DISSERTATION

TELOMERIC DOUBLE STRAND BREAKS UNDERGO RESECTION - BUT NOT REPAIR - IN G1 HUMAN CELLS

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ABSTRACT

TELOMERIC DOUBLE STRAND BREAKS UNDERGO RESECTION - BUT NOT REPAIR - IN G1 HUMAN CELLS

Telomeres are specialized G-rich repetitive regions at the ends of eukaryotic chromosomes ($TTAGGG_n$ in mammalian cells). Telomeres function to prevent double strand break (DSB) repair activities at chromosome ends, in order to avoid fusion events which result in lethal dicentric chromosomes. Telomeric repeats make up an appreciable amount of genomic DNA (1-15kb per chromosome end). Therefore, an interesting question becomes, how is the inevitable DSB occurring within a telomere dealt with by the cell? It has been suggested that DSBs within telomeric DNA may not be repaired at all, as DSB DNA damage response (DDR) foci at telomeres do not resolve following large amounts of global DNA damage (e.g. ionizing radiation). Such studies also suggest that telomere repair may be inhibited specifically in G1, as the majority of surviving cells with unresolved telomere damage responses were senescent (a G1 phenotype). On the other hand, studies on the fragmentation of telomeric DNA following cutting with a telomere-targeted endonucleases indicate that repair of telomere-specific DSBs involves Homologous Recombination (HR) and Break-Induced Replication (BIR). However, a marker of telomeric DSB DDRs was only observed in cells with BrdU incorporation, in support of the view that repair of telomeric DSBs is an S/G2-related process, which does not occur in G1.

To follow up on these studies, we investigated telomeric DDRs and DSB repair in individual G1 cells using ionizing radiation (IR) and a targeted telomere-cutting endonuclease. IR exposure could potentially induce loss of telomere function, such that persistent DDRs may

not represent actual DSBs. To rule out this possibility, we evaluated whether persistent telomeric DDRs following IR occurred at telomeres that were critically short or lacking TRF2. We found that persistent telomeric DDRs occurred at telomeres of normal length and TRF2 status, in support of the conclusion that G1 telomeric DSBs are irreparable.

Additionally, using the telomere-targeted endonuclease we observed that telomeric DSBs in G1 cells elicited a relatively conventional DSB DDR – with one important exception – G1 telomeric DDRs failed to recruit 53BP1, an event implicated in the completion of DSB repair by most pathways, but especially, canonical non-homologous end joining (cNHEJ). Further, shRNA knockdown and kinase inhibition of the cNHEJ factor DNA-PKcs, provided evidence that cNHEJ is not responsible for repair of telomeric DSBs, and that DNA-PKcs does not influence recruitment of 53BP1 to telomeric DSBs in G1. Partial deprotection of telomeres, achieved by siRNA depletion of TRF2, also failed to alleviate inhibition of 53BP1 recruitment to G1 telomeric DSBs, suggesting that 53BP1 recruitment to telomeric DSBs may require full deprotection of telomeres. However, as 53BP1 recruitment occurs at de-protected telomeres, this idea would be difficult to test.

Most likely related to the lack of 53BP1 recruitment, an abundance of bidirectionally occurring single-stranded DNA was observed at G1 telomeric DSBs, a characteristic of long-range repair-associated resection. In support of long-range resection, RPA70 and phospho-RPA32 were observed at G1 telomeric DSBs. Additionally, conventional DSB repair-associated resection machinery, including MRE11 and EXO1, but not the telomere processing exonuclease Apollo, promoted resection at telomeric DSBs. We then investigated whether long-range resection-dependent repair was occurring at G1 telomeric DSBs via RAD51 or RAD52 foci, and

DNA synthesis (S/G2 related processes). Despite activity resembling long-range repair-associated resection at G1 telomeric DSBs, no evidence for repair by these pathways was found.

Taken together, the results presented here provide strong evidence in support of the view that telomeric DSBs in G1 are unrepairable. Therefore, the extensive resection observed at telomeric DSBs must be reflective of an alternative, non-repair related function, perhaps related to structural end-protection. We speculate that resection at G1 telomeric DSBs may serve to prevent 53BP1 recruitment, thereby circumventing a full DDR and activation of cNHEJ, a scenario that would create a serious threat to genome stability.

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CHAPTER 1

Introduction

Telomeres

Telomeres are specialized repetitive regions at the ends of linear chromosomes first appreciated nearly 80 years ago [1-3]. Telomeres are hexameric 5'-TTAGGG-3' repeats in mammalian cells that, can vary in length from roughly 1-15kb per chromosome arm in human cells [4-6]. Structurally, telomeres end in single stranded G-rich overhangs (roughly 100-200 bases/telomere), that fold over and invade the duplex DNA forming a structure known as the T-loop [7-9]. Additionally, telomeres are bound by a six member complex of proteins known as shelterin. In human cells three of the core shelterin proteins directly bind to the telomere repeat sequence. First discovered of the telomere binding proteins was Telomere Repeat Factor 1 (TRF1), followed shortly thereafter by TRF2 [10-12]. Both TRF1 and TRF2 form homodimers and utilize MYB-like domains to bind telomeric DNA [12-14]. A third protein, Protection of Telomeres 1 (POT1), binds to the G-rich single stranded overhang via two oligosaccharide/oligonucleotide binding (OB) domains [15, 16]. The remaining members of the shelterin complex do not directly bind DNA. TRF1-Interacting Nuclear Protein 2 (TIN2) is recruited to telomeres by interaction with TRF1/2 [17-20]. Additionally, TIN2 interacts with Tripeptidyl Peptidase 1 (TPP1), which functions in the recruitment of POT1 [21-23]. The last shelterin protein, Repressor/Activator Protein 1 (Rap1), interacts directly with TRF2 with no evidence that it binds the other shelterin members [24, 25].

Telomere Function

The End Protection Problem

Telomeres are required to overcome two problems that arose with the advent of linear chromosomes. The first, known as "the end protection problem," is that chromosome ends must structurally or biochemically distinguish themselves from double-strand breaks (DSBs) [1-3, 26, 27]. Telomeres achieve this through direct inhibition of DSB DNA damage response (DDR) and repair proteins by shelterin [26, 28, 29]. Additionally, shelterin binding to telomeres coordinates higher order telomere structures that have been theorized to inhibit repair. These include the folding of the T-loop as well as chromatin compaction at the telomere [8, 9, 30-33].The fact that telomeres have evolved intricate mechanisms to prevent DSB repair activities (the full details of which will be discussed later) is testimony to the importance of solving the end protection problem for cell health.

When telomeres lack functional versions of many of the shelterin proteins, they are said to be "deprotected" and a DDR is activated [26, 34-38]. Similar to the responses that occur at DSBs, DDRs at deprotected telomeres can be detected cytologically, forming "telomere dysfunction induced foci" (TIFs) [26, 34-38]. Additionally, when telomeres become critically short as a result of the end replication problem (described below) they fail to solve the end protection problem, again forming TIFs [34, 39]. In cells with competent checkpoints, TIFs stimulate cell cycle arrest and eventually senescence or apoptosis [34, 40-43]. Alternatively, in cells that fail to activate a full checkpoint response, dysfunctional telomeres can be fused together forming dicentric chromosomes [44-48]. Telomere-telomere fusions primarily occur in G1 and become unstable during the following mitosis [44-48]. Telomere fusions contribute to genomic instability by activating cycles of breakage-fusion-bridge (BFB) wherein fused chromosomes form bridges across the mitotic spindle. Bridges are eventually broken apart at a new site, thereby

contributing a chromosome with non-telomeric sequence at its termini to each of the daughter cells. These ends are themselves prone to fusion leading to new cycles of BFB [48-49].

The End Replication Problem

The second problem of chromosome ends is "the end-replication problem." The end replication problem is an inability of the normal replication machinery to replicate the most terminal portion of the chromosome. The end-replication problem results from the fact that lagging-strand DNA synthesis requires an RNA primer, the most terminal of which can be variously placed, leaving no means of replicating to the very end of the telomere [50-51]. As a result, telomeres shorten with each round of replication by about 50-100bp [6, 46, 51-54]. Eventually, telomeres become critically short-defined as the point at which they are no longer long enough to "solve" the end protection problem (described above). Telomere shortening therefore contributes to the finite replicative lifespan of cells, a phenomenon known as the "Hayflick limit" [55].

The end replication problem has led to the concept of telomere shortening as a "mitotic clock" that underlies aging and its associated degenerative pathologies [56-58]. Indeed, the rate of telomere shortening is associated with replicative lifespan in cell culture and mortality in humans [59-60], and decreased telomere length has been associated with increased age, disease, stress and a myriad other negative health outcomes [58, 61-63].

How Telomeres Solve the End-Replication Problem

For organismal and species viability, telomere length must be maintained in stem and germ-line cells. The enzyme responsible for telomere length extensions is known as Telomerase, which was first discovered by Carol Greider and Elizabeth Blackburn in 1985 in *Tetrahymena thermophile* [64]. Telomerase is a reverse transcriptase composed of a catalytic core (TERT) and an RNA template component (TERC) [65]. Telomerase is expressed in

embryonic stem cells, adult stem cells, and germ-line cells but not in somatic cells [58, 61, 62].The reason somatic cells do not express telomerase likely relates to reducing potential for cancer development. Evidence for this idea comes from the fact that the majority of cancer cells must reactivate telomerase to achieve replicative immortality [66, 67].

Telomere Maintenance in Cancer Cells

Cancer development depends on sustained, uncontrolled proliferation. Therefore, telomere shortening and replicative senescence in somatic cells plays a tumor suppressive function [68]. In order to bypass telomere shortening-induced senescence, developing tumor cells must disrupt cell cycle regulation, most often by acquiring mutations in tumor suppressors such as TP53 [69]. However, when cells bypass senescence their telomeres continue to shorten eventually leading to a state known as “crisis.” [69]. Crisis is characterized by telomere fusions, BFB, and apoptosis [69]. To escape crisis and achieve proliferative immortality, cancer cells must activate a telomere lengthening mechanism, typically acquiring mutations that activate telomerase [66, 67, 69, 70].

A second strategy for telomere maintenance, known as Alternative Lengthening of Telomeres (ALT), is activated in a minority of cancers (~10%), and is more commonly associated with particular tumor types [71]. Telomere length maintenance in ALT cells depends on activation of recombinational repair at telomere ends (homologous recombination, break induced replication, or both- described under section heading *What we can learn about telomere DSB repair from ALT cells*). Repair activation at ALT telomeres suggests a structural or biochemical difference from normal telomeres that inhibit repair activity. In fact, both the chromatin state and shelterin binding have been found to be altered in ALT cells. While normal telomeres are associated with heterochromatin marks such as H3K9me3, H4K20me3 and low histone acetylation, ALT telomeres are characterized by more of a permissive, euchromatic chromatin landscape [72]. Additionally, ALT telomeres are enriched in alternative telomere

repeat sequences that contribute to a decreased presence of TRF1 and TRF2 [73]. ALT telomeres may therefore have decreased protection from repair processes as a result of an altered chromatin state and decreased shelterin abundance.

DNA Damage and Double-Strand Breaks

Introduction to DNA Damage

Damage to DNA occurs as a result of normal, daily cellular processes. These include reactions involving reactive oxygen and nitrogen species (ROS/NOS) generated by cellular metabolism, as well as reactions involving DNA itself (e.g. DNA synthesis). Additionally, damage occurs as a result of exogenous agents such as ionizing radiation (IR), ultraviolet radiation (UV), exogenous ROS and reactions with genotoxic chemicals [74-76].

The main types of DNA damage include oxidative and chemical damage to DNA bases, single (ss), and double-stand breaks (DSBs). Most repair of DNA damage relies on the intact opposing strand which can be used as a template. Base excision repair (BER) and nucleotide excision repair (NER) involve the removal of a single base or a stretch of single stranded DNA respectively, followed by template-mediated fill-in and lastly, ligation of the sugar-phosphate backbone. These pathways can repair the majority of base damage and ssDNA breaks quite reliably [74-76]. However, this type of repair strategy cannot be applied to DSBs, as both strands are altered, making DSBs particularly dangerous to the cell. The misrepair of DSBs can result in a range of mutations including point mutations, deletions, insertions, duplications, loss of heterozygosity, inversions, and translocations [74-76], which in turn can contribute to genomic instability and, malignant transformation, or cell death. On the other hand unrepaired DSBs can cause cell death or senescence as a result of sustained DDR signaling. Therefore, the accurate repair of DSBs is of the upmost importance for both the cell and the organism [74,

75]. As such, DSBs are repaired by a dedicated suite of tightly regulated pathways described under the section heading *DSB repair* pathways in mammalian cells.

DSB Detection and Cellular Response

In order for a cell to mount a damage response to a DSB, its presence must first be detected. Some contend that the initiation of DNA damage response (DDR) signaling at DSBs is prompted by relaxation of supercoils in the adjacent chromatin caused by the break itself [77-78]. However, this model has not been proven conclusively, and most reviews describe the recognition of a break as beginning when the broken ends are bound by protein complexes such as MRE11-RAD50-NBS1 (MRN) and Ku70/80 [79-81]. Following recognition of the DSB, members of the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family, Ataxia-telangiectasia mutated (ATM), Ataxia-telangiectasia and Rad3-related protein (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) become activated [78, 82]. While all three PIKKs are activated by randomly distributed DSBs (such as those induced by IR) differences in their kinase activation exist. ATM is thought to be strongly activated by any DSB, which initiates autophosphorylation of ATM at S1981 [78, 82, 83]. ATR activation seems to be triggered specifically by the presence of RPA coated single stranded DNA (such as that generated by resection) [78, 82-84]. Lastly, DNA-PKcs kinase activity depends on association with the Ku70/80 complex [82, 83, 85, 86]. The PIKK(s) then phosphorylate an array of targets that serve to activate both DNA repair and cell cycle checkpoints [78, 82, 87]

Interestingly, although ATM and ATR are activated by different DNA substrates there is significant evidence that they contribute to each other's activation. For instance, ATM promotes activation of end-resection, and production of single-stranded DNA, which is the ATR substrate [88, 89]. Further, ATM and ATR can redundantly phosphorylate some substrates and ATR has been shown to phosphorylate ATM [90].

The DDR to DSBs involves a few generic events notable for their usefulness in the study of DSBs and their repair. These include recruitment of MDC1 and 53BP1 to the break site, as well as ATM mediated phosphorylation of the histone variant H2AX on serine 139, forming what is known as (γ)-H2AX. γ -H2AX, MDC1, and 53BP1 are useful in that they occur in great abundance in the chromatin surrounding the break site almost immediately upon induction of the break, and they occupy a very large radius of the break (on the order of megabases) [91-95]. These properties make these three factors easily observable by immunofluorescence as discrete nuclear foci following DSB induction. As a result γ-H2AX and 53BP1 in particular are commonly regarded as markers of DSBs. Additionally, γ-H2AX is used to monitor repair kinetics as it becomes dephosphorylated with the completion of repair [91-95]. These same factors also constitute TIFs, co-localizing with deprotected telomeres [35]. It should be noted that while these DDR foci are commonly assumed to occur in response to all DSBs, this interpretation is somewhat equivocal particularly when it comes to 53BP1, which is notably absent during certain types of repair [96]. Additionally, the recruitment of 53BP1 is downstream of γ-H2AX and MDC1 and is not deterministically linked to them [97].

Checkpoint Activation and Consequences

Activation of cell cycle checkpoints by DDRs slow or prevent the progression through the cell cycle until damaged DNA is repaired. Phosphorylation of downstream effectors by PIKKs can trigger a checkpoint response in any phase of the cell cycle. However, depending on cell cycle phase the relative contribution of an individual kinase and the type of checkpoint response varies. ATM and ATR are the primary checkpoint activators with ATM doing most of the work in G1 and ATR being most important in S/G2 [90]. ATM phosphorylates the effector kinase CHK2 which coordinates the G1/S checkpoint, whereas ATR phosphorylates CHK1 which coordinates intra-S and G2/M checkpoints [78, 82, 90, 98]. While DNA-PKcs involvement in checkpoint

activation is less well established, it has been shown to activate CHK2 and may have redundancies with ATM [99]. CHK1 and CHK2 function to slow the progression of the cell cycle by directly and indirectly interfering with cyclin-dependent kinase activity [75, 76]. However, the persistent activation of a DDR and checkpoint signaling that presumably occurs as a result of unrepaired DSBs eventually leads to the activation of cell death via apoptosis or senescence [75].

Double Strand Breaks, Aging, and Cancer

DSBs and the outcomes of DSB repair impact human health and disease. This is exemplified by the many congenital disorders and cancer predispositions caused by mutations in DDR and repair genes. These range from the very severe, e.g. Nijmegen breakage syndrome and Ataxia telangiectasia, to the less so, e.g. enhanced breast and ovarian cancer susceptibilities conferred by BRCA1/2 mutations [75]. Even in a genetically normal person misrepair of DSBs contribute to the mutations that cause sporatic cancers in many of us [75]. Additionally, the accumulation of DNA damage, likely including DSBs, contribute to neurodegenerative diseases such as Parkinson's and Alzheimer's [75]. Finally, cell loss through apoptosis or functional loss through senescence are both potently triggered by DSBs and contribute to stem cell exhaustion which likely plays a role in diverse forms of aging associated degeneration and disease [75].

DSB Repair Pathways of Mammalian Cells

Canonical Non-Homologous End-Joining

The most commonly used DSB repair pathway in mammalian cells is canonical non-homologous end joining (cNHEJ) [100-102]. Conceptually, cNHEJ can be thought of as a blunt DNA ligation with no requirement for sequence homologies. cNHEJ is the dominant repair

pathway in the G1 phase when no sister chromatid is present, but also occurs in the G2 phase of the cell cycle. cNHEJ begins with binding of the broken DNA ends by the Ku70-Ku80 heterodimer, followed by recruitment of the catalytic subunit of DNA protein kinase (DNA-PK), DNA-PKcs [100-102]. DNA-PK then acts in concert with XRCC4, Ligase IV, and XLF alignment and ligation factors to join the DNA ends together [100-102]. cNHEJ can involve end modification by additional nucleases and polymerases (such as in cases when there is a short single- stranded overhanging sequence at a double-stranded end), resulting in small insertions and deletions at some C-NHEJ repair junctions. Therefore, despite having a major role in maintaining overall genome integrity, C-NHEJ is considered to be an “error-prone” pathway [100-103].

Homologous Recombination

Homologous recombination (HR) is the main process by which a homologous template can be used to faithfully restore the sequence at the site of a DSB. HR most often uses the sister chromatid as a template, allowing repair to be exact and considered “error free”. However, HR can also be mutagenic when a homologous chromosome, or a non-allelic site, are used as a template instead of a sister chromatid [104]. HR repair products sometimes involve “crossover,” wherein the portions of the chromatids distal to the break site are exchanged. If crossover occurs with anything other than the sister chromatid, this results in a translocation. Alternatively, non-crossover products occur, which amounts to a simple gene conversion, wherein only a short sequence from the template DNA is copied to the chromatid with the DSB [105-106]. Fortunately, gene conversion, and not crossover, dominates during mitotic HR [107-108]. Additionally, non-allelic HR rarely occurs, likely to be due in part to the tight cell cycle regulation of HR that favors HR only when a sister chromatid can be used as a template. Overall, HR is the second most common repair pathway in mammalian cells and is crucial for maintaining genomic integrity.

Mechanistically, HR begins when broken DNA ends are resected producing 3' ssDNA overhangs. Resection is initiated by the MRN complex (MRE11-RAD50-NBS1) and C-terminal binding protein interacting protein (CtIP) and is promoted by breast cancer susceptibility 1 (BRCA1) [105, 109] Once initial resection has begun, Exonuclease 1 (EXO1) and DNA2 helicase/nuclease extend the ssDNA even further. Next, Replication Protein A (RPA) binds the ssDNA. With the help of breast cancer susceptibility 2 (BRCA2) and other recombination mediators, RPA is eventually displaced by RAD51 recombinase forming a nucleoprotein filament [105, 109]. RAD51 coated DNA then invades a homologous template (most often a sister chromatid) and anneals to it, forming a holiday junction (HJ). Two main sub-pathways are responsible for the completion of HR. In DSB Repair (DSBR) the second broken end is captured forming a double HJ. DNA synthesis then occurs between the invaded strands. Finally, the double HJ is resolved forming either crossover or non-crossover products. In Synthesis-Dependent Stand-Annealing (SDSA), DNA synthesis extends the invaded strand. Next, the extended strand is removed and the newly synthesized portion anneals with the other broken strand at sites of homology. Repair is completed by fill-in of the remaining single stranded gap resulting exclusively in non-crossover products [105, 109].

Alternative End-Joining

In the mid 1990's to early 2000's experiments involving cells and mice lacking core cNHEJ factors (e.g. Ku70/Ku80), revealed that end-joining could still occur. These experiments were the first evidence for what is now known as alternative end-joining (Alt-NHEJ) [110-114]. Over the last two decades Alt-NHEJ has been further characterized to be less faithful then cNHEJ, often leading to chromosomal translocations [114-116]. However, the extent to which Alt-NHEJ promotes translocation is still being investigated with a recent report suggesting that cNHEJ and not Alt-NHEJ is the dominant mechanism of translocation in genetically normal human cells [117]. Additionally, Alt-NHEJ often occurs between regions of microhomology and

is sometimes referred to as microhomology-mediated end joining. Interestingly, while Alt-NHEJ has been considered a backup pathway, we now know that it occurs even in the presence of functional C-NHEJ during both V(D)J recombination and during repair of I-SCEI induced DSBs [118-120].

Alt-NHEJ begins with short-tract bidirectional 5'-3' resection, performed by the same proteins that initiate resection during HR [121-125]. Following resection, strand annealing occurs with the other DNA end, usually at a site of microhomology. Finally, 3' flap removal and ligation is performed. Only a few of the proteins and mechanistic details involved in Alt-NHEJ have been discovered to date. Evidence suggests that Poly(ADP-ribose) polymerase 1 (PARP1) is necessary for synapsis and annealing, while Pol θ, Ligase 3 and Ligase 1 have been implicated in completion of repair and ligation [126-131].

Recently, another resection dependent end-joining repair pathway has been described to occur at DSBs in G1 [132]. This pathway is distinct from Alt-NHEJ in that it relies on C-NHEJ repair proteins following resection, thus raising doubt that Alt-NHEJ plays a major role in DSB repair [132].

Single-Strand Annealing

When direct repeats flank the break site, a third homology dependent repair pathway known as Single-Strand Annealing (SSA) can be used. SSA is similar to Alt-NHEJ in that it involves DNA end resection followed by annealing at homologies, DNA ligation, and 3' flap removal [133]. However, the length of homologies used differs. Alt-NHEJ can be performed almost anywhere in the genome as it depends on only a few bases of homology, whereas SSA is much more limited requiring at least about 30 bases to be efficient in yeast [134, 135].

SSA shares resection machinery with HR, involving both initial resection by MRN/CtIP and long-range extension involving EXO1 and DNA2. Additionally, while SSA is mechanistically similar to Alt-NHEJ, it requires distinct proteins for strand annealing [136]. While Alt-NHEJ

depends on PARP1 and Pol θ for this activity, SSA requires the activities of Rad52 [96, 133, 137, 138]. Interestingly, the ability of Rad52 to promote strand annealing was initially assumed to play a part in HR, but chicken and mouse cells lacking RAD52 showed no HR repair defect. Therefore when SSA and BIR (discussed in the following section) were discovered to involve RAD52, this explained its evolutionary conservation and potent in-vitro activity [96, 133, 137-140]. Confounding this interpretation, a recent report using RAD52 deficient human cells suggests that SSA can occur without RAD52 and further, that HR is impaired without it [141].

Like Alt-NHEJ, SSA is an inherently mutagenic pathway as it results in deletion of the sequence intervening the homologies. Additionally, SSA has been implicated in translocation formations, however only between repetitive Alu elements, and its dependence on long homologies is thought to limit its potential for translocations [133-142].

Break-Induced Replication

Break-induced replication (BIR) occurs when a single-stranded DNA end (presumably a 3' end created by 5'-3' resection), invades a homologous template and primes DNA synthesis from the site of homology to the end of the chromosome [143]. Characterization of BIR has mostly been done in yeast where it has a limited role in DSB repair at endonuclease generated DSBs in wild type (wt) cells (roughly 1-2% of repair events in unselective conditions) [144-145]. However, BIR is hypothesized to function in replication restart of broken or collapsed replication forks, for which initial evidence in animals came from experiments in Xenopus extracts, as well as more recently in human cells [146-148]. Additionally, BIR seems to have a specific function at telomeres. Telomerase null survivors in budding yeast can activate BIR at telomeres to maintain their length [149-151].This scenario is similar to the ALT phenotype, which was also very recently shown to depend on BIR [152, 153].

Interestingly, BIR relies on RAD51 in 95% of cases using a chromosome fragmentation assay in yeast, wherein transformation of a partial chromosome can be stabilized by BIR [154].

However, BIR in Rad51 deficient cells has also been characterized [155]. In mammalian cells BIR seems to rely on the annealing and synapsis functions of RAD52, however whether RAD51 can mediate BIR without RAD52 has yet to be determined [139, 152, 156]. Most evidence suggests that DNA synthesis during BIR is dissimilar to normal DNA replication in S-phase, as it occurs within an HR-like D-loop structure that moves towards the telomere, a process known as "bubble migration" [157, 158]. Additionally, BIR depends on polδ, and particularly the nonessential polδ subunit pol32 as well as PIF1 helicase in yeast [150, 157-159]. Similarly, the mammalian homolog of pol32, POLD3 and another subunit of polδ, POLD4, are crucial for mammalian BIR [147, 152, 153].

DSB Repair Pathway Choice

Fast and Slow Components of DSB Repair

DSB repair in both G1 and S/G2 occurs with biphasic kinetics, consisting of a fast component (˜30min), in which a majority of the DSBs are repaired, and a slow component (~ 4-24hr) during which 10-20% of breaks are repaired [160-162]. Additionally, a small fraction of DSBs, which appear to be enriched at telomeres and likely promote the onset of senescence may not be repaired at all [163, 164].

The fast component of repair in both G1 and G2 is carried out by cNHEJ. Repair during the slow component in G1 is performed by an end joining pathway that involves cNHEJ factors as well as ATM and Artemis endonuclease [161]. Additionally, recent evidence suggests that the slow component NHEJ in G1 involves limited resection [132]. Repair during the slow component in G2, which also involves the activity of ATM and Artemis is performed by HR [162]. Finally, ATM-dependent slow component repair in G1 and G2 has been found to be the subset occurring in heterochromatic DNA [165].

The Role of 5'-3' Resection in Repair Pathway Choice

Each DSB repair pathway is characterized by the extent of single-stranded DNA exposed during resection. cNHEJ is characterized by a lack of resection while Alt-NHEJ involves short tracks of resection (˜20bp), produced by the "end-clipping," activities of MRE11 and CtIP [119, 166]. In contrast, HR, SSA, and likely BIR require long tracks of resection (hundreds of base pairs) produced by DNA2 and EXO1, with SSA requiring the largest amount of ssDNA [96, 166, 167]. Therefore, regulation of resection is thought to play a crucial role in determining repair pathway choice.

The Role of the Cell Cycle Phase in Repair Pathway Choice

The cell cycle phase greatly influences the balance between HR and cNHEJ. HR can only be accurate when an identical sister chromatid is available to be used as a template. Therefore, cells preferentially engage in HR post replication while cNHEJ is used throughout the cell cycle [168, 169]. Regulation of HR and cNHEJ is tied to the molecular regulation of the cell cycle itself. Specifically, Cyclin Dependent Kinases (CDKs) have been shown to selectively activate HR in S and G2 by phosphorylation of DNA end resection factors. Resection promoting phosphorylation events have been described for CtIP, EXO1, and NBS1 [170-172]. A more detailed regulatory circuit that prevents HR in G1 has also been worked out wherein ubiquitination of Palb2 in G1 blocks the interaction between BRCA1 with Palb2-BRCA2 [173]. Finally, post replicative chromatin may itself inhibit end joining in favor of HR. Evidence for this model comes from the discovery of a post-replicative chromatin mark (H4K20me0) that prevents the binding of 53BP1, a factor known to promote end joining and inhibit resection [174, 175].

53BP1 and BRCA1 in Repair Pathway Choice

53BP1 and BRCA1 promote cNHEJ and HR respectively. Mechanistically, 53BP1 favors cNHEJ by inhibiting resection [176, 177]. Resection inhibition is achieved via phosphorylation

dependent interaction of 53BP1 with RIF1 [178-180]. On the other hand, BRCA1 promotes resection at DSBs [181]. BRCA1 and 53BP1 may be in direct opposition to each other. This hypothesis is supported by the observation that defects in BRCA1 deficient cells, including impaired HR, are rescued by loss of 53BP1 [176]. Investigations have begun to elucidate the details of 53BP1-BRCA1 antagonism. BRCA1 has been found to be necessary for 53BP1 relocation to the periphery of DSB repair foci during HR [182, 183]. Additionally, BRCA1 and its interacting partner CtIP inhibit RIF1 foci formation in S/G2, which was recently shown to be driven by BRCA1 dependent dephosphorylation of 53BP1 [184, 185]. Reciprocally, 53BP1 prevents BRCA1 foci formation in G1 in a RIF1 dependent manner [184].

Usage of SSA, Alt-NHEJ, BIR

As C-NHEJ and HR are the two most frequently used DSB repair pathways much of the research on repair pathway choice has focused on the decision between them. This leaves open the question as to when or in what contexts the remaining characterized pathways are initiated. While this question is mostly unanswered, recent work has begun to shed some light on the physiological relevance of, Alt-NHEJ, SSA and BIR in human cells.

Both Alt-NHEJ and SSA may be used as backup pathways. Evidence, for this idea comes from observations in cNHEJ deficient cells, which undergo enhanced Alt-NHEJ, and HR deficient cells, which undergo enhanced SSA [110-112, 137]. Recent evidence has also suggested that SSA becomes dominant at highly resected DSBs, which occur when the number of breaks overwhelms the ability of 53BP1 to regulate resection [96]. Additionally, it has been hypothesized that DSBs occurring during early S-phase, a time when resection machinery is available but sister chromatids are not fully available for HR, may be particularly prone to repair by Alt-NHEJ or SSA [133]. Finally, BIR has also been proposed to be important for replication restart and for telomere elongation and/or repair [147, 148, 152, 153].

The State of DSB Research

The initial cellular response to a DSB is often viewed as a universally applicable signaling cascade. Likewise, DSB repair mechanisms are often portrayed as neatly fitting into one of a few well characterized pathways. In most cases, models are based on either the mean outcome of a large number of randomly distributed DSBs (such as those induced by ionizing radiation), or by enzymatically induced DSBs resulting from site-specific cleavage of genomically integrated reporter cassettes (such as those based on the homing nuclease I-SCEI). As a result, models do not necessarily reflect the full scope of DSB repair within diverse genomic loci.

We know from kinetic studies of repair that some DSBs are repaired quickly, while others are repaired more slowly and still others seem not to be repaired at all [160-164]. Additionally, certain genomic loci seem to be prone to misrepair, but the complete set of factors responsible for this enhanced mutagenicity is not yet clear [186, 187].We also know that different repair pathways have different potential mutational outcomes. However, studies on repair pathway choice have mostly been limited to the two most common pathways: cNHEJ and HR, or the choice to initiate 5'-3' resection. Only recently have differences in repair begun to be characterized at varying endogenous genomic loci, taking into account the role that sequence and chromatin context play.

The Genomic Context of Repair

Investigation into the influence of genomic context on DSB repair typically involves cutting with endonucleases, either within an integrated reporter cassette, or using site-specific endonucleases at endogenous loci (e.g. CRISPR-CAS9). Over the last few years this type of approach has begun to elucidate how repair varies within heterochromatin, in rDNA, as well as

at subtelomeres and telomeres. One pattern that has emerged is the migration of DSBs to nuclear domains conducive to repair by HR, whether it be the periphery of the nucleoli in the case of rDNA DSBs, or the periphery of heterochromatin when DSBs occur in pericentric heterochromatin or centromeres [188, 189]. Additionally, the cell cycle restriction of HR to S/G2 seems not to apply at certain sites including nucleoli and centromeres where HR repair activity can be detected in G1 [188, 189].

Extensive work on the DSB repair outcomes at human subtelomeres suggests that subtelomeres may be prone to misrepair mostly characterized by large deletions [190]. Additionally, subtelomeric DSBs are repaired by cNHEJ less frequently than observed at genomic loci [191, 192]. Finally, subtelomeric DSBs are prone to extensive resection which is dependent on MRE11 activity [192, 193].

The Inhibition of Repair at Normal Telomeres

The function of telomeres is largely to inhibit DSB repair activity at natural chromosomal DNA ends. How exactly telomeres achieve this is a question that has been the subject of intensive study. The majority of direct evidence points to the role of shelterin components in DSB inhibition. However, a handful of other factors seem to play a role as do certain structural properties of telomeres.

Inhibition of Repair Activities at Telomeres by Shelterin

TRF2 is the shelterin protein most strongly implicated in inhibition of DSB repair at telomeres. The presence of TRF2 at telomeres inhibits ATM signaling; dysfunctional telomeres lacking TRF2 trigger ATM mediated TIFs, followed by apoptosis or senescence [38, 41, 194]. TRF2 inhibits cNHEJ activities at telomeres as well, as a means of preventing telomere-telomere fusions in G1 [44, 45]. TRF2 is also at least partially responsible for inhibition of HR at

telomeres [195, 196]. However, most evidence supporting repression of HR at telomeres centers on the role that POT1 plays, which is mediated by inhibition of ATR signaling [37, 38, 195, 197]. TIN2 may also play a role in DSB repair inhibition at telomeres and a lack of functional TIN2 at telomeres causes the formation of TIFs [36, 198]. However, evidence suggests that inhibition of repair activities by TIN2 is a result of the fact that it stabilizes TRF2 and POT1 [19, 36, 198, 199]. Similarly TPP1 does not directly inhibit repair activities but is required for the repair inhibition functions of POT1 [21, 23]. Study of the protection of telomeres by Rap1 has produced many conflicting reports with evidence both for and against RAP1 in the prevention of both cNHEJ and HR at telomeres [25, 200-203]. However, it seems likely that RAP1 plays a secondary role in telomere protection as a result of its ability to stabilize TRF2 [204, 205]. Interestingly, while TRF1 is crucial for telomere integrity as it facilitates telomere replication, evidence is lacking for a role in telomere protection from repair [26, 206].

While it is clear that shelterin components are indispensable for the protection of telomeres, exact details of how repair inhibition is achieved by shelterin is less well understood. Most available evidence concerns what appears to be highly redundant mechanisms involving TRF2 based telomere protection. A commonly cited and highly speculative mechanism, is founded on the observation that TRF2 promotes formation of T-loop structures in telomeric DNA [8, 9, 31]. T-loops are proposed to provide an architectural solution to sequestration of chromosome ends, thereby preventing repair factors from detecting/signaling a break [8, 9, 31]. Others have suggested that repair inhibition by shelterin may come down to compaction of telomeric DNA [30, 32, 33]. However, two recent reports refute this claim and provide evidence that decompaction of telomeric DNA is not associated with TIF formation [207, 208]. TRF2 may also prevent NHEJ activities via inhibition of 53BP1 recruitment to telomeres [97]. 53BP1 has been shown to be essential in the formation of telomere-telomere fusions by cNHEJ, as it promotes the long range movement necessary for one chromosome end to find another [209]. A portion of the hinge domain of TRF2 termed the iDDR domain has been shown to prevent

53BP1 recruitment by blocking the E3 ubiquitin ligase RNF168 recruitment, which 53BP1 depends on [97]. Interestingly, chimeric TRF1 proteins that include the TRF2 iDDR domain have been found to partially complement a TRF2 knockout, preventing 53BP1 recruitment and telomere fusions, without preventing initial activation of the TIF response including γ-H2AX and MDC1 recruitment to telomeres [97].

The mechanistic details of HR inhibition at telomeres is less well understood. However, it has been suggested that POT1 occupancy of telomeric ssDNA excludes RPA, thus preventing activation of ATR signaling and downstream resection dependent repair pathways, including HR [210].

Inhibition of Repair Activities at Telomeres by Repair Proteins

Somewhat perplexingly, some DNA repair proteins, including the cNHEJ factors Ku70/80 and DNA-PKcs are required for protection of normal telomeres from repair activities [211, 212]. Ku70/80 is present at telomeres and has been found to contribute to the inhibition of telomeric HR [195, 213, 214]. This makes sense given that Ku70/80 inhibits HR at DSBs in favor of cNHEJ [215]. However, that Ku70/80 can persist at telomeres without engaging in cNHEJ is surprising. Recent evidence has suggested this may be due to TRF2 interaction/interferences with the α-helix 5 domain of Ku70, which is necessary for heterodimerization of Ku70 with Ku80 and end joining activity [214]. DNA-PKcs functions to partially inhibit telomere fusion [211, 216, 217]. The details of DNA-PKcs participation in telomeric end protection have not been fully determined, however its role has been shown to require DNA-PKcs catalytic activity [218]. Additionally, recent work suggests that DNA-PKcs mediated protection of telomeres involves the stabilization of TRF2 via the DNA-PKcs interacting protein, KIP [219].

Inhibition of Repair Activities by Telomeric Repeat Containing RNA (TERRA)

Even more surprising was the discovery that telomeres are transcribed into long-non coding RNAs termed telomeric repeat containing RNAs (TERRA) that form RNA-DNA hybrids at telomeres [220]. Interestingly, TERRA-DNA telomere hybrids have been shown to stall replication fork progression, creating telomeric DSBs during S-phase that are repaired by HR. In fact, this has been proposed as a potential mechanism of HR mediated telomere elongation, particularly in ALT cells where TERRA expression is greatly increased [220]. On the other hand, TERRA may play a role in telomere protection, as disruption of TERRA expression in human and mouse cells results in a TIF response [221, 222]. One possible explanation as to how TERRA protects telomeres comes from the fact that TERRA appears to be involved in post replication stabilization of the G-rich single-stranded overhangs at telomere ends, favoring POT1 binding over RPA [223]. However, whether TERRA plays a bona-fide role in telomere protection is currently unclear, as TIF responses following TERRA depletion may also be explained by the ability of TERRA to promote telomere extension/replication by HR at critically shortened telomeres [220].

DSB Repair within Telomeric DNA

DSB Repair Occurring at Telomeric DSBs following Global Damage

As normal telomeres inherently avoid DSB repair activities in order to preserve genome stability, a pertinent question becomes – what happens to DSBs that occur within telomeric DNA? Initial work tracking DNA damage responses following the induction of DSBs produced by IR or hydrogen peroxide found that while the majority of DSBs are repaired within 24 hours of the genotoxic insult, the remaining minority were heavily enriched at telomeres [163, 164]. Additionally, surviving cells experiencing persistent telomere damage responses became senescent. As senescence is a G1 phenomenon, together these results suggest that

irreparable telomeric DSBs were induced in G1 [163, 164]. Finally, it has been observed that DNA damage responses in aging mammals disproportionally occur at telomeres, suggesting that unrepaired telomere DSBs may contribute to the aging process itself [163].

These observations suggest that telomeric DSBs may be especially difficult to repair, or they may undergo unusually slow repair kinetics. However, interpretation remains challenging for several reasons. First, DSBs induced by high doses of IR or chemicals tend to be complex, often occurring near or overlapping with other types of DNA damage (clustered damage), including base damage and single strand damage [224, 225]. Additionally, the cellular phenotypes result from acute induction of copious amounts of global DNA damage may confound interpretation of sustained damage responses. In particular, senescence strongly induces endogenous ROS production and DNA breakage [226]. Another cause for concern is that supra-physiological levels of DNA damage may cause telomere shortening and/or loss of TRF2, which could lead to DSB DDR activation at unbroken telomeres. One attractive mechanism by which TRF2 could be lost following global DNA damage, involves its decreased binding affinity for telomeric DNA harboring oxidative base damage. [227].

Repair of Site-Specific Telomere DSBs

In order to dissect the damage response and repair at telomere DSBs a number of targeted approaches have been developed. These include TRAS1-EN-TRF1 (ENT), a fusion protein between the endonuclease domain of a telomere-specific non-long terminal repeat retrotransposon of the silkworm (*Bombyx mori*) and human TRF1. ENT is efficiently targeted to telomeres in human cells by TRF1, then generates nicks between the T and A of $(TTAGGG)_n$ and the C and T of $(CCTAAA)_n$, creating blunt-ended telomeric DSBs [228, 229]. ENT has been well characterized to efficiently cut telomeres both in-vitro and when transfected into human cells [228, 229]. ENT is dually specific to telomeres, as it relies not only on TRF1 targeting, but

sequence-specific cutting as well; no publications to date have described telomere repair following ENT expression.

Telomeric DSBs have also been produced using a CRISPR strategy (clustered regularly interspaced short palindromic repeats). In this approach, a guide RNA homologous to the human telomere repeat sequence targets Cas9 endonuclease to telomeres [230, 231]. It has been shown that Cas9 induced telomeric DSBs stimulate telomere HR in cycling cells. However, direct evidence for telomeric damage responses (53BP1 foci) were only detected in roughly 25% of the cells, suggesting that telomere repair may be cell cycle restricted [231].

Similar results have been described in mouse cells. Telomere specific DSBs were induced in mouse embryonic fibroblasts (MEFs) using the endonuclease domain of FokI, a type II restriction enzyme, fused to TRF1 (FokI-TRF1). The endonuclease domain of FokI cuts within any DNA sequence to produce a 4 base overhang at the DSB, FokI-TRF1 creates telomere specific DSBs by virtue of TRF1 mediated telomere recruitment [232. 233]. Telomeric DSBs produced by FOKI-TRF1 were found to be repaired by a combination of HR and Alt-NHEJ, but not cNHEJ in cycling cell populations. However, direct observation of damage responses occurring at individual telomeric DSBs (53BP1 foci) were again limited to a fraction of the cells – in this case identified as those in S-phase [234]. As 53BP1 is necessary for HR, Alt-NHEJ and C-NHEJ, these results suggest that repair outside of S-phase may be inhibited at telomeres, or that another DSB repair pathway such as SSA may be occurring [96, 132, 178, 180].

What We Can Learn about Telomere DSB Repair from ALT cells

Telomeres in ALT cells are typified by telomerase-independent engagement of recombinational repair as a means of maintaining telomere length [235,236]. Interestingly, the ALT phenotype has been associated with high frequencies of telomere sister chromatid exchange (T-SCE) [236-238], an indicator of resolution of recombination intermediates with crossovers; T-SCEs are detected utilizing the strand-specific methodology of Chromosome

Orientation Fluorescence In-situ Hybridization (CO-FISH) [237,238]. This suggests that telomere lengthening may be mediated by HR [235-239]. A mechanism involving telomere lengthening by HR has been proposed to depend on the unequal exchange between telomeres and non-random sister chromatid segregation [236].However, other recent reports suggest that ALT depends on BIR [152, 153]. Whether these two pathways cooperate to elongate telomeres in ALT cells or whether some ALT tumors favor one repair pathway over the other is yet to be determined.

It has recently been suggested that telomeric DSBs may be one of the factors that drive ALT. In support of such a proposition, site-specific cutting within ALT telomeres stimulates telomere clustering and BIR, which can extend individual telomeres [152, 240]. Interestingly, neither RAD51 recruitment, telomere clustering, nor signatures of BIR were found in non-ALT cells, which is in contrast to reports in normal cells described in the previous section [152, 231, 234, 240]. Thus, whether ALT cells are unique in their telomere repair capacity, or simply have a greater frequency of telomeric DSBs such that BIR mediated telomere elongation is a viable strategy, remains to be seen.

Influence of Chromatin Environment on Telomere DSB Repair

Telomeres exhibit high levels of H3K9m3 and H4K20me3 methylation, and low levels of histone acetylation, typical of heterochromatin [241]. It has recently been shown that telomere binding proteins serve to compact telomeric chromatin as well, and that sufficient telomere chromatin compaction is necessary to prevent DNA damage responses at normal telomeres [33]. This idea is supported by observations of ALT telomeres, in that ALT telomeres are less compacted, have fewer heterochromatic marks and higher histone acetylation levels than normal telomeres, and they exhibit activation of damage responses at telomeres [72, 236]. Further, treatment of ALT cells with Trichostatin A (TSA), a histone deacetylase inhibitor that

causes chromatin relaxation, leads to an enhancement of the ALT phenotype, which is likely contingent on an enhanced telomere damage response [242].

Cell Cycle Phase and Telomeric DSB Repair

There is significant evidence for some form of recombinational repair (HR or BIR) occurring at telomeric DSBs in both ALT and non-ALT cells, and limited evidence that Alt-NHEJ may repair some telomeric DSBs in normal cells [152, 231, 234, 240]. However, HR is thought to be limited to S and G2. Additionally, 53BP1 foci in non-ALT cells were found to be limited to telomeric DSBs in a subset of cycling cells, which were later identified as those in S-phase [231,234]. Taken together, these results suggest that repair of telomeric DSBs may be very limited outside of S-phase. Moreover, telomeres in G1 and early S are more repressive to damage response activation than in late S or G2 [243]. Therefore, it becomes apparent that very little is known in regard to how telomeric DSBs are dealt with throughout the cell cycle, particularly during G1 when recombinational repair is unlikely to be activated.

Summary/Project Overview

DNA DSBs occur normally throughout the life of a cell as a result of both exogenous (e.g. IR), and endogenous sources (e.g. DNA synthesis, metabolic ROS) [74-76]. The proper handling and correct repair of DSBs is crucial to maintenance of genomic stability and cell survival [74-76]. As such, DSBs are repaired by an assortment of highly regulated pathways. While the majority of DSBs are faithfully repaired, a small portion of DSBs remain unrepaired, which have been implicated in the onset of senescence [163-164]. Additionally, various types of mutations and structural variants can occur as a result of DSB misrepair, the consequences of which can be lethal, or tumor promoting [74-76]. The factors that contribute to successful, non-mutagenic DSB repair versus those that lead to unsuccessful or mutagenic repair are not well

understood. In particular, whether certain genomic regions are at enhanced risk for the negative outcomes of DSB repair has only begun to be investigated.

Randomly distributed DSBs produced by exogenous sources (e.g., ionizing radiation) in human cells are most often repaired by cNHEJ; a relatively simple re-ligation of broken ends [100-102]. Another prominent pathway for the repair of these breaks is HR; a homology directed repair process that uses information from a template strand to reconstitute the sequence at the break site [105, 109]. However, HR is primarily limited to post replicative DNA as the presence of a sister chromatid template greatly increases the probability for accurate repair. Additionally, HR may be particularly important in the repair of DSBs within heterochromatin [188, 189].

There are several lesser studied DSB repair pathways including Alt-NHEJ, SSA, and BIR. Alt-NHEJ and SSA both involve annealing of ssDNA at regions of homology, followed by deletion of the intervening sequence and ligation. Alt-NHEJ and SSA processes are orchestrated by distinct factors, with SSA relying on much longer regions of homology then Alt-NHEJ [136]. BIR is similar to HR in that it requires invasion of a homologous template, however in BIR, DNA synthesis copies the template from the site of the break to the telomere [143]. While the relative use of these pathways is somewhat unclear, work in recent years has identified particular contexts in which they are activated. Alt-NHEJ has been implicated in the repair of heterochromatic breaks in G1. On the other hand, SSA may become dominant following the induction of many DSBs by high doses of IR [96]. Finally, BIR contributes to the repair of stalled replication forks [147, 148].

Telomeres, the protective and repetitive ends of eukaryotic chromosomes, function to prevent activation of DSB repair processes, so as to avoid highly toxic chromosome end-to-end fusions [44-48]. Telomeres achieve repair inhibition via the redundant activities of the shelterin complex. Shelterin proteins both directly inhibit damage responses and repair (e.g., by dampening ATM signaling), as well as coordinate structural changes (e.g., T-loop formation), which likely render telomeric DNA unpermissive to repair [26, 28, 29, 30-33]. It stands to reason

that true DSBs occurring within telomeric DNA may be particularly onerous repair substrates and possibly prone to mutation. Therefore, experimental interrogation of the fate of DSBs occurring within telomeric DNA, and what/if any repair pathway(s) are potentially involved, is an important area of investigation, as it likely pertains to cell viability and tumor suppression.

Initial work on this topic tracked DNA damage responses following non-specific DSB induction by agents such as IR and hydrogen peroxide. Following large amounts of initial damage, DSBs and corresponding damage responses decreased over time. However, the fraction of remaining damage responses overlapping with telomeric DNA increased with time, suggesting that DSBs occurring within telomeric DNA may be unrepairable. Further, surviving cells harboring telomeric damage responses became senescent. Since senescence is a G1 initiated process, these results suggest that unrepaired telomeric DSBs were those that occurred in G1 [163-164]. However, non-specific damage induced at the extremely high doses necessary to produce telomeric damage response enrichment, could be explained by telomere deprotection via telomere shortening or loss of TRF2 [227]. To explore these possibilities, we investigated telomeres with persistent DDRs 10 days after a dose of 10Gy IR. However, telomeres that co-localized with persistent γ-H2AX signals were not shortened, as they were found by fluorescence in-situ hybridization (FISH) to be the same brightness as telomeres that did not co-localize with γ-H2AX. Additionally, telomeres with persistent DDRs, were just as likely as normal telomeres to co-localize with TRF2, and did not show a reduction in the intensity of TRF2. Therefore, persistent telomeric DDRs following global damage may represent true unrepaired DSBs. However, the limitations of this experimental design necessitate the use of another approach to validate the interpretation that telomeric DSBs are irreparable in G1.

One such alternative approach is the use of targeted endonucleases that can enzymatically cleave endogenous telomeric DNA. Using such an approach, telomeric DSBs were found to be repaired predominantly by HR and BIR, with only a minor role for Alt-NHEJ,

and no role at all for cNHEJ [152, 231, 234, 240]. Further, it appeared that telomere DSB repair is cell cycle regulated, occurring only in S-phase [231, 234]. This conclusion is based on the presence of RAD51 foci in only 25% of cycling cells harboring telomeric DSBs, as well as 53BP1 foci only occurring in cells that stain positive for BrdU following pulse labeling (s-phase cells) [231, 234]. Taken together with cell cycle availability of HR and BIR, it seems possible that DSB repair does not occur at G1 targeted telomeric DSBs, consistent with the results obtained using non-specific damage.

To investigate telomeric DNA DDR activation and repair in G1 cells, we transfected cells with the telomere cutting endonuclease TRAS1-EN-TRF1 (ENT) and monitored outcomes in G1 or S/G2 populations. We found that telomeric DSB induction initiated a DDR in G1 cells, as characterized by overlap with γ-H2AX and MDC1, but notably lacking 53BP1. Additionally, following laser micro-irradiation, staining for 53BP1, but not γ-H2AX, produced a decreased intensity at telomeres compared to random locations. However, telomeric DSBs in S/G2 cells were positive for all three DDR markers.

While 53BP1 was not recruited to telomeric DSBs in G1, the presence of other markers of a DSB DDR prompted us to hypothesize that some form of repair may be occurring. While 53BP1 is known to promote cNHEJ [176-178, 180, 209], the localization of Ku70/80 and DNA-PKcs to telomeres may facilitate cNHEJ at telomeric DSBs without the need to recruit 53BP1. However, results from cNHEJ kinase inhibition, which causes DNA-PKcs to remain at DSBs without activating repair and precludes activation of other pathways [244-246], did not change the extent of telomere DNA fragmentation. Additionally, knockdown of DNA-PKcs did not promote the recruitment of 53BP1, which may be expected to be necessary for activation of other pathways. Overall, these results suggest that in stark contrast to IR or enzymatically-induced genomic DSBs, cNHEJ is not responsible for repair of telomeric DSBs in G1 and DNA-PKcs does not inhibit 53BP1 recruitment to telomeric DSBs.

We reasoned that telomere protection mechanisms (shelterin, chromatin compaction), may prevent 53BP1 recruitment, and therefore cNHEJ at G1 telomeric DSBs. To test this idea we attempted to alleviate the inhibition on 53BP1 recruitment by partial (sub-TIF-inducing) depletion of TRF2, or by experimental decompaction of telomere DNA with the histone deacetylase Trichostatin A, as well as by exposure to hypotonicity. Much to our dismay, none of these experimental manipulations resulted in 53BP1 recruitment to telomeric DSBs.

Given that the mechanistic role of 53BP1 at DSB sites involves inhibition of DNA end resection [176-178, 180], we next investigated resection at telomeric DSBs induced by ENT in G1. Indeed, we observed a robust induction of ssDNA characteristic of bidirectional resection at telomeric DSBs, as well as RPA70 and RPA32 phosphorylated at serine 4 and 8 by PIKK kinases (phospho-RPA). Further, the presence of phospho-RPA at telomeric DSBs in G1 was found to be dependent on conventional DSB resection associated machinery including EXO1 and to a lesser extent MRE11. On the other hand, Apollo, the exonuclease implicated in leading-strand telomere overhang generation, was not involved in resection at telomeric DSBs.

These results would typically indicate that one of the DSB repair pathways requiring long range resection (e.g. HR, BIR, or SSA), were being activated. However, these pathways are thought to be down regulated in G1, and no evidence of their activation was observed at telomeric DSBs in G1, as neither RAD51, RAD52, nor DNA synthesis was detected at G1 telomeric DSBs. Therefore, our results are consistent with a lack of repair at telomeric DSBs in G1. We are currently investigating why G1 telomeric DSBs become resected and how this impacts telomeric repair. Or hypothesis is that single stranded DNA helps to stabilize telomere ends and prevents the recruitment of 53BP1 as well as repair by cNHEJ. We speculate that inhibition of cNHEJ may be important for the prevention of telomere fusion.

Table 1*:* DSB repair pathways are distinct in repair protein usage, mutational outcomes, and cell cycle availability. Our understanding of how DSB repair is regulated at different genomic loci is just beginning to take form. Recent evidence suggests that certain repair pathways (HR, Alt-NHEJ), may have chromatin-specific cell cycle regulation. Additionally, activation of some repair pathways may be enhanced within certain regions of the genome such as heterochromatin, subtelomeres, and telomeres.

	Resection Independent	**Short Track Resection**	**Extensive Resection**		
Repair Pathway	cNHEJ	Alt-NHEJ	HR	SSA	BIR
Discriminating Repair Proteins	DNA-PKcs, KU70/80, LigIV	PARP1, LigI,III, Pol θ	Rad51? (likely involved in BIR)	Rad52	Rad52, POLD 3, POLD4
Common outcomes	Accurate repair, small insertions or deletions. Can result in translocations, although rare.	Deletions are inherent, enhanced translocation risk.	Gene conversion, rarely causes LOH, translocations, deletions, duplications	Large deletions are inherent, translocations risk may be enhanced.	Not well studied in mammalian cells, Other than translocation, similar potential mutation spectrum as HR.
Availability in G1	Yes	Some evidence for activation in heterochromatin.	Unlikely, Some evidence for activation in heterochromatin.	Unlikely	Unlikely
Availability in S/G2	Yes	Likely	Yes	Yes	Yes
Genomic Compatibility	Most sites	Heterochromatin, most sites?	Most sites, dominant in Heterochromatin, Subtelomeres? Telomeres?	Direct Repeats (telomeres?)	Stalled Replication Forks, Telomeres?

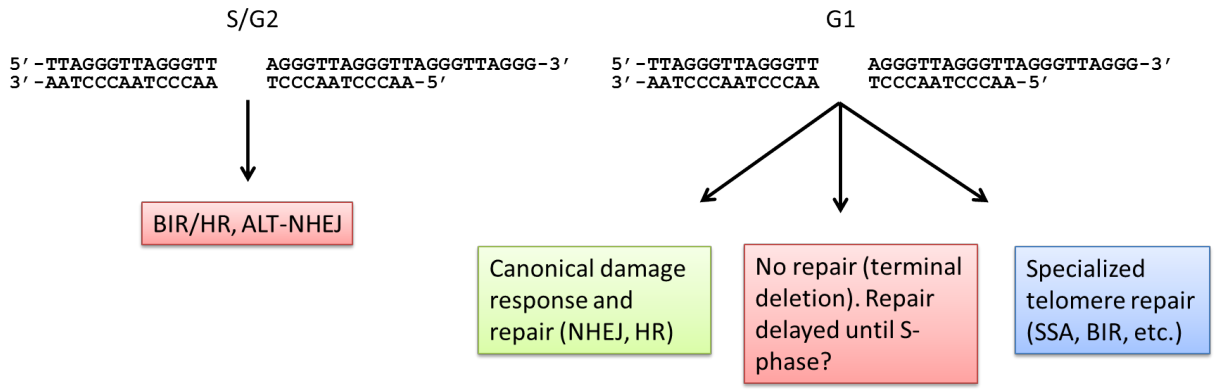

Figure 1: Potential outcomes of telomeric DSBs throughout the cell cycle. Recent publications have detected the use of HR, BIR, and to a lesser extent Alt-NHEJ at telomere DSBs in both ALT and non-ALT cells. However, in-situ evidence suggests that this activity is most likely restricted to S/G2. Several possibilities for G1 telomeric DSB repair exist including conventional DSB repair (cNHEJ, HR), specialized DSB repair (BIR, SSA), or no repair at all.

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CHAPTER 2

Damage Responses and Repair at G1 Telomeric DSBs

Introduction

Owing to an assortment of highly regulated and redundant DNA repair pathways, double-strand breaks (DSBs) are repaired accurately and efficiently the majority of the time [1-3]. Factors that influence outcomes of DSB repair are of significant relevance to human health and disease, as unrepaired or misrepaired DSBs pose a serious threat to cell viability, and genome stability, potentially contributing to degenerative pathologies including aging and cancer [4]. Aside from genetic background, perhaps the most obvious factor likely to play a role in the outcome of DSB repair is the genomic context of the break itself. Until recently however, the study of DSB repair has been limited to use of agents that randomly introduce damage throughout the genome (e.g., ionizing radiation), or within reporter cassettes integrated at a specific site within the genome (e.g., I-SCE1).

Recently, endonucleases that can cut at endogenous loci have greatly facilitated study of DSB responses and repair within different genomic contexts [5-9]. Researchers have begun to appreciate that repair varies dependent on genomic location of the DSB, such as within heterochromatin, rDNA repeats, or subtelomeres, and further that repair within these regions differs from conventional models of DSB repair [5, 6, 10-13]. In particular, repair in certain genomic contexts has been reported to exhibit HR in G1, relocation of the break, slow repair kinetics, as well as a much greater rate of deletion mutations [5, 6, 10-13].

Natural chromosomal DNA ends, or telomeres, are heterochromatic and repetitive features that represent intriguing substrates for DSB repair, as they actively inhibit repair activities to avoid detection and triggering of inappropriate DNA damage responses (DDR)

[14,15]. Active avoidance of DSB repair by telomeres involves the activities of telomere-specific binding proteins (e.g., TRF2), as well as higher order telomeric structural features (e.g., the T-loop) [14-17]. Whether DSBs that occur within telomeric DNA can be repaired, which pathways are involved, and what consequences may ensue, are questions that are just beginning to be addressed.

Most recently endonuclease-targeted telomere-specific DSBs have been utilized to identify HR and BIR as the two most prominent pathways used for telomeric DSB repair [8, 9]. However, substantiation for DDR activation at telomeric DSBs was only found in the fraction of cycling cells undergoing DNA replication, suggesting that cells outside of S-phase may be incapable of doing so [9]. Such a notion is consistent with earlier work describing a phenomenon wherein telomeric DDRs failed to resolve following sizeable amounts of global DNA damage, suggesting they were not repaired [2,3]. It has also been shown that surviving cells experiencing persistent telomeric DDRs became senescent, suggesting that the unrepaired telomere DSBs originated in G1 cells [2, 3]. Interesting distinctions between the telomere damage response initiated at targeted telomeric DSBs, versus those initiated following global DNA damage, also exist. While targeted telomeric DSBs fail to elicit 53BP1 foci outside of S-phase, global DNA damage results in persistent telomeric DNA damage responses involving both 53BP1 and γ-H2AX [2, 3].

As we have found that radiation shortens telomeres [18] and base damage has been shown to impair TRF2 binding to telomeres [19], it is possible that persistent telomeric DNA damage responses following global DNA damage may represent deprotected telomeres, rather than telomeric DSBs. This would be consistent with the presence and persistence of 53BP1 and γ-H2AX at telomeres following global damage, as well as the disparity in results following targeted induction of telomeric DSBs. To address this curious contradiction, we investigated persistent telomeric DNA damage responses following IR exposure. Importantly, ten days

following a 10 Gy dose (gamma-rays), persistent telomeric DNA damage responses were observed at telomeres that were normal in terms of telomere length and TRF2 binding, demonstrating that they were not deprotected.

To further clarify telomeric damage responses and repair throughout the cell cycle, the telomere cutting endonuclease (ENT) was employed. Our primary interest was in G1 telomeres, as previous work on telomeric DSBs, as well as evidence from the study of normal telomere protection, suggested that telomeres in G1 likely exhibit the strongest inhibition of normal repair mechanisms [2, 3, 8, 9, 20]. Following transfection with ENT we found that, telomeric DSBs triggered a damage response in G1 cells involving both γ-H2AX and MDC1. However, 53BP1 was not recruited to telomeric DSBs in G1, rather was only found in S/G2 populations. In support of these results, differential activation of γ-H2AX and 53BP1 at telomeres was also found immediately following spatially defined laser microirradiation.

53BP1 functions in part to facilitate cNHEJ by preventing DNA end resection, however, it also promotes telomere-telomere fusion by allowing long-range movement of double-stranded ends [21]. Interestingly, cNHEJ proteins (Ku70/80 and DNA-PKcs) are found at normal telomeres and like 53BP1, Ku70/80 can prevent resection [22-24]. Therefore, we speculated that locally concentrated cNHEJ factors may allow activation of cNHEJ at telomeric DSBs without requiring the activity of 53BP1. However, G1 telomeric DSBs were not found to recruit 53BP1 when DNA-PKcs was knocked down.

As an alternative explanation for why 53BP1 was not recruited to G1 telomeric DSBs we thought that telomere end protection mechanisms may be involved. However, neither partial TRF2 depletion (below the level that induces TIFs), nor chromatin relaxation treatments (Trichostatin A, Hypotonicity), were able to stimulate 53BP1 recruitment to G1 telomeric DSBs.

Nonetheless, that telomeric DSBs activate some form of a damage response in G1 suggested that repair might be occurring. As cNHEJ is by far the favored form of repair in G1 at

most DSBs, we explored the role of cNHEJ in telomeric DSB repair via kinase inhibition of DNA-PKcs. Results suggest that cNHEJ is likely *not* being activated at telomeric DSBs.

Other DNA repair pathways potentially involved in repair of telomeric DSBs in G1 involve an initial resection step, a process generally thought to be inhibited or greatly diminished in G1[25,26]. Additionally, 53BP1 plays an important role in limiting resection at DSBs and its striking absence at telomeric DSBs in G1 may indicate that resection is occurring [27-30]. To explore resection at telomeric DSBs in G1, we employed a non-denaturing single-stranded FISH assay, as well as immunostaining for RPA70 and phospho-RPA32. Indicative of resection, robust enhancement of single-stranded DNA and RPA70/phospho-RPA32, were observed at G1 telomeric DSBs. Further, single-stranded DNA tracts occurred bidirectionally, supportive of resection dependent repair.

To further characterize the single-stranded DNA present at telomeric DSBs in G1, phospho-RPA32 foci were monitored following MRE11 endonuclease activity inhibition or siRNA knockdown of EXO1. As expected, phospho-RPA was partially dependent on conventional DSB repair machinery. Further, knockout of Apollo, the exonuclease required for generation of single-stranded telomeric overhangs at leading-strand telomeres [31, 32], did not reduce the amount of resection at telomeric DSBs, supportive of the observed resection being DSB repair associated.

To gain mechanistic insight into what type of resection dependent repair was occurring at G1 telomeric DSBs, we investigated whether RAD51 protein recruitment indicated either HR or BIR was occurring. However, RAD51 recruitment to telomeric DSBs was not seen in G1, only in S/G2 phase cells. RAD52 recruitment in G1 was also investigated, which would provide indication of repair by BIR or SSA, but again we found no indication of induction at telomeric DSBs. Lastly, repair associated DNA synthesis that accompanies BIR and HR could not be detected. Taken together, these results suggest that while resection occurs at G1 telomeric DSBs (presumably due to absence of 53BP1), this response is not accompanied by active repair. Alternatively, we speculate that resection and regeneration of single- stranded overhangs

at broken telomere ends may serve to prevent 53BP1 recruitment and activation of risky end joining activities, which could result in telomere-telomere fusions, a hypothesis we continue to explore.

Materials and Methods

Cell Culture and Transfections

U20S cells, U20S RAD52-YFP (Obtained from Jiri Lucas, University of Copenhagen), and EJ-30 cells (obtained from Dr. John Murnane, UCSF) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS). BJ1 hTERT (ATCC), were cultured in Alpha-MEM (Hyclone) supplemented with 10% FBS. Transient transfections were carried out with Lipofectamine 3000 at 60-80% confluency in Opti-MEM (Gibco) for 20 minutes and replaced with normal media 8 hours later. Unless otherwise specified all experiments were carried out 48hrs post transfection.

Gamma Irradiation

While being constantly rotated cells were exposed to ^{137}Cs γ-rays in a Mark I irradiator (J.L. Shepherd; located at Colorado State University) at a dose rate of 2Gy per minute, and then allowed to recover for designated times.

Laser Micro-Irradiation

Laser micro-irradiations were performed with a Zeiss LSM880 confocal microscope (located at CSU), using a 405nm laser at 100% with settings of 50 iterations and a 15 us pixel dwell. Spatially defined stripes of damage were generated through the nuclei of cells followed by a recovery period of 30 min. Immunofluorescence and imaging of micro-irradiated cells was carried out as described below.

RNAi

SiRNA was initially delivered into cells using RNAiMAX in OptiMEM media according to manufactures instructions, followed by replacement with normal media 5 hours later. 24 hours following initial siRNA delivery, cells were co-transfected with ENT or TRF1 and appropriate siRNA in Lipofectamine 3000 according to manufactures instructions, and then fixed or harvested 48hrs later. siRNA sequences were as follows: TRF2: 5'-GAGGAUGAACUGUUUCAAGdtdt-3' (anti-sense also included 3' dtdt), EXO1: 5'-UGCCUUUGCUAAUCCAAUCCCACGC-3'. For DNA-PKcs shRNA stable cell line generation, we transduced BJ1 hTERTs shRNA against DNA-PKcs using MISSION® lentiviral transduction particles and selected in 1uM puromycin (TRCN0000006257, TRCN0000194719).

Chromatin Relaxation

Global chromatin relaxation was achieved by treatment with Trichostatin A at 0, 25, 50 or 100ng/ml for 24hrs or by treatment with a hypotonic solution composed of 75mM KCL or normal culture media and 75mM KCL at a 1:3 ratio for 1 hour prior to fixation.

Inhibitors

BJ1hTERTs were treated with either a DNA-PKcs kinase inhibitor that prevents autophosphorylation (NU7026, Sigma) or MRE11 endonuclease activity inhibitor (PFM01, Thermofisher). NU7026 was used at a concentration of 10uM for 24 hours prior to harvesting cells as per previous [33]. Alternatively PFM01 was used at a concentration of 100uM for 8 hours preceding fixation.

Western Blotting

Cell pellets were washed in phosphate buffered saline (PBS), and then incubated in lysis buffer for 10 minutes. Lysis buffer consisted of Mammalian Protein Extraction Reagent (MPER) with protease inhibitors (complete mini EDTA free, Sigma-Aldrich), and in cases when phosphorylated proteins were being detected, phosphatase inhibitors (PhosSTOP, Sigma-Alrdrich). Following isolation of protein, the Bradford assay was used to quantify protein (BioRad). 30-60ug of protein were loaded into precast SDS-PAGE gels (Mini-Protean TGX, 4-15%, BioRad) in Tris/Glycine/SDS buffer followed by electrophoretic separation for roughly 1.5 hours at 125V. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane in Tris/Glycine buffer with 10-15% methanol for 16-20hours at 30V at 4^{o}C. An even protein transfer was verified by reversibly staining membranes with Poncaeu S solution (Sigma-Aldrich, 0.1% w/v in 1% acetic acid). Next, membranes were blocked in 5% non-fat dry milk (NFDM), or bovine serum albumin (BSA) in 1X tris buffered saline with 0.1% Tween 20 (TBST) from 30 minutes to 1 hour with gentle shaking. Blocking solution was then replaced with fresh blocking solution containing the appropriate dilution of primary antibody and incubated from 2 hours to overnight with gentle shaking. Following primary antibody incubation, membranes were washed in 1X TBST for 4 washes of 10 minutes each with gentle shaking. Next, fresh blocking solution was added with the appropriate dilution of a horseradish peroxidase (HRP) labeled secondary antibody and incubated from 2 to 4 hours followed by another series of 4 washes in 1X TBST. Following the final wash, membranes were rinsed in PBS. To visualize proteins, membranes were treated with SuperSignal^{TM} West Pico Chemiluminscent Substrate according to the manufactures instructions (ThermoFisher) and imaged on a ChemiDoc^{TM} XRS+ imager with ImageLab^{TM} software (BioRad).

Antibodies and Concentrations for Western Blotting

Primary antibodies for western blotting included Rabbit Anti-phospho serine2056 DNA-PKcs (Abcam ab1249181, 1:2000), Mouse Anti-DNA-PKcs (ThermoFisher MS-423-P, 1:10000), Mouse Anti-TRF2 (SantaCruz sc-271710, 1:500), Mouse Anti-phospho serine1981 ATM (Upstate 05-740, 1:1000), Rabbit Anti-phospho Thr68 CHK2 (Cell signaling 2661, 1:1000)

HRP labeled secondary antibodies included Donkey Anti-Rabbit (Jackson ImmunoResearch 711-035-152, 1:20000), and Rabbit Anti-Mouse (ThermoFisher 816720, 1:10000).

Immunofluorescence

For the majority of immunofluorescence experiments, cells were grown on chamber slides, fixed in freshly prepared 4% paraformaldehyde (PFA) for 10 min at room temperature, and then permeabilized in 0.2% Triton X-100 in PBS for 4-10 minutes. Next, cells were blocked in 10% normal goat serum (NGS), or 5% BSA in 1xPBS for 40 minutes and then incubated with primary antibodies diluted in blocking solution for 1 hour at 37^{o}C or overnight at 4^{o}C. Following primary incubations cells were washed 3 times in 1xPBS at 42^{o}C. After washes cells were incubated with fluorophore-conjugated goat secondary antibodies for 30 minutes at 37^{o}C. Finally, cells were washed again as before and counterstained with prolong gold antifade reagent with DAPI (Invitrogen).

BrdU Incorporation Assay (Based on Protocol from ThermoFisher)

Cells were pulse labeled with BrdU for 2 hours (50mM) and then fixed for 15 minutes in 4% PFA at room temperature. Next cells were permeabilized for 20 min with 0.1% Triton x-100 in PBS, followed by DNA denaturation for 10 minutes on ice with 1N HCl and then 10 minutes at room temperature with 2N HCL. Cells were then washed with phosphate citric acid buffer pH 7.4 for 10 minutes at room temperature. Finally, cells were washed for 5 min in permeabilization

solution. Blocking was then carried out for 30 min at 37C in 5% NGS with 0.1% Triton X-100 in PBS. Finally, antibody incubations, washing steps, and counterstaining were carried out as described in the immunofluorescence section.

ImmunoFISH

Combined immunofluorescence and fluorescence in-situ hybridization (FISH) experiments were carried out on cells grown on chamber slides. Cells were initially fixed in 4% PFA for 5 minutes at room temperature. Next cells were permeabilized for 4 minutes in 0.2% Triton X-100 in PBS. Following permeabilization cells were blocked and immunostained as described in the immunofluorescence section. After the last washing step, cells were post fixed in 4% PFA for 15 minutes at room temperature. Next, cells were dehydrated in an ethanol series (75%, 85%, 95%) for 2 minutes each and allowed to air dry. When dry, DNA was denatured by submerging slides in 70% formamide in 2X SSC at 75^{o}C for 2 minutes followed immediately by another round in the ethanol dehydration series and air drying. While slides were air drying the hybridization solution was prepared by combining 36ul formamide, 12ul 0.05M Tris-HCL, 2.5ul 0.1M KCL, 0.6 ul 0.1M $MgCl_2$ and 0.5ul 0.5uM peptide nucleic acid telomere probe in 20% acetic acid (Biosynthesis TelC-Alexa488 or TelG-Cy3). Hybridization solution was then denatured at 85^{o}C for 10 min followed by cooling on ice. After cooling 50ul of hybridization solution was added per slide followed by a coverslip and slides were incubated at 37^{o}C in a humidified chamber for 6 hours. Following hybridization slides were washed twice in 50% formamide in 2X SSC (2.5 minutes 42^{o}C), twice in 2X SSC (2.5 minutes 42^{o}C) and twice in 2X SSC + 0.1% NP-40 (2.5 minutes 42^{o}C). Following the final wash cells were counterstained with prolong gold antifade reagent with DAPI.

For non-denaturing FISH, cells were only dehydrated once followed immediately by the addition of the cooled hybridization solution.

Antibodies and Concentration for Immunofluorescence

Primary antibodies and concentrations included: Rabbit Anti-53BP1 (Bethyl A300-272A, 1:800), Rabbit Anti- γ-H2AX (Bethyl A300-081, 1:1000), Mouse Anti-Flag (Sigma M2 F1804, 1:2000-4000), Rabbit Anti-RPA70 (Cell signaling #2267, 1:50), Rabbit Anti-phospho S4/S8 RPA32 (Bethyl A300-245A 1:2000), Mouse Anti-gammaH2AX (Millipore 05-636, 1:1500), Rabbit Anti-Cyclin A (Santa Cruz SC-751, 1:500), Rabbit Anti-MDC1 (Bethyl A300-051A, 1:1000), Rabbit Anti-RAD51 (H-92 SC-8349, 1:800), Sheep Anti-RAD52 (Kind gift from Jiri Lukas Lab, 1:100), Rat anti-BrdU (BioRad OBT0030, 1:200), Rabbit Anti-phospho S15 p53 (Abcam Ab18128-50, 1:500)

Secondary antibodies and concentrations included: Alexa-488 Goat anti-Mouse (ThermoFisher A11029, 1:750), Alexa-594 Goat anti-Mouse (ThermoFisher A11005, 1:750), Alexa-647 Goat anti-Mouse (ThermoFisher A21235, 1:350), Alexa-488 Donkey anti-Mouse (ThermoFisher 21202, 1:750), Alexa-488 Goat anti-Rabbit (ThermoFisher A11008, 1:750), Alexa-594 Goat anti-Rabbit (ThermoFisher A11012, 1:750), Alexa-555 Goat anti-Rat (ThermoFisher A21434, 1:750), Alexa-647 Donkey anti-Sheep (ThermoFisher A21448, 1:350), Alexa-488 Donkey anti-Mouse (ThermoFisher A21202 1:750)

Fluorescence Microscopy and Image Analysis

Images were acquired using a Zeiss Axio Imager.Z2 epi-fluorescent microscope using a 63X/1.4 N.A. objective (Plan-APOCHROMAT, Zeiss). For telomere length and TRF2 brightness experiments 11 Z-stacks per channel were taken at 0.2 μm intervals. Stacks were then compressed and thresholded at a constant value for each channel. Following thresholding, images were analyzed for intensity of TRF2 and telomeres as well as colocalization events between telomeres, TRF2 and γ-H2AX, using a custom journal written in Metamorph 7.7 (Molecular Devices). A similar approach was used to score other imaging experiments when

appropriate, albeit in single plane images. However, this journal did a poor job identifying foci that overlapped partially or in situations when discrete foci were clearly discernable but the background was relatively high or uneven. Therefore, for certain epitopes we resorted to a manual analysis approach. This approach involved blinded subjective thresholding of images followed by the generation of discrete borders around each object in Metamorph such that the boundaries of any foci could be clearly determined. Next, foci were subjectively determined to be overlapping if roughly 50% of one of the objects was observed to be inside the border of the other. Additionally, for RAD52-YFP, RPA and phospho-RPA foci analysis, cells tended to have very few or an abundance of foci and scoring was therefore done on the basis of whether a cell had > 4 foci.

Analysis of the laser microirradiation experiment involved first thresholding TRF2 foci using a fixed value for all images. Next, these thresholded foci were converted to regions in Metamorph and these regions transferred to γ-H2AX or 53BP1 images. Next the average intensity within the transferred regions was compared to that within pseudo-random regions of comparable dimensions generated by rotating TRF2 images by 90°

For BrdU foci analysis in BJ1 hTERTs, untransfected S-phase cells were excluded from analysis (identified by very bright pan nuclear staining). Similarly, although not well established, a subset of untransfected BJ1 hTERTs showed bright pan nuclear staining for RAD52, which presumably were those in S phase as well, and were also excluded from analysis.

Stochastic Optical Reconstruction Microscopy (STORM)

Acquisition was carried out on a Nikon Eclipse Ti Microscope (Nikon Inc) equipped with an Andor Clara camera (Andor Inc) and a 100X/1.42NA Planapochromat DIC, oil immersion lens (Nikon Inc). A cylindrical lens was placed between the camera and the left port to give three dimensional locations of telomeres within the nucleus. Cells were imaged in 2mL of STORM imaging buffer (50mM TRIS-HCl(pH 8.0) +10mM NaCl +

10% Glucose (supplemented with 70uL of 1M MEA solution and 7uL of GLOX solution per 620uL of the imaging buffer). STORMing of Alexa 647-FISH labeled telomeres was was performed by pulsing the 640 laser at 100% using 50ms exposure times. 500,000 events were collected per cell.

Image analysis included plotting fluorophores detected at individual telomeres by STORM in 3D within MeshLab software and using a convex hull algorithm to compute telomere volume. Further, density of fluorophores was calculated by dividing the number of fluorophores at an individual telomere by the telomere volume.

Telomere Restriction Fragment Southern Blots (TRF Assay)

The TRF assay was performed using the TeloTAGGG™ southern blotting kit (Roche) according to the manufactures instructions with some modifications, including a longer probe hybridization time (6hrs), as well as a longer incubation time with Anti-DIG antibody (1hr). 2ug of sample DNA were loaded per lane and blots were imaged on a ChemiDoc™ XRS+ imager with ImageLab™ software (BioRad). Quantitation of mean TRF length was performed using TeloTool software according to the manufacturer's protocol.

Plasmids

TRAS1-EN-TRF1 and TRF1 plasmids were constructed from a TRAS1-EN-TRF1 plasmid obtained from Dr. Haruhiko Fujiwara (University of Tokyo). Both constructs are driven by a CMV promoter and possess C-terminal flag tags for visualization.

Replication, Statistics

The majority of experiments involved 3 independent experiments for each condition and involved at least 30 cells per replicate for imaging experiments. The exceptions were for experiments in U20S cells, which were done in duplicate, as well as micro-irradiation

experiments, which were done in duplicate with 15 cells per replicate and for treatments with TSA or hypotonicity, which were done only once as there was no indication of any effect. Additionally, experiments in EJ-30 cells were also in triplicate, however, the number of cells imaged totaled at least 300 per condition to allow for DAPI intensity histogram generation. Error bars on bar graphs represent standard deviations, and p-values were computed when experiments were done in triplicate, and are provided in the text when less than 0.05 (significance threshold). When two groups were being compared p-values were generated via students T-tests, alternatively, when three or more groups were being compared an ANOVA with a Tukey's post hoc test was used. ANOVAs were either one way or two way depending on the number of categorical independent variables.

Results

Enrichment of Damage Responses Occurs at Telomeres of Normal Length and TRF2 Status.

High doses of ionizing radiation (IR) have been reported to cause a temporal enrichment of telomeric damage responses leading to the hypothesis that telomeric DSBs are not repaired [2,3]. In order to reproduce this result, we irradiated BJ1 hTERT fibroblasts with either 0.5Gy or 10Gy (Gamma rays), and then monitored induction of gamma (γ)-H2AX foci. 10 days following 10Gy exposure, total γ-H2AX foci were reduced to a level comparable to the number observed 30min after 0.5Gy IR (Fig. 2A). However, the fraction of damage responses at telomeres was much greater (30.4%) 10 days after 10Gy then 30min after 0.5Gy (8.99%), suggesting that telomeric DSBs become enriched following high doses of IR ($p = 0.00003$, Fig. 2A). However, since the doses required to achieve telomere enrichment of damage responses (10-20Gy) are quite large, it's possible that telomere damage responses may be a result of telomere deprotection, rather than true telomeric DSBs. This would most likely occur as a result of

telomere shortening or from a loss of TRF2 at telomeres [18, 19]. To investigate these possibilities, we evaluated enriched telomere damage responses 10 days after exposure to 10Gy for telomere length and for TRF2 protein levels. Telomere damage responses were not found to occur preferentially at short telomeres, nor at telomeres lacking TRF2 foci (Fig. 2B). Additionally, the intensity of TRF2 foci was found to be the same whether the telomeres were associated with persistent damage responses or not (not shown). These results suggests that telomeres may in fact represent particularly difficult DSB repair substrates.

Generation of Targeted Telomeric DSBs

To better understand the response to telomeric DSBs throughout the cell cycle, we sought to induce targeted DSBs in telomeric DNA. We employed a combination of cell lines, including U20S cells (ALT phenotype), in which we reproduced published results. We also used BJ1 hTERTs, an immortalized but non-tumorigenic human skin fibroblast cell line which performs well for fluorescence imaging. Finally, we used EJ-30 cells, a bladder carcinoma cell line that transfects and images well and has been used extensively to study subtelomeric DSB repair.

Following transient transfection of a plasmid encoding a telomere repeat specific endonuclease fused to the human TRF1 gene (TRAS1-EN-TRF1: Hereafter referred to as ENT) (Fig. 3A) [7], flag tagged ENT or TRF1 overexpression control, were both found to co-localize with telomere repeats (Fig. 3A-B). To validate induction of telomeric DSBs using this system, we performed the telomere restriction fragment (TRF) assay, and found that telomere fragmentation was detected following ENT expression, but not following TRF1 expression in EJ-30 cells (Fig. 3C). Additionally, to determine whether conventional DSB signaling was taking place, we performed western blots for the phosphorylated forms of ATM (pS1981), and CHK2 (pThr68), using lysates produced from cycling EJ-30 cells transfected with ENT, TRF1, or no treatment controls. While transfection alone induced some DSB signaling activity, as evidenced by the

increased intensity of phospho-ATM and phospho-CHK1 bands in TRF1 transfected samples relative to no treatment controls, transfection with ENT produced an even greater signal intensity for both targets, suggesting that telomere damage was activating DSB signaling (Fig. 3D).

ALT Cells Activate HR/BIR at Telomeric DSBs

As an additional validation of the ENT system, we sought to reproduce the finding that telomeric DSBs could stimulate a damage response and repair by some combination of HR and BIR in ALT cells [34, 35]. To this end, we found that U2OS cells (ALT) exhibited activation of telomere damage responses upon transfection with ENT, as evidenced by an increase in γ-H2AX foci compared to untransfected cells (Fig. 4A). A significant portion of these damage responses were found to occur at telomeres, as they overlapped with ENT foci (Fig. 4A). Additionally, following ENT transfection, U20S cells harbored elevated levels of Rad51 and Rad52-YFP foci (as well as endogenous RAD52 foci; not shown), which frequently overlapped with ENT foci, indicative of repair of telomeric DSBs by HR and BIR (Fig. 4B, C).

Strategies to Study Targeted Telomeric DSBs in Non-ALT G1 Cells

As various lines of evidence suggest that DSB repair may be non-conventional or even non-existent at the telomeres of normal cells in the G1 phase of the cell cycle [2,3,8,9, 20], we sought to investigate damage responses and repair at G1 telomeres directly. In order to study G1 telomeric DSBs in non-ALT EJ-30 cancer cells, we validated a high magnification (63X), DAPI intensity based approach as a means of distinguishing cell cycle phases, while retaining the ability to make accurate measurements on fluorescent foci. Cells in G1 form a clear peak in the lower intensity portion of a DAPI intensity histogram generated by even a relatively low number of EJ-30 cells (~300) (Fig. 5a). The specificity of the G1 DAPI intensity peak was validated via exclusion of cyclin A, which stains S and G2 cells (Fig. 5a). A similar DAPI

intensity histogram was produced to distinguish G1 from S /G2 in all other imaging experiments involving EJ-30 cells. Discrimination between S and G2 could not be achieved, so these populations were pooled throughout our analysis

We also wanted to evaluate telomeric DSBs in G1 BJ1 hTERT cells, an immortalized normal human fibroblast cell line. Transfection efficiency in BJ1 hTERT cells with either ENT or TRF1 was quite low (0.5-2% of cells). However, the low transfection efficiency of these cells was somewhat fortuitous as the vast majority of transfected BJ1 hTERTs did not exhibit pan-nuclear staining for cyclin A (ENT: 0%, TRF1: 3.2%, Fig. 4b), nor BrdU (Fig. 13C), following a 2 hour pulse label, indicating that cells were highly enriched in G1 48 hours post transfection. This finding was reproduced on several additional occasions and found to be highly repeatable. Therefore, transfected BJ1 hTERTs were assumed to be in G1 at 48hrs for further experiments.

Interestingly, following transfection with ENT or TRF1, a proportion of untransfected BJ1hTERT cells in the population did show nuclear positivity for Cyclin A and BrdU, indicating that these cells were still cycling (Fig 5b, 13c). However, the rate of growth was likely decreased compared to untreated controls, as the frequency of Cyclin A, and especially BrdU, positivity was much lower. Therefore, the stress of transient transfection likely played a role in the G1 enrichment of transfected cells. While it is possible that telomeric DSBs also triggered a G1 cell cycle arrest in ENT transfected cells, cells overexpressing TRF1 also showed significant enrichment in G1, suggesting that the main effect on the cell cycle of transfected cells was caused by transfection. In support of the idea that telomeric DSBs do not cause a G1 cell cycle arrest in normal cells, phospho-p53(S15) exhibits enhanced nuclear staining in ENT expressing U20S cells (~20%), but not in ENT expressing BJ1hTERTs (not shown).

Non-Canonical Damage Responses at G1 Telomeric DSBs

To investigate damage responses at individual telomeric DSBs, we evaluated the presence of γ-H2AX and 53BP1 at telomeres by immunofluorescence. Telomere-specific DSBs

were found to induce γ-H2AX in G1 BJ1 hTERT cells (p = 0.012), as well as in all phases of the cell cycle in EJ-30 cells (p = 0.0009 in G1 cells and 0.022 in S/G2, Fig. 6a). On the other hand, 53BP1 was induced at telomeric DSBs in S/G2 EJ-30 cells, but not G1 BJ1 hTERT or EJ-30 cells (p = 0.012 in S/G2 EJ-30 cells, Fig. 6B). To determine if other components of the early DNA damage response were activated by telomeric DSBs in G1, we also evaluated MDC1, an early mediator of the response to genomic DSBs that acts downstream of γ-H2AX, but upstream of 53BP1 in BJ1 hTERTs [36]. MDC1 foci were induced to a similar degree as γ-H2AX in response to G1 telomeric DSBs (p = 0.0007, Fig. 7a).

We next sought to determine whether our 53BP1 results were reproducible when telomeric DSBs were induced by other methodologies. To this end, we compared the intensity of γ-H2AX and 53BP1 between telomeres and random spots that occurred within spatially defined stripes of damage generated by laser microirradiation in BJ1 hTERT cells. Consistent with our results using ENT, 30 minutes after irradiation, the intensity of γ-H2AX was found to be similar at telomeres and random spots within the microirradiation stripe, while the intensity of 53BP1 was reduced at telomeres compared to random spots (Fig. 7b).

DNA-PKcs Does Not Impact 53BP1 Recruitment or Telomere Fragmentation Following Telomeric DSBs

While 53BP1 recruitment could not be detected at telomeric DSBs in G1, other components of a DSB response such as γ-H2AX and MDC1 were detected, suggesting that repair may still be occurring. Further, several of the major factors necessary for cNHEJ (Ku70/80, DNA-PKcs) are present at normal telomeres [37, 38]. In support of cNHEJ activity at telomeric DSBs, autophosphorylation of DNA-PKcs at serine 2056 was found to be somewhat increased in EJ-30 cells expressing ENT compared to cells expressing TRF1 or no treatment controls (Fig. 8a). However this could not be attributed to telomere DSB repair in a particular

phase of the cell cycle. We also tested whether DNA-PKcs autophosphorylation effected telomere DSB repair by comparing TRF blots in cells expressing ENT to those expressing ENT and treated with NU7026, a specific inhibitor of DNA-PKcs autophosphorylation (Fig. 8a, b). Chemical inhibition of DNA-PKcs autophosphorylation has been shown to prevent DSB repair by other pathways, as DNA-PKcs that cannot undergo autophosphorylation and engage in cNHEJ remains at the break site and prevents other types of repair from compensating [39-41]. Therefore, if cNHEJ was responsible for telomeric DSB repair, treatment with NU7026 would be predicted to decrease the average TRF size, as breaks would accumulate without being repaired. However, treatment with NU7026 in cycling EJ-30 cells for 24 hours did not change the TRF size relative to control (Fig. 8b).

We also reasoned that a lack of 53BP1 recruitment to G1 telomeric DSBs could be explained by a functional redundancy with cNHEJ factors, as both 53BP1 and cNHEJ factors are known to inhibit resection machinery at DSBs [22-24, 27-30]. Since cNHEJ factors are more readily available at telomeres than elsewhere in the genome, 53BP1 recruitment to telomere DSBs may be unnecessary. Interestingly, 53BP1 recruitment is required for most forms of repair (excluding SSA) [42-44]. Therefore, loss of cNHEJ activity might be expected to cause 53BP1 recruitment to telomeric DSBs such that repair can occur by another pathway. However, BJ1 hTERT cells with stable shRNA knockdown of DNA-PKcs did not show enhanced 53BP1 foci at telomeric DSBs (Fig. 8c).

Sub-TIF-Threshold TRF2 Depletion Does Not Affect 53BP1 Recruitment to Telomeric DSBs or Repair

It is likely that the lack of 53BP1 recruitment to G1 telomeric DSBs is what prevents cNHEJ, however, how 53BP1 is inhibited, and what the consequences are remain unknown. We believe the factors responsible for telomere protection may also underlie 53BP1 inhibition at G1 telomeric DSBs. To explore this possibility, we partially knocked down TRF2 protein using small

interfering (si)RNA. While a robust knockdown of TRF2 leads to telomere deprotection and TIF formation, including the recruitment of 53BP1 at normal telomeres, partial knockdowns of up to about 90% of the endogenous protein have been shown to elicit no phenotype [45]. We reasoned that a partial knockdown of TRF2, which was insufficient to initiate a TIF response itself, may when coupled with telomeric DSBs, result in 53BP1 recruitment and cNHEJ repair activation. Therefore, we selected an siRNA sequence against TRF2 published to achieve a partial TRF2 knockdown [46]. Using this TRF2 siRNA, we reproducibly reduced TRF2 levels by 50-75% of endogenous levels during co-transfection experiments with either ENT or TRF1 (Fig. 9A). In the context of TRF2 depletion, we then examined transfected G1 BJ1 hTERT cells for 53BP1 recruitment to ENT/TRF1 foci. However, we found no increase in 53BP1 foci in TRF2 depleted ENT transfected cells compared to controls (Fig. 9A). We also analyzed TRF size in cycling EJ-30 cells following ENT transfection and partial TRF2 depletion, and again found that partial TRF2 depletion did not produce a phenotype relative to transfection with ENT alone (Fig. 9b). Therefore, even in the context of a telomeric DSB, partial TRF2 depletion is not sufficient to alleviate inhibition of 53BP1 recruitment, nor does it promote telomere repair.

Treatment with TSA or Exposure to Hypotonicity Does not Influence 53BP1 Recruitment to Telomeric DSBs.

Recent evidence suggests that telomere chromatin compaction may facilitate the function of shelterin in prevention of repair activation at normal telomeres [47-49]. Therefore, we reasoned that chromatin compaction may regulate repair activity at telomeric DSBs, and underlie inhibition of 53BP1. To investigate this possibility, we treated ENT or TRF1 transfected cells with trichostatin A (TSA), a histone deacetylase inhibitor capable of global chromatin relaxation, as well as promotion of the ALT phenotype [51-53], at a range of concentrations (25, 50, 100 ng/ul) for 24 hours prior to fixation. Alternatively, we exposed cells to a hypotonic solution consisting of 75mM KCL, or 3 parts 75mM KCL and 1 part alpha-MEM with 10% Fetal

Bovine Serum (FBS) for 1 hour prior to fixation [53]. In either case telomere 53BP1 foci were not induced by chromatin decompaction (Fig 9C, not shown). Interestingly, we attempted to confirm telomeric DNA decompaction by either telomere volume or fluorophore density using Stochastic Optical Reconstruction Microscopy (STORM). However, our initial efforts were unsuccessful suggesting that well established chromatin relaxation treatments may be less effective at telomeres.

G1 Telomeric DSBs Harbor ssDNA Consistent with Resection

As 53BP1 is best known to play a role in the inhibition of 5'-3' end resection at genomic DSBs [27-30], we hypothesized that telomeric DSBs that fail to recruit 53BP1 may harbor single stranded (ss)DNA as a signature of resection. To investigate ssDNA at G1 telomeric DSBs, we performed fluorescence in situ hybridization (FISH) using a C-rich telomere probe, without denaturation of the DNA duplex, in G1 BJ1 hTERT cells transfected with ENT or TRF1. sstelomeric DNA was much greater in cells transfected with ENT as compared with TRF1 or no treatment controls ($p = 0.0002$, Fig. 10A). To determine if resection/ssDNA occurred bidirectionally, we also performed the ssFISH assay with a G-rich telomere probe to detect ssDNA in the C-rich strand of the telomere. Interestingly, hybridization with the G-rich probe produced more signals overall in all cell populations, but with the same general trend of many more signals in ENT transfected cells then in TRF1 transfected or no treatment controls ($p = 0.045$, Fig. 10B). Therefore, our results suggest that extensive resection at telomeric DSBs in G1 occurs bi-directionally, a finding consistent with DNA repair associated resection.

As a secondary indicator of repair associated resection, we also immunostained for RPA70 and phospho-RPA32 (S4/S8) in G1 BJ1 hTERTs transfected with ENT. Phosphorylated RPA is known to coat ssDNA initially and stabilize it prior to the template invasion/annealing steps in HR, BIR, and SSA [54-57]. Following generation of telomeric DSBs, phospho-RPA32 showed pronounced induction and very frequent overlapping with ENT-flag in G1 BJ1 hTERTs

(p = 0.0000046 Fig. 11A). Additionally, RPA70 foci were increased by expression of ENT, however this was not found to be significant compared to untransfected controls (Fig 11a). A more muted induction of RPA70 compared to phospho-RPA32 likely results from the fact that unphosphorylated RPA coats ssDNA during DNA synthesis as well as following resection at DSBs and S-phase cells are only found in the control population of BJ1 hTERTs.

ssDNA Production at G1 Telomeric DSBs Requires Conventional Resection Machinery but not the Telomere Processing Exonuclease Apollo.

As long range resection is thought to be inhibited in the G1 phase of the cell cycle [22-30] we were interested in whether or not the observed resection was conventional in terms of nuclease activity. If resection at telomeric DSBs functions not to promote repair, but instead to regenerate a normal ss-telomere overhang, Apollo exonuclease, which is necessary to initiate telomere overhang generation at blunt-ended leading-strand telomeres [31,32] may be involved. However, G1 Apollo$^{-/-}$ EJ-30 cells transfected with ENT exhibited only a slight reduction in ssAATCCC foci compared to wild type (WT) cells transfected with ENT (p = 0.37, Fig. 12A). Additionally, ENT expressing G1 Apollo$^{-/-}$ EJ-30 cells actually had more phospho-RPA32 foci then ENT expressing G1 WT EJ-30 cells (p = 0.099 Fig. 12B). Further, both measures of ssDNA were found to be slightly increased in the Apollo$^{-/-}$ S/G2 populations compared to WT cells (Not significant, Fig. 12A, B). Overall, these results suggest that Apollo exonuclease is not responsible for the resection observed at telomeric DSBs throughout the cell cycle, or possibly that its activities are redundant.

In contrast, conventional DNA repair related resection machinery was found to influence resection at G1 telomeric DSBs. Upon inhibition of MRE11 endonuclease activity in BJ1 hTERT cells, resection was slightly reduced at G1 telomeric DSBs, as phospho-RPA foci were reduced, albeit non-significantly (Fig. 11C). Phospho-RPA32 was further reduced when BJ1 hTERT cells were depleted of EXO1 by siRNA, which approached significance (p = 0.10 Fig. 11C). These

results suggest that conventional resection machinery involving MRE11 and EXO1, are responsible for resection at telomere DSBs, but they do not rule out the possibility that such resection serves to reconstitute ss-telomeric overhangs for end-structure/protection purposes. Further, it is possible that other enzymes, such as DNA2 and BLM may play an important role in the long-range resection observed at G1 telomeric DSBs.

Telomeric DSBs do not Undergo Long-Range Resection-Dependent Repair in G1

To determine whether the ssDNA generated at telomeric DSBs engaged in one of the known forms of long-range resection-dependent repair in G1, we performed a series of immunostaining experiments. HR (and BIR?) depends on the activities of RAD51 recombinase, which can be focally detected when this pathway is engaged [9, 34, 35]. Therefore, we immunostained for RAD51 in ENT and TRF1 transfected BJ1 hTERTs, as well as in ENT transfected EJ-30 cells. However, RAD51 foci were only present to a meaningful degree in EJ-30 S/G2 cells, and not in G1 BJ1 hTERT or G1 EJ-30 cells, suggesting that G1 telomeric DSBs do not undergo repair by HR (Fig. 13A). Additionally, Rad52, which plays an important role in the annealing step of both BIR and SSA [35, 43, 57-60], was not induced following telomeric DSBs in G1 BJ1 hTERTs (Fig. 13B). Finally, repair associated DNA synthesis, which occurs during HR and BIR, was not detected following induction of telomeric DSBs in G1 BJ1 hTERTs (Fig. 13C). Overall, these results suggest that conventional long-range resection-dependent repair does not occur at G1 telomeric DSBs.

Discussion

G1 telomeric DSBs have been hypothesized to be irreparable, as DSB DDRs generated globally by IR or other genotoxic agents fail to resolve when they occur within telomeric DNA, and cells become senescent (G1) [1,2]. However, interpretation of these experiments is difficult

because telomeres may also activate DDRs following high doses IR if they become de-protected. In support of G1 telomeric DSB irreparability, we have shown that unresolved DDRs at telomeres following 10Gy IR are normal in terms of telomere length and TRF2 status. This result strongly favors the interpretation that unresolved telomeric DDRs represent broken, and not deprotected telomeres. This approach still has limitations. For instance, telomeric DSBs would be predicted to be very rare even following the high doses of IR, making the number of unrepaired telomeric DSBs difficult to explain probabilistically. Additionally, IR generates many of other types of DNA damage in much greater quantities than DSBs, and these other lesions may influence the response to nearby DSBs [61-64]. Therefore, additional lines of evidence are needed to conclusively determine whether telomere DSBs are irreparable in G1.

Experiments in which targeted DSBs were produced at telomeres using site-specific endonucleases have suggested that HR and BIR pathways are responsible for repair [8, 9]. However, these pathways are generally known to be active only in S/G2 [40, 65, 66]. In support of this idea, when individual cells with targeted telomeric DSBs were examined by immunofluorescence for evidence of a DDR (53BP1) or RAD51 mediated repair (HR and BIR), only the subset of cycling cells in S-phase were found to exhibit foci at telomeres [9]. Overall, this work is consistent with the idea that G1 telomeric DSBs may be irreparable. However, only a very limited number of DDR/repair-related activities have been evaluated at telomeric DSBs to date. Therefore, we set out to fully characterize the cellular response to telomeric DSBs in G1. To do so, we utilized a recombinant telomere-cutting endonuclease (ENT). ENT was chosen as it is the only telomere-targeted endonuclease with dual specificity for telomere DNA, i.e., both the endonuclease domain and the targeting domain (TRF1), recognize telomeric DNA [7]. Additionally, ENT expression caused an increase in telomere fragmentation and triggered HR/BIR activity at ALT telomeres, suggesting that it effectively induced telomeric DSBs in our hands. While the decrease in mean telomere restriction fragment was only on the order of about

5-10%, this was expected given the transient transfection efficiency of EJ-30 cells (20-30%), and the number of DSBs estimated from telomeric DNA damage responses (discussed below).

Western blots of EJ-30 cell lysates for phospho-ATM (s1981), and phospho-CHK2 (Thr68) indicated that transfection of ENT into non-ALT cells initiated a DDR. However, this was difficult to distinguish from the stress of transient transfection as transfection with TRF1 alone caused an induction of phosho-CHK2, though not as great as that caused by ENT. Similarly, exposure to lipofectamine impacted the cell cycle progression, as even apparently untransfected BJ1 hTERT cells were somewhat less likely to display pan-nuclear staining for Cyclin A or BrdU. To facilitate a more straightforward examination of the cellular response to telomeric DSBs in G1 cells, we turned to single cell analyses.

By examining individual immunostained cells, we characterized the telomeric DDR in G1. We found that ENT triggered a relatively normal DDR, including γ-H2AX and MDC1 induction at telomeres in G1 cells, on the order of 6-8 foci per cell. However, there was one important exception, 53BP1 foci were not observed in response to telomeric DSBs in G1 cells. Therefore, we speculated that some form of repair may occur at G1 telomeric DSBs, albeit without the need for 53BP1. 53BP1 is best known to promote cNHEJ by preventing DNA ends from being resected, but it has also been implicated in the long range movement associated with telomere-telomere fusion [21]. Interestingly, both Ku70/80 and DNA-PKcs are found at normal telomeres and like 53BP1, Ku70/80 is known to inhibit resection [22-24]. We reasoned that a lack of 53BP1 recruitment to telomeric DSBs in G1 may reduce the likelihood of telomere-telomere fusion, while the local availability of cNHEJ factors at telomeres may facilitate repair and reduce the need for 53BP1 to inhibit resection. In support of cNHEJ at telomeric DSBs, enhanced autophosphorylation of DNA-PKcs could be detected by western blot in EJ-30 cells transfected with ENT.

If cNHEJ was indeed responsible for repair of telomeric DSBs, DNA-PKcs kinase inhibition following ENT transfection would be expected to decrease telomere fragmentation, as

it results in preservation of DNA-PKcs at the break site and prevention of repair by cNHEJ, or any other pathway [39-41]. However, in EJ-30 cells treated with a DNA-PKcs kinase inhibitor, we found no difference in the level of telomere fragmentation by southern blot. Additionally, if our hypothesis regarding the functional substitution of 53BP1 by local availability of cNHEJ factors was correct, one might expect recruitment of 53BP1 in the absence of cNHEJ factors. However, following stable shRNA knockdown of DNA-PKcs in BJ1hTERT cells, 53BP1 recruitment to G1 telomeric DSBs was still not observed. These results suggest that cNHEJ does not repair telomeric DSBs and further, that DNA-PKcs does not regulate 53BP1 recruitment to G1 telomeric DSBs

Alternatively, we hypothesized that telomere end-protection machinery such as the shelterin complex, may prevent 53BP1 recruitment to G1 telomeric DSBs. To explore this possibility, we manipulated telomere end-protection function without completely disrupting it, such that we might alleviate inhibition of 53BP1 recruitment to telomeric DSBs and avoid promoting the TIF response at undamaged telomeres. As a near complete siRNA knockdown of TRF2 is necessary to achieve a TIF response [45], we utilized an siRNA sequence that resulted in partial, sub-TIF-inducing, knockdown of TRF2 and combined this with ENT or TRF1 transfection in BJ1 hTERTs. While partial TRF2 knockdown did not result in a TIF response in untransfected cells, it also did not alleviate the inhibition of 53BP1 recruitment to G1 telomeric DSBs. Additionally, TRF2 siRNA did not affect telomere fragmentation in ENT transfected EJ-30 cells, indicating that this treatment did not impact telomere repair. These results do not completely rule out the possibility that TRF2 is responsible for inhibition of 53BP1 recruitment to G1 telomeric DSBs, but it may be difficult to relieve this inhibition without deprotection of telomeres.

Compaction of telomeric chromatin was recently proposed as a unifying physical mechanism by which shelterin protect telomeres from repair [47-49]. Therefore, we tested whether decompaction of genomic DNA could alleviate the repression of 53BP1 recruitment to

G1 telomeric DSBs. However, neither treatment of cells with Trichostatin A, as histone deacetylase inhibitor, nor exposure of cells to a hypotonic solution, resulted in recruitment of 53BP1 to G1 telomeric DSBs. Similar to our results with partial TRF2 knockdown, chromatin relaxation treatments did not cause a TIF response at normal telomeres. These results may suggest that an unknown mechanism prevents 53BP1 recruitment to G1 telomere DSBs, or simply that full telomere deprotection is required to relieve the inhibition of 53BP1 recruitment to G1 telomeric DSBs.

Both 53BP1 recruitment and cNHEJ impede DSB repair associated DNA resection. Since neither could be detected in response to G1 telomeric DSBs, we hypothesized that G1 telomeric DSBs may be subject to resection. Following induction of telomeric DSBs in G1, we detected sstelomeric DNA, RPA70 and Phospho-RPA32, indicating resection at G1 telomeric DSBs. Such resection was found to be bidirectional, as sstelomeric DNA could be detected on both strands, as well as extensive, as the detection limit of FISH is on the order of 0.5 Kb [67]. Further, we found that resection at G1 telomeric DSBs was dependent on conventional DSB resection machinery, including MRE11 and EXO1, but not on the telomere specific exonuclease, Apollo, which promotes ssoverhang generation at leading-strand telomeres [31, 32].

The most obvious explanation for these findings is that long-range resection-dependent repair occurs at G1 telomeric DSBs. However, we found no evidence for long-range resection-dependent repair, as neither RAD51 (HR/BIR), RAD52 (BIR/SSA), nor BrdU incorporation (HR/BIR), was detected at G1 telomeric DSBs. Alt-NHEJ is not a likely candidate for G1 telomeric DSB repair, as it utilizes only a few bases of homology (~20) [68, 69], and it is prevented by RPA binding to ssDNA [70].

It remains possible that some as of yet undescribed pathway repairs G1 telomeric DSBs. Most likely would be some form of RAD52-independent SSA, as SSA was recently shown to take place in RAD52$^{-/-}$ cells [71], and is the only form of long-range resection-dependent repair

that does not inherently require DNA synthesis [55]. However, we favor the possibility that G1 telomeric DSBs undergo extensive resection, but are not repaired. One plausible reason for this is that telomeric DSBs require reconstitution of G-rich single-stranded overhangs in order to prevent 53BP1 and cNHEJ, both of which would be expected to promote telomere-telomere fusion (Fig 14). Therefore, resection may serve to stabilize broken telomeres during G1. This idea is supported by the fact that naturally shortened telomeres do not undergo fusion until nearly all $TTAGGG_n$ repeats have been lost, suggesting that telomeres of nearly any length can be protected from repair activity [72]. Further, sstelomere overhangs have been implicated in protection from repair at normal telomeres [73]. As an extension of this model, resected telomeric DSBs may simply wait for S-phase, where telomeres shortened by DSBs may be extended via telomerase or recombination (Fig. 14).

In summary, consistent with the view of repressed repair at telomeric DSBs, here we provide strong evidence that telomeric DSBs are not repaired by any currently recognized repair pathway in G1. Even so, we find that G1 telomeric DSBs initiate at least a partial DDR, which may facilitate the observed extensive resection, without allowing for the activation of repair. We speculate that rather than for repair, such resection at G1 telomeric DSBs likely serves the purpose of telomere end stabilization and repair avoidance. These results have implications for both basic telomere and DNA repair research. Also intriguing is the possibility of exploiting the vulnerability of broken/damaged telomeres to improve radiation therapy strategies.

Future Directions

Telomeres damaged by global DNA damaging agents recruit 53BP1, whereas we find that 53BP1 recruitment does not occur at G1 telomeric DSBs induced by targeted enzymatic means, which instead undergo resection. It's possible that the complexity of telomeric DSB itself may influence repair outcomes and cellular consequences of G1 telomeric DSBs. Such a

possibility could be investigated using the recently described Killer Red (KR), a modified fluorescent protein, capable of producing site-specific reactive oxygen species (ROS), which, when fused to TRF1 (KR-TRF1), is sufficient to cause clustered lesions specifically at telomeres and including DSBs [74].

Of importance as well, we suspect that G1 telomeric repair avoidance protects telomeric DSBs from highly deleterious repair activities, such as telomere-telomere fusion. Testing this model is the subject of ongoing work. In particular, inhibition of resection is expected to promote 53BP1 foci and cNHEJ at telomeric DSBs and we suspect that this will result in telomere-telomere fusions, thus providing an explanation for why telomeric DSBs are not repaired by cNHEJ. For telomere-telomere fusion analysis we have attempted to achieve metaphase spreads of cells expressing ENT following transient transfection, however our efforts have been thwarted by an extremely low proportion of transfected cells making it to metaphase. Therefore, we are currently establishing a cell line that stably expresses ENT from which telomere fusions and other telomeric cytogenetic abnormalities may be evaluated.

Finally, we wish to investigate whether unrepaired telomeric DSBs can undergo delayed repair or elongation during S-phase, and whether normal telomere length can be reestablished following telomeric DSBs. To this end, cells expressing ENT may be synchronized at the G1/S boundary followed by release into S-phase and monitoring of telomere fragmentation over time.

Significance

The work presented here supports the idea that telomeric DSBs are not repaired in the G1 phase of the cell cycle. These findings are relevant to the field of DNA repair, which typically assumes that all DSBs are engaged by some form of repair, and may provide an explanation as to why a small fraction of DSBs appear to go unrepaired following global DNA damage [2, 3]. Additionally, results suggest some sort of G1 repair avoidance, potentially involving generation

of ssDNA by resection, which may be specific to telomeres, or possibly, a more general phenomenon. As such, other repetitive regions of the genome, which are plentiful (e.g. microsatellite DNA, centromeres, rDNA), may also be expected to exhibit similar G1 repair avoidance.

The cellular consequences of unrepaired DSBs are an important consideration as well. Studies utilizing global DNA damaging agents show that unresolved telomeric DDRs result in senescence, suggesting that telomeric DSBs may disproportionately contribute to aging and associated degenerative pathologies [2, 3]. Therefore, treatment strategies that facilitate resolution of telomeric DSBs, may be of clinical relevance, particularly in tissues with a large proportion of post mitotic cells, such as the heart and brain. Finally, unrepaired telomeric DSBs within G1 may help to explain why post mitotic cells still undergo telomere shortening [74, 75].

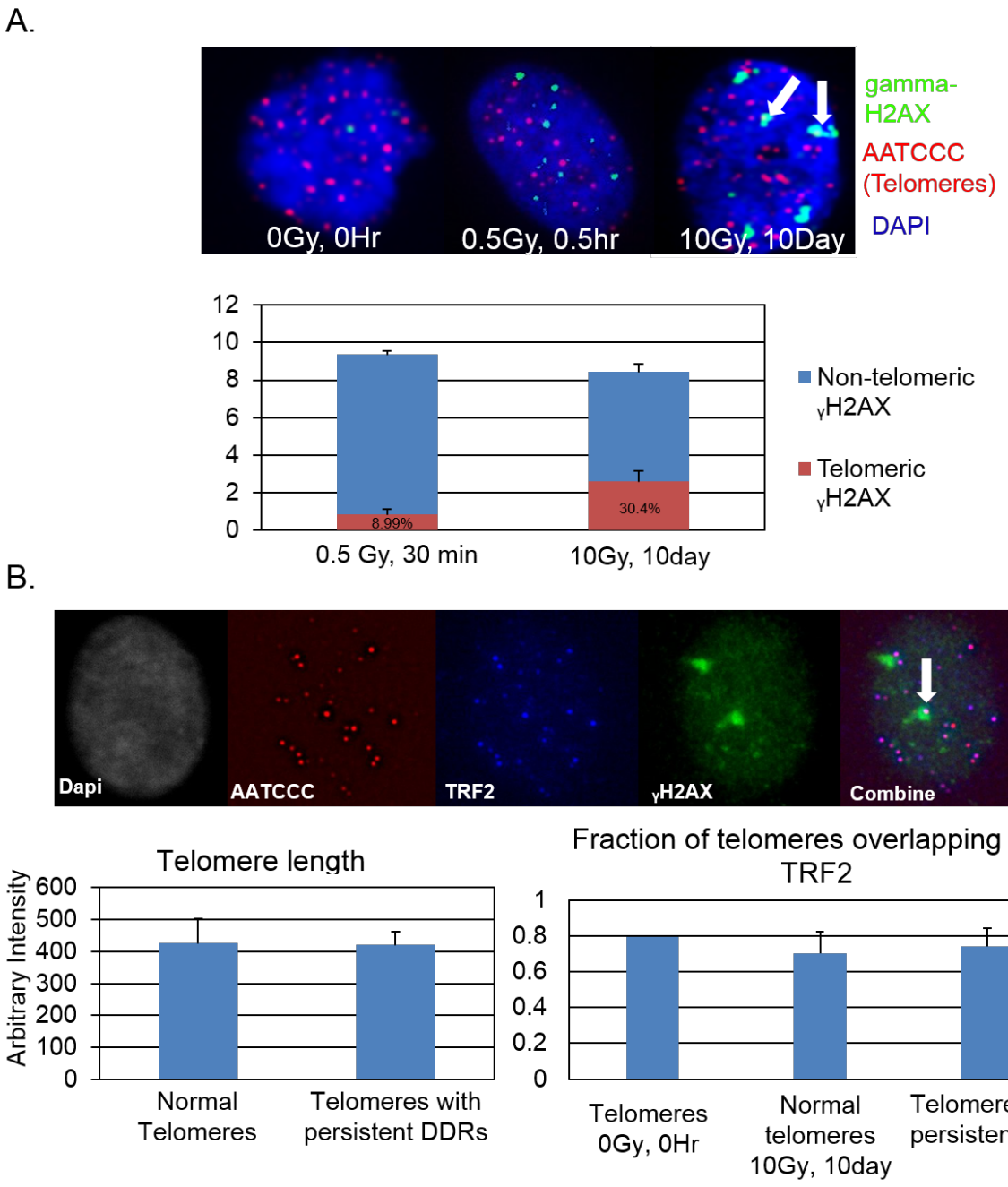


Figure 2: While the overall number of γ-H2AX foci was similar, an increased fraction were found to localize with telomeric DNA 10 days after 10Gy IR compared to 30 minutes after 0.5 Gy (A). Persistent telomeric γ-H2AX foci occurred at telomeres of normal length and TRF2 binding as measured by fluorescence intensity of telomere FISH probes or TRF2 immunostaining in compressed Z-stacks (B).

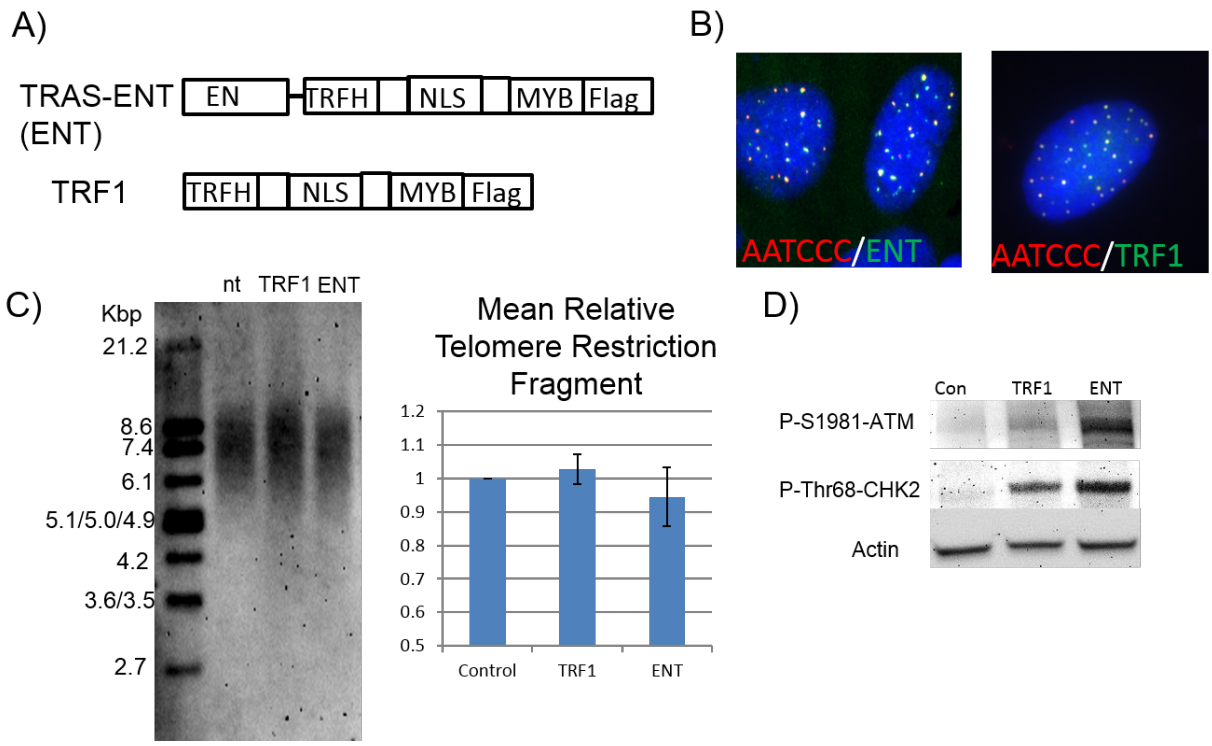


Figure 3: Schematic of the telomere cutting endonuclease TRAS-ENT (ENT) and TRF1 (A). Overexpressed ENT or TRF1 (flag tag) exhibited co-localization with telomere repeats (FISH signal) in BJ1 hTERT, EJ-30 and U20S cells (B). Expression of ENT in EJ-30 cells causes fragmentation of telomere DNA on a southern blot of telomeric restriction fragments (C), and activates DDR signaling including P-S1981-ATM and P-Thr68-CHK2 (D).

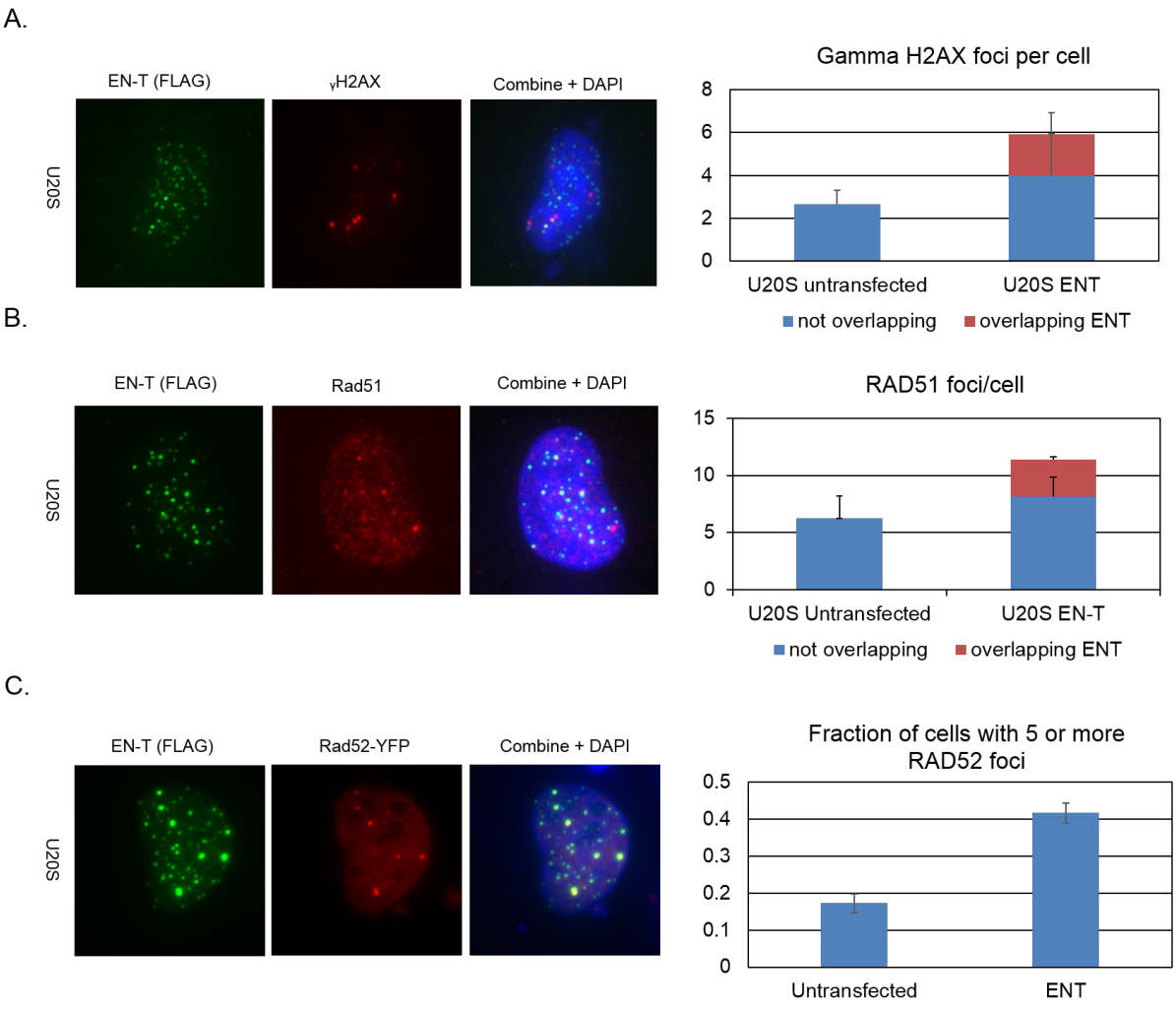
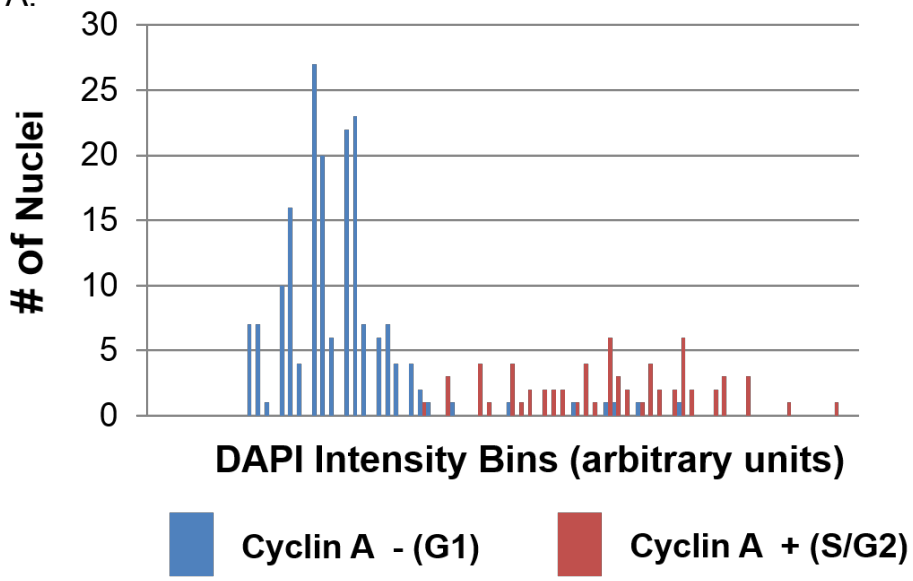


Figure 4: Transfection of ENT into U20S cells (ALT) stimulated a telomeric DDR in terms of γ-H2AX foci, which frequently overlapped with ENT-flag (A). Induced telomeric DSBs in U20S cells stimulated production of both RAD51 and RAD52 foci, mediators of HR and BIR respectively (B,C), and again, showed frequent overlapping with ENT-flag.

A.



B.

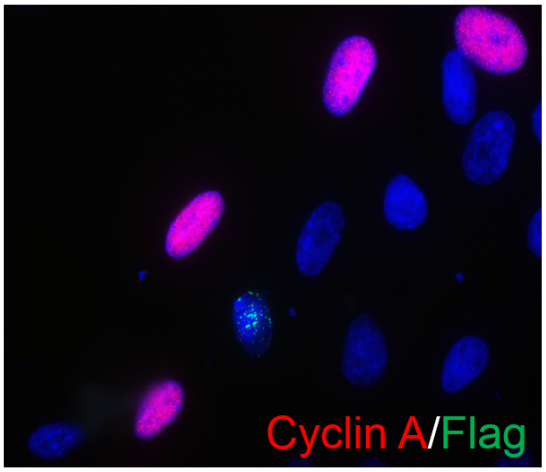


Figure 5: DAPI intensity histograms were generated from 63X images of roughly 300 cells for all experiments involving EJ-30 cells (A). Exclusion of Cyclin A (red bars, S/G2 cells) from the low DAPI intensity peak region of the histogram verified that these cells were in the G1 phase of the cell cycle (A). DAPI intensity histograms were not necessary to identify G1 BJ1 hTERT cells as ENT or TRF1 transfected BJ1 hTERTs were almost all negative for Cyclin A (and BrdU, not shown), indicating that they were highly enriched in G1 48 hours post transfection (B).

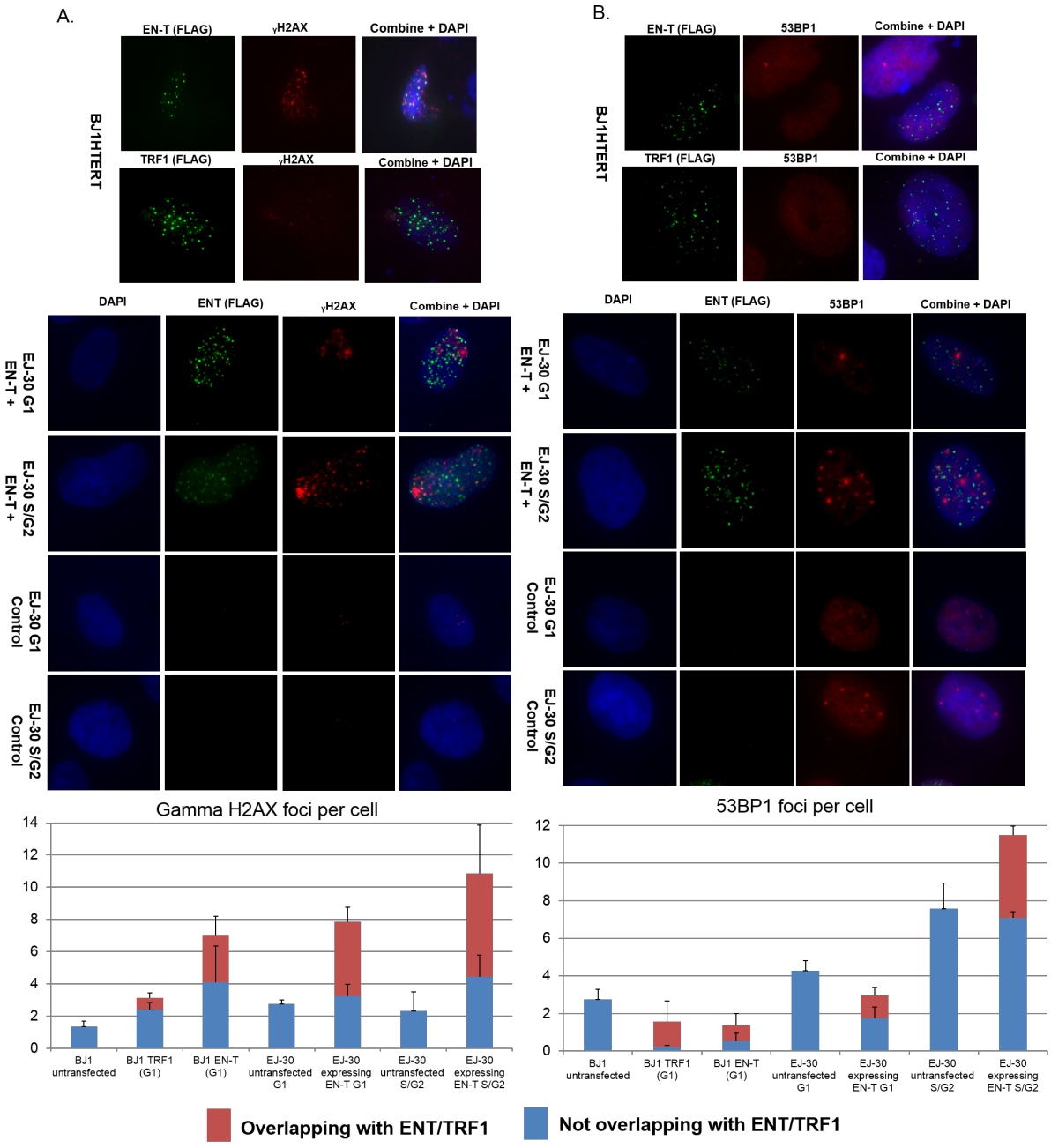


Figure 6: Cells transfected with ENT or TRF1 were immunostained for γ-H2AX and 53BP1 (A, B). γ-H2AX foci were induced and enriched at telomeres following transfection with ENT in G1 cells (BJ1 hTERT and EJ-30), as well as S/G2 cells (EJ-30), and often overlapped with ENT-flag (A). A similar induction of 53BP1 foci following ENT transfection in S/G2 EJ-30 cells was observed, which did not occur in BJ1 hTERT or EJ-30 G1 cells (B).

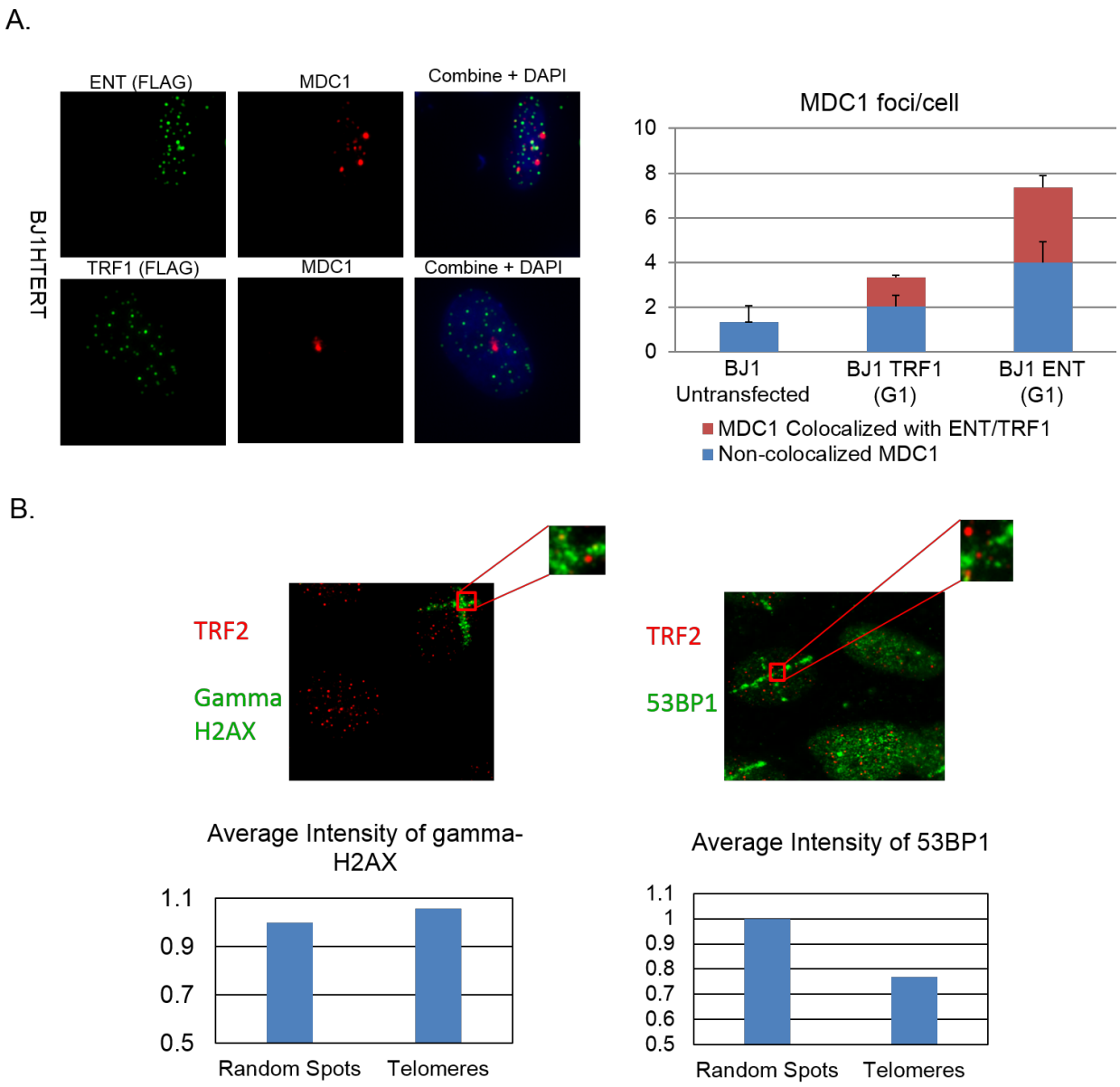


Figure 7: Transfection with ENT resulted in an increased number of MDC1 foci in BJ1 hTERT cells that often overlapped with ENT-flag (G1) (A). The intensity of γ-H2AX within microirradiation stripes was similar at telomeres and random spots within BJ1 hTERT cells (B). The intensity of 53BP1 within microirradiation stripes was decreased at telomeres relative to random spots (B).

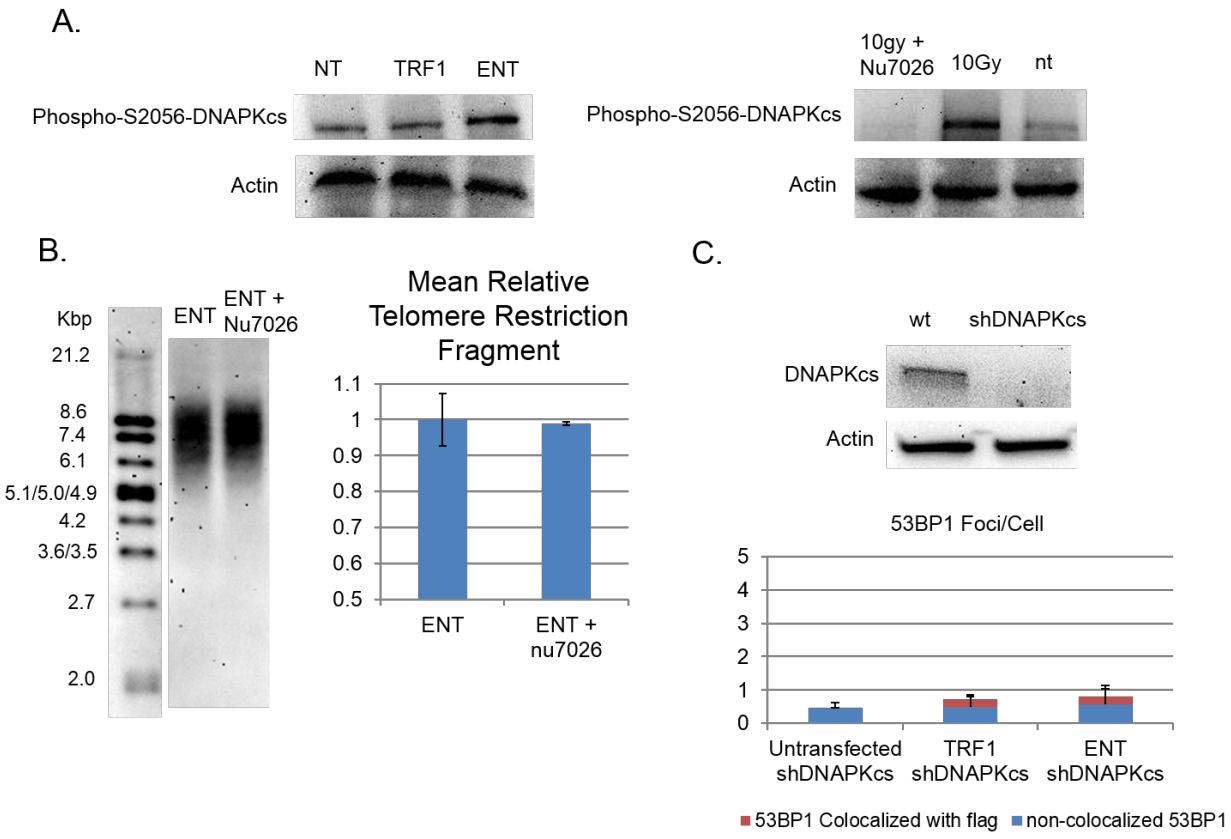


Figure 8: Autophosphorylation of DNA-PKcs at S2056 was induced following ENT transfection in EJ-30 cells (A). DNA-PKcs autophosphorylation following 10Gy IR was prevented with exposure of cells to a specific kinase inhibitor (NU7026) (A). Exposure to NU7026 did not influence mean TRF length in EJ-30 cells transfected with ENT (B). Further, shRNA knockdown of DNA-PKcs did not promote 53BP1 recruitment to telomeric DSBs in ENT transfected BJ1 hTERTs (C).

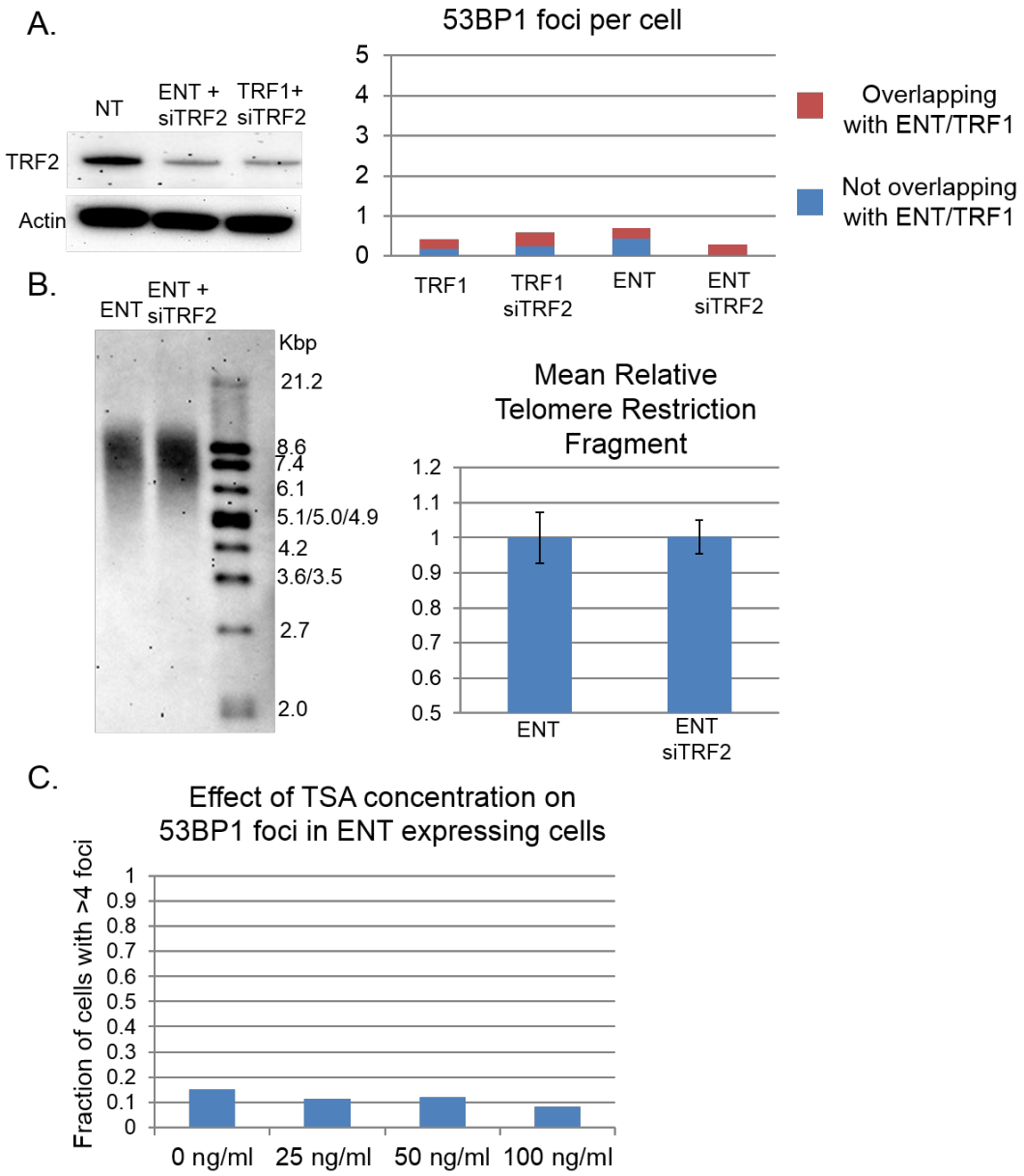


Figure 9: Partial siRNA knockdown of TRF2 (50-75%) did not promote 53BP1 recruitment to telomeric DSBs in BJ1 hTERT cells transfected with ENT (A). TRF2 knockdown did not impact mean TRF size (B). Exposure of cells to a range of concentrations of Trichostain A (TSA), also did not impact 53BP1 recruitment to telomeric DSBs in BJ1 hTERT cells (C).

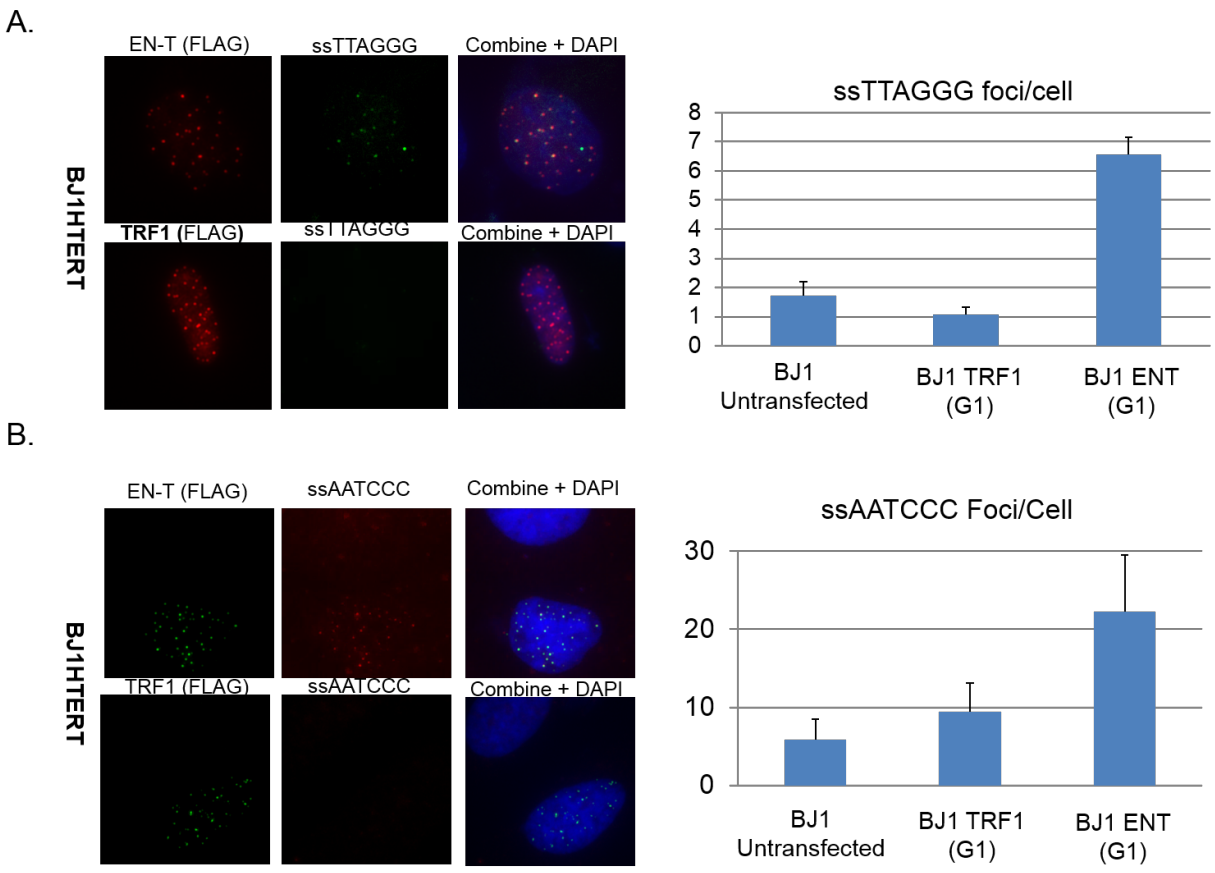


Figure 10: Transfection of BJ1hTERT cells with ENT promoted production of ssDNA on both the G-rich telomere strand (TTAGGG) (A), and the C-rich telomere strand (AATCCC) (B), consistent with bidirectional resection at G1 telomeric DSBs

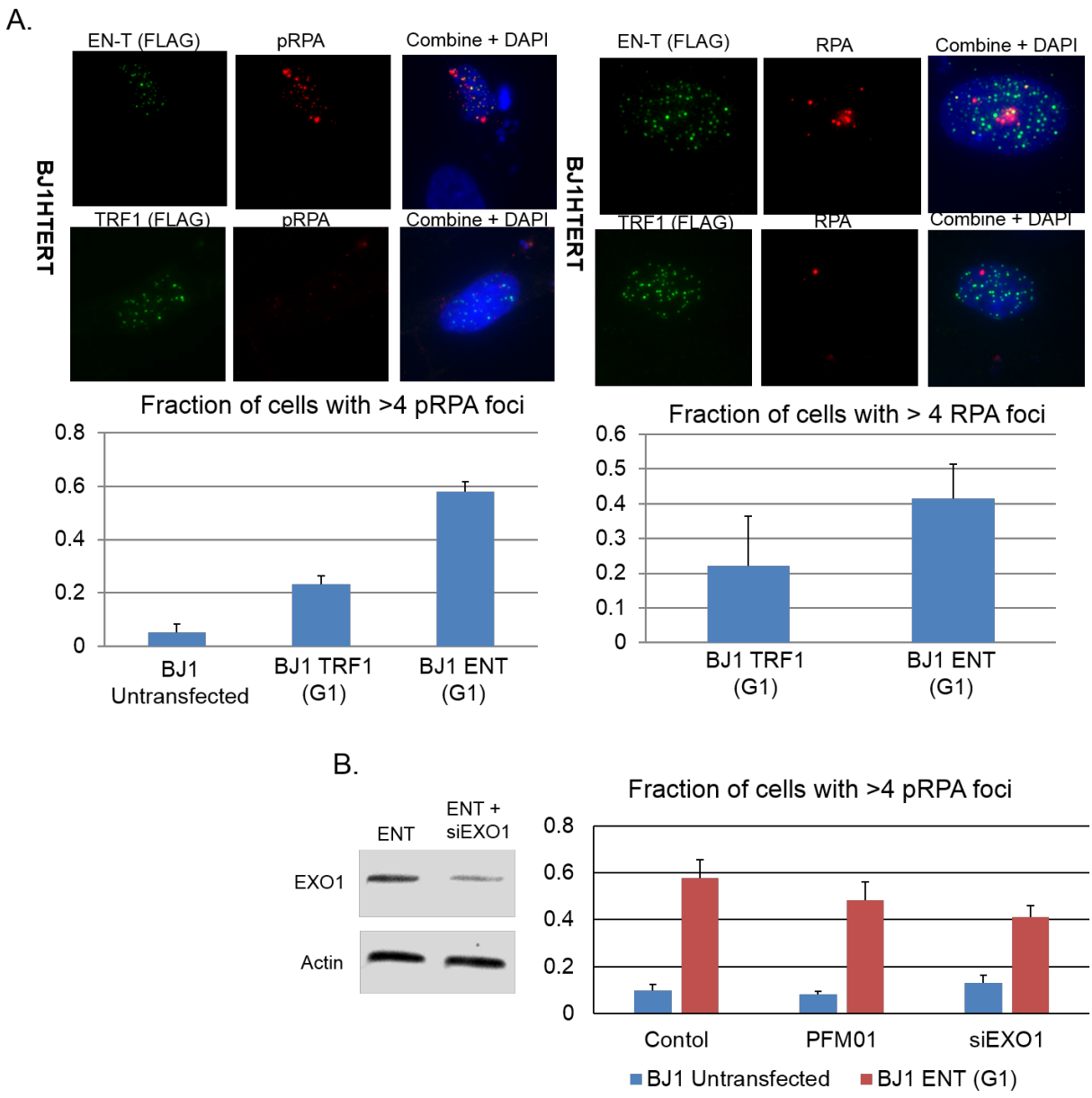


Figure 11: ENT expression induced both phospo-RPA32 and RPA70 foci that overlapped with ENT-flag (A). Phospho-RPA32 induction following ENT transfection was reduced by either inhibition of MRE11 endonuclease activity (PFM01) or partial siRNA knockdown of EXO1 (b).

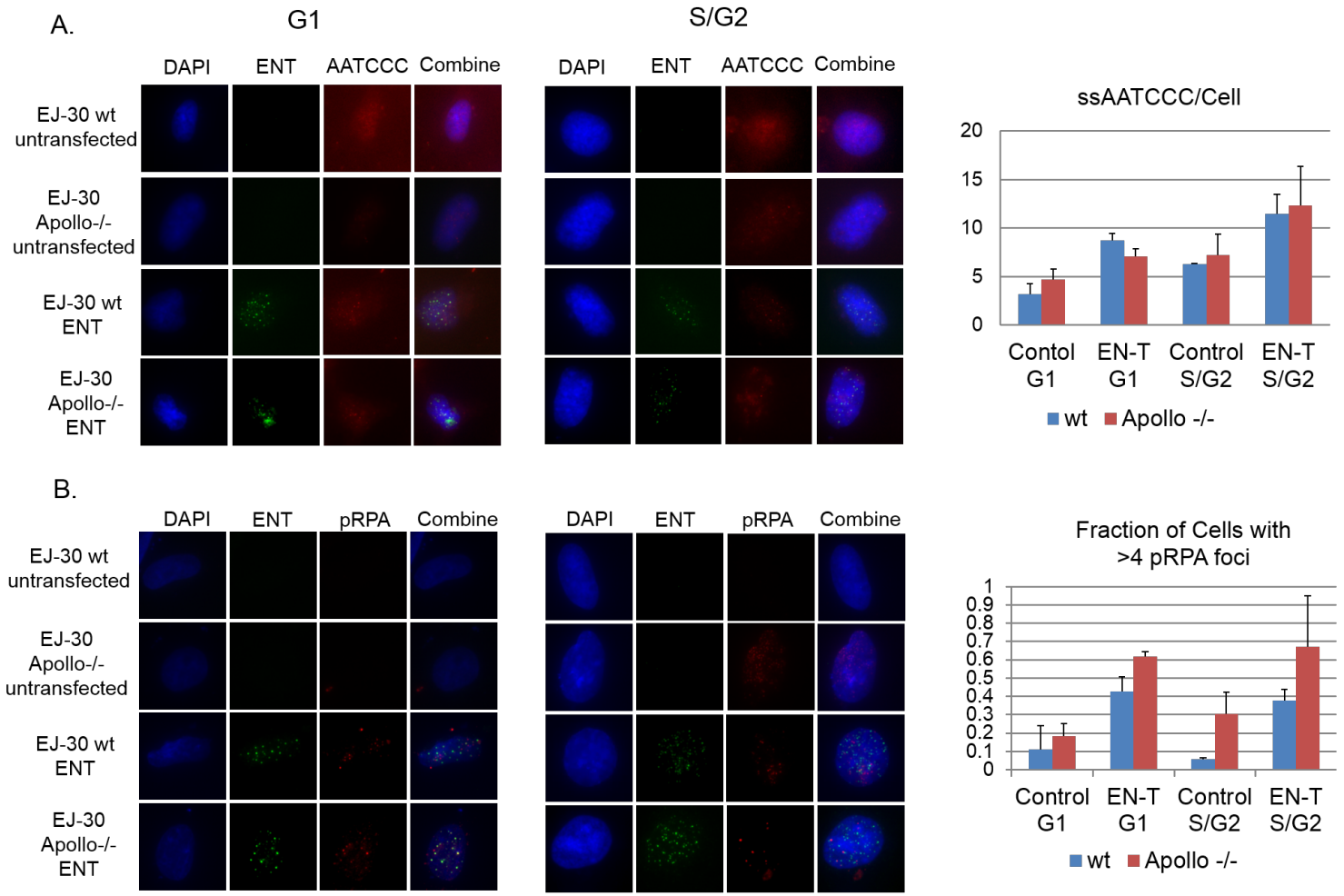


Figure 12: The role of Apollo endonuclease in the generation of ssDNA at telomeric DSBs was investigated. While sstelomeric DNA (AATCCC) was slightly reduced in ENT expressing Apollo$^{-/-}$ G1 EJ-30 cells relative to ENT expressing wild type (wt) EJ-30 cells, phospho-RPA32 foci were somewhat reduced by Apollo KO in G1 cells (A, B). These results suggest that Apollo does not have a major role in resection at G1 telomeric DSBs. Further, in S/G2 EJ-30 cells expressing ENT, Apollo KO, increased both sstelomeric DNA and phospho-RPA32 foci (A, B)

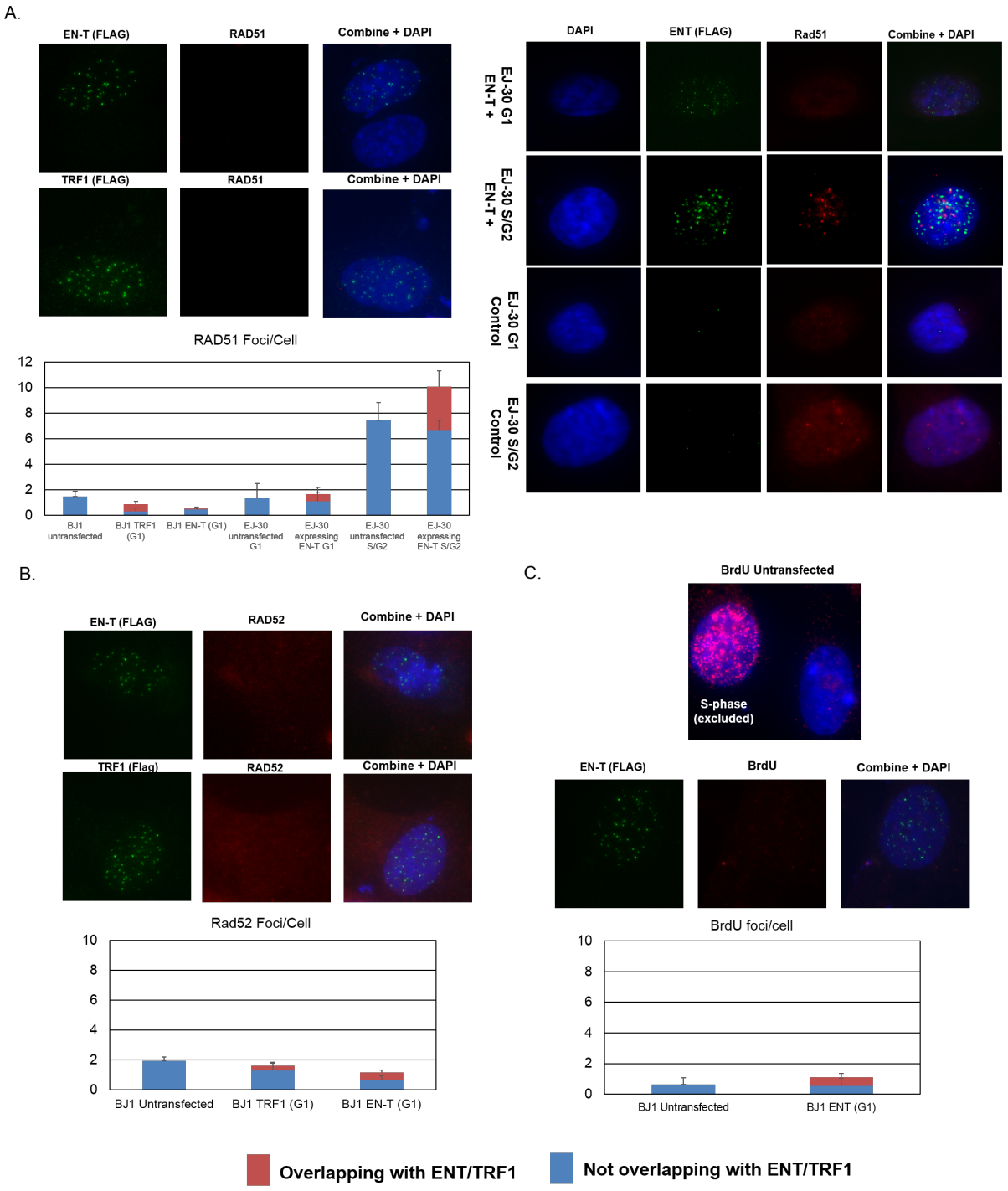


Figure 13: Transfection of G1 BJ1 hTERTs or EJ-30 cells with ENT did not induce RAD51 foci (A). However, RAD51 was induced in S/G2 EJ-30 cells following expression of ENT (A). Further, RAD52 was not induced in G1 BJ1 hTERTs (B). Following 2 hours pulse labeling with BrdU, immunostaining for BrdU produced pan-nuclear staining in a subset of untransfected cells (S-phase), but not in BJ1 hTERT cells transfected with ENT (C). Repair associated DNA synthesis (individual BrdU foci) was not observed in G1 BJ1 hTERTs (C).

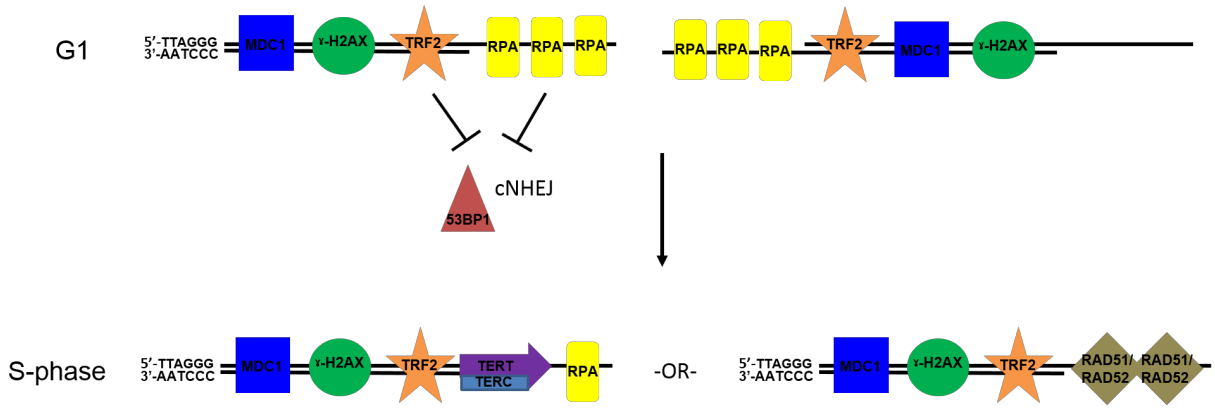


Figure 14: G1 telomeric DSBs do not recruit 53BP1, nor do they undergo cNHEJ. Extensive bidirectional resection does occur, producing sstelomeric overhangs coated with RPA, which are not involved in HR/BIR/SSA-related resection dependent repair (consistent with suppression of long-range resection-dependent repair in G1). We speculate that RPA coated sstelomeric DNA functions to prevent 53BP1 recruitment, thereby hindering cNHEJ as well (primary DSB repair pathway in G1). In this model, G1 telomeric DSBs are prevented from engaging in repair activities, being resected possibly to enable end-protection until they can be repaired/extended during S-phase by telomerase or recombinational repair.

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LIST OF ABBREVIATIONS

^{137}Cs	Cesium 137
53BP1	p53-Binding Protein 1
ALT	Alternative Lengthening of Telomeres
NHEJ	Alternative Non-Homologous End-Joining
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia-Telangiectasia and Rad3-Related Protein
BER	Base Excision Repair
BFB	Breakage-Fusion-Bridge
BIR	Break-Induced Replication
BRCA1	Breast Cancer Susceptibility 1
BRCA2	Breast Cancer Susceptibility 2
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CAS9	CRISPR associated protein 9
CDK	Cyclin Dependent Kinase
CHK1	Checkpoint Kinase 1
CHK2	Checkpoint Kinase 2
CMV	Cytomegalovirus
cNHEJ	Canonical Non-Homologous End-Joining
CO-FISH	Chromosome Orientation Fluorescence In-situ Hybridization
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CtIP	C-Terminal Binding Protein Interacting Protein
DDR	DNA Damage Response

DIG	Digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DNA2	Deoxyribonucleic Acid
DNA-PKcs	DNA Protein Kinase Catalytic Subunit
DSB	Double Strand Break
DSBR	Double-Strand Break Repair
ENT	TRAS1-EN-TRF1
EXO1	Exonuclease 1
FBS	Fetal Bovine Serum
FISH	Fluorescence In-situ Hybridization
HJ	Holliday Junction
HR	Homologous Recombination
HRP	Horseradish Peroxidase
iDDR	Inhibition of DNA Damage Response
IR	Ionizing Radiation
Kb	Kilobase
Lig IV	Ligase IV
MDC1	Mediator of DNA Damage Checkpoint 1
MEF	Mouse Embryonic Fibroblast
MPER	Mammalian Protein Extraction Reagent
MRN	MRE11-RAD50-NBS1
NFDM	Non-Fat Dry Milk
NGS	Normal Goat Serum
NOS	Reactive Nitrogen Species

OB	Oligosaccharide/Oligonucleotide Binding
PAGE	Polyacrylamide Gel Electrophoresis
PALB2	Partner and Localizer of BRCA2
PARP1	Poly(ADP-Ribose) Polymerase 1
PIF1	Petite Integration Frequency 1
PIKK	Phosphatidylinositol 3-Kinase-Related Kinases
Pol θ	DNA Polymerase Theta
POLD3	DNA Polymerase Delta 3, Accessory Subunit
POLD4	DNA Polymerase Delta 4, Accessory Subunit
polδ	Polymerase Delta
POT1	Protection of Telomeres
PVDF	Polyvinylidene Difluoride
RAP1	Repressor/Activator Protein 1
rDNA	Ribosomal Deoxynucleic Acid
RIF1	Replication Timing Factor 1
RNA	Ribonucleic Acid
RNF168	Ring Finger Protein 168
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RPA32	Replication Protein A 32 Kilodalton Subunit
RPA70	Replication Protein A 70 Kilodalton Subunit
SDSA	Synthesis-Dependent Strand-Annealing
shRNA	Short Hairpin Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
ss	Single Strand

SSA	Single-Strand Annealing
TERC	Telomerase Ribonucleic Acid Component
TERRA	Telomeric repeat-containing RNA
TERT	Telomerase Reverse Transcriptase
TIF	Telomere Dysfunction Induced Foci
TIN2	TRF1-Interacting Nuclear Protein 2
TP53	Tumor Protein p53
TPP1	Tripeptidyl Peptidase 1
TRF	Telomere Restriction Fragment
TRF2	Telomere Repeat Factor 2
TSA	Trichostatin A
T-SCE	Telomere Sister Chromatid Exchange
UV	Ultraviolet Radiation
WT	Wild Type
XLF	XRCC4-like factor
XRCC4	X-Ray Repair Cross Complementing 4
YFP	Yellow Fluorescent Protein