DISSERTATION

# Characterization the Role of Telomeric RNA (TERRA) in Telomeric DNA Double-Strand Break (DSB) Repair in Human ALT (Telomerase independent) Cells

Submitted by

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# ABSTRACT

Telomeres are specialized nucleoprotein complexes that protect natural chromosomal termini from degradation and prevent their detection as of DNA damage. Therefore, telomeres play critical roles in maintaining genomic stability. Telomeres are composed of tandem arrays of conserved repetitive sequences (TTAGGG in vertebrates), bound by a suite of proteins collectively termed "shelterin". Shelterin proteins are essential for telomere length regulation and end-capping structure/function. Due to their repetitive nature and together with telomeres possessing an abundance of heterochromatic marks, telomeres have long been regarded as silenced, non-transcribed features of the genome.

The relatively recent discovery of telomeric RNA (TElomere Repeat- containing RNA; TERRA) opened many new avenues of investigation. TERRA is a long, noncoding RNA (lncRNA) that serves a structural role at telomeres, as well as function in regulation of telomere length and telomerase activity, the specialized reverse transcriptase capable of elongating telomeres *de novo*. Further, TERRA participates in telomeric recombination in tumors that maintain telomere length in a telomerase independent fashion via the Alternative Lengthening of Telomeres (ALT) pathway. Emerging evidence also supports telomeric “DNA- TERRA hybrids” as indispensable for end protection and capping function; e.g., RNA interference mediated depletion of TERRA induced telomeric DNA damage responses (DDRs) and aberrations. Thus, TERRA participates in facilitating telomeric recombination and in preventing inappropriate telomeric DNA damage responses. We hypothesized that TERRA plays a critical role in the repair of telomeric DNA damage. To address the intriguing possibility that TERRA plays a role in the telomeric DNA damage response, we evaluated the colocalization of TERRA and γ-H2AX, a well-accepted

marker of double-strand breaks (DSBs), at broken telomeres in human Osteosarcoma U2OS-ALT cells in different phases of the cell cycle.

To test our hypothesis, we generated U2OS-ALT cells that stably expressed FUCCI green fluorescent signals to label cells in G2 phase. Telomeric DSBs were then induced in FUCCI-U2OS cells utilizing the ENT endonuclease fused to Telomere Repeat Factor 1 TRF1 (ENT-TRF1), and validated via colocalization of telomeres with γ-H2AX-FLAG. Forty-eight hours following transfection, FUCCI-U2OS cells were also treated with EdU to label cells in S phase. Cells negative for both FUCCI and EdU identified cells in G1 phase. Using this powerful strategy to distinguish cells in G1, S and G2 phases of the cell cycle, we showed that FUCCI-U2OS cells accumulate in G2 phase following transient transfection with ENT-TRF1. We validated that expression of ENT-TRF1 generates telomeric DSBs in U2OS-ALT cells through detection of telomere- γ-H2AX-FLAG colocalization events. Importantly, our data revealed that telomeric DSB induction triggers enrichment of TERRA in G2 phase. Taken together, these observations suggested that TERRA is increased in cells transfected with ENT-TRF1; i.e., in U2OS cells harboring telomere-specific DSBs.

TERRA recruitment to telomeric DSB damage sites in G2 was validated by assessing co-localization between TERRA and ENT (FLAG). Similarly, TERRA recruitment to telomeric DSBs in G1/S was also evaluated. Futhermore, non-denaturing Telomere DNA FISH was employed to visualize G-rich and C-rich single-stranded (ss)telomeric DNA. Treatment of U2OS ENT transfected cells with Rnase A and Rnase H to remove TERRA, uncovered elevated levels of resected 5’ C-rich (ss)telomeric DNA (complementary TERRA sequence), suggesting a potential role for TERRA in protecting resected telomeric DNA prior to cells entering G2 phase where Homologous Recombination (HR)-mediated elongation/repair would be possible.

Consistent with published reports, telomeric DSBs were also significantly induced in cells transfected with TRF1-only (positive control). However, although TERRA co-localized with FLAG/broken telomeres, resected (ss)telomeric DNA was not detected upon removal of TERRA. Therefore, our results further support induction of telomeric DSBs with overexpression of TRF1, and additionally indicate that they are repaired via a different pathway than those induced by ENT, potentially alternative End Joining (alt-EJ), as previously proposed.

In conclusion, our work revealed for the first time that the telomeric RNA TERRA, is enriched specifically at telomeric DNA DSB sites in U2OS (ALT) cells.

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Above all, I thank my God for watching over me through all the difficulties.

# DEDICATION

*I dedicate this work to*

*My parents who have scarified their lives for my siblings and myself*

*,and my son Abe, who taught me to enjoy the journey more than the end distention*

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# CHAPTER 1 INTRODUCTION

## Telomere structure and function

### *Telomere structure*

Telomeres are specialized nucleoprotein structures at the ends of linear chromosomes, composed of tandem arrays of repetitive sequence (5'-TTAGGG-3' in mammalian cells) [2] [3], and associated with a variety of proteins, six of which are collectively called shelterin [4]. While telomere-specific proteins Telomere Repeat Factor 1 (TRF1) and Telomere Repeat Factor 2 (TRF2) bind double-stranded telomeric DNA as homodimers [5-7], Protection of Telomeres 1 (POT 1) binds only single-stranded telomeric G-rich DNA [8]. Repressor-activator protein 1 (RAP1) binds telomeres via its interaction with TRF2 [9]. TRF1 and TRF2/RAP1 are linked through TRF1-Interacting Nuclear protein 2 (TIN2) [10]. TIN2- and POT1-organizing protein (TPP1) connects POT1 and TIN2 proteins [11]. A recent study discovered an additional regulator of telomere length, the telomeric zinc finger-associated protein (TZAP), which binds telomeric G-rich strands of long telomeres and trims them [12]. Telomeres can range in size from ~1Kbp-15Kbp [13].

Structural studies using Electron Micrographs in human cells revealed that chromosomal termini form large telomeric loops (T-loop), where the top single-strand (3' G rich overhang) loops back and hybridizes to the telomeric double-stranded DNA. The invading double-stranded telomeric DNA with G-rich single strand also creates a smaller Displacement loop (D-loop; **Figure 1.1**) [14-16]. In addition to T-loops, Nuclear Magnetic Resonance Spectroscopy (NMR) and X-ray crystallography studies support G-quadruplexes as a secondary telomeric structure in human cells. Each G-quadruplex consists of four planes of Guanines linked by Hoogsteen hydrogen bonds [17-19].

Due to the repetitive nature of telomeres, they were long regarded as silent heterochromatin. However, the discovery of long non-coding (lnc)RNA Telomeric Repeat-containing RNAs (TERRA 5'-UUAGGG-3') recently negated this notion [20-22]. It has also been observed that TERRA can directly bind telomeres via the C-rich strand, forming TeloDNA:TERRA hybrids, or indirectly by way of association with shelterin proteins [23, 24]. There is also a fraction of TERRA not bound to telomeres, or free TERRA [25] .

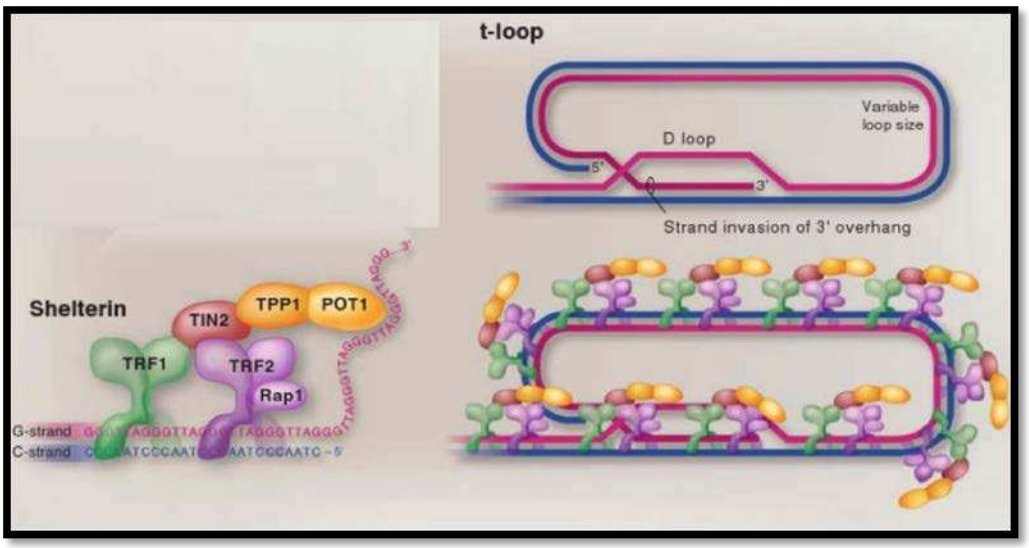



Figure 1.1: A schematic of mammalian telomere structure.

Homodimers of TRF1 and TRF2 bind directly to double-stranded telomeric DNA, while POT1 binds to single-stranded G-rich telomeric overhangs. Shelterin proteins interact with the telomere sequences indirectly via protein-protein interactions (lower left). The 3' G-rich overhang invades the telomeric duplex, forming a t-loop and D- loop (Upper right) [26].

### *Telomere Function*

Telomeres serve to solve two main problems resulting from the linearity of eukaryotic chromosomes; the challenge to replicate the very end of linear DNA molecules (the end replication problem) [27], and the threat of activating a DNA Damage Response (DDR) upon misidentifying the end of chromosomes as a double-strand break (DSB; the end protection problem) [26, 28].

### *The End Replication Problem*

In order to synthesize a new DNA strand from the parental strands, RNA primase binds the 3' end of the parental leading strand to provide a primer (short RNA sequence) with a free 3' OH group. DNA polymerase III requires that free 3' OH group on the primer to add new deoxyribose nucleotides dNTPs spontaneously in the 5' to 3' direction toward the replication fork. Due to the nature of the antiparallel double stranded DNA, RNA primase binds various sites along the parental lagging strand to provide small pieces of primers with 3' OH groups so DNA polymerase can add new dNTPs in the 3' direction in between the primers. Eventually, the RNA primers are removed and replaced with DNA sequences using DNA polymerase I [29, 30].

The DNA end -replication problem arises at the very end of telomeres after removing the 5' distal primer on the lagging-strand, which cannot be replaced with DNA sequence due to the lack of 3'OH group. Consequently, with every cell division telomere length progressively shortens by 30-200 base pairs [26, 31, 32]. Telomere shortening has been strongly associated with aging and age-related diseases [33]. Although the end replication problem is unavoidable, telomere shortening can be offset by telomerase, a specialized DNA polymerase capable of adding telomere sequence *de novo* onto newly replicated ends. However, telomerase is only sufficient to maintain telomere length in stem, germ-line, and cancer cells [34, 35].

### *Telomerase*

The discovery of telomerase in Tetrahymena contributed to better understanding of how cells maintain the length of telomeres [36]. Structurally, telomerase is a ribonucleotide enzyme comprised of three main subunits. First, the catalytic Telomere Reverse Transcriptase (TERT) protein encoded by the TERT gene in humans (hTERT). The second subunit is a long non-coding RNA called Telomerase RNA Component (TERC) with a sequence complementary to the G rich

telomeric strand (3'-AAUCCC-5'). A collection of proteins make up the third subunit, which play a role in telomerase stabilization and sequestration [37].

Telomerase is well appreciated for its role in providing a template for DNA replication in order to maintain the length of telomeres [35, 38]. The recruitment of telomerase to telomeres is regulated by telomere length and cell cycle phase. Studies have demonstrated that telomerase is preferentially recruited to short telomeres in S phase/during replication when the T-loop is unfolded. Additionally, some shelterin proteins participate in regulating telomerase-telomere binding. It has been proposed that long telomeres, which would be bound by more TRF1, would also recruit more PIN2 TRF1 Interacting Telomerase Inhibitor (PINX1), thereby blocking access of telomerase to the 3'end [39, 40].

On the other hand, the POT1-TPP1 complex facilitates telomerase-telomere localization via its telomerase N-terminus (TEN) domain [41, 42]. In germ-line, stem, and cancer cells, high levels of telomerase activity ensure telomere length maintenance. Conversely, normal human cells lack sufficient telomerase activity to restore lost nucleosides, and thereby limit the number of cell divisions (Hayflick number) [43]. Critical telomere shortening in normal human cells triggers replicative senescence or apoptosis, preventing further cell division and providing an effective tumor suppressor mechanism [44].

***The End-Protection Problem***

Functional telomeres cap linear chromosomes; they prevent recognition of natural DNA ends as DSBs by repair enzymes such as Ataxia-Telangiectasia-Mutated (ATM) and Ataxia Telangiectasia and Rad3- related (ATR) kinases, and Poly (ADP-Ribose) Polymerase 1 (PARP1), and so avoid triggering of inappropriate DDRs. Functional telomeres block DSB repair pathways classical Nonhomologous End Joining (c-NHEJ), alternative alt-EJ, and Homologous

Recombination (HR), and limit telomere hyper-resection [26, 45, 46]. Each repair pathway will be discussed in greater detail below.

Loss of telomere sequence or structure (e.g., the T-loop), and/or disrupting the shelterin complex results in compromised end-capping and inappropriate engagement of DSB repair pathways at telomeres. Telomere dysfunction-induced foci (TIF) are indicative of loss of end-protection, potential consequences of which include end-fusions between chromosomes and/or sister chromatids (setting up Breakage-Fusion-Bridges cycles) and induction of genomic instability [47, 48].

***Telomeres, immortality, and cancer***

Normal cells in long-lived mammalian species with short telomeres can stop dividing and remain in state of replicative senescence for years as a strategy to suppress carcinogenesis [44]. Human fibroblasts can escape the replicative senescence barrier and divide again by inactivating tumor suppressor genes such as p16INKa, Rb or p53. Some of the cells that emerge also pass a second barrier known as crisis [49], which involves loss of functional telomeres. Chromosomal fusions and rearrangements, genomic instability, telomerase activity upregulation, and ultimately cancer result [50, 51]. Ectopic expression of telomerase in telomerase silent human cell lines causes cells to bypass senescence and crisis, leading to immortalization - one of the hallmarks of cancer [52, 53].

Telomere Length Maintenance via Telomerase reactivation or Alternative Lengthening of Telomere pathway (ALT).

**Telomerase positive tumors**

The Telomerase Repeat Amplification Protocol (TRAP) assay demonstrated that 85-90% of human tumors rely on reactivation of telomerase to maintain telomere length and enable

replicative immortality [50, 54]. Upregulation of telomerase is attributed to mutations in the hTERT promoter in some types of cancers; e.g., 80-90% of gliomas, 70% of melanoma, and 60% of bladder cancers [55, 56]. Two single base-pair mutations located -146 and -124 upstream from the start codon of hTERT gene (c.−146C>T and c.−124C>T) have been reported to generate a new binding site for ETS transcription factor leading to upregulation of hTERT protein [57-59].

In addition to the ETS transcription factor, chromatin marked with histone H3 Lysine4 di-methylation H3K4me2/3 provides a site of GABPA/B1 transcription factors. GABPA/B1 transcription factors were also involved in hTERT upregulation. Intriguingly, H3K4me2/3 was absent from wild-type hTERT promoter and replaced with Histone H3 Lysin27 trimethylation H3K27me3, the epigenetic signature of gene silencing indicating that mutant promoter is also accountable for the epigenetic changes. However, it is still unknown if the GABPA/B1 alone is sufficient to activate telomerase transcription, or if the transcription requires ETS and GABPA/B [60, 61].

On the contrary, other types of cancer such as breast, ovarian, and gastric cancers have very low frequencies of hTERT promotor mutation; these cancers do not require promoter mutations to reactivate telomerase [62].

***Alternative Lengthening of Telomere pathway (ALT) Tumors***

The remaining ~10% of tumors utilize an Alternative Lengthening of Telomeres (ALT) pathway to maintain telomere length [63-66]. These tumors represent aggressive cancer types; e.g., 50% of osteosarcomas, 30% of soft tissue sarcomas, and 25% of primary brain tumors. Clinically, ALT tumors are less likely to metastasize, but a smaller fraction of patients survive compared to telomerase positive tumors [67, 68]. ALT cells can be identified by the presence of various characteristic hallmarks. In addition to telomerase activity being low or absent, telomere length is

very heterogeneous in ALT cells, as shown by Terminal Restriction Fragment (TRF) Southern blots [63]. ALT cells are also associated with discrete types of Promyelocytic leukemia (PML) nuclear bodies called ALT-associated PML bodies. ALT-associated PML bodies are found in the nucleus, are ~0.1–1.0 μm in diameter, and contain telomeric DNA, TRF1, TRF2, and DNA repair proteins [69]. Compared to telomerase positive cells, a significantly elevated number of telomere-sister chromatid exchange (T-SCE) events has been reported in ALT cells [64]. SCE is a common template-switching mechanism used by cells to resolve replication stress; SCE within telomeres can be detected by Chromosome Orientation Fluorescence In Situ Hybridization (CO-FISH) assay [70-72].

ALT cells are also associated with telomeric C-circles, which were first detected in osteosarcoma patients; C-circles are not present in telomerase positive cells, so they are specific for ALT tumors [73, 74]. A recent study showed that mutated α-thalassemia/mental retardation syndrome X-linked (ATRX) and death domain-associated protein (DAXX), which are associated with decompacted telomeric chromatin, are also hallmarks of ALT [75-78]. One possible explanation is that functional ATRX and its physical partner DAXX facilitate histone variant H3K9me3 deposition. Therefore, altered ATRX/DAXX hinders H3K9me3 incorporation, which can change the chromatin state [79, 80]. One potential consequence, increased transcription through telomeres, would result in elevated levels of telomeric RNA,TERRA when compared to telomerase positive tumors with functional ATRX/DAXX and compact chromatin [81-83].

***ALT-mediated telomeric DNA synthesis by Break-Induced Replication (BIR)***

Gaining mechanistic understanding of how cells maintain telomere length independently of telomerase activity, can lead to novel strategies for ALT cancer therapy. Over the last several decades, significant progress has been made in understanding the molecular mechanism of ALT

[84-86]. Appreciation for an ALT mechanism in human cancer and normal mouse cells was first gained using fluorescently tagged DNA sequences inserted into telomeres. FISH analysis showed that the passage doubling number of ALT cultures is positively correlated with the number of tagged telomeres. For instance, in human ALT cells with passage number 23, the fluorescent tagged DNA was found only on three telomeres. This cell culture was subcultured 40 times, the fluorescent tagged DNA was then detected on 5 telomeres indicating that telomeres can exchange their own sequences. However, the same observation was not detected when tagged DNA was inserted at centromeres, nor in telomerase-dependent cells [87-89]. This unique telomeric recombination event had been previously described in telomerase-independent *saccharomyces cerevisiae* yeast when Dunn et al [90] introduced a short linearized DNA fragment lacking telomere sequence. They found that short DNA fragments became longer and more stable by gaining yeast telomeric DNA sequence in the presence of RAD52.

Walmsley et al [91] also demonstrated that telomere maintenance can be accomplished by using another telomere as a template. In addition to the role of recombination in maintaining telomeres, investigation focused on the role of telomeric recombination in telomeric DSBs in *S. cerevisiae*. Bosco and Haber [92] used HO endonuclease to cut off telomeres on specific chromosomes, generating a one ended-DSB. They reported that the broken chromosome was repaired using either the homologous telomere sequence on the same chromosome in haploid genome, or the non-sister telomere in diploid genome only in the presence of RAD52, which is necessary for recombination. Furthermore, cutting telomeres using HO in *S. cerevisiae* showed that BIR requires polymerase Delta, pol δ, and its subunit pol32, enhanced by Pif1-PCNA complex, to primase DNA synthesis within the migrating D-loop [93, 94]. Collectively, these results suggest a model for the Break-Induced Replication (BIR) repair pathway. BIR is the recombination-

dependent DNA replication pathway in which RAD52 binds resected 3' ends and promotes the homologous telomeric double-stranded DNA invasion and formation of a D-loop, a critical step during BIR followed by DNA synthesis (**Figure 1.2**) [95].

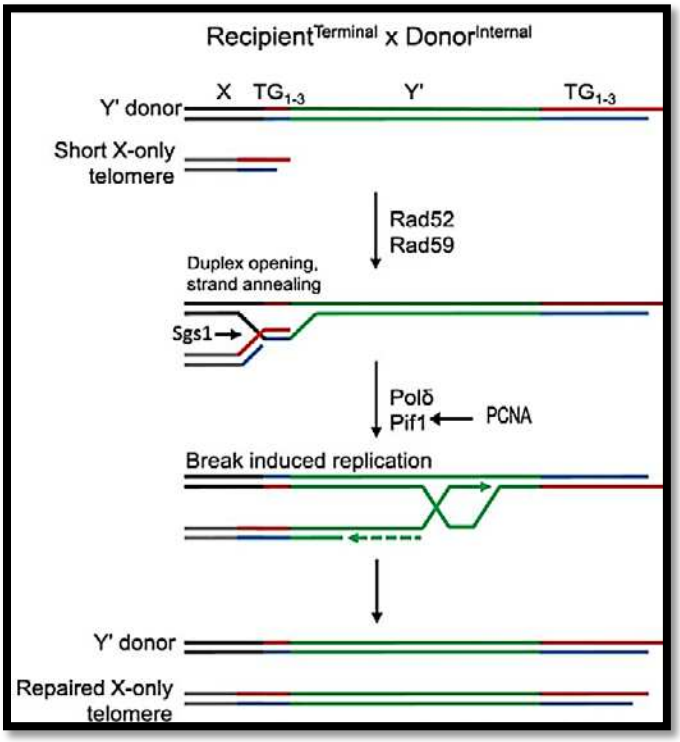



Figure 1.2: A model of telomere repair, via BIR pathway, in the absence of telomerase enzyme in *Saccharomyces cerevisiae.*

One ended-double strand break generated after inducing a site-specific HO. Rad52 and Rad59 are required for successful strand annealing and D-loop formation. The Pif1-PCNA complex primase DNA synthesis within the migrating D-loop [95].

A similar cleavage strategy has been used in ALT Osteosarcoma- U2OS mammalian cells by employing endonuclease protein FOKI fused to telomere shelterin protein TRF1 (FOKI -TRF1) [96]. Inducing telomeric-specific DSBs gave a direct answer as to whether the BIR pathway uses another homologous telomere as a template to repair the damage. This intriguing study used BrdU incorporation to measure telomeric DNA synthesis in cycling cells. Following induction of targeted telomeric DSBs, the length of nascent telomeres ranged between 5 - 70 kb in the presence

or absence of RAD51, suggesting two different BIR pathways; one being RAD51-dependent and one RAD51-independent. In the presence of RAD51, recombination between telomeres of different chromosomes was detected and contributed to DNA synthesis. In RAD51 deficient cells, BIR relied on the POLD3 subunit of human pol δ to synthesize DNA without detecting any recombination activity. Other supporting evidence includes telomerase positive cells treated with BIBR1532,telomerase inhibitor; over a six week times period, sustained telomeric DSBs showed longer telomeres compared to cells that were not treated with BIBR1532 [97].

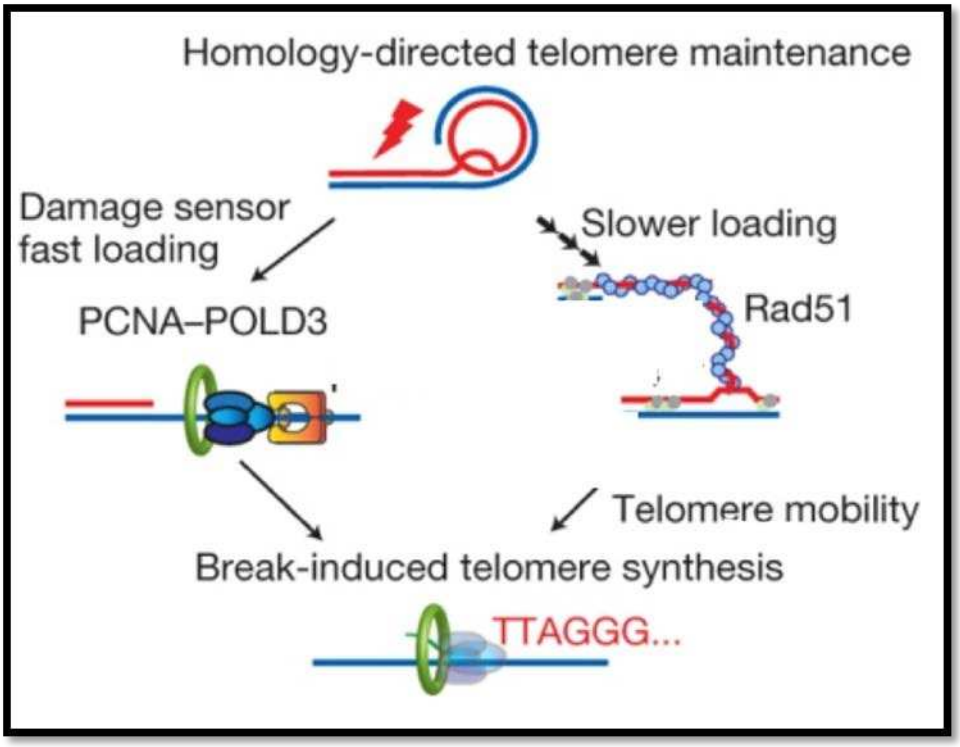



Figure 1.3: A model of telomere repair, via BIR pathway, in the absence of telomerase enzyme in ALT cancer cells.

A one ended-DSB generated after expressing telomere-specific endonuclease FokI is depicted above. Homology search in presence of Rad51 was detected between telomeres on different chromosomes. In absence of Rad51, PCNA-POLD3 promotes telomere DNA synthesis [96].

In addition to the role of RAD51 in BIR, a recent study revealed that the efficiency of BIR was drastically reduced in RAD52-KO G2 U2OS (ALT) clones, but not in RAD52-KO G2 telomerase positive clones [98]. In agreement with Dilley et al [96], they showed that BIR efficiency was not impaired in RAD51-KO G2 ALT clones in the presence of POLD3, confirming that RAD51-independent BIR pathway is mediated by pol δ in ALT.

Overall, the exploitation of developed experimental approaches has allowed for improved understanding of BIR as a mechanism of ALT in different organisms and different cell stages, as well as the roles of recombination and replication proteins in maintenance of telomere length [98].

***Chromatin landscape changes and increased Telo:TERRA hybrids mediate telomere elongation in ALT cells***

While endonuclease FOKI-induced telomeric DSBs has refined our understanding of how recombination mediated telomere synthesis occurs in ALT cells, the endogenous events that provoke ALT remain unclear. Most studies concede that modification of telomere chromatin conformation may be the main factor that initiates ALT. Specifically, it has been reported that inhibition of the histone chaperon Anti-Silencing Factor1 (ASF1) in telomerase positive cells represses expression of endogenous hTERT and promotes telomeric recombination [99].

Additionally, the telomere chromatin remodeler (ATRX) and its partner (DAXX) facilitate assembly of H3 histone variant H3.3 and H3 lysine 9 trimethylation (H3K9me3) to establish heterochromatin marks at telomeres. Mutation of the ATRX-DAXX complex, a hallmark of ALT, results in relaxed telomeric chromatin, facilitating access of BIR proteins to achieve telomere maintenance [79, 80]. Decompaction of telomeric DNA due to ATRX mutation has been associated with low levels of H3K9me3 and elevated levels of TERRA in ALT cells. In support

of such a role, RNAi KO of ATRX in telomerase positive cancer cells displays increased levels of TERRA [81-83].

The next question examined was whether ALT cells possess elevated levels of TERRA compared to telomerase positive cells. Fragmented DNA extracted from U2OS cells was subjected to DNA ImmunoPrecipitation (DIP) to detect Telo-RNA hybrids. This was done using S9.6 antibody to compare it to amount of TERRA in HeLa cells as a model of telomerase positive cells. The results showed that in contrast to telomerase positive cells, a significant increase of Telo:TERRA hybrids and elevated level of C-circles were observed. Interestingly, removing hybridized TERRA by overexpression of endogenous RnaseH1 in ALT cells resulted in telomere lengthening. These results support the contribution of elevated levels of Telo:TERRA hybrids in ALT-mediated BIR to maintain the length of telomeres [100]. TERRA has also been implicated in telomere mobility, in both ALT and telomerase positive cells, and to facilitate physical telomere-telomere interaction required for recombination [101, 102].

Increasing evidence suggests that Telo:TERRA hybrids are engaged in recombination-mediated telomere elongation in yeast. Depleted telomerase recruitment factor (Coiled-Coil protein Quantitatively ccq1) in *Schizosaccharomyces Pombe* cells have shown to have elevated levels of TERRA and Telo:TERRA hybrids associated with efficient telomeres recombination. [102]. On the other hand, repression of Telo:TERRA hybrids by way of overexpression RnaseH1 led to a sever growth crisis [102]. It has also been suggested that in *S. cerevisiae*, Telo:TERRA hybrids could contribute to stabilizing the D-loop to promote telomere recombination [103, 104]. Together, these observations suggest that Telo:TERRA hybrids can serve as substrates for recombination- mediated telomere elongation.

***Long Non-coding RNAs***

Over the past decade, molecular biology studies have discovered that 98% of the genome is transcribed into non-coding RNAs [105, 106]. Long non-coding (lnc)RNAs are a type of non-coding RNAs that interact with different proteins to achieve cellular functions [20, 21, 82, 107]. The telomeric lncRNA TERRA is important for telomere structure and function.

***Long non-coding Telomeric repeat-containing RNA: TERRA***

***TERRA*** Biogenesis

Telomeres were long thought to be transcriptionally silent due to their hypermethylated, inhibitive chromatin status [21, 107]. Discovery of the long non coding RNA, TERRA, challenged this historic and central tenet of telomere biology [20]. It is now known that TERRA transcription, driven by RNA polymerase II (RNAPII), can progress several kilobases through the C-rich telomeric strand before releasing the final product (**Figure 1.4**) [108]. Work In U2OS has shown that cohesion, and CCCTC-Binding Factor (CTCF) transcription factor work in concert to positively regulate TERRA transcription via their roles in RNA Pol II recruitment [109].

TERRA has been detected in yeast, mouse, human, zebrafish and plants. [20, 108, 110, 111]. Human TERRA is very heterogeneous in length, ranging from ~100bp to 9kb of 5'-UUAGGG-3' repeats, suggesting different promoter location sites [20, 108]. In support of this view, two sets of TERRA promoters within subtelomeres have been characterized. Detection of pSer2 and pSer5 (active form of RNA pol lI) binding sites 1Kb from telomere repeats helped identify the first set of TERRA subtelomeric promoters. Notably, these promoters are rich in dinucleotide CpG islands upstream of the Transcription Start Site (TSS) [109, 112]. RNA-seq approaches in HeLa cells revealed a second set of TERRA promoters located 5-10kb from telomeres on 10 different chromosomes [113].

Utilizing CRISPR-Cas9 genome editing to remove 8 kbp on the long arm subtelomere of chromosome number 20 in human U2OS cells resulted in significant TERRA depletion, indicating that a significant amount of TERRA is transcribed from one chromosome [114]. Similarly, the majority of mouse TERRA has been shown to be transcribed from chromosome number 18 [115]. Other recent work demonstrates that human TERRA is transcribed from many chromosomes [116]

***TERRA Structure***

Structurally, while the 5' end of TERRA is capped with 7-methylguanosin (m7G), a specific fraction of the 3' end of total TERRA is polyadenylated, leaving the remaining 3' ends nonpolyadenylated. Polyadenylation is an important factor in determining the half-life and the binding status of TERRA. That is, nonPoly A TERRA has a shorter half-life (3h), but localizes to telomere tracks forming Telo:TERRA hybrids (bound TERRA). This is in contrast to poly A TERRA, which has a longer half-life (8h), but does not associate with telomeres (free or unbound TERRA) [25].

***TERRA Decay***

Telo:TERRA hybrids can be advantageous to ALT cells; however, replication stress associated with Telo:TERRA hybrids in ALT and telomerase positive cells has been reported. Thus, regulation of TERRA levels and binding activities are important for maintaining stability.

For instance, in addition to RnaseH1, HeLa cells deficient in UP-Frameshift 1 (UPF1), a member of the nonsense-mediated mRNA decay pathway, display elevated levels of Telo:TERRA hybrids with no associated increase in TERRA transcription, revealing UPF1 as a negative regulator of bound TERRA [20]. A recent study identified TRF1, which facilitates replication through telomeres, as another negative regulator of bound TERRA [117]. In contrast to TRF1, depletion of TRF2 results in upregulation of TERRA transcription [113]. The NONO/SFPQ

heterodimer complex has also been proposed as a suppressor of undesirable levels of Telo:TERRA hybrids in U2OS cells [118].

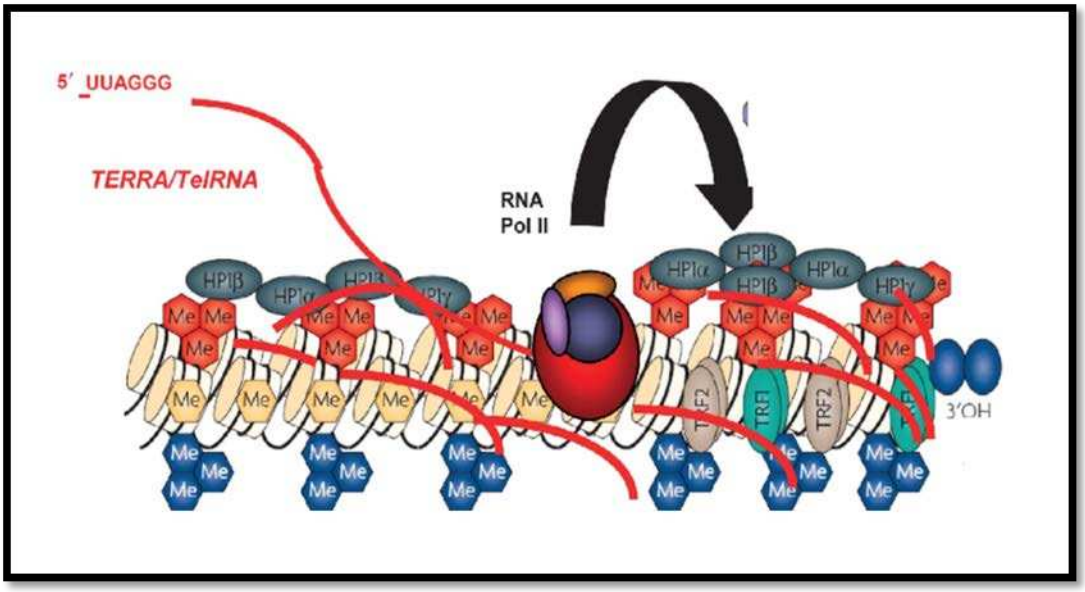



Figure 1.4: A model of TERRA transcription through telomere.

RNA Pol II drives transcription of UUAGGG long non coding telomeric RNA through telomeres [1]

***TERRA dynamics through the cell cycle in Telomerase positive and ALT cells***

Shortly after the discovery of TERRA, intense investigation into TERRA transcription and localization commenced, in both telomerase positive and ALT cells; reviewed in [119]. Porro et. al. evaluated TERRA levels from four different telomeres in synchronized HeLa (telomerase positive) G1/S cells, compared to TERRA levels from the same four telomeres in cells in S and G2/M phases. Results revealed that TERRA transcription is cell cycle dependent, with levels peaking in G1/S and decreasing through the cell cycle until cells reenter G1 and levels start to increase again [25].

Many reports gave insights into the necessity of having regulated levels of TERRA through the cell cycle in telomerase positive cells. Flynn et al observed that replication protein A (RPA) was able and sufficient to displace the shelterin POT1-TPP1 complex from telomeric ssDNA. However, the reverse experiment revealed that the POT1-TPP1 complex did not replace RPA

[120]. Knowing that RPA plays a role in ATR-mediated telomeric DNA repair activation, the investigation of how RPA released from telomeres was needed.

Biochemical studies showed that RPA is displaced from telomeric ssDNA when heterogeneous nuclear ribonucleoproteins (hnRNPs) bind telomeric ssDNA, which occurs only in the absence or low concentration of TERRA. Further investigation showed that elevated levels of TERRA bind and sequester hnRNPs, allowing RPA to displace POT1-TPP1 complex from ssDNA [120]. Taken together, published results provide an attractive model explaining why TERRA levels are tightly regulated through the cell cycle in telomerase positive cells (**Figure 1.5**).

In this model elevated levels of TERRA in G1 and G1/S sequester hnRNPs, allowing RPA to antagonize POT1-TPP1 complex binding to telomeres. Bound RPA is critical for DNA replication during S phase. Reduced levels of TERRA in S/G2 are insufficient to sequester hnRNPs, thereby facilitating RPA binding. An increase in TERRA during M/G1 leads to sequestering hnRNPs, again allowing the POT1-TPP1 complex to bind, cap telomeres and prevent the activation of ATR [119].

In conclusion, regulated levels of TERRA through the cell cycle in telomerase positive cells play an important role in telomere replication and end-capping [121]. In contrast, in ALT U2OS cells synchronized in S phase (via thymidine block), RNA FISH detected elevated levels of TERRA in S phase compared to what had been observed in telomerase positive HeLa cells. Furthermore, RNA dot blots of U2OS cells treated with a CDK1 inhibitor to enrich for cells in G2, did not show any reduction of TERRA levels as cells released from S into G2 [81]. These results indicate that in contrast to telomerase positive cells, TERRA levels in ALT cells are not regulated through cell cycle.

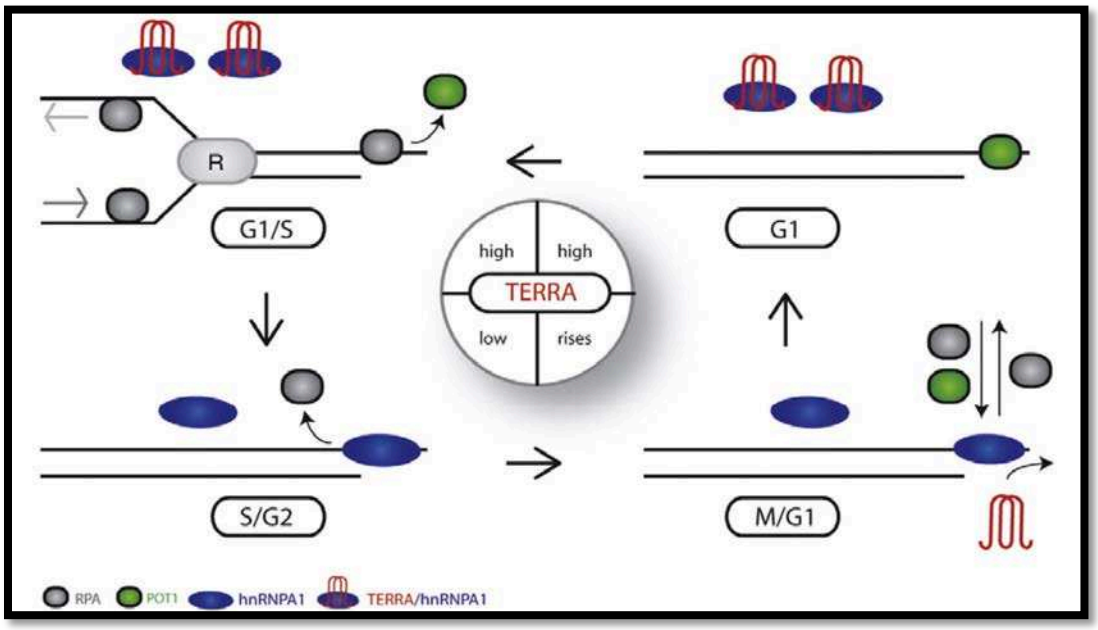



Figure 1.5: Regulated levels of TERRA through the cell cycle in telomerase positive cells is the main key to switch from (protected) closed to (replication) open status.

Increased levels of TERRA through G1 and S act to sequester hnRNP A1, allowing RPA to replace POT1 and bind telomeric ssDNA. Downregulation of TERRA through S and S/G2 phases releases hnRNPA1, which in turn displaces RPA by binding telomeric ssDNA. TERRA levels increase in M/G1 to repeat the cycle.

***TERRA is required to establish telomeric heterochromatin in ALT cells***

Telomerase positive cells rely on the ATRX/DAXX complex to deposit H3K9me3, the signature of telomeric heterochromatin [75-78]. The absence of functional ATRX/DAXX complexes in ALT cells raises the question of how ALT cells compact telomeric chromatin. Montero, J.J., et al [122] proposed a model in which the level of TERRA works as a sensor to compact the chromatin. TERRA binds Polycomb Repressive Complex 2 (PRC2) and guides it to telomeres. TERRA-PRC2 at telomeres induces H3K27 methylation, which in turn facilitates recruitment of H3K9me3, H4K20me3, and anchoring of HP1to telomeric chromatin. However, neither PRC2 nor any telomeric heterochromatin marks were observed on 20q telomeres in

TERRA-knockout U2OS clones. These findings suggest that TERRA interacts with a variety of players to ensure end-capping and protection of chromosome ends [122].

***TERRA maintain telomeres***

Emerging evidence suggests that the main role of regulated levels of TERRA is in telomere maintenance, as dysregulated levels of TERRA contribute to genome instability. To understand the role of TERRA in telomere maintenance, downregulation TERRA has been achieved in various cell lines utilizing a variety of approaches. The first attempt to deplete TERRA was in 2009, two years following its discovery. Small interference RNA (siRNA) mediated depletion of TERRA significantly reduced TERRA transcripts in U2OS cells measured by RNA-FISH, RNA dot-blot and Northern blot. [113]. In 2014 Lopez de Silanes, et al [115] employed LNA gapmeR AntiSense Oligonucleotide (ASO) to cleave mouse TERRA. Forty-eight hours post transfection with ASO, TERRA transcripts were measured by RT-qPCR and RNA dot-blot, which showed 50%reduction in total TERRA. The same group targeted the TERRA locus on subtelomere 20q in U2OS cells utilizing CRISPR/Cas9 technology. TERRA-20q U2OS clones displayed dramatic decreases in TERRA levels compared to parental U2OS cells [114]. In all mentioned studies [113] [114] [115], reduced levels of TERRA were associated with telomere fusions, telomere free ends, telomere duplications, telomere double minutes, and dysfunctional Telomere-Induced Foci (TIFs). Such observations support a protective role of TERRA at telomeres by inhibiting the telomeric DDR via its role in telomere capping; specifically, by facilitating heterochromatin mark deposition and displacing RPA with the POT1-TPP1 complex [119]. Paradoxically, it has been shown that TERRA is involved in the telomeric DDR upon TRF2 depletion in HeLa cells. In TRF2 deficient HeLa cells, it was observed that TERRA molecules interacted with Lysine-specific demethylase 1

(LSD1). TERRA-LSD1 complexes increased the affinity of MRE11 to bind telomeric damage sites, process 3' G overhangs, and sustain activation of ATM signals at uncapped telomeres [123].

***TERRA response to cellular stress***

Crosstalk between cellular stress and the DNA Damage Response (DDR) has been reported in many studies [124, 125]. In line with this notion, TERRA expression was induced in HeLa Wild Type (WT) cells upon incubating cells at 40 C° for an hour (Heat Shock). Strikingly, TERRA induction was dependent on recruiting the transcription factor Heat Shock 1 (HS1) to subtelomeres. However, TERRA induction was suppressed in HS1-knockout HeLa cells associated with telomeric DNA damage and detected TIF signals [126].

Moreover, nutrient deprivation (culturing human colon cancer HCT116 cells in serum-free media) has also been shown to promote TERRA expression. Similar to what has been shown in HeLa cells, TERRA induction in HCT116 cells was also a dependent behavior. TERRA induction with nutrient deprivation was associated with the presence of p53 transcription factor, the key protein in tumor suppression. Notably, depletion of p53 subtelomeric binding sites prevented TERRA induction and induced telomere damage [127]. Such evidence suggests that TERRA is part of tumor suppressor network in response to cellular stress [128]. In another study, treatment of adenocarcinomic human alveolar basal epithelial (A549) cells with Bleomycine, a radiomimetic drug that induces DSBs, and measuring TERRA expression by PCR demonstrated elevated levels of TERRA [129]. It remains to be investigated whether TERRA is induced in response to other DSB-inducing agents like ionizing radiation (IR). In conclusion, findings to date suggest that induced TERRA expression contributes to telomere protection.

***DNA Double Strand Break repair pathways***

Cells experience DNA DSBs with exposure to IR or treatment with site-specific endonucleases. DNA DSBs are appreciated as the most lethal form of DNA lesion, and they contribute to genome instability and thereby cancer (reviewed in [130, 131]). However, cells are equipped with an arsenal of different mechanisms to repair DSBs such as c-NHEJ, HR, alt-EJ and BIR reviewed in [132]. In the next section, we will briefly introduce each pathway and comment on their presence or absence when DSBs occur within telomeres.

***Classical non-homologous end joining (c-NHEJ)***

It has been well documented that c-NHEJ is the dominant DSB repair pathway in mammalian cells, particularly in G1 when a homologous template (sister chromatid) is not readily available. In S/G2 phases, c-NHEJ pathway proteins are expressed but the pathway itself is inhibited when Cell cYcle REgulator of NHEJ (CYREN) binds Ku70-Ku80 heterodimer (early c-NHEJ pathway proteins) [133]. In response to DSBs and in the absent of CYREN, Ku70-Ku80 heterodimers rapidly bind the DSB sites to stabilize the two ends and recruit the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Auto-phosphorylated DNA-PKcs phosphorylates ATM, and the histone variant H2AX on serine 139 to form γ-H2AX, the signature of DSBs.

These critical phosphorylation events are required to initiate and sustain the DNA damage response so that the cell cycle slows down [134]. Cells then commit to c-NHEJ when the 3′ to 5′ nucleolytic activity of MRE11, EXO1 and Artemis processes the two ends to generate two 3′ single stranded DNA overhangs, typically < 4bp. In c-NHEJ, extensive end resection is limited by binding of 53BP1, RIF1 and the shieldin complex [135]. Lastly, XRCC4 and Ligase IV align and ligate the two compatible ends. The c-NHEJ pathway results in deletion or insertion in the repaired product, and thus is considered as error- prone repair pathway.

Functional telomeres take advantage of shelterin proteins to prevent triggering of a telomeric DDR. TRF2 prevents ATM signaling and the c-NHEJ repair pathway via two mechanisms. TRF2 is sufficient for remodeling of the single-stranded telomeric G-rich overhang to form a T-loop – essential for preventing binding of the MRN complex and Ku70/80 heterodimer. TRF2 showed the ability to bind and block Ku70, preventing the tetramerization of Ku70/80 complex and thereby preventing initiation of c-NHEJ [132, 134].

***Homologous Recombination (HR)***

When sister chromatids are available after replication, 53BP1 is antagonized by BRCA1 allowing for extensive resection >100 bp [136]. Resection is promoted by the activity of endonuclease protein MRE11, one of the members of the MRN complex (MRE11-RAD50-NBS1) and the C-terminal binding protein interacting protein (CtIP), EXO1 and DNA2 helicase/nuclease. Following resection, the two ss-DNA ends become coated with RPA, which is eventually replaced with Rad51 [137]. Rad51-coated DNA invades and anneals to the homologous template forming holliday junctions. Lastly, DNA polymerase binds the 3' end of the invading allowing DNA synthesis and eventually resolving holliday junctions [138]. HR is considered less error-prone pathway that utilizes a homologous template. Comparison reports between telomerase positive cells and ALT cells have shown that HR is elevated in ALT cells due to reduced shelterin saturation [78, 132].

***Alternative-End Joining (alt-EJ)***

In addition to studies characterizing the pathways of C-NHEJ and HR, Liang and Jasin discovered alt-EJ as an alternative pathway to compensate for the loss of C-NHEJ pathway in XRCC4 or Ligase IV deficient cells [139, 140]. However, different studies have shown that Alt-EJ occurs in C-NHEJ proficient cells as well [141]. Mechanistically, Alt-EJ starts with processing

the two ends to expose microhomology that < 21 bp using the same proteins that used in HR. Next, the two main proteins in this pathway poly (ADP-ribose) polymerase 1 and polymerase θ (Polθ) facilitate DNA synapsis and strands annealing. Following the annealing, nuclease enzymes remove the non-complementary 3' flap and Ligase 1 and Ligase 3 ligate the two ends [141-143]. In cancer cells, Alt-EJ pathway have been implicated in insertions, deletions and chromosome fusions. In particular, recent studies have demonstrated that removing TRF2 or POT1/TPP1 shelterin protein from telomeres in Ku70/80 deficient cells results in a mild telomere fusions phenotype. Telomere fusions were observed involving every telomere after depleting all shelterin proteins in Ku70/80 deficient cells [4, 144].

***Break-induced Replication (BIR)***

BIR differs from the HR DSB repair pathway due to its unique ability in repairing DSBs with only one end. The BIR pathway has been discussed in a previous section.

The choice of the appropriate pathway can be determined firstly by the cell cycle phase in which the DSBs occurs [133]; the cell cycle takes a leading role in regulating the DNA end resection following the damage [145]. In G1 phase, the end- resection independent pathway NHEJ is the dominant pathway. Recent studies show that inhibition of resection in G1 has been associated with the activity of RPA-DNA Helicase B complex. This complex works, independently of 53BP1, to prevent the binding of exonuclease-mediated resection proteins including; EXO1, and BLM-DNA2 [146]. It is also well documented that expression of HR genes is downregulated in G1 phase [147]. In G2 phase, the opposite scenario was reported to promote the end- resection dependent pathways HR or alt-NHEJ [147].

In addition to cell cycle phase, accumulated evidence implies the sequence context at the DSB site can also impact the choose of the repair pathway [148]. NHEJ pathway has been shown

to be strictly blocked within telomeres through the cell cycle [96, 97, 130]. Alternatively , telomeric hyper resection HR and telomeric hypo resection alt-EJ pathways have been utilized by cells to repair the telomeric DSBs [96, 97, 130].

***The balance between telomere protection and telomere repair in mammalian cells***

The highly protective system of inhibiting DDRs and repair within telomeres made biologists question how telomeric DSBs are repaired following damage within telomeres themselves. Early experiments exposed proliferating fibroblasts to high doses of IR (10, 20 Gy, X-rays) to induce DSBs genome wide. [149]. Interestingly, while DNA damage at non-telomeric sites decreased over time, targeted telomeric DNA damage accumulated, indicating that telomeric DSBs are irreparable [149] . The main pitfall of using this approach is the need of extremely high doses of IR to generate DSBs within relatively rare telomeric DNA, which may alter gene expression and/or cause mitochondrial dysfunction/oxidative stress, known contributors to telomere damage [130, 131]. Thus, IR exposure cannot discriminate whether the detected telomeric damage response is a result of DSB within telomere sites or the indirect impact of high doses on telomeres. To address this obstacle and answer the question, telomeric site-specific endonucleases have been developed and employed in recent studies. Expression of FokI-TRF1, and Cas9 in mouse fibroblasts and human ALT cancer cells, results in TIFs (broken telomeres) with no associated loss of telomeres or telomere fusions in metaphases spreads, indicative of the ability of the cells to repair DSBs within telomeres, independent of c-NHEJ [96, 97, 150-152].

Other supportive evidence for the strict prevention of c-NHEJ following endogenous telomeric DSBs has been reported after expressing FokI-TRF1 in Lig4-/- mouse cells. Frequencies of TIFs in Lig4-/- mouse embryonic fibroblasts (MEFs) were not altered compared to TIFs following damage in WT-Lig4 control cells [151]. Our studies also found no evidence of c-NHEJ

following telomeric DSBs generated via transient transfection of the silkworm endonuclease TRAS1-EN (ENT) fused to TRF1 to target telomeres in human BJ1-hTERT fibroblasts and EJ-30 telomerase positive cancer cells (unpublished). Because c-NHEJ is the dominant repair pathway in G1, we monitored localization of 53BP1 at telomeric DSBs in G1. The lack of 53BP1 at telomeric damage sites, both in the presence of TRF2 and after depleting TRF2, supports suppression of c-NHEJ in G1 human fibroblasts and cancer telomerase positive cells. This hindrance to the c-NHEJ pathway within telomeres is attributed to the fact that c-NHEJ can contribute to genome instability [130, 153].

Since c-NHEJ is not the preferred pathway of DSB repair within telomeres, resection dependent repair pathways (HR, alt-EJ and BIR) were also investigated. Although we observed that fibroblast and telomerase positive cells undergo extensive resection in G1 following induced telomeric-specific damage, neither BIR-induced HR repair proteins, nor DNA synthesis was detected (unpublished).

While similar findings in G1 HeLa cells have been reported, sustaining TIF signals upon depleting PARP1 or Lig3 suggests potential participation of alt-EJ pathway at telomeric DSB in MEFs cells [151]. The repetitive nature of telomeric regions may make alt-NHEJ an attractive option for repairing telomeric DSBs.

In contrast to alt-EJ in fibroblasts, endonuclease-induced telomeric DSBs have been implicated in triggering extensive resection, homology-searching and pairing interaction between sister chromatids in S phase and non-sister telomeres in G1 phase and DNA synthesis in ALT cells [96, 97, 150]. Inhibiting or depleting Rad51 precludes the physical interaction between non-sister telomeres and thereby telomere shortening [96]. Cells exposed to long-term telomeric DSBs preferentially utilize recombination between non-sister telomeres, rather than sister chromatids, to

elongate telomeres [96, 97, 150]. Thus, telomeric recombination can occur between non sister telomeres in G1 when sister chromatids are not available.

At present, it appears that alt-EJ or BIR-induced HR may be involved in repair of telomeric DSBs in human ALT and non-ALT cells. However, whether TERRA plays a role in telomeric DSB repair has not been elucidated. Our work here revealed for the first time an increase in TERRA in response to IR exposure, as well as colocalization of TERRA at telomeric DSBs following induction of telomere-specific damage.

# CHAPTER 2 RESULTS

Telomeres are special nucleoprotein structures at the ends of linear chromosomes [2, 3]. In humans, telomeres consist of 5-15 kb of repetitive DNA sequence (5'-TTAGGG-3') bound by a specific protein-complex known as shelterin, made up of TRF1, TRF2, TIN2, TPP1, RAP1, POT1, and TZAP [8-12]. Due to the inability of conventional polymerases to replicate to the very end of linear DNA molecules (the end-replication problem), the telomeric G-rich strand ends with a 3' single-stranded (ss)overhang [16]. TRF2 has been shown to play a role in looping and facilitating invasion of the 3' (ss)overhang into telomeric double stranded (ds)DNA, forming a T-loop [15], an effective solution to the end-protection problem [26].

Telomerase is the specialized reverse transcriptase (TERT) capable of *de novo* elongation of telomeres [35]. Telomerase activity is significantly reduced in normal somatic mammalian cells [37]. Hence, telomeres shorten with cell division, and therefore with aging. Furthermore, as telomeres become critically short, they activate cell cycle checkpoints, cell cycle arrest, and senescence or apoptosis [52]. In contrast, ~85-90 % of cancers reactivate telomerase to maintain telomere length and enable unlimited cell division, some by way of mutations in the promoter of *TERT* [54]. The remaining ~10-15% of cancers maintain telomere length via an homologous recombination (HR)-mediated Alternative Lengthening of Telomeres (ALT) pathway [68, 81, 86].

Telomeres are transcribed into the telomeric long noncoding RNA TERRA, which is transcribed from start sites (CpG islands) originating within subtelomeric regions [20]. While a proportion of TERRA is found bound to telomeres, the remainder is not associated with telomeric chromatin [25, 154]. Interestingly, ALT cells have elevated levels of TERRA as compared to

telomerase positive cells [100]. *In vitro* and *in vivo* studies have shown that TERRAs is involved in maintaining both telomere length and telomere protection [113, 121, 122, 128].

It has also been demonstrated that conventional DNA repair is inhibited at telomeres [108]. Misrepair or non-repair of deleterious DSBs, such as those induced by ionizing radiation (IR) exposure, is known to give rise to chromosomal aberration and genomic instability[132] . This prompted us to investigate how human cells handle DSBs occurring within telomeres themselves. Understanding DSB repair within telomeres has become an intriguing topic, investigated by many research groups [96, 97, 130, 151]. However, the role of telomeric RNA (TERRA) is not well understood.

Here, we hypothesized that TERRA plays a role in repair of telomeric DSBs. To test this, we utilized human U2OS (ALT) cells, which have high levels of TERRA. Our aims included characterizing TERRA response to global DNA DSBs induced by IR, as well as to enzymatically-induced DSBs targeted specifically to telomeres.

**Increased TERRA following Ionizing Radiation exposure**

Increased TERRA transcription following treatment with Bleomycine, a radiomimetic drug that induces DSBs, has been demonstrated [129]. We investigated whether TERRA might also be influenced by IR exposure, a potent inducer of prompt DSBs. RNA FISH analysis revealed a significant increase in the number of TERRA foci in U2OS cells exposed to 2 Gy γ-rays compared to unirradiated controls (**Figure 2.1 A, B**). To validate these findings, irradiated and unirradiated U2OS cells were also treated with RnaseA and RnaseH to remove TERRA; TERRA foci were significantly diminished (**Figure 2.1 C,D**). Results demonstrate that TERRA responds to IR exposure and genome-wide induction of DSBs.

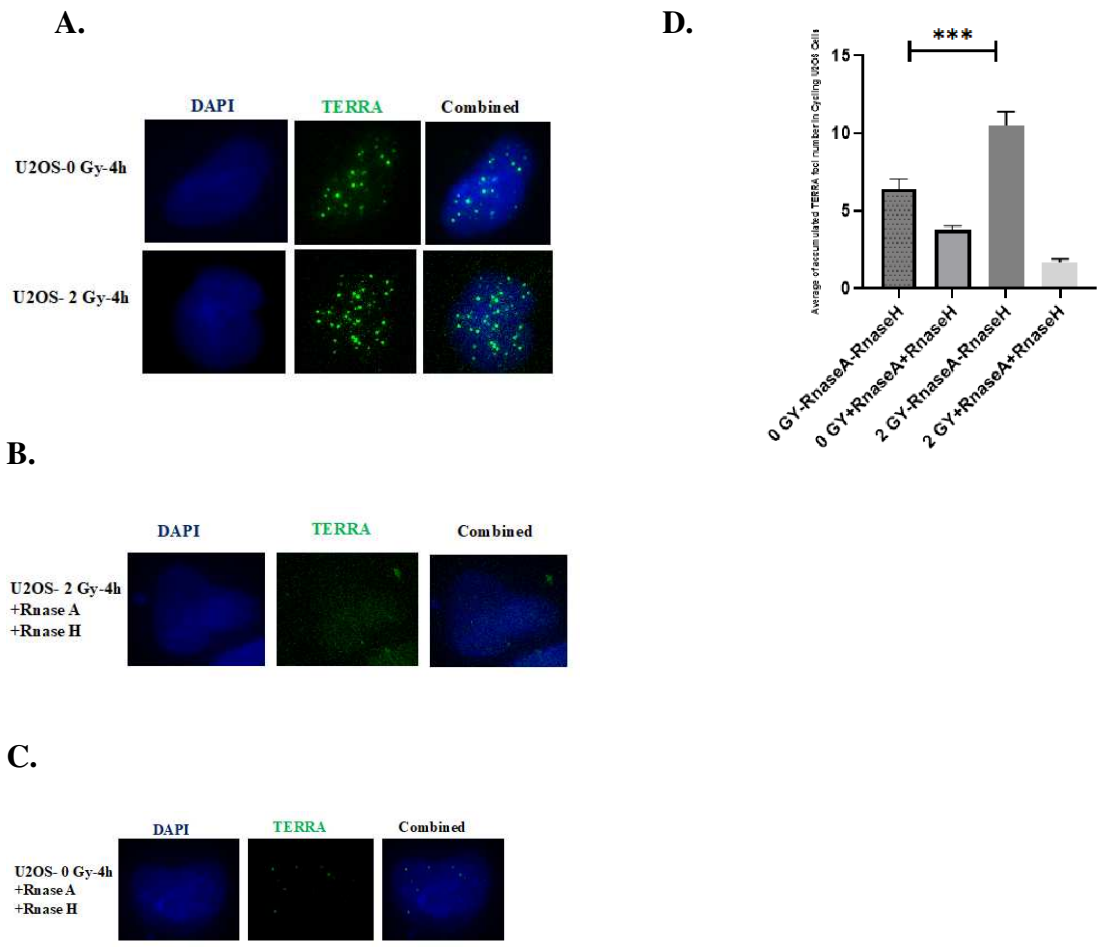



Figure 2.1: TERRA and IR in cycling U2OS (ALT) cells.

**A, B, C.** TERRA foci were significantly increased four hours after IR exposure (2 Gy) compared to 0 Gy controls. Treatment with RnaseA + RnaseH removed TERRA from irradiated and unirradiated cells. **D,** Quantification of nuclear TERRA foci; 3 experiments each ($n = 50$), error bars represent SEM. T test $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

Telomere-specific DSBs induce telomeric DDR involving TERRA

To better understand ALT DDRs involving TERRA at telomere-specific DSBs, we employed our validated system utilizing transient transfection of a plasmid encoding a flag-tagged telomere repeat-specific endonuclease fused to the human TRF1 gene (TRAS1-EN-TRF1: hereafter referred to as ENT-TRF1) that produces blunt ended DSBs within telomeres [155, 156]. Here, we used the EN-T system in U2OS (ALT) cells to characterize the telomeric DDR to targeted

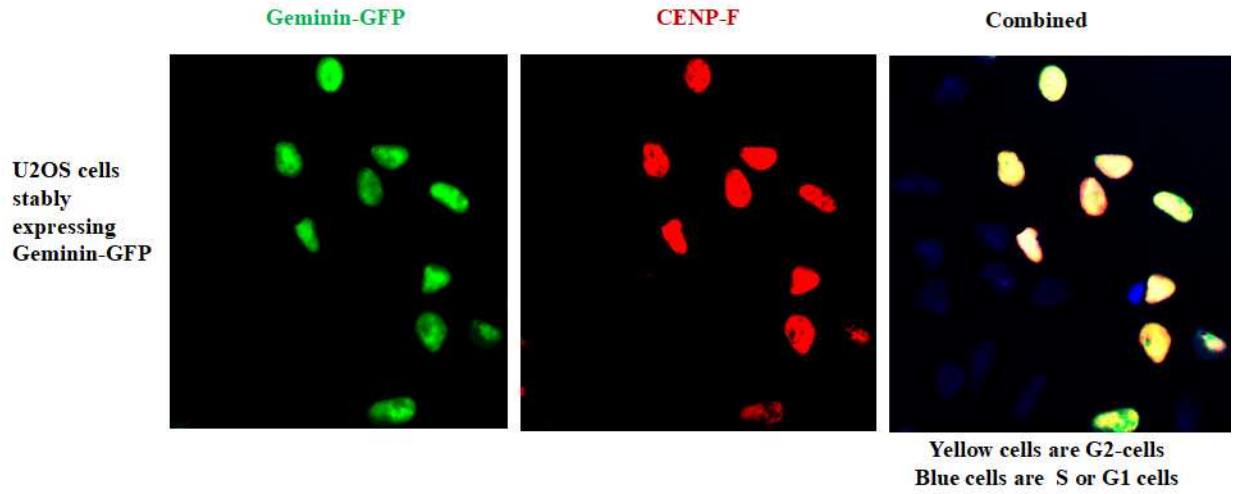
DSBs. For controls, U2OS cells were transfected with plasmids that expressed TRF1-only, empty vector, or were un-transfected. In experiments interrogating TERRA, a combination of Rnase A and Rnase H was used to remove TERRA as a negative control.

First, we sought to establish transfection efficiencies in U2OS cells, determine phase of the cell cycle in which cells accumulate following transfection, and establish TERRA response to cellular stress of transfection. Additionally, we confirmed and quantified ENT-TRF1 induction of telomeric DSBs via co-localization of telomeres, EN-T (FLAG) and the DSB marker γ-H2AX, evaluated whether TERRA co-localized at telomeric DSB sites via co-localization of TERRA and FLAG, and evaluated potential role of the cell cycle on TERRA recruitment to damaged telomeres.

Distinguishing cell cycle phase in FUCCI - U2OS interphase cells

In order to monitor the effects of transfection on TERRA distribution throughout the cell cycle, we employed a Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) strategy to readily distinguish G1, S, and G2 phase in interphase nuclei. A stable U2OS cell line expressing Geminin protein fused to Green Fluorescence Protein (Geminin-GFP) [157] was generated to positively identify cells in G2 phase. Colocalization of the G2 markers Geminin-GFP and Centromere protein F (CENP-F) confirmed that the FUCCI plasmid had not been lost, and that absence of Geminin-GFP signals in each negative (unlabeled) cell represented a cell in G1 or S phase. Results also confirmed that cells labeled with CENP-F were also positive for Geminin-GFP and so were in G2, and cells negative for CENP-F did not express Geminin-GFP and so were in G1/S (**Figure 2.2 A).** To definitely label cells in S phase, cells were incubated with EdU for 30 min, after transfection; cells negative for Geminin-GFP and EdU were in G1 (**Figure 2.2 B**).

**A.**




**B.**

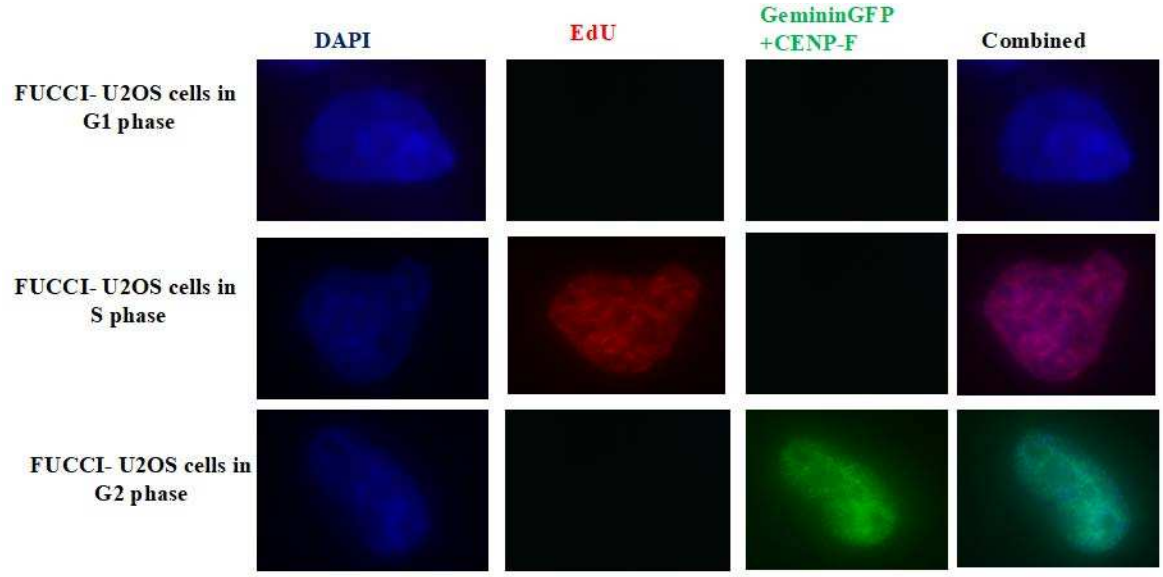



Figure 2.2: FUCCI – U2OS cell line to identify cell cycle phase.

**A.** Co-localization of nuclear CENP-F protein (red) and Geminin-GFP (green) in U2OS cells stably expressing Geminin-GFP confirms that the FUCCI plasmid had not been lost, and that negatively staining cells (blue) were in G1 or S. **B.** Simple schema for discriminating cell cycle in interphase nuclei: G1 cells negative for EdU and Geminin-GFP/CENP-F (blue); S phase cells positive for EdU (red); G2 cells positive for Geminin-GFP/CENP-F (green).

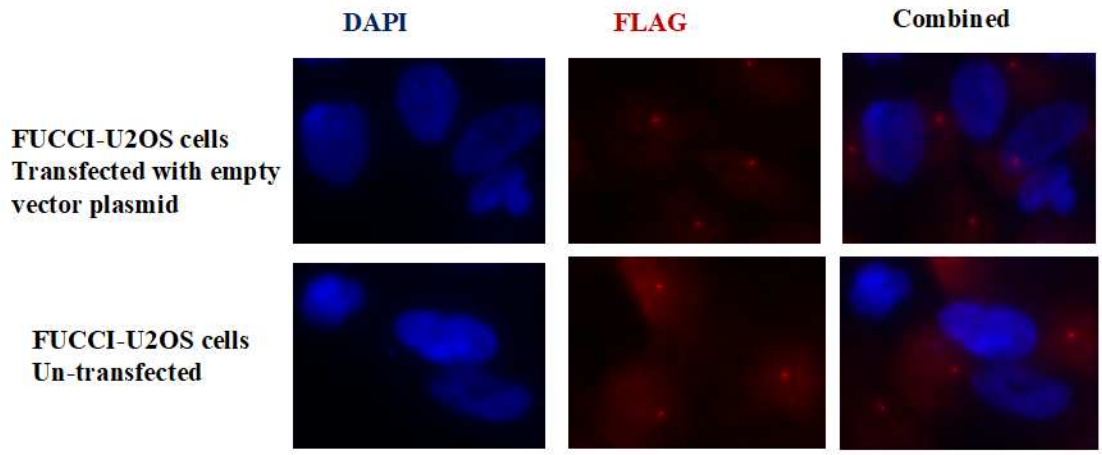
### Transfection efficiencies in FUCCI- U2OS cells

Utilizing lipofectamine, populations of U2OS cells expressing FUCCI were transfected with either ENT-TRF1, TRF1-only, or empty vector, and as a negative control, no transfection.

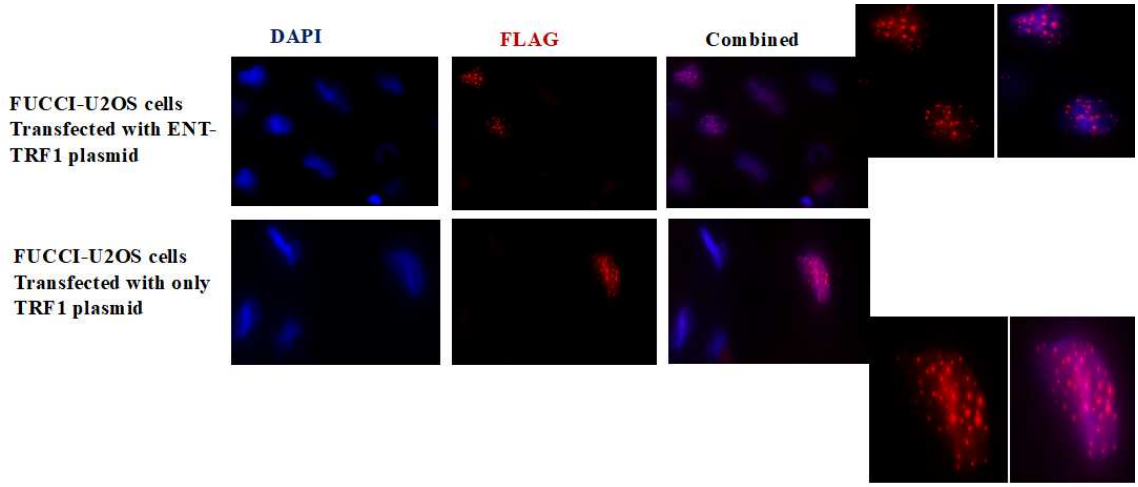
Following transfection (48 hr), FUCCI-U2OS cells were fixed, and Anti- FLAG antibody used to evaluate transfection efficiencies. Transfection efficiencies of ~25-30% were observed (**Figure 2.3A**). There were no significant differences in average number of FLAG foci in cells transfected with ENT-TRF1 or TRF1-only (**Figure 2.3B**).

The low efficiency of transient transfection in U2OS cells may be attributable to the particular cell line, and/or the lipid-based delivery method used, which does have lower efficiency compared to viral-based delivery approaches. Another consideration is that cells were fixed 48 hours after transfection using Lipofectimine 2000, while expression of the gene starts 6-8 hours after transfection. The number of expressed FLAG foci in FUCCI-U2OS cells transfected with ENT-TRF1 or TRF1-only did not show any significant difference, indicating that the two different populations were exposed to the same level of stress (size of plasmid, transfection reagent).

**A.**


DAPI
FLAG
Combined
FUCCI-U2OS cells
Transfected with empty
vector plasmid
FUCCI-U2OS cells
Un-transfected

**B.**


DAPI
FLAG
Combined
FUCCI-U2OS cells
Transfected with ENT-
TRF1 plasmid
FUCCI-U2OS cells
Transfected with only
TRF1 plasmid

**C.**

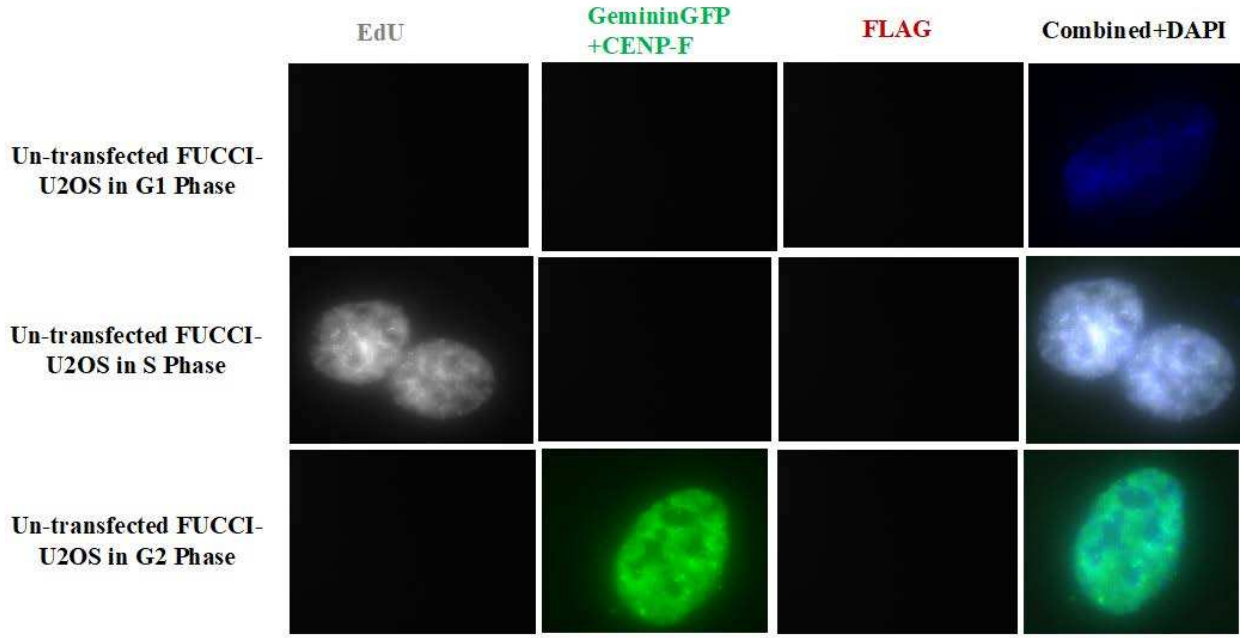

Figure 2.3: FUCCI-U2OS transfection efficiencies.

**A**. Representative images of transiently transfected FUCCI-U2OS cells co-stained for nuclei (DAPI; blue) and (FLAG; red). **B.** Quantitation of average number FLAG foci/cell. Data represent 3 experiments each; error bars are SEM (*n* = 12-200). Significance of one-way ANOVA and Holm-sidak Test ***p,0.001; **p<0.01; *P<0.05. ns, not significant

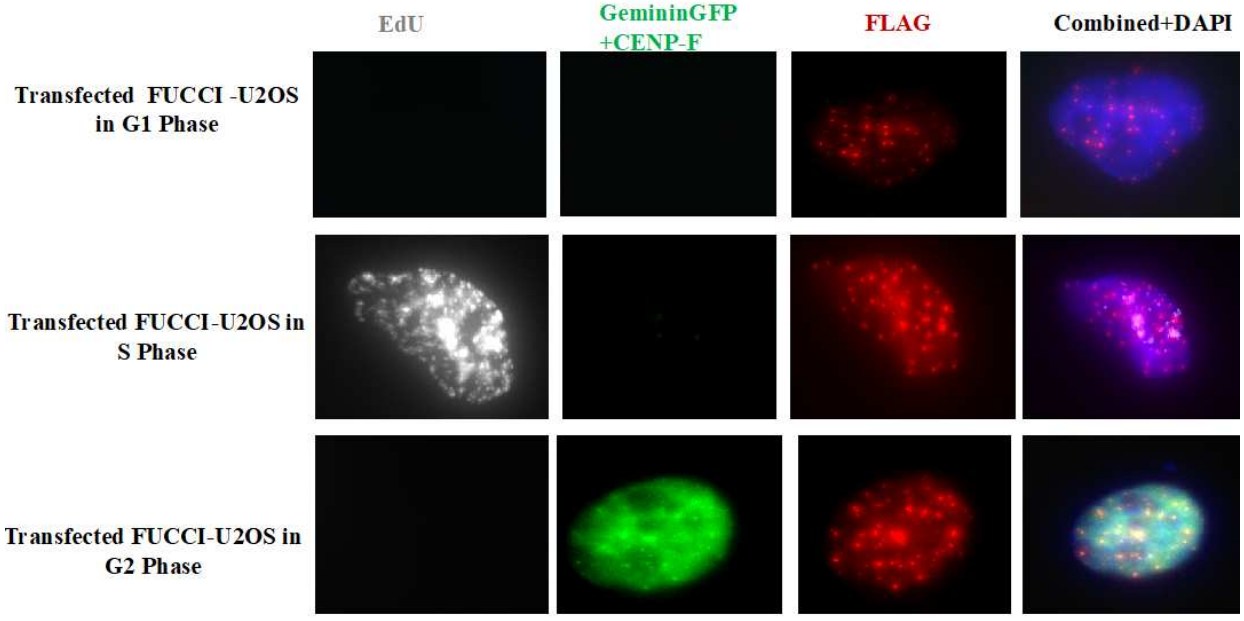
**FUCCI-U2OS cells accumulate in G2 following transfection**

We have previously shown that normal human, non-ALT BJ1-hTERT fibroblasts transfected with ENT-TRF1 or TRF1-only arrest in G1 (manuscript under review). Here we find that FUCCI-U2OS cells transiently transfected with ENT-TRF1, TRF1- only, or empty vector accumulate in G2 compared to untransfected cells; specifically, 48 hour following transfection the fraction of cells in G2 increased (**Figure 2.4 A, B**). The enrichment of positively transfected cells in G2 phase suggests that transfected cells undergo DNA damage and /or cellular stress response. These results are consistent with U2OS cells having a permissive G1 checkpoint [158, 159] .

**A**.


EdU
GeminingGFP +CENP-F
FLAG
Combined+DAPI
Un-transfected FUCCI-U2OS in G1 Phase
Un-transfected FUCCI-U2OS in S Phase
Un-transfected FUCCI-U2OS in G2 Phase

**B.**


EdU
GeminingGFP +CENP-F
FLAG
Combined+DAPI
Transfected FUCCI -U2OS in G1 Phase
Transfected FUCCI-U2OS in S Phase
Transfected FUCCI-U2OS in G2 Phase

**C.**

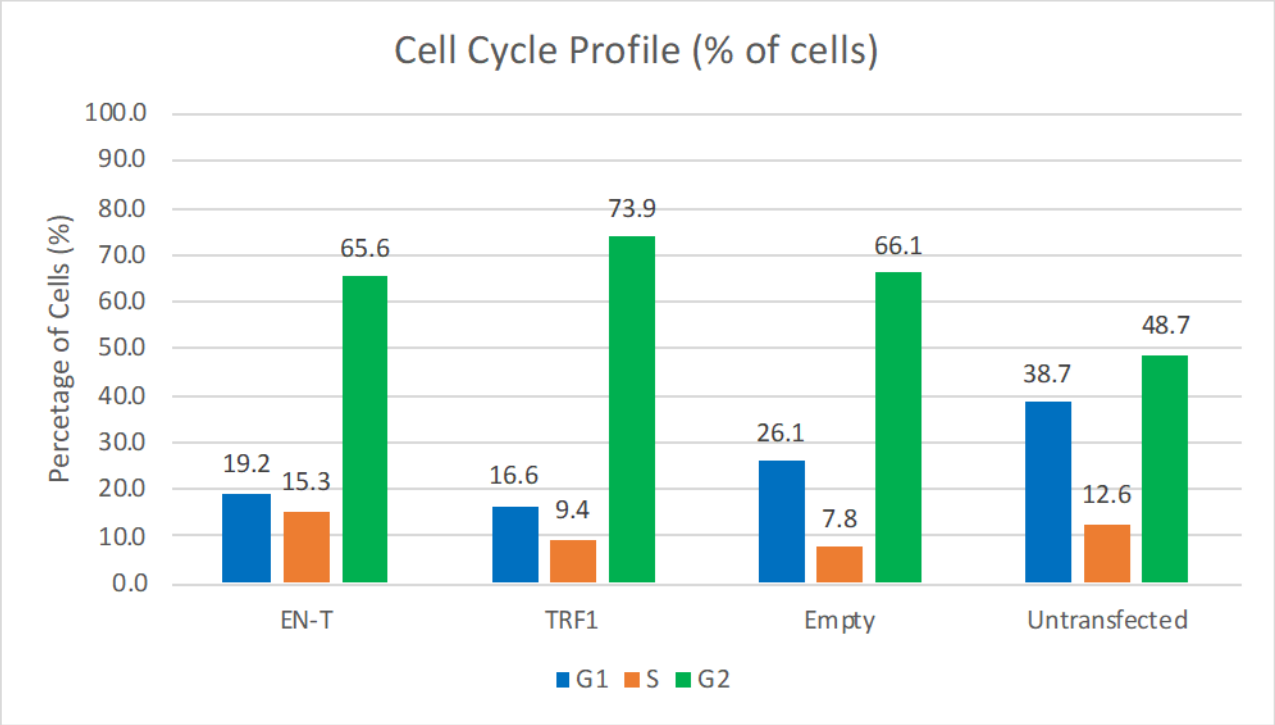



Figure 2.4: FUCCI-U2OS cell cycle distribution following transfection.

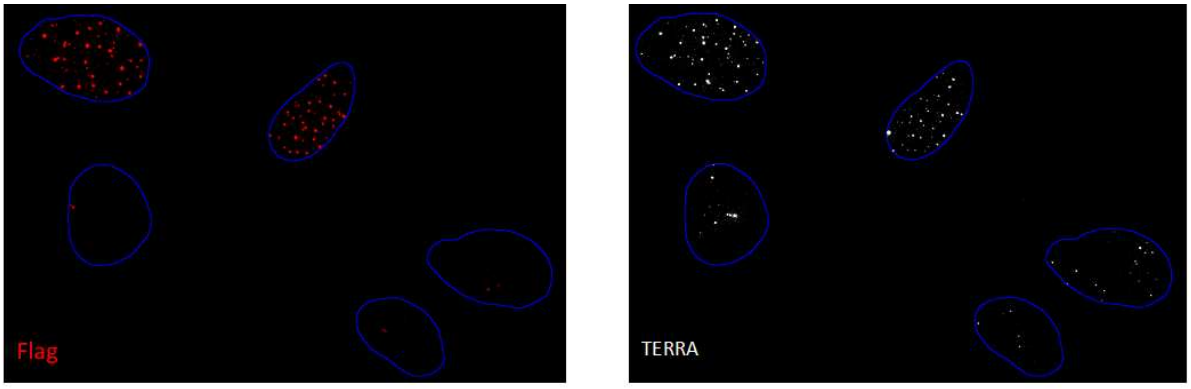
**A.** Representative images of untransfected FUCCI-U2OS cells labeled with only DAPI (G1 phase), EdU (S phase), Geminin-GFP and CENP-F (G2 phase), FLAG (untansfected), merged views with DAPI (blue) **B.** Representative images of transiently transfected FUCCI-U2OS cells labeled with with only DAPI (G1 phase), EdU (S phase), Geminin-GFP and CENP-F (G2), FLAG (transfected), and merged views with DAPI (blue). **C.** Histogram represents the relevant percentage of FUCCI-U2OS cells in each cell cycle phase with and without transfection. Data was analyzed from 3 experiments.

### TERRA distribution in FUCCI-U2OS cells following transfection

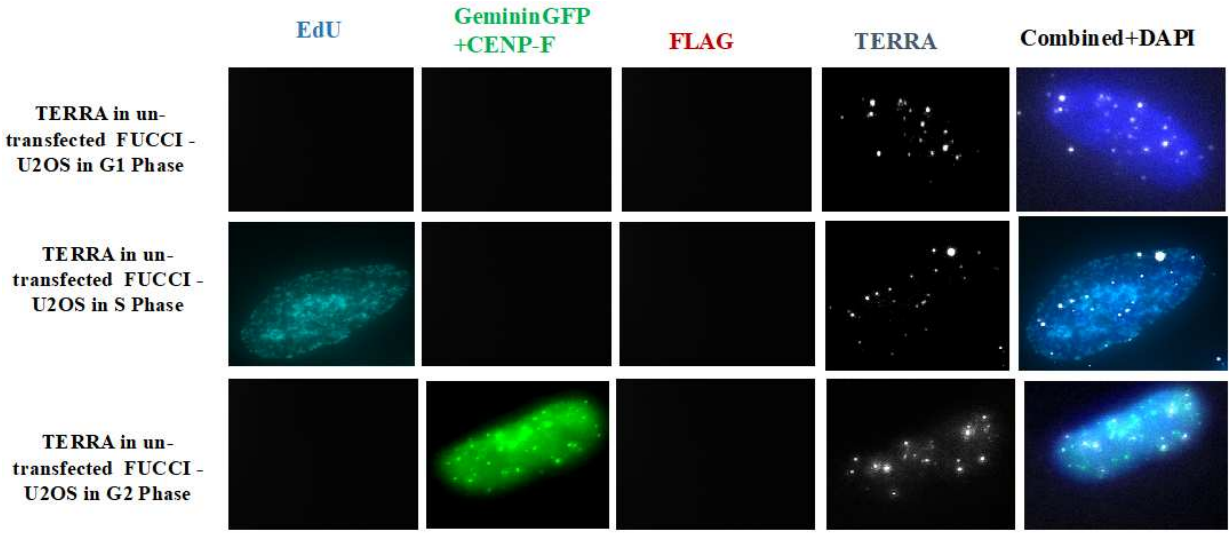
Next, we examined the effect of transient transfection of ENT-TRF1, TRF1-only, and empty vector on the distribution of TERRA through the cell cycle in FUCCI-U2OS cells. Total TERRA foci were evaluated by RNA-FISH in positively (25-30%) and negatively (65-70%) transfected cells for each treatment and compared to un-transfected controls. Consistent with the cell cycle profiles, TERRA foci were highly enriched in G2 compared to un-transfected cells (**Figure 2.5 A, B, C).** Treatment with RnaseA and RnaseH diminished TERRA signals, confirming

TERRA presence (**Figure 2.5 D, E)**. The accumulated levels of total TERRA in G2 positively transfected as compared to un-transfected control cells suggest TERRA involvement in the DNA damage and/or cellular stress response.

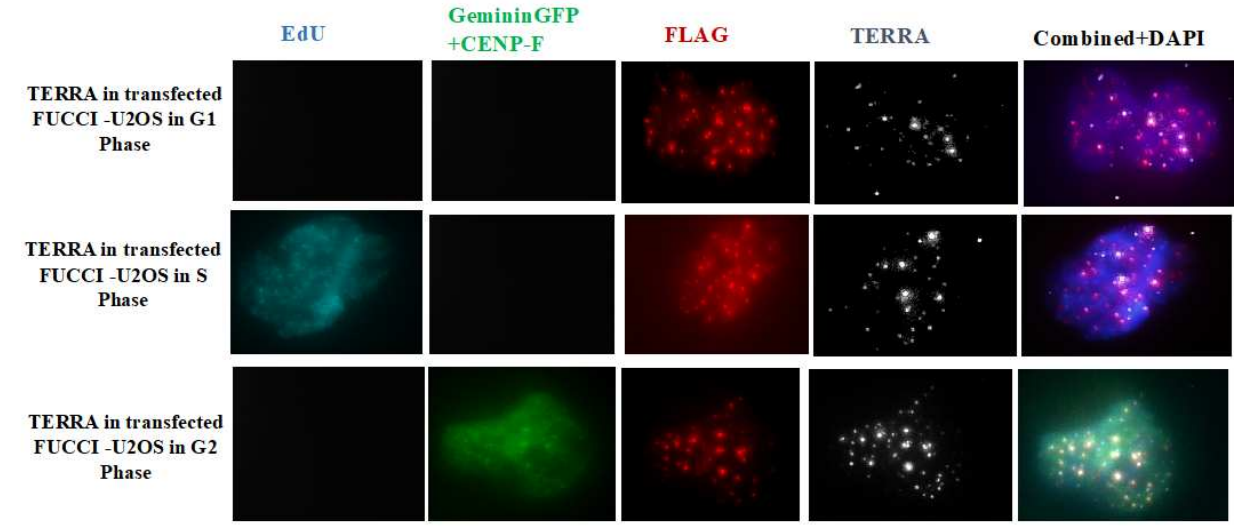
A.



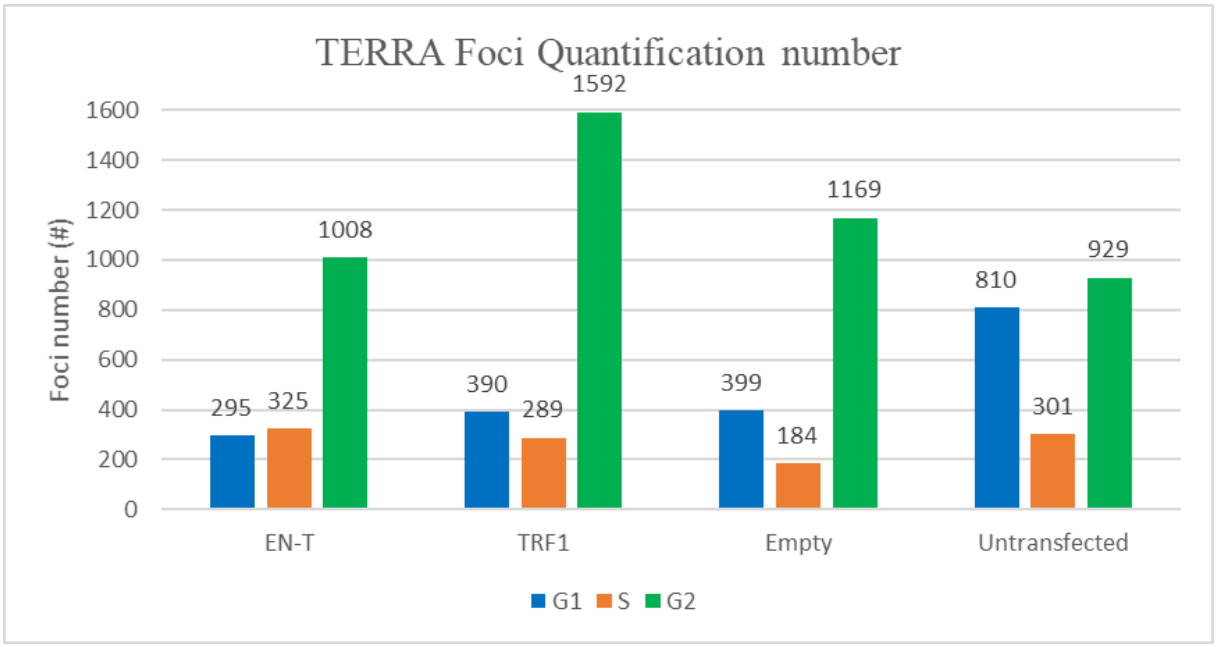

**B.**



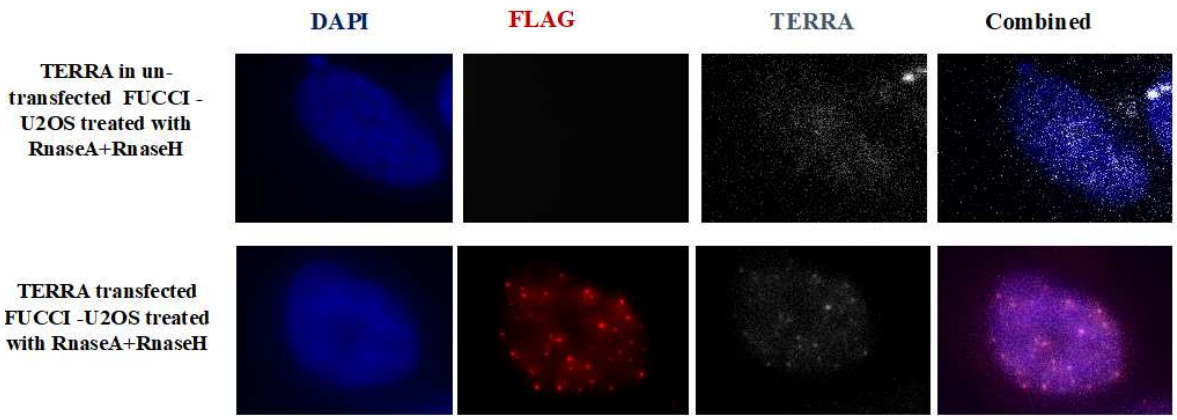
**C.**


EdU
GeminGFP +CENP-F
FLAG
TERRA
Combined+DAPI
TERRA in transfected FUCCI -U2OS in G1 Phase
TERRA in transfected FUCCI -U2OS in S Phase
TERRA in transfected FUCCI -U2OS in G2 Phase

**D.**


TERRA Foci Quantification number
Foci number (#)
1600
1400
1200
1000
800
600
400
200
0
295
325
1008
390
289
1592
399
184
1169
810
301
929
EN-T
TRF1
Empty
Untransfected
G1
S
G2

**E.**




**F.**

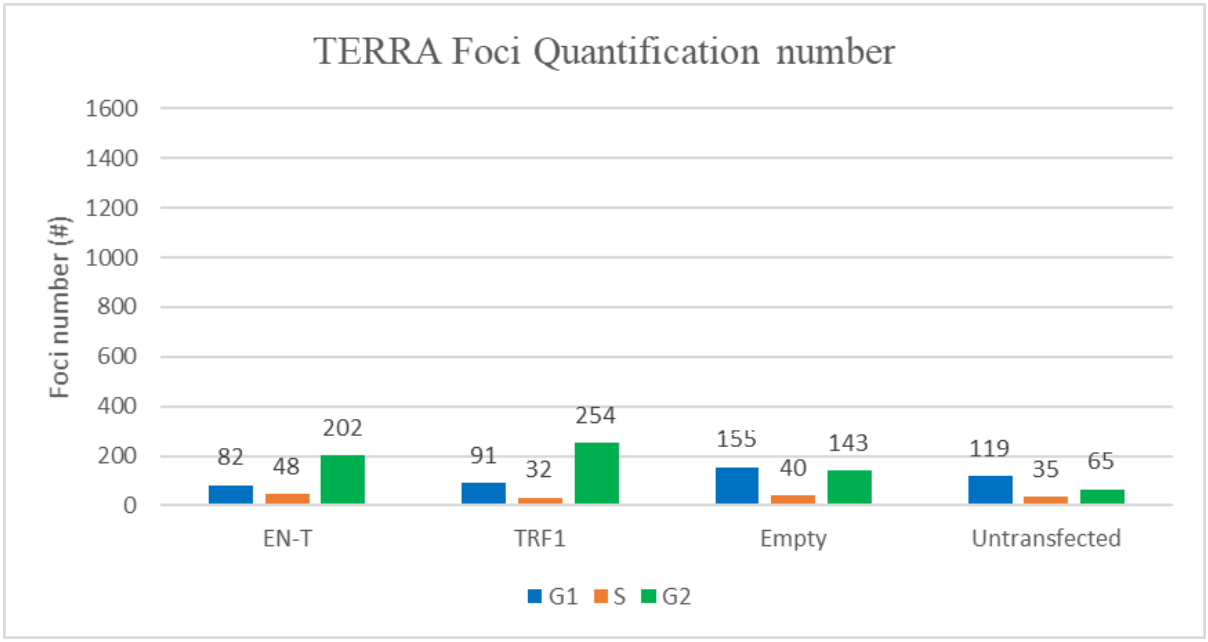


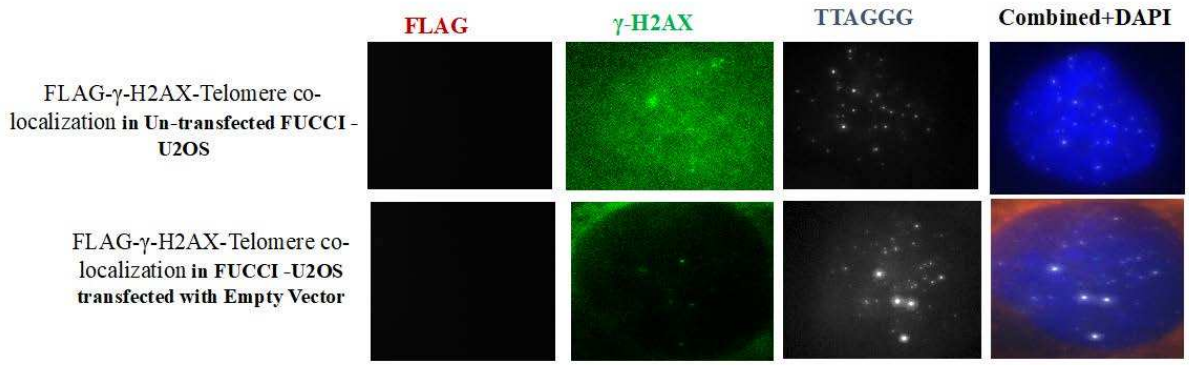

Figure 2.5: TERRA accumulates in G2 following transfection**.**

**A.** Representative images of transiently transfected FUCCI-U2OS cells labeled for FLAG and TERRA. Total TERRA was scored in positively (25-30%) and negatively (65-70%) transfected cells from each treatment **B.** Representative images of transiently transfected FUCCI-U2OS cells stained for EdU (S phase), Geminin-GFP and CENP-F (G2), FLAG, or TERRA, and merged views with DAPI (blue). **B.** Representative images of transiently transfected FUCCI-U2OS cells stained for EdU (S phase), Geminin-GFP and CENP-F (G2), FLAG, or TERRA, and merged views with DAPI (blue). **C.** Histogram shows the corresponding percent of TERRA in each cell phase with and without transfection. **D.** Representative images of transiently transfected FUCCI-U2OS cells, treated with Rnase A and Rnase H and labeled for EdU, Geminin-GFP and CENP-F, FLAG, TERRA and merged views with DAPI. **F.** Histogram shows the corresponding percentage of TERRA in each cell phase with and without transfection. Data was analyzed from 3 experiments.

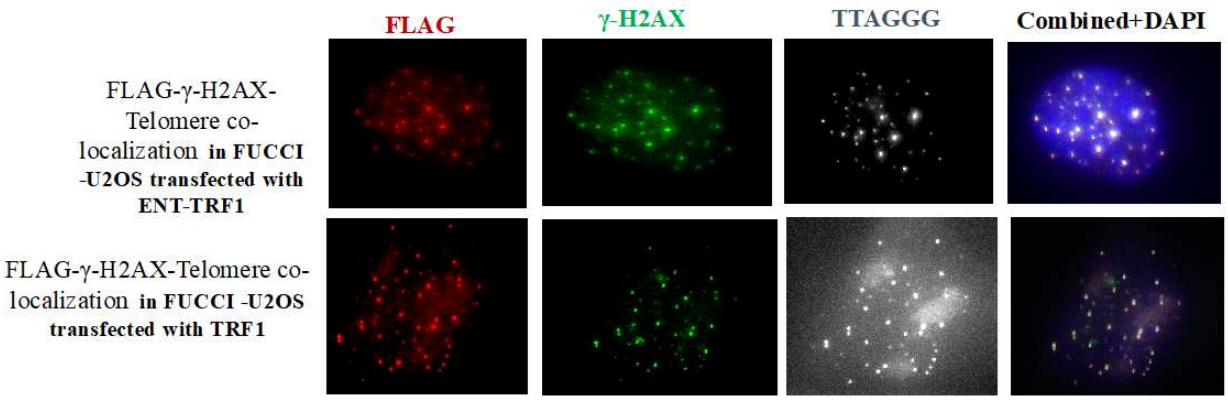
**Telomere-specific DSBs in FUCCI-U2OS cells**

The enrichment of transfected cells and accumulation of total TERRA in G2 phase provided evidence that transfected cells experienced DNA damage. Combining telomere FISH with fluorescence immunostaining against FLAG and the DNA DSB marker γ-H2AX (FLAG/γ-H2AX/Telomere co-localized foci) confirmed that ENT-TRF1 localized to telomeres and induced telomere-specific DSBs. Consistent with previous reports in telomerase positive cells [160-162] , we also observed that overexpression of TRF1 induced telomeric DSBs in ALT cells. Only cells with > 20 FLAG foci were counted as positive transfected cells (**Figure 2.6 A, C**). Taken together with the absence of γ-H2AX foci in cells transfected with empty vector or untransfected cells (**Figure 2.6 B**), results validated that expression of ENT-TRF1, or TRF1-only, in FUCCI-U2OS cells induces telomere-specific DSBs. Consistent with expectation, treatment with RNAse A + H did not influence results (**Figure 2.6 C**).

**A.**



**B.**




**C.**

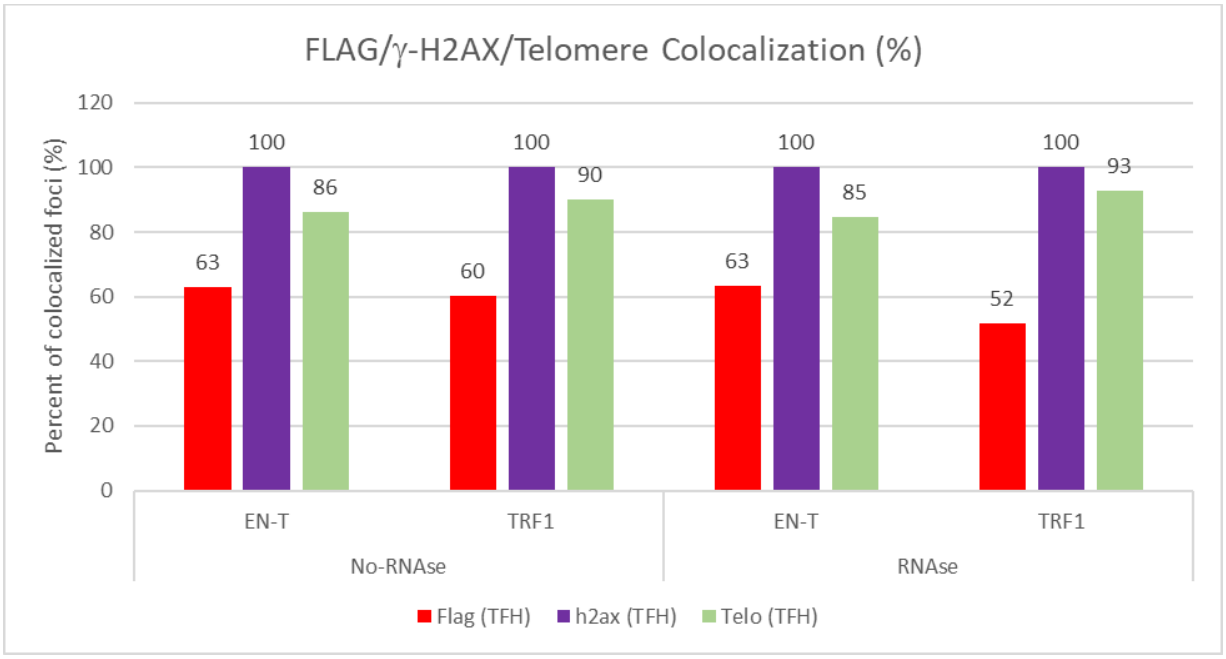


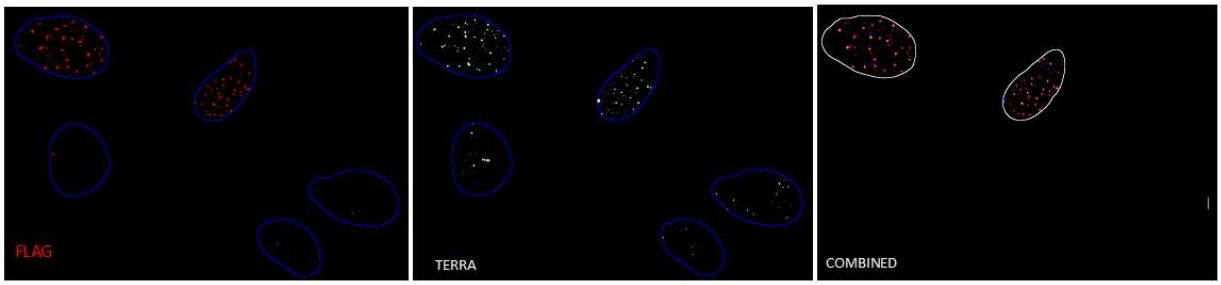

Figure 2.6: Telomere-specific DSBs in FUCCI-U2OS cells.

**A.** Representative images of FUCCI-U2OS cells transfected with empty vector and untransfected cells, and then labeled for FLAG, γ-H2AX- and telomeres, and merged images with DAPI (blue) confirming induction of telomeric DSBs. **B.** Representative images of FUCCI-U2OS cells transfected with either ENT-TRF1 or TRF1 only, and then labeled for FLAG, γ-H2AX- and telomeres, and merged images with DAPI (blue) confirming induction of telomeric DSBs. **C.** Histogram represents the corresponding percentages of colocalized foci in cells with >20 FLAG foci (n= 30). Treatment with RNAse did not influence results.

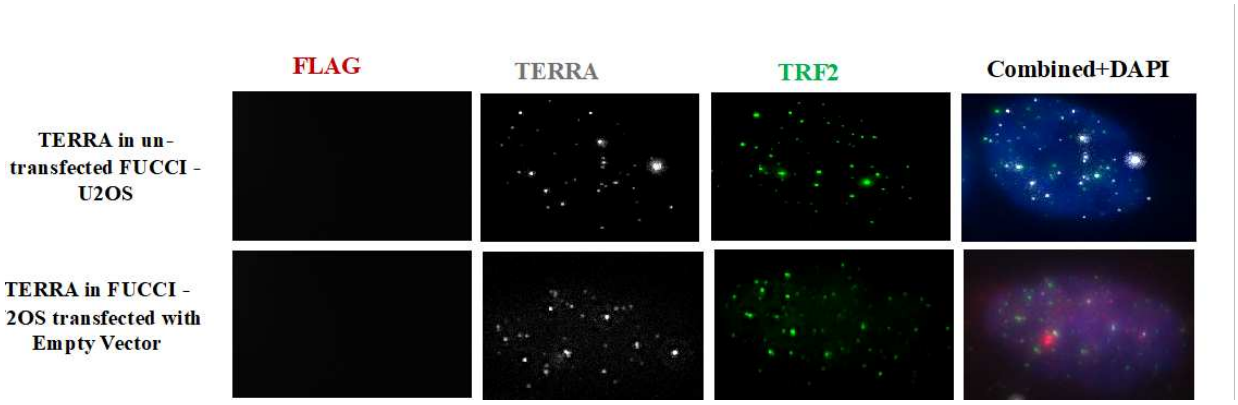
**TERRA accumulates at telomere-specific DSBs**

We then evaluated the question of whether TERRA participated in the telomeric DDR by responding to telomeric DSBs in FUCCI-U2OS cells. To ensure that TERRA-FLAG colocalization events (broken telomeres) were induced by ENT-TRF1 or TRF1-only, scoring was restricted to positively transfected ENT-TRF1 and TRF1-only cells (**Figure 2.7A)**. In order to visualize TERRA at telomeres in cells transfected with empty vector or un-transfected cells that lack FLAG, another means of detecting telomeres was necessary. The telomere-specific binding factor TRF2 was selected, as this approach was compatible with RNA-FISH (**Figure 2.7 B**). Although FLAG signals from ENT-TRF1 and TRF1-only populations colocalized often with telomeres (**Figure 2.6 C, D)**, fewer telomeric FLAG signals were detected using TRF2 (**Figure 2.7 C**). This can likely be attributed to the observation of faint or absent TRF2 fluorescent signals in some positively transfected ENT-TRF1 and TRF1-only cells compared to TRF2 signals in controls (**Figure 2.7 C).** Three distinct TERRA colocalization events were detected in ENT-TRF1 and TRF1-only populations: TERRA-TRF2, FLAG-TRF2(telomere)-TERRA, and FLAG-TERRA (**Figure 7 C, D).** Results clearly demonstrated that TERRA accumulates at telomeric DSBs, a finding supported by treatment with RnaseA and RnaseH, which removed TERRA signals (**Figure 2.7 E).** Summing up the average number of foci for the three different types of TERRA events (**Figure 2.7 F),** our data provides direct evidence of TERRA at telomeric DSBs.

**A.**


FLAG
TERRA
COMBINED

**B.**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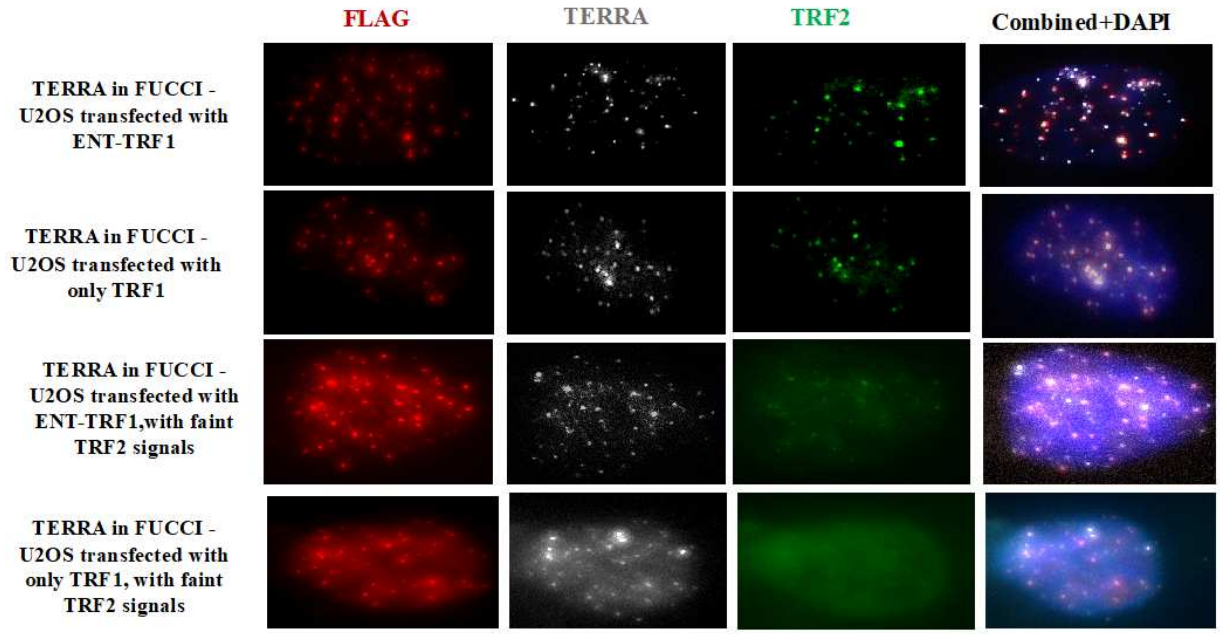
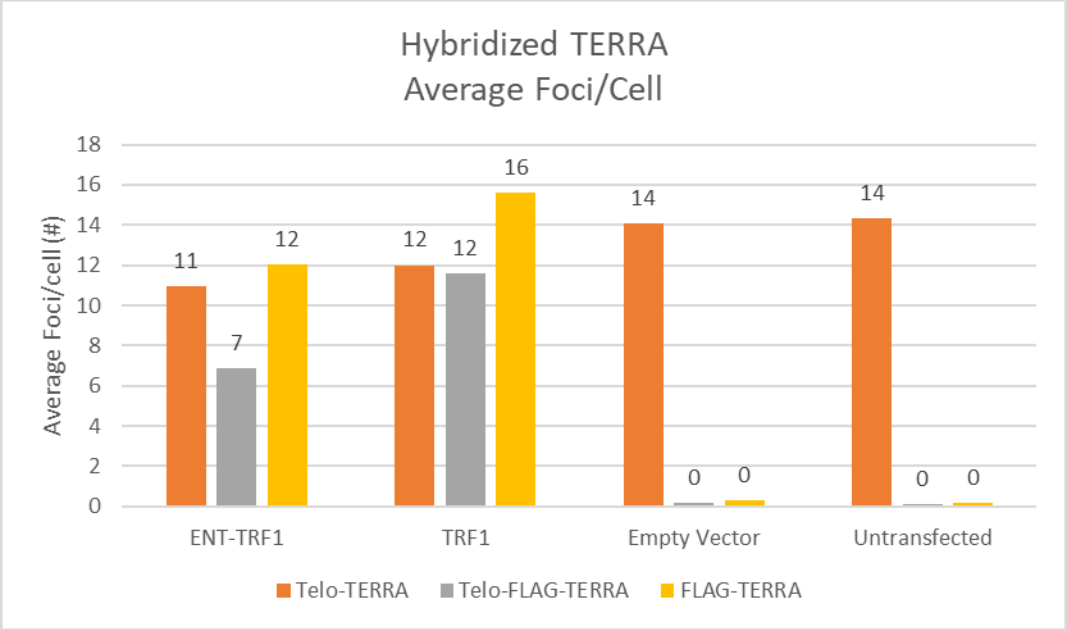
TRF2
Combined+DAPI
TERRA in un-transfected FUCCI - U2OS
TERRA in FUCCI - 2OS transfected with Empty Vector

**C.**


FLAG
TERRA
TRF2
Combined+DAPI
TERRA in FUCCI - U2OS transfected with ENT-TRF1
TERRA in FUCCI - U2OS transfected with only TRF1
TERRA in FUCCI - U2OS transfected with ENT-TRF1,with faint TRF2 signals
TERRA in FUCCI - U2OS transfected with only TRF1, with faint TRF2 signals

**D.**


Hybridized TERRA
Average Foci/Cell
Average Foci/cell (#)
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16
14
12
10
8
6
4
2
0
11
7
12
12
12
16
14
0
0
14
0
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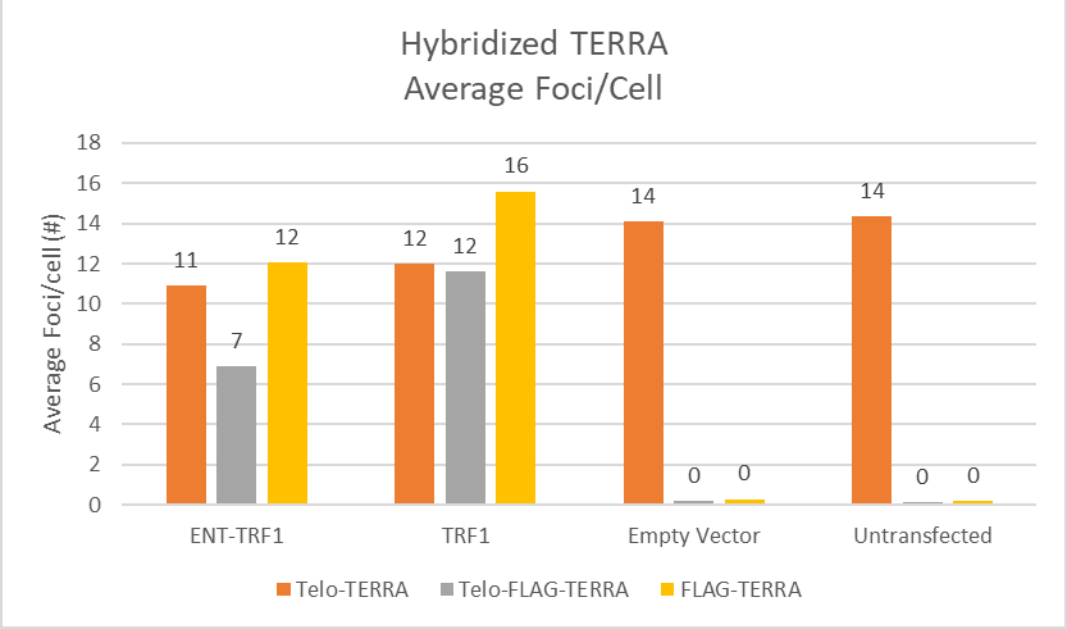
ENT-TRF1
TRF1
Empty Vector
Untransfected
Telo-TERRA
Telo-FLAG-TERRA
FLAG-TERRA

**E.**


Hybridized TERRA
Average Foci/Cell
Average Foci/cell (#)
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16
14
12
10
8
6
4
2
0
11
7
12
12
12
16
14
0
0
14
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ENT-TRF1
TRF1
Empty Vector
Untransfected
Telo-TERRA
Telo-FLAG-TERRA
FLAG-TERRA

**F.**

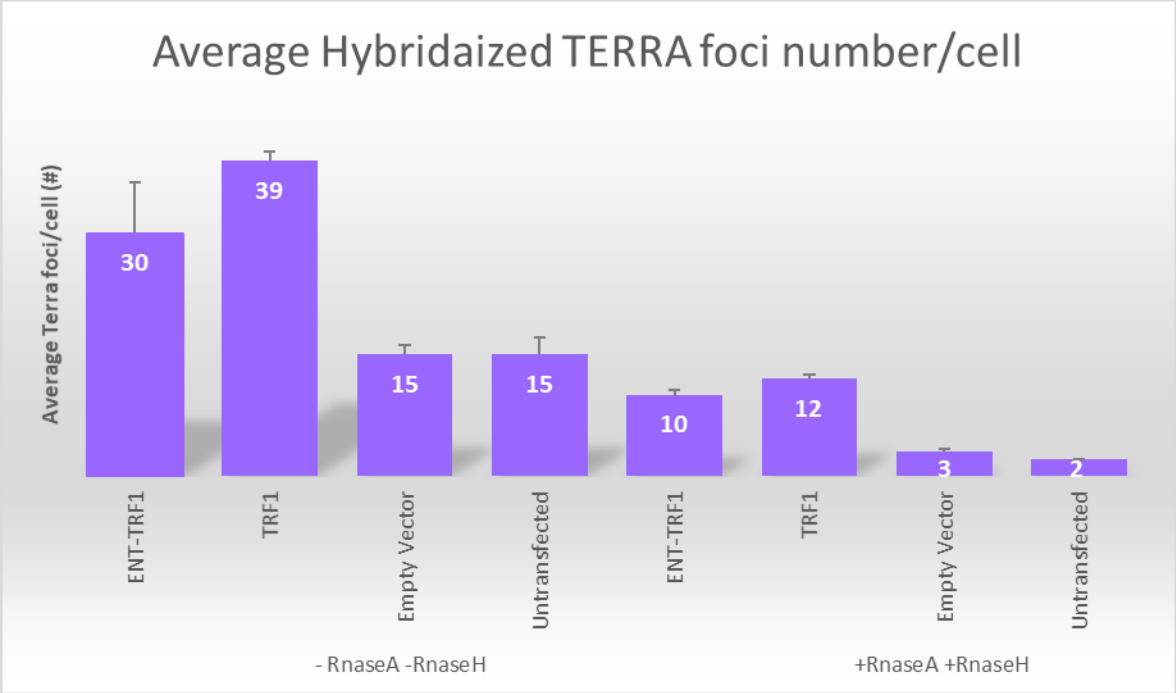


Figure 2.7: ERRA accumulates at telomeric DSBs**.**

**A.** Representative images of transiently transfected FUCCI-U2OS cells labeled for FLAG and TERRA. Hybridized TERRA was scored in only positively (25-30%) transfected cells from each treatment **B**. Representative images of untransfected and empty vector transfected FUCCI-U2OS cells labeled for FLAG, TERRA (grey), and TRF2 (telomeres, green), and merged views with DAPI (blue). **C.** Representative images of transiently transfected FUCCI-U2OS cells labeled for FLAG (red), TERRA (grey), and TRF2 (telomeres, green), and merged views with DAPI (blue). **D.** Histogram represents the average number of foci (TERRA-TRF2, FLAG-TRF2-TERRA, or FLAG-TERRA)/cell. **E.** Histogram represents the average number of foci/cell following treatment with Rnase A and H. Data was analyzed from 3 experiments. **F.** Histogram represents the average number of hybridized TERRA/cell.

**TERRA plays a protective role at telomeric DSBs**

Our unpublished work showed that induction of targeted telomeric DSBs in normal human G1 non-ALT fibroblasts (BJ1-hTERT), which have low levels of TERRA compared to ALT (U2OS) cells, was associated with a significant increase in 5' C-rich single-stranded (ss)telomeric DNA (manuscript in preparation/under review). Interestingly, extensive resection was not detected in BJ1-hTERT cells transfected with TRF1-only. Here, we present evidence that transfecting FUCCI-U2OS cells with either ENT-TRF1 or TRF1-only induces telomeric DSBs, and further that TERRA accumulates at the damage sites (**Figure 2.6 B, C**). Utilizing native (non-denaturing) DNA FISH to detect 5' C-rich (ss)telomeric DNA in FUCCI-U2OS transfected cells, combined with immunostaining to detect FLAG in cells presumably in G1 (negative for green FUCCI, so not in G2), no induction of C-rich (ss) telomeric DNA was observed (**Figure 2.8 A, C**). However, treatment with RnaseA and RnaseH to remove TERRA (5'-UUAGGG-3') revealed a significant increase in telomeric resected C-strands (5'-CCCTAA-3') in cells transfected with ENT-TRF1, but not with TRF1-only (**Figure 2.8 B, C**). Taken together, these results suggest a protective role for TERRA at telomeric DSB sites in G1, as well as a different mechanism of telomeric repair in ALT (U2OS) vs non-ALT (BJ1-hTERT) cells dependent on the particular type of telomeric DSB.

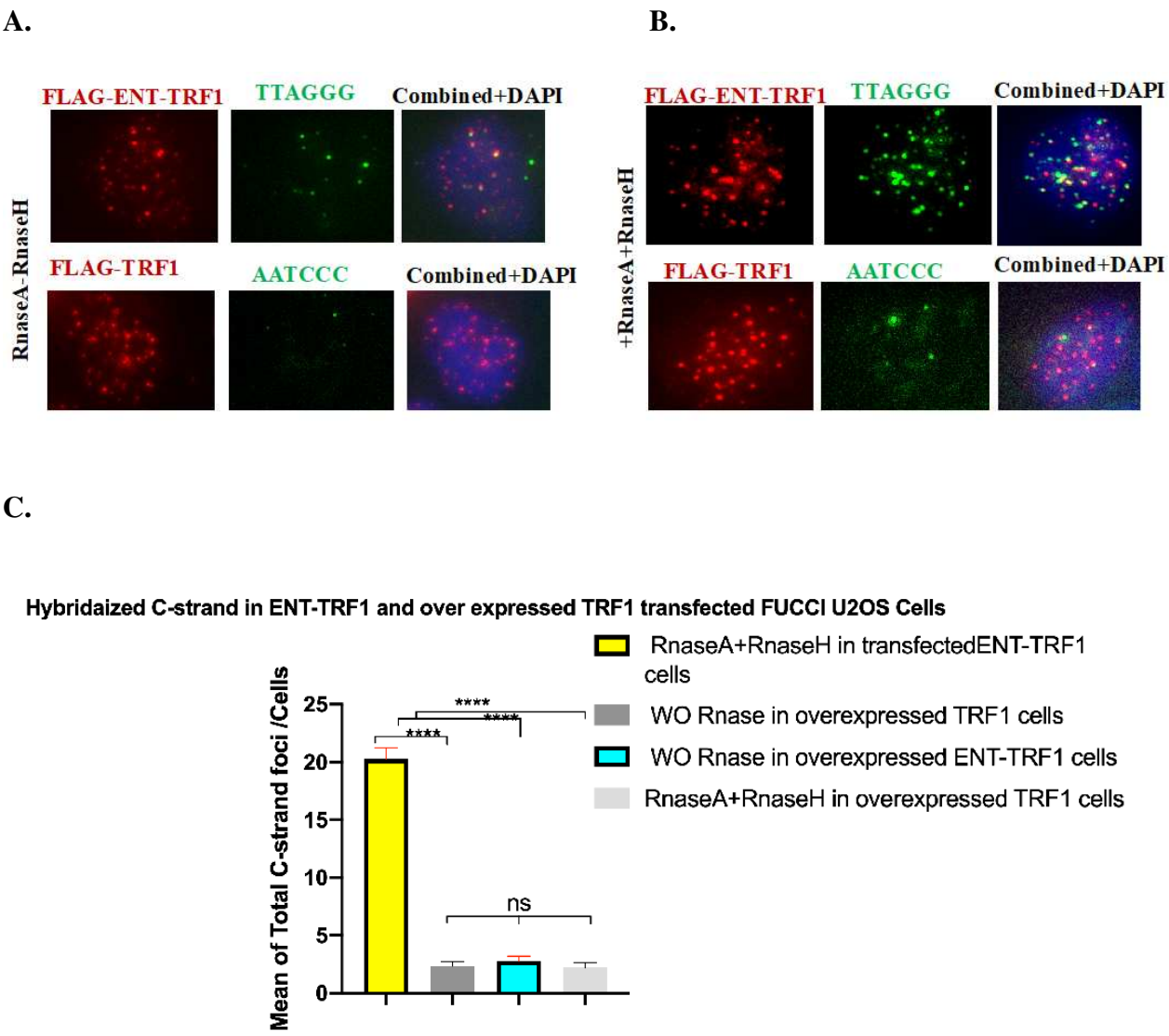


Figure 2.8: Transfection of FUCCI-U2OS cells with ENT-TRF1 and removing TERRA revealed significant induction of 5' C-rich single-stranded (ss)telomeric DNA

**A.** Representative images of transiently transfected FUCCI-U2OS cells labeled for ENT-TRF1 or TRF1 only using FLAG (red), C-strand telomeric sequence (AATCCC) using G-rich probe (green), and merged views. **B.** Representative images of transiently transfected FUCCI-U2OS cells, treated with RnaseA+RnaseH, and labeled for ENT-TRF1 or TRF1 only using FLAG (red), C-strand telomeric sequence (AATCCC) using G-rich probe (green), and merged views. **C.** Quantitation of average number telomeric C-strand foci. Data represents three experiments and values are expressed as SEM (*n* = 120-200). One-way ANOVA and Holm-sidak Test ***p,0.001; **p<0.01; *P<0.05. ns, not significant

# CHAPTER 3 DISCUSSION AND CONCLUSION

Over the last decade, transcription of telomeres has been intensely investigated. Since its discovery, heterogeneous lengths of the telomeric RNA TERRA (3'-UUAGGG-5') have been visualized in yeast, human and mouse [20, 108, 110]. The discovery of TERRA was corroborated by Chromatin immunoprecipitation analysis, which also revealed RNA pol II binding at various subtelomeric promoter sites [109, 112]. Functionally, TERRA has been shown to be involved in telomere replication [81, 120], telomere protection [109, 114, 115, 122], telomeric heterochromatin mark deposition [23] , and telomere length maintenance by homologous recombination (HR) [100]. Human cancer cells that maintain their telomeres using the telomerase-independent ALT pathway are characterized by a number of hallmarks [163], including elevated levels of TERRA [81-83], and telomere length maintenance via a HR-dependent mechanism [96, 97, 150]. Indeed, work by *Arora et al* has shown that TERRA promotes HR in human osteosarcoma U2OS (ALT) cells [100]. However, the role of TERRA in response to DNA damage, and specifically in response to telomeric DSBs, has not been elucidated.

TERRA in response to genomic DNA DSBs

Ionizing radiation (IR) is a well-established inducer of prompt DSBs. We investigated the response of TERRA to genomic DNA DSBs resulting from IR exposure (2Gy; γ-rays) in U2OS (ALT) cells. Interestingly, TERRA foci were significantly increased in irradiated vs. non-irradiated control cells **(Figure 2.1)**. This finding is consistent with a recent study reporting that genomic DSBs induced by the radiomimetic drug Bleomycine showed elevated levels of TERRA [129].

However, considering the small fraction of the genome that telomeric DNA represents, this relatively low dose of IR would be expected to directly "hit" and "break" telomeres only very rarely. Therefore, we employed an approach to enzymatically-induce telomere-targeted DSBs using the endonuclease ENT fused to the telomeric protein TRF1, which was developed and validated in U2OS cells [164].

TERRA in response to transient transfection

We employed a FUCCI-based system to positively identify cell cycle phase of interphase nuclei, which revealed that the stress of transient transfection significantly influenced U2OS cell cycle profiles. Cell cycle analyses demonstrated that transfected cells accumulate in G2 **(Figure 2.4** ), similar to the G2 arrest observed following exposure to IR [165], or treatment with the radiomemetic drug-neocarzinostain [166, 167]. A linear correlation between the amount of DNA damage induced, and the stringency of the activated G2 checkpoint/halting of cell cycle progression, has been previously demonstrated in U2OS cells [166, 167]. A permissive G1/S checkpoint in U2OS cells, resulting from truncated WIP1(wild-type p53-induced phosphatase 1) and p16 deficiency (a cyclin-dependent kinase inhibitor essential for regulating the cell cycle), also likely contributes to the enrichment of G2 cells observed [158, 159].

We then evaluated whether TERRA levels were elevated in G2 phase following transfection. Consistent with the cell cycle analysis, the distribution of total TERRA shifted, accumulating in G2 in response to induction of telomeric DSBs and/or the stress stimuli of transfection **(Figure 2.5)**. It has been previously shown that adenovirus-mediated transfection of U2OS cells with ENT-TRF1 activates a p53-dependent pathway [164], and that activation of subtelomeric p53 in response to cellular stress can induce TERRA transcription [127]. A more recent study demonstrated a direct role for long non-coding (lnc)RNA p53-induced NC RNA

(PINCR) in G1 arrest in colorectal cancer cells; PINCR was found to regulate the expression of genes implicated in G1 arrest [168]. Several p53-induced lncRNAs, including LED, p53-regulated lncRNA (PR lncRNA), and lncRNA p21, have been shown to be required for checkpoint activation and cell cycle arrest; reviewed in [169]. Considering that U2OS cells are p53 positive [170] and have a disrupted G1 checkpoint [158, 159], accumulation of TERRA in G2 following transfection (ENT-TRF1 or TRF1-only) supports TERRA responsiveness to p53, as well as suggests a potential role for TERRA in activation of the G2 checkpoint in U2OS (ALT) cells.

TERRA in response to telomere-specific DSBs

We validated the telomere-targeting/DSB-inducing capabilities of the ENT-TRF1 system in U2OS (ALT) cells [164]. Since ENT-TRF1 also increases endogenous levels of TRF1, FUCCI-U2OS cells transfected with a TRF1-only plasmid that overexpressed TRF1 were used as a control. Consistent with previous reports, TRF1-only also induced co-localization with γ-H2AX, indicative of telomeric DSB induction **(Figure 2.6 ),** which could be due to the fact that TRF1 plays a critical role in telomere protection and telomere length regulation [160-162]. In particular, telomerase positive cells overexpressing TRF1 experience telomere shortening [160], telomere bridges, and DNA DSBs [171]. In telomerase independent ALT cells, it has been shown that non telomeric phosphorylated (T371)TRF1 is crucial for promoting homologous recombinational (HR) repair upon induction of global DNA damage (IR exposure) [172]. Furthermore, overexpression of TRF1 in TRF1 knockdown ALT cells suppressed the reduction of C-circles [173], which have been associated with HR activity [97]. Our results provide additional support for a role of TRF1 in promoting telomeric HR following induction of telomeric DSBs**.**

Our controls (empty vector and un-transfected) required a different shelterin component in order to visualize potential hybridization of TERRA to telomeres in control populations; the

telomere repeat factor 2 (TRF2) was selected. Again, a significant increase of TERRA co-localized at broken telomeres was observed in U2OS cells expressing ENT-TRF1 and TRF1-only **(Figure 2.7)**. It is also worth noting that the TERRA detected here was hybridized (co-localized) at telomeric DSBs **(Figure 2.7)**. Hybridized TERRA at telomeric DSBs is distinct from free/unbound TERRA (foci not co-localized at telomeres), which was detected in response to transfection **(Figure 2.4)** and in cells transfected with empty vector.

**TERRA is required to protect telomeric DSBs in G1 cells**

Our previous work in normal G1-human fibroblasts, which have low levels of TERRA, demonstrated increased frequencies of 5' C-rich single-stranded (ss)telomeric DNA (indicative of extensive resection) at telomeric DSB sites (manuscript under review). In light of the complementary sequence of TERRA, we hypothesized that hybridized TERRA at telomeric DSBs in ALT cells may well protect the exposed C-rich (ss)overhangs from being extensively resected. Consistent with such a notion and with our previous findings, a significant induction of 5' C-rich (ss)telomeric DNA was observed in G1 U2OS (ALT) cells experiencing ENT-TRF1 induced DSBs **(Figure 2.8)**, **(Figure 3.1)**.

Emerging evidence, in both yeast and mammalian cells, supports a link between RNA:DNA hybrids and DNA end resection. For example, inhibiting DNA-end resection by knocking down either EXO1 or CtIP after inducing a site-specific DSB in U2OS cells impairs formation of RNA:DNA hybrids [174]. In *Schizosaccharomyces pombe,* RNA:DNA hybrids at DSBs have been shown to limit resection of ssDNA and regulate the binding of replication protein A (RPA) [175]; overexpression of Rnase H1 stabilized RNA:DNA hybrids and impaired recruitment of RPA [175]. Phosphorylated RPA at (ss)telomeric DSB sites in G1 human cells was also observed in our previous work. Of relevance in this regard, a recent work identified RPA as a

sensor of RNA:DNA hybrids in non-S phase HeLa cells [176]; RPA enhanced the binding of RnaseH1 and RNA:DNA hybrids [176]. Additionally, stabilized RNA:DNA hybrids at I-SceI-induced DSBs in U2OS cells have been observed to avert RPA binding [177]. Removal of RNA from RNA:DNA hybrids significantly reduced BRCA1 and RAD51 recruitment in G2 [174].

Interestingly, in contrast to the extensive resection observed at ENT-TRF1 induced telomeric DSBs and the evidence in support of HR-mediated repair in G2, only minimal resection was detected at broken telomeres induced by TRF1-only, potentially implicating alt-EJ in their repair [130]. In either case, hybridized TERRA would be expected to protect the vulnerable (ss)telomeric overhangs.

Although the role of long noncoding (lnc)RNA in response to genomic DSBs has been recently investigated, our data are the first to demonstrate a role for the telomeric lncRNA, TERRA, in telomeric DSB repair. We propose that TERRA binds exposed 5' C-rich (ss)telomeric DNA at extensively resected telomeric DSBs, presumably to preserve/protect them until S/G2 when HR-mediated elongation/repair is possible, perhaps by providing a homologous template.

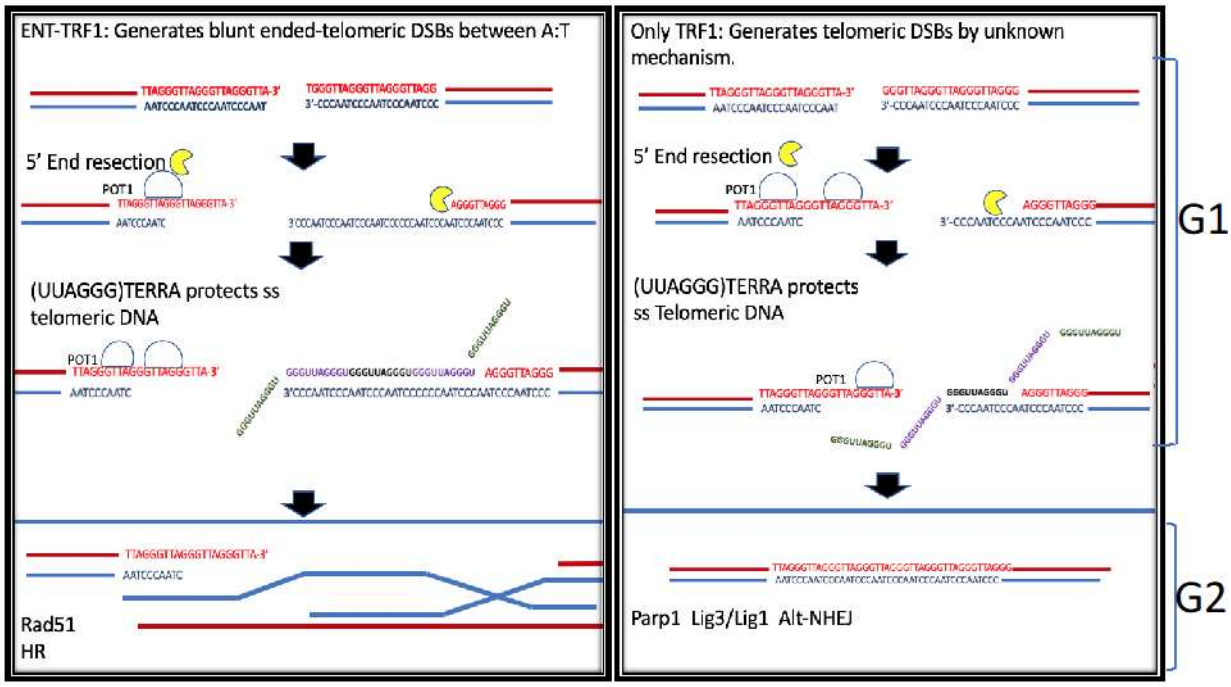


Figure 3.1: Telomeric RNA TERRA protects exposed ss telomeric DNA**.**

Expression of ENT-TRF1 induces blunt ended telomeric DSBs in U2OS (ALT) cells results in extensive resection and generating C-rich overhangs which protected by pairing to telomeric RNA (TERRA) in G1. Previous work demonstrated that telomeric DSB-induced hyper resection can be repaired via HR [151]. Our model here also showed that over expression TRF-only induces telomeic DSBs by unknown mechanism. Although hybridized TERRA was accumulated at the damage sites, the C-rich overhangs was not detected in G1. The accumulated TERRA and undetectable resection provide another strong evidence for what has been reported; that U2OS-ALT cells repair telomeric DSBs damage in G2 via alt-EJ pathway which require limited resection and perfect homology within telomeres sequences [151]. Finally, while POT1was suggested to protect G-strands [151]our model illustrates the role of TERRA in protecting C-strands in G1.

## Conclusion

In summary, the work presented here provides evidence supportive of a role for the telomeric RNA TERRA in response to telomeric DNA DSBs in U2OS (ALT) cells. IR-induced genomic DSBs increased frequencies of TERRA foci. Furthermore, enzymatically-induced telomere-specific DSBs resulted in increased co-localization of (bound) TERRA to broken telomeres. ALT (U2OS) cells experiencing telomeric DSBs accumulated in G2, as did TERRA; cells also accumulated in G2 in response to the cellular stress of transfection. TRF1-only induced telomeric DSBs resulted in TERRA accumulation at the break sites, but no (ss)telomeric DNA was

observed. A protective role of hybridized/bound TERRA at resected telomeric DSBs in G1 human cells is proposed, which likely contributes to preserving vulnerable (ss)telomeric DNA until G2 when HR-mediated elongation/repair is a possibility. Our work also provides additional support for overexpression of TRF1 inducing telomeric DSBs in U2OS (ALT) cells.

# CHAPTER 4 FUTURE DIRECTIONS

The work reported here characterizes the response of the telomeric lncRNA, TERRA, in the DNA damage response, specifically to DSBs induced by either ionizing radiation (globally) or targeted specifically to telomeres (ENT-TRF1) in human U2OS (ALT; telomerase independent) cells. TERRA levels were elevated following induction of DSBs by both treatments. Our results revealed that TERRA associates with telomeric DSB sites (suggestive of TERRA: telomere DNA hybrids), and that cells suffering telomeric DSBs accumulate in G2. We also found evidence in support of a role for hybridized TERRA in protecting (ss)telomeric DNA in G1.

Methodological limitations of the current study include low transfection efficiencies in U2OS cells utilizing the ENT-TRF1 plasmid-based approach, and losing expression of the vector in a majority of cells 72 hours after transfection. Therefore, establishment of a tetracycline (Tet)-inducible (On/Off) U2OS (ALT) cell line expressing ENT-TRF1 would represent a valuable improvement, as it would facilitate stable levels of transfection and control of expression.

Although treatment with RnaseA and RnaseH in combination as performed here effectively removed TERRA, more permanent depletion of TERRA would also be an important next step in analyzing the response to telomeric DSBs – in both ALT and telomerase positive cancer cells. Development of CRISPR-Cas9 genome editing strategies against TERRA would be an important advancement, as it would facilitate additional analyses on repair pathway choice (e.g., HR-BIR, alt-EJ) and telomere length dynamics (changes over time).

In light of recent controversy regarding the chromosomal origin(s) of TERRA [114, 116] it would be of particular interest to characterize and identify specific TERRAs responding to telomere DNA damage; are such TERRA transcripts unique? It might be possible to identify them

based on unique sub-telomeric sequences they possess, which could be determined using state-of-the-art RNA-seq approaches.

Together, such studies would improve mechanistic understanding and provide insights into the potential of TERRA as a novel therapeutic target for a variety of different cancers.

# CHAPTER 5 MATERIALS AND METHODS

## Cell culture and Transfections

U2OS cell lines and Fucci- U2OS cells were used in this study. The two cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Hyclone) with 10% Fetal Bovine Serum (FBS) and 3% GlutaMAX-100X (Gibco). The cells were cultured at 5% $CO_2$,37C and 95% humidified incubator.

## Plasmids

In this study two plasmids were used to generate stable cell lines: Fucci-S-G2-M-Green plasmid (AM-V9016, Amalgaam)[157] obtained from Dr. Jac Nickoloff (Colorado State University). For transient transfection, TRAS1-EN-TRF1 plasmid obtained from Dr. Haruhiko Fujiwara (University of Tokyo), TRF1 plasmid constructed from TRAS1-EN-TRF1 plasmid, and mutated Cas9 pSpCas9m(BB)-2A-Puro (PX462) V2.0 a gift from Feng Zhang (Addgene plasmid #62987 ; http://n2t.net/addgene:62987 ; RRID:Addgene_62987) and obtained from Dr. Claudia Wiese (Colorado State University) were used.

## Transfections

Stable Transfection to generate FUCCI-U2OS: U2OS expressing FUCCI-Germinin (Green for S-G2 and M-phase) and U2OS expressing Fucci-G1-Orange were established by transfecting cells with 0.5 ug of Kan-Fucci-Green (S-G2-M) plasmid. Plasmid were delivered using Lipofectamine 3000 ( Invitrogen) following the manufacture's instruction. Eight hours following transfection, Opti-MEM media was replaced with fresh DMEM media. A week later, cells were trypsinized and each individual cell was seeded in 96-well plate. A positive single clone was identified, using the fluorescence Microscope, and expanded in presence of 800 μg/ml G-418

sulfate (GoldBio). After reaching 90% confluency, cells were splited into 24 well plate, 10 days later into 12 well plate, a week after into 6 well plate. Then, the cells were transferred into T-25 flask and finally into a T-75 flask after 6 days. The DMEM media containing 800 μg/ml G-418 sulfate was changed every 2 days.

Transient transfections: Fucci-U2OS transiently expressing TRAS1-EN-TRF1 or TRF1:

U2OS cells stably expressing FUCCI G2-Green were transfected with TRAS1-EN-TRF1 or TRF1 using Lipofectamine 2000 (Invitrogen) following the manufacture's instruction. Eight hours following transfection, Opti-MEM media was replaced with fresh DMEM media. Then, cells were fixed 48 h post transfection.

**Gamma Irradiation**

U2OS cells were seeded in 4 well chamber slides (Nunc Lab-Tek II 154526) at a density of 50,000 cell/ well. In a Mark I irradiator at Colorado State University, cells were rotated and exposed to $^{137}$Cs γ-rays at a dose of 2Gy. Cells then were placed back in $CO_2$ incubator for Four hours preceding fixation.

**Single Stranded telomeric DNA fluorescence in-situ hybridization ssTelo-FISH and RNA fluorescence in-situ hybridization RNA FISH**

For combined Telomeric C rich strand detection and flag staining, U2OS expressing Fucci-S-G2-M-Green cells were grown on 4 well chamber slides (MilliCell EZ). 48 hours following transfection, slides were washed with Cytoskeleton (CSK) buffer (100 mM NaCl, 1 mM EGTA, 10 mM PIPES pH 6.8, 3 mM MgCl2, 300 mM sucrose) containing 200mM Vanadyl for 30 second. Cells were then fixed in freshly prepared 3% Paraformaldehyde in 10X PBS for 12 minutes at room temperature. Next cells were permeabilized with 0.5% Triton X-100 in CSK containing 200 mM Vanadyl for 7 minutes at room temperature. Following permeabilization, cells were washed

with 70% Ethanol and dehydrated in a graded series of alcohol (85%,95% and 100% Ethanol). For C -rich strand (CCCTAA) detection, 0.5uM (TelG-Cy3, Bio-Synthesis) Peptide Nucleic Acid (PNA-GGGATT) telomere probe 0.5uM (TelC-Alexa 488, Bio-Synthesis) or (TelC- Alexa 647, Bio PNA 1013) Peptide Nucleic Acid (PNA-CCCTAA) telomere probe was added to the hybridization buffer for TERRA (UUAGGG) detection in 50% (vol/vol) formamide, 2X(vol/vol) Saline Sodium Citrate (SSC) hybridization buffer and 200mM Vanadyl) hybridization buffer was denaturated at 85ºC for 10 min and cooled on ice. 200 ul hybridization buffer was added to each slide and slides then were placed in humidified chamber and incubated at 37ºC for 6 hours. Slides then were washed in twice in 50% formamide in 2X SSC (2.5 minutes 42ºC), twice in 4X SSC (2.5 minutes 42ºC) and once in 2X SSC + 0.1% NP-40 (2.5 minutes 42ºC).

**Pre-hybridization Rnase A and Rnase H treatment**

To remove TERRA molecules, fixed and permeabilized U2OS and U2OS expressing Fucci-S-G2-M-Green cells were treated with (1mg/ ml Rnase A and 15 Unit Rnase H) in 1X Rnase H buffer at 37ºC for one hour.

**Immunofluorescence staining**

Blocking nonspecific immunoglobulin binding was carried out, following FISH, with 10% Goat Normal Serum (GNS) for 30 min in humidified chamber at room temperature. Following blocking, slides were incubated with primary antibody in 5% GNS overnight at 4C. slides were washed 3 times with 1XPBS at room temperature. protein signals were visualized by incubating slides with fluorophore-conjugated secondary antibody for 40 min in humidified chamber at room temperature and washed again 3 times in 1XPBS. Lastly, slides were mounted and counterstained with prolong gold antifade reagent with DAPI (Invitrogen). Primary antibodies and concentrations used in this study: Mouse Anti-Flag (Sigma M2 F1804, 1:2000), Rabbit Anti-Human FLAG

(1:300), Mouse Anti-Human TRF2 (Santa Cruz B-5, 1:200) and Mouse Anti-Human Mitosin (BD Biosciences 610768, 1:500). Secondary antibodies and concentrations used in this study: Alexa-647 Goat anti-Mouse (ThermoFisher A21236, 1:750), Alexa-594 Goat anti-Mouse (ThermoFisher A11005, 1:750), Alexa-488 Goat anti-Rabbit (ThermoFisher A11008, 1:750) and Alexa-488 Goat anti-Mouse (ThermoFisher A11005, 1:750). For EdU detection, the click-iT EdU Alexa Flour 555 was used as described by the manufacture.

**Cell cycle analysis**

The detection of cells in S phase was achieved through the incorporation of the thymidine analog 5-Ethynyl-2´-deoxyuridine (EdU) into DNA. Thirty min before fixation, 10uM EdU (Click-iT, Invitrogen) was added into the culture at 37C. Cells were considered to be in G2 when it is positive to FUCCI or when it is positive to FUCCI and EDU. Cell was considered to be in S phase cells were identified when they showed only positive stain for EdU. Finally, cells were considered to be in G1 when they were negative for both FUCCI and EdU.

To evaluate the enrichment of transfected U2OS cells in G1, G2 and S phase; U2OS cells labeled with EdU represent S phase. Likewise, any transfected cell negative for green FUCCI and EdU was considered cell in G1 phase. The colocalization between FLAG and DAPI (G1 cells), FLAG and EdU, (S cells), and FLAG and Geminin-GFP (G2 cells), represented transfected cells in each phase of the cell cycle analyzed.

**Data analysis**

Telomeric RNA FISH (TERRA) analysis following IR treatment: TERRA foci number was counted in treated and control groups using Metamorph 7.7. The student t test was performed to calculate the statistical significance of number of TERRA foci between treated and control groups. Statistical analysis was performed with Microsoft Office Excel, $n=50$**.**

ENT-TRF1 and TRF1 transfection analysis: For (FLAG-DAPI) immunostaining analysis, cells were considered to be positively transfected when they had >20 FLAG foci. One-way ANOVA and Holm-Sidak test (multiple comparison test of means with 95% confidence interval), using the statistical software GraphPad Prism 8.0

Immunofluorescence plus Telomeric DNA FISH and immunofluorescence plus telomeric RNA FISH: For (FLAG - γ-H2AX- C-strand) and (TRF2-TERRA, FLAG-TERRA, FLAG-TRF2 -TERRA) immunostaing analysis, the number of multiple colocalization events were counted from the sum of 21 Z-stacks. Quantitative analysis was performed using imageJ and Cell Profiler. One-way ANOVA was used to evaluate Statistical analysis using the statistical software GraphPad Prism 8.0

Native telomeric C-Strand FISH analysis following ENT-TRF1 transfection, with and without RNAse treatment: C-strand foci number were counted in cellular objects using Cell Profiler image analysis software 3.1.5. using designed segmentation-based pipeline [178]. Our pipeline designed to create mask around nuclei positively transfected with FLAG and identify and quantify the C-strand in the same nuclear region. One-way ANOVA and Holm-Sidak test (multiple comparison test of means with 95% confidence interval), using the statistical software GraphPad Prism 8.0

**Microscopy and Quantitative Analysis**

Images were captured blindly using 63X/1.4 N.A objective in a Zeiss Axio Imager .Z2 epi-fluorescent microscope using Zen Blue software. Image for colocalizations were captured in 0.2 intervals in a total of 21 z-stacks in combinations of 4 colors at the time. Quantitative analysis was performed using Fiji (ImageJ2) and Cell Profiler using customized pipeline for reconstructions in 3D and simultaneous triple and dual colocalizations of the different markers.

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