controls, triplicate cultures were grown in the presence of DMSO alone.

For the analysis of small and large deletions, triplicate cultures of the clones of GFP-6D1 and GFP-7F1 were grown for 12 days in the presence of the Shield1 (0.5 μ M) and TA (100 nM) ligands, with medium changes every two days. For controls, triplicate cultures were grown in the presence of DMSO alone. The frequency of large deletions at the *I-SceI*-induced DSB was measured by determining the frequency of GFP-negative cells at days 0, 4, 8 and 12 using a Cellometer Vision (Nexcelcom), as previously described [31, 32]. The frequency of small deletions at the *I-SceI*-induced DSB was analyzed by isolating genomic DNA from the same cultures used for analysis of large deletions (days 0, 4, 8 and 12). Small deletions were determined by first generating a PCR product spanning the *I-SceI* site in the integrated pEJ5-GFP plasmid, and then digesting the PCR product with *I-SceI* endonuclease, as previously described [31, 32]. PCR was performed using Taq 2 $\times$ Master Mix (New England Biolabs) with primers GFP-1 (5'-GCGGGGTTTCGGCTTCTGG-3') and GFP-2

(5'-TCGGGCATGGCGGACTTG-3') located to either side of the I-SceI site, which amplifies a 440 bp fragment. PCR involved 94°C for 2 minutes, then 40 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds. 25 µl of the PCR product was then digested with 20 units of I-SceI endonuclease (New England Biolabs) in 1× CutSmart buffer (New England Biolabs) at 37°C overnight, and the products were run on 3% agarose gels. After staining with ethidium bromide, digital images were analyzed using Image J software (<http://www.versiontracker.com/dyn/moreinfo/macosx/37303>) to calculate the intensity of the bands.

The fraction of cells containing small deletions (SD) at the I-SceI site was determined by dividing the intensity of the uncut band (UC) by the combined intensity of the cut (C) and uncut bands. Values were corrected for background of uncut PCR fragment in untreated controls, apparently due to small leakiness in the system, by subtracting the fraction of uncut DNA in cultures at time 0. Because the PCR product is generated from the pooled DNA from all of the cells in the population, the frequency of PCR product not cut by I-SceI represents the frequency of small deletions at the I-SceI site. However, as we have previously shown [30–32, 35], the loss of the plasmid sequences (primarily through large deletions) in some cells will result in an overestimation of the frequency of small deletions, because cells containing large deletions at the I-SceI site will not generate a PCR product. The fraction of uncut PCR product was therefore multiplied by 1 minus the fraction of cells with large deletions (1-LD), as determined in our large deletion assay. The final equation for the fraction of cells with small deletions is therefore: $SD = UC/UC+C \times (1-LD)$. Although other types of rearrangements at the I-SceI site can also prevent small deletions, this was not corrected for because the frequency of these events is too low to significantly affect our results. The validity of this correction was previously demonstrated by the analysis of the frequency of small deletions in 100 individual subclones selected at random [35].

2.3 Chromatin immunoprecipitation

I-SceI-induced and control (DMSO) cells (1.0×10^6 /flask) were fixed with 1% formaldehyde (PFA) for 10 min at room temperature (RT), followed with 2.5 M glycine (1 M final) for 5 min. The cells were then washed twice with cold phosphate-buffered saline (PBS), and scrapped from the flask in PBS containing proteases inhibitors (Roche). The cells were then spun and lysed with RIPA buffer (10% sodium deoxycholate, 10% SDS, 10% NP-40, 1× PBS). To obtain 0.5–1.0 kbp DNA fragments, the chromatin suspension was sonicated using a Fisher Scientific Model 100 with 8 pulses for 20 seconds each time at a setting of 3, and with 1-minute intervals on ice. The suspension was then spun to remove debris, and aliquot samples (1.0×10^5 cells/sample) were snap frozen in liquid nitrogen and stored at –80°C until further use. Sheared aliquots were incubated overnight at 4°C with the following antibodies: anti-53BP1 (Novus Biologicals), anti-ATM (Novus Biologicals), anti-γH2AX (Upstate), anti-BRCA1 (Genetex), anti-Rad51 (Santa Cruz), anti-Ku70 (Santa Cruz), anti-CtIP (Abcam), anti-ATR (Abcam), or no antibody (mock). Protein magnetic beads (Life Technologies) were blocked at 4°C with 1 ml IP dilution buffer (1 M Tris-HCl pH 8.0, 5 M NaCl, 10% Triton-X 100, 0.5 M EDTA, plus protease inhibitors (Roche)) for 1 hour. Magnetic beads were then incubated with the sheared chromatin along with antibodies for 2 hours at 4°C. The immunoprecipitates were washed five times for five minutes with wash

buffer (100 mM Tris pH 7.5, 500 mM LiCl, 1% NP-40 (v/v), 1% sodium deoxycholate (v/v)), and eluted with ChIP elution buffer (1% SDS (v/v), 0.1 M NaHCO₃, filtered with a 0.2 micron filter). Samples were treated with proteinase K and incubated overnight at 65°C to reverse crosslinking. Immunoprecipitated DNA was purified using a PCR purification kit with buffer PM (Qiagen), and quantified using SYBR Green (Invitrogen). The amount of various proteins bound to the plasmid sequences containing the DSB was then determined by real time quantitative PCR using a StepOnePlus real time PCR machine (AB applied Biosystems). The PCR primers used for ChIP analysis amplify a 228 bp region that is 4040 bp from the I-SceI-induced DSB in the integrated pGFP-ISceI plasmid, and were as follows: forward primer, 5'-GAAGTGGTGGCCTAACTACGG; reverse primer, 5'-TCGTTCCACTGAGCGTCAG. All ChIP data was normalized to the mock (no antibody) control. Samples were analyzed in triplicate. Error bars represent standard deviation of samples.

2.4 Cell sorting

I-SceI-induced and control (DMSO) cells were labeled with Vybrant® DyeCycle™ blue (Life technologies) for sorting as described in manufacture's protocol. G1 cells were sorted using a BD FACSARIA-2 (BD Biosciences, San Jose, CA). One million cells per sample of the clones GFP-6D1 and GFP-7F1 (I-SceI-induced and DMSO controls) were collected and spun at 4°C. Samples were fixed for 10 minutes with 1% PFA, washed with PBS, and split into aliquots. Samples were then processed for ChIP as described above.

3. Results

3.1 Generating DSBs with an inducible I-SceI endonuclease in a human cancer cell line

Human tumor cell clones GFP-6D1 and GFP-7F1, which contain the pGFP-ISceI plasmid integrated at telomeric or interstitial sites, respectively, were used for analysis of proteins associated with I-SceI-induced DSBs. We have previously used these cell clones to show that DSBs near telomeres result in a much higher frequency of large deletions and GCRs than interstitial DSBs [30–32, 35]. I-SceI-induced DSBs in these clones were generated using a second stably integrated plasmid, ddSceGR, which contains a gene for I-SceI endonuclease that is activated by the addition of Shield1 and TA (Figure 1A, Supplemental Figure 1A). We were unable to demonstrate activation of the I-SceI endonuclease by detection of DSBs, possibly because DSBs at the I-SceI site were too infrequent, and/or quickly repaired. However, we were able to monitor the activity of I-SceI endonuclease after addition of Shield1 and TA through the appearance of small and large deletions at the I-SceI site (Figure 1A). As in our earlier studies [30–32, 35], the frequency of small deletions was analyzed by first using PCR to amplify the DNA at the site of the I-SceI-induced DSB, followed by digestion of the PCR product with I-SceI endonuclease (see Materials and Methods). Consistent with our earlier studies using I-SceI endonuclease introduced by transduction with a retrovirus vector [30–32, 35], the frequency of small deletions in our inducible system was similar at the interstitial and subtelomeric I-SceI-induced DSB (Figure 1B, Table 1, Supplemental Figures 1B and C). The percent of small deletions increased with time at both interstitial and subtelomeric DSBs, reaching 14.9 % in both clone GFP-7F1 and clone GFP-6D1 following 12 days of treatment with Shield 1 and TA.

As in our earlier studies [30–32, 35], the frequency of large deletions at the I-SceI site was monitored by the loss of GFP fluorescence (Figure 1C). Consistent with our earlier studies, the generation of DSBs following the addition of Shield1 and TA resulted in a much greater frequency of large deletions (GFP negative cells) in clone GFP-6D1 with a subtelomeric DSB than in clone GFP-7F1 with an interstitial DSB (Figure 1C). Following an initial delay due to the relatively long half-life of GFP [40, 41], cells with large deletions (GFP-negative) increased to 36.7 % of the population in clone GFP-6D1 by day 12, compared with 5.2 % of the population in clone GFP-7F1. Based on these results, we concluded that small deletions and large deletions are generated through two separate mechanisms. We therefore proposed that small deletions were generated during NHEJ, which is functional near telomeres, whereas large deletions were generated by excessive processing during HRR.

3.2 Comparison of DDR and repair proteins at interstitial and subtelomeric DSBs

To examine the presence of DDR and DNA repair proteins at I-SceI-induced DSBs, we performed ChIP experiments using a PCR product located 4040 bps from the DSB. This long distance from the DSB was chosen to minimize the underestimation of the amount of protein associated with the subtelomeric DSB due to the increased frequency of large deletions at DSBs near telomeres.

Unlike large deletions, our previous study found that small deletions were identical at interstitial and subtelomeric DSBs and were formed during C-NHEJ, leading us to conclude that the reason for the sensitivity of subtelomeric regions to DSBs is not due to a deficiency in C-NHEJ [32]. To further test this hypothesis, we analyzed the amount of Ku70 protein, which is specific for the C-NHEJ pathway, that was bound to either interstitial or subtelomeric DSBs. We observed no difference in the amount of Ku70 bound to interstitial or subtelomeric DSBs (Figure 2), supporting our earlier conclusion that the sensitivity of telomeric regions to DSBs is not due to a deficiency in C-NHEJ.

We next investigated the abundance of the phosphorylated form of histone H2AX (γ H2AX) and the repair protein 53BP1, well-known DSB markers, at interstitial or subtelomeric DSBs (Figure 3A, B). Unlike Ku70, which is specific to the C-NHEJ pathway, the phosphorylation of H2AX and 53BP1 can occur in both the C-NHEJ (by DNA-PKcs) and HRR (by ATM) pathways [42, 43]. Our results show that unlike Ku70, γ H2AX and 53BP1 are more abundant at subtelomeric DSBs than at interstitial DSBs. In view of the similar amounts of Ku70 protein (Figure 2) and the similar frequencies of small deletions at subtelomeric and interstitial DSBs [30–32, 35], this increase in γ H2AX and 53BP1 at subtelomeric DSBs cannot be due to a greater number of DSBs introduced by I-SceI at subtelomeric sites. Therefore, there is either an increased amount of γ H2AX and 53BP1 at subtelomeric DSBs, or subtelomeric DSBs are more slowly repaired than interstitial DSBs. In the latter case, the persistent DSBs would appear to be those being processed for HRR, since as discussed above, the frequency of repair of DSBs by C-NHEJ is similar at both locations.

To investigate whether the defect in repair of subtelomeric DSBs is associated with processing during HRR, we next analyzed the amount of BRCA1, Rad51 and CtIP at either interstitial or subtelomeric DSBs (Figure 3C, D, E). BRCA1, Rad51 and CtIP are involved in the HRR pathway [44–46], while CtIP and BRCA1 have also been shown to play a role in

the DSB processing involved in the A-NHEJ pathway [12, 29, 45, 47, 48]. Consistent with the presence of persistent processed DSBs near telomeres, we observed an increased amount of BRCA1, Rad51 and CtIP at subtelomeric DSBs compared to the interstitial DSBs. Importantly, the increased amount of BRCA1, RAD51 and CtIP associated with subtelomeric DSBs exceeds the increased amount of γ H2AX and 53BP1 found at subtelomeric DSBs. Our combined results therefore show that a protein specific for C-NHEJ (Ku70) is not increased at subtelomeric DSBs, proteins associated with both C-NHEJ and HRR (γ H2AX and 53BP1) show an intermediate increase at subtelomeric DSBs, and proteins specific for processing of DSBs during HRR (BRCA1, Rad51 and CtIP) are greatly increased at subtelomeric DSBs. Combined, these results suggest that resected DSBs persist in an unrepaired state when they occur near telomeres.

3.3 Comparison of ATM and ATR associated with interstitial and subtelomeric DSBs

To further understand the unique response to DSBs near telomeres, we also investigated two important kinases involved in the DDR, ATM and ATR. Unlike HRR proteins, we found the amount of ATM to be lower at subtelomeric DSBs compared to interstitial DSBs (Figure 4A). This could be because telomeres are protected from the DDR by TRF2, a protein component of the telomere protein complex, which is involved in suppressing ATM at telomeres [49]. However, an alternative explanation is that this decrease in ATM at subtelomeric DSBs is due to excessive processing, because DSBs transition from ATM to ATR following processing [23, 24]. Consistent with this later possibility, we found ATR levels to be higher at subtelomeric DSBs compared to interstitial DSBs (Figure 4B). This result demonstrates that DSBs near telomeres preferentially activate the ATR pathway. This result is consistent with our results above, that proteins involved in the processing of DSBs are greatly increased at subtelomeric DSBs compared to interstitial DSBs. Combined, these data further support the model that DSBs near telomeres undergo excessive processing.

3.4 Comparison of 53BP1 at interstitial and subtelomeric DSBs in G1 phase

To investigate how the cell cycle impacts the repair of DSBs at interstitial and subtelomeric sites, we performed ChIP experiments to assess the amount of 53BP1 associated with I-*SceI*-induced DSBs in G1 phase-sorted cells. The results show that similar to asynchronous cells, cells in G1 phase show a higher abundance of 53BP1 at subtelomeric DSBs than at interstitial DSBs (Supplemental Figure 2). Therefore, as discussed above for asynchronous cells, subtelomeric DSBs appear to persist for longer periods of time than interstitial DSBs in cells in G1 phase. In view of our results showing extensive processing of these persistent DSBs, these results suggest that subtelomeric DSBs are processed in G1, a time when processing does not normally occur because HRR is not possible due to the absence of a sister chromatid [50]. These results are consistent with the persistence of DSBs near telomeres in cells in G1 phase demonstrated by analysis of non-dividing cells following exposure to ionizing radiation [37]. The sensitivity of telomeric regions to DSBs may therefore be due to the inappropriate processing of DSBs in G1 phase, since these processed DSBs cannot be repaired by either C-NHEJ or HRR.

4. Discussion

DSBs near telomeres result in an increased frequency of large deletions, gross chromosome rearrangements (GCRs), and eventually chromosome instability, compared to DSBs at interstitial sites [30–32, 35]. Previous results from our laboratory and other laboratories have shown that the majority of mutagenic events at interstitial I-*SceI*-induced-DSBs are small deletions, while large deletions are relatively rare [30, 31, 35, 51–53]. In contrast, at subtelomeric DSBs, although the frequency of small deletions is similar to that at interstitial DSBs, the frequency of large deletions is greatly increased [30–32, 35]. As a result, the frequency of large deletions at subtelomeric DSBs is much higher than the frequency of small deletions [30–32, 35], demonstrating important differences in the mechanisms of repair of DSBs near telomeres. We have previously proposed that this difference in repair near telomeres is due to the extensive processing of DSBs near telomeres, which indirectly inhibits C-NHEJ [32]. This conclusion was based upon our previous genetic studies, which showed that the inhibition of the 3′-5′ exonuclease activity of MRE11 partially inhibits the formation of large deletions and GCRs at both interstitial and telomeric DSBs [32].

In the current study, we investigated the mechanism responsible for the sensitivity of telomeric regions to DSBs through analysis of proteins found in proximity to an I-*SceI*-induced DSBs at interstitial or subtelomeric sites. The results shown here are consistent with our earlier hypothesis based on genetic studies that small deletions occur during C-NHEJ, in that we now find no difference in the amount of Ku70 at interstitial and subtelomeric I-*SceI*-induced DSBs (Figure 2). Our current results also support the hypothesis that DSBs near telomeres undergo more extensive processing than interstitial DSBs, based on the increased recruitment of the RAD51, BRCA1, and CtIP repair proteins at subtelomeric DSBs. Although the reason for this excessive processing of DSBs near telomeres remains to be determined, our results with G1 phase cells suggests a problem in regulating the timing of the processing of subtelomeric DSBs in the cell cycle, since DSBs processed in G1 phase cannot be repaired by either C-NHEJ or HRR.

BRCA1 and CtIP are known to be involved in the processing of DSBs [48, 54], while RAD51 binds to the single-stranded tail and facilitates strand exchange during HRR [55]. RAD51-mediated strand exchange during HRR occurs using the sister chromatid as a template [56]; however, when HRR cannot occur, such as with DSBs processed in G1 phase, the single-stranded DNA of the processed DSBs can join with other processed DSBs through A-NHEJ [57]. A-NHEJ is known to be a major contributor of chromosome translocations [29] and chromosome instability [4, 58], although C-NHEJ can also contribute to the formation of chromosome translocations [59]. A role for A-NHEJ in the eventual repair of excessively processed DSBs near telomeres is suggested by the presence of microhomology at the sites of junctions of chromosome fusions resulting from spontaneous or DSB-induced telomere loss [31, 60, 61].

The BRCA1 and 53BP1 repair proteins can help to determine repair pathway choice by either promoting (BRCA1) or preventing (53BP1) processing and resection of DSBs [62–64]. Our results show 53BP1 is increased at DSBs near telomeres relative to interstitial DSBs (Figure 3B). 53BP1 is involved in repair of DSBs in heterochromatin during G1 phase

[65, 66], and can promote C-NHEJ by preventing the binding of CtIP and end processing [57]. When 53BP1 is down-regulated, DSBs are processed and resected extensively and are no longer repaired by C-NHEJ, leading to chromosome rearrangements [67]. In view of the role of 53BP1 in preventing the processing and resection of DSBs, it is unclear why we observe an increased amount of 53BP1 at telomeres. One possibility is that although 53BP1 is increased at subtelomeric DSBs, the amount of BRCA1 that is bound at subtelomeric DSBs is much greater (Figure 3C), and therefore is likely to counteract any protective effects of 53BP1. A second possibility is that the 53BP1 at DSBs near telomeres is not phosphorylated, and therefore does not function to protect DSBs. Although phosphorylation of 53BP1 is required to prevent processing of DSBs, it is not required to 53BP1 binding to DSBs [67]. Unfortunately, we were unable to obtain antibodies to phosphorylated 53BP1 that would function in a ChIP assay, and therefore could not address this possibility. Finally, it has also been shown that 53BP1 can promote the processing of DSBs in G1 phase [68], which would lead to A-NHEJ, since HRR cannot occur in G1 phase.

The decreased ATM and increased ATR at subtelomeric DSBs relative to interstitial DSBs in our studies is also consistent with excessive processing of DSBs, since following processing, DSBs transition from ATM to ATR [23, 24, 69]. CtIP is then directly phosphorylated by ATR, leading to processing and resection of DSBs [25, 70]. Furthermore, ATM activity has been shown to be reduced at DSBs undergoing end-resection during HRR [71]. However, it is also possible that the low levels of ATM present at DSBs near telomeres DSBs is due to the presence of the telomere protein TRF2 near the DSB. TRF2, which is a key component of the shelterin complex that protects the telomere, suppresses ATM at telomeres, thus leading to the inhibition of C-NHEJ repair [72–74].

Although we were not able to measure the frequency of DSBs directly, these differences in DDR proteins at interstitial and subtelomeric DSBs cannot be explained by an increased frequency of DSBs generated by *I-SceI* endonuclease at the two locations. Although RAD51, BRCA1, CtIP, and ATM are increased at subtelomeric DSBs, ATR is decreased, and Ku70 shows no difference compared to interstitial DSBs. Similarly, our earlier studies have clearly shown differences in repair of DSBs at interstitial and subtelomeric sites, in that large deletions and GCRs are increased in frequency at DSBs near telomeres, while small deletions and HRR are unchanged, and distal NHEJ is decreased in frequency [30–32]. Furthermore, the large deletions at DSBs near telomeres are much greater in size than those at interstitial DSBs [30, 31]. Finally, our earlier studies demonstrated important differences in the role of ATM and MRE11 in the formation of large deletions, GCRs, small deletions, and distal NHEJ at interstitial and subtelomeric sites.

Combined, our current results add further support to our hypothesis that DSBs near telomeres undergo prolonged processing and remain unrepaired for long periods of time, resulting in excessive degradation and/or GRCs at the site of the DSB. Ultimately, our results suggest that telomeres are sensitive to DSBs because excessive processing inhibits repair by C-NHEJ, leading to large deletions, GCRs, and chromosome instability involving eventual repair through A-NHEJ.

Supplementary Material

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Highlights

- Nonhomologous end-joining protein Ku70 is similar at interstitial and subtelomeric DSBs
- Repair proteins RAD51, BRCA1 and CtIP are greatly increased at subtelomeric DSBs
- ATM is reduced at subtelomeric DSBs compared to interstitial DSBs
- ATR is increased at subtelomeric DSBs compared to interstitial DSBs
- DSBs near telomeres are more likely to be inappropriately processed

Figure 1. Monitoring the frequency of small and large deletions at interstitial and subtelomeric DSBs following inducible expression of I-SceI endonuclease

(A) The plasmid pGFP-ISceI is stably integrated at a subtelomeric site in clone GFP-6D1 or an interstitial site in clone GFP-7F1. pGFP-ISceI contains a recognition site for I-SceI endonuclease that is located between a GFP gene and its promoter. DSBs at the I-SceI site are generated through the addition of Shield 1 and TA, which activates the I-SceI endonuclease that is expressed from the ddSceGR plasmid that is also stably integrated in these cell clones. Small deletions at the I-SceI site are monitored by the loss of the I-SceI site in a PCR product that is generated using primers located to either side of the I-SceI site (red arrows). Large deletions at the I-SceI site are monitored by the loss of GFP expression. Cells with large deletions do not produce a PCR product due to loss of sequences complementary to the primers. As a result, the amount of uncut PCR product must be

adjusted to account for the presence of large deletions to determine the frequency of small deletions (see Materials and Methods. **(B)** The frequency of cells with small deletions at the I-SceI site in clones GFP-6D1 (6D1) and GFP-7F1 (7F1) in cultures untreated (DMSO) or treated (I-SceI) with Shield1 and TA over time (days). Control cultures treated with DMSO alone showed no change in GFP expression during the same period (not shown). Error bars represent the standard deviation of experiments performed in triplicate. **(C)** The frequency of cells with large deletions at the I-SceI site in clones GFP-6D1 (6D1) and GFP-7F1 (7F1) in cultures with Shield1 and TA over time (days). Control cultures treated with DMSO alone showed no change in GFP expression during the same period (not shown). Error bars represent standard deviation of experiments performed in triplicate.

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Figure 2. Binding of Ku70 is the same at subtelomeric and interstitial I-SceI-induced DSBs (A). Graph of the percent of Ku70 protein in ChIP input in clones GFP-6D1 (6D1) and GFP-7F1 (7F1) untreated [(-), DMSO] or treated (+) with Shield1 and TA for a period of 4 days. Samples were incubated with Ku70 antibody or no antibody (mock), and ChIP was performed as described in Materials and Methods. N = 3 independent experiments (mean $\pm$ s.d.).

Figure 3. Recruitment of DSB repair proteins at subtelomeric and interstitial I-SceI-induced DSBs

(A) Graphs of the percent of various repair proteins in ChIP input in clones GFP-6D1 (6D1) and GFP-7F1 (7F1) untreated [(-), DMSO] or treated (+) with Shield1 and TA for a period of 4 days. Samples were incubated with anti-γH2AX (A), anti-53BP1 (B), anti-BRCA1 (C), anti-RAD51 (D), anti-CtIP (E), or no antibody (mock). N = 3 independent experiments (mean ± s.d.).

A

B

Figure 4. Presence of ATM and ATR at subtelomeric and interstitial I-SceI-induced DSBs
 Graphs of the percent of ATM or ATR in ChIP input in clones GFP-6D1 (6D1) and GFP-7F1 (7F1) untreated [(-), DMSO] or treated (+) with Shield1 and TA for a period of 4 days. Samples were incubated with anti-ATM (A), anti-ATR (B), or no antibody (mock). I-SceI-induced DSBs at an interstitial site show higher abundance of ATM compared to a subtelomeric site ($p < 0.05$), whereas an I-SceI-induced DSB near a telomere shows a higher

abundance of ATR compared to a subtelomeric site ($p < 0.05$). $N = 3$ independent experiments (mean $\pm$ s.d.).

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Table 1

Percent small deletions at different times after activation of I-SceI.

Clone	Days	% Uncut [§]	% Lg del	% Sm del ^{//}
6D1	4	4.2 ± 0.9	5.8 ± 1.0	4.0
	8	11.7 ± 0.4	19.3 ± 0.3	9.4
	12	23.5 ± 2.3	36.7 ± 3.4	14.9
7F1	4	3.6 ± 1.2	0	3.6
	8	9.3 ± 0.6	1.9 ± 0.8	9.1
	12	15.7 ± 1.0	5.2 ± 1.1	14.9

[§]Corrected for background by subtracting the percent of uncut DNA at time 0.

^{//}The frequency of small deletions is calculated by multiplying the frequency of uncut PCR fragment by 1 minus the frequency of large deletions to correct for the fact that cells with large deletions do not produce a PCR fragment (see Materials and Methods).