THESIS

TELOMERE RECOMBINATION AND REGULATION

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Abdunaser Omar Eadaim

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Master's Committee:

Advisor: Susan M. Bailey

Paul Laybourn
Marvin R. Paule

ABSTRACT

TELOMERE RECOMBINATION AND REGULATION

Telomeres, tandem arrays of repetitive G-rich sequence at the physical ends of linear chromosomes, serve to protect chromosomal termini from enzymatic activity and prevent recognition of natural DNA ends as double strand breaks (DSBs). The telomeric Shelterin complex and other associated proteins play critical roles in maintaining stability of the telomere. Telomere length can be maintained by telomerase enzymatic activity or by an alternative lengthening of telomere (ALT) recombination based mechanism, which has been characterized by increased frequencies of telomere sister chromatid exchange (T-SCE). Telomeres have been shown to be especially sensitive to oxidative stress and ultraviolet (UV)-induced DNA damage, for example cyclobutane pyrimidine dimers (CPDs) between two adjacent pyrimidines.

In this project, exposure to ultraviolet C (UVC) was evaluated for its ability to induce sister chromatid exchange (SCE) in cell lines with different telomerase status. Our results showed that sister chromatid exchanges, both genome-wide (G-SCE) and within telomeres (T-SCE), were increased in a dose-dependent manner following UVC exposure in telomerase negative normal human fibroblast (BJ1) and ALT human dermal fibroblasts. However, in telomerase positive human fibroblasts, while G-SCE frequencies increased in response to UVC, T-SCE frequencies did not. These results have important implications not only for aging, but for carcinogenesis as well, since UV exposure from the sun (and tanning beds) is linked to increased risk of both aging of the skin and skin cancer. The susceptibility of telomeric DNA to oxidative stress and the dampened DNA damage response in this region provide likely explanations for the increased frequencies of T-SCE observed following UV exposure. Although certainly not the

only contributor, T-SCE themselves provide intriguing insight into possible mechanisms of increased telomere shortening, senescence, and carcinogenesis, and may therefore represent informative biomarkers of aging and cancer.

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Introduction/Background

1. Telomeres: A Historical Perspective

Telomeres are unique structures located at the ends of linear chromosomes in eukaryotic organisms composed of DNA-protein complexes. Telomeres are characterized by an abundance of G and C nucleotides, which in humans and all vertebrates studied to date, are tandem arrays of the repetitive DNA sequence, TTAGGG/CCCTAA (Meyne, Ratliff, & MoYzIs, 1989; Moyzis et al., 1988) and are protected by the core-protein complex termed Shelterin, as well as numerous telomere associated proteins (De Lange, 2005). Telomere sequence and size is species specific, with human telomeres ranging in size from 4 to 15 Kb dependent on age and disease state (Lea, 2004; von Zglinicki, 2002). A critical feature of telomeres is a G-rich, 3'single-stranded overhang, which is estimated to range from 100-150 to as many as 400 base pairs in length (E.H. Blackburn, 1991). The G-rich overhang plays an important role in capping or protecting the telomere from degradation and/or fusion, as it facilitates formation of a telomere (t)-loop, a higher order structure which serves to differentiate the telomere from broken DNA double strand breaks (DSBs) (Griffith et al., 1999).

There are a variety of proteins that bind telomeric DNA, which play a variety of roles in their function. The primary, or core complex associated with telomeres was originally identified as the telosome (D. Liu, O'Connor, Qin, & Songyang, 2004), and more recently termed Shelterin (De Lange, 2005). Shelterin is composed of the protein components: Telomere Repeat Binding Factors 1 and 2 (TRF1 and TRF2 respectively), and Protection of Telomeres Protein (POT1), which bind directly to telomeric DNA (Hockemeyer, Daniels, Takai, & de Lange, 2006). In addition to TRF1, TRF2, and POT1, other Shelterin components include the repressor and

activator protein 1 (RAP1), TRF1- interacting nuclear protein 2 (TIN2), and POT1-interacting protein (TPP1), all of which associate with the directly binding telomere proteins via protein-protein interactions and serve various roles in maintaining telomere function (O'Connor, Safari, Xin, Liu, & Songyang, 2006). In general, the shelterin complex acts to safeguard and protect natural chromosome DNA ends from recombination and end-joining activities, thereby preventing inappropriate fusion of telomeres to other telomeres or to broken DNA ends (DSBs), which occur both from natural endogenous sources such as replication, and from exogenous sources such as exposure to ionizing and non-ionizing radiations (E.H. Blackburn, 1991).

Telomeres were first described by Hermann Joseph Muller in the fruit fly, Drosophila melagaster, and Barbara McClintock in maize (McClintock, 1941; Muller, 1938). Muller and McClintock, in separate but contemporary experiments, observed that the natural ends of chromosomes did not normally fuse to broken chromosome ends induced by ionizing radiation. They postulated that a protective structure at the end of every chromosome interfered with binding to interstitial chromosome breaks. Muller coined the term “telomere”, a compound Greek word meaning “end part”; telo= end and mere= part (Muller, 1938).

Telomere research languished somewhat until 1960, when Leonard Hayflick reported that normal human diploid cells were only able to grow and divide for a limited number of divisions, generally estimated to be around 60 population doublings (Hayflick & Moorhead, 1961). He observed that non-immortalized cells were capable of dividing a finite number of times, known as the Hayflick limit, at which point the proliferative process arrests and cells enter an irreversible growth arrest called replicative senescence.

In the 1970s it was recognized that because conventional DNA polymerases were incapable of replicating to the very 5' terminal end of chromosomes, telomeres would be expected to shorten as a consequence of the end-replication problem (Watson, 1972) (Olovnikov, 1973). During lagging strand DNA synthesis, the requirement for an RNA primer would result in the loss of estimated 30-150 base pairs of the C-rich telomeric DNA sequence with each cell division. It was later observed that telomeres do indeed shorten with cell division (Harley, Futcher, & Greider, 1990). Thus, mechanism for the Hayflick limit was proposed and pointed towards the telomere as the answer.

It was later postulated that since not all organisms suffered telomere shortening, there must exist a specific enzyme to compensate for the end-replication problem. Blackburn and Gall had successfully cloned the telomere sequence of one such organism, *Tetrahymena thermophile,* in the mid-1970s by fragmenting ribosomal RNA (rDNA) with a restriction endonuclease that gave fragment repeat sequences of different lengths. These sequences were then used as a template and artificially synthesized using DNA polymerase I of *Escherichia coli* (Elizabeth H. Blackburn & Gall, 1978). Then, on Christmas morning 1984, Carol Greider, a graduate student of Elizabeth Blackburns at the time, identified the enzyme responsible for *de novo* telomere elongation, which they named telomerase (Greider & Blackburn, 1985, 1987). It is now appreciated that telomerase plays critical roles in both cancer and aging, the importance of which was recently recognized with the 2009 Nobel Prize in Medicine.

In 1989, Morin et. al. found that normal somatic cells lacked telomerase activity, and so suffered telomere shortening; in contrast cancer cells possessed telomerase activity and maintained telomere length . In 1990, the Blackburn team reported that *Tetrahymena* telomeres required the enzymatic activity of telomerase to enable immortality; in contrast, repression of

telomerase enzymatic activity resulted in critically shortened telomeres and triggering of senescence. In the 1990s, Shay and Harley reported telomerase activity in 90 of 101 human tumor cell samples, while 50 normal somatic samples were telomerase negative (Kim et al., 1994). These seminal findings, together with others such as the telomerase knockout mouse suffering critically shortened telomeres and loss of cell viability (Blasco et al., 1997) , and reintroduction of telomerase into primary human fibroblasts indefinitely extending lifespan in culture (Bodnar et al., 1998), have convincingly established the underlying molecular mechanism of replicative senescence as telomere shortening.

2. Telomere Structure and Function

Critical features of functional telomeres are extensive 3' G-rich single stranded overhangs (Makarov, Hirose, & Langmore, 1997; McElligott & Wellinger, 1997). Electron microscopy has revealed large telomere lariat structures, which have been termed t-loops (Griffith et al., 1999). It is hypothesized that t-loops form via the looping back of the single-stranded overhang and invasion along the double stranded telomeric DNA tract, resulting in the formation of a displacement loop (D-loop) where the G-overhang invades, and forming the overall structure of a loop. T-loops are thought to play an important structural or architectural role in the protection of chromosome ends, physically hiding telomeres from DNA damage machinery and preventing inappropriate fusion events. Interestingly, t-loop size varies dramatically between particular cells within different tissues and between different organisms (Griffith et al., 1999).

The Shelterin complex of six core telomere proteins (TRF1, TRF2, RAP1, POT1, TIN2 and TPP1) facilitates formation of the t-loop structure (Palm & de Lange, 2008). TRF1 and TRF2 specifically bind the double stranded telomeric DNA, while POT1 has affinity for single

stranded telomeric DNA. TIN2 forms a “bridge” between TRF2 and POT1. RAP1 directly binds TRF2 and performs various functions that remain poorly understood (O'Sullivan & Karlseder, 2010).

Somewhat paradoxically, many telomere accessory proteins are also major players in various DNA repair pathways. One such example would be the DNA- dependant protein kinase (DNA-PK) holoenzyme, made up of the DNA-PK catalytic subunit (DNA-PKcs) and the Ku70/80 heterodimer, which is central both to the end-capping function of mammalian telomeres (S. M. Bailey et al., 1999), and to the accurate repair of broken DNA ends (DSBs) via non-homologous end joining (NHEJ) (G. B. Celli, E. L. Denchi, & T. de Lange, 2006) . Another telomere associated DNA repair complex, Mre11, RAD50, Nbs1 (MRN), is an important factor involved in the repair of DSBs breaks via homolgous recombination (HR). Proteins involved in a variety of other DNA repair pathways have also been found associated with telomeres, including PARP1/2 (involved in base excision repair), Tankyrase1/2 (with roles in NHEJ), ATM (damage detection), ERCC/XPF (nucleotide excision repair), as well as WRN and BLM (both RecQ like helicases that aid in the replication of DNA during S-phase) (Karlseder et al., 2004) . Loss or disruption of proteins in the shelterin complex or involved in DNA repair have been shown to lead to telomere dysfunction, which can manifest either as premature and/or excessive degradation/loss of telomere *sequence*, or as loss of “end capping” function/*structure*, resulting in inappropriate fusion events; e.g., telomere-telomere and telomere-double strand break fusions (S. M. Bailey & Murnane, 2006).

3. Telomeres in Aging and Cancer

In order to protect chromosome ends, functional telomeres must be of sufficient length to bind all necessary shelterin components and accessory proteins (Palm & de Lange, 2008). Telomeres are susceptible not only to replication-induced erosion and exonuclease-mediated degradation, but to a multitude of other insults as well, including oxidative stress resulting from both endogenous and exogenous sources (Thomas, 2002) . Telomere length maintenance mechanisms (TMM) act to balance these attacks and maintain homeostasis. In most normal human somatic tissues, telomerase is repressed, therefore telomeres shorten with every cell division, a process that eventually triggers replication induced senescence and contributes to aging phenotypes (Itahana, Campisi, & Dimri, 2004).This hypothesis of aging has been supported by numerous studies, including the demonstration of degeneration resulting from loss of tissue and organ function in telomerase negative mice (Armanios et al., 2009).

It is important to appreciate that telomere shortening with age and replication-induced senescence acts as a barrier to unlimited growth, and so also represents a very effective tumor suppressor mechanism (Itahana et al., 2004). Loss of telomere sequence and/or end capping capabilities results in the inappropriate recognition of telomeres as broken DNA ends (DSBs), activation of DNA damage machinery including ATM and p53 and initiating cell cycle arrest, and triggering senescence or apoptosis (di Fagagna et al., 2003). Cellular senescence can be bypassed if the p53 and Rb proteins are inactive (X. Wu et al., 2011).Cells can escape crisis and become immortalized if telomere length is stabilized by the activation of a TMM, but they do so at the risk of massive genetic alteration and increased tumorigenesis (Bodnar et al., 1998; Counter et al., 1992).

4. Telomere length maintenance pathways

4.1. Telomerase

Telomerase is a RNA-dependent, reverse transcriptase that adds species-specific telomere repeat sequences *de novo* to the 3' end of chromosomes(Autexier & Lue, 2006; Collins, 2006). In humans and most vertebrates, the highly conserved telomerase holoenzyme consists of two primary parts, the telomerase reverse transcriptase (TERT) catalytic protein subunit, which is encoded by the *hTERT* gene located on chromosome 5p15.33 (Harrington et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997), and the telomerase RNA component (TR or TERC), which is encoded by the *hTR* gene located on chromosome 3q21-q28 (Feng, Funk, Wang, Weinrich, Avilion, Chiu, Adams, Chang, Allsopp, & Yu, 1995). Dyskerin, an accessory component of the human telomerase holoenzyme encoded by the *DKC1* gene located on the X chromosome is thought to aid in proper folding and stabilization of the TERC subunit (Wong & Collins, 2006).Dyskerin has also been isolated as part of the active telomerase complex (Cohen et al., 2007; Fu & Collins, 2003). TERT, like other reverse transcriptases (RT), has a RT domain composed of a prototypical RT motif (Autexier & Lue, 2006). TERT contains two additional domains that flank the RT domain, a large N-terminal extension (NTE) and short C-terminal extension (CTE). The NTE has two RNA interacting domains; one with high affinity for binding RNA, RID2, and one with low affinity for RNA, RID1(Morin, 1989).

The telomerase RNA component is characterized by its species-specific telomere length, and in humans contains 11 base pairs in the active site (Feng, Funk, Wang, Weinrich, Avilion, Chiu, Adams, Chang, Allsopp, Yu, et al., 1995). Telomere sequence and structure differences between organisms have been identified, yet, all eukaryotic TERC subunits are single stranded at

the active site of DNA synthesis and are complementary to a specific telomere DNA repeat sequence (Theimer & Feigon, 2006). To initiate replication, telomerase is recruited to the telomeric G-rich single-stranded overhang, which it uses as a substrate (Dai et al., 2010) , a process likely regulated by the shelterin complex, however the exact details of this process remain largely unknown (Xin et al., 2007).

4.1. a. Regulation of telomerase activity

Differential expression of human telomere reverse transcriptase (hTERT) plays an important role in regulating telomerase activity. hTERT expression is minimal or absent in most normal somatic cells, but is high in stem cells and the majority of tumors (Shay & Wright, 2006). In contrast, the RNA component (hTERC) is expressed in all cells (Cairney & Keith, 2008). Additionally, post-transcriptional modification can influence telomerase activity; e.g., alternative splicing of TERT, and/or phosphorylation and ubiquitination of the enzyme (Aisner, Wright, & Shay, 2002; Cairney & Keith, 2008).

Recruitment of telomerase to and accessibility of chromosomal termini can also affect telomerase activity. In mammalian cells, TERT recruitment to telomeres is regulated by the cell cycle, in that it is only present at chromosome ends during S phase (Tomlinson, Ziegler, Supakorndej, Terns, & Terns, 2006). There is some evidence that recruitment of telomerase to telomeres is controlled by telomere binding proteins. For example, in *S.cerevisiae*, the number of molecules of the double-stranded telomere binding protein, Rap1, that bind the telomere helps determine the length of the telomere, which in turn effects telomerase recruitment and subsequent action (Marcand, Gilson, & Shore, 1997; Smogorzewska & de Lange, 2004) . Protection of Telomeres (POT) 1 specifically binds single-stranded telomeric DNA, the substrate

for telomerase (Dai et al., 2010). POT1 is regarded as a negative regulator of telomere length, as deletion of POT1, or mutant variations that cannot bind single-stranded DNA, enhance telomerase binding to telomeres and telomere lengthening (Loayza & De Lange, 2003; Ye et al., 2004). Competition between POT1 and telomerase for binding the G-rich single-stranded telomeric overhang has been observed *in vitro* (Kelleher, Kurth, & Lingner, 2005; Lei, Zaug, Podell, & Cech, 2005). Although POT1 interferes with telomerase binding the G-rich single-stranded overhang, it has also been shown that POT1 binding to TPP1, another member of the shelterin complex, especially at internal sites, can stimulate telomerase activity; TPP1 can also bind telomerase directly (Xin et al., 2007).

4.2. Alternative lengthening of telomeres (ALT)

Telomere length maintenance in telomerase negative backgrounds was first described in yeast (Lundblad & Blackburn, 1993). It was later reported that although a majority of human tumors express telomerase, an estimated 5-10% of them do not (Kim et al., 1994), indicating that some tumors utilize an different mechanism for maintaining telomere length, which has been termed Alternative lengthening of telomeres (ALT) (T. Bryan, Englezou, Gupta, Bacchetti, & Reddel, 1995; T. M. Bryan, Marusic, Bacchetti, Namba, & Reddel, 1997; Hande, Samper, Lansdorp, & Blasco, 1999; Niida et al., 2000; Rogan et al., 1995). Several lines of evidence indicate that the human ALT pathway is homologous recombination based (Henson, Neumann, Yeager, & Reddel, 2002; J. Murnane, L. Sabatier, B. Marder, & W. Morgan, 1994; R. R. Reddel, Bryan, Colgin, Perrem, & Yeager, 2009). Interestingly, some tumors appear to have both telomerase and ALT mediated telomere maintenance mechanisms (Ulaner et al., 2003). It has also been suggested that ALT is not the only telomerase independent pathway of telomere length maintenance in human and other species (Nabetani & Ishikawa, 2011).

Mutations in the telomerase components TR and TERT that reduce telomerase activity result in telomere shortening in both humans and mice, and TERT has been shown to be limiting and necessary for telomerase function *in vivo* (Blasco et al., 1997; Y. Liu et al., 2000) . Consistent with such observations, are reports of the absence of hTR and low levels of full length hTERT in ALT cells (T. M. Bryan, Englezou, Dalla-Pozza, Dunham, & Reddel, 1997; Kilian et al., 1997; Y. Liu et al., 2000). When telomerase activity was reconstituted in ALT cells, evidence of the existence of both telomere length maintenance mechanisms was found (Cerone, Londono-Vallejo, & Bacchetti, 2001; Ford et al., 2001; Grobelny, Kulp-McEliece, & Broccoli, 2001; Perrem, Colgin, Neumann, Yeager, & Reddel, 2001). It has also been shown that critically short telomeres undetectable by FISH can be rescued by ectopic expression of telomerase, producing telomeres of heterogeneous lengths (Henson et al., 2002; Perrem et al., 2001). Heterogeneous telomere lengths have also been observed in backgrounds experiencing over-expression of hTERT and hTR (Cao et al., 2008; Pickett, Cesare, Johnston, Neumann, & Reddel, 2009).

4.2. a. Hallmarks of ALT

ALT is characterized by the absence of telomerase activity (T. Bryan et al., 1995) and the presence of heterogeneous telomere lengths (Perrem et al., 2001). However, telomere length heterogeneity itself is not a definitive marker of ALT, as some telomerase positive cells experience similar variation in telomere length as a result of high expression levels of hTR or hTERT (Cao et al., 2008; Pickett et al., 2009). The ALT phenotype is also associated with Promyeloctytic Leukemia (PML) bodies,, which provide an additional marker for confirming ALT and distinguishing from telomerase positive tumors (Tarsounas et al., 2004). ALT-associated PML bodies (ABPs) are aggregations of PML protein and numerous other nuclear proteins (Lang et al., 2010) including some involved in DNA replication and homologous

recombination (Tarsounas et al., 2004; Yeager et al., 1999). Importantly, APBs also contain telomeric DNA, and thereby provide a template for homologous recombination (Draskovic et al., 2009).

4.2.b. Mechanisms of ALT

4.2. b. 1. Inter-telomeric recombination

Inter-telomeric recombination, i.e., recombination between telomeres of different chromosomes, has been proposed as a mechanism of telomere length maintenance in mammalian ALT cells (R. Reddel, Bryan, & Murnane, 1997). A marker near the telomere (sub-telomeric DNA) was seen to multiply and jump from chromosome to chromosome with increasing cell division (Muntoni, Neumann, Hills, & Reddel, 2009; J. P. Murnane, L. Sabatier, B. A. Marder, & W. F. Morgan, 1994). In contrast, a marker placed near the centromere of ALT or telomerase positive cells did not multiply or move (M. A. Dunham, A. A. Neumann, C. L. Fasching, & R. R. Reddel, 2000).

4.2. b. 2. T-Loops

The t-loop represents a recombination intermediate and so may also participate in telomere length maintenance, a function possibly regulated by the telomere binding protein TRF1(Baumann & Cech, 2001).Telomere shortening could result from resolution of cross over events (J. Murnane et al., 1994). Telomere lengthening could also occur following self (or other telomere) strand invasion and template copying (M.A. Dunham, A.A. Neumann, C.L. Fasching, & R.R. Reddel, 2000), resulting in synthesis of the G-rich strand and elongation (Nosek, Rycovska, Makhov, Griffith, & Tomaska, 2005). .

4.2. b. 3. Rolling circles

Rolling circle replication can occur if the G-rich overhang migrates and forms a holiday junction , a process that requires XRCC3(R. C. Wang, Smogorzewska, & de Lange, 2004). G- and C-circles have been observed in ALT cells, with C-circles being much more frequent than G-circles (Henson et al., 2009). C-circles were not detected in telomerase positive cells or mortal cells (Grudic et al., 2007).

4.2. b. 4. Extra chromosomal telomeric repeats (ECTRs)

ECTRs, small double-stranded linear telomeric DNA fragments, have been purified from ALT cell extracts (Ogino et al., 1998) and observed cytogenetically using telomere FISH on ALT metaphase chromosome spreads (Tokutake et al., 1998). ECTRs could be used to maintain telomere length via direct fusion with a shortened telomere, or a telomere could use the ECTR as a template for elongation (Grobelny, Godwin, & Broccoli, 2000; G. Wu, Lee, & Chen, 2000).

5. Telomere sister chromatid exchange (T-SCE)

Sister chromatid exchange (SCE) involves the exchange of genetic/chromosomal material between two identical sister chromatids and is a general phenomenon of eukaryotic chromosome (S. Wolff, 1977). SCE was first visualized using a radioactive thymidine analogue such that one chromatid was labeled and the other was not, which was subsequently detected by autoradiography (Taylor, 1958). Routine detection of SCE did not become practical until the advent of the Fluorescence plus Giemsa (FPG) technique and differential staining of sister chromatids to produce “harlequin” chromosomes (Perry & Wolff, 1974) which made visualization of SCE a simple matter of a color “switch” from one chromatid to the other.

Evidence suggests that SCE occurs during DNA replication (S phase of the cell cycle) when lesions that stall and/or collapse the replication fork are encountered (Wilson III & Thompson, 2007), and is a process dependent on homologous recombination (S. M. Bailey, M. A. Brenneman, & E. H. Goodwin, 2004). Reported spontaneous background frequencies of SCE in human cells range from 3-5 exchanges per cell per cycle (Kato, 1974; Pinkel, Thompson, Gray, & Vanderlaan, 1985; Stoilov, Wojcik, Giri, & Obe, 2002; SH Wolff & Perry, 1975), Hot spots for SCE have also been reported and include heterochromatic regions around the centromere (Morgan & Crossen, 1977). Application of the strand-specific technique of Chromosome Orientation FISH (CO-FISH), which requires a single round of replication in the presence of the thymidine analogue BrdU followed by preferential degradation of the newly synthesized DNA strands (S. Bailey, Goodwin, Meyne, & Cornforth, 1996), lead to the observation of high rates of SCE recombination, especially in subtelomeric regions of chromosomes (Cornforth & Eberle, 2001).

CO-FISH is unique in its capability to evaluate SCE recombination within telomeric DNA itself by detecting events termed telomere SCE (T-SCE), where the single-sided CO-FISH telomere signal is split between the two sister chromatids, the frequencies of which are elevated compared to genomic SCE (G-SCE) (S.M. Bailey, M.A. Brenneman, & E.H. Goodwin, 2004). Interestingly, high levels of T-SCE have also been associated with the ALT phenotype (telomerase negative) and so, T-SCE were deemed a marker of ALT that may serve as a means of recombination-based telomere length maintenance (S.M. Bailey et al., 2004; Bechter, Zou, Walker, Wright, & Shay, 2004; Londoño-Vallejo, Der-Sarkissian, Cazes, Bacchetti, & Reddel, 2004). However, high T-SCE frequencies have also been reported in telomerase positive cell (Vera, Canela, Fraga, Esteller, & Blasco, 2008).Further, recent modeling has led to the

proposition that hyper telomere recombination actually accelerates replicative senescence and may therefore promote premature aging phenotypes (e.g., Werner's Syndrome), rather than extend replicative lifespan (Blagoev, Goodwin, & Bailey, 2010; Hagelstrom, Blagoev, Niedernhofer, Goodwin, & Bailey, 2010).

Investigations into the regulation of T-SCE frequencies have involved many telomeric binding proteins, including mammalian Ku, TRF2, POT1, and RAP1. Although Ku deletion alone does not significantly affect T-SCE frequencies, when combined with TRF2 or POT1 depletion, increased T-SCE frequencies resulted, suggesting they normally act to suppress this type of recombination activity at telomeres (G.B. Celli, E.L. Denchi, & T. de Lange, 2006; Palm, Hockemeyer, Kibe, & De Lange, 2009; Sfeir, Kabir, Van Overbeek, Celli, & De Lange, 2010). Accessibility to and/or ease of replication of the telomere are affected by its more "open" or "closed" chromatin state, thus likely influencing recombination rates (Jegou et al., 2009). It has also been shown that deficiency of the WRN helicase in a background of short telomeres due to the absence of telomerase activity, resulted in elevated frequencies of T-SCE and premature aging phenotypes associated with human Werner's Syndrome (Laud et al., 2005), while G-SCE frequencies were not significantly affected (Hagelstrom et al., 2010). In contrast, depletion of the BLM helicase in telomerase negative backgrounds resulted in significantly elevated frequencies of both T-SCE and G-SCE (Hagelstrom et al., 2010).

It is well established and accepted that oxidative stress (e.g., reactive oxygen species; ROS) enhances telomere shortening (Von Zglinicki, Petrie, & Kirkwood, 2003). The increased sensitivity of G-rich telomere sequence to oxidative stress (Grygoryev & Zimbrick, 2010; Rochette & Brash, 2010) also represents a likely explanation for its increased susceptibility to T-SCE formation, as the altered bases 8-oxo-7,8- dihydroguanine (8-oxoG) and 2,6-diamino-4-

hydroxy-5-formamidopyrimidine (FapyG) that result from oxidative stress are lesions that stall replication forks (Z. Wang et al., 2010). It has been shown that 8-oxoG in telomeric DNA can affect telomere protein binding ability (Opresko, Fan, Danzy, Wilson III, & Bohr, 2005), which can stimulate homologous recombination specifically at the telomere (G. B. Celli et al., 2006; Zhu et al., 2003). Additionally, the oxidative lesion 8-oxoG can be removed by the DNA glycosylase OGG1, without which T-SCE frequencies are increased (Z. Wang et al., 2010).

Exposure of DNA to ultravoilet (UV) light results in formation of cyclobutane pyrimidine dimers (CPDs) between two adjacent pyrimidines (BRUNK, 1973) (S. Y. Wang, 1976), photoproducts that can be removed by the nucleotide excision repair (NER) DNA repair pathway (Thoma, 1999). Following exposure to UVC (254 nm), Rochette and Brash (Rochette & Brash, 2010) found that pyrimidine rich telomeric DNA was much more sensitive to UV- induced DNA damage compared to the rest of the chromosome, evidenced by increased CPDs in telomeric DNA..They also reported reduced ability of telomeric DNA to remove CPDs, which interfere with telomeric replication, consistent with reports of repressed DNA repair at telomeres (Rochette & Brash, 2010).

HYPOTHESIS

We hypothesized that telomerase status and exogenous DNA damaging agents (specifically UVC exposure) influence T-SCE frequencies independently of their influence on G-SCE frequencies.

SPECIFIC AIMS

Aim 1. Determine spontaneous T-SCE frequencies in human cell lines with various telomerase status; specifically, BJ-1 (normal human fibroblasts; telomerase negative), BJ-hTERT (normal human fibroblasts; telomerase positive), Li Fraumeni (p53 mutant fibroblasts; ALT).

Aim 2. Determine induced T-SCE frequencies (in same cell lines) following exposure to exogenous DNA damaging agents (specifically UVC).

Aim 3. Determine spontaneous and induced G-SCE frequencies (in same cell lines) with same exposures.

RESULTS:

UVC exposure elevated BOTH genomic and telomeric sister chromatid exchange (G-SCE and T-SCE, respectively) frequencies in telomerase negative normal human fibroblasts

Spontaneous (background; 0 dose) G-SCE and T-SCE frequencies were determined in telomerase negative normal human fibroblasts (BJ1). Consistent with previous reports (Fackel, Dertinger, & Wolff, 1998) exposure to UVC (254 nm wavelength) elevated G-SCE frequencies in a dose dependent manner (Figure 1); doses above 30 J/m^2 were lethal. G-SCE per cell increased from 3.73 in untreated samples to 13.46 in $30J/m^2$. We also found that UVC exposure increased T-SCE frequencies in this telomerase negative background in a dose dependent manner; spontaneous ($0J/m^2$) T-SCE per cell of 1.37 increased with UVC dose to 6.7 T-SCE per cell following a $30J/m^2$ exposure. Thirty metaphases per condition were scored. Error bars were calculated using SEM, and SCE frequencies were calculated on a per cell basis. Three

independent experiments and scoring of SCE frequencies were done. Illustrative examples are provided (Figures 2-5).

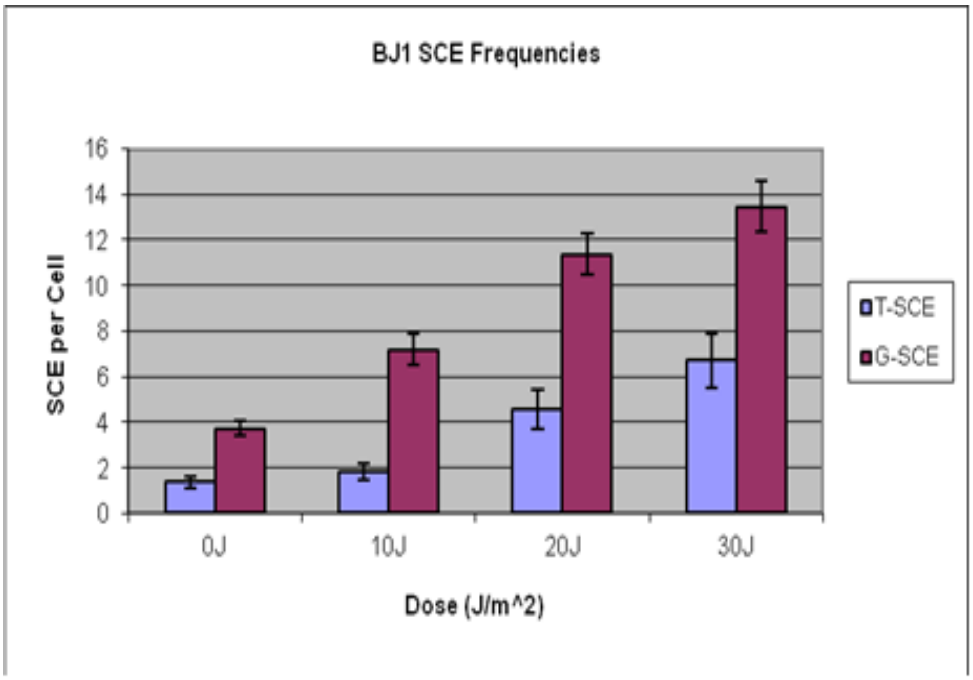

Figure 1. Genomic and Telomeric SCE frequencies in telomerase negative BJ-1 fibroblasts.

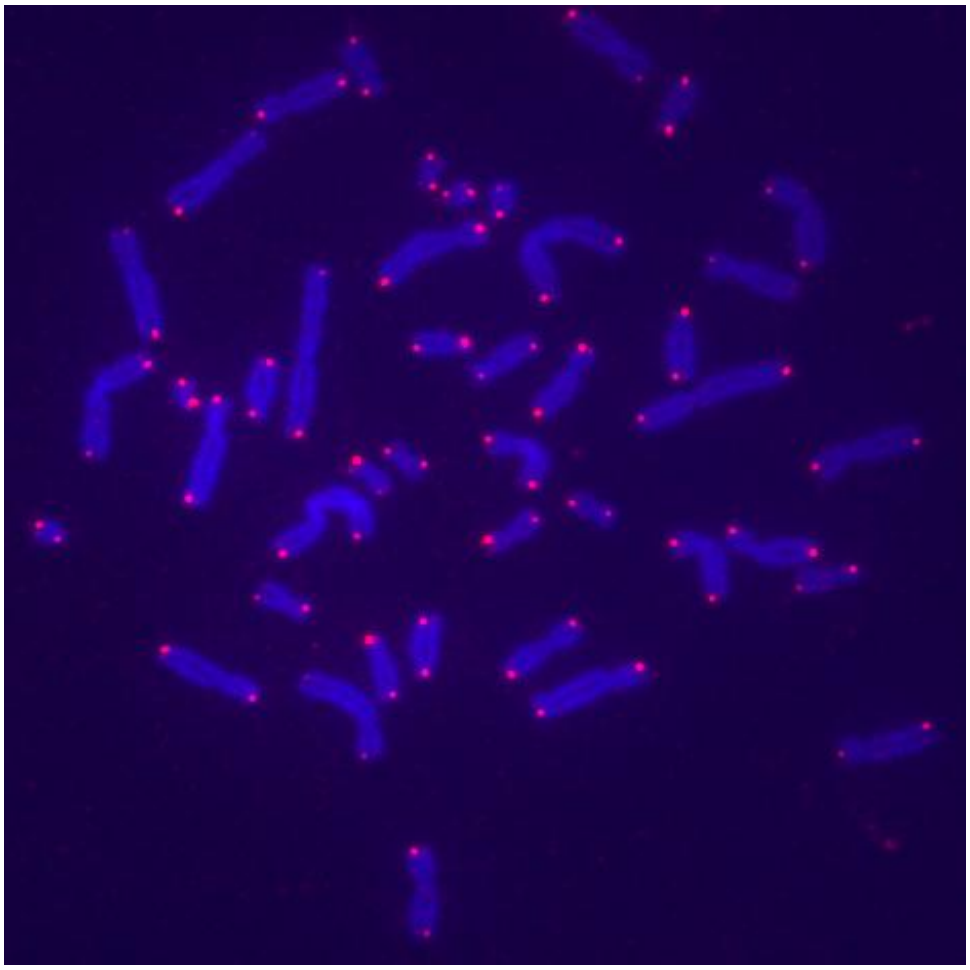

Figure 2: T-SCE detection Normal human fibroblast, untreated (0J/m²) UVC

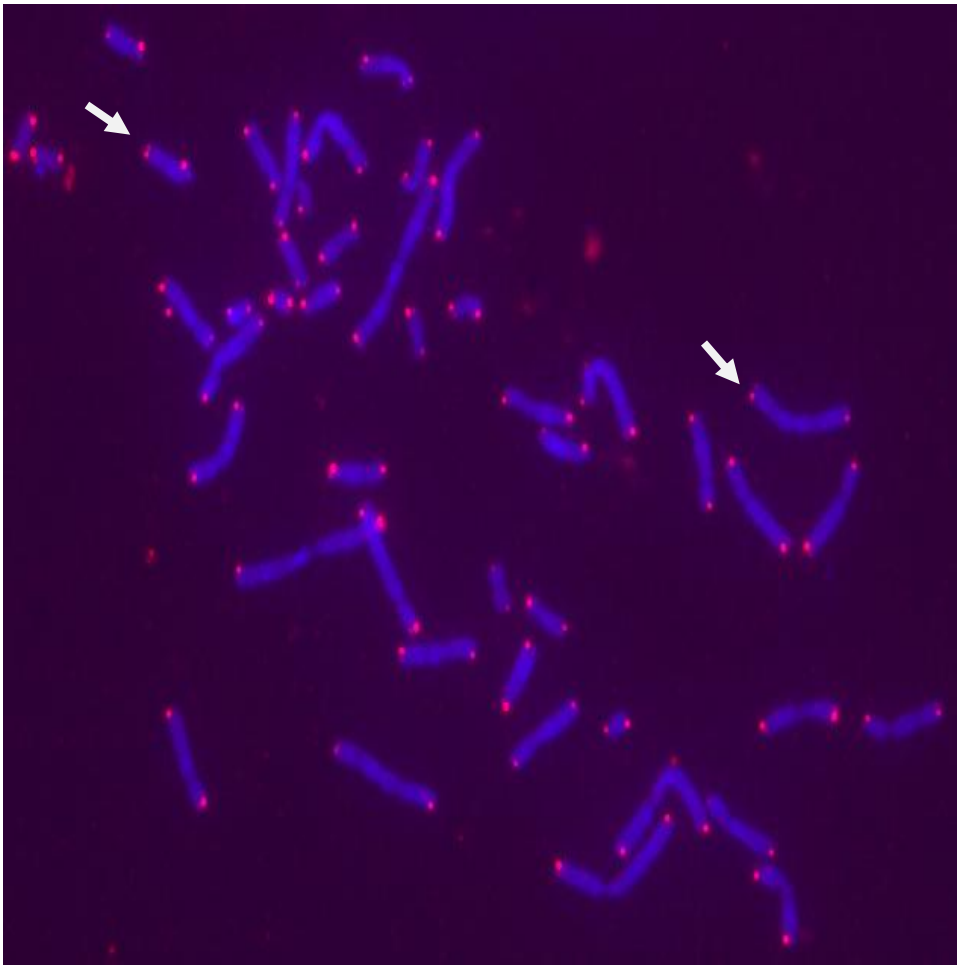

Figure 3: T-SCE detection. Normal human fibroblast following 30J/m² UVC

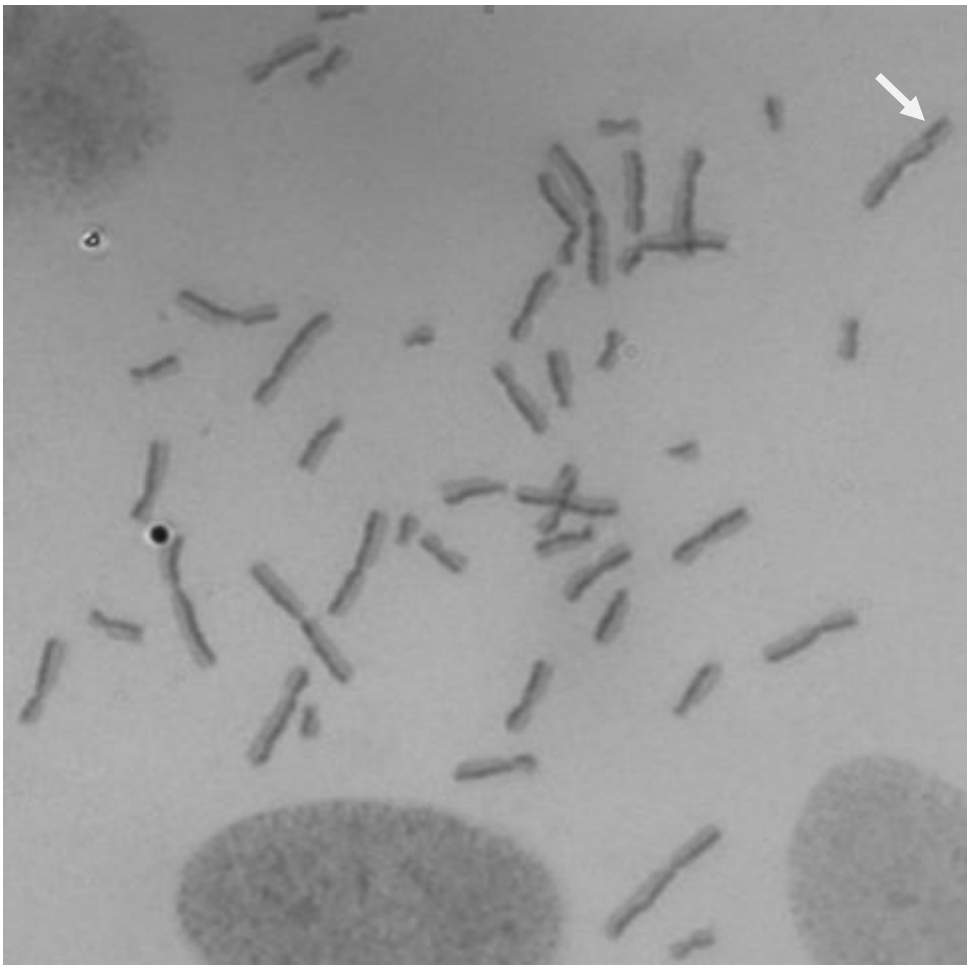

Figure 4: G-SCE detection. Normal human fibroblast, untreated (0J/m²) UVC

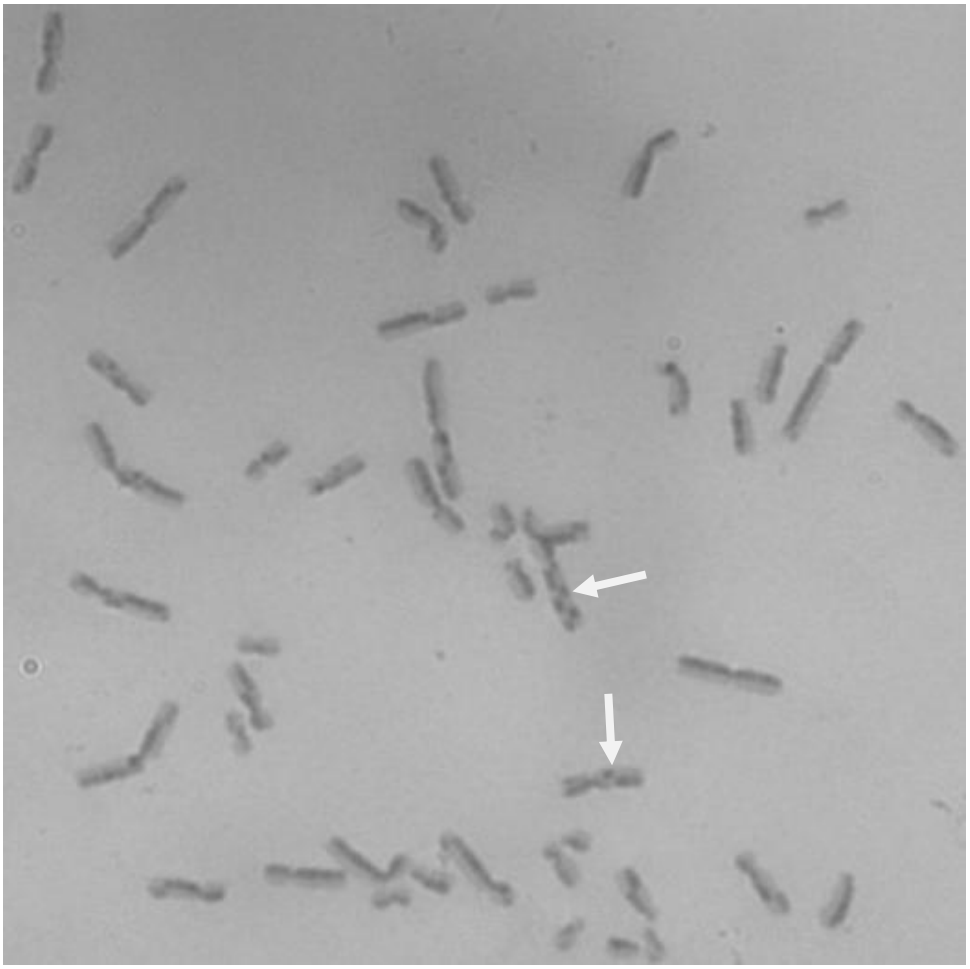

Figure 5: G-SCE detection. Normal human fibroblast metaphase spread following 30J/m² UVC

UVC exposure elevated G-SCE, but NOT T-SCE frequencies in telomerase positive human fibroblasts

Spontaneous (background; 0 dose) G-SCE and T-SCE frequencies were determined in telomerase positive normal human fibroblasts (BJ-hTERT). Again, exposure to UVC (254 nm wavelength) elevated G-SCE frequencies in a dose dependent manner (Figure 6); doses above 30 J/m^2 were lethal. G-SCE per cell increased from 4.93 in untreated samples to 11.36 following UVC doses of $30J/m^{2.}$ Thirty metaphases per condition were scored. Error bars were calculated using SEM, and SCE frequencies were calculated on a per cell basis. The saving and scoring of SCE frequencies were repeated three times.

In striking contrast, it is clear that T-SCE frequencies are not influenced by UVC exposure in this telomerase positive background, averaging 0.167 T-SCE per cell at $0J/m^2$. This result is consistent with our previous reports of a lack of T-SCE response in the presence of telomerase following various treatments (Dregalla et al., 2010). Illustrative examples are provided (Figures 7-10).

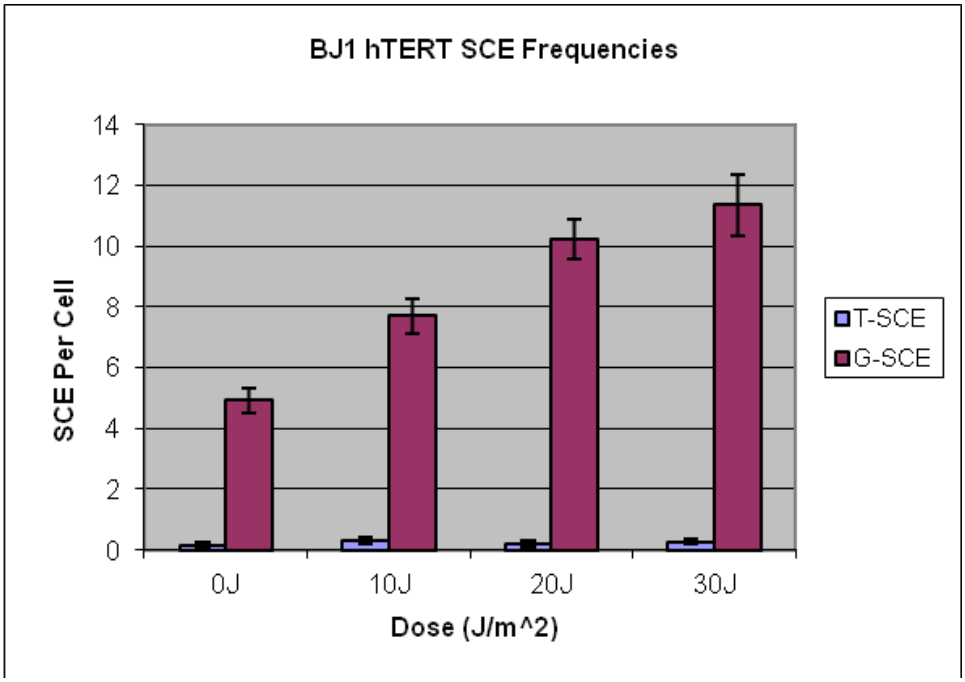

Figure 6: Genomic and Telomeric SCE frequencies in telomerase positive BJ-hTERT fibroblasts.

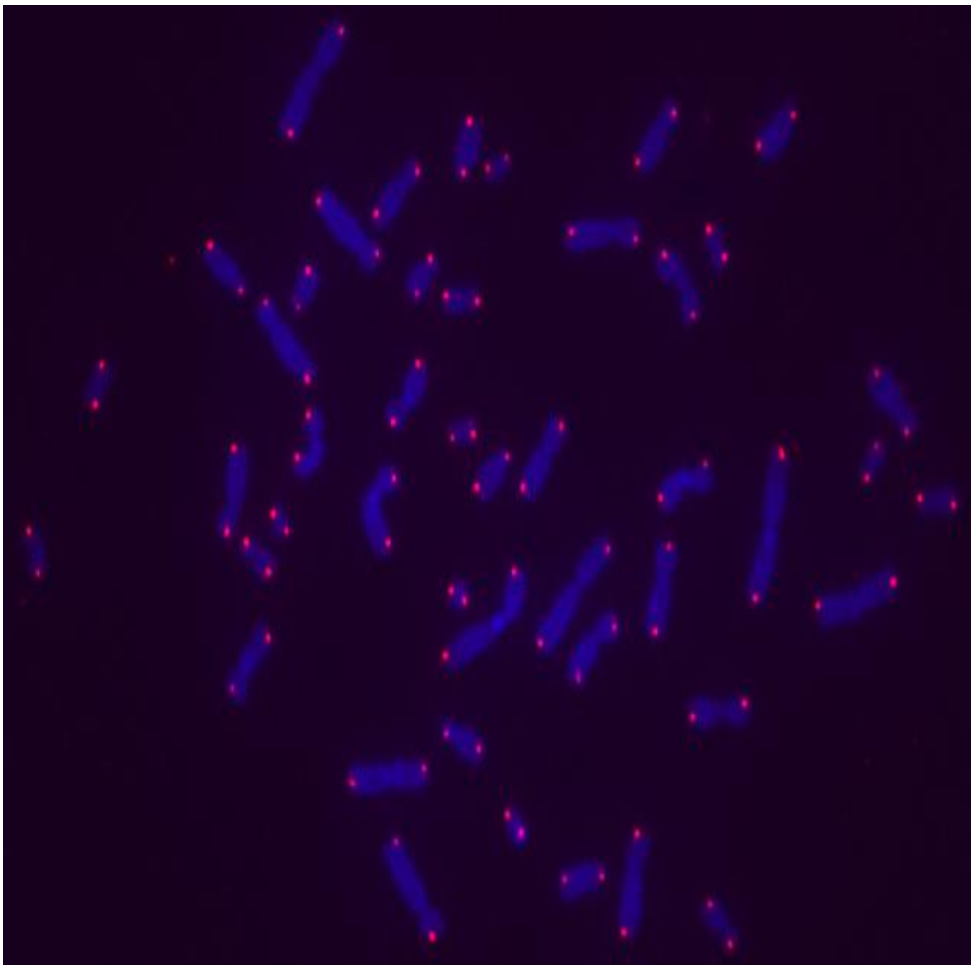

Figure 7: T-SCE detection. Telomere positive human metaphase spread, untreated (0J/m²) UVC

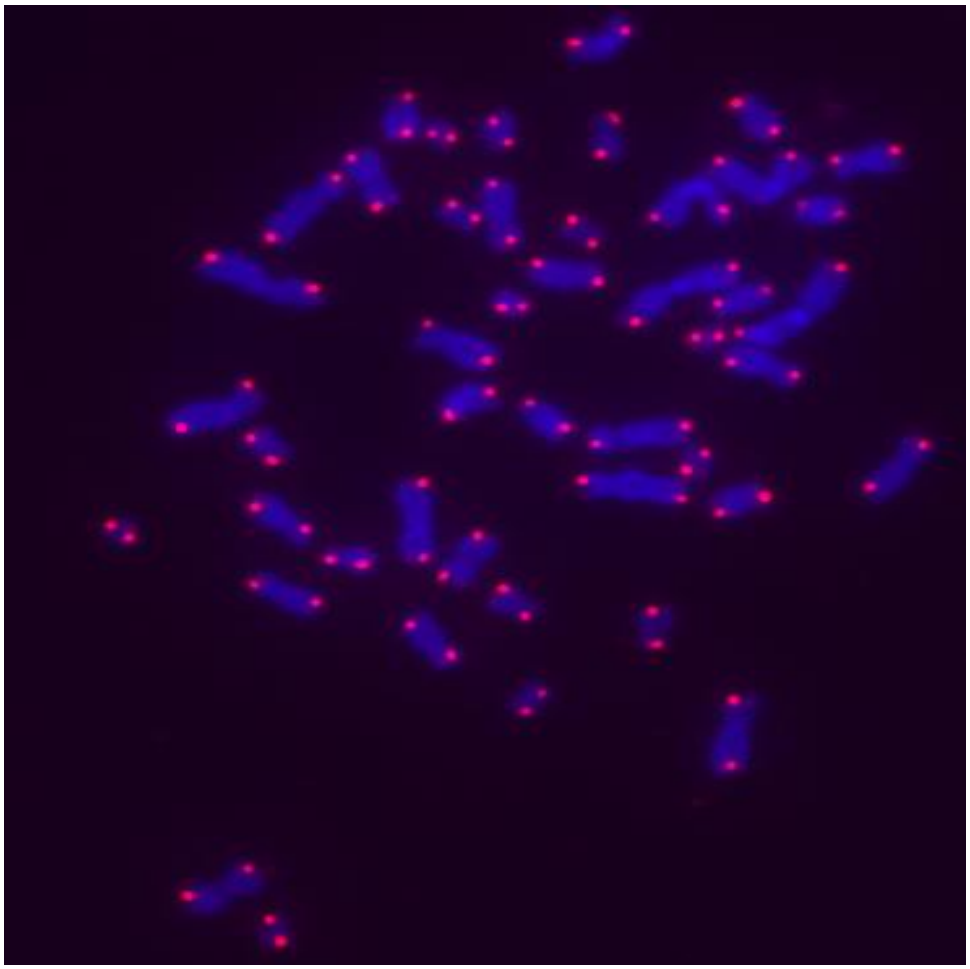

Figure 8: T-SCE detection. Telomere positive human metaphase spread following 30J/m² UVC

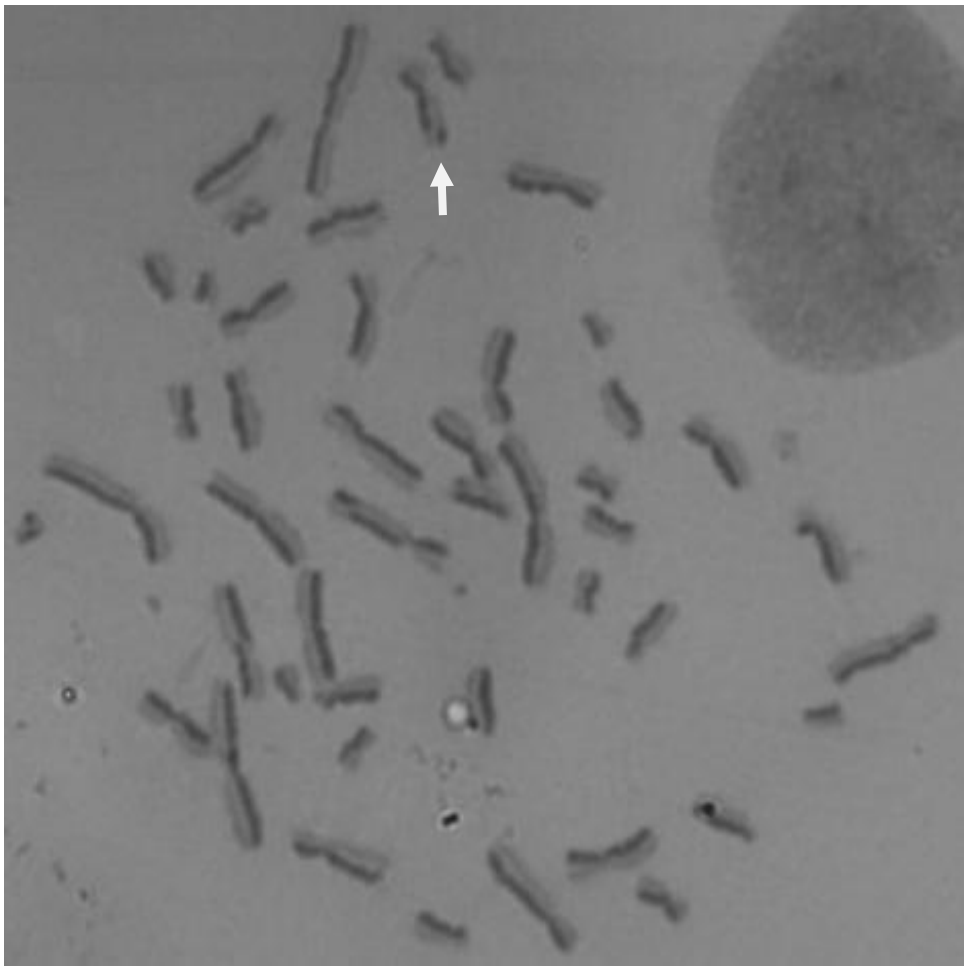

Figure 9: G-SCE detection. Telomere positive human metaphase spread, untreated (0J/m²)

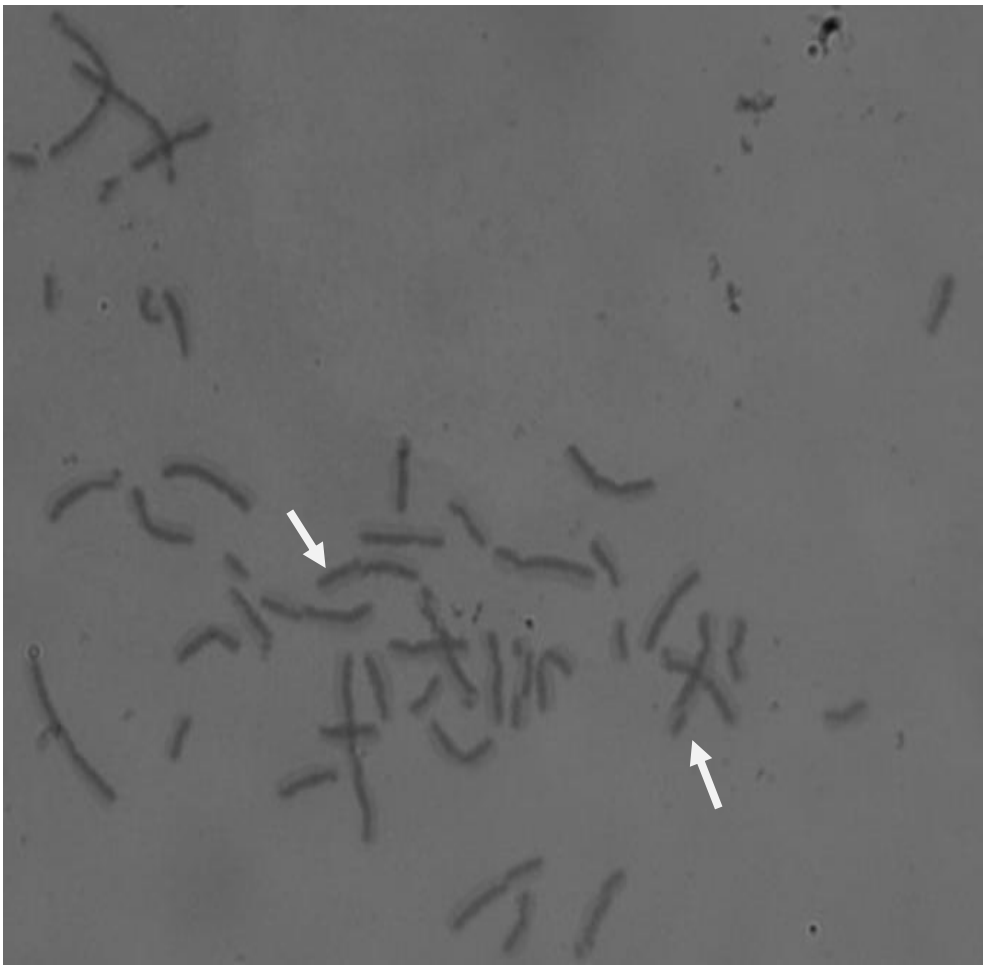

Figure 10: G-SCE detection. Telomere positive human metaphase spread following 30J/m² UVC

UVC exposure elevated G-SCE, and dramatically elevated T-SCE frequencies in human ALT background

Spontaneous (background; 0 dose) G-SCE and T-SCE frequencies were determined in the Li Fraumeni telomerase negative ALT cell line 087. Exposure to UVC (254 nm wavelength) elevated G-SCE frequencies in a dose dependent manner (Figure 11); doses above 30 J/m^2 were lethal. G-SCE per cell increased from 2.4 in untreated samples to 6.96 following UVC doses of $30J/m^{2.}$ Thirty metaphases per condition were scored. Error bars were calculated using SEM, and SCE frequencies were calculated on a per cell basis. Three independent experiments and scoring of SCE frequencies were done. Illustrative examples are provided (Figures 12-15).

UVC exposure dramatically increased T-SCE frequencies in this telomerase negative ALT background in a dose dependent manner. Spontaneous ($0J/m^2$) T-SCE per cell of 3.06 increased with UVC dose to 60.53 T-SCE per cell following a $30J/m^2$ exposure.

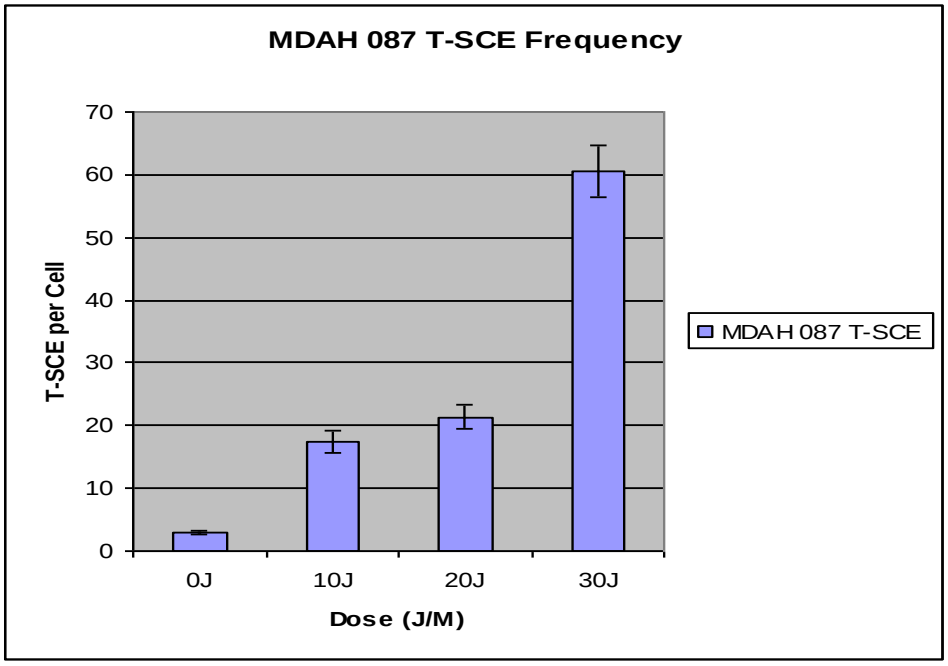

Figure 11 A: Telomeric SCE frequencies in telomerase negative ALT fibroblasts.

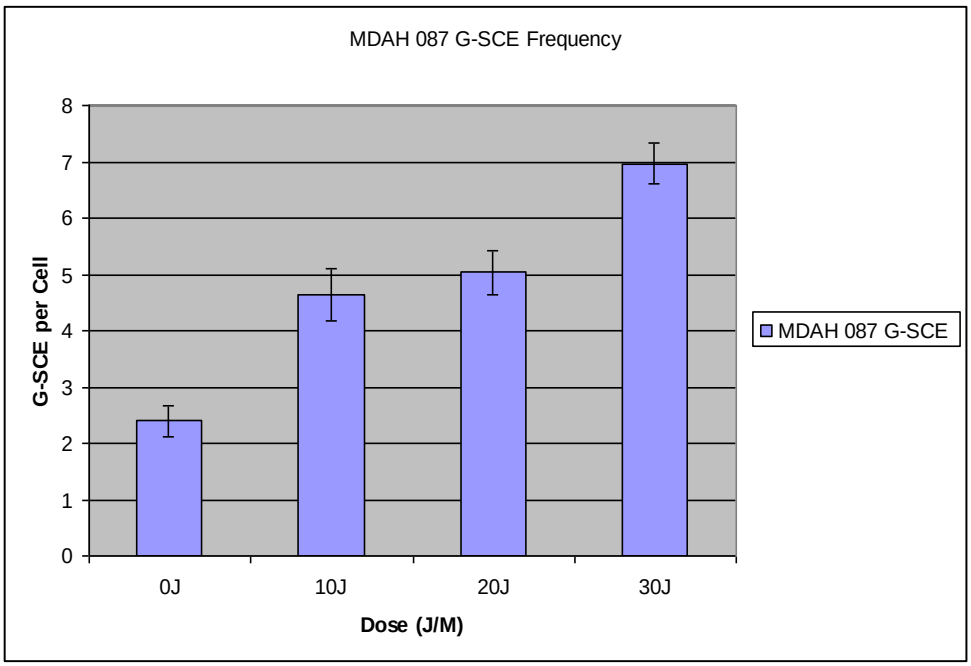

Figure 11B: Genomic SCE frequencies in telomerase negative ALT fibroblasts

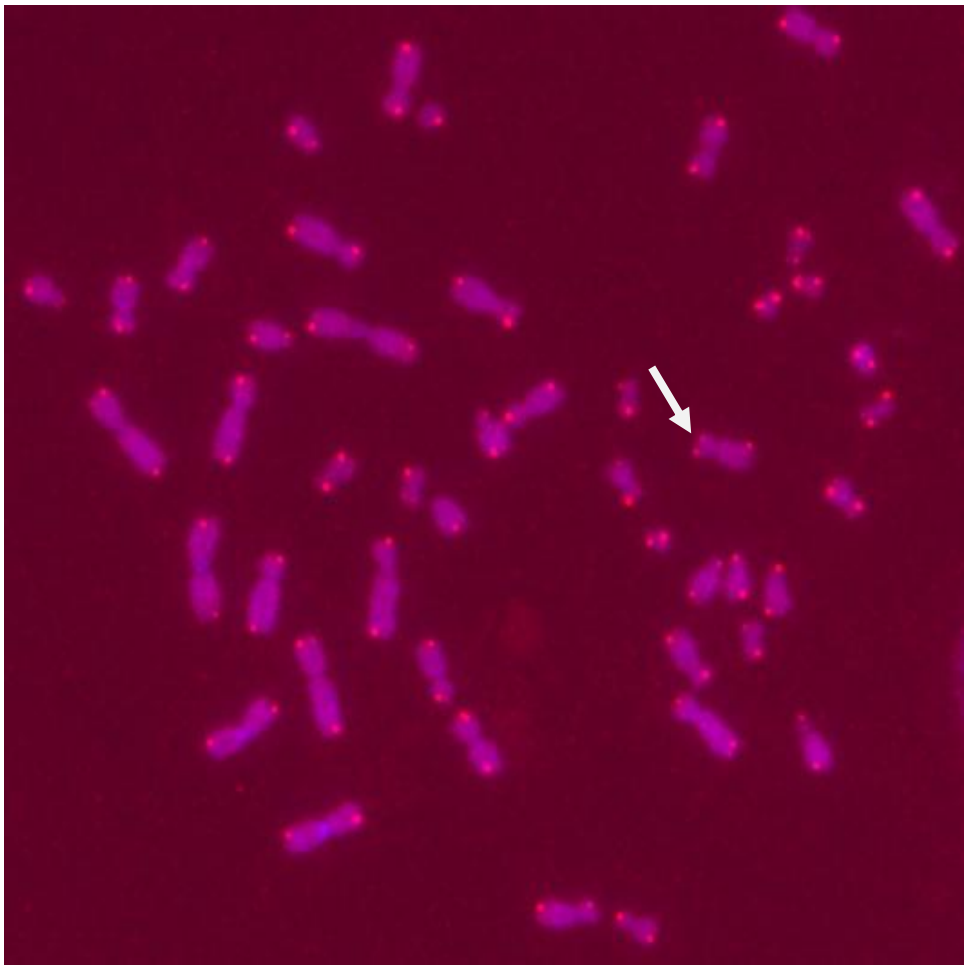

Figure 12: T-SCE detection Human ALT metaphase spread, untreated (0J/m²) UVC

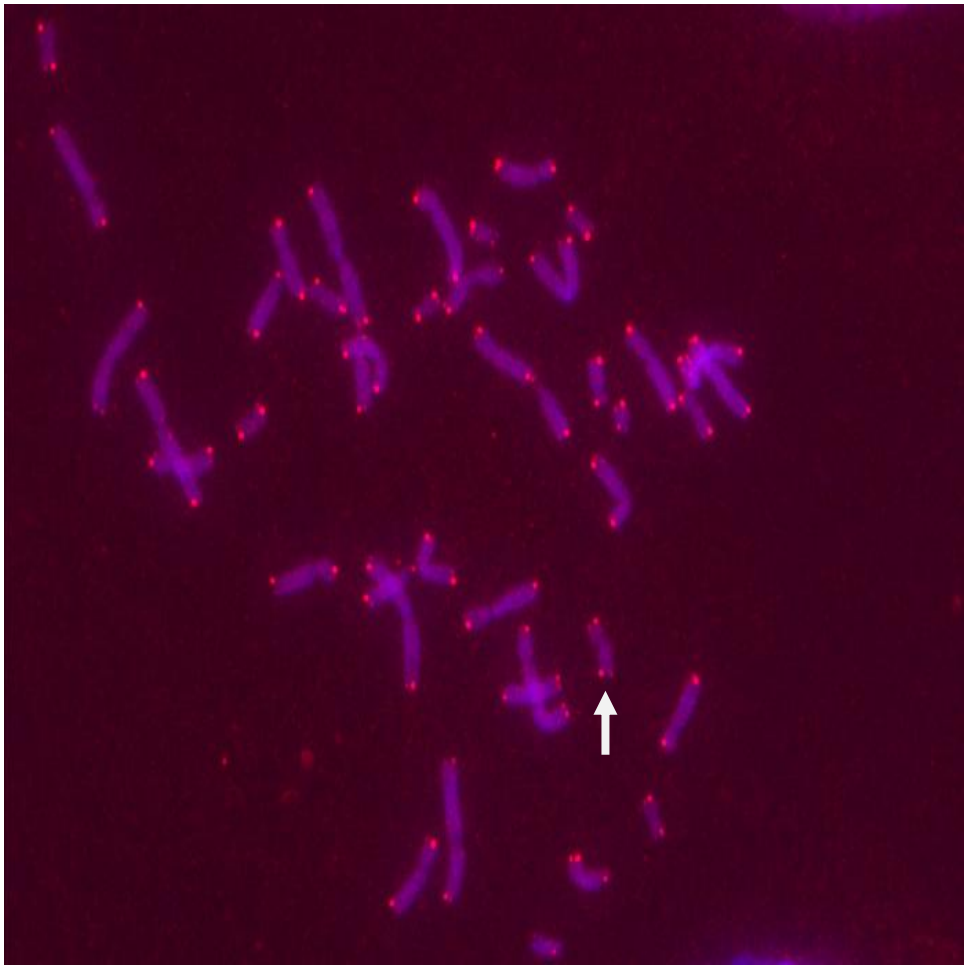

Figure 13: T-SCE detection Human ALT metaphase spread, untreated (10J/m²) UVC

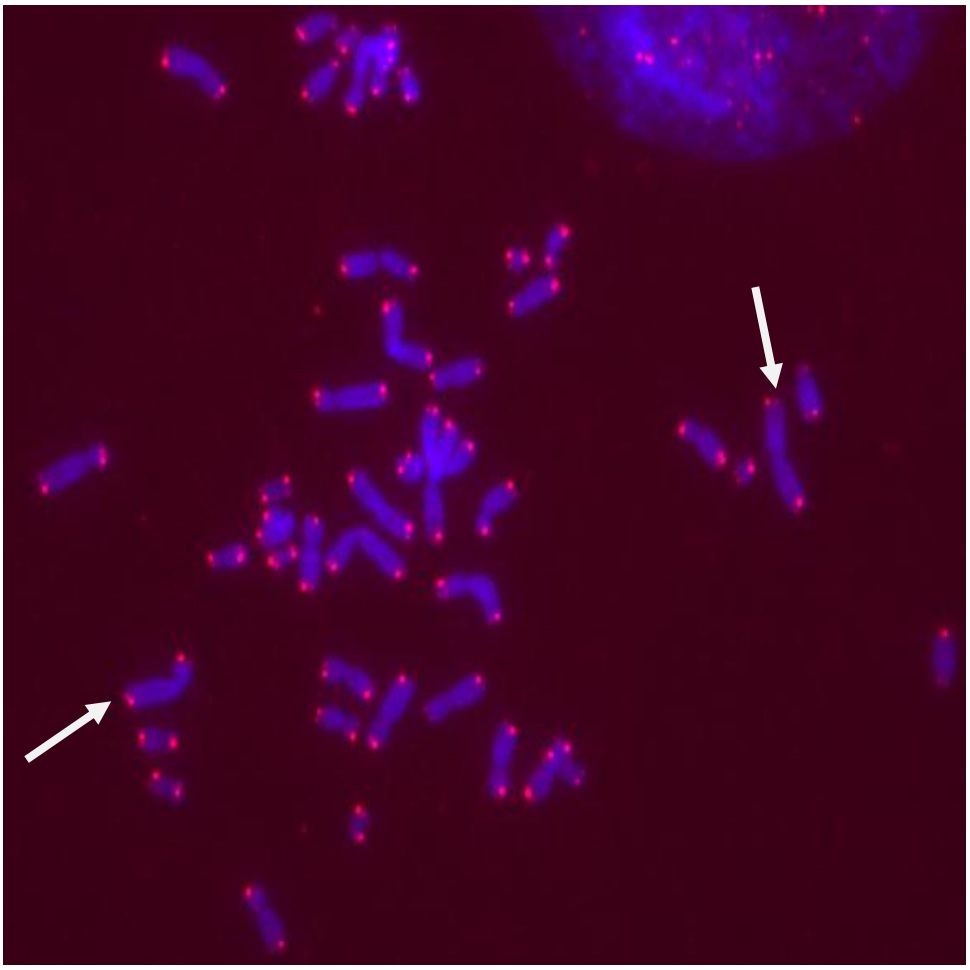

Figure 14: T-SCE detection Human ALT metaphase spread, untreated (20J/m²) UVC

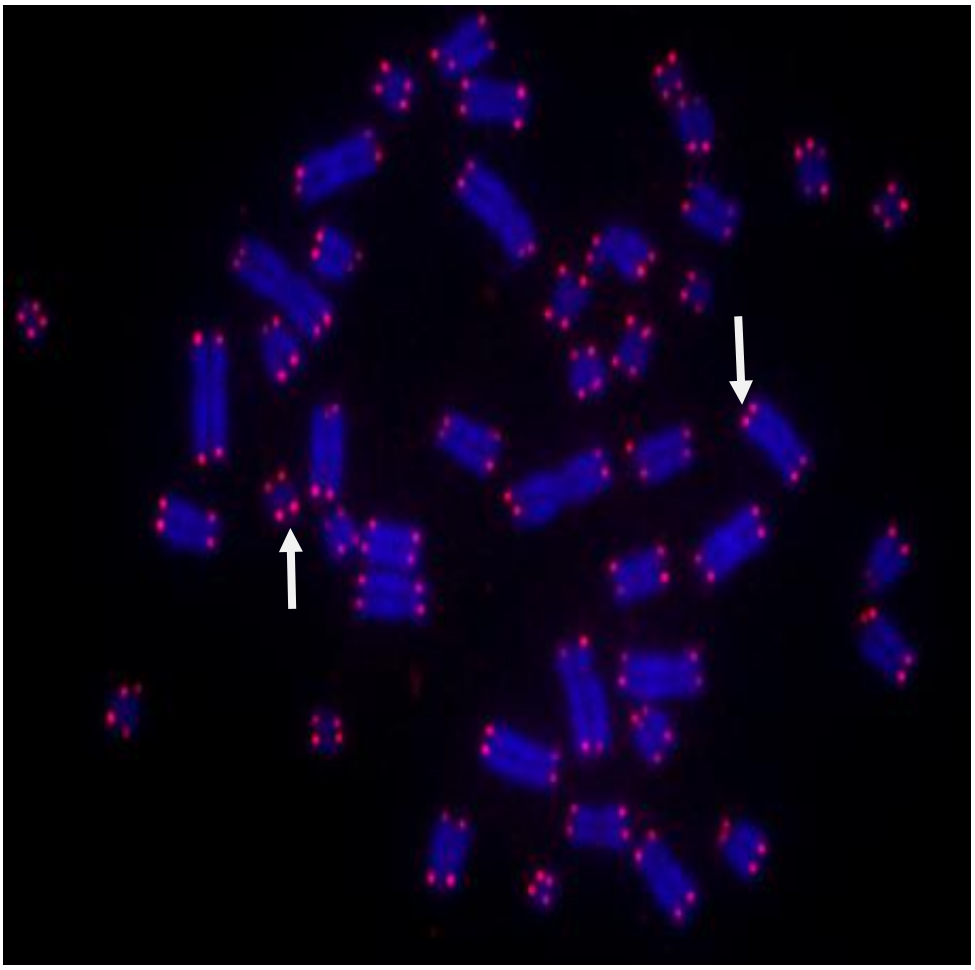

Figure 15: T-SCE detection. Human ALT metaphase spread following 30J/m² UVC

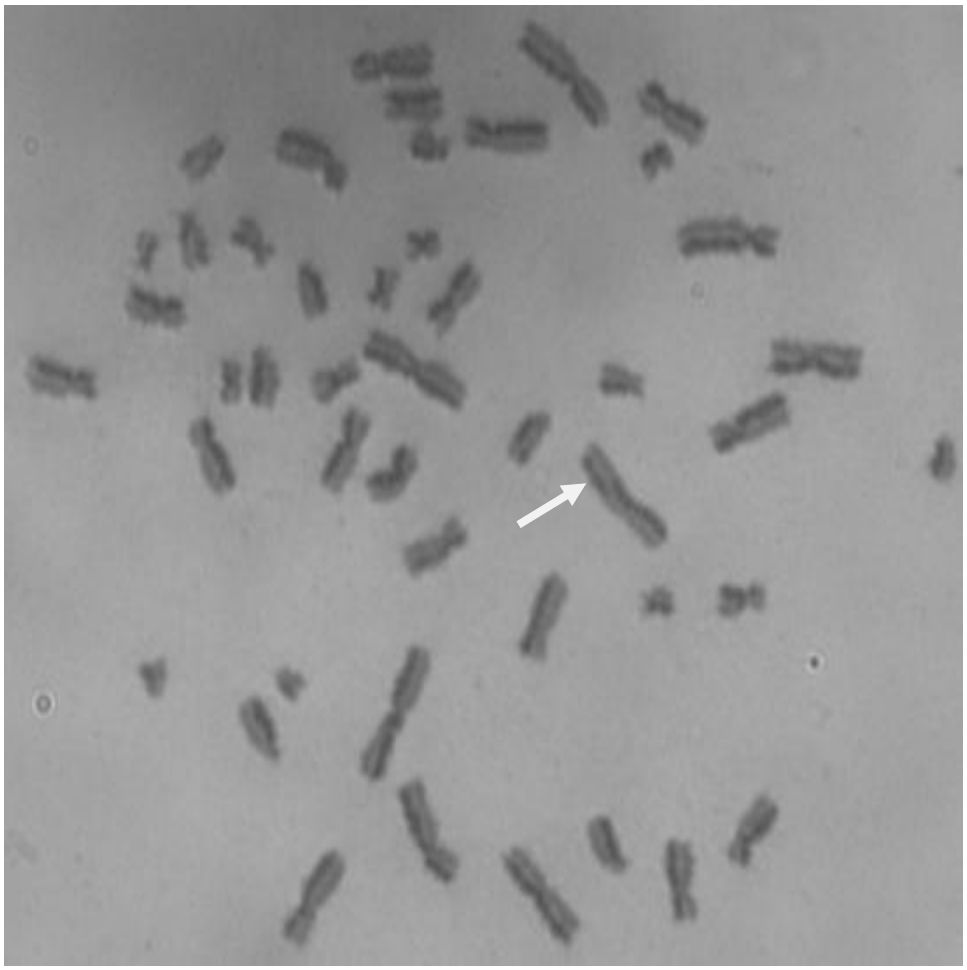

Figure 16: G-SCE detection. Human ALT metaphase spread, untreated (0J/m²) UVC

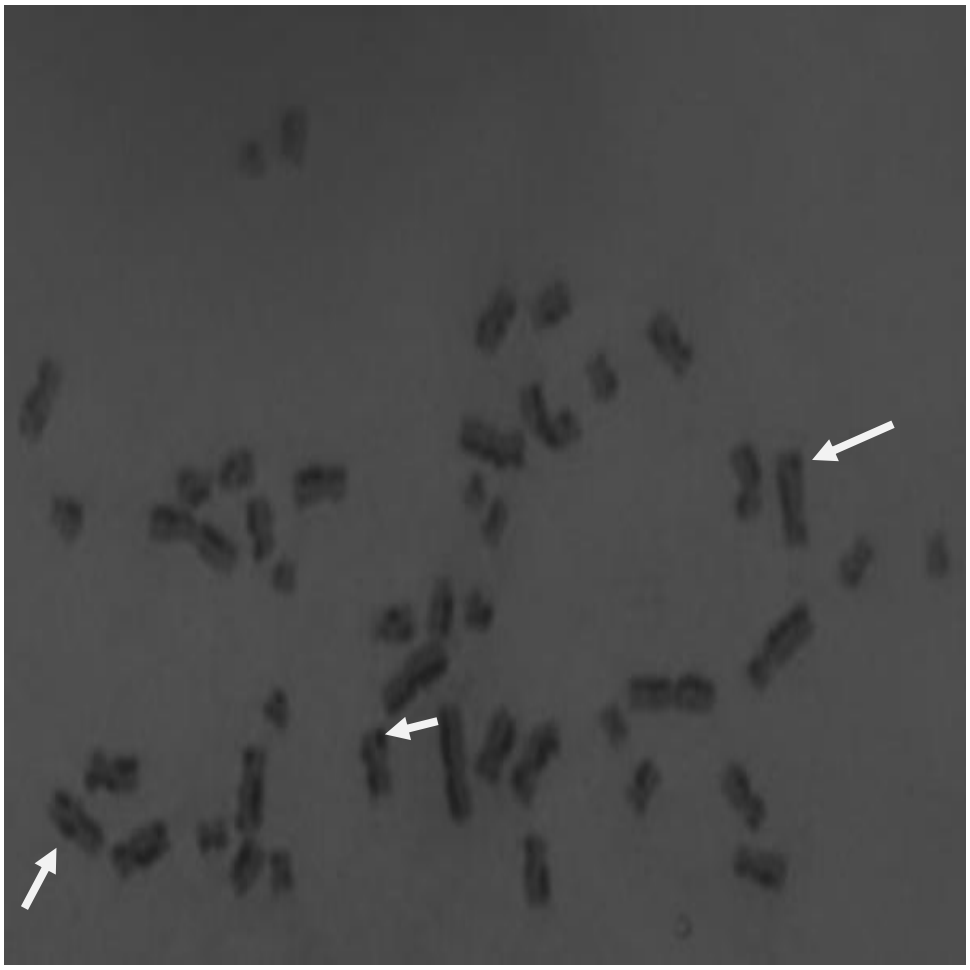

Figure 17: G-SCE detection. Human ALT metaphase spread following 30J/m² UVC exposure

DISSCUSSION

Human telomeres are protective structures at the ends of chromosomes composed of tandem arrays of the repeat sequence TTAGGG (Moyzis et al., 1988). The length of telomere is maintained by the reverse transcriptase telomerase (Greider & Blackburn, 1985, 1987). This pyrimidine-rich environment is especially susceptible to insult from oxidative stress, such as that produced by exposure to ultraviolet (UV) light, since cyclobutane pyrimidine dimers (CPDs) are the primary DNA damage caused by UV irradiation (Cadet, Sage, & Douki, 2005; Douki, Reynaud-Angelin, Cadet, & Sage, 2003). The most common CPDs occur between two adjacent thymines , thus producing thymidine dimmers (Haseltine et al., 1980). When encountered during replication, CPDs block and/or stall replication forks, thereby activating ATM signaling and DNA damage responses (Batista, Kaina, Meneghini, & Menck, 2009; Paulsen & Cimprich, 2007). CPDs are repaired by the nucleotide excision repair (NER) DNA repair pathway, which acts to excise the damaged nucleotides and replace them (BRUNK, 1973). CPDs occurring in the telomeric region present a special challenge, however, as DNA repair pathways, including NER, are repressed at telomeres (Rochette & Brash, 2010). Stalled replication forks, regardless of their location, can also be dealt with by recombination based mechanisms such as sister chromatid exchange (SCE) to "bypass" the offending lesion (Wilson III & Thompson, 2007).

We hypothesized that telomerase status and exogenous DNA damaging agents (specifically UVC exposure) influence telomeric recombination (T-SCE) frequencies independently of their influence on SCE recombination elsewhere in the genome (G-SCE). We

found that exposure of telomerase negative normal human fibroblasts to UVC (254nm) elevated G-SCE frequencies as previously reported (Fackel et al., 1998) and also significantly elevated T-SCE levels. Considering that the amount of telomeric DNA is considerably less than the total amount of the remainder of the genomic DNA, our results support the suppositions that telomeres are hot-spots for such recombination events (S. M. Bailey et al., 2004), and that telomeres are especially susceptible to oxidative damage (Rochette & Brash, 2010).

In contrast, UVC exposure of telomerase positive human fibroblasts elevated G-SCE, but *not* T-SCE frequencies, indicating that telomerase status influences telomeric recombination, a conclusion consistent with our previous work (Dregalla et al., 2010). We speculate that in the presence of telomerase when replication forks stall at oxidative lesions and collapse, unable to proceed, telomerase promptly adds sequence onto the end of the chromosome de novo, obviating the need for SCE recombination. It has also been suggested that telomerase may play a critical role in removing CPDs by facilitating DNA repair, specifically NER (Shin et al., 2004).

High levels of T-SCE have been previously reported and associated with the telomerase negative, immortalized ALT phenotype, and so, T-SCE were deemed an important marker of ALT that may serve as a means of recombination-based telomere length maintenance (S. M. Bailey et al., 2004; Bechter et al., 2004; Londoño-Vallejo et al., 2004). Here, we found that UVC exposure of human Li Fraumeni (p53 mutant) ALT cells (Tsutsui et al., 2003) elevated G-SCE levels as expected, but also dramatically elevated T-SCE frequencies, providing additional support for the relevance of T-SCE in telomerase negative backgrounds. However, rather than extending proliferative lifespan as originally proposed (S.M. Bailey et al., 2004), more recent modeling suggests that hyper telomere recombination more likely acts to accelerate replicative

senescence and may therefore promote premature aging phenotypes (e.g., Werner's Syndrome) (Blagoev et al., 2010; Hagelstrom et al., 2010).

These results have important implications not only for aging, but for carcinogenesis as well, since UV exposure from the sun (and tanning beds) is linked to increased risk of both aging of the skin and skin cancer (Stern, Weinstein, & Baker, 1986). The susceptibility of telomeric DNA to oxidative stress and the dampened DNA damage response in this region provide likely explanations for the increased frequencies of T-SCE we observed following UV exposure. Although certainly not the only contributor, T-SCE themselves provide intriguing insight into possible mechanisms of increased telomere shortening, senescence, and carcinogenesis, and may therefore represent informative biomarkers of aging and cancer.

Materials and Methods

Cell lines and culture conditions

All cell lines were cultured under standard conditions. Human foreskin fibroblasts (BJ1) and telomerase immortalized BJ1 foreskin fibroblasts (BJ1 hTERT) (Pedro de Magalhães et al., 2004)(gifts from Dr. Jerry Shay, UT Southwestern**)** were grown in a 4:1 ratio of D-MEM high glucose media and M-199 media (HyClone) supplemented with 10% fetal bovine serum and 1.4% L-Glutamine, at 37^{o}C in a humidified incubator with 5% CO_2. MDAH 087 are spontaneously immortalized, ALT human dermal fibroblasts isolated from a Li Fraumeni patient(Tsutsui et al., 2003), which were grown in alpha-MEM media (life technologies) supplemented with 15% fetal bovine serum and 1.4% L-Glutamine, at 37^{o}C in a humidified incubator with 5% of CO_2.

Ultraviolet (UVC) irradiations

Cells were grown on 100 mm tissue culture dishes to confluence to achieve contact inhibition. Immediately prior to irradiation, media was aspirated and 1.5 ml of cold 1x phosphate buffered saline (PBS) was added to each dish. Cells were exposed using a UVC irradiator described previously (Elkind, 1979) outfitted with 254 nm UVC bulbs, to acute doses of 0, 10, 20, 30J/m^2 at a dose rate of 1.1J/m^2 per second. Following irradiation, cells were promptly rinsed with cold 1x PBS and cells in the potentially unirradiated "shadow region" along the perimeter of the dish were removed using a rubber scraper. Cells were again rinsed in 1X PBS, then trypsinized and transferred to cell culture flasks for expansion, treatment and cytogenetic analysis.

Preparation of metaphase chromosomes

Cell suspensions irradiated with different doses of UVC were transferred to T-75 tissue culture flasks (Nunc) containing media with 7.5 x 10^{-6} M Bromodeoxy Uridine (BrdU) and 2.5 x 10^{-6} M Bromodeoxy Cytidine (BrdC) (Sigma). Flasks were incubated at 37 °C for either one (for CO-FISH analysis) or two (for FPG analysis) cell cycles in 1 x 10^{-5} M BrdU. To enrich for cells in mitosis, Colcemid (0.1 µg/ml) was added for 3.5 hours prior to harvest. Media was collected and cells were trypsinized, then centrifuged at 1000 RPM for 10 minutes at 4°C in 50 mL conical tubes. Supernatant was aspirated and the cell pellet was resuspended in 8 mL of 0.075 M KCl, which was transferred to a 15 mL centrifugation tube and placed at 37°C for 30 minutes. One mL of a 3:1 ratio of fresh methanol to acetic acid solution was added to each tube and mixed adequately. Samples were then incubated at room temperature for 5 minutes and centrifuged at 1000 RPM for 10 minutes. Supernatant was aspirated and the cell pellets were resuspended in 10 mL of 3:1 methanol to acetic acid and stored at -20°C. Before dropping the cell suspensions onto clean slides, cells were washed 2-3 times with freshly prepared fixative to remove any remnants of debris.

Chromosome orientation fluorescence in situ hybridization (CO-FISH)

CO-FISH has been extensively described(S. Bailey, Cornforth, Kurimasa, Chen, & Goodwin, 2001), and was used here with some modification to evaluate T-SCE. Briefly, slides with metaphase chromosomes that had gone through one round of replication in the presence of BrdU were incubated in 1% formaldehyde for 10 min at room temperature, then washed with 1x PBS and dehydrated in cold ethanol series (75%, 80%, 100%; 2 min each) and incubated in 37^{o}c drying oven for 10 min to completely dry the chromosomes. Dried slides were stained in

Hoechst 33258/2X SSC for 15 min at room temperature to increase sensitivity of newly synthesized, BrdU-substituted DNA to UV light. The slides were rinsed in deionized, distilled water (ddH_2O) and flooded with 2xSSC cover slipped (No. 1) and exposed to UV light (Stratalinker outfitted with 365 nm bulbs) for various times depending on desired dose and prior dosimetry (Williams, Cornforth, Goodwin, & Bailey). Slides were rinsed in ddH_2O to remove coverslip and avoid scratching and allowed to air dry. Slides were then incubated with exonuclease III (3U/ul in buffer stored in -20,Promega) for 40 min at 25^{o}C, which acts on single-stranded nicks created by the UV exposure and effectively digests the newly replicated strands of DNA. Slides were rinsed in ddH_2O and allowed to air dry. Slides were then denatured in 70% formamide/2xSSC for 2 min at 75^{o}C and immediately dehydrated through a cold ethanol series (75%, 80%, 100%; 2 min each) and allowed to air dry.

Telomere Probe Hybridization

Probe hybridizations were performed in the dark due to the sensitivity of fluorescently labeled probes to light. A hybridization mixture (40-50ul) containing Cy3 labeled G-rich telomere ($TTAGGG_3$) PNA probe (3ug/ml) was prepared and placed on each slide, which was then cover slipped (No.1) and placed in a humidified hybridization chamber for 1.5 - 2 hrs. in the dark at 37^{o}C. Slides were then washed through the following series at 43^{o}C for 4 min each: 50%formamide/2xSSC; 2xSSC and 2xssc + 0.1% NP-40. Slides were allowed to air dry, 20-25ul Anti-fade Gold Reagent with DAPI [Life technologies] was applied, and each slide was cover slipped. The slides were kept in at 20^{o}C overnight before examining metaphase spreads and scoring T-SCE using fluorescence microscopy.

Fluorescence plus Giemsa (FPG)

FPG was performed according to the original protocol of Perry and Wolff (Perry & Wolff, 1974). Slides with metaphase chromosomes that had been through two rounds of replication in the presence of BrdU [2,7 mM, Sigma] were incubated with freshly prepared Hoechst 33258 [0.5ug/ml, Sigma] for 15 min at room temperature. Slides were rinsed in ddH_2O and allowed to air dry; they were then flooded with 2xSSC and cover slipped (No. 1), and exposed to UV light (Stratalinker outfitted with 365 nm bulbs) for 30 min. Slides were rinsed briefly in ddH_2O to remove the coverslip and then placed in 2XSSC at 60^oc for 30 min Slides were rinsed well in ddH_2O and allowed to air dry.Giemsa stain [5%; Sigma] was freshly prepared and slides were stained for 15 min at room temperature. Slides were rinsed in ddH_2O and allowed to air dry, then evaluated for quality metaphase spreads and scored for G-SCE using bright field microscopy.

Scoring criteria and microscope information

Metaphase chromosomes were examined and images captured and analyzed using a Zeiss Axioskop2Plus microscope equipped with a Photometrics CoolsnapES2 camera and Metavue 7.1 software. T-SCE was visualized using the TRITC (red; telomere signal) and DAPI (blue; chromosomes) filters and scored as a split telomere signal between the two sister chromatids (S. M. Bailey et al., 2004). FPG harlequin chromosome staining was visualized using bright field microscopy and G-SCE were scored at each “color switch” between the two sister chromatids (Perry & Wolff, 1974).

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