DISSERTATION

TELOMERE LENGTH, TELOMERASE ACTIVITY, AND STRUCTURAL VARIANTS AS BIOMARKERS OF EXTREME ENVIRONMENTS

Submitted by

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ABSTRACT

TELOMERE LENGTH, TELOMERASE ACTIVITY, AND STRUCTURAL VARIANTS AS BIOMARKERS OF EXTREME ENVIRONMENTS

Mammals, and in particular humans, are masterful at overcoming and adapting to extreme environments. Whether astronauts in low earth orbit aboard the International Space Station (ISS) or U.S. military veterans exposed to nuclear fallout from atomic weapons testing, humans can persist through a wide range of physical, psychological, and environmental stressors. The overall goal of the studies presented here was to evaluate the biological influences of extreme environments not commonly experienced by the general population. Whether spaceflight or exposure to nuclear fallout, results improve our understanding of short- and long-term effects of low gravity environments, exposure to ionizing radiation (IR) of mixed qualities, as well as low dose and low dose effects of IR. We explored these scenarios by evaluating biomarkers of stress, specifically telomere length dynamics, and biomarkers of DNA damage, specifically transmissible structural rearrangements. Telomeres are not only regarded as valuable biomarkers of aging and age-related degenerative pathologies like cardiovascular disease and cancer, and so are reflective of overall health status, they also serve as "hallmarks" of radiosensitivity. Stable chromosomal structural rearrangements (translocations and inversions) persist with time and so provide informative signatures of IR exposure as well.

During the 1950's United States military personnel and weathermen, collectively known as the atomic veterans, were unintentionally exposed to nuclear fallout during atomic bomb testing following WWII. Here, directional Genomic Hybridization (dGH) for high-resolution detection

of IR-induced chromosomal inversions and translocations was assessed as a more sensitive, quantitative retrospective biodosimetry approach. The influence of IR exposure on telomere length dynamics was also evaluated to determine the long-term influence of such exposures. Our results illustrate that even for nuclear events that occurred six decades in the past, evidence of exposure is still present. We find that although translocations and inversions are reliable biodosimeters independently, a combined approach provides a more sensitive measurement of past radiation exposure. We also report, for the first time, the influence of age and smoking on background inversion frequencies. Furthermore, telomere length was inversely related to IR dose, suggesting that a single acute exposure to nuclear fallout may lead to persistent long-term effects on overall health.

Telomere length dynamics and structural rearrangements were also monitored longitudinally in monozygotic twin and unrelated astronauts. NASA astronauts are a unique group of individuals who experience an extreme environment that the human body is not adapted for. Little is known about the biological health effects of a low gravity environment with increased IR exposure including galactic cosmic rays (GCRs), solar particle events, and secondary neutrons. Not only do astronauts have shorter telomeres than age-/gender-matched controls at baseline (pre-flight), but a transient increase in telomere length during space flight was also observed. Results suggest this unexpected finding may be due to an upregulation of telomerase, the enzyme responsible for maintaining telomere length. Moreover, telomerase activity also increased post-flight in both twins, Scott and Mark Kelly. Although not spaceflight specific, this is the first report of telomerase upregulation in humans due to a psychologically traumatic event. A dose dependent increase in inversions, and to a lesser extent, translocations, as a consequence of IR exposure on the ISS was also seen in Scott Kelly.

Collectively, the studies presented here demonstrate a profound influence of extreme environments, particularly those involving low dose IR, on human biological responses. Telomere length dynamics and chromosome aberration frequencies (e.g. translocations and inversions) provide insight into the long-term health effects and implications of spaceflight and exposure to nuclear events.

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CHAPTER 1

INTRODUCTION

Telomeres and Telomerase

Telomere Structure and Function

Telomeres are natural ends of linear chromosomes that serve to protect coding DNA from inappropriate DNA damage responses and truncation, thus insuring genomic stability (O'Sullivan and Karlseder, 2010; Webb et al., 2013). In mammals, specifically vertebrates, these chromosome termini end in repetitive DNA repeats, 5'-TTAGGG$_n$-3' (Moyzis et al., 1988). Although estimates vary based on tissue type, the average telomere length in humans is between 10-15kb (Cairney and Keith, 2008; Okuda et al., 2002), 12-23 kb in dogs (Nasir et al., 2001), and upwards of 50kb in laboratory mice (Zijlmans et al., 1997). As originally proposed by Muller and McClintock in the late 1930's, telomeres were suspected to cap, and therefore protect, the ends of linear chromosomes (McClintock, 1941; Muller, 1938). Subsequent studies confirmed their observations, including those of Griffith et al. in 2005 and discovery of the T-loop. Using electron microscopy, Griffith noted that telomeres end in a 3' single-stranded DNA (ssDNA) overhang that displaces a portion of the double stranded sequence, forming the displaced loop (D-loop), and ultimately leading to the formation of the telomere loop (T-loop) (Griffith et al., 1999). The T-loop structure fundamentally forms a cap to prevent chromosome termini from being recognized as a double strand DNA break (DSB) and minimize aberrant repair (Longhese, 2008; Mao et al., 2016).

With each cellular division, telomeres naturally shorten due to the end-replication problem. This phenomenon was first described by Watson (1972) and Olovnikov (1973) whereby during discontinuous lagging-strand replication, the removal of RNA primers and Okazaki fragment formation prevent replication of the very ends of chromosomes (Olovnikov, 1973; Watson, 1972). In humans, telomeres are estimated to shorten by 50-100 bp per cell division if they lack the ability to maintain telomere length (Allsopp et al., 1992; Harley et al., 1990). In the absence of a telomere maintenance mechanism, telomeres will reach a critically short length *in vitro*, and the cells will enter replicative senescence (Shay et al., 1991). This limit is known as the Hayflick limit, first described by Leonard Hayflick, whereby cell lines derived from human fetal tissue stopped dividing after 40-50 cell cycles (Hayflick and Moorhead, 1961).

Additionally, telomeres are bound by several telomere-specific proteins, termed shelterin, forming a protein complex along the telomere DNA track. The shelterin complex is composed of six proteins, TRF1, TRF2, POT1, TPP1, TIN2, and Rap1, which collectively form a protective structure, or scaffold, "sheltering" telomeric DNA and forming the T-loop (de Lange, 2005). Notably, telomere repeat factors 1 and 2 (TRF1 and TRF2) bind the double stranded DNA telomere track and provide structural integrity for the T-loop (Bilaud et al., 1997; Chong et al., 1995); however, they also play crucial roles in telomere maintenance regulation. TRF1 is responsible for regulating telomere length by modulating access of telomerase, the enzyme responsible for *de novo* synthesis of telomere sequence (Smogorzewska et al., 2000). TRF2 complexed with Rap1 inhibits classical non-homologous end joining (NHEJ) at telomeres (Bae and Baumann, 2007), and prevents telomere fusions (Bailey et al., 2001; van Steensel et al., 1998). Although not a shelterin protein, the recently identified telomere zinc-finger associated protein (TZAP), may play an important role in telomere length maintenance. TZAP was shown to bind preferentially to long

telomeres, specifically when shelterin protein (e.g. TRF1 and TRF2) concentrations are low, and elicit telomere trimming (Li et al., 2017). This suggests that while telomerase access to telomeric DNA is limited by TRF1, there may be a secondary mechanism to maintain telomere homeostasis. Since first hypothesized by Muller and McClintock, we've only recently begun to fully appreciate the vital role of telomeres in genomic stability.

Telomerase and Telomere Length Maintenance

Without the ability to maintain telomere length, telomeres will continually shorten with each cell division eventually leading to replicative senescence (Shay et al., 1991). In 1985, Drs. Carol Greider and Elizabeth Blackburn discovered the telomere specific terminal transferase in the ciliated protozoan, *Tetrahymena*, which was conveniently named telomerase. While in the presence of synthetic telomere primers, $TTGGGG_n$ in the case of *Tetrahymena*, cell free extracts containing the terminal transferase enzyme were able to add tandem telomere repeats and effectively elongate the primers (Greider and Blackburn, 1985). Soon thereafter, telomerase activity was detected in cell extracts from the human cervical cancer cell line, HeLa, and sequence analysis revealed the telomere sequence to be TTAGGG (Morin, 1989; Moyzis et al., 1988). While this was not the first documentation of telomerase activity, it is an important finding since human cells contain significantly lower levels of telomerase activity than *Tetrahymena* (Corey, 2009). These discoveries led to the first identification of a telomere length maintenance mechanism.

Telomerase is a specialized ribonucleoprotein complex composed of the catalytically active subunit, telomerase reverse transcriptase (TERT) protein, and the telomerase RNA component (TERC) (Greider and Blackburn, 1987; Shippen-Lentz and Blackburn, 1990). The holoenzyme is responsible for *de novo* synthesis of telomere repeats at the terminus of the single

stranded 3' G-rich DNA strand by forming a RNA/DNA hybrid with TERC priming for reverse transcription (Podlevsky and Chen, 2012). In humans, telomerase is thought to only be present at sufficient levels to maintain telomere length in germline, stem, and cancerous cells (Shay and Wright, 2010). Conversely, normal somatic cells are low expressers of telomerase; therefore, they lack the ability to maintain telomere length, which has been proposed as an effective tumor suppressor mechanism. Dysfunctional or critically short telomeres elicit a DNA damage response and checkpoint activation leading to replicative senescence or apoptosis (Deng et al., 2008). Several lines of evidence indicate that telomerase is necessary for cell immortalization, specifically in cancer, which can promote tumor progression. In support of this notion, telomerase negative mice crossed with Ink4a/Arf null mice effectively lead to a hybrid that is telomerase negative and null for the tumor suppressor proteins p16 and p19Arf. These mice display less tumor formation and a longer latency period compared to their telomerase positive counterparts (Artandi and DePinho, 2010; Khoo et al., 2007).

In addition to telomerase, a second means of telomere length maintenance was discovered in 1994. Simian virus 40 (SV40) transformed human cells containing critically short telomeres demonstrated a rapid, heterogeneous increase in length in the absence of telomerase (Murnane et al., 1994), suggesting a telomerase independent method of telomere maintenance. This alternative maintenance mechanism, known as alternative lengthening of telomeres or ALT, was confirmed by Bryan and Reddel in 1995 while evaluating whether telomerase activity was necessary for cell immortalization. Out of 35 immortalized cell lines, 15 were telomerase negative and displayed signs of long heterogeneous telomere lengths. Concluding that in fact telomerase was not always necessary for immortalization. Their finding supported the idea of a second telomere length maintenance method (Bryan et al., 1995).

While telomerase activity is the predominant telomere maintenance mechanism, being expressed in an estimated 85% of cancers, the remaining 10-15% achieve immortalization through ALT (Heaphy et al., 2011; Reddel, 2003; Shay and Bacchetti, 1997). Although not fully understood, ALT typically occurs in tumors of mesenchymal origin, and research suggests that telomerase is repressed more in mesenchymal as compared to epithelial tissues, potentially promoting the ALT phenotype (Atkinson et al., 2005; Henson et al., 2002). Several mechanisms have been proposed to explain ALT, but the most practical and supported theory is that ALT maintains telomeres through homologous recombination-mediated DNA replication (Cesare and Reddel, 2010; Henson et al., 2005; Reddel, 2014). Characteristics of ALT include heterogeneous telomere length (HTR) (Henson et al., 2002), an abundance of ALT-associated promyelocytic leukemia bodies (APB) (Osterwald et al., 2015), extrachromosomal telomeric DNA including C-circles (Cesare and Griffith, 2004; Ogino et al., 1998), as well as increased telomeric sister chromatid exchanges (T-SCE) (Bailey et al., 2004).

Although it is well accepted that both telomerase and ALT can lead to replicative immortalization of cells, one of the hallmarks of cancer (Hanahan and Weinberg, 2000, 2011), telomerase has also been shown to play multiple non-canonical roles, independent of telomere length maintenance, in normal and cancerous cells. Several reports have associated the protein catalytic subunit, TERT, with transcriptional regulation, angiogenesis, cell proliferation, genome instability, and immune responses (Low and Tergaonkar, 2013). Specifically, TERT was shown to function in a similar manner as Myc and Wnt in the transcriptional regulation of development in epithelial cells. To test the transcriptional regulation capabilities of TERT, Choi and colleagues inserted catalytically inactive TERT mutants into either TERT -/- mouse embryonic fibroblasts (MEF) or primary human fibroblasts. Their results suggest that the catalytically inactive TERT

mutant was sufficient for increasing keratinocyte proliferation, as well as activating hair follicle stem cells. Microarray analyses of the transcriptional response to catalytically inactive TERT display a rapid change in gene expression similar to that of natural hair follicle cycling in mice, suggesting that TERT displays similar control over progenitor cells as Myc and Wnt (Choi et al., 2008a). Likewise, TERT can regulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) through a feed forward loop, promoting factors such as tumor necrosis factor α (TNFα), which is responsible for pro-inflammatory responses (Ghosh et al., 2012; Yin et al., 2000). Such influences over transcriptional regulation may promote or enhance the pathways required for oncogenesis.

Lifestyle Factors Influencing Telomere Dynamics

Telomeres are essentially a mitotic clock for aging, and aging is the single greatest contributing factor to telomere erosion. With each cellular division telomere length shortens in normal somatic cells until they enter a state of replicative senescence. As cells reach senescence, tissue function declines, contributing to the physical manifestation of aging (Aubert and Lansdorp, 2008). Importantly, the *rate* at which telomeres shorten can provide an informative marker of aging and age-related degenerative pathologies. Although telomeres are thought to be stable structures, there are several physical, environmental, and psychological factors that influence telomere dynamics, and therefore telomerase activity.

A prominent theme across the scientific community is that in addition to a reduced risk for cardiovascular disease, individuals that lead physically active lifestyles typically display a positive correlation with increased telomere length (Cherkas et al., 2008; Mora et al., 2006). Conversely, sedentary lifestyles, high body mass index (BMI), and obesity are negatively correlated with

telomere length (Cherkas et al., 2008; Rode et al., 2014). Moreover, nutrition plays a critical role in telomere maintenance and telomerase activity. A high concentration of vitamin B9 (folate) in plasma, commonly found in beans, lentils and spinach, is positively correlated with telomere length. Folate is involved in nucleotide synthesis and potentially influences DNA methylation patterns regulating telomere length (Paul et al., 2009). Deficiencies in folate also lead to improper incorporation of uracil into DNA (Blount et al., 1997). Uracil incorporation at telomeres may cause inappropriate end-capping and therefore chromosome fusions and genomic instability. Similarly, nicotinamide found in chicken and tuna, as well as zinc, are shown to reduce telomere attrition, as well as increase telomerase activity and replicative capacity *in vitro* (Kang et al., 2006; Paul, 2011; Poiesz et al., 1974). A balanced diet and adequate exercise can slow the rate of telomere attrition, and therefore aging, further preserving tissue function.

While physical activity and nutrition can modulate telomere length maintenance, there are many environmental influences on telomere maintenance as well. A prominent negative factor is cigarette smoking or second hand smoke. Cigarette smoke can significantly decrease telomere length in chronic smokers in a dose-dependent manner (e.g. pack-years) (Morlá et al., 2006). When combined with obesity, there is a cumulative effect leading to drastically shorter telomeres than healthy non-smokers or each covariate separately (Valdes et al., 2005). Both obesity and chronic cigarette smoking lead to accelerated aging and reduced tissue function.

In 2013, a meta-analysis examined how environmental and occupational exposures impact telomere length. Of those evaluated, some of the most common chronic environmental exposures including traffic related air pollution, polycyclic aromatic hydrocarbons (PAHs) which are released during high temperature cooking (e.g. grilling), pesticides, and lead have been shown to increase the rate of telomere erosion (Zhang et al., 2013). Not surprisingly, many of these

exposures have been associated with a variety of diseases. Chronic, high exposures to PAHs can cause a 20-40% increase in risk for fatal ischemic heart disease (Burstyn et al., 2005). A recent report associated with the Agricultural Health Study evaluated >57,000 pesticide applicators and the incidence of lung cancer. Although further study is necessary, this report provided evidence for the association between increased lung cancer risk and exposure to specific pesticides (Bonner et al., 2017).

As with physical and environmental influences, psychological stress can also negatively influence telomere length and telomerase activity by modulating the rate of cellular aging. One of the earliest reports providing mechanistic evidence for telomere shortening due to life stress examined healthy premenopausal women with various stages of stress. Results indicated that women with high perceived stress and/or chronic stress displayed lower levels of telomerase activity and increased oxidative stress in peripheral blood mononuclear cells (PBMC), both of which lead to increased rates of telomere attrition and cellular senescence (Epel et al., 2004). Additionally, a study evaluating telomere length in 3,000 individuals from southern Germany suggest that posttraumatic stress disorder (PTSD) increased the rate of telomere erosion. Interestingly, this same study did not find any correlation between depression and telomere length (Ladwig et al., 2013). Perceived stresses, such as temperament, and chronic stresses, such as chronic childhood psychological stress and severe psychological trauma, have all been associated with increased telomere erosion. While these stresses have been correlated with shorter telomeres, it is important to keep in mind that they are also associated with altered lifestyle and behavioral influences, which may further influence telomere dynamics and the progression of cardiovascular disease and poor immune function (Lin et al., 2012).

Ionizing Radiation and Telomere Dynamics

Ionizing radiation (IR), by definition, is any electromagnetic (e.g. X and γ-rays) or particulate (e.g. α-particles, protons, and high-energy and atomic number (HZE) particles) possessing enough energy to remove electrons from atoms they encounter. This energy transfer is referred to as linear energy transfer (LET) and is described as the amount of energy deposited by a particle or wave per unit length of track. Photon ionizing events, such as X- and γ-rays, classically interact with water molecules within a cell producing hydroxyl radicals or reactive oxygen species (ROS). ROS in close proximity to DNA produce single-stranded (ss) or double-stranded breaks (DSBs) (Lieber, 2010). Therefore, photons indirectly create DNA damage, and are considered low LET events. Particles, however, interact directly with DNA producing complex, clustered DNA damage (Hada and Georgakilas, 2008), and for the most part are high LET radiations.

Like chemical exposures, ionizing radiation can modulate telomerase activity as well as alter telomere dynamics. Most IR exposures occur from natural background sources such as the sun, galactic cosmic radiation (GCR) or radioactive elements in the soil such as uranium or plutonium. Although in this context, individuals receive relatively low doses, along the order of 3-4 mSv per year in the United States (NRC, 2015), accidental, occupational or medical exposures account for large chronic or acute doses of localized or whole body irradiations. With a reduced repair capacity, telomeres are particularly susceptible to oxidative stress, and therefore damage caused by these ionizing events.

Depending on the dose (high or low) and dose-rate (acute or chronic), the influence of IR exposure on telomere dynamics seems to vary, as radiation has been shown to both increase and decrease telomere length. According to a study by Reste and colleagues, retrospective analysis of Chernobyl nuclear power plant clean-up workers found that individuals exposed to higher levels

of radiation typically displayed longer telomeres, but also had a higher incidence of cancer and degenerative diseases. Most likely this correlation is due to an activation of telomerase in response to DNA damage leading to poor regulation of the enzyme and ultimately cancer (Reste et al., 2014). In a similar fashion, whole human blood exposed to a low dose rate (LDR) of 1 Gy cesium-137 (^{137}Cs) prior to stimulating in culture was shown to elongate telomeres 2 days post IR. However, when exposed to 1 Gy acute ^{137}Cs, telomere length decreased at 2 and 5 days post-irradiation (Sishc et al., 2015).

Until recently, little was appreciated and even less known about mechanisms associated with telomere dynamics and radiation exposure. One of the most common lesions formed by ionizing radiation are 8-oxoguanine (8-oxoG) damaged bases. Due to a low redox potential, guanine is the most susceptible base to oxidation by free radicals or ROS (Aguiar et al., 2013; Singh et al., 2011). Telomeres are especially susceptible to damage by IR due to their high guanine content. The presence of 8-oxoG sites destabilizes the formation of G-quadruplex structures, which are believed to sequester and foster the 3'-end of telomeres, however this is dependent on the location of the lesion (Vorlícková et al., 2012). In the absence of 8-oxoG sites, partial resolution of the G-quadruplex structure is necessary for telomerase to access the 3'-end during telomere elongation (Moye et al., 2015).

Of importance, the presence of 8-oxoG sites or 8-oxo-guanine triphosphates (8-oxo-GTP) in the nucleotide pool can modify telomerase activity. In 2016, Fouquerel and colleagues noticed that during telomere elongation, if telomerase attempted incorporation of 8-oxo-GTP into the telomere sequence, elongation was aborted. The premature termination of telomere maintenance due to the presence of oxidized guanine can accelerate the rate of telomere erosion. In contrast, if 8-oxoG bases are present in the sequence prior to telomere elongation, telomerase no longer needs

to resolve or partially resolve G-quadruplex structures, and so can more readily access and elongate telomeres (Fouquerel et al., 2016). The dual nature of oxidized guanines in the context of telomerase may explain why reports of IR exposure vary based on radiation quality and dose rate.

Telomeres, Telomerase and Disease

Telomeres and telomerase have been implicated in a host of diseases ranging from cardiovascular disease and reduced immune function due to chronic stress, to psychological disorders and cancer. These relationships further support the role of telomere maintenance as an overall biomarker of health. That is, the rate at which telomeres lengthen or shorten may be prognostic of an early disease state.

Cardiovascular disease (CVD) accounts for 1 in 4 deaths each year in the United States, and includes a range of conditions involving structural abnormalities of the heart, poorly functioning vessels and blood clotting (CDC, 2015a). It is worth noting that men have a higher mortality rate from CVD than women, suggesting that there may be a connection between gender and CVD. Indeed, estrogen has been shown to have protective effects on tissue function. In addition to reducing LDL cholesterol and increasing HDL cholesterol, estrogen is a vasodilator that also reduces smooth cell proliferation (Chen et al., 1996; Lieberman, 1994; Serrano and Andrés, 2004). Additionally, estrogen can influence telomerase regulation. Estrogen receptor (ER) positive cells treated with 17β-estradiol, a selective agonist of the ER, display an up-regulation of TERT mRNA and an increase in telomerase activity (Kyo et al., 1999). This may provide a potential explanation for the decreased CVD mortality rate in women. However, aging, accelerated telomere erosion, and decreased telomerase activity in endothelial and smooth muscle cells can

lead to senescence-associated atherosclerotic lesions (Minamino et al., 2002; Yeh and Wang, 2016), which may weaken or rupture arterial walls.

Similarly, accelerated telomere erosion has been associated with reduced immune function, which is commonly influenced by chronic stress. One such report examined depressive symptoms, PBMC function, and telomere length in caregivers of Alzheimer's patients under chronic stress. Compared to age- and sex-matched controls, these individuals displayed significantly shorter telomeres, reduced T-cell proliferation *in vitro*, and an increase in inflammatory cytokines. Although these caregivers displayed an increase in telomerase activity compared to controls, telomerase levels were not sufficient to limit telomere erosion (Damjanovic et al., 2007). Conversely, chronic stress is also associated with increased cortisol levels (Schulz et al., 1998), and elevated cortisol levels have been shown to reduce telomerase activity in CD4 and CD8 T-cells (Choi et al., 2008b).

Interestingly, schizophrenia, a mental disorder associated with the inability to properly perceive, think or act, leads to the early onset of cardiovascular disease and premature aging. In cases of schizophrenia, individuals have been shown to have accelerated rates of telomere shortening. Galletly and colleagues pursued a case-control study evaluating male subjects between the ages of 25-35 years and noted significantly shorter telomeres in schizophrenic patients compared to controls (Galletly et al., 2017). As with most diseases associated with decreased telomere length, this is thought to be associated with oxidative stress (Bitanihirwe and Woo, 2011). Furthermore, it is believed that protein kinase B is downregulated and glycogen synthase kinase 3 beta expression is increased leading to a decrease in β-catenin and ultimately a decrease in TERT transcription in schizophrenic patients (Polho et al., 2015).

In addition to schizophrenia, telomere maintenance is likewise implicated in Alzheimer's disease. In recent years, Alzheimer's disease has been likened to an aberrant pro-inflammatory response. Chronically activated microglia in response to amyloid beta (Aβ) peptide, a component of amyloid plaque, elicits a large cytokine, chemokine and macrophage inflammatory protein release resulting in a chronic inflammatory environment (Akiyama et al., 2000). Chronic cytokine exposure can modulate telomerase activity (Akiyama et al., 2002), and therefore influence telomere length. Additionally, several reports have correlated shortened telomere length in white blood cells (Hochstrasser et al., 2012; Panossian et al., 2003), as well as significantly longer telomeres in hippocampal cells, in Alzheimer's patients (Thomas et al., 2008).

In contrast to most diseases, telomere maintenance in cancer is rather diverse. Depending on cancer type, telomeres have been reported to vary in length. For instance, shorter telomeres have been associated with head and neck, bladder, lung and renal cell cancers (Wu et al., 2003), while osteosarcomas have been associated with heterogeneous telomere length presumably due to ALT telomere maintenance (Ulaner et al., 2003). Interestingly, both long and short telomeres in peripheral blood cells have been implicated in breast cancer risk and survival (Shen et al., 2007; Svenson et al., 2008). Although telomerase has been implicated in a host of non-canonical roles, telomere maintenance is its principal function in carcinogenesis through reactivation (Parkinson et al., 1997; Shay and Wright, 1996). To avoid cellular senescence, cancer cells must acquire several oncogenic hallmarks for tumor development, including immortalization (Hanahan and Weinberg, 2000, 2011). To achieve immortalization and therefore avoid senescence, oncogenic cells must, at a minimum, maintain telomere length above a critically short length or point where crisis occurs.

While most somatic cells downregulate telomerase transcription in embryogenesis during asynchronous stem cell division, neoplastic cells must overcome this obstacle. Carcinogenesis requires critical driver mutations that disrupt regulation of cell cycle control and proliferation. Loss-of-function mutations commonly occur in tumor suppressor genes, such as BRCA1, BRCA2, and retinoblastoma (RB), or gain-of-function mutations in proto-oncogenes such as genes in the Ras family of GTPases or Raf serine/threonine protein kinases. However, the most reported gene mutation in cancer cells is TP53 (Kamb et al., 1994). The p53 protein is a tumor suppressor responsible for triggering cell cycle arrest, to allow for DNA repair, and/or apoptosis due to DNA damage and cellular stress. Loss of function mutations or complete loss of p53 causes aberrant checkpoint control and ultimately uncontrollable cell division and evasion of programmed cell death, or apoptosis (Rivlin et al., 2011). Uncontrolled cellular division leads to critically short telomeres and mutations through breakage-fusion-bridge cycles, further propagating genomic instability (Lo et al., 2002). The loss of telomerase repression in cells no longer capable of cell cycle control rescues cells from the crisis phase of telomere attrition and ultimately gives rise to cellular immortality.

DNA Damage, Repair and Genomic Instability

Sources of DNA Damage

The human genome is under constant stress from both endogenous and exogenous sources of DNA damage. It is estimated that over 20,000 DNA lesions occur per cell per day in normal functioning cells from endogenous sources alone (Preston et al., 2010). These lesions form through hydrolysis reactions, oxidative stress, and improper methylation. Inappropriate repair of these

lesions can lead to mismatched bases, polymerase fork stalling, and replication errors or aborted replication. The most prominent source of endogenous DNA damage comes from hydrolysis reactions, or the cleavage of the glycosidic bond between DNA bases and deoxyribose by a water molecule (De Bont and van Larebeke, 2004). These include depurination, or the removal of guanine or adenine from the DNA sugar backbone and depyrimidination, which is the removal of a cytosine or thymine. These processes lead to the formation of abasic sites, also known as apurinic/apyrimidinic sites (AP sites), which are estimated to cause 10,000 lesions per cell per day (Lindahl, 1993). If not repaired, AP sites can prevent DNA replication and transcription (Boiteux and Guillet, 2004).

In addition to hydrolysis of DNA, oxidation is responsible for a considerable amount of DNA damage. While endogenous sources of oxidative stress are commonly created during metabolic processes, the nuclear compartmentalization of DNA minimizes exposure to reactive oxygen species (Chen et al., 2006; Jones and Go, 2010). Nonetheless, free radicals produce an estimated 3,000 lesions per cell per day (Preston et al., 2010). On the contrary, exogenous or environmental sources of free radicals are damaging to DNA regardless of compartmentalization. The formation of free radicals can be influenced by chemical agents commonly found in air pollution and smoking, as well as UV and ionizing radiations. For example smoking has been shown to induce both mitochondrial and nuclear DNA damage, but reports have also suggested that this damage in male germline cells could lead to an increase in offspring childhood cancers and infertility (Aitken and Krausz, 2001; Mak et al., 2000; Sun et al., 1997). Moreover, a study evaluating the influence of traffic related air pollution on Taiwanese traffic controllers noted a positive correlation between particulate matter (PM) and polycyclic aromatic hydrocarbons (PAHs) and DNA damage. Huang and colleagues sampled ambient PM and PAH levels and

monitored DNA strand breaks through the comet assay and 8-oxoG levels from urine in 91 traffic controllers and 53 indoor office workers. They concluded that there was a strong statistical correlation between PM/PAH levels, 8-oxoG and DNA strand breaks suggesting that air pollution does in fact induce oxidative DNA damage (Huang et al., 2012).

In addition to producing oxidative stress, ultraviolet (UV) radiation penetrating the earth's atmosphere produces highly mutagenic cyclobutane-pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs) (Mouret et al., 2006; Sinha and Häder, 2002). UV-A and UV-B interacts with the carbon-carbon double bonds in pyrimidines coupling the two bases together forming CPDs (Mouret et al., 2006; Setlow, 1966). On the other hand, 6-4PPs are formed by linking carbon 6 of one pyrimidine to carbon 4 of the adjacent pyrimidine (Rubin, 1998). Even though 6-4 photoproducts occur less frequently than CPDs, they are considered more mutagenic (Yokoyama and Mizutani, 2014). That is to say that thymidine-thymidine 6-4PPs cause a thymidine to cytosine transition during DNA replication (LeClerc et al., 1991). Regardless of the specific UV-induced mutation, if the lesions are not properly repaired, DNA strands are distorted and the replication fork can be blocked leading to premature termination of replication. Defects in repair of such lesions, such as the inability to perform nucleotide excision repair on CPDs as seen in xeroderma pigmentosum, leads to extreme UV sensitivity and early onset of skin cancer.

Furthermore, there are many factors that can influence larger structural rearrangements in DNA. For instance, translocations, a specific type of structural variant that occurs between two independent chromosomes, are influenced by a wide range of lifestyle factors. A study from Dr. James Tucker's laboratory evaluated the effects of age and lifestyle factors on the accumulation of translocations. Blood samples from a total of 91 individuals ranging in age from new born to 79 years old were collected and analyzed by chromosome painting. By far, aging was the greatest

contributing factor spontaneous translocations. However, environmental or life decisions such as tobacco use, exposure to asbestos and coal based products, as well as diet drinks and sweeteners likewise contributed to an increase in translocation frequencies (Ramsey et al., 1995).

Radiation-Induced Structural DNA Damage

Low doses of IR can be carcinogenic, while high doses are widely utilized as in cancer therapy regimes to target solid tumors. Ionizing radiation is a very efficient modality for inducing a variety of sub-lethal and lethal DNA damage, including prompt double-strand breaks (DSBs). Estimates suggest that 1 Gy of gamma radiation, the equivalent to 1 joule of energy released per kilogram of matter (J/kg), induces 1,000 base lesions, 1,000 single strand DNA (ssDNA) breaks, and 30-40 double strand DNA breaks (Thompson and Limoli, 2013; Ward, 1998). The majority of base lesions and ssDNA breaks are repaired with high efficiency in mammalian cells, with a half-life ($T_{1/2}$) of 10-11 minutes (Affentranger and Burkart, 1992). Single particle tracks, as well as clustered ionizing events creating two ssDNA breaks in close proximity to one another, are capable of producing DSBs (Freifelder and Trumbo, 1969; Lieber, 2010), lesions that are much more difficult to repair. Misjoining of DSBs can result in structural rearrangements, and indeed chromosome aberration frequencies have been shown to be influenced by radiation exposure in a dose-dependent manner (RERF, 2007a).

There are two main classifications of chromosome aberrations that form as a result of two (or more) IR-induced DSBs, which can be visualized cytogenetically in metaphase, where each chromosome consists of two sister chromatids. First, chromo*some*-type rearrangements form in G_0 or G_1 phase of the cell cycle, so that following replication both sister chromatids are affected. These events include dicentric chromosomes (DCs) and reciprocal translocations, as well as centric

rings, interstitial deletions, and *para*-/*peri*-centric inversions. Second, chroma*tid*-type aberrations form in S or G_2 phase of the cell cycle, after DNA replication, such that only one of the two sister chromatids is affected (Savage, 1976, 1999).

Since these aberrations are visualized post-ligation, they can be classified by the location of the breaks on each chromosome arm. Per Dr. John Savage, interchange events, such as dicentrics and translocations, occur if the breaks are situated on opposite sister chromatids of different chromosomes. *Inter*-arm intrachanges, such as *peri*centric inversions, occur if breaks are on opposite arms of the same chromosome, and *intra*-arm intrachanges like *para*centric inversions, occur if the two breaks are on the same arm of a chromosome (Savage, 1976, 1999; Savage and Tucker, 1996). Importantly, structural rearrangements that lead to the loss of genetic information are usually lethal to the cell. For the most part, these are known as asymmetrical exchanges producing deletion events or dicentric chromosomes with acentric fragments (Savage, 1976). Due to the lethality of asymmetrical exchanges, they are not considered persistent.

Alternatively, symmetrical exchanges such as reciprocal translocations, are persistent with time since there is no net loss of genetic information (Tawn and Whitehouse, 2003; Tucker et al., 2005). Translocations have been noted to decrease in human peripheral blood within the first two years post-exposure before stabilizing in the population, likely due to the lingering presence of deleterious structural rearrangements in the cell, such as dicentric chromosomes (Lindholm et al., 1998). Potential drawbacks to the use of translocations for measurement of biological response to radiation exposure (biodosimetry) are lifestyle factors such as aging, tobacco use, and diet drinks and sweeteners, which can cause an increase in translocation frequency (Ramsey, 1995). Like translocations, inversions are a form of symmetrical exchange. Inversions were first identified in drosophila while studying linkage and crossover events (Sturtevant, 1921). These spontaneous

inversions have been proposed as a mechanism for reorganizing the genome and suppression of recombination (Kirkpatrick, 2010; Kirkpatrick and Barton, 2006). However, inversions are also induced by exposure to ionizing radiation.

IR-induced inversions can form either as the result of one DSB on each side of the centromere, creating a *peri*centric inversion, or by two DSBs occurring on a single chromosome arm, but not encompassing the centromere, creating a *para*centric inversion (Kaiser, 1984). The DNA between the two breaks is reinserted in a reverse orientation to maintain a 5' to 3' polarity (Kirkpatrick, 2010; Ray et al., 2013). Although both pericentric and paracentric inversions are induced by IR exposure, reports of their frequencies vary (Dutrillaux et al., 1986; Pleshkova and Plevako, 1982). This is likely due in part, to the resolution limits of standard cytogenetic approaches to inversion detection. However, radiation quality, that is, low versus high LET radiation, can also influence inversion induction. Human cells exposed to high LET particles, such as 600 MeV ^{56}Fe and 74 MeV ^{16}O *in vitro,* displayed a significantly higher inversion rate per unit dose compared to cells exposed to ^{137}Cs gamma rays (Ray et al., 2014). This phenomenon is most likely explained by clustered damage produced by high LET ionizing radiation (Lomax et al., 2013; Ray et al., 2014). In addition to the DSBs produced by high LET radiation, failed repair of clustered damage sites may further increase the inversion frequency (Sutherland et al., 2001). Interestingly, both high and low LET radiations have been shown to produce inversions more frequently per unit dose than translocations (Pleshkova and Plevako, 1982; Ray et al., 2014), presumably because the damage sites are in closer proximity to each other than those responsible for inter-chromosomal exchanges, like reciprocal translocations.

DNA Damage Repair Mechanisms for Structural Rearrangements

Mammalian cells have multiple DNA damage repair (DDR) mechanisms to preserve genomic stability and ensure cellular survival. The two main DSB repair pathways are homologous recombination and non-homologous end joining. Homologous recombination (HR) requires a template strand of DNA to facilitate homology mediated repair of DSBs. Briefly, the DSB is first identified by ataxia telangiectasia mutated (ATM), which phosphorylates histone H2AX (γH2AX) to signal and recruit repair proteins. The break is then resected by the MRE11-RAD50-NBS1 (MRN) complex followed by strand invasion of the template DNA, facilitated by RAD51. DNA polymerases, Pol μ and Pol λ, extend the broken DNA strands, and the double Holliday junctions are resolved (Mazón et al., 2010; Takata et al., 1998; Wyman et al., 2004).

Non-homologous end joining (NHEJ), the primary DSB repair pathway in mammalian cells, is considered to be homology independent, although microhomology (1-2 bp) is occasionally observed (Pannunzio et al., 2014). During NHEJ, donut-shaped Ku70/80 dimers recognize and slide onto each of the two ends of a DSB, which recruits the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). Once bound to the DNA, DNA-PKcs autophosphorylates, activating Artemis, the nuclease responsible for processing the DNA ends of a break site. The 5' and 3' DNA overhangs are cleaved to create blunt ends. The DNA strands are ultimately ligated together by the XRCC4-DNA ligase IV complex (Burma et al., 2006; Hefferin and Tomkinson, 2005; Lieber, 2010). What determines pathway choice for repair of DSBs, i.e., HR vs. NHEJ, is an area of active investigation.

What is known, is that the decision to use one mechanism over the other appears to be heavily cell cycle dependent. Homologous recombination is preferred in S and G2 phase of the cell cycle due to the presence of a template strand of DNA provided by the sister chromatid

(Brandsma and van Gent, 2012). Additionally, for efficient resection by the MRN complex, NBS1 requires phosphorylation by cyclin dependent kinase 1 (CDK1) / cyclin B, which are only present in S and G2 phase (Branzei and Foiani, 2008; Falck et al., 2012). Non-homologous end joining, however, is prominently favored in G_0 and G_1 phase, but may still function in G2 and S (Branzei and Foiani, 2008; Mao et al., 2008). Since there is most often not a template strand present in G1, there is little use for homology mediated repair. Thus, even though HR mediated repair has high fidelity, it is limited by the cell cycle. On the contrary, NHEJ is considered more error prone, and aberrant repair of DSBs, especially those induced by IR, can result in structural rearrangements/chromosome aberrations (Simsek and Jasin, 2010).

Radiation Biodosimetry

Early Exposure Biodosimetric Assessment

Radiation biodosimetry is the measurement of biological markers in human or mammalian tissues as a signature of IR exposure for dose reconstruction (NCRP, 2009). More often than not, obtaining physical dose estimates is challenging or impossible after an exposure event; e.g., A-bomb survivors. In the case of occupational exposures, external dosimeters such as film-badges or thermoluminescent dosimeters (TLDs), may vary in accuracy (Simon et al., 2010; Suntharalingam and Cameron, 1966). Biodosimetry is important for determining an individual's exposure in order to accurately assess need of short-term care to address acute radiation syndrome, as well as potential long-term effects, such as cancer.

Historically, IR-induced dicentric chromosomes and micronuclei (MN) have been the "gold standard" for early exposure biodosimetric assessments (Rothkamm et al., 2013; Vaurijoux

et al., 2012). Micronuclei are extra-nuclear, miss-segregated whole chromosomes or chromosome fragments (e.g. acentric fragments) commonly associated with DC formation (Fenech et al., 2011). A key component to a quality biodosimetry assay is a low limit of radiation detection. Depending on the radiation source, the lower limit of detection for DC and MN assays are reported to range from 10-20 mGy for neutrons to 100 mGy for γ-rays (Lloyd, 1997).

However, for retrospective assessment of exposures that took place in the past, such as occupational exposures that go undetected, DC and MN assays suffer a serious shortcoming; they are not persistent with time (Tucker et al., 2005). According to a study evaluating accidental radiation exposure to industrial radiographers, cytogenetic assessment of dicentric chromosomes in PBMCs suggested a half-life of 1 year *in vivo* (Cho et al., 2015). Consistent with DCs, micronuclei have been reported to possess a similar half-life (Vral et al., 2011). Therefore, for the purpose of biodosimetry, estimates are only considered accurate up to one year post-exposure (Lloyd et al., 1998). However, if a half-life correction factor is utilized, it may be possible to have accurate assessments up to 3-4 years post-exposure (Beaton-Green et al., 2016; Lloyd et al., 1980). Utilizing the half-life correction, dose estimations for a single individual accidentally exposed to gamma-rays four years prior, provided comparable estimations to more advanced techniques (Beaton-Green et al., 2016).

Similarly, γH2AX, a marker of DSBs, has been explored as a potential early exposure biodosimetry method. Rapid protocols have been developed to process large numbers of samples for mass exposure events, requiring only a small amount (e.g. > 1.0 mL) of blood (Moquet et al., 2014). However, most DSBs are repaired within 4 hours after exposure (Horn et al., 2011), and at 24 hours post exposure (1.8 Gy cobalt-60), γH2AX foci in whole blood samples were shown to decrease by 85% (Moroni et al., 2013).

Importantly, dose estimates determined with these assays were shown to vary considerably between multiple laboratories. Blood samples irradiated with 10 increasing doses of X-rays were analyzed by 11 independent institutions using the dicentric chromosome assay, cytokinesis-blocking micronucleus assay, and the γH2AX assay. Analysis of their results exhibited a 2.5–4-fold difference in accuracy from the true doses between laboratories (Rothkamm et al., 2013). Additionally, in cases where exposures occurred several years ago, these assays were no longer capable of producing accurate results.

Assessment of Stable Aberrations for Retrospective Biodosimetry

For past IR exposures, stable and persistent chromosome aberrations are necessary for biodosimetry estimates. An important aspect of cytogenetic-based biodosimetry is that normal chromosomes have a reproducible and identifiable morphological appearance. During the late 1960's, while studying chromosome differentiation in *Vicia faba* (the broad bean), Dr. Torbjörn Caspersson noted that metaphase chromosomes stained with quinacrine mustard produced a banding pattern (Caspersson et al., 1969). This discovery lead to development of techniques such as Q- and G-banding, which stain chromosomes based on adenine and thymine (AT)-rich and guanine and cytosine (GC)-rich DNA regions, giving a "banded" appearance (Schreck and Distèche, 2001). These techniques allow for ready identification of persistent, or long-lived, symmetrical rearrangements, and is especially useful for retrospective biodosimetry.

One of the most common chromosome aberrations used for retrospective biodosimetry are reciprocal translocations. Non-reciprocal translocations are typically not taken into consideration for retrospective biodosimetry because they are not stable with time (Gregoire et al., 2006; Rodríguez et al., 2004). While quinacrine banding (Q-banding) was the first developed banding

technique, Giemsa banding or G-banding (Seabright, 1971) is the most commonly used banding technique for detecting translocations after radiation exposure. Retrospective analysis of Hiroshima atomic bomb (A-bomb) survivors analyzed reciprocal translocations, in addition to insertions, complex exchanges, and inversions, noted that not only did translocation rates increase with dose, but they were the most predominantly identified rearrangement of those observed (Ohtaki, 1992). The most likely reason for translocations being the most identified rearrangement via G-banding is due to the limit of resolution for banding techniques. Subtle rearrangements, such as small inversions or insertions, often go unnoticed by banding since the limit of detection is 5-10 Mb (Riegel, 2014; Shaffer and Bejjani, 2004). Through the advent of fluorescent methods such as spectral karyotyping (SKY) or multicolor-FISH (mFISH), resolution has been greatly increased, but still, the detection of *intra*-chromosomal rearrangements (e.g. insertions, deletions and inversions) is limited, if impossible (Imataka and Arisaka, 2012).

More recently, a methodology termed Directional Genomic Hybridization (dGH) was developed, which combines the strand specificity of chromosome orientation fluorescence *in situ* hybridization (CO-FISH) with sophisticated bioinformatics-driven probe design for the detection of structural rearrangements, including inversions (Ray et al., 2013; Robinson et al., Submitted). Notably, dGH based oligonucleotides are strand- and orientation-specific, so they only bind to a single sister chromatid at a precise location. Based on these design parameters, dGH can detect translocations, insertions and deletions, but the power of the technology lies in its ability to reliably detect inversions simply by signal "switching" between sister chromatids (Ray et al., 2013, 2014). In the context of retrospective biodosimetry, dGH can detect most, if not all, structural rearrangements with high accuracy, and it opens a door for exploring inversions as a biomarker of radiation exposure.

Interstitial deletions are the most common radiation induced mutation (National Research Council, 2006), however inversions are alternative products of the same recombinational process. By unifilar incorporation of 5-bromo-2'-deoxyuridine (BrdU) into metaphase chromosomes exposed to X or γ-rays, Mühlmann-Díaz and Bedford concluded that in fact inversions and interstitial deletions occur at a ~1:1 ratio (Mühlmann-Díaz and Bedford, 1995). This suggests that inversions should be induced at a greater rate per unit dose than translocations. Indeed, dGH analysis of human cell lines exposed to increasing doses of IR does suggest that inversions increase at a greater rate per unit dose than translocations and further, that HZE particles are more efficient at inducing inversions than photons (Figure 1.1) (Ray et al., 2014). However, in contrast, inversions also appear to have a higher spontaneous background rate (Ray et al., 2014). Though little is known about covariate influence (e.g. age and smoking) on inversion frequency, inversions may still be a more informative biological marker of radiation exposure due to their high induction rate.

Role of Telomeres in Retrospective Biodosimetry

Owing to their reduced repair capacity, telomeres have been proposed as biomarkers of radiosensitivity. Several reports suggest that short telomeres tend to be highly sensitive to IR exposure (Goytisolo et al., 2000; Wong et al., 2000). According to a study in the Journal of Experimental Medicine, fifth generation telomerase null mice possessing telomeres 40% shorter than wild-type mice appear to be hypersensitive to IR exposure, with over half of the sample population dying from acute radiation toxicity (Goytisolo et al., 2000). Importantly, ionizing radiation may also alter telomere maintenance, further influencing genomic stability (Ayouaz et al., 2008; Wong et al., 2000).

Moreover, telomeres may also be utilized to help identify terminal rearrangements for the purpose of biodosimetry. Telomeres have a rich history with the CO-FISH application. They have been utilized to monitor elevated sister chromatid exchange (SCE) rates, which are indicative of increased rates of mitotic recombination at chromosome termini (Cornforth and Eberle, 2001). Additionally, telomere CO-FISH has been used to monitor recombination of telomeric DNA (T-SCE) (Bailey et al., 2004), as well as leading and lagging strand synthesis and post replicative processing of mammalian telomeres (Bailey et al., 2001). Recently, we proposed combining dGH methodology with telomere CO-FISH (Telo-dGH) to distinguish terminal rearrangements, specifically terminal inversions and sister chromatid exchange (McKenna and Bailey, 2017; McKenna et al., 2017). By combining the two methodologies we can further improve the resolution of Directional Genomic Hybridization for biodosimetry.

Sister chromatid exchange (SCE) occur during DNA replication when ssDNA breaks are encountered. This leads to fork stalling and collapse followed by homology mediated recombination repair, which is responsible for crossover events and template switching (Wilson III and Thompson, 2007). Even though inversions and SCE form by two independent mechanisms, terminal breakpoints in or near the telomere render them indistinguishable from each other by dGH without the telomere probe. On a normal chromosome, devoid of rearrangements, the telomere probe binds in a *trans*-configuration on opposite sister chromatids. However, if a SCE is present, the telomere signals appear on the same sister chromatid producing a *cis*-configuration (Cornforth and Eberle, 2001). In the instance of a terminal inversion, the telomere probes will remain in the *trans*-configuration, but the dGH chromatid paint will "switch" to the opposite sister chromatid (McKenna and Bailey, 2017; McKenna et al., 2017). Figure 1.2 illustrates the various signal patterns identified by Telo-dGH. Overall, this methodology will improve resolution for

retrospective biodosimetry, and allow for the simultaneous evaluation of multiple stable symmetrical exchanges.

Biological Influence of Extreme Environments

Astronauts Aboard the International Space Station

Space travel is on the forefront of future endeavors as the world prepares for long-duration missions (>1 year) aboard the International Space Station (ISS), as well as the prospect of deep space travel to Mars and beyond. Though possible, mammalian physiology and biology is poorly equipped to deal with such extreme environments. To ensure success, it is vital to better understand the implications of space travel for human health, and identify potential mitigation strategies.

The ISS orbits at an altitude of 220 miles above the earth's surface, traveling at speeds upwards of 17,500 miles per hour (Wall, 2015). At this altitude, astronauts experience a microgravity environment with less shielding from IRs than at the earth's surface. The space radiation environment is composed of protons, photons, heavy ions and secondary neutrons, originating from the sun (e.g. solar events) or across the galaxy, collectively known as galactic cosmic radiation (GCR). These forms of radiation exposure, independent of microgravity, can have profound implications on DNA and tissue damage, even at low fluence. Physical and biological dose assessments for a 6 month mission estimate an average exposure of 81-85 mGy (Cucinotta et al., 2008). One of the most studied phenomena due to space radiation is the early appearance and increased incidence of cataracts in astronauts. Astronauts have long reported seeing flashes of light when they close their eyes, which is caused by particle tracks passing through their eyes (Pinsky

et al., 1974). Epidemiological studies suggest that even low doses of IR (~8 mSv) to the lens of the eye are sufficient to cause cataracts (Chylack et al., 2009; Cucinotta et al., 2001).

Microgravity further complicates spaceflight for astronauts. As early as the Apollo missions, studies have reported a decrease in astronaut bone density and demineralization (Mack and Vogt, 1971; Mack et al., 1967). This is, at least in part, caused by reduced mechanical load from the weightless environment. However, improved nutrition and physical exercise regiments have been shown to reduce bone loss during extended missions. In 2008, NASA implemented a new resistance exercise machine and an improved nutrition plan that increased caloric intake and vitamin D supplementation. Astronauts that utilized the improved resistance exercise machine and followed the dietary plan maintained pre-flight body mass and bone density during 4-6 month missions. Additionally, these crew members had a higher percent lean mass and decreased fat mass (Smith et al., 2012).

Microgravity also underlies fluid shifts that astronauts experience during flight. Though fluid shifts have been associated with bone loss (McCarthy, 2005), the primary concern is increased intracranial pressure and ocular refraction. After a single six-month mission, seven astronauts were evaluated for the influence of fluid shifts on physical changes to their eyes. All seven presented with various ophthalmic findings, but the most severe changes included increased ocular nerve fiber thickening and decreased near vision (Mader et al., 2011).

Aside from physiological effects and biodosimetry assessments, little is known about the impact of spaceflight at a molecular or '-omics' level. Most of what we do know has been discovered through animal or cell culture models, and while that is a start, the results may not be directly applicable to humans. For instance, a study examining immune responses in rats, specifically T-cell proliferation and interleukin-2 (IL2) production, suggest that spaceflight has no

influence on the immune system when compared to ground-based controls (Nash et al., 1992). Yet, the few studies that have evaluated immune function in astronauts indicate this may not be the case. Evaluation of 16 astronauts pre- and post-flight identified a decrease in thymopoiesis (Benjamin et al., 2016), as well as a reduction in the phagocytic index of monocytes in 25 astronauts (Kaur et al., 2005) immediately upon return from six-month missions.

Nonetheless, animal and cell models are valuable and can provide interesting insights when human volunteers are not a possibility. The first, and to my knowledge only, account of telomere length dynamics associated with spaceflight utilized *Caenorhabditis elegans*. After a short, 11-day spaceflight, *C. elegans* exhibited an increase in telomere length (Zhao et al., 2006). Though a fascinating finding, this is contradictory to many ground-based studies. Under normal atmospheric and gravitational conditions, radiation exposure (Lustig et al., 2016), oxidative stress (Kawanishi and Oikawa, 2004), and physical and psychological stress (Lin et al., 2012), all of which are experienced by astronauts, have been shown to result in telomere attrition, not elongation.

Taken together, results from human, animal, and cell based models provide adequate data to support the need for further evaluation of the effects of spaceflight on human health. Included in this, is the assessment of telomere length changes, as seen in the C. *elegans*, which has been associated with genomic stability, and a whole host of diseases ranging from cardiovascular disease to cancer. Additionally, since astronauts are exposed to more damaging high energy particles than on the earth's surface, evaluating chromosome inversion rates may be of particular value for biodosimetry and genomic stability.

United States Veterans Exposed to Nuclear Fallout (The Atomic Vets)

Incidental radiation exposure is an unfortunate and unavoidable consequence as society has drifted towards higher energy demands, advanced warfare, and greater use of radiation in diagnostics and medical treatment. During the early 1940's, as World War II (WWII) began and tensions peaked with Japan, the United States and its allies embarked on the Manhattan Project, which was the first nuclear weapons program in the world. Since then, our ability to harness the energy produce by radioactive material has momentously influenced the energy industry and warfare. However, with great advancements, usually come considerable consequences.

In an effort to end WWII, the U.S. released two atomic bombs, one on Hiroshima and one on Nagasaki, resulting in one of the greatest human radiation exposure events in history. Additionally, these events provided an opportunity for epidemiological studies on the effects of nuclear fallout. Those that were not lost by the initial blast, but were close enough to receive a dose of >~1.0 Sievert (Sv) were at risk of acute radiation syndrome (ARS) or radiation toxicity (NRC, 2015). Depending on the magnitude of the dose, symptoms ranged from nausea and vomiting to depletion of hematopoietic and circulating blood cells, and severe gastrointestinal complications (CDC, 2015b). Without proper care (e.g. antibiotics and blood transfusions), survival times ranged from days to months (CDC, 2015b; NRC, 2015).

Of concern for those exposed individuals that did not suffer from ARS, were early-onset cancers, particularly leukemia, lymphoma, and thyroid cancer. The first report of excess leukemia morbidity and mortality associated with the atomic bombs was published in 1952. Comparison of leukemic cases and mortalities in exposed and non-exposed populations of Hiroshima and Nagasaki illustrated a highly significant increase in leukemia morbidity and mortality for those closer than 2,000 meters to the blast, and little evidence of radiation induced leukemias greater

than 2,000 meters (Folley et al., 1952). The Life Span Study, which continues to this day and evaluates the life-long effects of radiation exposure among A-bomb survivors, suggests an average attributable risk of 46% for leukemia with exposures between 0.005 Gy and >2.0 Gy (RERF, 2007b). In addition to leukemia, the first early-onset solid tumor to appear in A-bomb survivors was thyroid cancer (Boice, 2006; Wood et al., 1969).

A multitude of radioactive isotopes are released during a nuclear event, but the radioactive isotope, iodine-131 (^{131}I), is of particular concern for children. Even though thyroid cancer is rarely fatal, ^{131}I is responsible for radiation-induced thyroid cancer (Robbins and Schneider, 2000). The thyroid gland sequesters iodine for the production of thyroid hormones, and is especially crucial during development (Boyages, 1993; Glinoer, 2004). Importantly, the human body cannot distinguish between iodine and the radioactive isotope, so contaminated food and water/milk supplies, as well as inhalation of radioactive particles lead to ^{131}I uptake by the thyroid gland.

Roughly 5-10 years after exposure to the fallout, thyroid cancer in excess of the expected incidence, began appearing in children and adults (Wood et al., 1969). Interestingly, females >20 years of age at the time of exposure appeared to have a higher incidence of thyroid cancer than males. Likewise, proximity, and therefore IR dose, played a significant role in this occurrence. However, those under 20 years old at the time of exposure were the most affected population, but there were no differences in incidence rate between sexes, suggesting that children were equally affected (Wood et al., 1969). An important contributing factor, which became apparent after the atomic bombs and Chernobyl power plant disaster, was the correlation between iodine deficiency and thyroid cancer incidence (Boice, 2006).

According to the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), an estimated 6,848 cases of thyroid cancer emerged between 1991-1995 in children

under the age of 18 years during the Chernobyl accident (UNSCEAR, 2008). Not surprisingly, an evaluation of children >18 years old in the Bryansk region of Russia after the accident reported a 5.5-fold increase in thyroid cancer incidence compared to the national average, with excess relative risk (ERR) increasing with dose (Shakhtarin et al., 2003). However, multiple studies have reported an inverse relationship between thyroid cancer and increased urinary excretion of iodine compared to regions associated with iodine deficiencies, which had a higher incidences of thyroid cancer (Cardis et al., 2005; Shakhtarin et al., 2003).

Radiation-induced cancers are likely the outcome of damaged DNA and subsequent structural/chromosomal rearrangement during repair. For example, molecular analysis of IR-induced papillary thyroid cancer found that the most common rearrangement involved a fusion product between the proto-oncogene, RET, with the receptor genes PTC1 and PTC3 (Nikiforov, 2002; Tronko et al., 2010), substantiating the need for not only biodosimetry, but also molecular assessment of persons post-exposure. To date, numerous biodosimetry and molecular studies have been conducted on both the Chernobyl and A-bomb survivor populations, however many civilian-based studies lack crucial information for accurate dose assessment. Complicating issues for dose reconstruction range from loss of medical records, as was the case for the A-bomb survivors (Folley et al., 1952), to inability to accurately recall location or shielding (e.g. inside a building).

Most of what we currently know about the effects of acute and chronic low-dose radiation on humans revolve around large epidemiological studies of the atomic bomb survivors and Chernobyl population. However, much is still to be learned about low dose and low dose rate effects. As Dr. James Tucker pointed out, epidemiological studies are beneficial for an entire population, however, they do not take into account individual radiosensitivity or risk (Tucker, 2008). Additionally, these study populations are primarily of Slavic or Asian descent, and ethnic

background may influence a population's radiosensitivity, as similarly reported in predisposition to disease states (Manolio et al., 2009; West and Barnett, 2011). Genetics, structural variants present in a population prior to exposure, as well as cultural dietary trends may all alter biodosimetry and radiosensitivity outcomes.

More recently, the National Committee on Radiation Protection and Measurements (NCRP) began the Million Worker Study to evaluate the effects of low dose and low dose effects on United States radiation workers and military personnel. This study includes workers from the Manhattan Project, nuclear utility workers, military veterans (atomic veterans), and medical workers. In particular, the atomic veterans were military personnel accidentally exposed to nuclear fallout during atomic bomb testing during the 1950's. Though few WWII veterans remain today, this unique group of men provide an opportunity to study the effects of acute low dose (< 1.0 Gy) radiation through retrospective biodosimetry (>60 years since exposure), as well as telomere length maintenance. This study will improve our understanding of the genetic effects of low dose IR exposure by evaluating biomarkers of DNA damage misrepair (e.g. inversions and translocations), as well as the influence of IR on telomere length maintenance.

Conclusions

The overall goal of the studies presented here was to evaluate the influence of extreme environments, such as spaceflight and nuclear fallout exposure, on telomere length dynamics and telomerase activity, as well as genomic stability through retrospective biodosimetry. Combined, results will help improve our understanding of the effects of environments not commonly experienced by the general population. Notably, these studies are the first of their kind. We had

the unique opportunity to monitor the influence of spaceflight on monozygotic twin astronauts, Scott and Mark Kelly. While Mark remained on earth, Scott Kelly spent almost a full year in space, which is the longest consecutive time aboard the ISS of any NASA astronaut. Since Mark and Scott are identical twins, the role of genetics was controlled as much as possible and observed changes were specific to spaceflight. Additionally, we have, and are currently, evaluating a cohort of unrelated astronauts and their age matched controls. Results from these individuals allow for conclusions about the role of individual genetics and susceptibilities. In addition to the astronauts, we evaluated atomic veterans exposed to nuclear fallout during A-bomb testing in the 1950's. This was an exceptional opportunity to not only assess a new retrospective biodosimetry approach, but also to determine the long-term (>60 years ago) influence of acute IR exposure events on telomere length dynamics.

We first sought to explore the utility of telomere length maintenance as a biomarker of overall health. Since telomere dynamics are influenced by a host of conditions ranging from radiation exposure, physical and psychological stress, to nutrition and exercise, we anticipated capturing the influence of these extreme environments above normal life events, such that results would shed light on the overall health effects. In the context of astronauts, an increase in telomere length may represent a greater risk for cancer, particularly if due to improper regulation of telomerase, as postulated from results of Chernobyl cleanup workers (Reste et al., 2014). Conversely, shorter telomeres may represent a greater risk for cardiovascular disease (Yeh and Wang, 2016). Additionally, we evaluated the long-term effects of nuclear fallout on telomere length in US Atomic Veterans, which few studies to date have had the opportunity to assess.

These study groups also provided opportunities to evaluate the utility of Directional Genomic Hybridization (dGH) and high resolution inversion detection, as a new approach for

retrospective biodosimetry. The atomic veterans allowed us to evaluate the sensitivity of inversions as a biodosimeter, but also to begin to understand the influence of covariates, such as age and smoking, on inversion rates. Similarly, evaluating the astronauts improved past and current biodosimetry efforts by including inversions as a biomarker of radiation exposure. It's important to appreciate that astronauts are exposed to a significantly different composition of ionizing radiations as compared to the atomic veterans; specifically, astronauts are exposed to more high energy radiations. Therefore, we can begin to compare and contrast the effects of different radiation qualities on human cohorts *in vivo*.

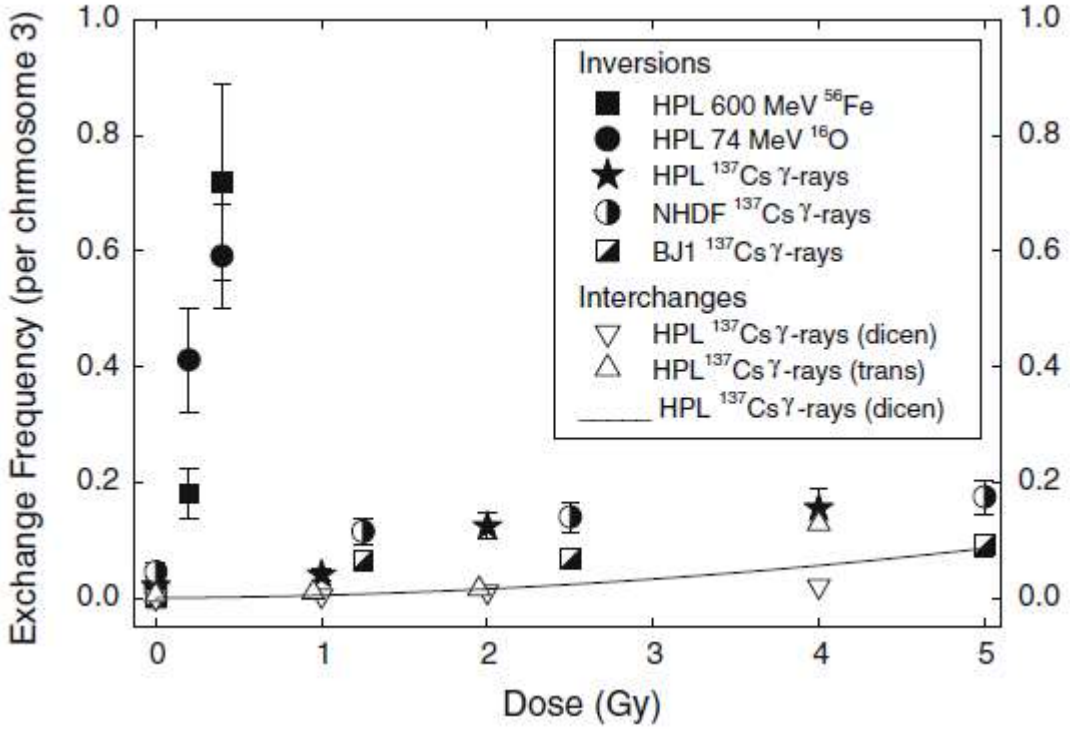

Figure 1.1 dGH analysis of radiation induced structural rearrangements using a chromosome 3 paint. Inversions are induced at a great rate per unit dose of ^{137}Cs γ-ray ionizing radiation than translocations. High atomic number and high energy particles (600 MeV ^{56}Fe and 74 MeV ^{16}O) are more efficient at inducing inversions per unit dose than ^{137}Cs γ-rays (Ray et al., 2014)

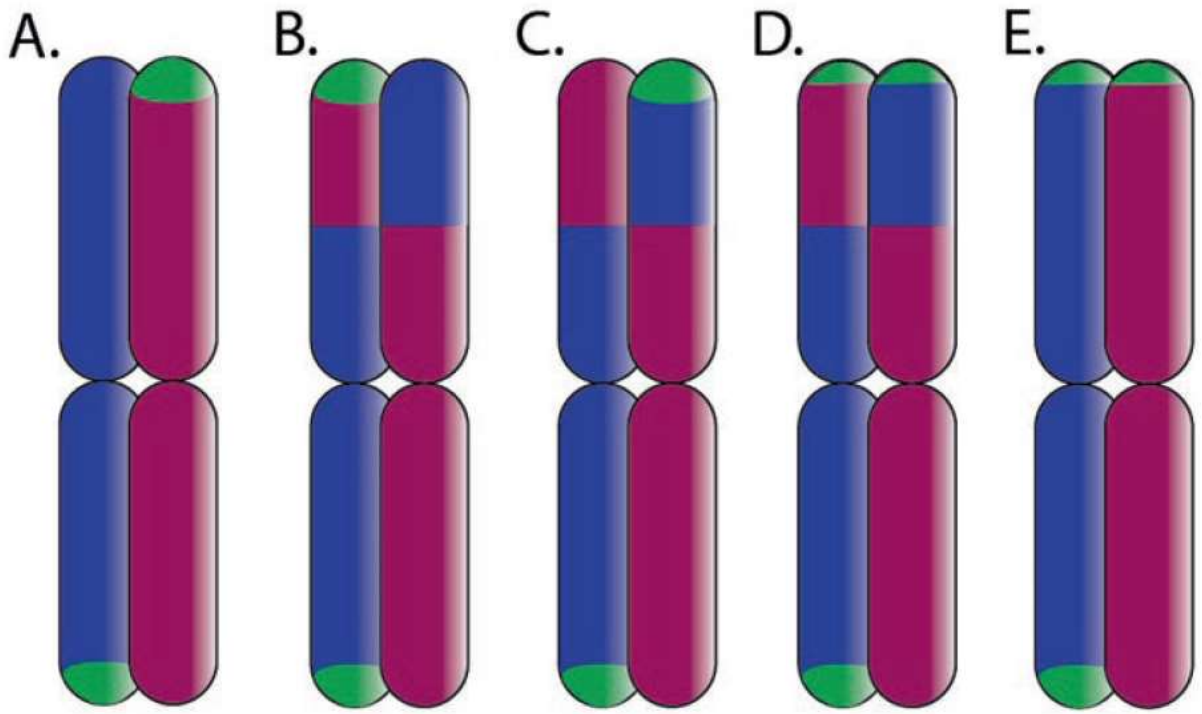

Figure 1.2 Telo-dGH discrimination of SCE vs. terminal inversion. On a normal chromosome (A), the telomere signals are in *trans*-configuration with the dGH paint on a single sister chromatid. If a SCE occurs (B), the telomere signal will appear in *cis*-configuration with a portion of the dGH paint "switching" to the opposite sister chromatid. In the case of an inversion (C and D), the telomere signal will remain in the *trans*-configuration unless the breakpoint occurs in the telomere signal, which splits the signal. The dGH paint will switch to the opposite sister chromatid. If a T-SCE occurs (E), the telomere signal will split between sister chromatids and the dGH paint will remain on one sister chromatid (McKenna et al., 2017).

REFERENCES

Affentranger, M.I., and Burkart, W. (1992). Assessment of radiation-induced DNA strand breaks and their repair in unlabeled cells. Cytometry *13*, 31–38.

Aguiar, P.H.N., Furtado, C., Repolês, B.M., Ribeiro, G.A., Mendes, I.C., Peloso, E.F., Gadelha, F.R., Macedo, A.M., Franco, G.R., Pena, S.D.J., et al. (2013). Oxidative stress and DNA lesions: the role of 8-oxoguanine lesions in Trypanosoma cruzi cell viability. PLoS Negl. Trop. Dis. *7*, e2279.

Aitken, R.J., and Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. Reproduction *122*, 497–506.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G.M., Cooper, N.R., Eikelenboom, P., Emmerling, M., Fiebich, B.L., et al. (2000). Inflammation and Alzheimer's disease. Neurobiol. Aging *21*, 383–421.

Akiyama, M., Hideshima, T., Hayashi, T., Tai, Y.-T., Mitsiades, C.S., Mitsiades, N., Chauhan, D., Richardson, P., Munshi, N.C., and Anderson, K.C. (2002). Cytokines modulate telomerase activity in a human multiple myeloma cell line. Cancer Res. *62*, 3876–3882.

Allsopp, R.C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E.V., Futcher, A.B., Greider, C.W., and Harley, C.B. (1992). Telomere length predicts replicative capacity of human fibroblasts. Proc. Natl. Acad. Sci. U. S. A. *89*, 10114–10118.

Artandi, S.E., and DePinho, R.A. (2010). Telomeres and telomerase in cancer. Carcinogenesis *31*, 9–18.

Atkinson, S.P., Hoare, S.F., Glasspool, R.M., and Keith, W.N. (2005). Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the htr and htert gene promoters. Cancer Res. *65*, 7585–7590.

Aubert, G., and Lansdorp, P.M. (2008). Telomeres and aging. Physiol. Rev. *88*, 557–579.

Ayouaz, A., Raynaud, C., Heride, C., Revaud, D., and Sabatier, L. (2008). Telomeres: Hallmarks of radiosensitivity. Biochimie *90*, 60–72.

Bae, N.S., and Baumann, P. (2007). A rap1/trf2 complex inhibits nonhomologous end-joining at human telomeric dna ends. Mol. Cell *26*, 323–334.

Bailey, S.M., Cornforth, M.N., Kurimasa, A., Chen, D.J., and Goodwin, E.H. (2001). Strand-specific postreplicative processing of mammalian telomeres. Science *293*, 2462–2465.

Bailey, S.M., Brenneman, M.A., and Goodwin, E.H. (2004). Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. Nucleic Acids Res. *32*, 3743–3751.

Beaton-Green, L.A., Barr, T., Ainsbury, E.A., and Wilkins, R.C. (2016). Retrospective biodosimetry of an occupational overexposure—case study. Radiat. Prot. Dosimetry *172*, 254–259.

Benjamin, C.L., Stowe, R.P., St. John, L., Sams, C.F., Mehta, S.K., Crucian, B.E., Pierson, D.L., and Komanduri, K.V. (2016). Decreases in thymopoiesis of astronauts returning from space flight. JCI Insight *1*.

Bilaud, T., Brun, C., Ancelin, K., Koering, C.E., Laroche, T., and Gilson, E. (1997). Telomeric localization of TRF2, a novel human telobox protein. Nat Genet *17*, 236–239.

Bitanihirwe, B.K.Y., and Woo, T.-U.W. (2011). Oxidative stress in schizophrenia: An integrated approach. Neurosci. Biobehav. Rev. *35*, 878–893.

Blount, B.C., Mack, M.M., Wehr, C.M., MacGregor, J.T., Hiatt, R.A., Wang, G., Wickramasinghe, S.N., Everson, R.B., and Ames, B.N. (1997). Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: Implications for cancer and neuronal damage. Proc. Natl. Acad. Sci. U. S. A. *94*, 3290–3295.

Boice, J.D. (2006). Thyroid disease 60 years after Hiroshima and 20 years after Chernobyl. JAMA *295*, 1060–1062.

Boiteux, S., and Guillet, M. (2004). Abasic sites in DNA: repair and biological consequences in Saccharomyces cerevisiae. DNA Repair *3*, 1–12.

Bonner, M.R., Freeman, L.E.B., Hoppin, J.A., Koutros, S., Sandler, D.P., Lynch, C.F., Hines, C.J., Thomas, K., Blair, A., and Alavanja, M.C.R. (2017). Occupational exposure to pesticides and the incidence of lung cancer in the agricultural health study. Environ. Health Perspect. *125*, 544–551.

Boyages, S.C. (1993). Clinical review 49: Iodine deficiency disorders. J. Clin. Endocrinol. Metab. *77*, 587–591.

Brandsma, I., and van Gent, D.C. (2012). Pathway choice in DNA double strand break repair: observations of a balancing act. Genome Integr. *3*, 9.

Branzei, D., and Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. Nat. Rev. Mol. Cell Biol. *9*, 297–308.

Bryan, T.M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R.R. (1995). Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J. *14*, 4240–4248.

Burma, S., Chen, B.P.C., and Chen, D.J. (2006). Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA Repair *5*, 1042–1048.

Burstyn, I., Kromhout, H., Partanen, T., Svane, O., Langård, S., Ahrens, W., Kauppinen, T., Stücker, I., Shaham, J., Heederik, D., et al. (2005). Polycyclic aromatic hydrocarbons and fatal ischemic heart disease. Epidemiol. Camb. Mass *16*, 744–750.

C. Lloyd J. E. Moquet S. Oram A. A. Edwards J. N. Lucas, D. (1998). Accidental intake of tritiated water: a cytogenetic follow-up case on translocation stability and dose reconstruction. Int. J. Radiat. Biol. *73*, 543–547.

Cairney, C.J., and Keith, W.N. (2008). Telomerase redefined: Integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. Biochimie *90*, 13–23.

Cardis, E., Kesminiene, A., Ivanov, V., Malakhova, I., Shibata, Y., Khrouch, V., Drozdovitch, V., Maceika, E., Zvonova, I., Vlassov, O., et al. (2005). Risk of thyroid cancer after exposure to 131I in childhood. J. Natl. Cancer Inst. *97*, 724–732.

Caspersson, T., Zech, L., Modest, E.J., Foley, G.E., Wagh, U., and Simonsson, E. (1969). Chemical differentiation with fluorescent alkylating agents in Vicia faba metaphase chromosomes. Exp. Cell Res. *58*, 128–140.

CDC (2015a). Heart disease facts & statistics. cdc.gov.

CDC (2015b). Acute radiation syndrome: a fact sheet for physicians. cdc.gov.

Cesare, A.J., and Griffith, J.D. (2004). Telomeric dna in alt cells is characterized by free telomeric circles and heterogeneous t-loops. Mol. Cell. Biol. *24*, 9948–9957.

Cesare, A.J., and Reddel, R.R. (2010). Alternative lengthening of telomeres: models, mechanisms and implications. Nat. Rev. Genet. *11*, 12.

Chen, S.-J., Li, H., Durand, J., Oparil, S., and Chen, Y.-F. (1996). Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery. Circulation *93*, 577–584.

Chen, Y., Cai, J., and Jones, D.P. (2006). Mitochondrial thioredoxin in regulation of oxidant-induced cell death. FEBS Lett. *580*, 6596–6602.

Cherkas, L.F., Hunkin, J.L., Kato, B.S., Richards, J.B., Gardner, J.P., Surdulescu, G.L., Kimura, M., Lu, X., Spector, T.D., and Aviv, A. (2008). The association between physical activity in leisure time and leukocyte telomere length. Arch. Intern. Med. *168*, 154–158.

Cho, M.S., Lee, J.K., Bae, K.S., Han, E.-A., Jang, S.J., Ha, W.-H., Lee, S.-S., Barquinero, J.F., and Kim, W.T. (2015). Retrospective biodosimetry using translocation frequency in a stable cell of occupationally exposed to ionizing radiation. J. Radiat. Res. (Tokyo) *56*, 709–716.

Choi, J., Southworth, L.K., Sarin, K.Y., Venteicher, A.S., Ma, W., Chang, W., Cheung, P., Jun, S., Artandi, M.K., Shah, N., et al. (2008a). Tert promotes epithelial proliferation through transcriptional control of a myc- and wnt-related developmental program. PLOS Genet. *4*, e10.

Choi, J., Fauce, S.R., and Effros, R.B. (2008b). Reduced telomerase activity in human T lymphocytes exposed to cortisol. Brain. Behav. Immun. *22*, 600–605.

Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., and de Lange, T. (1995). A human telomeric protein. Science *270*, 1663.

Chylack, L.T., Peterson, L.E., Feiveson, A.H., Wear, M.L., Manuel, F.K., Tung, W.H., Hardy, D.S., Marak, L.J., and Cucinotta, F.A. (2009). NASA Study of Cataract in Astronauts (NASCA). Report 1: cross-sectional study of the relationship of exposure to space radiation and risk of lens opacity. Radiat. Res. *172*, 10–20.

Corey, D.R. (2009). Telomeres and telomerase: from discovery to clinical trials. Chem. Biol. *16*, 1219–1223.

Cornforth, M.N., and Eberle, R.L. (2001). Termini of human chromosomes display elevated rates of mitotic recombination. Mutagenesis *16*, 85–89.

Cucinotta, F.A., Manuel, F.K., Jones, J., Iszard, G., Murrey, J., Djojonegro, B., and Wear, M. (2001). Space radiation and cataracts in astronauts. Radiat. Res. *156*, 460–466.

Cucinotta, F.A., Kim, M.-H.Y., Willingham, V., and George, K.A. (2008). Physical and biological organ dosimetry analysis for international space station astronauts. Radiat. Res. *170*, 127–138.

Damjanovic, A.K., Yang, Y., Glaser, R., Kiecolt-Glaser, J.K., Nguyen, H., Laskowski, B., Zou, Y., Beversdorf, D.Q., and Weng, N. (2007). Accelerated telomere erosion is associated with a declining immune function of caregivers of Alzheimer's disease patients. J. Immunol. *179*, 4249–4254.

De Bont, R., and van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis *19*, 169–185.

Deng, Y., Chan, S.S., and Chang, S. (2008). Telomere dysfunction and tumour suppression: the senescence connection. Nat. Rev. Cancer *8*, 450–458.

Dutrillaux, B., Sabatier, L., Al Achkar, W., Muleris, M., Aurias, A., Couturier, J., Dutrillaux, A.M., Gerbault-Seureau, M., Hoffschir, F., and Lamoliatte, E. (1986). Radiation induced inversions in human somatic cells. Ann. Genet. *29*, 189–194.

Epel, E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Morrow, J.D., and Cawthon, R.M. (2004). Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. U. S. A. *101*, 17312–17315.

Falck, J., Forment, J.V., Coates, J., Mistrik, M., Lukas, J., Bartek, J., and Jackson, S.P. (2012). CDK targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination. EMBO Rep. *13*, 561–568.

Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J.W., Parry, J., Norppa, H., Eastmond, D.A., Tucker, J.D., and Thomas, P. (2011). Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis *26*, 125–132.

Folley, J.H., Borges, W., and Yamawaki, T. (1952). Incidence of leukemia in survivors of the atomic bomb in Hiroshima and Nagasaki, Japan. Am. J. Med. *13*, 311–321.

Fouquerel, E., Lormand, J., Bose, A., Lee, H.-T., Kim, G.S., Li, J., Sobol, R.W., Freudenthal, B.D., Myong, S., and Opresko, P.L. (2016). Oxidative guanine base damage regulates human telomerase activity. Nat. Struct. Mol. Biol. *23*, 1092–1100.

Freifelder, D., and Trumbo, B. (1969). Matching of single-strand breaks to form double-strand breaks in DNA. Biopolymers *7*, 681–693.

Galletly, C., Dhillon, V.S., Liu, D., Balzan, R.P., Hahn, L.A., and Fenech, M.F. (2017). Shorter telomere length in people with schizophrenia: A preliminary study from Australia. Schizophr. Res.

Ghosh, A., Saginc, G., Leow, S.C., Khattar, E., Shin, E.M., Yan, T.D., Wong, M., Zhang, Z., Li, G., Sung, W.-K., et al. (2012). Telomerase directly regulates NF-κB-dependent transcription. Nat. Cell Biol. *14*, 1270–1281.

Glinoer, D. (2004). The regulation of thyroid function during normal pregnancy: importance of the iodine nutrition status. Best Pract. Res. Clin. Endocrinol. Metab. *18*, 133–152.

Goytisolo, F.A., Samper, E., Martín-Caballero, J., Finnon, P., Herrera, E., Flores, J.M., Bouffler, S.D., and Blasco, M.A. (2000). Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals. J. Exp. Med. *192*, 1625–1636.

Gregoire, E., Sorokine-Durm, I., Bertho, J.M., Jacquet, N., Delbos, M., Demarquay, C., Voisin, P., and Roy, L. (2006). Follow-up of stable chromosomal aberrations in gamma-ray irradiated non-human primates. Int. J. Radiat. Biol. *82*, 493–502.

Greider, C.W., and Blackburn, E.H. (1985). Identification of a specific telomere terminal transferase activity in tetrahymena extracts. Cell *43*, 405–413.

Greider, C.W., and Blackburn, E.H. (1987). The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell *51*, 887–898.

Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H., and de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. Cell *97*, 503–514.

Hada, M., and Georgakilas, A.G. (2008). Formation of clustered DNA damage after high-LET irradiation: a review. J. Radiat. Res. (Tokyo) *49*, 203–210.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646–674.

Harley, C.B., Futcher, A.B., and Greider, C.W. (1990). Telomeres shorten during ageing of human fibroblasts. Nature *345*, 458–460.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp. Cell Res. *25*, 585–621.

Heaphy, C.M., Subhawong, A.P., Hong, S.-M., Goggins, M.G., Montgomery, E.A., Gabrielson, E., Netto, G.J., Epstein, J.I., Lotan, T.L., Westra, W.H., et al. (2011). Prevalence of the Alternative Lengthening of Telomeres Telomere Maintenance Mechanism in Human Cancer Subtypes. Am. J. Pathol. *179*, 1608–1615.

Hefferin, M.L., and Tomkinson, A.E. (2005). Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair *4*, 639–648.

Henson, J., Neumann, A., Yeager, T.A., and Reddel, R.R. (2002). Alternative lengthening of telomeres in mammalian cells. Oncogene *21*, 13.

Henson, J.D., Hannay, J.A., McCarthy, S.W., Royds, J.A., Yeager, T.R., Robinson, R.A., Wharton, S.B., Jellinek, D.A., Arbuckle, S.M., Yoo, J., et al. (2005). A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. Clin. Cancer Res. *11*, 217–225.

Hochstrasser, T., Marksteiner, J., and Humpel, C. (2012). Telomere length is age-dependent and reduced in monocytes of Alzheimer patients. Exp. Gerontol. *47*, 160–163.

Horn, S., Barnard, S., and Rothkamm, K. (2011). Gamma-H2AX-based dose estimation for whole and partial body radiation exposure. PLOS ONE *6*, e25113.

Huang, H.-B., Lai, C.-H., Chen, G.-W., Lin, Y.-Y., Jaakkola, J.J.K., Liou, S.-H., and Wang, S.-L. (2012). Traffic-related air pollution and DNA damage: a longitudinal study in Taiwanese traffic conductors. PLOS ONE *7*, e37412.

Imataka, G., and Arisaka, O. (2012). Chromosome analysis using spectral karyotyping (SKY). Cell Biochem. Biophys. *62*, 13–17.

Jones, D.P., and Go, Y.-M. (2010). Redox compartmentalization and cellular stress. Diabetes Obes. Metab. *12*, 116–125.

Kaiser, P. (1984). Pericentric inversions. Hum. Genet. *68*, 1–47.

Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R.S., Johnson, B.E., and Skolnick, M.H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science *264*, 436–440.

Kang, H.T., Lee, H.I., and Hwang, E.S. (2006). Nicotinamide extends replicative lifespan of human cells. Aging Cell *5*, 423–436.

Kaur, I., Simons, E.R., Castro, V.A., Ott, C.M., and Pierson, D.L. (2005). Changes in monocyte functions of astronauts. Brain. Behav. Immun. *19*, 547–554.

Kawanishi, S., and Oikawa, S. (2004). Mechanism of telomere shortening by oxidative stress. Ann. N. Y. Acad. Sci. *1019*, 278–284.

Khoo, C.M., Carrasco, D.R., Bosenberg, M.W., Paik, J.-H., and DePinho, R.A. (2007). Ink4a/Arf tumor suppressor does not modulate the degenerative conditions or tumor spectrum of the telomerase-deficient mouse. Proc. Natl. Acad. Sci. *104*, 3931–3936.

Kirkpatrick, M. (2010). How and why chromosome inversions evolve. PLOS Biol. *8*, e1000501.

Kirkpatrick, M., and Barton, N. (2006). Chromosome inversions, local adaptation and speciation. Genetics *173*, 419–434.

Kyo, S., Takakura, M., Kanaya, T., Zhuo, W., Fujimoto, K., Nishio, Y., Orimo, A., and Inoue, M. (1999). Estrogen activates telomerase. Cancer Res. *59*, 5917–5921.

Ladwig, K.-H., Brockhaus, A.C., Baumert, J., Lukaschek, K., Emeny, R.T., Kruse, J., Codd, V., Häfner, S., Albrecht, E., Illig, T., et al. (2013). Posttraumatic stress disorder and not depression is associated with shorter leukocyte telomere length: findings from 3,000 participants in the population-based KORA F4 study. PLOS ONE *8*, e64762.

de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev. *19*, 2100–2110.

LeClerc, J.E., Borden, A., and Lawrence, C.W. (1991). The thymine-thymine pyrimidine-pyrimidone(6-4) ultraviolet light photoproduct is highly mutagenic and specifically induces 3' thymine-to-cytosine transitions in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. *88*, 9685–9689.

Li, J.S.Z., Miralles Fuste, J., Simavorian, T., Bartocci, C., Tsai, J., Karlseder, J., and Lazzerini Denchi, E. (2017). TZAP: A telomere-associated protein involved in telomere length control. Science.

Lieber, M.R. (2010). The mechanism of double-strand dna break repair by the nonhomologous DNA end joining pathway. Annu. Rev. Biochem. *79*, 181–211.

Lieberman, E.H. (1994). Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. Ann. Intern. Med. *121*, 936.

Lin, J., Epel, E., and Blackburn, E. (2012). Telomeres and lifestyle factors: Roles in cellular aging. Mutat. Res. Mol. Mech. Mutagen. *730*, 85–89.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709–715.

Lindholm, C., Tekkel, M., Veidebaum, T., Ilus, T., and Salomaa, S. (1998). Persistence of translocations after accidental exposure to ionizing radiation. Int. J. Radiat. Biol. *74*, 565–571.

Lloyd, D.C. (1997). Chromosomal analysis to assess radiation dose. STEM CELLS *15*, 195–201.

Lloyd, D.C., Purrott, R.J., and Reeder, E.J. (1980). The incidence of unstable chromosome aberrations in peripheral blood lymphocytes from unirradiated and occupationally exposed people. Mutat. Res. *72*, 523–532.

Lo, A.W. l., Sabatier, L., Fouladi, B., Pottier, G., Ricoul, M., and Mumane, J.P. (2002). DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. Neoplasia *4*, 531–538.

Lomax, M.E., Folkes, L.K., and O'Neill, P. (2013). Biological consequences of radiation-induced DNA damage: relevance to radiotherapy. Clin. Oncol. *25*, 578–585.

Longhese, M.P. (2008). DNA damage response at functional and dysfunctional telomeres. Genes Dev. *22*, 125–140.

Low, K.C., and Tergaonkar, V. (2013). Telomerase: central regulator of all of the hallmarks of cancer. Trends Biochem. Sci. *38*, 426–434.

Lustig, A., Shterev, I., Geyer, S., Shi, A., Hu, Y., Morishita, Y., Nagamura, H., Sasaki, K., Maki, M., Hayashi, I., et al. (2016). Long term effects of radiation exposure on telomere lengths of leukocytes and its associated biomarkers among atomic-bomb survivors. Oncotarget *7*, 38988–38998.

Mack, P.B., and Vogt, F.B. (1971). Roentgenographic bone density changes in astronauts during representative Apollo space flight. Am. J. Roentgenol. Radium Ther. Nucl. Med. *113*, 621–633.

Mack, P.B., LaChance, P.A., Vose, G.P., and Vogt, F.B. (1967). Bone demineralization of foot and hand of gemini-titan IV, V and VII astronauts during orbital flight. Am. J. Roentgenol. Radium Ther. Nucl. Med. *100*, 503–511.

Mader, T.H., Gibson, C.R., Pass, A.F., Kramer, L.A., Lee, A.G., Fogarty, J., Tarver, W.J., Dervay, J.P., Hamilton, D.R., Sargsyan, A., et al. (2011). Optic disc edema, globe flattening, choroidal folds, and hyperopic shifts observed in astronauts after long-duration space flight. Ophthalmology *118*, 2058–2069.

Mak, V., Jarvi, K., Buckspan, M., Freeman, M., Hechter, S., and Zini, A. (2000). Smoking is associated with the retention of cytoplasm by human spermatozoa. Urology *56*, 463–466.

Manolio, T.A., Collins, F.S., Cox, N.J., Goldstein, D.B., Hindorff, L.A., Hunter, D.J., McCarthy, M.I., Ramos, E.M., Cardon, L.R., Chakravarti, A., et al. (2009). Finding the missing heritability of complex diseases. Nature *461*, 747–753.

Mao, P., Liu, J., Zhang, Z., Zhang, H., Liu, H., Gao, S., Rong, Y.S., and Zhao, Y. (2016). Homologous recombination-dependent repair of telomeric DSBs in proliferating human cells. Nat. Commun. *7*, 12154.

Mao, Z., Bozzella, M., Seluanov, A., and Gorbunova, V. (2008). DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. Cell Cycle Georget. Tex *7*, 2902–2906.

Mazón, G., Mimitou, E.P., and Symington, L.S. (2010). Snapshot: homologous recombination in dna double-strand break repair. Cell *142*, 648.e1-648.e2.

McCarthy, I.D. (2005). Fluid shifts due to microgravity and their effects on bone: a review of current knowledge. Ann. Biomed. Eng. *33*, 95–103.

McClintock, B. (1941). The stability of broken ends of chromosomes in Zea mays. Genetics *26*, 234–282.

McKenna, M.J., and Bailey, S.M. (2017). Chromosomal and telomeric biomarkers of normal tissue injury to evaluate risk of degenerative health effects (secondary malignancy, cardiovascular disease) post radiation therapy. Transl. Cancer Res.

McKenna, M.J., Robinson, E., Goodwin, E.H., Cornforth, M.N., and Bailey, S.M. (2017). Telomeres and NextGen CO-FISH: Directional Genomic Hybridization (Telo-dGH^{TM}). In Telomeres and Telomerase: Methods and Protocols, Z. Songyang, ed. (New York, NY: Springer New York), pp. 103–112.

Minamino, T., Miyauchi, H., Yoshida, T., Ishida, Y., Yoshida, H., and Komuro, I. (2002). Endothelial cell senescence in human atherosclerosis. Circulation *105*, 1541–1544.

Moquet, J., Barnard, S., and Rothkamm, K. (2014). Gamma-H2AX biodosimetry for use in large scale radiation incidents: comparison of a rapid “96 well lyse/fix” protocol with a routine method. PeerJ *2*, e282.

Mora, S., Lee, I.-M., Buring, J.E., and Ridker, P.M. (2006). Association of physical activity and body mass index with novel and traditional cardiovascular biomarkers in women. JAMA *295*, 1412–1419.

Morin, G.B. (1989). The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell *59*, 521–529.

Morlá, M., Busquets, X., Pons, J., Sauleda, J., MacNee, W., and Agustí, A.G.N. (2006). Telomere shortening in smokers with and without COPD. Eur. Respir. J. *27*, 525–528.

Moroni, M., Maeda, D., Whitnall, M.H., Bonner, W.M., and Redon, C.E. (2013). Evaluation of the gamma-H2AX assay for radiation biodosimetry in a swine model. Int. J. Mol. Sci. *14*, 14119–14135.

Mouret, S., Baudouin, C., Charveron, M., Favier, A., Cadet, J., and Douki, T. (2006). Cyclobutane pyrimidine dimers are predominant DNA lesions in whole human skin exposed to UVA radiation. Proc. Natl. Acad. Sci. *103*, 13765–13770.

Moye, A.L., Porter, K.C., Cohen, S.B., Phan, T., Zyner, K.G., Sasaki, N., Lovrecz, G.O., Beck, J.L., and Bryan, T.M. (2015). Telomeric G-quadruplexes are a substrate and site of localization for human telomerase. Nat. Commun. *6*, 7643.

Moyzis, R.K., Buckingham, J.M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, R.L., and Wu, J.R. (1988). A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. U. S. A. *85*, 6622–6626.

Mühlmann-Díaz, M.C., and Bedford, J.S. (1995). Comparison of gamma-ray-induced chromosome ring and inversion frequencies. Radiat. Res. *143*, 175–180.

Muller, H.J. (1938). The remaking of chromosomes. Collect. Net *13*, 181–195.

Murnane, J.P., Sabatier, L., Marder, B.A., and Morgan, W.F. (1994). Telomere dynamics in an immortal human cell line. EMBO J. *13*, 4953–4962.

Nash, P.V., Konstantinova, I.V., Fuchs, B.B., Rakhmilevich, A.L., Lesnyak, A.T., and Mastro, A.M. (1992). Effect of spaceflight on lymphocyte proliferation and interleukin-2 production. J. Appl. Physiol. *73*, S186–S190.

Nasir, L., Devlin, P., Mckevitt, T., Rutteman, G., and Argyle, D.J. (2001). Telomere lengths and telomerase activity in dog tissues: a potential model system to study human telomere and telomerase biology. Neoplasia *3*, 351–359.

National Research Council (2006). Health risks from exposure to low levels of ionizing radiation: BEIR VII Phase 2 (Washington, DC: The National Academies Press).

NCRP (2009). Report No. 163 - radiation dose reconstruction: principles and practices.

Nikiforov, Y.E. (2002). RET/PTC rearrangement in thyroid tumors. Endocr. Pathol. *13*, 3–16.

NRC (2015). NRC: backgrounder on biological effects of radiation.

Ogino, H., Nakabayashi, K., Suzuki, M., Takahashi, E., Fujii, M., Suzuki, T., and Ayusawa, D. (1998). Release of telomeric DNA from chromosomes in immortal human cells lacking telomerase activity. Biochem. Biophys. Res. Commun. *248*, 223–227.

Ohtaki, K. (1992). G-banding analysis of radiation-induced chromosome damage in lymphocytes of Hiroshima A-bomb survivors. Jpn. J. Hum. Genet. *37*, 245–262.

Okuda, K., Bardeguez, A., Gardner, J.P., Rodriguez, P., Ganesh, V., Kimura, M., Skurnick, J., Awad, G., and Aviv, A. (2002). Telomere length in the newborn. Pediatr. Res. *52*, 377–381.

Olovnikov, A.M. (1973). A theory of marginotomy. J. Theor. Biol. *41*, 181–190.

Osterwald, S., Deeg, K.I., Chung, I., Parisotto, D., Wörz, S., Rohr, K., Erfle, H., and Rippe, K. (2015). PML induces compaction, TRF2 depletion and DNA damage signaling at telomeres and promotes their alternative lengthening. J. Cell Sci.

O'Sullivan, R.J., and Karlseder, J. (2010). Telomeres: protecting chromosomes against genome instability. Nat. Rev. Mol. Cell Biol. *11*, 171–181.

Pannunzio, N.R., Li, S., Watanabe, G., and Lieber, M.R. (2014). Non-homologous end joining often uses microhomology: implications for alternative end joining. DNA Repair *17*, 74–80.

Panossian, L.A., Porter, V.R., Valenzuela, H.F., Zhu, X., Reback, E., Masterman, D., Cummings, J.L., and Effros, R.B. (2003). Telomere shortening in T cells correlates with Alzheimer's disease status. Neurobiol. Aging *24*, 77–84.

Parkinson, E.K., Newbold, R.F., and Keith, W.N. (1997). The genetic basis of human keratinocyte immortalisation in squatnous cell carcinoma development: The role of telomerase reactivation. Eur. J. Cancer *33*, 727–734.

Paul, L. (2011). Diet, nutrition and telomere length. J. Nutr. Biochem. *22*, 895–901.

Paul, L., Cattaneo, M., D'Angelo, A., Sampietro, F., Fermo, I., Razzari, C., Fontana, G., Eugene, N., Jacques, P.F., and Selhub, J. (2009). Telomere length in peripheral blood mononuclear cells is associated with folate status in men. J. Nutr. *139*, 1273–1278.

Pinsky, L.S., Osborne, W.Z., Bailey, J.V., Benson, R.E., and Thompson, L.F. (1974). Light flashes observed by astronauts on Apollo 11 through Apollo 17. Science *183*, 957–959.

Pleshkova, G.N., and Plevako, N.G. (1982). Types, frequency and interchromosomal distribution of radiation-induced chromosome rearrangements in the malaria mosquito Anopheles messeae. Tsitologiia *24*, 206–210.

Podlevsky, J.D., and Chen, J.J.-L. (2012). It all comes together at the ends: Telomerase structure, function, and biogenesis. Mutat. Res. *730*, 3–11.

Poiesz, B.J., Seal, G., and Loeb, L.A. (1974). Reverse transcriptase: correlation of zinc content with activity. Proc. Natl. Acad. Sci. U. S. A. *71*, 4892–4896.

Polho, G.B., De-Paula, V.J., Cardillo, G., dos Santos, B., and Kerr, D.S. (2015). Leukocyte telomere length in patients with schizophrenia: a meta-analysis. Schizophr. Res. *165*, 195–200.

Preston, B.D., Albertson, T.M., and Herr, A.J. (2010). DNA replication fidelity and cancer. Semin. Cancer Biol. *20*, 281–293.

Ramsey, M.J., Moore, D.H., Briner, J.F., Lee, D.A., Olsen, L.A., Senft, J.R., and Tucker, J.D. (1995). The effects of age and lifestyle factors on the accumulation of cytogenetic damage as measured by chromosome painting. Mutat. Res. *338*, 95–106.

Ray, F.A., Zimmerman, E., Robinson, B., Cornforth, M., Bedford, J., Goodwin, E., and Bailey, S. (2013). Directional genomic hybridization for chromosomal inversion discovery and detection. Chromosome Res. *21*, 165–174.

Ray, F.A., Robinson, E., McKenna, M., Hada, M., George, K., Cucinotta, F., Goodwin, E.H., Bedford, J.S., Bailey, S.M., and Cornforth, M.N. (2014). Directional genomic hybridization: inversions as a potential biodosimeter for retrospective radiation exposure. Radiat. Environ. Biophys. *53*, 255–263.

Reddel, R.R. (2003). Alternative lengthening of telomeres, telomerase, and cancer. Cancer Lett. *194*, 155–162.

Reddel, R.R. (2014). Telomere maintenance mechanisms in cancer: clinical implications. Curr. Pharm. Des. *20*, 6361–6374.

RERF (2007a). Chromosome aberrations - Radiation Effects Research Foundation.

RERF (2007b). Leukemia risks among atomic-bomb survivors - Radiation Effects Research Foundation.

Reste, J., Zvigule, G., Zvagule, T., Kurjane, N., Eglite, M., Gabruseva, N., Berzina, D., Plonis, J., and Miklasevics, E. (2014). Telomere length in Chernobyl accident recovery workers in the late period after the disaster. J. Radiat. Res. (Tokyo) *55*, 1089–1100.

Riegel, M. (2014). Human molecular cytogenetics: from cells to nucleotides. Genet. Mol. Biol. *37*, 194–209.

Rivlin, N., Brosh, R., Oren, M., and Rotter, V. (2011). Mutations in the p53 tumor suppressor gene. Genes Cancer *2*, 466–474.

Robbins, J., and Schneider, A.B. (2000). Thyroid cancer following exposure to radioactive iodine. Rev. Endocr. Metab. Disord. *1*, 197–203.

Robinson, E., McKenna, M.J., Bedford, J.S., Goodwin, E.H., Cornforth, M.N., Bailey, S.M., and Ray, F.A. (Submitted). Directional Genomic Hybridization (dGH®) for detection of intra-chromosomal rearrangements. Radiation Cytogenetics: Methods in Molecular Biology.

Rode, L., Nordestgaard, B.G., Weischer, M., and Bojesen, S.E. (2014). Increased body mass index, elevated C-reactive protein, and short telomere length. J. Clin. Endocrinol. Metab. *99*, E1671-1675.

Rodríguez, P., Montoro, A., Barquinero, J.F., Caballín, M.R., Villaescusa, I., and Barrios, L. (2004). Analysis of translocations in stable cells and their implications in retrospective biological dosimetry. Radiat. Res. *162*, 31–38.

Rothkamm, K., Beinke, C., Romm, H., Badie, C., Balagurunathan, Y., Barnard, S., Bernard, N., Boulay-Greene, H., Brengues, M., De Amicis, A., et al. (2013). Comparison of established and emerging biodosimetry assays. Radiat. Res. *180*, 111–119.

Rubin, C.M. (1998). The genetic basis of human cancer. Ann. Intern. Med. *129*, 759.

Savage, J.R. (1976). Classification and relationships of induced chromosomal structual changes. J. Med. Genet. *13*, 103–122.

Savage, J.R.K. (1999). An introduction to chromosomal aberrations. Atlas of Genetics and Cytogenetics in Oncology and Haematology.

Savage, J.R.K., and Tucker, J.D. (1996). Nomenclature systems for FISH-painted chromosome aberrations. Mutat. Res. Genet. Toxicol. *366*, 153–161.

Schreck, R.R., and Distèche, C.M. (2001). Chromosome banding techniques. Curr. Protoc. Hum. Genet. *Chapter 4*, Unit4.2.

Schulz, P., Kirschbaum, C., Prüßner, J., and Hellhammer, D. (1998). Increased free cortisol secretion after awakening in chronically stressed individuals due to work overload. Stress Health *14*, 91–97.

Seabright, M. (1971). A rapid banding technique for human chromosomes. The Lancet *298*, 971–972.

Serrano, A.L., and Andrés, V. (2004). Telomeres and cardiovascular disease. Circ. Res. *94*, 575–584.

Setlow, R.B. (1966). Cyclobutane-type pyrimidine dimers in polynucleotides. Science *153*, 379–386.

Shaffer, L.G., and Bejjani, B.A. (2004). A cytogeneticist's perspective on genomic microarrays. Hum. Reprod. Update *10*, 221–226.

Shakhtarin, V.V., Tsyb, A.F., Stepanenko, V.F., Orlov, M.Y., Kopecky, K.J., and Davis, S. (2003). Iodine deficiency, radiation dose, and the risk of thyroid cancer among children and adolescents in the Bryansk region of Russia following the Chernobyl power station accident. Int. J. Epidemiol. *32*, 584–591.

Shay, J.W., and Bacchetti, S. (1997). A survey of telomerase activity in human cancer. Eur. J. Cancer *33*, 787–791.

Shay, J.W., and Wright, W.E. (1996). The reactivation of telomerase activity in cancer progression. Trends Genet. *12*, 129–131.

Shay, J.W., and Wright, W.E. (2010). Telomeres and telomerase in normal and cancer stem cells. Febs Lett. *584*, 3819–3825.

Shay, J.W., Wright, W.E., and Werbin, H. (1991). Defining the molecular mechanisms of human cell immortalization. Biochim. Biophys. Acta BBA - Rev. Cancer *1072*, 1–7.

Shen, J., Terry, M.B., Gurvich, I., Liao, Y., Senie, R.T., and Santella, R.M. (2007). Short telomere length and breast cancer risk: a study in sister sets. Cancer Res. *67*, 5538–5544.

Shippen-Lentz, D., and Blackburn, E.H. (1990). Functional evidence for an RNA template in telomerase. Science *247*, 546–552.

Simon, S.L., Bouville, A., and Kleinerman, R. (2010). Current use and future needs of biodosimetry in studies of long-term health risk following radiation exposure. Health Phys. *98*, 109–117.

Simsek, D., and Jasin, M. (2010). Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation. Nat. Struct. Mol. Biol. *17*, 410–416.

Singh, S.K., Szulik, M.W., Ganguly, M., Khutsishvili, I., Stone, M.P., Marky, L.A., and Gold, B. (2011). Characterization of DNA with an 8-oxoguanine modification. Nucleic Acids Res. *39*, 6789–6801.

Sinha, R.P., and Häder, D.P. (2002). UV-induced DNA damage and repair: a review. Photochem. Photobiol. Sci. Off. J. Eur. Photochem. Assoc. Eur. Soc. Photobiol. *1*, 225–236.

Sishc, B.J., Nelson, C.B., McKenna, M.J., Battaglia, C.L.R., Herndon, A., Idate, R., Liber, H.L., and Bailey, S.M. (2015). Telomeres and telomerase in the radiation response: implications for instability, reprogramming, and carcinogenesis. Front. Oncol. *5*.

Smith, S.M., Heer, M.A., Shackelford, L.C., Sibonga, J.D., Ploutz-Snyder, L., and Zwart, S.R. (2012). Benefits for bone from resistance exercise and nutrition in long-duration spaceflight: Evidence from biochemistry and densitometry. J. Bone Miner. Res. *27*, 1896–1906.

Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M.R., Schnapp, G., and de Lange, T. (2000). Control of human telomere length by TRF1 and TRF2. Mol. Cell. Biol. *20*, 1659–1668.

van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell *92*, 401–413.

Sturtevant, A.H. (1921). A case of rearrangement of genes in Drosophila. Proc. Natl. Acad. Sci. U. S. A. *7*, 235–237.

Sun, J.G., Jurisicova, A., and Casper, R.F. (1997). Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol. Reprod. *56*, 602–607.

Suntharalingam, N., and Cameron, J.R. (1966). A comparison of TLD and film for personnel dosimetry. Health Phys. *12*.

Sutherland, B.M., Bennett, P.V., Schenk, H., Sidorkina, O., Laval, J., Trunk, J., Monteleone, D., and Sutherland, J. (2001). Clustered DNA damages induced by high and low LET radiation, including heavy ions. Phys. Medica PM Int. J. Devoted Appl. Phys. Med. Biol. Off. J. Ital. Assoc. Biomed. Phys. AIFB *17 Suppl 1*, 202–204.

Svenson, U., Nordfjäll, K., Stegmayr, B., Manjer, J., Nilsson, P., Tavelin, B., Henriksson, R., Lenner, P., and Roos, G. (2008). Breast cancer survival is associated with telomere length in peripheral blood cells. Cancer Res. *68*, 3618–3623.

Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., and Takeda, S. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J. *17*, 5497–5508.

Tawn, E.J., and Whitehouse, C.A. (2003). Persistence of translocation frequencies in blood lymphocytes following radiotherapy: implications for retrospective radiation biodosimetry. J. Radiol. Prot. Off. J. Soc. Radiol. Prot. *23*, 423–430.

Thomas, P., O' Callaghan, N.J., and Fenech, M. (2008). Telomere length in white blood cells, buccal cells and brain tissue and its variation with ageing and Alzheimer's disease. Mech. Ageing Dev. *129*, 183–190.

Thompson, L.H., and Limoli, C.L. (2013). Origin, recognition, signaling and repair of DNA double-strand breaks in mammalian cells. Landes Bioscience.

Tronko, M., Bogdanova, T., Voskoboynyk, L., Zurnadzhy, L., Shpak, V., and Gulak, L. (2010). Radiation induced thyroid cancer: fundamental and applied aspects. Exp. Oncol. *32*, 200–204.

Tucker, J.D. (2008). Low-dose ionizing radiation and chromosome translocations: a review of the major considerations for human biological dosimetry. Mutat. Res. Mutat. Res. *659*, 211–220.

Tucker, J.D., Cofield, J., Matsumoto, K., Ramsey, M.J., and Freeman, D.C. (2005). Persistence of chromosome aberrations following acute radiation: I, PAINT translocations, dicentrics, rings, fragments, and insertions. Environ. Mol. Mutagen. *45*, 229–248.

Ulaner, G.A., Huang, H.-Y., Otero, J., Zhao, Z., Ben-Porat, L., Satagopan, J.M., Gorlick, R., Meyers, P., Healey, J.H., Huvos, A.G., et al. (2003). Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res. *63*, 1759–1763.

UNSCEAR (2008). Annex D: Health effects due to radiation from the Chernobyl accident.

Valdes, A.M., Andrew, T., Gardner, J.P., Kimura, M., Oelsner, E., Cherkas, L.F., Aviv, A., and Spector, T.D. (2005). Obesity, cigarette smoking, and telomere length in women. Lancet Lond. Engl. *366*, 662–664.

Vaurijoux, A., Gruel, G., Voisin, P., and Roch-Lefèvre, S. (2012). Biological dosimetry of ionizing radiation. INTECH Open Access Publisher.

Vorlícková, M., Tomasko, M., Sagi, A.J., Bednarova, K., and Sagi, J. (2012). 8-Oxoguanine in a quadruplex of the human telomere DNA sequence. FEBS J. *279*, 29–39.

Vral, A., Fenech, M., and Thierens, H. (2011). The micronucleus assay as a biological dosimeter of in vivo ionising radiation exposure. Mutagenesis *26*, 11–17.

Wall, J. (2015). What is the International Space Station?

Ward, J.F. (1998). Nature of lesions formed by ionizing radiation. in DNA damage and repair, J.A. Nickoloff, and M.F. Hoekstra, eds. (Humana Press), pp. 65–84.

Watson, J.D. (1972). Origin of concatemeric T7DNA. Nature *239*, 197–201.

Webb, C.J., Wu, Y., and Zakian, V.A. (2013). DNA repair at telomeres: keeping the ends intact. Cold Spring Harb. Perspect. Biol. *5*.

West, C.M., and Barnett, G.C. (2011). Genetics and genomics of radiotherapy toxicity: towards prediction. Genome Med. *3*, 52.

Wilson III, D.M., and Thompson, L.H. (2007). Molecular mechanisms of sister-chromatid exchange. Mutat. Res. Mol. Mech. Mutagen. *616*, 11–23.

Wong, K.K., Chang, S., Weiler, S.R., Ganesan, S., Chaudhuri, J., Zhu, C., Artandi, S.E., Rudolph, K.L., Gottlieb, G.J., Chin, L., et al. (2000). Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. Nat. Genet. *26*, 85–88.

Wood, J.W., Tamagaki, H., Neriishi, S., Sato, T., Sheldon, W.F., Archer, P.G., Hamilton, H.B., and Johnson, K.G. (1969). Thyroid carcinoma in atomic bomb survivors Hiroshima and Nagasaki. Am. J. Epidemiol. *89*, 4–14.

Wu, X., Amos, C.I., Zhu, Y., Zhao, H., Grossman, B.H., Shay, J.W., Luo, S., Hong, W.K., and Spitz, M.R. (2003). Telomere dysfunction: a potential cancer predisposition factor. J. Natl. Cancer Inst. *95*, 1211–1218.

Wyman, C., Ristic, D., and Kanaar, R. (2004). Homologous recombination-mediated double-strand break repair. DNA Repair *3*, 827–833.

Yeh, J.K., and Wang, C.Y. (2016). Telomeres and telomerase in cardiovascular diseases. Genes *7*.

Yin, L., Hubbard, A.K., and Giardina, C. (2000). NF-κB regulates transcription of the mouse telomerase catalytic subunit. J. Biol. Chem. *275*, 36671–36675.

Yokoyama, H., and Mizutani, R. (2014). Structural biology of DNA (6-4) photoproducts formed by ultraviolet radiation and interactions with their binding proteins. Int. J. Mol. Sci. *15*, 20321–20338.

Zhang, X., Lin, S., Funk, W.E., and Hou, L. (2013). Republished: Environmental and occupational exposure to chemicals and telomere length in human studies. Postgrad. Med. J. *89*, 722–728.

Zhao, Y., Lai, K., Cheung, I., Youds, J., Tarailo, M., Tarailo, S., and Rose, A. (2006). A mutational analysis of Caenorhabditis elegans in space. Mutat. Res. Mol. Mech. Mutagen. *601*, 19–29.

Zijlmans, J.M.J.M., Martens, U.M., Poon, S.S.S., Raap, A.K., Tanke, H.J., Ward, R.K., and Lansdorp, P.M. (1997). Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. Proc. Natl. Acad. Sci. U. S. A. *94*, 7423–7428.

CHAPTER 2

THE ATOMIC VETERANS: RETROSPECTIVE BIODOSIMETRY TO ASSESS NUCLEAR FALLOUT EXPOSURE

Summary

Shortly after World War II, United States military personnel and weather observers, collectively known as the atomic veterans, were exposed to prompt radiation as well as debris from nuclear tests in the form off fallout during atomic bomb testing at two separate sites; the Marshall Islands and the Nevada Test Site (NTS). The circumstances of exposures occurred in the line of duty, i.e., during participation in military maneuvers to test the capability of servicemen performing duties on the battlefield following a nuclear detonation, airborne reconnaissance and surveillance of the fallout clouds, and observations of weather. The present study is part of the National Cancer Institute's study of exposures of atomic veterans. That study is separate but loosely associated with the Million Workers Study to evaluate low dose and low dose effects on Manhattan Project workers, nuclear utility workers, military veterans (atomic veterans), and medical workers. Our overall goal was to improve understanding of long-term (>60 years) biological signatures of ionizing radiation (IR) exposure following a nuclear detonation, as well as provide a means of validating physical dosimetry done at the time. Chromosome aberrations, specifically dicentrics, have long been the gold standard of biodosimetry. Here, the utility of Directional Genomic Hybridization (dGH) for high-resolution detection of IR-induced chromosomal inversions and translocations was assessed as a more sensitive, quantitative

retrospective biodosimetry approach. The influence of IR exposure on telomere length dynamics was also evaluated, as telomeres are informative biomarkers of exposure as well, serving as "hallmarks" of radiosensitivity and reflective of overall health status.

Standard calibration (dose response) curves for inversions and translocations were established by exposing whole peripheral blood to ^{137}Cs γ-rays from six male volunteers in their mid-20's, to reflect age at exposure for the veterans. Calibration curves were constructed using six dose points (0, 0.15, 0.25, 0.50, 1.0 and 2.0 Gy) to encompass the estimated dose range of exposure. Chromosome aberrations and telomere length were then assessed on peripheral blood samples from 12 exposed veterans and an equal number of unexposed, age-matched controls (>80 yrs). Explicit one- on- one matching of exposed and control subjects was not applied because the design was an analytical epidemiology study. Rather, the control subjects were used to establish average values of chromosome aberration rates that could be compared with each exposed subject.

Confounding factors (e.g. age and smoking) were accounted for to adjust any effect on aberration rates. Though covariate influences on translocation rates are well established, this is the first report of smoking and age associated increases in background inversion rates. Dose reconstruction was performed for each veteran using translocation and inversion rates independently, however combining them was found to generally improve the accuracy of dose estimates as judged by a comparison with analytical dose reconstruction. Of the 12 exposed veterans, 4 individuals were well below the limit of detection, most likely due to the high background of spontaneous inversions and translocations with age. Although age was the greatest contributing factor to telomere shortening as expected, there was a slight inverse relationship between telomere length and IR exposure, even though such a small cohort was investigated. Interestingly, smokers in the non-exposed veteran cohort displayed similar telomere lengths as

those in the non-smoker, exposed veteran group, suggesting that chronic smoking had as profound of an impact on telomere length as the single, acute exposure to nuclear fallout. Lastly, no evidence of an additive effect on telomere erosion for smokers that were exposed to nuclear fallout was observed.

Introduction

Much of what we know about direct or indirect exposures to ionizing radiation (IR) from nuclear detonation events stem from studies of the Japanese atomic bomb survivors. In this context, physical dose modeling may be limited after mass exposures due to missing information or incorrect personal recounts of location and shielding at time of exposure, especially for exposures that occurred many years in the past. Moreover, civilians do not typically possess or routinely wear external dosimeters such as film-badges or thermoluminescent dosimeters (TLDs). Even when present, external dosimeters often vary in accuracy (Simon et al., 2010; Suntharalingam and Cameron, 1966). Thus, unfortunate mass exposure events necessitate the need for biodosimetry-based dose reconstruction to assist in determining short-term care, acute radiation syndromes (ARS), and long-term preventative measurements (Rea et al., 2010).

Evaluation of atomic bomb survivor mortality rates between 1950 and 1997, found a total of 41,216 deaths among a cohort of 86,572. Of those involved in the study, greater than 60% received a dose greater than 5 millisieverts (mSv), and radiation exposure contributed to an estimated 5.8% of the total deaths (Preston et al., 2003). The majority of mortalities were attributable to IR-induced cancers, and to a lesser extent, noncancer diseases such as thyroid

disease, chronic liver disease and myocardial infarction, among others (Preston et al., 2003; Yamada et al., 2004).

While the information gained from those events have proven invaluable for improving understanding of the biological effects of radiation exposure on humans, and for setting of radiation protection standards, they do not fully represent the demographic exposed, and therefore risk of long-term effects, if a subsequent nuclear catastrophe occurred in the United States. In the present study, we present the findings on estimating the dose to U.S. military personnel and weather observers from exposure to nuclear detonations by biological dosimetry amongst a small subset of veterans selected because they are those living veterans with the largest recorded doses by the Department of Defense (DOD). The participants in this study were exposed to fallout at either the Rongerik Atoll, a site downwind of the Bikini atoll test site in the Marshall Islands, at the Nevada Test Site in the 1950's, or who participated in multiple events. These individuals provide a unique opportunity to assess quantitative retrospective biodosimetry and evaluate the effects of IR exposures that took place more than 60 years ago to a U.S. population.

The most common biodosimetry methodologies for relatively recent exposures (< 1 year) evaluate frequencies of dicentric chromosomes or micronuclei (Rothkamm et al., 2013; Vaurijoux et al., 2012). Though sensitive biomarkers of radiation exposure (Lloyd, 1997), both aberrations reflect loss of genetic information and so are lethal events that do not persist with time (Cho et al., 2015; Tucker et al., 2005). Reciprocal translocations on the other hand *are* persistent with time, and so are used for exposures that occurred several years in the past for retrospective biodosimetry (Tawn and Whitehouse, 2003). However, increases in translocation background frequencies due to lifestyle (e.g. smoking) and environmental (e.g. pesticides) factors can complicate analyses (Ramsey et al., 1995).

Chromosomal inversions (inverted segments *within* chromosomes) were recently proposed as potential retrospective biodosimeters (Ray et al., 2014). Like reciprocal translocations (rearrangements *between* chromosomes), inversions are symmetrical (i.e., they are balanced), so they persist with time. However, due to low-resolution banding approaches historically used for their detection, appreciation for the value of inversions in biodosimetry and prevalence in disease is currently limited. The strand-specificity of the emerging cytogenomics-based methodology of Directional Genomic Hybridization (dGH) enables detection of inversions at much higher resolution than previously possible (Ray et al., 2013), while simultaneously also detecting translocations. Indeed, analyses of *in vitro* irradiated human cell lines suggest that inversions are induced at a greater rate per unit dose than translocations and further, high LET particles are more efficient at inducing inversions than γ-rays (Ray et al., 2014).

Telomeres, tandem TTAGGG repeats that cap and protect chromosome termini, have also been proposed as biomarkers of radiation sensitivity (Goytisolo et al., 2000; Wong et al., 2000). Telomere length dynamics can be influenced by a wide variety of lifestyle (e.g. smoking, nutrition, and exercise) and environmental (e.g. ionizing radiations) factors, thus providing a valuable indicator of overall health (Cherkas et al., 2008; Reste et al., 2014; Valdes et al., 2005). Monitoring telomere length together with chromosome aberrations, particularly inversions, has been proposed as a means of evaluating radiation toxicity and normal tissue damage (and therefore risk of degenerative effects including second malignancy) post-radiation therapy (McKenna and Bailey, 2017). In the context of the atomic veterans, telomere length measurements may shed light on long-term health effects of acute, low dose IR exposure from nuclear detonation events.

In the current study, we hypothesized that inclusion of inversions would improve retrospective biodosimetry for estimating doses of IR exposures that took place over 6 decades in

the past, reasoning that the persistence of inversions and their higher induction rate per unit dose would be favorable and exploitable qualities for dose reconstruction. Furthermore, we speculated that telomere length might also reflect these past exposures, thereby providing insight into the long-term biological/overall health implications. Taken together, results improve retrospective biodosimetry efforts and dose estimation, as well as enhance understanding of low dose IR exposure in humans and potential long-term health effects.

Materials and Methods

Study Cohort

The study group consisted of 9 young adult males (20-29 years of age) and 32 male, U.S. military veterans >80 years of age at time of enrollment. Each young adult was considered generally healthy with no history of smoking or radiation exposure (e.g. radiation therapies or CT scans). Three of the 9 young adult males were excluded from the study due to the inability to stimulate their blood samples in culture. Hence, the Young adults, enrolled for purposes of generating calibration curves, totaled six .

Of the 32 U.S. veterans, 4 veterans were removed from the study, and therefore their age-matched controls were removed as well, due to illness or death. Of the 24 veteran samples collected, 6 were exposed to nuclear fallout at the Rongerik Atoll from the 1954 BRAVO detonation on Bikini Atoll, 6 were exposed at the Nevada Test Site, and 12 were unexposed age-matched veteran controls. Among those selected, 14 veterans (7 exposed to nuclear fallout and 7 unexposed controls) were classified as chronic smokers.

Sample Collection and Blood Stimulation

Peripheral blood from consenting volunteers was drawn and collected in 10 mL sodium heparin tubes (Becton, Dickinson, and Co #367874) in accordance with NIH institutional review board approval (NIH Protocol 14CN170-C, Steven L. Simon, PI). Samples were shipped at ambient temperature and received within 24 hours of blood draw, then stimulated for 48-56 hours at 1:9 split in Gibco PB-Max Karyotyping Medium (ThermoFisher #12557021) supplemented with phytohaemagglutinin A (PHA) by the manufacturer. For Directional Genomic Hybridization (dGH®), 5.0 mM 5-bromo-deoxyuridine (BrdU) and 1.0 mM 5-bromo-deoxycytidine (BrdC) were added to the medium as previously described by Ray and Robinson (Ray et al., 2013; Robinson et al., Submitted). Four hours prior to harvest, KaryoMax Colcemid (ThermoFisher #15210040) was added at 0.1 μg/mL. The stimulated blood was then harvested and metaphase chromosome spreads prepared using standard cytogenetic techniques (Howe et al., 2014).

Irradiations for Calibration (Dose Response) Curves

Blood samples from the 6 young adult males were exposed to acute doses of ^{137}Cs γ-rays in a Mark I irradiator (J.L. Shepherd) located at Colorado State University. The calibration curves were constructed using doses of 0, 0.15, 0.25, 0.50, 1.0, and 2.0 Gy, at a dose rate of 2.5 Gy/min prior to stimulating. Unirradiated controls were kept under similar conditions (e.g. room temperature) prior to stimulating.

Directional Genomic Hybridization (dGH) and imaging

To identify inversions and translocations, dGH was employed using single color whole chromosome 1, 2 and 3 paints (KromaTiD Inc., Ft. Collins, CO), and performed as described by

Ray and Robinson (Ray et al., 2013; Robinson et al., Submitted). Briefly, slides containing metaphase spread preparations substituted with bromonucleotides (BrdU and BrdC) are submersed in Hoechst 33258 (Millipore Sigma #B1155) for 15 minutes, selectively photolysed using a SpectroLinker UV Crosslinker equipped with 365nm UV bulbs for 35 minutes, followed by exonucleolytic degradation of the nicked DNA with Exonuclease III (New England Biolabs #M0206L) for 30 minutes. The hybridization mixture containing single-color (Cyanine-3) chromosome 1, 2 and 3 paints was provided by KromaTiD, Inc. (Fort Collins, CO) and applied to the slides. Cover slips were then applied, sealed using rubber cement, and denatured at 68°C for 3 minutes. The slides were hybridized overnight at 37°C followed by five washes in 2x SSC at 43°C prior to imaging. Metaphase spreads were imaged on a Nikon Eclipse Ni-U epifluorescent microscope equipped with an Andor Zyla 5.5 sCMOS camera and SpectraX LED light source. Stacked images were taken of each metaphase spread composed of 5 images per stack with a step size of 0.3 μm. Over 200 metaphase spreads per veteran and 300 metaphase spreads per young adult were imaged and analyzed. Any clonal rearrangement that appeared two or more times were only scored once.

DNA Isolation

DNA was isolated from a minimum of $5x10^5$ peripheral blood mononuclear cells (PBMCs) with the DNeasy Blood and Tissue Kit (Qiagen #69504). PBMCs were initially incubated in proteinase K for 3 hours at 37°C, rather than 10 minutes at 56°C as specified by the kit's protocol. An average of 19.15 ± 3.41 ng/μLof DNA was isolated from each sample.

Multiplexed Quantitative PCR Telomere Length Measurement

Multiplexed quantitative PCR measurements of telomere length was carried out as previously described by Richard Cawthon (2009). Briefly, a 22 μL master mix was prepared using SYBR green GoTaq qPCR master mix (Promega #A6001) combined with the telomere forward primer (TelG; 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'), telomere reverse primer (TelC; 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'), albumin forward primer (AlbU; 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG-3'), albumin reverse primer (AlbD; 5'-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3') at 10 μM per primer (Integrated DNA Technologies), and RNase/DNase free water. To the master mix, 3 μL of DNA at 3.33 ng/uL was added for a final volume of 25 μL. The TelG/C primers were at a final concentration of 900nM and the AlbU/D primers at 400nM.

Telomere length measurements were carried out using a Bio-Rad CFX-96 qPCR machine. The cycle design was as follows: Step 1- 1 cycle at 95°C for 3 min. to heat inactivate the Taq polymerase; step 2- 2 cycles at 94°C for 15 sec. and 49°C for 15 sec. to anneal and extend the telomere primers; step 3- 32 cycles at 94°C for 15 sec., 62°C for 10 sec., 74°C for 15 sec., 84°C for 10 sec., and 88°C for 15 sec. to melt the early-amplified telomere products, followed by annealing and extension of the albumin primers. The melt curve was established by a 72°C to 95°C ramp at 0.5°C/sec. increase with a 30 second hold. Multiplexing both telomere and albumin primers using a single fluorescent DNA-intercalating dye is possible because the telomere primers are amplified at a lower quantification cycle (Cq) than the albumin primers. Standard curves were prepared using human genomic DNA (Promega) with 3-fold dilutions ranging from 50 ng to 0.617 ng in 3 μL per dilution. Negative controls include a no-template TelG/C only and AlbU/D only,

and a combined TelG/C and AlbU/D control. Samples were normalized across plates using a human genomic DNA standard. Each sample was run in triplicate on a 96-well plate format and relative telomere length was established using a telomere (T) to albumin (A) ratio.

Statistical Methods

Assessment of covariates: Statistical significance was established using a two-tailed Student's t-test on Graph Pad Prism 5 software for Windows. The normality assumption was tested before using the t-test. Pearson's correlation coefficient (r) was used to determine the strength of correlation between two variables.

Extrapolation to Whole-Genome Equivalency for Interchanges (translocations): The extrapolation considers exchanges between painted and unpainted (counterstained) chromosomes, as well as exchanges taking place among the uniquely painted chromosomes. Values for the genomic content of chromosomes used in the following derivation previously described by Loucas et al. (2016) are from Mayhall and Morton (Mayall et al., 1984; Morton, 1991). Its derivation, as applied to our experimental system, is as follows: Let f_p represent the fractional sum of the genome covered by the individual chromosomes 1, 2 and 3, where $f_1 = 0.080$; $f_2 = 0.079$; $f_3 = 0.064$, $f_p = (f_1+f_2+f_3) = 0.223$ (equation 1), and the unpainted (DAPI-counterstained) fraction then becomes $(1-f_p) = 0.777$ (equation 2). For whole chromosome painting, the frequency of visible interchanges in the genome that can occur between painted and unpainted chromosomes (FP) is given by the cross product of the binomial expansion $(p+q)^2 = p^2 +2pq + q^2$ - namely 2pq - where $p = (f_p)$ and $q = 1-(f_p)$. Substituting values in equation 2 gives the following expression: $F_p = 2pq = 2f_p(1-f_p) = 0.346$ (equation 3).

Extrapolation to Whole-Genome Equivalency for Intrachanges (inversions): The correction factor is the inverse of $f_p = (f_1+f_2+f_3) = 0.223$.

Estimating Dose and Related Uncertainty: Dose estimations based on chromosome aberration rates (translocations or inversions) were made using the equation $D_i = (R_i – B_i) / CF$; where D_i is the dose (Gy) of person i, R_i is the observed aberration rate, B_i is the average background rate among the group of age-matched controls, and CF is the calibration factor obtained from the slope of the calibration curves (dose, Gy, per aberration rate). Since each variable is associated with error, an uncertainty analysis was conducted the estimate the total error of the estimated dose. In this case, a Monte Carlo simulation was used to propagate uncertainty with 50,000 simulations. Covariates (e.g. age and smoking status) were considered before propagating the uncertainty. Even though the chromosome 1, 2, and 3 paints account for only 22.3% of the genome, we elected to not extrapolate aberration rates to whole genome equivalencies as recently proposed (Loucas et al., 2016), since translocations and inversions were simultaneously analyzed on the same chromosomes.

Best Linear Unbiased Estimator: For dose reconstruction utilizing both translocation and inversion rates simultaneously, a weighted dose model was used to derive an unbiased dose estimate. The inverse variance method was used which weights each estimate (in this case, the translocation-based estimate and the inversion-based estimate) by the inverse variance from simulation of each dose giving greater weight to the overall average by the more precise estimate.

Estimation of the minimum detectable dose (MDD): In this case, the MDD can be defined as the dose that represents a valid, detectable dose at a specified confidence level. Here, the MDD is calculated as: MDD (Gy) = 3 σ CF; where σ is the standard deviation of the background rate and CF is the aberration rate to dose conversion factor determined by the calibration experiment.

Results

Establishing Calibration (Dose Response) Curves

To establish the IR-induced dose response of translocations and inversions, whole peripheral blood from six Young adults were exposed to escalating acute doses of ^{137}Cs γ-rays ranging from 0 Gy (controls) to 2.0 Gy. Chromosome aberrations (translocations and inversions) were assessed on metaphase spreads hybridized with dGH whole chromosome 1, 2, and 3 paints. On normal chromosomes devoid of structural rearrangements, the dGH chromosome paints label a single sister chromatid (Figure 2.1 left panel); inversions are detected as a signal "switch" between sister chromatids (Figure 2.1 middle panel), and translocations are identified by signal exchanges between chromosomes (Figure 2.1 right panel).

At the dose range interrogated, both inversions and translocations displayed a linear dose response (Figure 2.2). Consistent with previous *in vitro* findings (Ray et al., 2014), inversions increased at a greater rate per unit dose than translocations, and also exhibited a higher spontaneous background rate. No translocations were identified in any of the young adult unirradiated control samples analyzed, but inversions were identified at a background rate of 0.223 ± 0.124 aberrations per cell equivalent (CE).

Covariate Influence on Background Aberration Rates

Once aberration rates were established in the young adult and veteran age-matched controls, covariates (e.g. smoking and age) were adjusted for to determine the contribution of IR-induced aberrations to the exposed cohort. Table 2.1 summarizes the relationships between age, smoking and aberration frequencies. Positive correlations of age and smoking with translocation

rates are well established, aging being the greatest contributor to increased background translocation rates (Ramsey et al., 1995; Sigurdson et al., 2008). However, little is known about the influence of age and environmental or lifestyle factors on inversion frequencies. Considering the influence of age alone between the young adult and age-matched controls (Figure 2.3), there is a statistically significant increase in both inversion and translocation rates. Inversions increased by 0.289±0.035 per CE ($p = 0.0013$) and translocations increased by 0.065±0.434 ($p = 0.0024$).

Likewise, smoking was found to increase both inversion and translocation rates. Comparing the ratio of aberration rates between chronic smokers to non-smokers demonstrated a 15% increase in background inversions and 3.77% increase in background translocation rates. Comparisons were only made in the age-matched veteran cohort since none of the Young adults reported smoking. Also, reports suggest that there are no statistically significant changes between smokers and non-smokers in their mid-20's (Sigurdson et al., 2008). Figure 2.4 displays the proportional change in inversion and translocation frequencies with background aberration rates in the non-smoking population held to 1.

Biodosimetry Assessment of Radiation Exposure to the Atomic Veterans

To establish dose estimations based on inversion and translocation rates for the atomic veterans, the covariate influences on background rates were removed. Using the basic equation for dose estimation, $D_i = (R_i - B_i) / CF$, as described in the methods section, estimated doses for each exposed veteran were established based on observed inversion or translocation rates independently (Figure 2.5). Since each variable in the equation for dose estimation is associated with error, uncertainty analysis using the Monte Carlo method with 50,000 simulations was used to propagate the uncertainty. Estimates with a negative dose value are considered to be well below the limit of

detection. Translocations appear to have, in general, less variance than inversions suggesting a more reliable dose estimate, although four of the twelve atomic veterans fell in the non-detectable range. Interestingly, while many of the inversion and translocation rates overlapped for individual veterans, suggesting the dose estimates derived from the two aberration types were are statistically comparable, the error bars for the aberration-specific dose estimates for three veterans did not overlap, an anomaly perhaps due to differences in individual susceptibilities.

In an effort to improve dose estimates for exposures that occurred over six decades ago, we combined aberration rates for inversions and translocations since both resulted from IR exposure (Figure 2.6). To do so, we used a best linear unbiased estimation approach to determine a weighted dose estimate. The inverse variance of each aberration rate was used to emphasize uncertain dose. In other words, more weight was put on the translocation rates than inversion rates since inversions were the more variable aberration. Though the weighted dose estimation did not significantly improve the limit of detection, it considerably improved the correlation with best estimated physical dose reconstructions. The correlation coefficient between the physical dose reconstruction and translocation dose ($r=0.330$) and inversion dose ($r=0.277$) increased to $r=0.552$ when the dose estimates were combined using the best linear unbiased estimator. These results suggest that for IR exposures that occurred several decades in the past, a retrospective biodosimetry approach combining inversion and translocation frequencies can provide a more reliable and possibly accurate estimation of dose than from translocations or inversions independently.

Estimation of minimum detectable dose (MDD)

The estimation of a minimum detectable dose follows the logic presented by the US Department of Commerce (1961) on estimating minimum detectable activity (MDA). A limiting factor in estimating low dose is the same as in estimating low levels of activity, i.e., the variability of the background. In the case of radioactivity measurements, background is related to the performance of the detector system without a sample present. Background measurements, because of their statistical nature, can vary. Similarly, in biodosimetry, the background is the rate of aberration occurrence without any exposure (or at least, with only exposure due to natural radiation that might be considered relatively equal among most people).

There are various ways to estimate MDA with the simplest being the activity estimated at three-times the background standard deviation. That definition was originally put forth by the National Bureau of Standards (1961). More sophisticated methods have since been derived for estimating detection limits for the purpose of minimizing Type I and II statistical errors (Curie 1968, Altshuler and Pasternack 1963). For simplicity, the method of the NBS is used though some relaxation of the confidence level constraints are applied. Three sigma (σ = standard deviation of background) represents a confidence level of 99.7%. Less restrictive constraints, as illustrated by table 2.2, are 95% confidence (1.96 σ) and 99% confidence (2.575 σ).

In this work, multiple assays of the background rate of aberrations, either translocations or inversions, were made from samples taken from six, mid-20-year-old males. The standard error of the mean aberration rate was determined as an estimate of the standard deviation of the "mean sampling distribution." This analysis suggests that the MDD for the exposed veterans varies for the experimental design described in this text from about 0.21 to 0.4 Gy for translocations depending on the certainty level and smoking status, and from about 0.16 to 0.24 Gy for inversions,

also depending on certainty level and smoking status (Table 2.2). It should be understood that the MDD will vary in other studies depending on the experiment design for obtaining background aberration rates.

Influence of Age, Smoking and Radiation Exposure on Telomere Length

Telomeres are an informative biomarker for the atomic veterans because most, if not all, lifestyle and environmental influences are captured as changes in telomere length. Therefore, telomeres represent a particularly relevant biomarker of overall health (Shammas, 2011). Of interest, is the role of low dose ionizing radiation in telomere maintenance. However, little is known in regard to the long-term effects of IR exposure in general on telomere length, a reality highlighted by conflicting results in the few studies that have been reported (Lustig et al., 2016; Reste et al., 2014).

Using a multiplexed quantitative PCR (qPCR) based approach as previously described (Cawthon, 2009), telomere length is normalized to a single-copy gene, albumin, to establish a relative telomere length measurement for each sample. Comparison of young adult controls to age-matched, unexposed veterans suggest that age contributes the most to telomere erosion irrespective of smoking or IR exposure (Figure 2.7). A 35.3% and 39.4% decrease in telomere length was observed between the unexposed (0 Gy) young adult controls and the unexposed age-matched veterans ($p = <0.0001$), and exposed veterans ($p=<0.0001$), respectively. Although not significant, a 6.3% decrease occurred between the 0 Gy and 2.0 Gy γ-ray exposed young adult control samples. However, these samples were only in culture for a single round of replication (~48-56 hours). Likewise, a 6.4% decrease was observed between the unexposed and exposed veterans.

Assessment of smoking status on telomere length between the unexposed and exposed veterans was rather interesting. Age-matched veteran controls that reported a history of chronic smoking displayed a similar decline in telomere length (T/A ratio = 0.874) as exposed veterans who were non-smokers (T/A ratio 0.871), compared to non-smoker age-matched controls (T/A ratio = 0.991) (Figure 2.8). These results infer that chronic smoking over several decades produce a similar attrition rate as those exposed to a low dose nuclear event. Additionally, there did not appear to be an additive effect between a history of chronic smoking and IR exposure.

Furthermore, there is a weak negative correlation (r=-0.399) between telomere length and the weighted combined translocation and inversion doses (Figure 2.9). Atomic veterans with a negative dose estimation, and therefore below the limit of detection, were removed from the analysis. This result supports the notion that IR exposures associated with nuclear detonation events more than six decades ago have lingering effects on general health.

Discussion

The present study of United States WWII veterans exposed to radiation from nuclear testing in the 1950's reveals that in addition to chromosomal translocations, the long-lasting signature of such an IR event also includes inversions and, to a lesser extent, an inverse relationship with telomere length. Consistent with a report from Japanese fishermen exposed during atomic bomb testing in the Bikini Islands (Tanaka et al., 2016), stable aberrations (e.g. translocations) remained elevated above background levels even 60 years post-exposure. Though translocations have been utilized in this context for atomic bomb survivors (Kodama et al., 2001), Chernobyl clean-up workers (Snigiryova et al., 1997), and occupational exposures (Cho et al., 2015), this is

the first report of pericentric and paracentric chromosome inversions as a biomarker for retrospective biodosimetry.

Calibration curves for translocation and inversion rates using peripheral blood samples from six young-adult males in their mid-20's were established with escalating doses up to 2 Gy of γ-rays. Inversions were induced at a greater rate per unit dose (0.4037/CE/Gy) than translocations (0.1281/CE/Gy). However, while no translocations were identified in the unirradiated samples, inversions appeared at a background rate of 0.223 ± 0.124 per cell equivalent. A more recent report of symmetrical rearrangements in human fibroblast cell lines (NHDF4012 and BJ-1 hTERT) also noted a greater induction of inversions per unit dose and a high background rate in the NHDF4012's (Ray et al., 2014). Though it was thought this background rate was inherent to the NHDF4012 cell line, our results suggest spontaneous inversions occur frequently *in vivo*. This may be a reflection of the role spontaneous inversions play in reorganizing the genome, as previously hypothesized (Kirkpatrick, 2010). Additionally, interstitial deletions are the most common IR-induced mutation (National Research Council, 2006), and inversions are an alternative outcome of the same recombinational process (McKenna and Bailey, 2017). Indeed, inversions and interstitial deletions are the two most common mutation signatures associated with radiation-induced secondary malignancies (Behjati et al., 2016). Furthermore, inversions and interstitial deletions occur at a 1:1 ratio (Mühlmann-Díaz and Bedford, 1995), so it is not surprising that inversions are induced at a greater rate per unit dose than translocations.

Moreover, age and smoking status influenced both inversion and translocation rates. Compared to the young-adults, the age-matched veteran controls had a 0.289±0.035/CE increase in background inversions and 0.065±0.043/CE in translocations. Inversions increased by 2.3-fold between the young-adults and >80-year-old individuals, which has never been previously reported.

Likewise, smoking status was directly correlated with background aberration frequencies. No young-adult controls reported a history of smoking, however according to Sigurdson et al. (2008), smoking status did not impact translocation frequencies within this age-range. In our veteran cohort, seven of the atomic veterans and seven age-matched controls reported a history of chronic smoking. A 15% (0.0706/CE) increase in inversion rates and a 3.77% (0.0024/CE) increase in translocation rates was observed due to smoking alone.

To reconstruct dose using independent aberration rates (e.g. translocations or inversions) for each atomic veteran, both age and smoking were accounted for prior to estimating dose and propagating uncertainty by determining the baseline translocation and inversion rates amongst ever-smokers and never-smokers independently. Both translocations and inversions produced dose estimates for 8 out of 12 individuals. Also, 9 of 12 analyzed samples had overlapping mean values and/or variances suggesting that both aberrations were statistically comparable. However, three individuals did not have overlapping variances, suggestive of differences in individual susceptibilities. To improve sensitivity and minimize variance, both translocations and inversion dose estimates were combined by a best linear unbiased estimator.

This was accomplished using a weighted combined dose that utilizes the inverse of the variance for translocations and inversions to establish the weighting. Since translocations had a lower variance across all samples, more weight was placed on the translocation rate than on the inversion rate. By doing so, Pearson's Correlation Coefficient between weighted combined aberration dose and the best estimated physical dose reconstruction improved to r=0.552 compared to r=0.330 for translocations and r=0.277 for inversions alone. Even for the three individuals that did not have overlapping variances for the individual aberration dose estimates, the weight inverse variance dose estimation increased the correlation for the physical dose reconstruction. These

results suggest that for exposures that occurred over six decades in the past, combined translocation and inversion rates provide more reliable and possibly accurate dose estimations for retrospective biodosimetry than independently.

Furthermore, telomere length measurements were assessed for all 30 individuals in the study. It is well established that telomere length erosion occurs with cellular division, and therefore with aging, limiting the replicative lifespan of somatic cells. This reduced replicative capacity has been proposed as a tumor suppressor mechanism preventing aberrant cell survival (Deng et al., 2008). Not only are telomeres associated with a host of diseases, such as cardiovascular disease (Serrano and Andrés, 2004) and cancer (Artandi and DePinho, 2010), but they are also indicative of radiosensitivity (Goytisolo et al., 2000; Wong et al., 2000), further increasing the value of telomeres as a biomarker of overall health.

Not surprisingly, the greatest contribution to telomere length erosion in our cohorts was age. A 35.3% and 39.4% decrease was noted between the unirradiated young adult samples and the age-matched and atomic veterans, respectively (Figure 2.7). Additionally, comparison of smoking status in the age-matched control veterans suggested that long-term, chronic smoking reduced telomere length as much as in veterans exposed to nuclear fallout with no history of smoking (Figure 2.8). The T/A ratio for chronic smokers was 0.874, and for exposed veterans who were non-smokers was 0.871. Interestingly, there appears to be no additive effect between smoking status and exposure for the atomic veterans.

Consistent with reports from atomic bomb survivors (Lustig et al., 2016), our results suggest an inverse relationship between telomere length and exposure to nuclear fallout (Figure 2.9). Telomere length averaged across all exposed samples, that is all young adult samples exposed to 2 Gy γ-rays or the atomic veterans, suggest only a 6.3% and 6.4% decrease, respectively,

compared to their unexposed controls (Figure 2.7). Importantly, when we evaluated telomere length for each individual compared to their estimated weighted combined average, the inverse relationship with escalating dose was apparent. Individuals with negative dose estimations were excluded from the analysis.

Overall, these results verify that stable reciprocal translocations and peri-/para-centric inversions are persistent even six decades after exposure to a nuclear detonation event. With regards to retrospective biodosimetry, under detectable dose conditions, a weighted combined dose using both inversion and translocation rates is more sensitive than either biomarker independently. Additionally, this is the first report of age- and smoking-status influencing background inversion rates, even more than for translocations. While this may slightly hinder their utility as a biomarker for retrospective biodosimetry, their induction rate per unit dose is much greater than translocations. Conditions permitting, if background rates can be accounted for in the same individual, say prior to occupational or therapeutic exposure to IR, inversions may prove to be a more sensitive biomarker of radiation exposure. Lastly, our telomere length results support the idea of telomeres as a biomarker of overall health, especially in the context of radiation exposure. Not only did long-term, chronic smoking shorten telomeres as much as a single IR exposure event, but an inverse relationship between telomere length and weighted combined dose estimates was established. These results further exemplify that despite smoking and exposure, age was still had the greatest influence on telomere shortening.

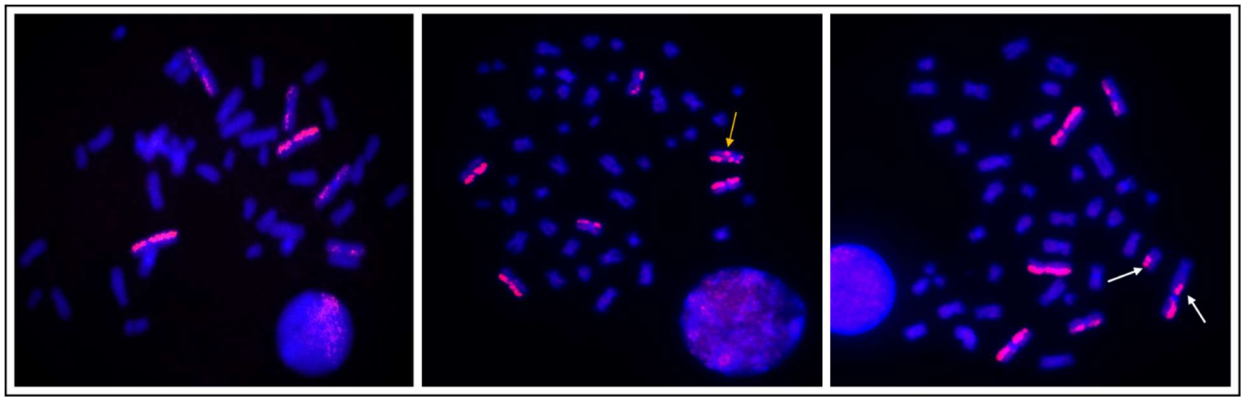

Figure 2.1 Representative images of metaphase spreads labeled with whole chromosome 1, 2 and 3 paints in cyanine-3 (Cy3) by dGH and counter stained with DAPI. Left panel – a normal metaphase spread free of any structural rearrangements. Chromosome paints uniformly label a single sister chromatid of a chromosome. Middle panel- an inversion occurred on chromosome 1 as denoted by the yellow arrow, and the presence of a sister chromatid exchange (SCE). Right panel – a translocation involving chromosome 1 and a second, unpainted chromosome highlighted by the white arrows.

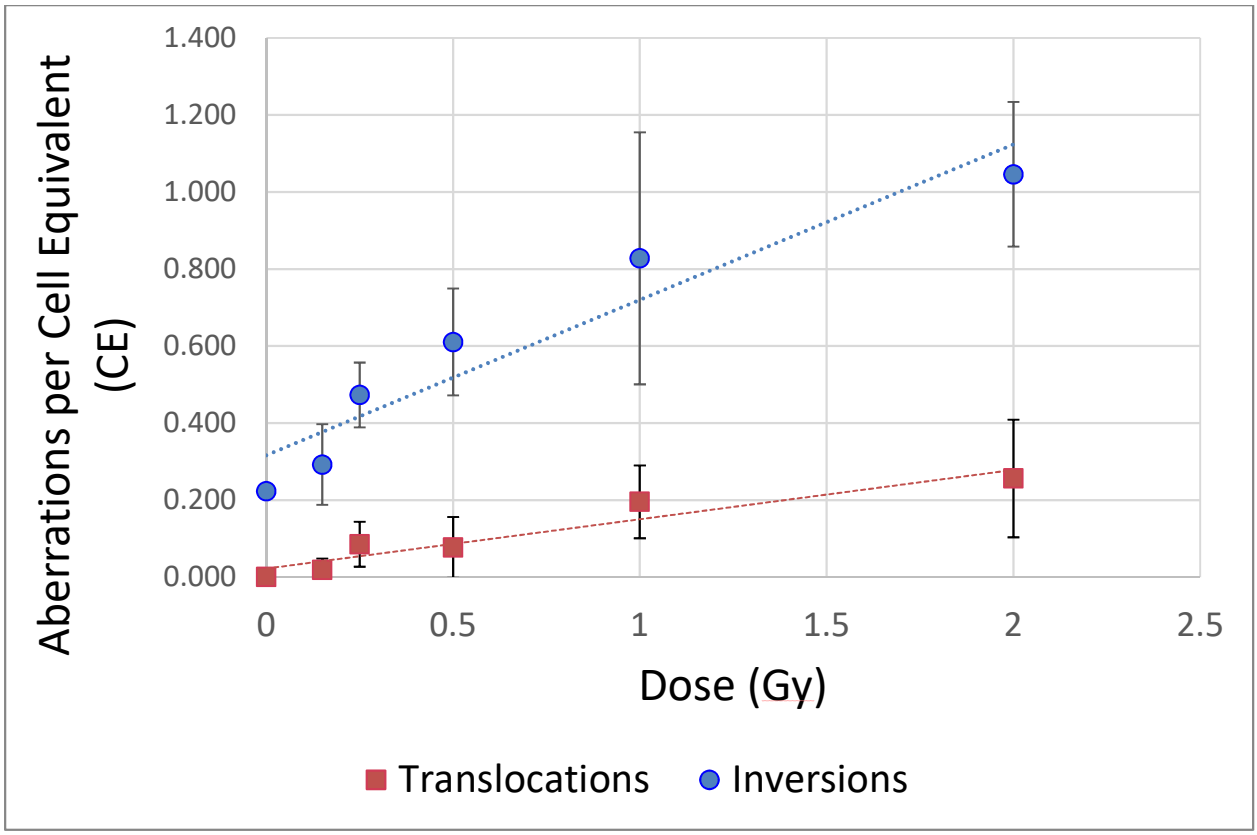

Figure 2.2 Calibration (dose response) curves generated from stimulated peripheral blood exposed to ^{137}Cs γ-rays. Inversions (blue circles) were induced at a greater rate per unit dose than translocations (red squares). However, inversions had a greater spontaneous background rate and more variance associated per dose point. The linear equations were y=0.4037x + 0.3162 for inversions and y=0.1281x+0.022 for translocations. R-squared values were 0.9098 and 0.9091 for inversions and translocations, respectively. Error bars represent the standard deviation.

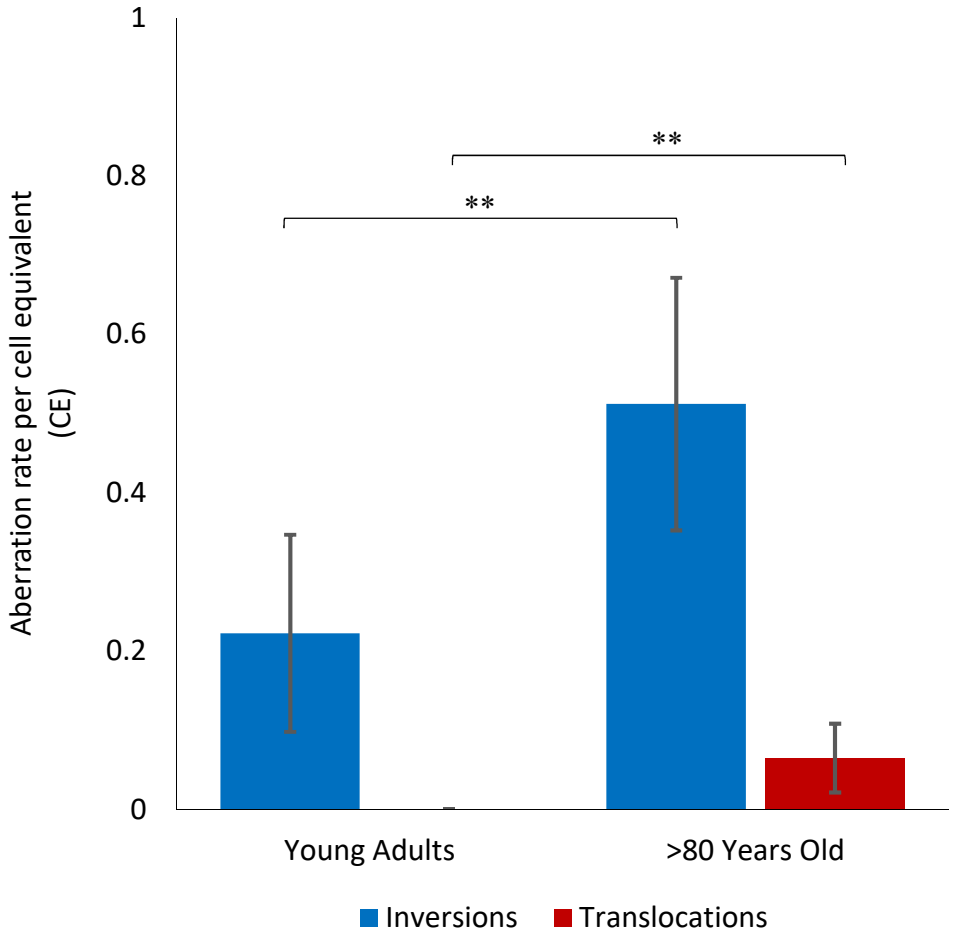

Figure 2.3 Influence of age on background inversion and translocation rates. Inversions (blue) increased by 0.289 ± 0.035 per CE and translocations (red) increased by 0.065 ± 0.434 per CE. Significance was established using a Student's t-test, p=0.0013 and p=0.0024, respectively.

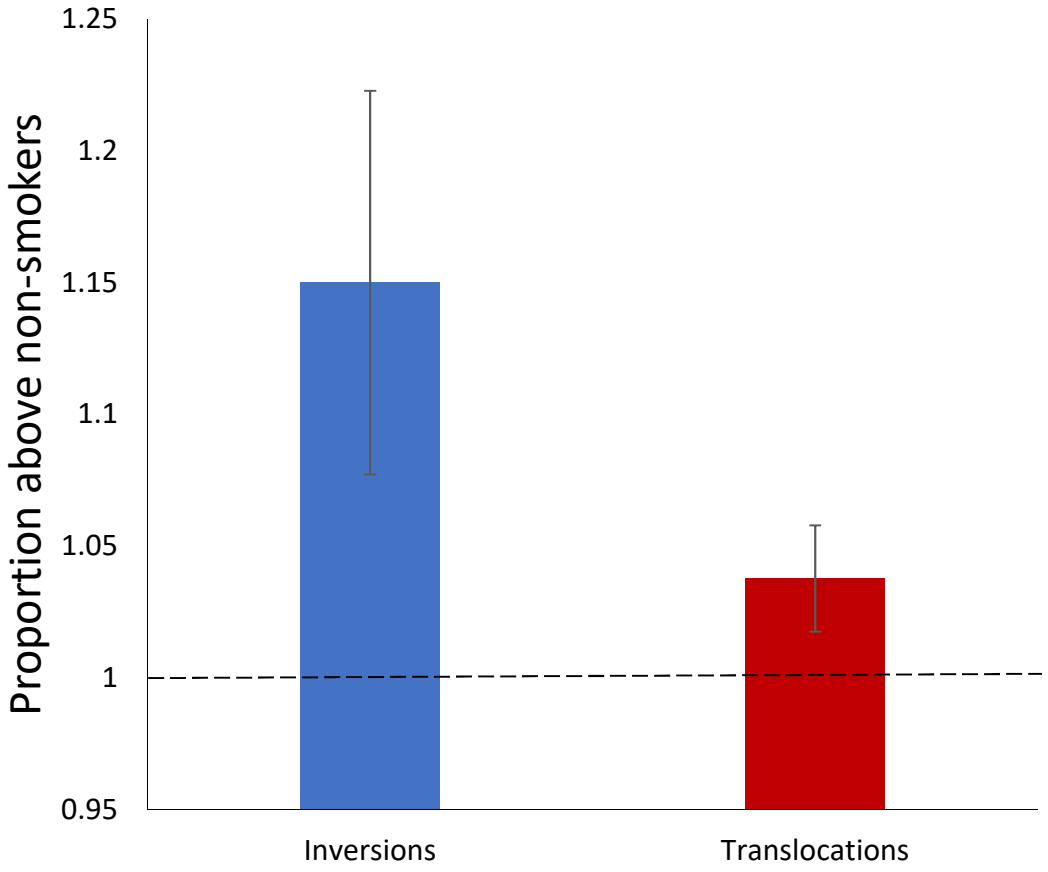

Figure 2.4 Influence of smoking on background inversion and translocation rates. The ratio of aberration rates between smokers and non-smokers in the veteran age-matched control cohort was used to obtain the proportional change in inversions and translocations. Non-smokers were held to 1 (dashed line), Inversions increased by 0.15 and translocations increased by 0.0377. Error bars represent the standard error of the mean.

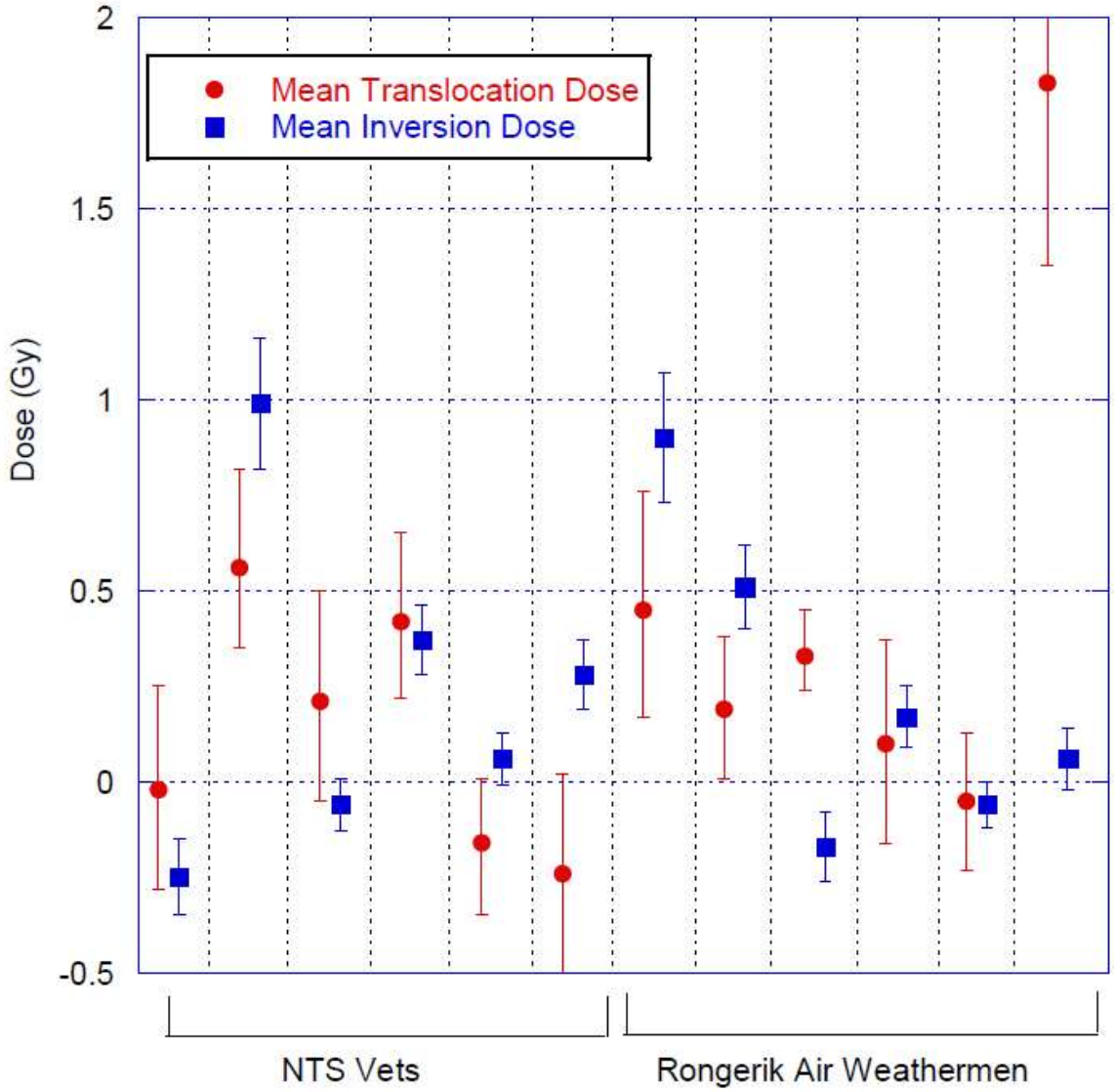

Figure 2.5 Mean dose estimations and 90% confidence intervals for the NTS and Rongerik Atoll veterans. Each vertical dashed line represents a single individual. Dose estimates (Gy) for inversions (blue) and translocations (red) were established independently.

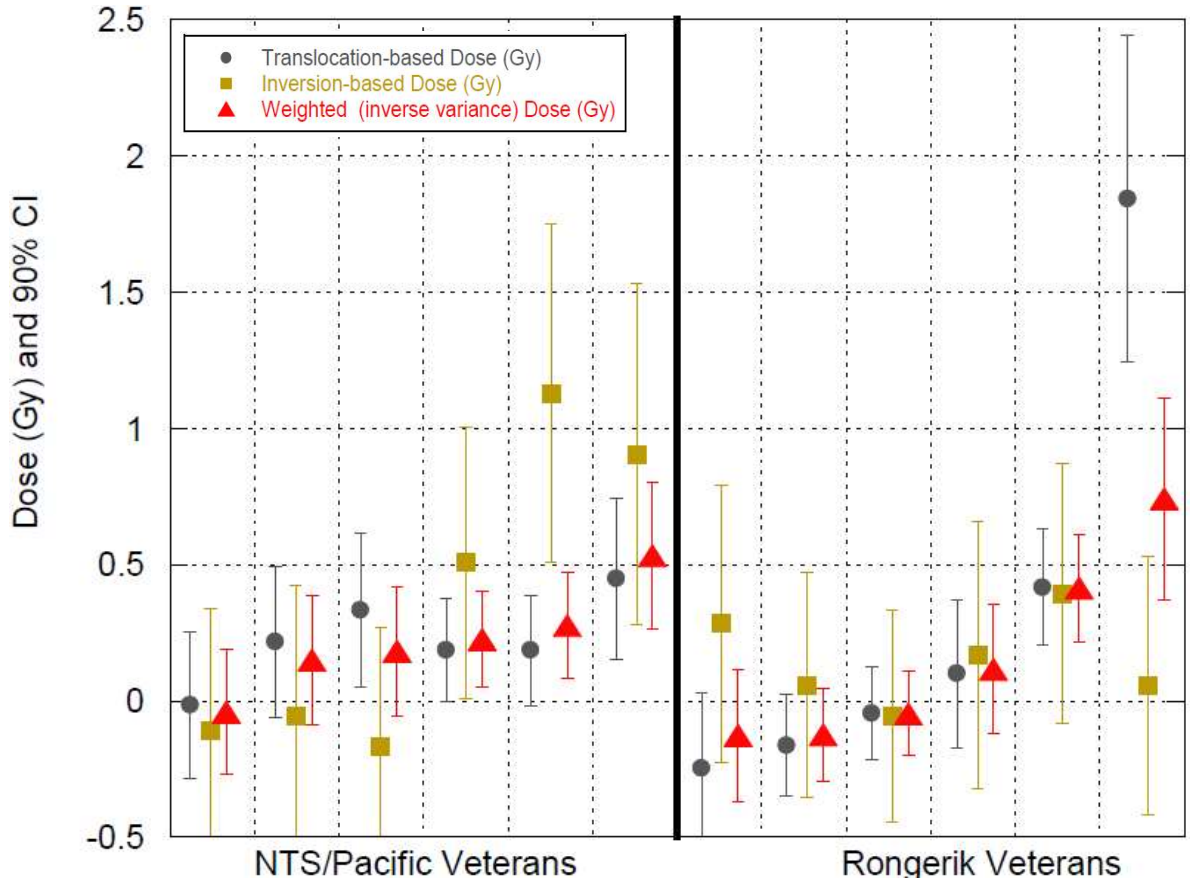

Figure 2.6 Dose estimations and 90% confidence intervals for the NTS and Rongerik Atoll veterans. Each vertical dashed line represents a single individual. Dose estimates (Gy) for inversions (gold) and translocations (gray) were first established independently. A weighted estimation (red) combining inversion and translocation rates considered the inverse variance of each aberration for combined dose estimates.

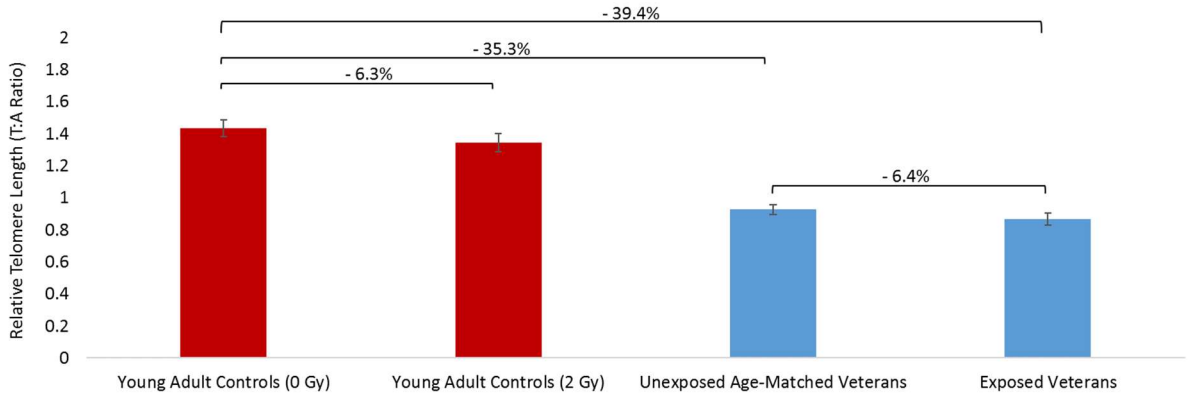

Figure 2.7 Relative telomere length measurements between cohorts based on age and radiation exposure. Values between bars represent percent change between groups. Age contributed a 35.3% and 39.4% decrease in telomere length between unexposed (0 Gy) young adult controls and the unexposed age-matched veterans ($p =< 0.0001$) and exposed veterans ($p =< 0.0001$), respectively. A 6.3% decrease in telomere length were noted between the unexposed (0 Gy) and exposed (2 Gy) young adult controls and 6.4% decrease between unexposed age-matched veterans and exposed veterans. Error bars represent the SEM.

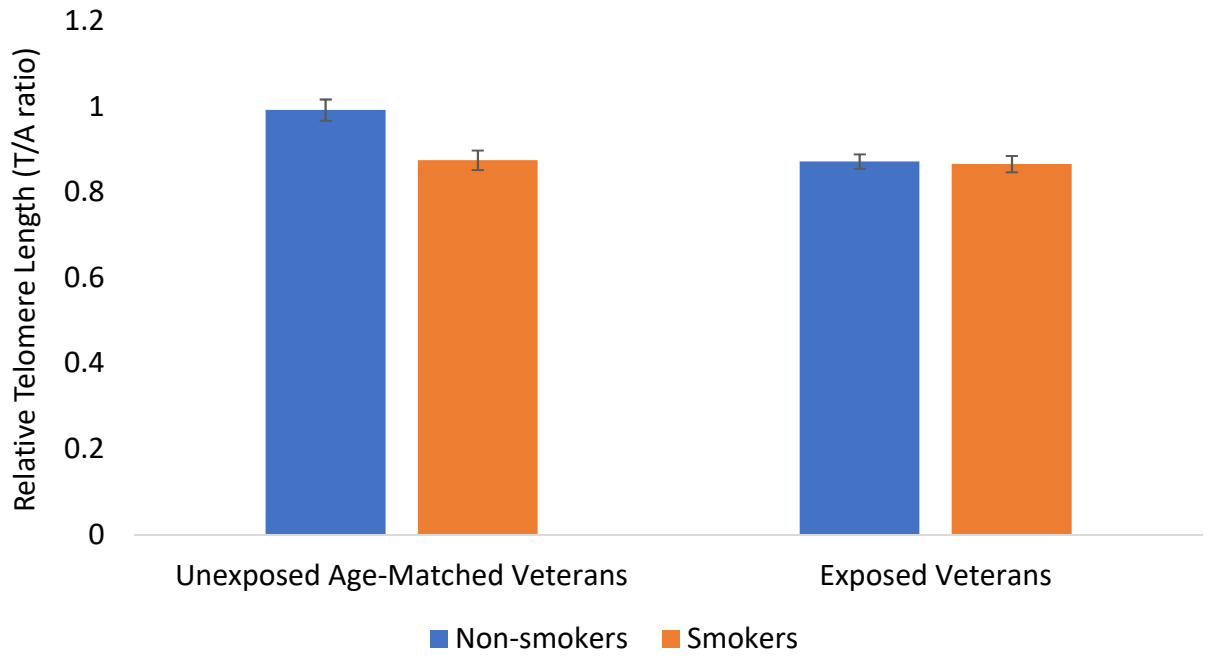

Figure 2.8 Evaluation of telomere length and smoking status and/or IR exposure in the veteran cohorts. Unexposed age-matched veterans with a history of smoking display a similar decrease in telomere length as both exposed, non-smoker and smoker veteran groups. No difference was observed between smokers and non-smokers among the exposed veterans. Error bars represent the SEM.

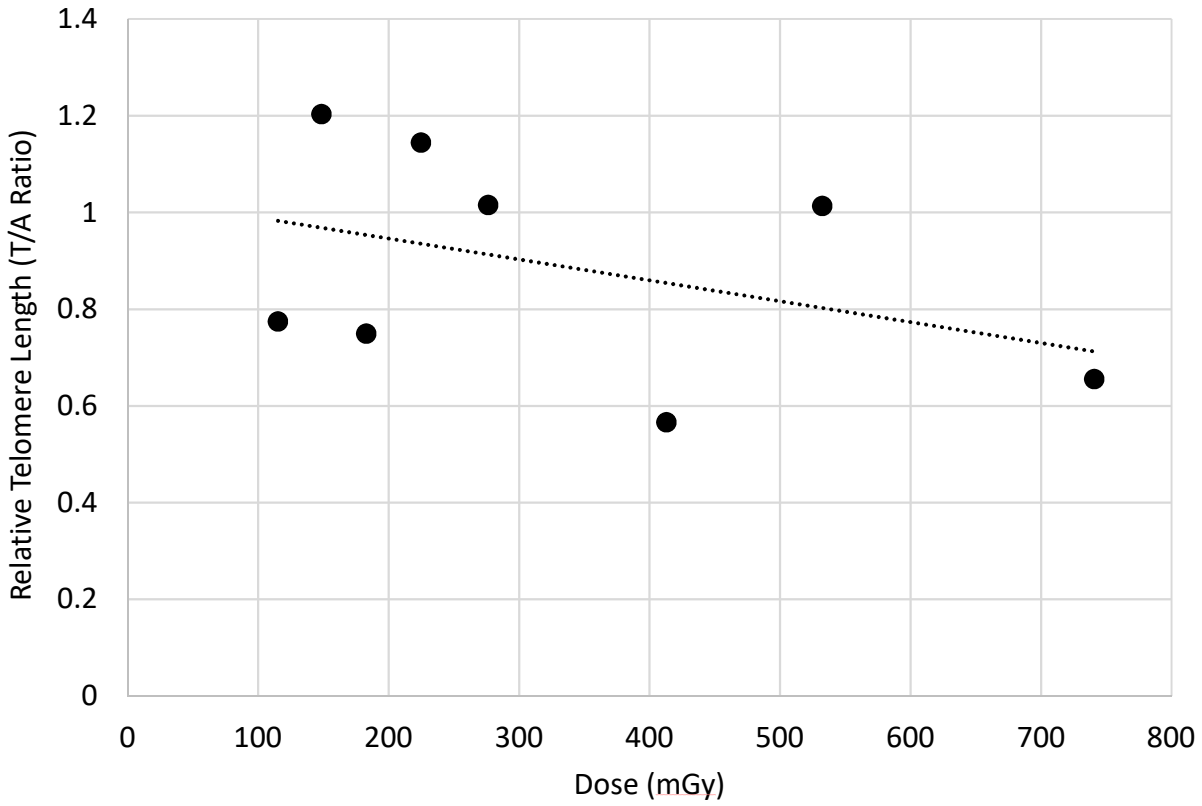

Figure 2.9 Relative telomere length as a function of weighted combined aberration dose among exposed veterans. A weak correlation exists between telomere length and weighted combined translocation and inversion dose (r= -0.399). Veterans with a negative dose estimation were removed from the analysis. The linear equation is y=-0.0004x+1.0324 and $R^2 = 0.1599$.

Table 2.1 Summary of control cohort demographics and covariate influence on background translocation and inversion rates. Aberration rates are expressed as per cell equivalent (CE) as described by Loucas et al., 2016.

	Average Age (years)	Smokers	Non-smokers	Chronic smokers		Non-smokers	
				Translocations per CE	Inversions per CE	Translocations per CE	Inversions per CE
Young adults	27 ± 2.3	0	6	n/a	n/a	0	0.223 ± 0.124
Veteran age-matched controls	85.75 ± 2.76	7	5	0.066 ± 0.053	0.541 ± 0.192	0.0636 ± 0.030	0.471 ± 0.104

Table 2.2 MDD (Gy) as a function of age group, smoking status, and certainty level. [a]Variation of calibration factors for 80-90-year-old males was determined amongst the 25-year old males to simulate exposure at that age. The MDD applies specifically to this experimental design.

	25-year old Males		80-90-year-old Males[a]			
	Never-smokers		Never-smokers		Ever-smokers	
	95% (1.96 σ)	99% (2.575 σ)	95% (1.96 σ)	99% (2.575 σ)	95% (1.96 σ)	99% (2.575 σ)
Translocations	0.00	0.00	0.21	0.27	0.31	0.40
Inversions	0.50	0.66	0.16	0.21	0.18	0.24

REFERENCES

Altshuler B, Pasternack, B (1963) Statistical measures of the lower limit of detection of a radioactivity counter. Health Physics. 9, pp. 293-298.

Artandi, S.E., and DePinho, R.A. (2010). Telomeres and telomerase in cancer. Carcinogenesis *31*, 9–18.

Behjati, S., Gundem, G., Wedge, D.C., Roberts, N.D., Tarpey, P.S., Cooke, S.L., Loo, P.V., Alexandrov, L.B., Ramakrishna, M., Davies, H., et al. (2016). Mutational signatures of ionizing radiation in second malignancies. Nat. Commun. *7*, 12605.

Cawthon, R.M. (2009). Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. *37*, e21–e21.

Cherkas, L.F., Hunkin, J.L., Kato, B.S., Richards, J.B., Gardner, J.P., Surdulescu, G.L., Kimura, M., Lu, X., Spector, T.D., and Aviv, A. (2008). The association between physical activity in leisure time and leukocyte telomere length. Arch. Intern. Med. *168*, 154–158.

Cho, M.S., Lee, J.K., Bae, K.S., Han, E.-A., Jang, S.J., Ha, W.-H., Lee, S.-S., Barquinero, J.F., and Kim, W.T. (2015). Retrospective biodosimetry using translocation frequency in a stable cell of occupationally exposed to ionizing radiation. J. Radiat. Res. (Tokyo) *56*, 709–716.

Curie LA. (1968). Limits of qualitative detection and quantitative determination. Analytical Chemistry. vol. 40, no.3, pp. 586-593.

Deng, Y., Chan, S.S., and Chang, S. (2008). Telomere dysfunction and tumour suppression: the senescence connection. Nat. Rev. Cancer *8*, 450–458.

Goytisolo, F.A., Samper, E., Martín-Caballero, J., Finnon, P., Herrera, E., Flores, J.M., Bouffler, S.D., and Blasco, M.A. (2000). Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals. J. Exp. Med. *192*, 1625–1636.

Howe, B., Umrigar, A., and Tsien, F. (2014). Chromosome preparation from cultured cells. J. Vis. Exp. JoVE.

Kirkpatrick, M. (2010). How and why chromosome inversions evolve. PLOS Biol. *8*, e1000501.

Kodama, Y., Pawel, D., Nakamura, N., Preston, D., Honda, T., Itoh, M., Nakano, M., Ohtaki, K., Funamoto, S., and Awa, A.A. (2001). Stable chromosome aberrations in atomic bomb survivors: results from 25 years of investigation. Radiat. Res. *156*, 337–346.

Lloyd, D.C. (1997). Chromosomal analysis to assess radiation dose. Stem Cells *15*, 195–201.

Loucas, B.D., Shuryak, I., and Cornforth, M.N. (2016). Three-color chromosome painting as seen through the eyes of mfish: another look at radiation-induced exchanges and their conversion to whole-genome equivalency. Front. Oncol. *6*.

Lustig, A., Shterev, I., Geyer, S., Shi, A., Hu, Y., Morishita, Y., Nagamura, H., Sasaki, K., Maki, M., Hayashi, I., et al. (2016). Long term effects of radiation exposure on telomere lengths of leukocytes and its associated biomarkers among atomic-bomb survivors. Oncotarget *7*, 38988–38998.

McKenna, M.J., and Bailey, S.M. (2017). Chromosomal and telomeric biomarkers of normal tissue injury to evaluate risk of degenerative health effects (secondary malignancy, cardiovascular disease) post radiation therapy. Transl. Cancer Res.

Mühlmann-Díaz, M.C., and Bedford, J.S. (1995). Comparison of gamma-ray-induced chromosome ring and inversion frequencies. Radiat. Res. *143*, 175–180.

National Research Council (2006). health risks from exposure to low levels of ionizing radiation: BEIR VII Phase 2 (Washington, DC: The National Academies Press).

Preston, D.L., Shimizu, Y., Pierce, D.A., Suyama, A., and Mabuchi, K. (2003). Studies of mortality of atomic bomb survivors. Report 13: solid cancer and noncancer disease mortality: 1950–1997. Radiat. Res. *160*, 381–407.

Ramsey, M.J., Moore, D.H., Briner, J.F., Lee, D.A., Olsen, L.A., Senft, J.R., and Tucker, J.D. (1995). The effects of age and lifestyle factors on the accumulation of cytogenetic damage as measured by chromosome painting. Mutat. Res. *338*, 95–106.

Ray, F.A., Zimmerman, E., Robinson, B., Cornforth, M., Bedford, J., Goodwin, E., and Bailey, S. (2013). Directional genomic hybridization for chromosomal inversion discovery and detection. Chromosome Res. *21*, 165–174.

Ray, F.A., Robinson, E., McKenna, M., Hada, M., George, K., Cucinotta, F., Goodwin, E.H., Bedford, J.S., Bailey, S.M., and Cornforth, M.N. (2014). Directional genomic hybridization: inversions as a potential biodosimeter for retrospective radiation exposure. Radiat. Environ. Biophys. *53*, 255–263.

Rea, M.E., Gougelet, R.M., Nicolalde, R.J., Geiling, J.A., and Swartz, H.M. (2010). Proposed triage categories for large-scale radiation incidents using high-accuracy biodosimetry methods. Health Phys. *98*, 136–144.

Reste, J., Zvigule, G., Zvagule, T., Kurjane, N., Eglite, M., Gabruseva, N., Berzina, D., Plonis, J., and Miklasevics, E. (2014). Telomere length in Chernobyl accident recovery workers in the late period after the disaster. J. Radiat. Res. (Tokyo) *55*, 1089–1100.

Robinson, E., McKenna, M.J., Bedford, J.S., Goodwin, E.H., Cornforth, M.N., Bailey, S.M., and Ray, F.A. (Submitted). Directional Genomic Hybridization (dGH®) for detection of intra-chromosomal rearrangements. Radiation Cytogenetics: Methods in Molecular Biology

Rothkamm, K., Beinke, C., Romm, H., Badie, C., Balagurunathan, Y., Barnard, S., Bernard, N., Boulay-Greene, H., Brengues, M., De Amicis, A., et al. (2013). Comparison of established and emerging biodosimetry assays. Radiat. Res. *180*, 111–119.

Serrano, A.L., and Andrés, V. (2004). Telomeres and cardiovascular disease. Circ. Res. *94*, 575–584.

Shammas, M.A. (2011). Telomeres, lifestyle, cancer, and aging. Curr. Opin. Clin. Nutr. Metab. Care *14*, 28–34.

Sigurdson, A.J., Ha, M., Hauptmann, M., Bhatti, P., Sram, R.J., Beskid, O., Tawn, E.J., Whitehouse, C.A., Lindholm, C., Nakano, M., et al. (2008). International study of factors affecting human chromosome translocations. Mutat. Res. Toxicol. Environ. Mutagen. *652*, 112–121.

Simon, S.L., Bouville, A., and Kleinerman, R. (2010). Current use and future needs of biodosimetry in studies of long-term health risk following radiation exposure. Health Phys. *98*, 109–117.

Snigiryova, G., Braselmann, H., Salassidis, K., Shevchenko, V., and Bauchinger, M. (1997). Retrospective biodosimetry of Chernobyl clean-up workers using chromosome painting and conventional chromosome analysis. Int. J. Radiat. Biol. *71*, 119–127.

Suntharalingam, N., and Cameron, J.R. (1966). A comparison of TLD and film for personnel dosimetry. Health Phys. *12*.

Tanaka, K., Ohtaki, M., and Hoshi, M. (2016). Chromosome aberrations in Japanese fishermen exposed to fallout radiation 420-1200 km distant from the nuclear explosion test site at Bikini Atoll: report 60 years after the incident. Radiat. Environ. Biophys. *55*, 329–337.

Tawn, E.J., and Whitehouse, C.A. (2003). Persistence of translocation frequencies in blood lymphocytes following radiotherapy: implications for retrospective radiation biodosimetry. J. Radiol. Prot. Off. J. Soc. Radiol. Prot. *23*, 423–430.

Tucker, J.D., Cofield, J., Matsumoto, K., Ramsey, M.J., and Freeman, D.C. (2005). Persistence of chromosome aberrations following acute radiation: I, PAINT translocations, dicentrics, rings, fragments, and insertions. Environ. Mol. Mutagen. *45*, 229–248.

U.S. Dept. of Commerce, NBS Handbook #80, A manual of radioactivity procedures, p. 27, 1961.

Valdes, A.M., Andrew, T., Gardner, J.P., Kimura, M., Oelsner, E., Cherkas, L.F., Aviv, A., and Spector, T.D. (2005). Obesity, cigarette smoking, and telomere length in women. Lancet Lond. Engl. *366*, 662–664.

Vaurijoux, A., Gruel, G., Voisin, P., and Roch-Lefèvre, S. (2012). Biological dosimetry of ionizing radiation. INTECH Open Access Publisher.

Wong, K.K., Chang, S., Weiler, S.R., Ganesan, S., Chaudhuri, J., Zhu, C., Artandi, S.E., Rudolph, K.L., Gottlieb, G.J., Chin, L., et al. (2000). Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. Nat. Genet. *26*, 85–88.

Yamada, M., Wong, F.L., Fujiwara, S., Akahoshi, M., and Suzuki, G. (2004). Noncancer disease incidence in atomic bomb survivors, 1958-1998. Radiat. Res. *161*, 622–632.

CHAPTER 3

ASSESSING TELOMERE LENGTH, TELOMERASE ACTIVITY AND BIODOSIMETRY IN TWIN AND UNRELATED ASTRONAUTS

Summary

Human chromosomes are capped by telomeres, tandem arrays of repetitive DNA sequence and associated shelterin proteins. Functional telomeres prevent these natural DNA ends from being recognized as broken DNA, triggering aberrant DNA damage responses and propagating instability. With cellular division, and therefore aging, telomere length erodes due to the end-replication problem. However, it is becoming increasingly appreciated that telomere length is also influenced by a host of lifestyle factors including nutrition, stress (e.g., physical and psychological), infection, inflammation, as well as environmental exposures (e.g., cigarette smoke, UV, and ionizing radiations). Telomerase is the specialized reverse transcriptase responsible for telomere maintenance via template-mediated addition of repeats onto newly replicated chromosome ends; however, it is only sufficiently active in germline, stem, and cancer cells to do so. Telomere maintenance is a key component of the cumulative effects of genetic, environmental, and lifestyle factors. Over time, telomere dynamics can provide an integrative biomarker of aging and age-related pathologies, ranging from reduced immune function and loss of fertility, to cardiovascular disease and cancer.

Telomere maintenance represents a relevant and informative biomarker of risk for astronauts, as it reflects the combined exposures and experiences encountered during spaceflight.

Genetic susceptibilities, exposure to galactic cosmic rays (GCRs), physical and psychological stressors, are all captured as changes in telomere length. Our overall objective was to identify and define the risk of accelerated telomere shortening and changes in telomerase activity associated with spaceflight aboard the International Space Station (ISS). Chromosome aberrations were also assessed as a means of evaluating the biological effects of ionizing radiation (IR) exposure. Previous biodosimetry studies have monitored translocation frequencies in the astronauts, however, ours is the first to also consider inversion rates, a valuable biomarker of high LET radiation exposure and signature of radiation-induced cancers.

For ISS missions ranging from ~six months to one year, we evaluated twin (Scott and Mark Kelly) and unrelated astronauts pre-flight (to establish baselines), in-flight (to evaluate short-term, transient changes), and post-flight (to evaluate long-term, permanent changes). For the first time, this study demonstrates changes in human telomere dynamics associated with spaceflight. Results suggest that astronauts in general have shorter telomeres and lower levels of telomerase activity than healthy age/gender-matched controls. Further, space flight transiently increased telomere length, which returned to baseline levels within days to weeks upon return to earth. Biodosimetry analyses of Scott Kelly's chronic, low-dose rate IR exposure during 340 days spent in space, revealed increased frequencies of translocations and inversions, with inversions being particularly informative. Mr. Kelly's cumulative physical dose was measured at 76 mGy, which corresponded to an effective dose equivalent of 146.34 mSv when corrected for exposure to mixed IR qualities (high LET protons, secondary neutrons and HZE particles).

Introduction

The ends of human chromosomes are capped by telomeres, tandem arrays of repetitive DNA sequence (5'-TTAGGG$_n$-3') bound by a plethora of associated proteins, which serve to protect chromosomal termini from inappropriate degradation and loss (Moyzis et al., 1988; Webb et al., 2013). Telomeres also function in preserving genomic stability by preventing natural chromosomal ends from being recognized as broken DNA (double-strand breaks; DSBs) and triggering inappropriate DNA damage responses (Longhese, 2008; O'Sullivan and Karlseder, 2010). It is well established that telomere length erodes with cellular division, and therefore aging, due to the end-replication problem, a scenario that causes telomeres to shorten until reaching a critically short length, at which point a permanent cell cycle arrest, known as cellular senescence, occurs (Olovnikov, 1973; Watson, 1972). Healthy lifestyles and diets are positively correlated with telomere length (Cherkas et al., 2008), while chronic physical (e.g. oxidative) and psychological stress and are negatively correlated (Cherkas et al., 2008; Epel et al., 2004). Indeed, recent research supports telomere maintenance as a key integrating component for the cumulative effects of genetic, environmental, and lifestyle factors on aging (Aubert and Lansdorp, 2008). That is, the *rate* at which telomeres shorten provides an informative biomarker of biological aging (Epel et al., 2004). Further, telomere dysfunction and/or decline of telomere length can be linked to age-related degenerative pathologies, ranging from reduced immune function and dementia (Damjanovic et al., 2007), to cardiovascular disease (CVD) and cancer (Serrano and Andrés, 2004; Svenson et al., 2008).

Moreover, radiation exposures (Sishc et al., 2015), and healthy diet and lifestyles (Paul, 2011), have also been shown to modulate telomerase activity. Telomerase is the specialized reverse

transcriptase capable of *de novo* addition of telomeric repeats onto the ends of newly synthesized telomeres. However, while telomerase activity is prominent in highly proliferative cells like stem, germ-line and cancer, it is virtually absent in normal somatic cells leading to telomere erosion (Shay and Wright, 2010). Of relevance in this regard are our demonstrations of elevated telomerase activity following ionizing radiation (IR) exposure, which was associated with enrichment of critical stem cell compartments, suggestive of an important role in carcinogenesis (Sishc et al., 2015). Further, and consistent with recent reports of long term alterations in telomere length maintenance in Chernobyl recovery workers (Reste et al., 2014) and A-bomb survivors (Lustig et al., 2016), telomere shortening was observed in peripheral blood mononuclear cells (PBMCs) at times post IR exposure (Sishc et al., 2015). Such findings also have implications for a variety of degenerative late effects, particularly CVD, as an inverse relationship between telomere length in peripheral blood cells and coronary heart disease has been demonstrated (Haycock et al., 2014). A large remaining puzzle for NASA astronauts are the relationships between telomere length, telomerase activity and aging, and a host of space-flight specific factors.

We speculate that for the astronauts, telomere maintenance represents a particularly relevant and informative biomarker of risk, as it reflects the combined exposures and experiences encountered during spaceflight. That is, an individual's genetic susceptibilities, exposures to galactic cosmic radiation, the nutritional, physical and psychological stressors encountered, are all integrated and captured as changes in telomere length. Indeed, our on-going work is – *for the first time* – demonstrating changes in human telomere dynamics associated with space flight, supporting the view of telomeres as informative and integrative biomarkers**.** We hypothesized that changes in telomere length and telomerase activity will be associated with spaceflight in a time dependent manner.

Telomere length and telomerase activity were assessed in astronauts and healthy age/gender-matched controls over time; i.e., pre-flight (to establish baseline), in-flight (to evaluate short-term/temporary changes), and post-flight (to evaluate long-term/permanent changes). Importantly, such changes were monitored in monozygotic twin astronauts, Scott and Mark Kelly. The NASA Twins Study is the first biological study to evaluate the influence of spaceflight on twins – individuals of similar "nature and nurture". In a collaborative effort, 11 universities and institutions monitored both twins during the one-year ISS mission for changes not only in telomere and telomerase dynamics, but also the influence of spaceflight on the genome, transcriptome, proteome, metabolome, microbiome, as well as fluid shifts and cognitive function. Scott Kelly was the first NASA astronaut to spend almost a full year in space. His total duration aboard the ISS was 340 days, for a combined career total of 520 days. Mark Kelly has a career total of 54 days logged between four space shuttle missions, and remained on earth during the one-year mission. Results from the space- and ground-based twins were compared to identify spaceflight specific factors, independent of genetic susceptibilities, that influence telomere maintenance, and therefore aging and disease risk. Though Scott Kelly started with shorter telomeres than Mark prior to the one year mission, a transient increase in telomere length was identified mid-flight, which returned to near his pre-flight levels within days to weeks. In support of such an unexpected result, the same transient, space-flight specific increase in telomere length was seen in unrelated astronauts as well, though the extent of change varied by individual, which indicates a genetic component of susceptibility. Lastly, biodosimetric analyses of chromosome aberration rates demonstrate a convincing dose-dependent increase in inversion frequencies, which was not as prominent when considering translocation rates. These results are consistent with previously reported higher

induction rates of inversions than translocations per unit dose, as well as higher induction of inversions by high LET particles (Ray et al., 2014).

Materials and Methods

Study Cohort and Design

The astronaut cohort is composed of 10 unrelated astronauts, 2 twin astronauts, and 12 age- and gender-matched controls. Of the astronauts, 2 were females and 10 were males. All individuals are considered generally healthy and were required to pass annual physical examinations per NASA regulations. Two to three peripheral blood samples were collected from each astronaut and their control on average every 90-120 days prior to launch (L-270, L-180 and L-60). Two mid-flight peripheral blood samples were collected for three of the twelve astronauts, and then immediately upon returning to earth (R+2-7 days post-flight) followed by every 60-90 days out to 270 days post-flight (R+60, R+180, and R+270). Collections for the unrelated astronauts are currently ongoing and will be completed in 2018.

Sample Collection and Blood Stimulation for Telo-FISH and dGH

Peripheral blood from consenting astronauts and age-/gender-matched controls were drawn and collected in one 10 mL sodium heparin tube (Becton, Dickinson, and Co #367874) and one 10 mL ethylenediaminetetraacetic acid (K_2EDTA) tube (Becton, Dickinson, and Co #368589) in accordance with institutional review board approval. Samples were shipped under ambient conditions and received at Colorado State University within 24 hours of blood draw. The heparinized blood was split into two T-25 flasks and stimulated for 48 hours at 1:9 split in Gibco

PB-Max Karyotyping Medium (ThermoFisher #12557021) supplemented with phytohaemagglutinin A (PHA) by the manufacturer. For Directional Genomic Hybridization (dGH®), 5.0 mM 5-bromo-deoxyuridine (BrdU) and 1.0 mM 5-bromo-deoxycytidine (BrdC) were added to the medium as described by Ray and Robinson (Ray et al., 2013; Robinson et al., Submitted). The BrdU and BrdC analogs were not added to the medium for telomere FISH (Telo-FISH) on metaphase spreads. Four hours prior to harvest, KaryoMax Colcemid (ThermoFisher #15210040) was added at 0.1 μg/mL. The stimulated blood was then harvested and metaphase chromosome spreads prepared using standard cytogenetic techniques (Howe et al., 2014).

Blood Processing for qPCR Telomerase Activity and Telomere Length

Blood samples collected in K¬2EDTA tubes were transferred to CPT mononuclear cell preparation tubes (Becton, Dickinson, and Co # 362761) and centrifuged at 1700xg for 20 minutes. The peripheral blood mononuclear cells (PBMCs) were collected and washed in PBS. Any remaining red blood cell contamination was removed using ACK lysis buffer (CSHP, 2014). The PBMCs were then separated for either quantitative RT-PCR based telomerase activity or qPCR based-telomere length measurements at $1x10^6$ cells / mL. PBMC protein lysates were prepared in mammalian protein extraction buffer (M-PER) (ThermoFisher #78503) containing cOmplete Protease Inhibitor Cocktail (Millipore Sigma #11836170001) and RNasin Ribonuclease Inhibitors (Promega #N2515) at $1x10^6$ cells per 100 μL. Lysates were cleared by incubating on ice for 10 min. followed by centrifugation at 18,000 RPM for 15 minutes at 4ºC, aliquoted, and stored at -80ºC. Protein concentration was determined using the Bio-Rad Protein Assay Kit (Bradford Assay) (Bio-Rad # 5000001). DNA extraction for qPCR telomere length measurements was performed using the DNeasy Blood and Tissue Kit (Qiagen #69504). PBMCs were initially incubated in

proteinase K for 3 hours at 37°C, rather than 10 minutes at 56°C as specified by the kit's protocol. Additionally, DNA isolated from CD4+ and CD8+ T-cells, CD19+ B-cells, lymphocyte depleted (LD), and whole PBMC populations at multiple timepoints for the twin subjects were appreciatively obtained from Dr. Christopher Mason at Weill Cornell Medical College.

Telomere Fluorescence in situ Hybridization (Telo-FISH)

Slides prepared for metaphase chromosome spreads were washed in PBS for 5 min., dehydrated through a cold ethanol series (75%, 85% and 100%) for 2 min. each, and air dried. The slides were then denatured in a 70% formamide/2x sodium chloride and sodium citrate (2xSSC) solution at 75°C for 2 min. followed by a second cold ethanol series. A G-rich $TTAGGG_3$ peptide nucleic acid (PNA) telomere probe labeled in Cyanine-3 (Cy3; Biosynthesis) was prepared by diluting 0.5 µL of probe in 36 µL of formamide, 12 µL of 0.5 M pH 7.5 Tris-HCl, 2.5 µL of 0.1 M KCl, and 0.6 µl of 0.1 M $MgCl_2$ for a final probe concentration of 5nM. The hybridization mixture was then incubated at 75°C for 5 minutes, and 50 µL was directly added to each slide. Coverslips were added and sealed with rubber cement then incubated at 37 °C for 18 hours. After hybridizing, the slides were washed in a series of 43.5 °C washes for 3 min. each: washes 1 and 2: 50% formamide in 2x sodium chloride and sodium citrate (2xSSC); washes 3 and 4: 2x SSC; and washes 5 and 6: 2x SSC plus 0.1% Nonidet P-40. Slides were counterstained with 50 µL of DAPI in Prolong Gold Antifade (ThermoFisher #P36931), coverslipped, and stored at 4°C.

Metaphase spreads (30-50 per sample) were imaged on a Nikon Eclipse Ni-U epifluorescent microscope equipped with an Andor Zyla 5.5 sCMOS camera and SpectraX LED light source. Stacked images were taken of each metaphase spread composed of 5 images per stack with a step size of 0.3 µm. Fluorescence was then quantified using the ImageJ (NIH) plugin,

Telometer (de Marzo Lab) and normalized using the mouse lymphoma control cell line, LY-S, due to their stable telomere length (Wong and Slijepcevic, 2004).

Directional Genomic Hybridization (dGH)

To identify inversions and translocations simultaneously, Directional Genomic Hybridization utilizing single color whole chromosome 1, 2 and 3 paints (KromaTiD Inc., Ft. Collins, CO) was performed as described by Ray and Robinson (Ray et al., 2013; Robinson et al., Submitted). Briefly, slides containing metaphase spread preparations substituted with bromonucleotides (BrdU and BrdC) were submersed in Hoechst 33258 (Millipore Sigma #B1155) for 15 minutes, selectively photolysed using a SpectroLinker UV Crosslinker equipped with 365 nm UV bulbs for 35 minutes, and the nicked DNA was exonucleolytically degraded with Exonuclease III (New England Biolabs #M0206L) for 30 minutes. The hybridization mixture containing single-color (Cyanine-3) chromosome 1, 2 and 3 paints was provided by KromaTiD, Inc. (Fort Collins, CO) and applied to the slides. Cover slips were applied and sealed using rubber cement then denatured at 68°C for 3 minutes. The slides were hybridized overnight at 37°C followed by five washes in 2x SSC at 43°C prior to imaging. Metaphase spreads were imaged on a Nikon Eclipse Ni-U epifluorescent microscope equipped with an Andor Zyla 5.5 sCMOS camera and SpectraX LED light source. Stacked images were taken of on average 50 metaphase spreads per sample composed of 5 images per stack with a step size of 0.3 μm.

Quantitative Real Time PCR (q-RT PCR) Telomere Repeat Amplification Protocol (TRAP Assay)

Telomerase activity was monitored using the quantitative real time polymerase chain reaction (q-RT PCR) telomere repeat amplification protocol (TRAP) assay as previously described

(Herbert et al., 2006; Hou et al., 2001). The protein lysates were suspended at 0.20 µg in 25 µL of SYBR green GoTaq qPCR master mix (Promega #A6001) containing 0.20 µg T4 gene 32 protein (New England Biolabs #M0300S), 0.10 µg of primers TS (5'-AATCCGTCGAGCAG AGTT- 3') and ACX (5'-GCGCGG(CTTACC)$_3$CTAACC-3') (Integrated DNA Technologies) and RNase/DNase free water. The q-RT PCR reactions took place using a Bio-Rad CFX-96 qPCR machine using the following steps: Step 1- telomerase elongates the TS primer for 1 cycle at 25℃ for 20 min.; Step 2- telomerase is then heat-inactivated for 1 cycle 95℃ for 3 min.; Step 3- PCR amplification and detection for 40 cycles of 95℃ for 20 sec., 50℃ for 30 sec. and 72℃ for 1min. 30 sec.; Step 4- establish a melt curve and prevent primer dimerization for 80 cycles for 10 sec. per cycle. Each sample was run in triplicate on a 96-well plate format allowing for an average Cq to be obtained per sample. Relative telomerase activity was then established using the Delta Delta Ct method (Livak and Schmittgen, 2001). In addition to the test samples, the following controls were included: no template controls with TS only, ACX only, and TS/ACX primers combined, as well as a HeLa heat inactivated control to determine background noise, and HeLa cell lysate as a positive control since HeLa cells are notorious for high telomerase activity.

Multiplexed Quantitative PCR Telomere Length Measurement

Multiplexed quantitative PCR measurements of telomere length were carried out as previously described by Richard Cawthon (2009). Briefly, a 22 µL master mix was prepared using SYBR green GoTaq qPCR master mix (Promega #A6001) combined with the telomere forward primer (TelG; 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'), telomere reverse primer (TelC; 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCT AACA-3'), albumin forward primer (AlbU; 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGA

AATGCTGCACAGAATCCTTG-3'), albumin reverse primer (AlbD; 5'-GCCCGGCCCGCCG CGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3') at 10 μM per primer (Integrated DNA Technologies), and RNase/DNase free water. To the master mix, 3 μL of DNA at 3.33 ng/uL was added for a final volume of 25 μL. The TelG/C primers were at a final concentration of 900 nM and the AlbU/D primers at 400 nM.

Telomere length measurements were carried out using a Bio-Rad CFX-96 qPCR machine. The cycle design was as follows: Step 1- 1 cycle at 95°C for 3 min. to heat inactivate the Taq polymerase; step 2- 2 cycles at 94°C for 15 sec. and 49°C for 15 sec. to anneal and extend the telomere primers; step 3- 32 cycles at 94°C for 15 sec., 62°C for 10 sec., 74°C for 15 sec., 84°C for 10 sec., and 88°C for 15 sec. to melt the early-amplified telomere products, followed by annealing and extension of the albumin primers. The melt curve was established by a 72°C to 95°C ramp at 0.5°C/sec. increase with a 30 second hold. Multiplexing both telomere and albumin primers using a single fluorescent DNA-intercalating dye is possible because the telomere primers are amplified at a lower quantification cycle (Cq) than the albumin primers. Standard curves were prepared using human genomic DNA (Promega) with 3-fold dilutions ranging from 50 ng to 0.617 ng in 3 μL per dilution. Negative controls include a no-template TelG/C only and AlbU/D only, and a combined TelG/C and AlbU/D control. Samples were normalized across plates using a human genomic DNA standard. Each sample was run in triplicate on a 96-well plate format and relative telomere length was established using a telomere (T) to albumin (A) ratio.

Statistical Methods

Statistical significance was established using a two-tailed Student's t-test or one-way ANOVA for multiple comparisons on Graph Pad Prism 5 software for Windows. Normality was assessed prior to running the t-tests.

Results

Telomerase Activity in Astronauts and Age-Matched Controls

Telomerase activity was monitored pre- and post-flight for all 12 astronauts and their age matched controls. Unfortunately, telomerase activity was lost in all mid-flight samples collected from three of the astronauts; other in-flight sampling was unexpectedly and abruptly cancelled by NASA. Loss of activity was most likely due to transit conditions (e.g. time and temperature) between blood collection on the ISS and processing at Johnson Space Center (JSC) (Figure 5.1). Although not statistically significant, astronauts in general had slightly lower levels of telomerase activity than their controls, and telomerase activity was slightly lower post-flight than pre-flight (Figure 3.1). This may, in part, be due to the rigorous physical and psychological training regimens astronauts constantly endure.

Comparisons between the twins, Scott and Mark Kelly, showed similar trends. Scott appears to have slightly lower levels of telomerase activity pre-flight and post-flight, compared to his identical twin brother, Mark. Interestingly, the twins experienced an emotional personal life event shortly prior to the R+270 blood collection. Figure 3.2A illustrates the dramatic increase in post-flight telomerase activity in both individuals, attributable to the R+270 samples. Since this increase in telomerase activity was noted 270 days after return from space, and was apparent in

both twins, it was not associated with spaceflight. Importantly however, this is the first report of a significant telomerase response to psychological trauma in humans. Upon omission of the R+270 sample, no statistically significant differences in telomerase activity between or within the twins' sample collections were observed (Figure 3.2B).

Influence of Spaceflight on Telomere Length Dynamics

Telomere length dynamics are an informative biomarker for the combined exposures and experiences during spaceflight. Like telomerase activity, overall telomere length in the astronaut cohort appeared to be slightly shorter than their age- and gender-matched controls pre-flight ($p=0.023$) (Figure 3.3). Intriguingly, a transient *lengthening* of telomeres occurred for all mid-flight samples, as assessed by both qPCR and TeloFISH. The qPCR based assay provided average telomere length estimates of whole PBMC populations (Figure 3.3A), while Telo-FISH enabled cell-by-cell analyses of telomere length dynamics specifically in T-cell populations, due to preferential stimulation of T-cells with phytohemagglutinin A (PHA) (Figure 3.3B). In-flight telomere lengthening returned to baseline levels within days to weeks of returning to earth, and was again evident with both assays. Since mid-flight telomerase activity was lost due to transit conditions, DNA integrity was assessed by gel electrophoresis. There was no significant DNA degradation of the mid-flight samples compared to pre- or post-flight samples (Figure 5.2).

Analysis of telomere length dynamics in the twin astronauts, show similar trends as seen in the unrelated astronaut cohort. Prior to flight, Scott Kelly displayed very similar telomere length as his brother Mark (Figure 3.4A/B), suggesting a strong genetic component. Spaceflight resulted in a transient increase in telomere length, which then returned to near baseline levels post-flight. Results from PBMC telomere length analyses suggest statistically significant increases in Scott

telomere length compared to his pre-flight sample collections (p=0.048), and to Mark's relative telomere length (p=0.007). Though not statistically significant, analysis of T-cells supports similar trends. By monitoring both twins' telomere length over time (Figure 3.4C/D), we see that telomere length remained elevated during Scott's time in space, which then retuned to baseline levels by 60 days after flight (R+60). Results from unrelated astronauts suggest a rapid decrease (within days) in telomere length once back on earth (data not shown). While it may be possible that such changes in telomere length for Scott Kelly mid-flight could be due to cell population dynamics (e.g. a change in the ratio of circulating cell populations), we believe evidence suggests otherwise.

Comparison of Scott Kelly's pre-flight (L-180) vs. mid-flight (FD140) telomere length distributions, generated from Telo-FISH cell-by-cell analyses, provides evidence of two concurrent dynamics (Figure 3.5). First, there is a dramatic reduction in the number of cells possessing short telomeres mid-flight (FD140), denoted by the non-overlapping relative fluorescence intensity (RFI) of the telomere populations on the left side of the histogram (short telomeres); i.e., a shift to the right (higher RFI) occurs at FD140. Interestingly, cells containing short telomeres have been known to be more radiosensitive than those with longer telomeres (Goytisolo et al., 2000; Wong et al., 2000), potentially explaining the loss of cells with short telomeres; i.e., they do not survive and so are lost from the population. Secondly, there is a slight increase in the number of cells with longer telomeres mid-flight, seen as a shift to the right (higher RFI) for FD140. This rightward shift may be due to new cells entering circulation, e.g. asymmetrically dividing stem cells would possess longer telomeres than more differentiated cells that have divided multiple times, or telomerase upregulation/activation may be acting to lengthen telomeres of circulating cells.

In an effort to validate our results and rule out any possibility that mid-flight blood samples were somehow compromised during transit or from processing, and so were giving false positive/increased telomere length values, an independent lab well versed in qRT-PCR telomere length measurements on large cohorts also ran our samples (Twins investigators Rana and De Vivo); similar trends of increased telomere length in-flight were observed. Furthermore, we performed qPCR telomere length measurements on additional Twins PBMC samples (isolated DNA) collected by an independent lab at Weill Cornell Medical College (Twins investigator Mason). Frozen samples from Cornell were combined with ambient return samples prepared at Colorado State University (CSU) for both twins (Figure 3.6). While Mark Kelly's telomere length remained relatively steady over the course of the study, Scott Kelly's telomere length increased in-flight at all ten time points analyzed (FD14 – FD334), then shortened immediately upon return to earth, rebounding to near baseline levels over the following months. Collectively, these results support increased telomere length in-flight as a real phenomenon, and not an artifact of processing or transit.

Telomere Length in Circulating Blood Cell Sub-Populations in Twin Astronauts

To address what mechanisms might underlie the transient, space-flight specific increase in telomere length observed, telomere length was measured in individual circulating cellular sub-populations at multiple time points for both twins. Lymphocyte sub-populations have been shown to possess different telomere lengths (e.g. T-cells have shorter telomeres than B-cells), as well as differences in telomere length dynamics; i.e., T-cells have more stable telomere lengths than B-cells over time (Lin et al., 2016). Even though TeloFISH examines telomere length specifically in T-cells, we sought to confirm that the observed change in telomere length was not due to changes

in cell sub-population ratios. To do so, we received DNA isolations from CD4+ and CD8+ T-cells, CD19+ B-cells and lymphocyte depleted (LD) sub-populations for Scott Kelly from Twins investigator Dr. Christopher Mason (Weill Cornell Medical College).

Our results confirm that CD19+ B-cells, in general, possess the longest telomeres of all circulating blood cells, and that both CD4+ and CD8+ cells possess shorter telomeres than CD19+ B-cells or the lymphocyte depleted population (LD) (Figure 3.7). Additionally, analysis of Scott Kelly's individual circulating cell populations show that telomere length is increased in both CD4+ and CD8+ T-cells, as well as in the LD population, but not in CD19+ B-cells (Figure 3.8). This advocates against increased telomere length mid-flight being an artifact of changes in circulating blood cell ratios, and supports a selective influence of each individual population, collectively being responsible for increased telomere length. Preliminary comparisons with results from additional investigations within the Twins Study intriguingly suggest that the promoter region of the telomerase reverse transcriptase gene is hypomethylated mid-flight, suggestive of potential upregulation/activation of telomerase, and an associated increase in transcription/gene expression. A decrease in transcription of TZAP, which is responsible for trimming telomeres (Li et al., 2017) is also suggested. Although further analyses are needed and on-going, results to date support a model in which increased telomere length in-flight, as definitively demonstrated by the right shift in fluorescence intensity for FD140 (Figure 3.5), is a consequence of increased telomerase activity and decreased telomere trimming, rather than major mobilization of stem cells possessing longer telomeres.

Assessment of IR Exposure to Scott Kelly During the One Year Mission

Though previous biodosimetry studies have evaluated chromosomal translocations as biomarkers of radiation exposure for astronauts (Cucinotta et al., 2008), here we report the use of inversions as informative biomarkers of radiation exposure during the one year ISS mission. Scott Kelly spent a collective 340 days in space, which was the longest consecutive time spent in space by any NASA astronaut. During his mission, Mr. Kelly received a total measured physical dose of 76 mGy, or 146.34 mSv dose equivalent from exposures to galactic cosmic rays (GCRs), high LET secondary neutrons and photons from solar particle events. Analysis of metaphase chromosome spreads pre-, mid- and post-flight using Directional Genomic Hybridization (dGH) demonstrated a dose dependent increase in inversions, and to a lesser extent, translocations (Figure 3.9). Aberration frequencies are expressed as aberrations per chromosome 1, 2 and 3 (not as whole genome equivalents). Background aberration rates for Scott Kelly were slightly elevated (0.136 inversions/C1,2,3 and 0.0227 translocations/C1,2,3), as compared to his twin brother Mark (0.110 inversions/C1,2,3 and 0.0220 translocations/C1,2,3), presumably due to his previous flight history. Consistent with previous reports (Ray et al., 2014), chromosomal inversions were induced at a greater rate per unit dose than translocations, particularly for high LET radiation exposures. Post-flight dose reconstruction taking both inversions and translocations into account, appears to underestimate total physical dose received. Translocation rates decreased from 0.00081 translocations/mSv midflight, to 0.00014 translocations/mSv post-flight, and inversions decreased from 0.00051 inversions/mSv to 0.00035 inversions/mSv, respectively.

Discussion

NASA astronauts are a very exclusive group of highly disciplined and trained individuals, who are exposed to an extreme environment for which we have little knowledge of adverse biological or health effects, particularly for long duration missions. We evaluated telomerase activity and telomere length dynamics in an ISS astronaut cohort consisting of two monozygotic twins, one of whom spent 340 consecutive days in space, and ten unrelated astronauts, as well as their age- and gender-matched controls. This is the first report of spaceflight influencing telomere length dynamics (changes over time) in astronauts.

Telomerase activity was monitored pre-, mid-, and post-flight in three of the twelve astronauts, and pre- and post-flight for the remaining nine. Unfortunately, telomerase activity was lost from all mid-flight samples. In order to preserve telomerase activity, blood samples must remain at ambient conditions between the time of collection (e.g., on the ISS), and processing at either Johnson Space Center or Colorado State University, which ideally would occur within 24 hours of draw. Thus, unavoidable transit conditions for in-flight samples (e.g., time and/or temperature fluctuation during return to earth via Soyuz capsule) likely contributed to loss of telomerase activity. All other collections were successfully collected, shipped and processed. Astronauts in general have slightly lower levels of telomerase activity than their controls at base-line (pre-flight) and post-flight, although not quite statistically significant (Figure 3.1). Consistent with the unrelated astronauts, Scott Kelly also had slightly lower levels of telomerase activity than his brother, Mark. Unexpectedly, both twins experienced a dramatic increase in telomerase activity 270 days post-flight (Figure 3.2). Since this increase in telomerase activity occurred 9 months after Scott's return to earth from the ISS, and was observed in both Scott and Mark, the effect was not

considered spaceflight specific. However, this observation did correlate with the timing of a traumatic life event that affected both of them just prior to their R+270 collection. Presumably, the increase in telomerase activity was a response to considerable psychological stress experienced by both twins, a finding that has not been reported in humans, but previously reported in rats (Beery et al., 2012).

Similar to telomerase activity, telomere length was significantly shorter ($p=0.028$) in astronauts compared to their healthy controls at baseline, prior to flight (Figure 3.3). This most likely reflects the combined physical and psychological stresses experienced by astronauts during intensive training regimens. Indeed, chronic physical and psychological stress has been shown to increase the rate of telomere attrition (Lin et al., 2012). The most striking finding, however, was a rapid and significant lengthening of telomeres mid-flight. Importantly, all astronauts for which blood samples were collected during their mission, experienced a space-flight specific increase in telomere length (Figure 3.3). Telomere lengthening was a consistent observation across both measurement approaches used; qPCR, which provided a relative, average telomere length in whole PBMC populations, and TeloFISH, which provided a cell-by-cell analysis of T-cells. Scott Kelly's average telomere length values (Figure 3.4) were significantly longer mid-flight than his pre-flight ($p=0.048$), and his brother's mid-flight ($p=0.0073$). To our knowledge, only one additional report of increased telomere length due to spaceflight has been documented. After a short, 11 day, flight in space, C. *elegans* were documented to have elongated telomeres (Zhao et al., 2006).

Due to the somewhat surprising and unanticipated nature of our results, we sought to further validate that increased telomere length in-flight was a real effect, and not due to sample instability or artifact. Fortunately, we were able to obtain DNA isolated from frozen PBMCs collected from Scott Kelly at multiple time points during his mission by Twins Study investigator

Dr. Chris Mason (Weill Cornell Medical College). DNA from CSU and Cornell were run in parallel via qPCR to avoid potential batch effects. Results from both independent sample collections verified increased telomere length in-flight at multiple time points (Figure 3.6).

Telo-FISH determination of individual telomere length distributions pre-flight versus mid-flight provided additional supporting evidence. First, mid-flight samples displayed a decreased number of cells with short telomeres (Figure 3.5), a finding consistent with increased radiosensitivity (increased cell death in space radiation environment) in cells containing short telomeres (Goytisolo et al., 2000; Wong et al., 2000). Secondly, mid-flight samples also had an increased number of cells with long telomeres, as shown by the shift to the right at FD140 (Figure 3.5). This result suggests that either stem cells (longer telomeres) are being mobilized to replenish depleted cell populations (perhaps akin to wound healing), and/or telomerase is upregulated/activated, which serves to elongate telomeres in somatic cells due to the influence of spaceflight.

It is also possible that the transient increase in mid-flight telomere length may be due to changes in cell population dynamics. In other words, changes in the ratio of circulating cell sub-populations may influence overall telomere length measurements. To address this possibility, telomere length was evaluated in DNA isolated from CD4+ and CD8+ T-cells, CD19+ B-cells and lymphocyte depleted (LD) populations at multiple timepoints throughout Scott Kelly's mission. Relative telomere length measurements in CD4+ and CD8+ T-cells, as well as the LD population, showed increased telomere length mid-flight compared to pre- and post-flight (Figure 3.8), suggesting that cell population dynamics are not the underlying cause of the observed increase in telomere length mid-flight. A recent report from Elizabeth Blackburn's lab illustrates that telomere length and telomere length dynamics vary between lymphocyte subsets, in that T-cells

possess shorter telomeres than B-cells, however T-cell telomere length is more stable over time (Lin et al., 2016). Our results also provide additional confirmation of these variations in lymphocyte sub-populations (Figure 3.7).

To investigate whether space-flight specific increases in telomere length were due to stem cell mobilization or upregulated telomerase expression in somatic cells, we identified correlations with other endpoints being established within the Twins Study. Preliminary results suggest that the promoter region of the telomere reverse transcriptase (TERT) gene is hypomethylated mid-flight, suggestive of increased transcription and expression. Additionally, transcriptional regulation of TZAP, the protein responsible for trimming long telomeres (Li et al., 2017), appears to be down regulated mid-flight. Taken together, these mid-flight differences suggest that, at least in part and for whatever reason, telomerase activity may be increased in somatic cells during spaceflight.

For biodosimetry and to assess genomic stability, chromosomal translocation and inversion rates were measured in Scott Kelly pre-, mid-, and post-flight. Historically, translocation frequencies have been used to cytogenetically monitor radiation exposure in the astronauts (Cucinotta et al., 2008; George et al., 2013). Here, we evaluated the utility of inversions as a biomarker of radiation exposure for the astronauts, as inversions have been shown to be particularly prevalent following high LET exposures (Ray et al., 2014), and inversions have also been deemed a “radiation signature” for radiation therapy-induced second cancers (Behjati et al., 2016).

Scott Kelly spent 340 consecutive days aboard the ISS, accumulating a total physical dose of 76 mGy, which represents a dose equivalent of 146.34 mSv when corrected for combined GCR, secondary neutron, and photon exposure. Scott had slightly elevated background inversion and

translocation rates than his brother, Mark (Figure 3.9). Prior to the one year ISS mission, Scott spent almost four times longer in space than Mark, which could account for this difference. Furthermore, both inversion and translocation rates increased at FD140 (mid-flight), and then again after return to earth (post-flight). Inversions increased at a greater rate per unit dose (and time) than translocations, which was also noted in pervious *in vitro* studies (Ray et al., 2014). Post-flight dose reconstruction combining both inversions and translocation appear to underestimate total physical dose received. Translocation rates decreased from 0.00081 translocations/mSv midflight to 0.00014 translocations/mSv post-flight and inversions decreased from 0.00051 inversions/mSv to 0.00035 inversions/mSv, respectively. Reports have suggested that translocation rates (and similarly inversion rates), may decrease after IR exposure due to the presence of deleterious/lethal mutations (e.g. dicentric chromosomes) in the cell population (Lindholm et al., 1998). Dicentric chromosomes were seen (but not scored) here, providing a likely explanation for the decrease in both frequencies observed. Results, especially for translocations, may also be influenced by the low number of cells scored (~50/time point), since translocations occur much less frequently than inversions. Thus, in scenarios with limited sample availability such as with the astronauts, inversions may be a more appropriate biodosimeter, as they require fewer cells to be scored per individual.

In conclusion, this is the first report of spaceflight specific influences on telomere length dynamics and telomerase activity in astronauts. While some inter-individual variation existed between the unrelated astronauts, as well as between the twins themselves, telomere length was consistently increased for all astronauts for which mid-flight samples were collected. Although telomerase and telomeres have been proposed as potential "fountains of youth" (de Magalhães and Toussaint, 2004), changes in telomere length and telomerase activity are also associated with

disease. Telomerase is upregulated in an estimated 85% of all cancers (Artandi and DePinho, 2010; Shay and Wright, 1996). Changes in telomere length dynamics are likewise associated with cancer (Shen et al., 2007; Wu et al., 2003), and non-cancer diseases such as cardiovascular disease (Haycock et al., 2014; Serrano and Andrés, 2004). Therefore, drastic changes in telomere length and maintenance, whether increasing or decreasing, is cause for concern. Moreover, ionizing radiation exposure not only influences telomere dynamics, but is also well recognized for its ability to produce structural rearrangements. We find that the use of chromosomal inversions as biodosimeters may be a more sensitive indicator of exposure than translocations, especially when baseline samples are available for comparison, or when sample availability is limited. We propose that assessing telomere length dynamics and inversion frequencies should be routine for astronauts, as a means of monitoring overall health during spaceflight.

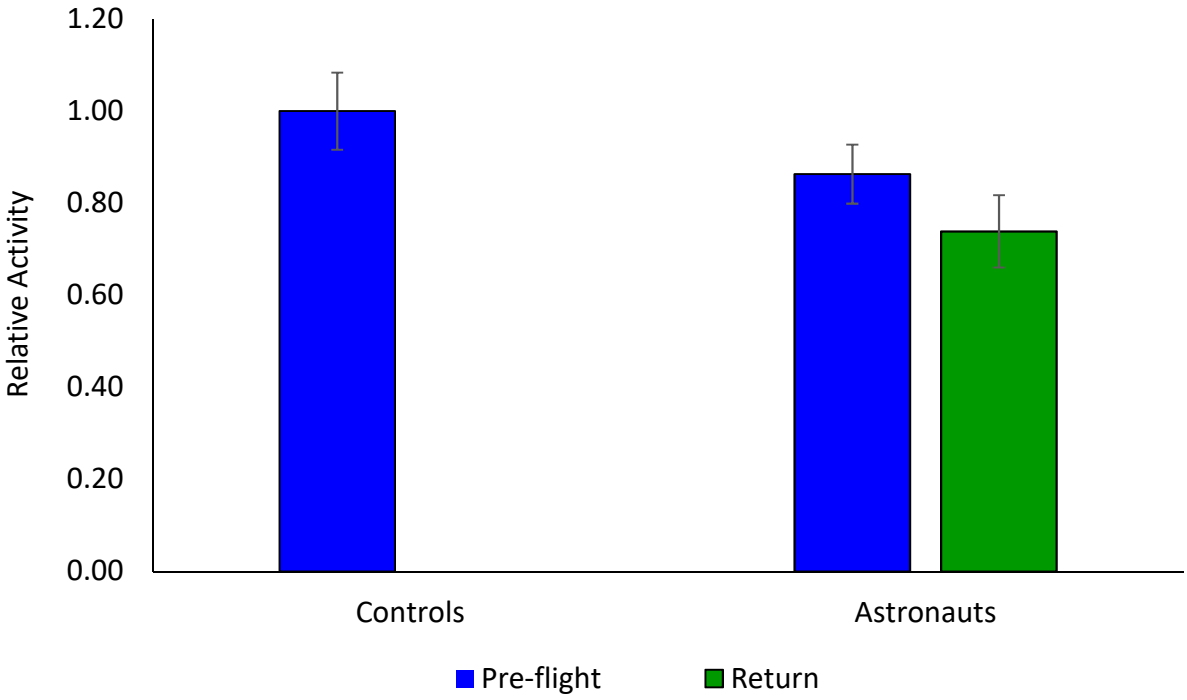

Figure 3.1 Relative telomerase activity of all astronauts compared to their age- and gender-matched controls. Astronauts appear to have slightly lower levels of telomerase activity, pre-flight, than their controls; not statistically significant. Mid-flight telomerase activity was lost due to transit conditions. Upon returning to earth, telomerase activity decreased slightly more than pre-flight levels; not statistically significant. Error bars represent the SEM.

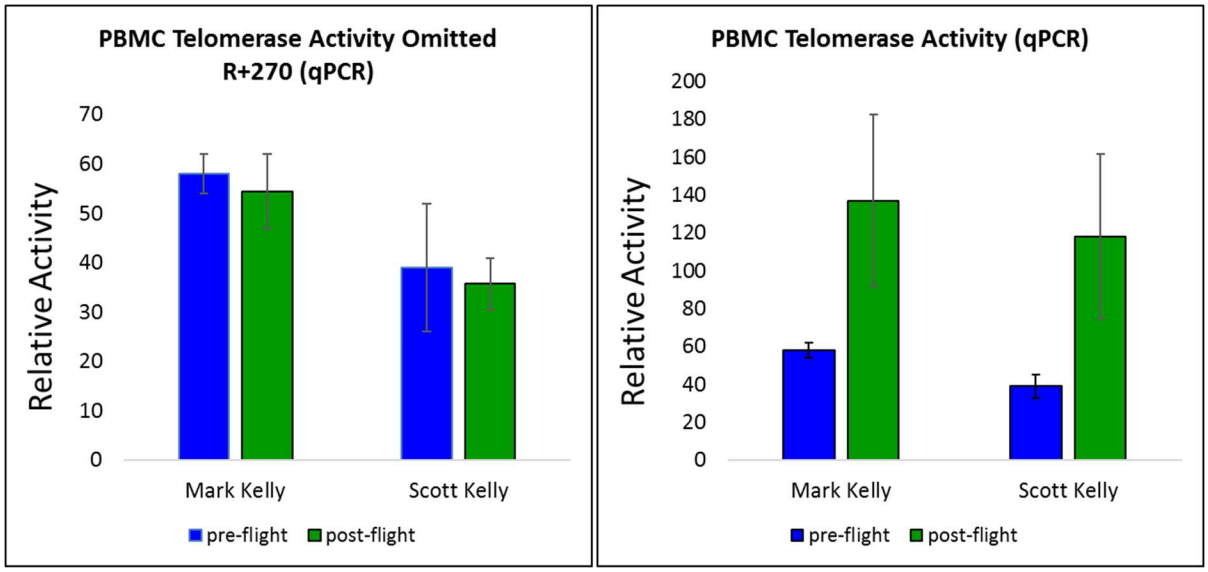

Figure 3.2 Relative telomerase activity for monozygotic twins. **A**) R+270 samples omitted from the data set. Scott Kelly displayed slightly lower levels of telomerase activity across all time points. **B**) Combined pre-, mid-, and post-flight telomerase activity. Post-flight R+270 telomerase activity significantly elevated compared to pre-flight for Mark (earth bound twin; p=0.006) and Scott (space bound twin; p=0.012) Kelly. Mid-flight telomerase activity collected on the ISS was lost. Error bars represent the SEM.

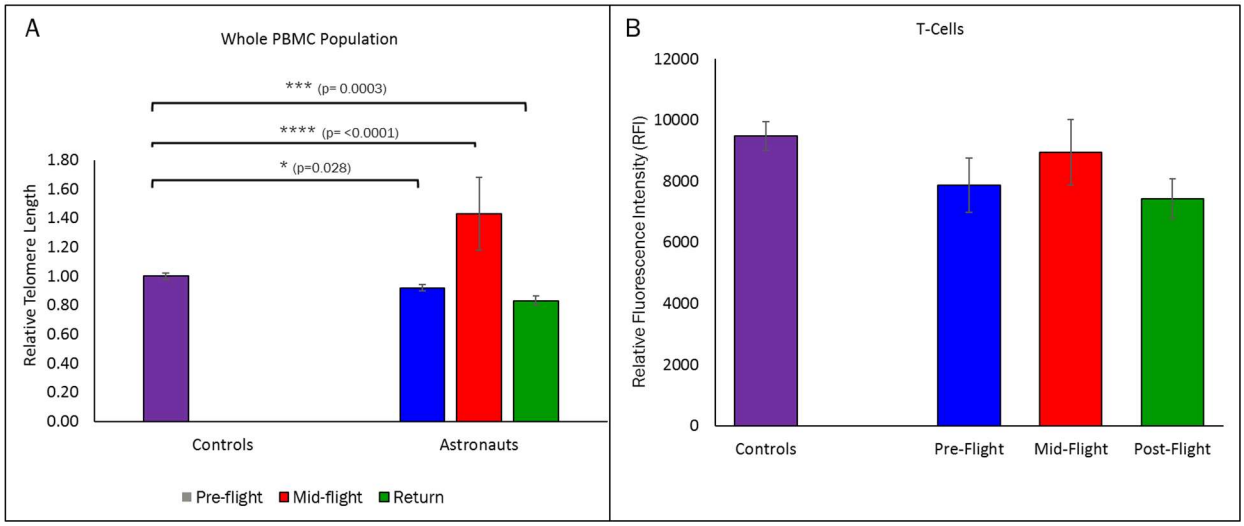

Figure 3.3 Pre-, mid-, and post-flight telomere length measurements for both whole PBMC populations and T-cells. **A**) qPCR based-telomere length measurements for astronauts and their age- and gender-matched controls. Overall astronauts have shorter telomeres pre-flight than their controls (p=0.028). Telomeres transiently lengthened mid-flight (p= <0.0001) and decreased back to relatively baseline levels upon return to earth (p=0.0003). **B**) TeloFISH measurements of telomere length in stimulated T-cells. Like qPCR, though not as drastic, astronauts appear to have shorter telomeres than their controls pre-flight, which are then elevated mid-flight and return to baseline levels within days to weeks of returning to earth. Error bars represent the SEM.

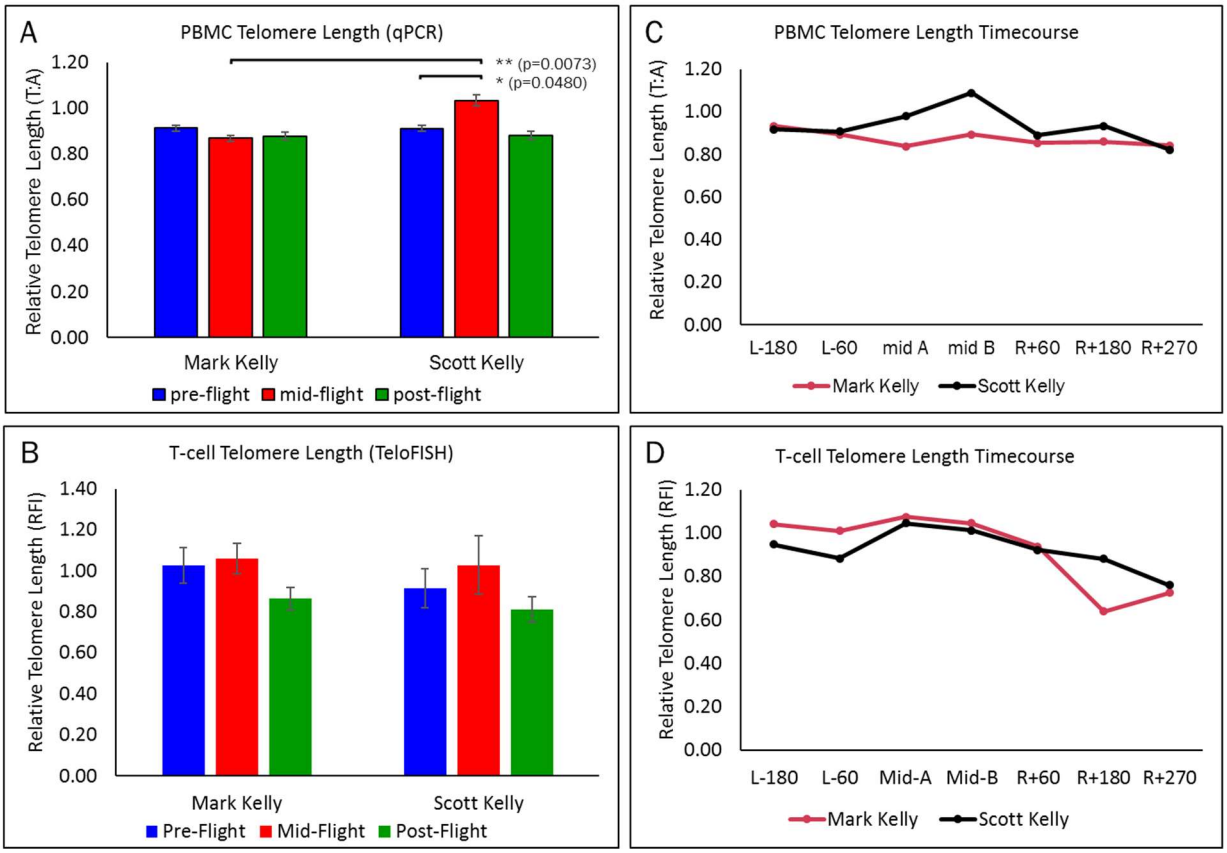

Figure 3.4 PBMC and T-cell telomere length dynamics for the twin astronauts. **A**) PBMC telomere length pre-, mid-, and post-flight for Scott and Mark Kelly. Mark Kelly's telomere length remained stable over time. Scott Kelly had a statistically significant transient increase in telomere length mid-flight compared to his pre-flight (p=0.048) and between he and his brother (p=0.007). **B**) T-cell telomere length analysis display a similar trend as the whole PBMC population, but differences are not statistically significant. **C/D**) PBMC (C) and T-cell (D) relative telomere length time course. Both twins start and end with similar telomere lengths. Scott Kelly had a transient increase in telomere length that remained elevated through the duration of his mission, but returned to baseline levels with 60 days of returning to earth. Error bars represent the SEM.

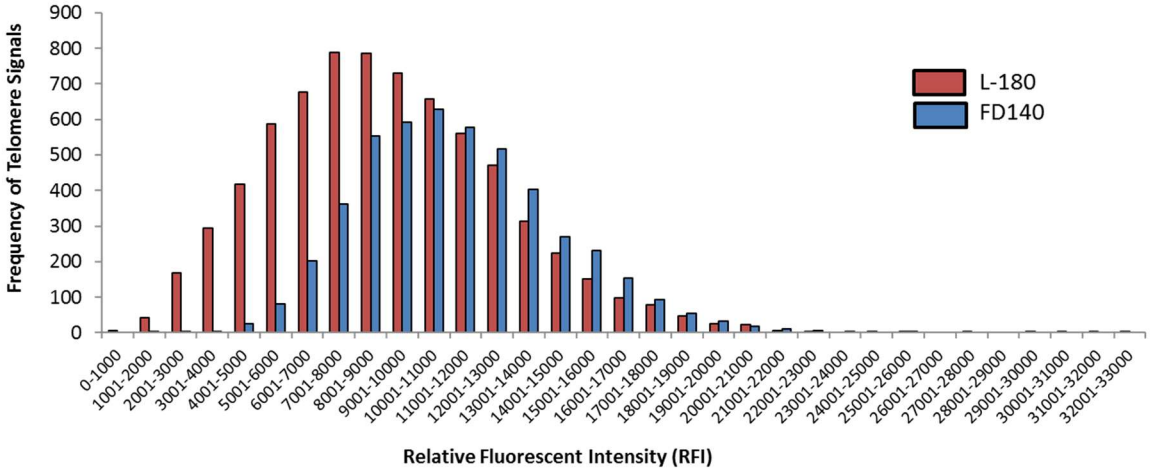

Figure 3.5 Histogram of telomere length distributions (Telo-FISH cell-by-cell analyses) for Scott Kelly at L-180 days (red) prior to flight, and at FD140 days (blue) during flight. FD140 demonstrates a dramatic reduction in the number of cells with short telomeres compared to L-180, denoted by the shift to the right (higher RFI) for FD140. There is also a concurrent increase in the number of cells with longer telomeres.

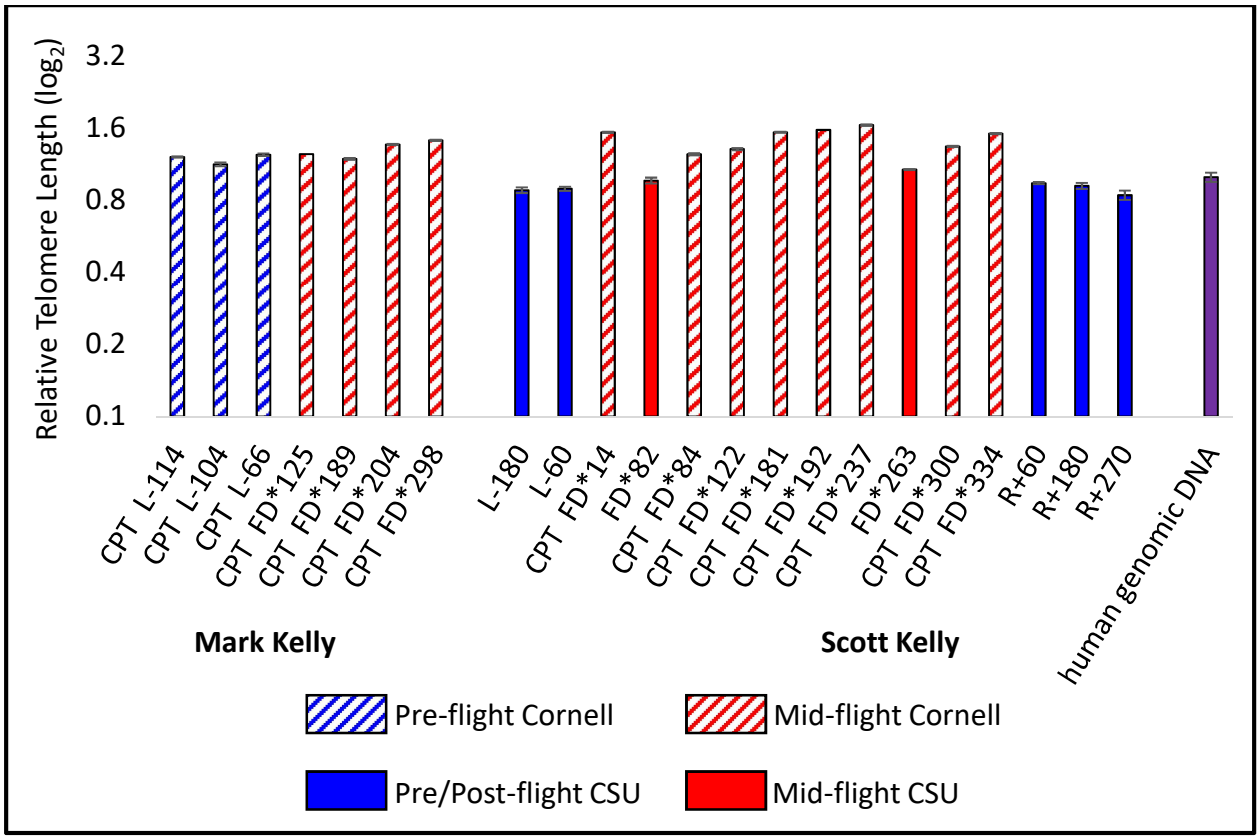

Figure 3.6 Relative telomere length from samples processed at Colorado State University and Weill Cornell Medical College. Mark Kelly's telomere length remained relatively stable across multiple time points, while a notable increase in telomere length was seen in PBMC DNA samples for both CSU and Cornell samples. All samples were normalized to a human genomic DNA control. Error bars represent the SEM.

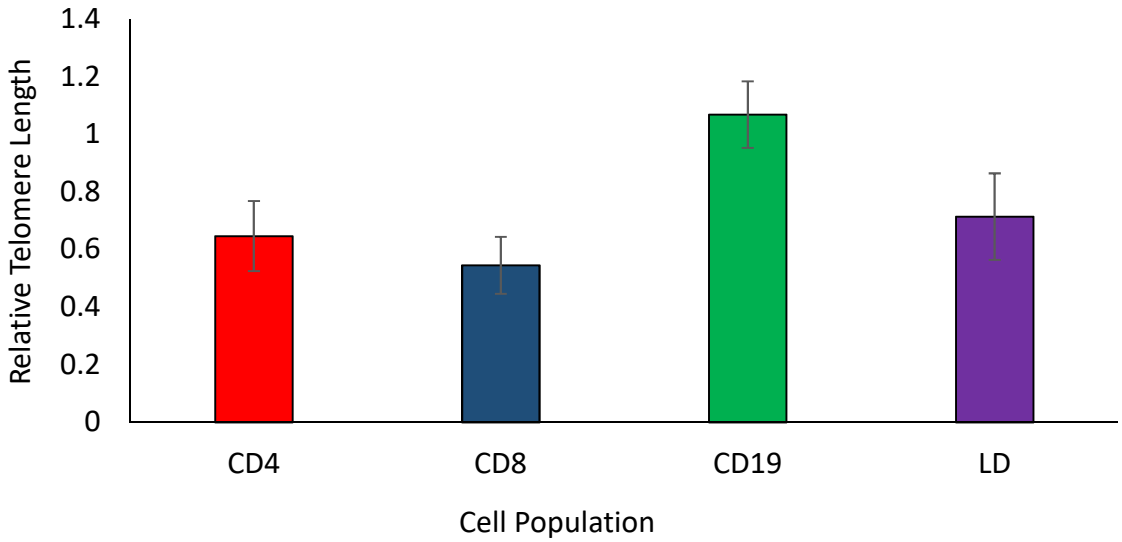

Figure 3.7 Relative telomere length of individual circulating cell populations across all sample collections. CD4+ and CD8+ T-cells, in general, have shorter telomeres than CD19+ B-cells. The lymphocyte depleted population (LD) possess longer telomeres than T-cells, but slightly shorter than B-cells. Error bars represent the standard deviation (SD)

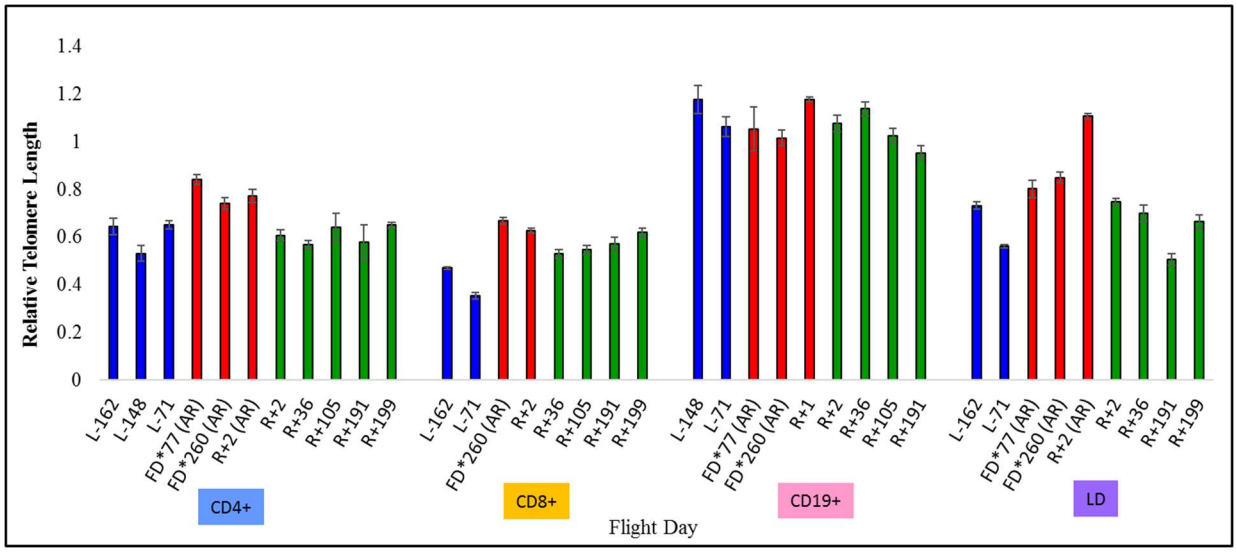

Figure 3.8 Relative telomere length of circulating cell sub-populations in peripheral blood collections from Scott Kelly. Relative telomere length was monitored over time in CD4+ and CD8+ T-cells, CD19+ B-cells, and the lymphocyte depleted (LD) populations. An increase in telomere length was observed in CD4+, CD8+ and LD populations mid-flight compared to pre- and post-flight. Error bars represent the SEM.

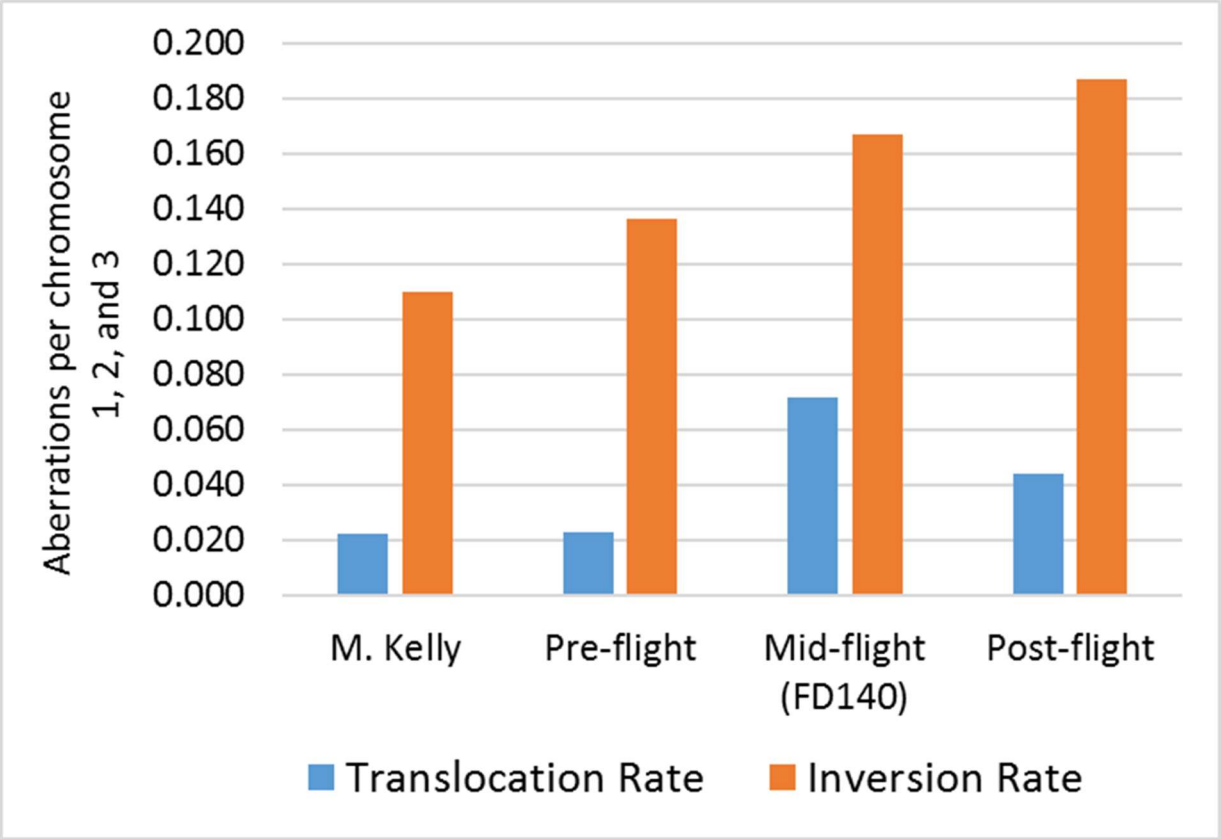

Figure 3.9 Biodosimetry for Scott Kelly after one year aboard the ISS using dGH. Inversion and translocation rates using whole chromosome 1, 2 and 3 paints. Though inversions had a higher background rate, they increased at a greater rate per unit dose, and therefore with time, than translocations. Translocation frequencies are much more variable per time point than inversions, presumably because of the low number of cells scored.

REFERENCES

Artandi, S.E., and DePinho, R.A. (2010). Telomeres and telomerase in cancer. Carcinogenesis *31*, 9–18.

Aubert, G., and Lansdorp, P.M. (2008). Telomeres and aging. Physiol. Rev. *88*, 557–579.

Beery, A.K., Lin, J., Biddle, J.S., Francis, D.D., Blackburn, E.H., and Epel, E.S. (2012). Chronic stress elevates telomerase activity in rats. Biol. Lett. *8*, 1063–1066.

Behjati, S., Gundem, G., Wedge, D.C., Roberts, N.D., Tarpey, P.S., Cooke, S.L., Loo, P.V., Alexandrov, L.B., Ramakrishna, M., Davies, H., et al. (2016). Mutational signatures of ionizing radiation in second malignancies. Nat. Commun. *7*, 12605.

Cherkas, L.F., Hunkin, J.L., Kato, B.S., Richards, J.B., Gardner, J.P., Surdulescu, G.L., Kimura, M., Lu, X., Spector, T.D., and Aviv, A. (2008). The association between physical activity in leisure time and leukocyte telomere length. Arch. Intern. Med. *168*, 154–158.

Cucinotta, F.A., Kim, M.-H.Y., Willingham, V., and George, K.A. (2008). Physical and biological organ dosimetry analysis for international space station astronauts. Radiat. Res. *170*, 127–138.

Damjanovic, A.K., Yang, Y., Glaser, R., Kiecolt-Glaser, J.K., Nguyen, H., Laskowski, B., Zou, Y., Beversdorf, D.Q., and Weng, N. (2007). Accelerated telomere erosion is associated with a declining immune function of caregivers of Alzheimer's disease patients. J. Immunol. *179*, 4249–4254.

Epel, E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Morrow, J.D., and Cawthon, R.M. (2004). Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. U. S. A. *101*, 17312–17315.

George, K.A., Rhone, J., Chappell, L.J., and Cucinotta, F.A. (2013). Cytogenetic biodosimetry using the blood lymphocytes of astronauts. Acta Astronaut. *92*, 97–102.

Goytisolo, F.A., Samper, E., Martín-Caballero, J., Finnon, P., Herrera, E., Flores, J.M., Bouffler, S.D., and Blasco, M.A. (2000). Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals. J. Exp. Med. *192*, 1625–1636.

Haycock, P.C., Heydon, E.E., Kaptoge, S., Butterworth, A.S., Thompson, A., and Willeit, P. (2014). Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis. BMJ *349*, g4227.

Herbert, B.-S., Hochreiter, A.E., Wright, W.E., and Shay, J.W. (2006). Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. Nat. Protoc. *1*, 1583–1590.

Hou, M., Xu, D., Björkholm, M., and Gruber, A. (2001). Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. Clin. Chem. *47*, 519–524.

Howe, B., Umrigar, A., and Tsien, F. (2014). Chromosome preparation from cultured cells. J. Vis. Exp. JoVE.

Li, J.S.Z., Miralles Fuste, J., Simavorian, T., Bartocci, C., Tsai, J., Karlseder, J., and Lazzerini Denchi, E. (2017). TZAP: A telomere-associated protein involved in telomere length control. Science.

Lin, J., Epel, E., and Blackburn, E. (2012). Telomeres and lifestyle factors: Roles in cellular aging. Mutat. Res. Mol. Mech. Mutagen. *730*, 85–89.

Lin, J., Cheon, J., Brown, R., Coccia, M., Puterman, E., Aschbacher, K., Sinclair, E., Epel, E., and Blackburn, E.H. (2016). Systematic and cell type-specific telomere length changes in subsets of lymphocytes. J. Immunol. Res. *2016*, 5371050.

Lindholm, C., Tekkel, M., Veidebaum, T., Ilus, T., and Salomaa, S. (1998). Persistence of translocations after accidental exposure to ionizing radiation. Int. J. Radiat. Biol. *74*, 565–571.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif *25*, 402–408.

Longhese, M.P. (2008). DNA damage response at functional and dysfunctional telomeres. Genes Dev. *22*, 125–140.

Loucas, B.D., Shuryak, I., and Cornforth, M.N. (2016). Three-color chromosome painting as seen through the eyes of mFISH: another look at radiation-induced exchanges and their conversion to whole-genome equivalency. Front. Oncol. *6*.

Lustig, A., Shterev, I., Geyer, S., Shi, A., Hu, Y., Morishita, Y., Nagamura, H., Sasaki, K., Maki, M., Hayashi, I., et al. (2016). Long term effects of radiation exposure on telomere lengths of leukocytes and its associated biomarkers among atomic-bomb survivors. Oncotarget *7*, 38988–38998.

de Magalhães, J.P., and Toussaint, O. (2004). Telomeres and telomerase: a modern fountain of youth? Rejuvenation Res. *7*, 126–133.

Mayall, B.H., Carrano, A.V., Moore, D.H., Ashworth, L.K., Bennett, D.E., and Mendelsohn, M.L. (1984). The DNA-based human karyotype. Cytometry *5*, 376–385.

Morton, N.E. (1991). Parameters of the human genome. Proc. Natl. Acad. Sci. *88*, 7474–7476.

Moyzis, R.K., Buckingham, J.M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, R.L., and Wu, J.R. (1988). A highly conserved repetitive DNA sequence,

(TTAGGG)n, present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. U. S. A. *85*, 6622–6626.

Olovnikov, A.M. (1973). A theory of marginotomy. J. Theor. Biol. *41*, 181–190.

O'Sullivan, R.J., and Karlseder, J. (2010). Telomeres: protecting chromosomes against genome instability. Nat. Rev. Mol. Cell Biol. *11*, 171–181.

Paul, L. (2011). Diet, nutrition and telomere length. J. Nutr. Biochem. *22*, 895–901.

Ray, F.A., Zimmerman, E., Robinson, B., Cornforth, M., Bedford, J., Goodwin, E., and Bailey, S. (2013). Directional genomic hybridization for chromosomal inversion discovery and detection. Chromosome Res. *21*, 165–174.

Ray, F.A., Robinson, E., McKenna, M., Hada, M., George, K., Cucinotta, F., Goodwin, E.H., Bedford, J.S., Bailey, S.M., and Cornforth, M.N. (2014). Directional genomic hybridization: inversions as a potential biodosimeter for retrospective radiation exposure. Radiat. Environ. Biophys. *53*, 255–263.

Reste, J., Zvigule, G., Zvagule, T., Kurjane, N., Eglite, M., Gabruseva, N., Berzina, D., Plonis, J., and Miklasevics, E. (2014). Telomere length in Chernobyl accident recovery workers in the late period after the disaster. J. Radiat. Res. (Tokyo) *55*, 1089–1100.

Robinson, E., McKenna, M.J., Bedford, J.S., Goodwin, E.H., Cornforth, M.N., Bailey, S.M., and Ray, F.A. (Submitted). Directional Genomic Hybridization (dGH®) for detection of intra-chromosomal rearrangements. in radiation cytogenetics: Methods in Molecular Biology.

Serrano, A.L., and Andrés, V. (2004). Telomeres and cardiovascular disease. Circ. Res. *94*, 575–584.

Shay, J.W., and Wright, W.E. (1996). The reactivation of telomerase activity in cancer progression. Trends Genet. *12*, 129–131.

Shay, J.W., and Wright, W.E. (2010). Telomeres and telomerase in normal and cancer stem cells. Febs Lett. *584*, 3819–3825.

Shen, J., Terry, M.B., Gurvich, I., Liao, Y., Senie, R.T., and Santella, R.M. (2007). Short telomere length and breast cancer risk: a study in sister sets. Cancer Res. *67*, 5538–5544.

Sishc, B.J., Nelson, C.B., McKenna, M.J., Battaglia, C.L.R., Herndon, A., Idate, R., Liber, H.L., and Bailey, S.M. (2015). Telomeres and telomerase in the radiation response: implications for instability, reprogramming, and carcinogenesis. Front. Oncol. *5*.

Svenson, U., Nordfjäll, K., Stegmayr, B., Manjer, J., Nilsson, P., Tavelin, B., Henriksson, R., Lenner, P., and Roos, G. (2008). Breast cancer survival is associated with telomere length in peripheral blood cells. Cancer Res. *68*, 3618–3623.

Watson, J.D. (1972). Origin of concatemeric T7DNA. Nature *239*, 197–201.

Webb, C.J., Wu, Y., and Zakian, V.A. (2013). DNA repair at telomeres: keeping the ends intact. Cold Spring Harb. Perspect. Biol. *5*.

Wong, H.-P., and Slijepcevic, P. (2004). Telomere length measurement in mouse chromosomes by a modified Q-FISH method. Cytogenet. Genome Res. *105*, 464–470.

Wong, K.K., Chang, S., Weiler, S.R., Ganesan, S., Chaudhuri, J., Zhu, C., Artandi, S.E., Rudolph, K.L., Gottlieb, G.J., Chin, L., et al. (2000). Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. Nat. Genet. *26*, 85–88.

Wu, X., Amos, C.I., Zhu, Y., Zhao, H., Grossman, B.H., Shay, J.W., Luo, S., Hong, W.K., and Spitz, M.R. (2003). Telomere dysfunction: a potential cancer predisposition factor. J. Natl. Cancer Inst. *95*, 1211–1218.

Zhao, Y., Lai, K., Cheung, I., Youds, J., Tarailo, M., Tarailo, S., and Rose, A. (2006). A mutational analysis of Caenorhabditis elegans in space. Mutat. Res. Mol. Mech. Mutagen. *601*, 19–29.

CHAPTER 4

CONCLUSIONS AND DISCUSSION

Overview

Telomeres have long been appreciated as a "mitotic clock" of aging, as with each cellular division telomere length shortens due to the end-replication problem (Olovnikov, 1973; Watson, 1972). In recent years, telomeres and telomere maintenance have also become valued as biomarkers of overall health, as a host of lifestyle factors (smoking, diet, exercise) and environmental exposures (pollution and ionizing radiation) are captured as changes in telomere length dynamics (Cherkas et al., 2008; Reste et al., 2014; Valdes et al., 2005).

Moreover, telomeres have also been proposed as "hallmarks" of radiosensitivity (Ayouaz et al., 2008). For example, mice possessing short telomeres due to loss of a telomere maintenance mechanism, have been shown to be hypersensitive to ionizing radiation (IR) exposure, with over half of the population dying from acute radiation toxicity (Goytisolo et al., 2000). Critically short telomeres elicit a DNA damage response and checkpoint activation, ultimately leading to apoptosis or senescence in normal somatic cells, which is believed to act as a tumor suppressor mechanism (Deng et al., 2008). On the other hand, telomerase activity is sufficient to maintain telomere length only in stem, germline, and cancerous cells (Shay and Wright, 2010), but has been shown to be IR responsive in peripheral blood mononuclear cells (PBMCs) (Sishc et al., 2015), thereby potentially increasing cancer risk following exposure. Therefore, telomeres are particularly relevant

biomarkers of health effects in extreme environments/situations such as during spaceflight or nuclear fallout scenarios.

While localized, high dose acute radiation exposure is commonly utilized in therapeutic settings for cell killing, more chronic low dose IR exposures are considered mutagenic (Sykes et al., 2006); e.g., in the context of radiation exposure experienced during space flight, or environmental IR exposure resulting from nuclear detonation events (whether intentional or accidental). Biodosimetry measures biological response (e.g, chromosome aberrations) as a means of evaluating IR dose in such situations, and can also be used to monitor genomic stability (McKenna and Bailey, 2017). Telomere dysfunction also contributes to/propagates instability, e.g., via breakage-fusion-bridge cycles (Lo et al., 2002). Here, we evaluated telomere length maintenance and structural rearrangements (translocations and inversions) retrospectively in U.S. veterans exposed to nuclear fallout several decades in the past, and longitudinally in twin and unrelated astronauts aboard the International Space Station (ISS).

The overall goal of the present studies was to evaluate the biological response to extreme environments not commonly experienced by the general population. Whether exposure to the space radiation environment or to nuclear fallout, results presented here improve understanding of short- and long-term effects of exposure to IR of mixed qualities, as well as of low dose and low dose rate effects. Indeed, with deep space travel progressing steadily toward reality, and the threat of nuclear events ever increasing, better understanding of the associated radiobiological responses - in human populations - is essential for development of effective mitigation strategies.

The Atomic Veterans: Retrospective Biodosimetry to Assess Nuclear Fallout Exposure

Biodosimetry-based dose reconstruction is a critical component of short-term care for acute radiation syndromes (ARS), as well as for long-term preventative measures after an IR exposure event (Rea et al., 2010). We aimed to develop and test a robust and sensitive biodosimetry assay, first by evaluating the utility of various biomarkers of low dose IR exposures that took place several decades in the past. Chromosomal translocations have long been regarded an informative biomarker for retrospective biodosimetry since they persist with time (Tawn and Whitehouse, 2003). However, translocation background rates are influenced by lifestyle and environmental factors such as age, smoking, and pesticide exposure (Ramsey et al., 1995), which confound estimations. More recently, inversions have been proposed as a potential biomarker of radiation exposure since they too persist with time, and *in vitro* results suggest inversions increase at a greater rate per unit dose of IR than translocations (Ray et al., 2014).

We utilized directional genomic hybridization (dGH) to simultaneously evaluate translocations and inversions in U.S. veterans exposed to nuclear fallout in the 1950's. Estimated IR doses were then reconstructed based on comparison of observed aberration frequencies to *in vitro* calibration (dose response) curves, established from peripheral blood collected from males in their mid-20's (to correct for age at exposure). Background aberration frequencies for the exposed veterans were corrected using age-matched controls (unexposed veterans). Relative telomere length was also evaluated for each participant. Telomeres have been proposed as biomarkers for radiosensitivity, which may influence the long-term effects of IR exposure (Ayouaz et al., 2008).

For the first time, our results demonstrate that, like translocations, both age and smoking status influence background inversion frequencies. Independent of IR exposure or smoking status, comparisons between young adults and veteran age-matched controls suggest an increase in spontaneous background aberrations due to age alone of 0.289±0.035 inversions per cell equivalent (CE) and 0.065±0.434 translocations per CE. Similarly, smoking increased background inversion rates by 15% and translocation rates by 3.77%. While estimates vary in regard to the influence of smoking on background translocation rates, many are based on 'ever or never' smoking status (Sigurdson et al., 2008). The veterans who reported smoking in our study, were typically chronic, long-term habits.

Dose estimations for the exposed veterans were initially determined using translocation and inversion rates independently of one another. Compared to best estimated physical dose reconstructions, chromosome aberration dose estimates were only weakly correlated. However, combining observed inversion and translocation dose estimations using a best linear unbiased estimation approach, where more weight was placed on the aberration with less variance (e.g. translocations), correlation to estimated physical dose reconstructions greatly improved. We speculate that for retrospective biodosimetry, especially for exposures that took place many years in the past, combining both translocation and inversion rates using a weighted approach provides the most accurate biological dose estimation.

Interestingly and even though a small cohort, relative telomere length inversely correlated, although weakly, with the weighted biological dose estimations, indicating that the effect of acute, low-dose exposure to nuclear fallout was still detectable – six decades later. These results provide additional support for long term effects of IR exposure on telomere length of blood leukocytes established in atomic bomb survivors (Lustig et al., 2016). Radiation dose also appears to influence

telomere dynamics. While low doses, such as those experienced by our veterans and the atomic bomb survivors have been shown to decrease telomere length, reports from Chernobyl cleanup workers who experienced much higher doses, had an increase in leukocyte telomere length (Snigiryova et al., 1997).

As expected, the greatest contribution to telomere attrition was age. Comparisons between the mid-20-year-old controls and the age-matched veterans displayed a 35.3% decrease in telomere length, far succeeding the influence of IR or smoking status independently. However, veteran controls who reported chronic smoking habits displayed a similar overall decrease in telomere length as exposed veterans without a history of smoking, suggesting that habitual smoking reduces telomere length as much as a low dose IR exposure to nuclear fallout.

Implications and Future Directions

While translocations are the gold standard for retrospective biodosimetry, we present a new methodology and strategy that utilizes both inversion and translocation rates. Historically, inversions have been notoriously difficult to reliably detect with standard cytogenetic techniques, but dGH circumvents this shortcoming. Using a combined approach, more accurate dose estimations can be made than each independently. Moreover, telomere length dynamics represent an important biomarker not only for radiosensitivity, but also as an indicator of overall health (Cherkas et al., 2008; Reste et al., 2014; Valdes et al., 2005). We recently proposed a combined approach, Telo-dGH, to simultaneously detect translocations, inversions, and monitor telomere length in a single assay (McKenna and Bailey, 2017; McKenna et al., 2017). This not only has profound implications for biodosimetry studies, but also for evaluating off-target effects of radiotherapy and risk of secondary malignancies and late-radiation toxicity (McKenna and Bailey,

2017). In support of such a strategy, inversions were recently shown to be a radiation signature of radiation therapy-induced secondary malignancies (Behjati et al., 2016). Therefore, telomeres in combination with dGH could prove to be a valuable diagnostic monitoring tool for personalized medicine prior to and following cancer therapy.

Assessing Telomere Length, Telomerase Activity and Biodosimetry in Twin and Unrelated Astronauts

NASA astronauts are a unique and highly disciplined group of individuals who experience an environment that the human body is not adapted for. Little is known about the health effects of a low gravity environment with increased IR exposure, including galactic cosmic rays (GCRs), solar particle events, and secondary neutrons. We aimed to assess telomere length dynamics and telomerase activity, as well as biodosimetry for twin and unrelated astronauts. Peripheral blood samples were collected pre-flight (to establish a baseline), mid-flight (to determine transient changes), and post-flight (to evaluate long-term, permanent changes).

The monozygotic twins, Scott and Mark Kelly, were a remarkable opportunity to control for individual genetic susceptibilities, as much as possible, making any observed variations spaceflight-specific. While Mark Kelly remained on earth, Scott participated in the one year mission, which was the longest time aboard the ISS of any NASA astronaut. Comparisons with and between unrelated astronauts and their age-/gender-matched controls allowed us to evaluate the role of individual susceptibilities as well.

Telomere length is associated with a variety of diseases, and of particular concern for the astronauts are cardiovascular diseases (CVD) and cancer. CVD is associated with shortening of

telomeres (Serrano and Andrés, 2004), and cancer, is associated with both increased and decreased telomere length and upregulation of telomerase activity (Shay and Wright, 2010). Overall, astronauts appear to have slightly shorter telomeres and lower levels of telomerase activity, prior to their missions than their controls. This is most likely due to the highly stressful and physically intensive training regimens. Both physical and psychological stress have been shown to decrease telomere length (Lin et al., 2012). The most striking observation, however, was a significant increase in telomere length mid-flight. This was observed in three of the nine astronauts, for which mid-flight samples were collected, which then returned to near baseline levels within days or weeks of returning to earth. This unexpected finding was observed both by qPCR, which analyzed relative average telomere length for entire PBMC populations, and by Telo-FISH, a cell-by-cell approach that specifically evaluates T-cells. The qPCR results for Scott Kelly were also verified by independent investigators (Drs. Rana and de Vivo, UCSD) involved with the Twins Study.

Telomere dynamics vary depending on the PBMC sub-population being analyzed; B-cells have longer telomeres than T-cells, but T-cell telomere length is more stable with time (Lin et al., 2016). To evaluate whether space-flight specific increases in telomere length were due to cell population dynamics, CD4+ and CD8+ T-cells, CD19+ B-cells and lymphocyte depleted (LD) populations for Scott Kelly were obtained from twins investigator, Dr. Christopher Mason (Weill Cornell Medical College). Increased telomere length mid-flight was observed in CD4+ and CD8+ T-cells, as well as the LD population, suggesting that this was not an artifact of cell population dynamics, but was the result of some other spaceflight associated factor(s).

Other potential contributing mechanisms include the possibility that spaceflight may mobilize stem cells to repopulate dying lymphocyte populations, a response akin to wound healing (Sishc et al., 2015). Cells possessing short telomeres are hypersensitive to IR exposure (Goytisolo

et al., 2000), and so increased cell death may trigger repopulation. To further evaluate the underlying cause for the transient increase in telomere length, preliminary correlations between our results and those of other Twin Study investigations were made. Notably, the promoter region for the telomerase protein (TERT) was hypomethylated in-flight, a finding suggestive of increased transcription and expression of telomerase. Additionally, TZAP, the protein responsible for maintaining telomere length homeostasis by trimming long telomeres (Li et al., 2017), was transcriptionally repressed in-flight. Taken together, these results suggest that increased telomere length during spaceflight was most likely associated with an upregulation of telomerase activity, rather than mobilization of stem cells. Interestingly, increased telomere length has also been reported in C. *elegans* during a short, 11 day, spaceflight (Zhao et al., 2006). Though not associated with spaceflight, increases in telomere length have also been observed in Chernobyl clean-up workers exposed to high doses of IR (Snigiryova et al., 1997), atomic bomb survivors exposed to <0.5 Gy IR (Yoshida et al., 2016), as well as exposures to high LET protons (Sgura et al., 2006).

Telomerase activity was compromised in our mid-flight samples, most likely due to transit conditions (e.g. time and temperature). However, a dramatic increase in telomerase activity for *both* Scott and Mark Kelly was observed at 270 days post-flight, so was not associated with spaceflight. Prior to the collection time, the twins experienced an emotionally traumatic event in their lives, which was likely responsible for the increase in telomerase activity, a response to psychological stress that has been previously reported in rats (Beery et al., 2012), but this is the first documented response in humans.

Biodosimetric assessment of the twin astronauts also revealed an in-flight dose dependent increase in inversions and translocations for Scott Kelly. While Scott did have an elevated background aberration rate compared to his brother at baseline, this was attributed to Scott's

previous missions and extended time in space compared to Mark. Scott Kelly spent almost four-fold longer in space through various missions than Mark prior to the one year mission. Inversions increased at a greater rate per unit dose than translocations as previously reported (Ray et al., 2014), which was especially apparent due to the mixed radiation qualities. Consistent with previous translocation studies (George et al., 2013), a gradual increase in inversion frequencies post-flight (>180 days) was also observed, a finding that may reflect damage inflicted on stem-cells in the bone marrow and so be associated with an increased risk for cancer (George et al., 2013).

Implications and Future Directions

Combined these results suggest that there are spaceflight specific influences on telomere length, telomerase activity and biodosimetry. Though telomere length transiently increased mid-flight, our preliminary interrogations suggest that this is due to an increase in telomerase activity, a finding with implications for increased disease risk, as telomerase upregulation is associated with 85% of cancers (Shay and Wright, 2010). Additional comparisons are being pursued with other Twins Study investigators to further substantiate our results. Biodosimetry results from Scott Kelly will be compared with the unrelated astronauts to develop a time and dose response relationship with spaceflight. We are also attempting to obtain calibration curves from previous astronauts (including Mr. Kelly) to determine the dose estimation from structural rearrangements.

We believe that telomere length represents a particularly relevant – and integrative – biomarker as it represents the combined exposures and experiences encountered during spaceflight. In conjunction with biodosimetry, telomere length should be included in routine monitoring of astronauts' overall health, for future spaceflight endeavors, such as Mars. Moreover,

telomere length measurements may also be relevant for individuals exposed to additional types of extreme environments, such as fighter pilots, submariners, and radiological workers.

Implications and Future Directions

Collectively, these results demonstrate that there is a profound influence of extreme environments, particularly those involving low dose IR, on biological responses. Telomere length dynamics and aberration frequencies (e.g. translocations and inversions) shed light on the long-term effects and implications of spaceflight and exposure to nuclear detonation events. We believe that monitoring these informative biomarkers will improve development of mitigation strategies for spaceflight and fallout exposure, and have the potential to be utilized in a variety of different situations as well. For instance, we employed a similar strategy to monitor aquanauts involved in extended deep sea dives for the NASA Extreme Environment Mission Operations (NEEMO). Astronaut candidates spent 9-16 days 62ft. underwater in a high barometric pressure environment. Since little radiation exposure is associated with underwater dives, these studies provide an interesting control. Our very preliminary results suggest that telomere length and telomerase activity decreased for two individuals and increased for the third (Figure 4.1). Additionally, and certainly not limited to, Telo-dGH measurements can be used to evaluate individual radiosensitivity and late-radiation toxicity, as well as risk of secondary malignancies associated with radiation therapy (e.g, IMRT, protons, carbon ions).

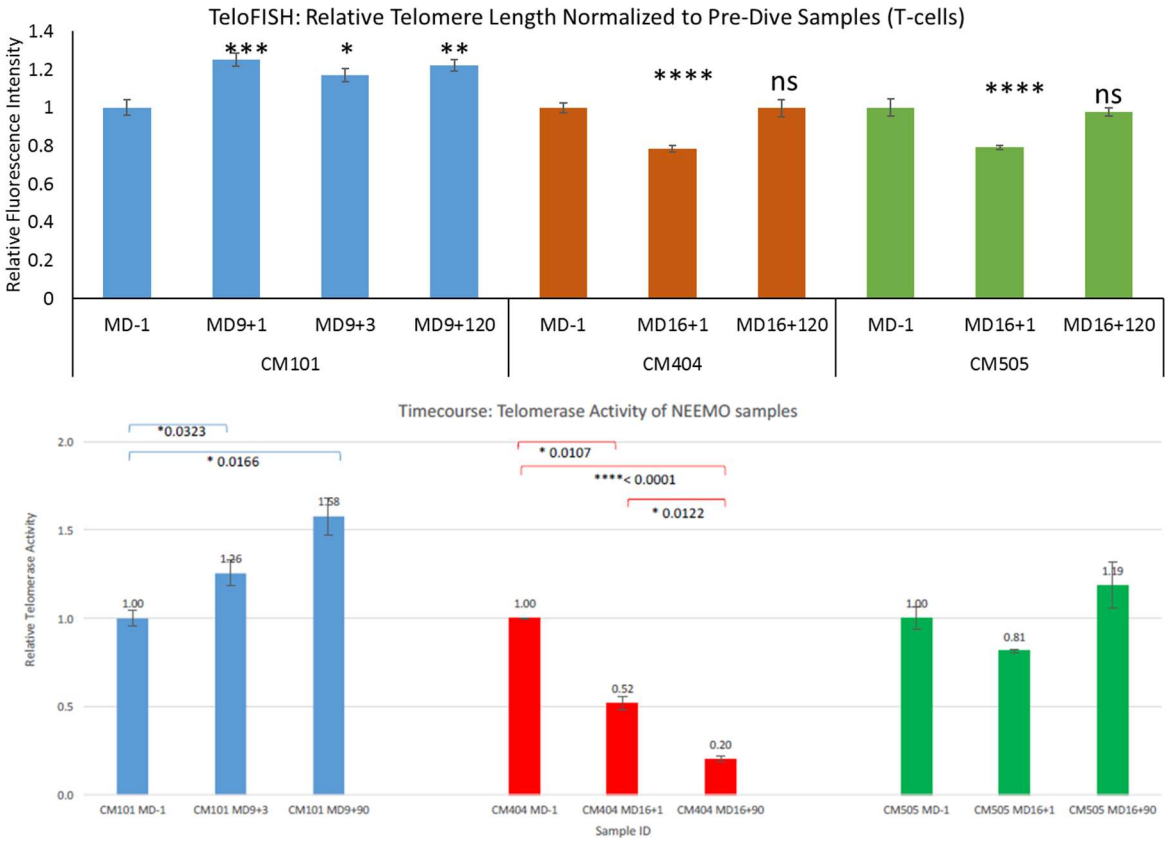

Figure 4.1 Assessment of telomere length and telomerase activity for NEEMO aquanauts. Top panel: Telomere length measurements pre- and post-dive. Telomere length increased after 9 days under water for CM101 and remained elevated up to 120 days post-dive. CM404 and CM505 stayed under water for 16 days and experienced an initial decrease in telomere length that then rebounded back to normal levels post-dive. Bottom panel: Telomerase activity pre- and post-dive. Telomerase activity increased post-dive for CM101, but decreased for both CM404 and CM505. Error bars represent SEM.

REFERENCES

Ayouaz, A., Raynaud, C., Heride, C., Revaud, D., and Sabatier, L. (2008). Telomeres: hallmarks of radiosensitivity. Biochimie *90*, 60–72.

Beery, A.K., Lin, J., Biddle, J.S., Francis, D.D., Blackburn, E.H., and Epel, E.S. (2012). Chronic stress elevates telomerase activity in rats. Biol. Lett. *8*, 1063–1066.

Behjati, S., Gundem, G., Wedge, D.C., Roberts, N.D., Tarpey, P.S., Cooke, S.L., Loo, P.V., Alexandrov, L.B., Ramakrishna, M., Davies, H., et al. (2016). Mutational signatures of ionizing radiation in second malignancies. Nat. Commun. *7*, 12605.

Cherkas, L.F., Hunkin, J.L., Kato, B.S., Richards, J.B., Gardner, J.P., Surdulescu, G.L., Kimura, M., Lu, X., Spector, T.D., and Aviv, A. (2008). The association between physical activity in leisure time and leukocyte telomere length. Arch. Intern. Med. *168*, 154–158.

Deng, Y., Chan, S.S., and Chang, S. (2008). Telomere dysfunction and tumour suppression: the senescence connection. Nat. Rev. Cancer *8*, 450–458.

George, K.A., Rhone, J., Chappell, L.J., and Cucinotta, F.A. (2013). Cytogenetic biodosimetry using the blood lymphocytes of astronauts. Acta Astronaut. *92*, 97–102.

Goytisolo, F.A., Samper, E., Martín-Caballero, J., Finnon, P., Herrera, E., Flores, J.M., Bouffler, S.D., and Blasco, M.A. (2000). Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals. J. Exp. Med. *192*, 1625–1636.

Li, J.S.Z., Miralles Fuste, J., Simavorian, T., Bartocci, C., Tsai, J., Karlseder, J., and Lazzerini Denchi, E. (2017). TZAP: A telomere-associated protein involved in telomere length control. Science.

Lin, J., Epel, E., and Blackburn, E. (2012). Telomeres and lifestyle factors: Roles in cellular aging. Mutat. Res. Mol. Mech. Mutagen. *730*, 85–89.

Lin, J., Cheon, J., Brown, R., Coccia, M., Puterman, E., Aschbacher, K., Sinclair, E., Epel, E., and Blackburn, E.H. (2016). Systematic and cell type-specific telomere length changes in subsets of lymphocytes. J. Immunol. Res. *2016*, 5371050.

Lo, A.W. l., Sabatier, L., Fouladi, B., Pottier, G., Ricoul, M., and Mumane, J.P. (2002). DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. Neoplasia *4*, 531–538.

Lustig, A., Shterev, I., Geyer, S., Shi, A., Hu, Y., Morishita, Y., Nagamura, H., Sasaki, K., Maki, M., Hayashi, I., et al. (2016). Long term effects of radiation exposure on telomere lengths of leukocytes and its associated biomarkers among atomic-bomb survivors. Oncotarget *7*, 38988–38998.

McKenna, M.J., and Bailey, S.M. (2017). Chromosomal and telomeric biomarkers of normal tissue injury to evaluate risk of degenerative health effects (secondary malignancy, cardiovascular disease) post radiation therapy. Transl. Cancer Res.

McKenna, M.J., Robinson, E., Goodwin, E.H., Cornforth, M.N., and Bailey, S.M. (2017). Telomeres and NextGen CO-FISH: Directional Genomic Hybridization (Telo-dGH^{TM}). Telomeres and Telomerase: Methods and Protocols, Z. Songyang, ed. (New York, NY: Springer New York), pp. 103–112.

Olovnikov, A.M. (1973). A theory of marginotomy. J. Theor. Biol. *41*, 181–190.

Ramsey, M.J., Moore, D.H., Briner, J.F., Lee, D.A., Olsen, L.A., Senft, J.R., and Tucker, J.D. (1995). The effects of age and lifestyle factors on the accumulation of cytogenetic damage as measured by chromosome painting. Mutat. Res. *338*, 95–106.

Ray, F.A., Robinson, E., McKenna, M., Hada, M., George, K., Cucinotta, F., Goodwin, E.H., Bedford, J.S., Bailey, S.M., and Cornforth, M.N. (2014). Directional genomic hybridization: inversions as a potential biodosimeter for retrospective radiation exposure. Radiat. Environ. Biophys. *53*, 255–263.

Rea, M.E., Gougelet, R.M., Nicolalde, R.J., Geiling, J.A., and Swartz, H.M. (2010). Proposed triage categories for large-scale radiation incidents using high-accuracy biodosimetry methods. Health Phys. *98*, 136–144.

Reste, J., Zvigule, G., Zvagule, T., Kurjane, N., Eglite, M., Gabruseva, N., Berzina, D., Plonis, J., and Miklasevics, E. (2014). Telomere length in Chernobyl accident recovery workers in the late period after the disaster. J. Radiat. Res. (Tokyo) *55*, 1089–1100.

Serrano, A.L., and Andrés, V. (2004). Telomeres and cardiovascular disease. Circ. Res. *94*, 575–584.

Sgura, A., Antoccia, A., Berardinelli, F., Cherubini, R., Gerardi, S., Zilio, C., and Tanzarella, C. (2006). Telomere length in mammalian cells exposed to low- and high-LET radiations. Radiat. Prot. Dosimetry *122*, 176–179.

Shay, J.W., and Wright, W.E. (2010). Telomeres and telomerase in normal and cancer stem cells. Febs Lett. *584*, 3819–3825.

Sigurdson, A.J., Ha, M., Hauptmann, M., Bhatti, P., Sram, R.J., Beskid, O., Tawn, E.J., Whitehouse, C.A., Lindholm, C., Nakano, M., et al. (2008). International study of factors affecting human chromosome translocations. Mutat. Res. Toxicol. Environ. Mutagen. *652*, 112–121.

Sishc, B.J., Nelson, C.B., McKenna, M.J., Battaglia, C.L.R., Herndon, A., Idate, R., Liber, H.L., and Bailey, S.M. (2015). Telomeres and telomerase in the radiation response: implications for instability, reprogramming, and carcinogenesis. Front. Oncol. *5*.

Snigiryova, G., Braselmann, H., Salassidis, K., Shevchenko, V., and Bauchinger, M. (1997). Retrospective biodosimetry of Chernobyl clean-up workers using chromosome painting and conventional chromosome analysis. Int. J. Radiat. Biol. *71*, 119–127.

Sykes, P.J., Day, T.K., Swinburne, S.J., Lane, J.M., Morley, A.A., Hooker, A.M., and Bhat, M. (2006). In vivo mutagenic effect of very low dose radiation. Dose-Response *4*, 309–316.

Tawn, E.J., and Whitehouse, C.A. (2003). Persistence of translocation frequencies in blood lymphocytes following radiotherapy: implications for retrospective radiation biodosimetry. J. Radiol. Prot. Off. J. Soc. Radiol. Prot. *23*, 423–430.

Valdes, A.M., Andrew, T., Gardner, J.P., Kimura, M., Oelsner, E., Cherkas, L.F., Aviv, A., and Spector, T.D. (2005). Obesity, cigarette smoking, and telomere length in women. Lancet Lond. Engl. *366*, 662–664.

Watson, J.D. (1972). Origin of concatemeric T7DNA. Nature *239*, 197–201.

Yoshida, K., Misumi, M., Kubo, Y., Yamaoka, M., Kyoizumi, S., Ohishi, W., Hayashi, T., and Kusunoki, Y. (2016). Long-term effects of radiation exposure and metabolic status on telomere length of peripheral blood t cells in atomic bomb survivors. Radiat. Res. *186*, 367–376.

Zhao, Y., Lai, K., Cheung, I., Youds, J., Tarailo, M., Tarailo, S., and Rose, A. (2006). A mutational analysis of Caenorhabditis elegans in space. Mutat. Res. Mol. Mech. Mutagen. *601*, 19–29.

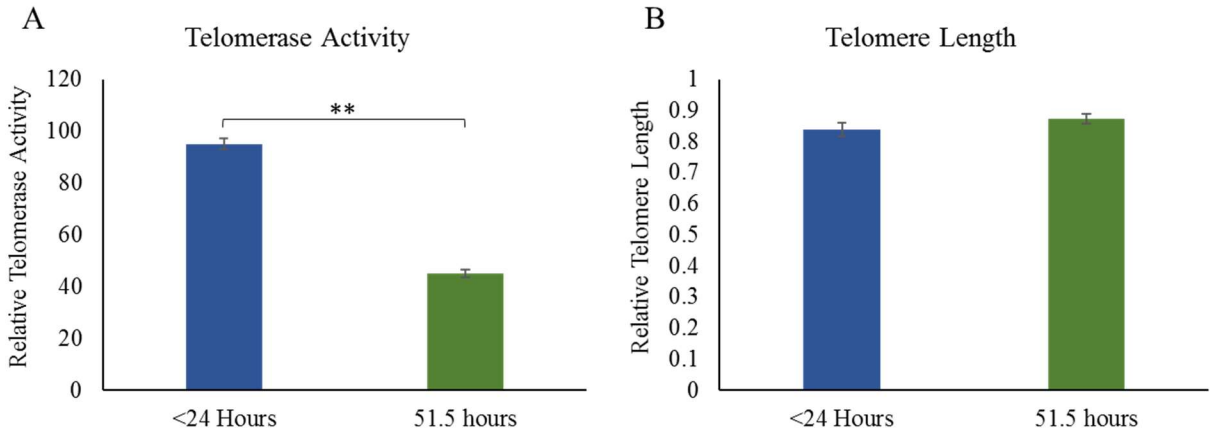

Figure 5.1 Influence of transit time on relative telomerase activity and telomere length for samples in transit less than 24 hours and 51.5 hours prior to processing. A) A significant decrease in telomerase activity was observed in the sample processed at 51.5 hours compared to less than 24 hours (p=0.002). B) No significant change in telomere length was observed between both samples.

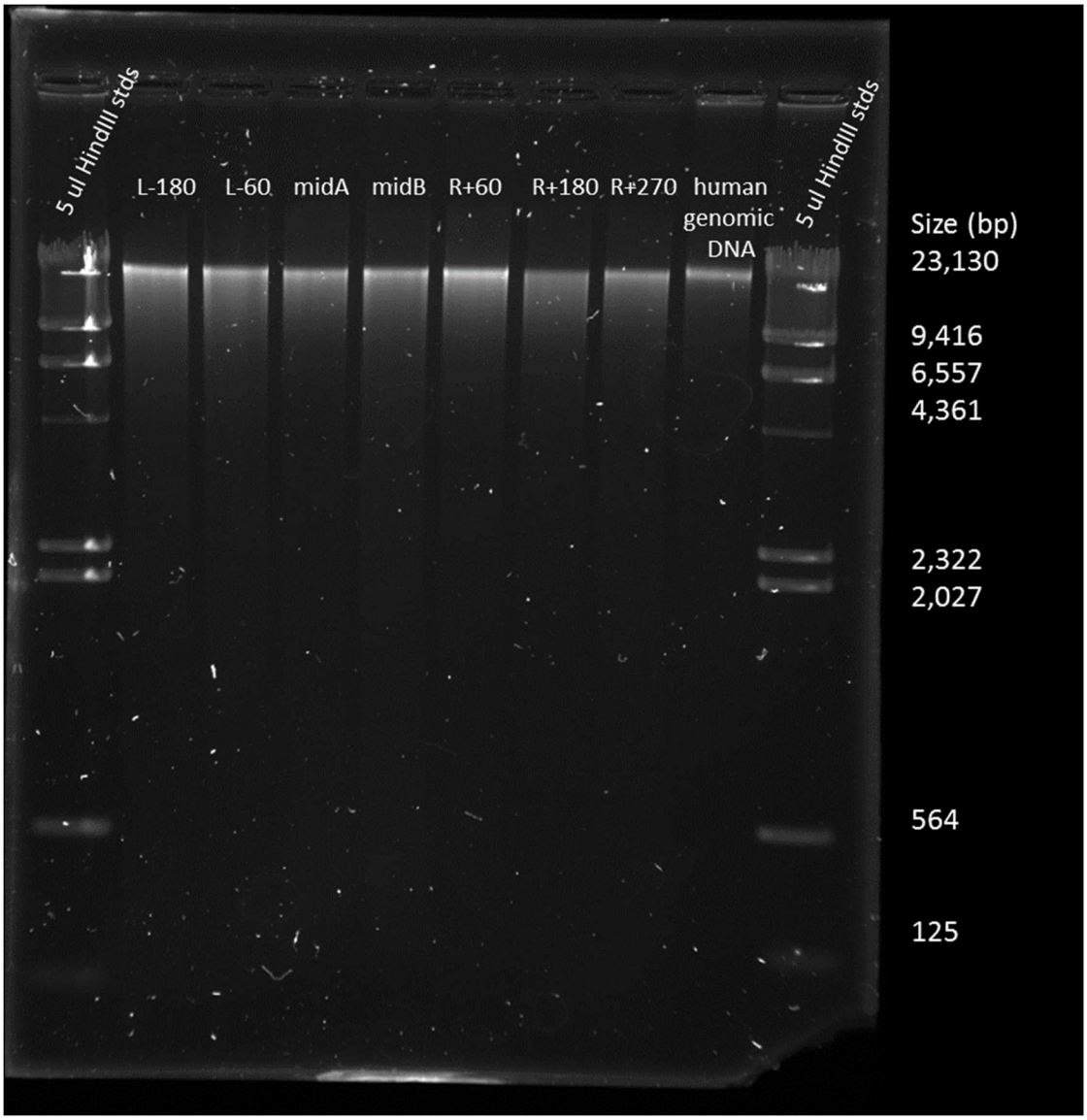

Figure 5.2 Assessment of DNA integrity for mid-flight samples. A 0.8% agarose gel loaded with 37 ng per sample of DNA was run for 3.3 hours at 60 V and stained with 1x SYBR gold. Though telomerase activity was lost during flight, DNA from mid-flight samples was stable and undegraded for telomere length measurements.