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The Effect of Long-Duration Spaceflight on DNA Methylation of Longevity-Related Genes in a One-Year Astronaut Study

Erin Yael Sharoni

A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2021

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Abstract

The aging global population presents one of the most significant challenges of the 21st century, precipitating humanitarian, economic, and environmental burdens. Space provides a unique environment for studying longevity given the extreme hazards it poses to human health. Genomic damage and epigenetic changes have been observed in astronauts and animal models during spaceflight. Space agencies plan to launch long-duration missions (> 6 months) to the Moon and Mars within the next decade. The aim of this investigation is to interrogate the effects of a 340-day spaceflight mission on the methylation of genes associated with two hallmarks of aging, genomic instability and cellular senescence. This aim is based on the premise that spaceflight hazards like radiation induce the accumulation of epigenetic noise, and that epigenetic noise is both a feature and a driver of aging (Sinclair & LaPlante, 2019). The NASA Twins Study is the first year-long mission featuring genetically matched twin astronaut subjects and it provides a unique multidimensional molecular dataset for studying the effects of space on human aging (Garrett-Bakelman et al., 2019). In this investigation, longitudinal genome-wide methylation data obtained from the NASA Twins Study was intersected with a set of 30 longevity-related candidate genes to uncover differential DNA methylation and biological age estimates were obtained using computational tools. Analysis of mean differential methylation results across preflight, inflight, and postflight mission periods revealed negligible significant changes in DNA methylation of candidate genes in the space-bound twin and a number of significant changes in the earthbound twin. Analysis

of biological age using an epigenetic clock algorithm revealed no significant change in rate of aging in the space-bound twin. The paradoxical results of this investigation raise novel questions about the role of DNA methylation as a marker of aging in space and underscore the need for additional longitudinal studies in larger groups of astronauts with longer postflight follow-up periods.

Dedication

For my late grandfather, Shalom, and my late grandmother, Frances, both of whom were creative thinkers far ahead of their time.

Acknowledgments

I would like to extend my gratitude to the individuals who made this work possible. At Harvard, my thesis director Dr. Edward Pace-Schott and research advisor Dr. James Morris provided invaluable academic guidance and encouragement throughout a long process amid the extraordinary challenge of a global pandemic. Dr. David Sinclair's Information Theory of Aging inspired me to pursue this investigation and I am grateful to him for his early positive feedback on a radical hypothesis that I have included in the Future Directions section of the thesis. At the Mason Lab at Weill Cornell Medical College, Dr. Jonathan Foox generously shared his informatics prowess and provided critical support in data analysis and interpretation, and Dr. Christopher Mason graciously allowed me to work with his laboratory on the NASA Twins Study dataset. Dr. Mason's openness to collaboration and the pursuit of creative research is a shining example of scientific leadership. None of this work would have been possible without retired NASA astronaut twins Scott and Mark Kelly and all those before them who dedicated their bodies to science, exploration, and the advancement of humankind. Many thanks to my beloved safta who always sees the positive in the greatest of challenges; my sister who keeps me laughing; KM, IM, CS, GL, DD, JG, MK, SB, KJ whose friendship buoyed me in countless ways throughout this journey; and JF, without whose help and companionship I would be lost. Most importantly, a heartfelt thanks to my parents, who have always supported my dreams and long ago taught me to reach for the stars.

Table of Contents

Dedication v

Acknowledgments vi

List of Tables ix

List of Figures x

Chapter I. Introduction 1

Background of the Problem 5

DNA Damage and Cellular Senescence 10

The Information Theory of Aging 12

Biological Age 15

DNA Methylation as a Marker of Biological Age 16

Health Risks of Spaceflight 19

The NASA Twins Study 20

Genes of Interest 23

Question and Hypothesis 24

Significance of Research 25

Chapter II. Materials and Methods 27

Materials 27

Subject Consent, Sample Collection, and Approvals 27

T Lymphocyte-Derived Cell Lines 28

Methods 28

Methods for sample collection....28
Analysis....28
Ensembl BioMart release 101....28
BWA-Meth....29
MethylDackel....29
MethylKit....29
Python Script....29
methDiff and Differentially Methylated Cytosines (DMCs)....30
DNA Methylation Age Calculator....31
Chapter III. Results....32
Differential DNA Methylation....32
DNA Methylation Age Calculations....36
Chapter IV. Discussion....41
Explaining the Paradox....47
Study Limitations....51
Future Directions....53
Conclusions....59
Appendix A. Candidate Genes....61
Appendix B. All Significant Differentially Methylated CpGs....71
Appendix C. Python Script....76
Appendix D. Definition of Terms....79
References....82

List of Tables

Table 3. Summary of differentially methylated genes and direction of change. 36

Table 4. Summary of DNAmAge results.....40

Table 1. Candidate genes used in this investigation and descriptions of their longevity-related functions.....61

Table 2. CD4+ and CD8+ T cell sample collection dates. 67

Table 5. DNAmAge vs. chronological age across all sample collection dates for both subjects.....68

Table 6. Significant methDiff across individual CpGs.....71

List of Figures

Figure 1. Percentage of world population aged 60 years or over. 6

Figure 2. Crude birth rate worldwide.....7

Figure 3. The nine hallmarks of aging. 15

Figure 4. Statistically significant differential methylation results for HR. 33

Figure 5. Statistically significant differential methylation results for TW. 34

Figure 6. Frequency of genes with statistically significant differential methylation. 35

Figure 7. DNA methylation age (DNAmAge) results. 38

Figure 8. Average Δ between DNAmAge and chronological age. 39

Chapter I.

Introduction

On November 13th, 1789, Benjamin Franklin wrote a letter that birthed a popular idiom: Nothing in life is certain but death and taxes. Barring some dystopian intervention lifted from the pages of a science fiction novel, all organic life does indeed die. And on our way to death, we age. While death is inevitable, Mr. Franklin ascribed no such certainty to aging. According to geneticist and longevity expert David Sinclair, "Aging is a disease, and that disease is treatable" (Sinclair & LaPlante, 2019). The recent work of Sinclair and others marks a paradigm shift in the field of longevity science, prompting humanity to reconsider what we once accepted as an unhappy fate. This revelation comes at a critical point in history, as the aging global population on this planet presents one of the most significant challenges of the 21st century (United Nations Population Fund [UNFPA], 2012). Within the next few decades, up to 20% of the world's population will be over the age of 60 (Hansen & Kennedy, 2016).

In childhood, we come to understand that aging is associated with the functional decline of biological systems over time. We watch as grandparents grow increasingly frail and parents sprout gray hair; we mourn the loss of family pets. We form a view of aging: it is painful, ugly, and inevitable. We are told that the worst of it can be diminished or delayed if we sleep more, eat better, avoid smoke, exercise, meditate, and avoid loneliness. Indeed, the biomolecular hallmarks of aging can be improved by some of these basic interventions, and their underlying mechanisms continue to be elucidated

(Sinclair & LaPlante, 2019). Two such hallmarks of aging are cellular senescence and DNA damage. Like most markers of aging, they are not mutually exclusive, but related, with DNA damage serving as an inducer of senescence, and transcriptional repression of DNA repair genes being a feature and cause of senescence itself (Collin et al., 2018). Cellular senescence is widely described as a state of permanent cell cycle arrest. While timely cellular senescence is an evolutionarily conserved feature that can be protective to the organism by arresting the growth of deleterious cells, chronic senescence that occurs with normal aging or prematurely in response to environmental stress induces DNA damage and accelerates aging and disease (Collin et al., 2018).

DNA damage is caused by a variety of factors, including oxidative stress, exposure to chemical toxins, oncogene signaling, and radiation exposure. There are a few places on Earth where humans have been exposed to these factors to extreme degrees, the most notable being the nuclear disaster sites of Hiroshima and Chernobyl. However, a unique setting for observing the aging hallmarks of DNA damage and cellular senescence exists off Earth—during spaceflight. Astronauts on long-duration spaceflight missions are prime candidates for studying the effects of DNA damage on biological aging. Astronauts are exposed to greater amounts of radiation than the average human being, including space radiation from solar particle events (SPE) and galactic cosmic rays (GCR) (Luxton et al., 2020). Even in lower-Earth orbit on the International Space Station (ISS), astronauts are exposed to ionizing radiation (IR), a more damaging form of radiation than the non-ionizing kind (Chancellor et al., 2014). IR causes more damage to cells that proliferate the most, such as intestinal epithelial cells, skin epithelial cells, and fetal tissue cells. Studies in mouse models have revealed that IR exposure in spaceflight can induce a

persistent stress response and increases senescence signaling, especially in tissues with highly proliferative cells that are vulnerable to radiation, like the intestinal epithelium (Kumar et al., 2018). Upregulation of senescence markers in hematopoietic stem cells during spaceflight has been previously reported, along with increased genetic expression of senescence-associated pathways in mice (Blaber, Pecaut, & Jonscher, 2017). It is well-documented that IR levels of 50 millisieverts (mSv) and above increase the risk of cancer. According to NASA, astronauts are exposed to roughly 50 to 2,000 mSv of IR on six-month-long missions aboard the ISS (Perez, 2017). Statistical models have predicted significantly increased cancer risks from exposure to space radiation for astronauts engaged in multiple missions of 18 months or longer in total duration (Cucinotta, 2014). In addition to radiation, astronauts are uniquely exposed to other chronic stressors such as microgravity, physical and social isolation, dietary changes, psycho-cognitive performance demands, and the extreme physical stress of the return to Earth (Luxton et al., 2020). The NASA Twins Study (One-Year Mission) is an unprecedented investigation of astronaut Scott Kelly and his earthbound twin brother, former astronaut Mark Kelly, over the course of a 340-day long ISS mission, that provides novel insights using a multidimensional biomolecular dataset to observe the impact of spaceflight on the human body (Garrett-Bakelman et al., 2019). This seminal research has yielded a vast array of biological data and results to interrogate and indeed, additional papers have been published in the past year revealing novel insights—especially where DNA damage and implications for lifespan are concerned (Kim, 2021).

It is the aim of this thesis to analyze longitudinal genetic datasets obtained from the One-Year Mission to establish whether or not there is a significant relationship

between long-duration spaceflight and accelerated biological aging, by interrogating differential methylation of longevity-related genes involved in processes of DNA damage response (DDR) and cellular senescence. DNA methylation is an epigenetic signaling mechanism that plays an important role in both controlling gene expression and maintaining chromosome stability (Phillips, 2008). Comparing differences in gene methylation status between mission time periods—e.g., preflight versus postflight—can help elucidate the impact of the space environment on longevity. Importantly, initial findings of the One-Year Mission revealed that changes in transcriptional expression of genes and increased DNA damage occurred inflight and persisted for up to 6 months after astronaut Scott Kelly returned to Earth (Garrett-Bakelman et al., 2019).

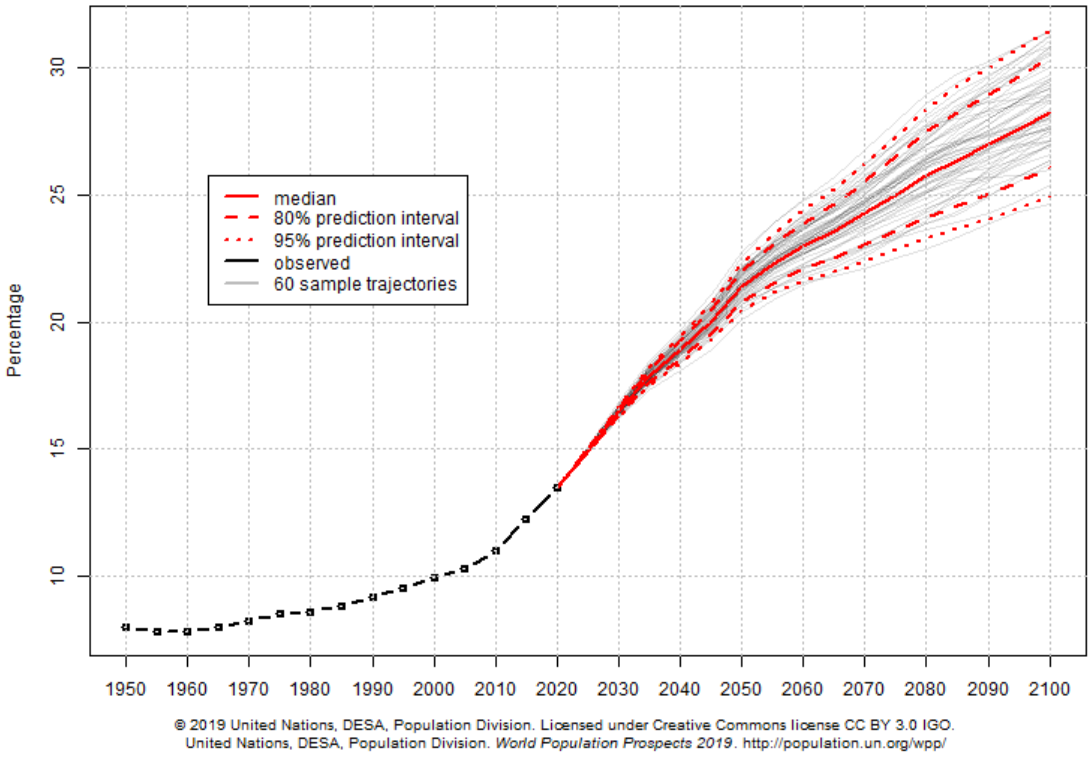
In this investigation, I aim to determine whether genes central to the maintenance of genomic stability and the process of cellular senescence were differentially methylated across three longitudinal time periods (preflight, inflight, and postflight), and to determine if these results correlate with an estimate of biological age. I hypothesize that long-duration human spaceflight > 6 months induces changes in the methylation of these genes, and that these changes contribute to accelerated epigenetic aging in astronauts. This hypothesis is based on initial findings from epigenome-wide interrogation in the One-Year Mission study conducted by Garrett-Bakelman et al. (2019) and on David Sinclair's proposed Information Theory of Aging. The Information Theory of Aging asserts that damage to the epigenome causes a loss of epigenetic information over time and results in observable aging phenotypes (Sinclair & LaPlante, 2019). To this end, I employ analysis of DNA methylation data from the One-Year Mission and estimate changes in biological age using an epigenetic clock. My investigation presents an

opportunity to probe a unique set of longitudinal epigenomic data to uncover novel relationships between spaceflight and biological aging that is immediately significant given NASA's plans to send humans to the Moon by 2024 and Mars by the 2030s, and given the urgent need to sustain an aging human population on a planet with diminishing resources.

Background of the Problem

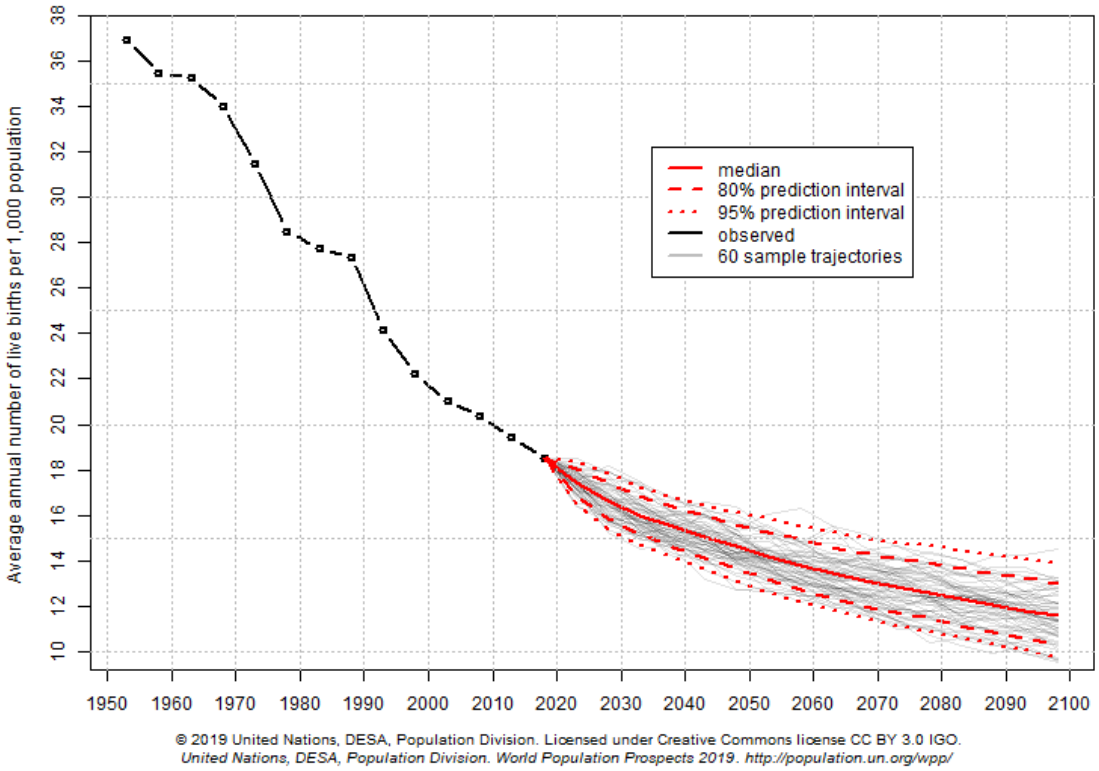
According to census predictions, there are now more people over the age of 65 than under the age of five living on this planet for the first time in recorded history (World Health Organization [WHO], 2015). As illustrated in Figure 1, worldwide aging is accelerating. That our Earth is ferrying an increasingly aging population around the sun should come as no surprise given the global birth rate—over the last fifty years, it has halved (Figure 2). However, while humans are living longer on average than ever before, they are also getting sicker with more chronic, degenerative diseases than ever before (WHO, 2015). According to molecular biologist Aubrey de Grey, aging is the leading cause of death in the developed world, a claim aligned with the position that aging itself should be classified as a treatable disease (Sinclair & LaPlante, 2019; Smith, 2010). This is partly a consequence of the fact that the longer a body remains alive, the more its various systems deteriorate. Evolutionary geneticists would say we have antagonistic pleiotropy to thank for this effect—that which benefits an organism's wellness and survival early on in life may become detrimental as the organism ages beyond its reproductive years (Williams & Day, 2003). Regardless, the significance of the trend should not be underestimated. Age-related decline in health is a costly problem that precipitates significant social and economic burdens.

Figure 1. Percentage of world population aged 60 years or over.



Worldwide aging is accelerating (United Nations, 2019).

Figure 2. Crude birth rate worldwide.



Worldwide crude birth rate has halved since 1950 and a continued decline is projected (United Nations, 2019).

According to the United Nations Population Fund, the aging global population is one of the most significant challenges of this century, as it is intricately tied to many other major challenges we face, like climate change and equitable economic development (United Nations Population Fund [UNFPA], 2012). In the US alone, annual GDP growth is expected to decrease by ~1.2% per year due to population aging (Maestas & Mullen, 2016). While medicine and technology have extended life expectancy, they have not guaranteed equivalent improvements in healthspan. Healthspan is a measure of the

functional period of life that is free of major physiological impediments like disease, pain, and impaired mobility. The costs of a rapidly growing aged population are already increasing the burden on healthcare systems and if we do not work to better understand and address the biomolecular drivers of aging, this trend will become unsustainable (Hansen & Kennedy, 2016). As an example, The Organisation for Economic Co-operation and Development reports that up to 50% of total healthcare spending in Europe is already allocated to people over 65 (OECD/EU, 2018). That will likely increase as the aged population grows and birth rates continue their downward trend.

A key finding of the Second World Assembly on Aging offers hope: so long as healthcare and socioeconomic infrastructure remain robust, people over 60 are incredibly productive (UNFPA, 2012). People in this age group can bring invaluable experience and wisdom to the table when it comes to devising solutions for large global problems, and so it remains critically important to improve healthspan (Sinclair & LaPlante, 2019). A recent economic analysis of the value of extending both human lifespan and healthspan concluded that increasing life expectancy by one year is worth $38 trillion, while an increase of ten years is worth $367 trillion (Ellison, Scott, & Sinclair, 2021). Indeed, aging will impact the health of the majority of the global population and will have an unprecedented effect on myriad social, economic, and ecological systems, from the workforce to the stability of our biosphere and all the life residing within it. This forces us to consider our mortality as a species: How will we manage a global population with more sick, old people than young, healthy ones? How much longer can our planet with its fragile, failing ecosystems, sustain life as we know it? The consequences of aging are

staggering, and major efforts to better elucidate and treat its mechanistic drivers of aging are underway.

Perhaps nowhere is there more room for accelerated scientific innovation and learning than in space, where organisms are subjected to highly controlled, physically extreme environments. Space is also a place we must look to as the future of our planetary home hangs in the balance. Certainly, the significance of space exploration itself cannot be understated. From cultivating novel scientific findings and driving the development of new technologies on Earth to providing humanity with a "plan B" should Earth fail to support life, it is imperative that we improve the potential for prolonged human spaceflight. Ensuring the safety and efficacy of manned missions means we must better understand how spaceflight affects the lifespan and healthspan of its intrepid participants.

Spaceflight presents unique challenges to human physiology, some of which have been obvious from the early days of space travel, like radiation exposure and microgravity, but the full consequences of which researchers are only just beginning to elucidate. As rich new datasets emerge, like those from the One-Year Mission, and as newer tools and technologies are improved, such as whole-genome bisulfite sequencing (WGBS), striking discoveries are being made that underscore the need for further research (Ziller et al., 2016). For example, changes in telomere length, chromosomal inversions, microbiome alterations, mitochondrial dysfunction, and differences in epigenetic aging have been observed in data collected from astronauts on long-duration spaceflight missions and it is clear that DNA damage is pervasive and persistent (Luxton et al., 2020a; Nwanaji-Enwerem et al., 2020). Many of these findings have been

published within the past year. It is an active field of study as it is critical to better understand the consequences of off-world travel if we are to ensure the viability of a future space program. Taken together, addressing the problems unique to astronauts in spaceflight and those we face on Earth due to aging may help us solve both urgent challenges more efficiently.

DNA Damage and Cellular Senescence

DNA damage is a key driver of aging in humans. It is defined as a change in the basic DNA structure, resulting in a break, inversion, translocation, or other chemical disruption that is carried over when the damaged DNA is replicated, resulting in a mutation (Bernstein et al., 2012). Properly functioning DNA damage repair mechanisms are essential for ensuring that mutations are avoided, and these mechanisms are compromised during the course of aging, as well as by exposure to exogenous and endogenous hazards, like radiation and cancer. The effect can be bidirectional: impaired damage repair function leads to more DNA damage, and DNA damage can lead to impaired repair function. Cellular senescence plays a role in this process. Senescence is best described as a state of permanent growth arrest; all somatic cells are subject to cellular senescence under the right conditions, dividing until their cell-cycle is irreversibly halted. In young, healthy bodies, senescence is followed by clearance of the growth-arrested cells via autophagy and then tissue regeneration (Muñoz-Espín & Serrano, 2014). However, in aging and disease, uncleared senescent cells languish in a zombie-like state and secrete proinflammatory signals known as senescence-associated secretory phenotype, or SASP (Campisi & di Fagagna, 2007; Sinclair & LaPlante, 2019). Senescent cells may be either a cause or a consequence of aging, or both, and clearance

of senescent cells results in a slowing down of aging (Barker et al., 2016). Indeed, senescence plays several important but paradoxical roles, with the end result being a manifestation of an aging phenotype (Campisi, 2011; Rodier & Campisi, 2011).

Cellular senescence likely evolved as a protective mechanism to the young organism, but its effects cease to be protective over time—following Peter Medawar's theory of antagonistic pleiotropy, senescence is something that helps us survive our reproductive years but loses its beneficial effect as we age and accumulate damage, having become evolutionarily dispensable (Gavrilov, L.A. & Gavrilova, 2002). Senescence is essential to tumor suppression and acts as an irreversible stop-gap measure to halt proliferation if a cell experiences highly corrupting DNA mutations, epigenetic aberrations, exposure to toxins, dysregulation of telomerase, or erosion of telomeres (Campisi & di Fagagna, 2007). Apart from DNA damage and oncogene activation, senescence can be induced by replicative exhaustion when a cell reaches its natural division limit (van Deursen, 2014).

However, while senescence may suppress tumor proliferation, it also contributes to tumor promotion and bodily aging (Rodier & Campisi, 2011). When cells senesce, they both deplete tissues of stem cells and exhibit SASP, an inflammatory cocktail of secretions that includes cytokines, chemokines, and various growth factors (Rodier & Campisi, 2011; Watanabe et al., 2017). In turn, those secretions, stimulated by the NFκB signaling pathway, contribute to the physical deterioration that we describe as aging and drive age-related disease. Interestingly, the One-Year Mission findings revealed pro-inflammatory cytokines including IL-6 and TNF-α were upregulated both inflight and postflight. Previous studies have also shown that these same cytokines can activate the

NFκB signaling pathway and increase the proliferation of reactive oxygen species (ROS) that cause DNA damage (Ask et al., 2018). Taken together, this illustrates the relationship between cellular senescence and the DNA damage accrued during long-duration spaceflight missions that forms the basis for the hypothesis of this investigation.

The Information Theory of Aging

The Information Theory of Aging (ITA) is described in detail by David Sinclair, Ph.D. in his 2019 book, *Lifespan: Why We Age—and Why We Don't Have To*. The ITA is a novel and compelling epigenetics-focused theory which posits that damage to the epigenome causes a loss of epigenetic information over time and ultimately results in the observable phenotype of aging (Sinclair & LaPlante, 2019). In other words, a root cause of biological aging may be a loss of epigenetic information. The epigenome is a collection of chemical marks on an organism's DNA and histone proteins that regulates gene expression, differentiation, and development, and these marks can be described as information that is both heritable and altered by the environment (Heard & Martienssen, 2014; Yang et al., 2019). As we age, we accrue DNA damage; specifically, double-stranded breaks. These breaks commonly occur under normal conditions and in a healthy person they are readily repaired by cellular machinery. However, when enough double-stranded breaks accrue—either over the natural course of time or accelerated by exposure to intrinsic and extrinsic factors that damage the genome, such as stress, diet, and radiation—the repair process may fail and result in a loss of epigenetic information (Lu et al., 2020).

To illustrate this phenomenon, Sinclair employs a clever metaphor to describe the ITA, based on the premise that there are two main types of information stored in the

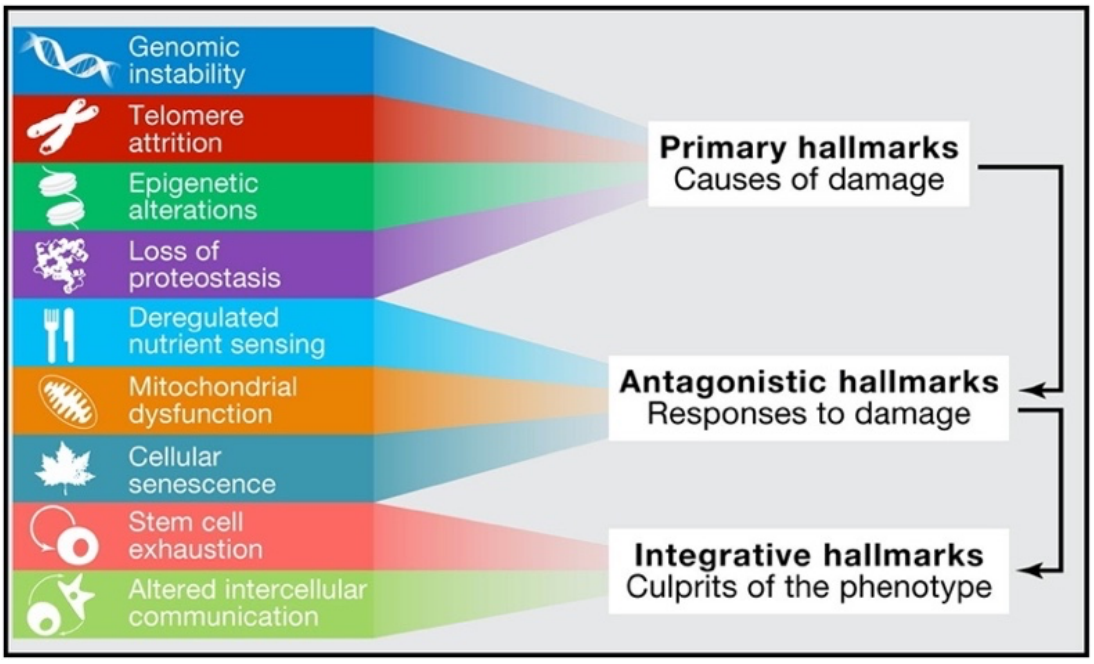
body: digital and analog. The genome itself is a receptacle of information, like a compact disc, where the DNA is stored as digital information and the epigenome is the analog reader of that information (Sinclair & LaPlante, 2019). Anyone old enough to have owned a Sony Discman will recall that when a CD is scratched, the music doesn't play correctly. It skips or stops altogether, leaving the listener with either a disrupted song or no sound at all. The digital information encoded on the CD remains intact, but the ability to play it is lost. Such is the case with our genome—when the disc is scratched, it can't be properly read. DNA is like digital information that lasts far longer than a typical human lifespan, but the analog mechanism that reads it, the epigenome (the cellular structure that allows genes to be turned on and off) fails much earlier because it is much harder to preserve. As such, the expected remedy for aging would involve finding a way to polish the disc and erase the scratches.

How do the epigenomic scratches on this DNA disc occur? In keeping with the analogy, if one handles their CDs recklessly, exposing them to the environment without protection, then one should expect the CDs to accrue damage and not play properly. Similarly, poor lifestyle habits, environmental stressors, radiation exposure, and the natural process of aging itself will induce DNA damage. A family of proteins called sirtuins are responsible for maintaining genomic stability and regulating gene expression, and they go to work repairing the damage at the site of the DNA break. In a young, healthy organism, sirtuins can find their way back to their home position after making such repairs, but in an aging or unhealthy organism where excessive damage has accrued, sirtuins may become distracted and unable to resume their regular duty, resulting in altered gene expression and a loss of cellular identity (Oberdoerffer et al., 2008; Sinclair

& LaPlante, 2019). This loss of cellular identity and perturbed gene expression pattern induces the aging phenotype (Lu et al., 2020). Sirtuins and the SIRT genes which encode them are thus essential to lifespan regulation, as they play a central role in mediating genetic damage accrued over time as we age. In this investigation, we interrogate epigenetic alterations in SIRT genes and other DDR genes.

Genomic instability due to DNA damage is one of nine key hallmarks of aging. The nine hallmarks of aging are not unique to Sinclair's theory, but they are central to it. These hallmarks are important to understand in the context of this investigation, as it focuses on two in particular—genomic instability and cellular senescence. The focus on these two hallmarks is due to the nature of our dataset and the health risks unique to spaceflight, which are outlined in detail later in this introduction. First described by López-Otín et al., (2013) the nine hallmarks of aging are: Genomic instability due to DNA damage, telomere attrition, epigenetic changes, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Figure 3). As illustrated, these hallmarks can be placed into three buckets: Primary hallmarks that are causes of damage, antagonistic hallmarks that are responses to damage, and integrative hallmarks that are culprits of the phenotype (López-Otín et al., 2013).

Figure 3. The nine hallmarks of aging.



Reprinted from López-Otín et al. (2013) with permission from Elsevier, license #4997691040229.

Biological Age

Importantly, the hallmarks of aging have been used in many different models of biological age. Biological age generally describes the difference between an individual's chronological age and their physiological age based on an evaluation of age-associated factors (Jackson, Weale & Weale, 2003). The assessment of biological age has evolved over time as new mechanisms are elucidated and new tools are built. Before the sequencing of the human genome, biological age calculations relied on physical parameters such as frailty tests, cognitive assessment, and blood biomarkers, and investigation of biological age was largely limited to the arena of gerontology (Borkan &

Norris, 1980). With the advent of next-generation sequencing (NGS) and advancements in imaging technologies, biological age calculations in the 21st century have become increasingly complex and accurately predictive, and are in demand across disciplines.

Current models of biological age incorporate epigenetic information. The epigenome controls gene activity and function without modifying the underlying DNA; it is both heritable and changeable during an organism's lifetime in response to intrinsic and extrinsic factors (Moore, Le, & Fan, 2012). Conrad Waddington coined the term epigenetics and in 1957 proposed the idea of an "epigenetic landscape" to describe the process of cellular differentiation during embryonic development in multicellular organisms (Goldberg, Allis, & Bernstein, 2007). In this respect, methylation patterns are heritable and stable, allowing a cell to become its fate. At the same time, the methylome is also a dynamic landscape that can change over time and in response to certain stimuli. It now is widely agreed that *de novo* changes in the epigenome are central to the process of aging, as these changes mediate cellular function and stress response (Booth & Brunet, 2016). Indeed, the ITA is predicated upon this very concept: when the epigenome is perturbed by stressors or by age, cells may forget their fate. Measuring the age of the epigenome can thus estimate the biological age of the organism.

DNA Methylation as a Marker of Biological Age

There are various actors on the proverbial epigenetic stage, and DNA methylation (DNAm) plays a major role. Because DNAm is a more robust measurement than RNA gene expression, and because DNAm patterns are both inherited and reflective of accumulated environmental exposures, it is commonly used to estimate biological age. Whole-genome bisulfite sequencing (WGBS) and the development of DNAm microarray

platforms have allowed researchers to observe methylation patterns in the epigenome and paved the way for the creation of epigenetic clocks. Unlike chronological age, which measures the amount of time an organism has lived since its day of birth, epigenetic clocks aim to measure the biological rate of aging, and the two measures may conflict—the number of candles on a birthday cake might be too many or too few, as it turns out. Steve Horvath, a geneticist and biostatistician at University of California, Los Angeles, is credited with developing the first high-fidelity, multi-tissue epigenetic clock in 2013 (Horvath, 2013). Age-associated changes in DNAm have been well-described and are widely understood to be one mechanism of epigenetic control (Booth & Brunet, 2016). As an analogy, epigenetic clocks can be compared to the carbon-dating process in that they aim to roughly predict an organism's biological age at a given point time. The clocks may also predict the rate of aging and even time to death when information on blood cell counts is incorporated (Chen et al., 2016). Since Horvath's original publication, novel species-specific, multi-tissue epigenetic clocks have continued to emerge, each attempting to achieve greater fidelity. The breakthrough Horvath multi-tissue clock was able to determine a set of 393 CpG sites that could predict an individual's biological age within an error margin of 3.6 chronological years and a correlation of 0.96 (Jung and Pfeifer, 2015). Horvath's DNAm age clock has been updated since its inception and remains a gold standard for universal measurement of epigenetic age, and as such, it is the clock used in this investigation (Ecker & Beck, 2019).

The role of DNAm in the regulation of eukaryotic gene expression can be described as a signaling tool wherein a methyl group (CH_3) is transferred onto the C5 position of a cytosine base, resulting in a conversion to 5-methylcytosine by DNA

methyltransferase (DNMT) enzymes (Moore, Le, & Fan, 2012; Lim et al., 2019). In mammals, methylation nearly always occurs in CpG dinucleotides, which are usually found in CG-rich regions of the genome known as CpG islands (CGI). In human genes approximately 70% of promoters, located upstream of DNA near transcription start sites, contain CGI and these CGI are where gene transcription is initiated (Deaton & Bird, 2011). Simply put, methylation can transcriptionally silence or turn off a gene, whereas hypomethylation (de-methylation) can turn a gene on. It is by this method that methylation controls gene expression, and while a specific CpG site can be either methylated or unmethylated, it is the proportion of cells in a sample in which a CpG is methylated that is used to assess epigenetic age (Ecker & Beck, 2019). In this investigation, DNAm measurements are drawn from the bulk signal of many thousands of cells rather than a single cell alone.

Interestingly, both hypomethylation and hypermethylation are observed during the aging process in a variety of species, across cell and tissue types (Horvath, 2013). Decreased methylation is linked to enhanced cell proliferation and is commonly found in cells with a high replication rate and in immortal cancer cells (Salhab et al., 2018). Tumor suppressor genes like p53 are commonly hypermethylated—silenced—in cancer cells, as are genes involved in DDR. Cellular senescence plays an important role here too, as senescence is responsible for inducing methylation changes that silence tumor-promoting genes. The relationship between senescence and methylation is dynamic and continues to be elucidated (Xie, Baylin, and Easwaran, 2019).

Taken together, it is clear that DNAm and key hallmarks of aging have strong bidirectional associations. This investigation probes a longitudinal dataset from the

NASA One-Year Mission for the percentage of differentially methylated cytosines (DMCs) between mission stages in genes associated with these hallmarks, with the expectation that both DNAm and epigenetic age will be significantly different in the astronaut due to the unique molecular risks of spaceflight.

Health Risks of Spaceflight

How does spaceflight affect the human body? New and exciting answers to this question continue to emerge. The short of it is what one would expect—space is hard on the body. It should come as no surprise that the environment aboard a spacecraft is physiologically and psychologically challenging. Microgravity causes changes in pressure, fluid distribution, and digestion; cellular circadian rhythms are disrupted due to shifts in light; the sterile spacecraft environment induces microbiome alterations; stress and isolation take a toll, and the various types of radiation astronauts are exposed to are hazardous to cells. In low-Earth orbit aboard the ISS, these effects are due to the main hazards of microgravity and IR, but health risks are sure to increase as spaceflight missions target the Moon and Mars in coming decades (Malkani et al., 2020).

Hallmark health risks of spaceflight include but are not limited to DNA damage, mitochondrial damage, altered telomere dynamics, anemia, microbiome alterations, bone degradation, skeletal muscle loss, ophthalmologic problems, and significant disruptions to the circulatory, respiratory, metabolic, nervous, immune, gastrointestinal, and reproductive systems (Evans & Ball, 2001; Garrett-Bakelman et al., 2019). Some of these effects are acute and resolve upon return to Earth, such as fluid retention and muscle atrophy, but some appear to persist, such as loss of bone density, telomere alterations, cognitive function, and DNA damage. Additionally, effects are more pronounced and

slower to resolve given long-duration missions > 6 months, such as those involving the cardiovascular and musculoskeletal systems (Convertino, 2011). In the One-Year Mission, researchers observed persistent increased DNA damage due to chromosome inversions in astronaut Scott Kelly upon return to Earth, and his immune system remained in a hyperactive state with upregulated expression of some immune-related genes (Garrett-Bakelman et al., 2019). Microgravity affects the bone marrow and thymus, and as all progenitor T cells originate in bone marrow and develop in the thymus, it is unsurprising that the acquired immune system would be disrupted (Akiyama et al., 2020).

The NASA Twins Study

The NASA Twins Study (One-Year Mission) has provided an unprecedented opportunity to analyze the multidimensional effects of long-duration spaceflight through the lens of identical twin astronauts Scott Kelly (TW) and Mark Kelly (HR). In this mission, TW spent 340-days aboard the ISS and earth-bound twin HR served as a genetically matched ground control subject. TW lifted off on March 27, 2015 and returned to Earth on March 1, 2016. Biological samples from both TW and HR were simultaneously collected over 25 months, across preflight, inflight, and postflight stages. While HR served as ground control, he is also a former NASA astronaut and before the study had spent a career total of 54.1 days in space versus TW's 519.7 career total days spent in space (Garrett-Bakelman et al., 2019). This shared rare experience further underscores the unique nature of these study subjects.

Pertaining to the domain of longevity, implications of the One-Year Mission study include the identification of reversible causes of DNA damage in spaceflight, quantification of biological aging in space, the establishment of gene expression patterns

mediated by spaceflight, and novel insights into how genetically matched subjects age differently in different environments. During the mission, TW performed three spacewalks for a total of 18 hours and 20 minutes spent on extravehicular activity (EVA). EVA is conducted outside the protective shielding of the ISS and results in significantly increased exposure to harmful space radiation and increased cumulative radiation dose to the astronaut (Chappell et al., 2017; Welsh et al., 2019). One important result, touted by global media, was the unexpected lengthening of TW's telomeres. On the surface, this outcome appears inconsistent with the fact that spaceflight damages the body—telomeres shorten with age, disease, and stress. However, as with senescence, telomere dynamics are not binary. While longer telomeres are often associated with longevity, immortal cancer cells also exhibit longer telomeres (McNally, Luncsford, & Armanios, 2019). Longer telomeres as a result of exposure to radiation and microgravity likely do not indicate lifespan extension; indeed, upon return to Earth, TW's telomeres immediately shortened within less than 48 hours of returning to Earth and TW retained more short telomeres overall in the postflight period than at baseline (Garrett-Bakelman et al., 2019). Several publications in recent months have attempted to diagnose the mechanisms behind this confounding result, and additional studies are required to observe longer-term effects. The One-Year Mission yielded a trove of results showing significant and pervasive multisystem changes in spaceflight, the full scope of which are described in the initial publication by Garrett-Bakelman et al. (2019). The biological data obtained from the study continues to be mined, such as in this investigation.

Significant findings from the One-Year Mission include pervasive chromosomal inversions and double-stranded DNA breaks, altered telomere dynamics, and variability

of gene expression in TW, all of which persisted for up to six months after the mission (Garrett-Bakelman et al., 2019; Luxton et al., 2020). Additionally significant is the fact that differential gene expression during the 6-12 month inflight period was six-fold greater than during the first 6 months inflight, suggesting that global gene regulation is increasingly affected by spaceflight duration. Furthermore, those genes with altered expression inflight were found to be significantly enhanced in DDR and mitochondrial function pathways (Garrett-Bakelman et al., 2019). It is important to note that gene expression findings published in the One-Year Mission study are based on RNA sequencing data, not DNAm data, whereas this investigation seeks to probe DNAm as a deeper marker of epigenetic alteration in a specific subset of longevity-associated genes. Differentially expressed genes (DEGs) and differentially methylated genes (DMGs) are not the same things. While DNAm can affect transcription of RNA and thus plays a key role in gene expression, the two are different measures of epigenetic activity and methylation of individual CpG sites does not always correlate with transcriptional expression outcomes. Genes may be over- or under-expressed for different biological reasons, or individual CpGs may not receive sufficient coverage in the bisulfite libraries generated from sequencing. Moreover, in human peripheral blood mononuclear cells (PBMCs) like the CD4+ and CD8+ cells used in this investigation, it has been suggested there is a weaker correlation between gene expression and DNA methylation changes (Jung & Pfeifer, 2017). Initial analysis of DNAm by Garrett-Bakelman et al. (2019), revealed that global mean methylation variation and methylation stochasticity was similar in TW and HR throughout the study, with slightly more variation in HR. However, loci

associated with DNA repair and senescence were not specifically interrogated and thus form the basis for this investigation.

Genes of Interest

Candidate genes were selected for DNA methylation difference (methDiff) comparisons across longitudinal time points, preflight, inflight, and postflight. While myriad genes are related to the hallmarks of aging, the hypothesis of this thesis is predicated on the concepts outlined in the ITA, where genomic instability due to DNA damage induces epigenetic aging, and on the cellular senescence which results from that damage. Thus, 30 candidate genes were selected as a subset of those which are expected to be involved in DDR and cellular senescence, as well as genes more broadly associated with lifespan regulation (e.g. *FOXO*, *MTOR,* and *AMPK* genes). Of the 30 candidate genes selected for differential methylation investigation, only three did not appear as differentially expressed genes (DEGs) in TW in any time period in the RNA-seq results of the One-Year Mission published by Garrett-Bakelman et al. (2019)— *DAO*, *EPN3*, and *PARBP*. Nonetheless, they are included as candidate genes in this investigation due to their important roles in cellular senescence and DDR; specifically, *PARPBP* is necessary for the suppression of inappropriate homologous recombination in the process of DNA damage repair (Chen et al., 2020). As previously discussed, methylation of individual CpGs does not always correlate with transcriptional expression outcomes and genes may be over- or under-expressed for different reasons, but it is interesting to note these differences nonetheless. This candidate gene selection is neither exhaustive nor conclusive but allows for a customized snapshot of methDiff with respect to specific

aging-associated processes. Table 1 (Appendix A) lists the 30 candidate genes included in this investigation, along with a brief explanation of each gene's role in DDR, senescence, and lifespan.

Question and Hypothesis

Do the stressors unique to long-duration spaceflight mediate DNA methylation as a measure of biological age? It is the aim of this thesis to analyze longitudinal genetic datasets obtained from NASA's One-Year Mission to establish whether there is a significant association between long-duration spaceflight and epigenetic aging, using differential methylation of DDR genes and senescence-associated genes and a high-fidelity epigenetic clock to estimate changes in biological age. Additionally, it is relevant that this investigation uses methylation data obtained from sorted CD4+ and CD8+ T cells, as it has been demonstrated that T cells with dysfunctional mitochondria accelerate the onset of cellular senescence by triggering an increase in circulating cytokines and the chronic inflammation that drives aging (Desdín-Micó et al., 2020). Mitochondrial dysfunction is a health risk of spaceflight and ROS-induced mitochondrial dysfunction was observed in TW by Garrett-Bakleman et al. (2019). While T cell senescence was not measured in the original study, a hallmark of senescent T cells is the shortening of telomeres, and TW experienced an immediate and significant shortening of telomeres upon his return to Earth. I hypothesize that long-duration human spaceflight > 6 months induces changes in the methylation of genes associated with DNA damage repair and senescence in CD4+ and CD8+ T cells, and that these changes contribute to accelerated epigenetic aging in astronauts.

Significance of Research

This investigation presents an unanswered question resulting from the One-Year Mission. As with all studies involving humans in space, the significance of this work can be placed into two categories: significance to human spaceflight and exploration and significance to the complex challenges faced here on Earth. This duality is part of what makes such research so compelling. By better understanding the problems human bodies face in space, we can both improve opportunities for future space travel while also improving life on Earth. Indeed, many of the results from experiments conducted aboard the ISS have resulted in novel applications, from water purification technologies to advancements in cancer surgery.

Further elucidating the biomolecular mechanisms that drive aging may be achieved by observing human beings in spaceflight, a highly controlled, extreme environment where whole-body stress is increased and DNA damage is accelerated. The research proposed here and conducted in the One-Year Mission study may advance our understanding of not only aging but also cancer and circadian rhythm, which are known to exhibit epigenomic and epi-transcriptomic differences. Additionally, by observing how identical twins age differently in response to different environments, we may uncover novel mechanisms involved in human longevity. Novel discoveries from this investigation may contribute to the development of small-molecule targets for therapies to eliminate senescent cells, potential targets for future CRISPR therapies for aging, and non-invasive preventative interventions that can be readily adopted by younger populations. Given the plans of NASA and other international space agencies to send human beings on long-duration spaceflight missions over the next two decades, and in

light of the urgent economic, ecological, and healthcare challenges driven by Earth's aging global population, the implications of this research are far-reaching.

Chapter II.

Materials and Methods

This research was conducted in collaboration with the Mason laboratory at Weill Cornell Medical College Institute for Computational Biomedicine, led by Christopher Mason, Ph.D. I worked directly with Jonathan Foox, Ph.D, a postdoctoral associate in the Mason laboratory. The laboratory contributed to the landmark Garrett-Bakelman et al. (2019) NASA Twins Study publication by conducting WGBS and transcriptome library preparation, sequencing, and analysis of subjects' genomes. The Mason laboratory collaborates with NASA to support long-term human spaceflight by elucidating key molecular and genetic foundations through better understanding astronaut genomes, epigenomes, and transcriptomes. The materials and methods employed in this investigation are a result of that ongoing work.

Materials

Subject Consent, Sample Collection, and Approvals

Study subjects' consent and blood sample collection are described by Garrett-Bakelman et al. (2019), and protocols were approved by the NASA Flight IRB (protocol number Pro1245) and all participating institutions. I was added to the IRB by the Mason laboratory at Weill Cornell Medical College Institute for Computational Biomedicine.

T Lymphocyte-Derived Cell Lines

CD4+ and CD8+ T cell isolated data from TW and HR was collected at 20 time points longitudinally across the preflight, inflight, and postflight periods comprising the 25-month longitudinal study as described by Garrett-Bakelman et al. (2019). Sample collection times are shown in Table 2 (Appendix A).

Methods

Methods for sample collection

Cell sorting, processing, sequencing, and WGBS library generation using NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, USA) was conducted as described by Garrett-Bakelman et al. (2019).

Analysis

To obtain results, the following software packages and programs were used in the order in which they are listed here.

Ensembl BioMart release 101

Ensembl is a system for retrieving genome annotation data, such as genes, proteins, and transcripts, from model organisms and humans, and BioMart is a customizable web-based tool used for mining Ensembl data (Yates et al., 2020). After identifying candidate genes, I used BioMart to export a file with information necessary to run an intersectional analysis with astronaut WGBS data, including transcript regions and locations of all candidate genes.

BWA-Meth

For primary analysis, the original WGBS Illumina sequence data were aligned using the BWA-Meth software program. In bisulfite conversion, any methylated cytosine is protected and any unmethylated cytosine is converted to uracil; reducing the number of nucleotides from four to three poses algorithmic challenges. Thus, the function of this program is to take short sequence reads and align them against a reference genome (Pedersen et al., 2014).

MethylDackel

To call the proportion of methylation in each CpG, per dinucleotide, MethylDackel software was used (https://github.com/dpryan79/MethylDackel).

MethylKit

To visualize the distribution of methylation across the genome, calculate coverage per CpG, and to conduct differential analysis (methDiff) across samples, the R package MethylKit was used (Akalin et al., 2012). The MethylKit outputs a global map of all DMCs across the genome.

Python Script

Lastly, a Python script was used to parse raw DMC data derived from WGBS results and intersect it with CpGs in candidate genes. The script is available in Appendix C. Results were then filtered for statistically significant DMCs. For statistical significance, the following thresholds were applied: (1) p-value < 0.05; (2) q-value < 0.05; (3) methDiff $\geq +25\%$ or $\leq -25\%$. Rationale for these thresholds is commensurate with standard statistical applications of p-value and q-value; in this investigation, the application of q-value is essential to reduce the likelihood of false positives given the vast number of cell samples

analyzed. In current research, the standard methDiff threshold applied to DMCs compared between time points is 25%. The 25% threshold is generally expected to indicate a large enough shift in methylation to be significant both biologically and statistically. Accordingly, this investigation looks for DMC results between time points greater than or equal to +25% and less than or equal to -25%.

methDiff and Differentially Methylated Cytosines (DMCs)

For this investigation, differential methylation at each CpG (DMC) was obtained as a bulk signal within a population of cells at each longitudinal time point (Table 2). The distribution of CpG methylation in a population of cells is generally bimodal (i.e., 0% and 100%), but methyltransferases may work differentially and result in clonal expansion in either direction (J. Foox, personal communication, February 12, 2021). Thus, methDiff reflects the percentage of difference in mean methylation at an individual CpG site as compared between time points (e.g., postflight vs. preflight). For example, a methDiff of +30% at a specific CpG site could indicate that 70% of cells were methylated in the postflight period and 40% of cells were methylated in the preflight period; a methDiff of -30% could indicate that 40% of cells were methylated in the postflight period and 70% of cells were methylated in the preflight period. We can thus view methDiff as a measure of the mean amplitude and direction of the bulk signal.

There are approximately 27 million CpGs in the human genome and 21 million of them were captured with satisfactory coverage in the samples from TW and HR, allowing for a significant intersectional analysis to be conducted. There were approximately 20-30k overlaps per sample, per time point, for each TW and HR. A list of transcript start

and end sites for each candidate gene used in this analysis can be easily reproduced using Ensembl BioMart version 101 but is too extensive to publish as an appendix to this paper.

DNA Methylation Age Calculator

DNA methylation age (DNAmAge) for both TW and HR was calculated across longitudinal time points (Table 2) using WGBS data from the Mason laboratory and an epigenetic age calculator with methods and R function as described by Horvath (2013). Developed by Steve Horvath and made freely available for research, the DNAmAge clock is a high-fidelity, multi-tissue biological clock used to estimate the epigenetic age of a subject with data derived from most tissues and cell types. However, it is important to note that the DNAmAge calculator is built on microarray data, estimating fluorescence of probe-based capture and methylation. Thus, this method yields an association with directional change in biological age, not a precise prediction. For this investigation, NEBNext Enzymatic Methyl-seq (EM-seq™) WGBS libraries were merged per sample to obtain results. WGBS output was intersected with the 21,000 markers used by the DNAmAge calculator by aligning each genomic coordinate with CpG identifiers in DNAmAge.

Chapter III.

Results

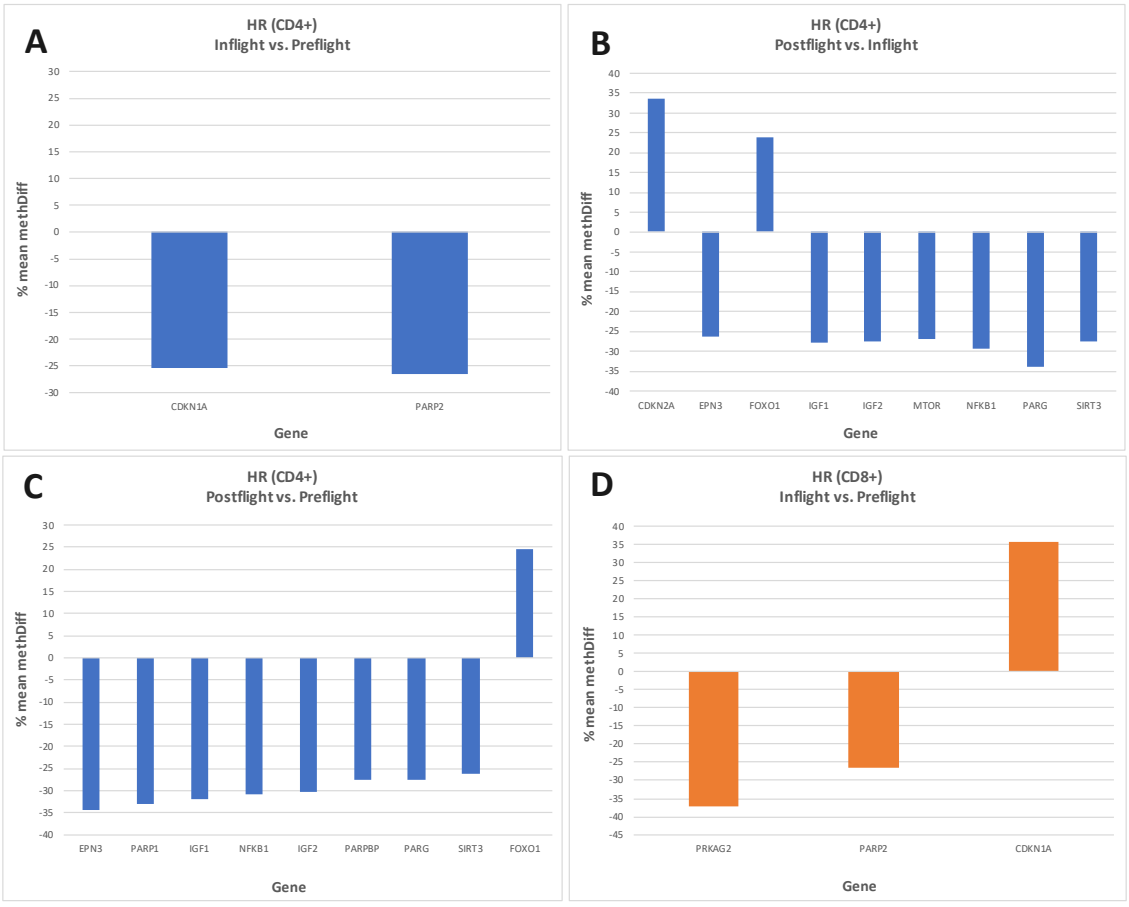
Differential DNA Methylation

Figures 4 and 5 illustrate the percentage of mean methDiff per gene for HR and TW, where only those genes with statistically significant shifts in differentially methylated cytosines (DMCs) between mission periods are included in the results. Therefore, not all periods and cell types are shown. As described in the Materials and Methods section, a statistically significant methDiff result for a DMC is $\geq +25\%$ or $\leq -25\%$ with a p-value and q-value of < 0.05. For each gene with multiple statistically significant DMCs, the mean was taken to summarize the direction and magnitude of methylation shift across the gene. A comprehensive table of methDiff results showing all statistically significant DMCs across mission periods and cell types is included in Table 6 (Appendix B). In total, 16 out of 30 candidate genes were significantly differentially methylated across all periods, cell types, and subjects (Figure 6). Mean methDiff per gene was used to generate these results. *FOXO1* was the most frequently differentially methylated gene, with three instances of significant mean methDiff across all results—two instances in HR and one instance in TW.

The number of significantly differentially methylated genes and the proportion of direction of change were summarized across periods, cell types, and subjects (Table 3). Mean methDiff was used to tabulate these results. Overall, most of the significant methDiff was observed in HR's CD4+ cells. Decreased methylation was more common than increased methylation. Across all results, the average proportion of decreased methylation was 87% and the average proportion of increased methylation was 22%.

There were no significant methylation differences observed in TW's CD4+ cells. The only significant difference (q-value < 0.05) in methylation for TW was observed in five genes in the postflight vs. inflight period in CD8+ cells, where all statistically significant changes showed a decrease in methylation. Notably, the methDiff threshold was relaxed to ≥ +15% or ≤ -15% for this TW sample. At the standard +/-25% threshold, there were no statistically significant methDiff results in any samples at any time points for TW.

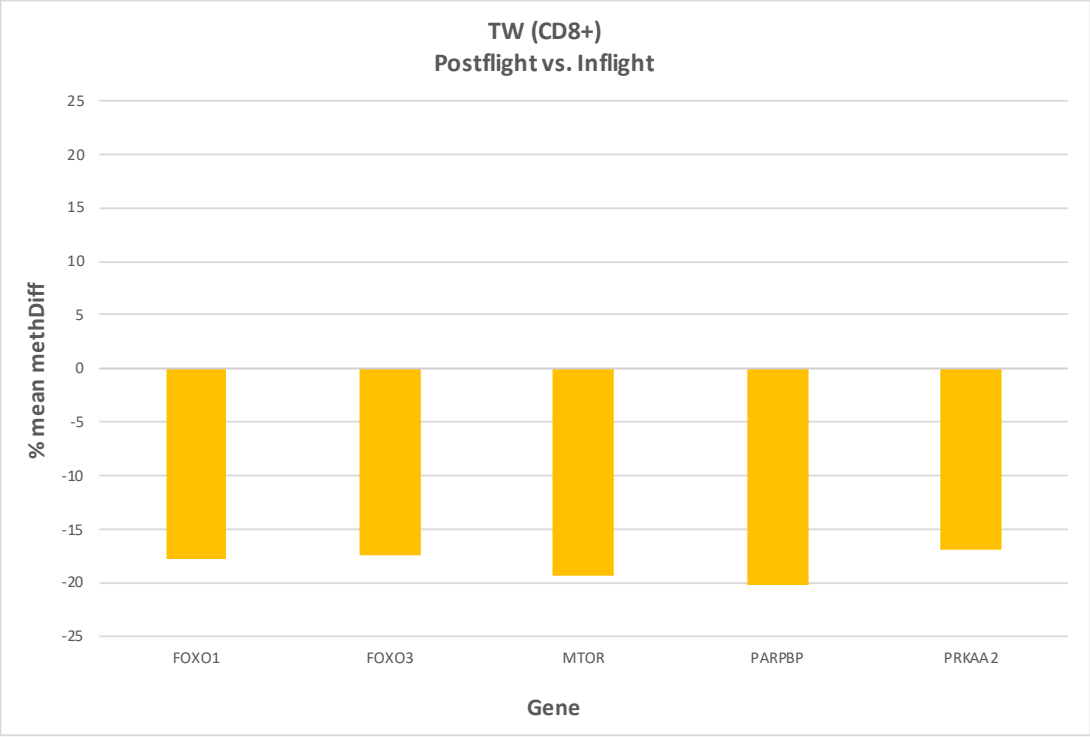
Figure 4. Statistically significant differential methylation results for HR.



Statistically significant differential methylation (methDiff) results for HR illustrate the percentage of mean methDiff per gene for different mission periods and cell types. The

mean was derived from all individually changed CpGs (i.e., differentially methylated cytosines, aka DMCs) in each gene. Each gene had multiple DMCs, and the mean of those DMCs was calculated to determine the net direction and amplitude of the change. Only those genes with statistically significant mean methDiff were included—the cutoff for statistically significant mean methDiff was ≥ +25% or ≤ -25% with a p-value and q-value of < 0.05. CD4+ cells showed significant methDiff in all mission period comparisons and CD8+ cells showed significant methDiff only in the inflight vs. preflight period. The y-axis indicates the percentage of mean methDiff and the x-axis depicts the gene name. ***(a)*** *Percentage of statistically significant mean methDiff per gene for CD4+ cells in the inflight vs. preflight period.* ***(b)*** *Percentage of statistically significant mean methDiff per gene for CD4+ cells in the postflight vs. inflight period.* ***(c)*** *Percentage of statistically significant mean methDiff per gene for CD4+ cells in the postflight vs. preflight period.* ***(d)*** *Percentage of statistically significant mean methDiff summarized per gene for CD8+ cells in the inflight vs. preflight period. methDiff, the percentage of differential methylation per gene between mission periods; HR, earthbound twin Mark Kelly; TW, astronaut twin Scott Kelly.*

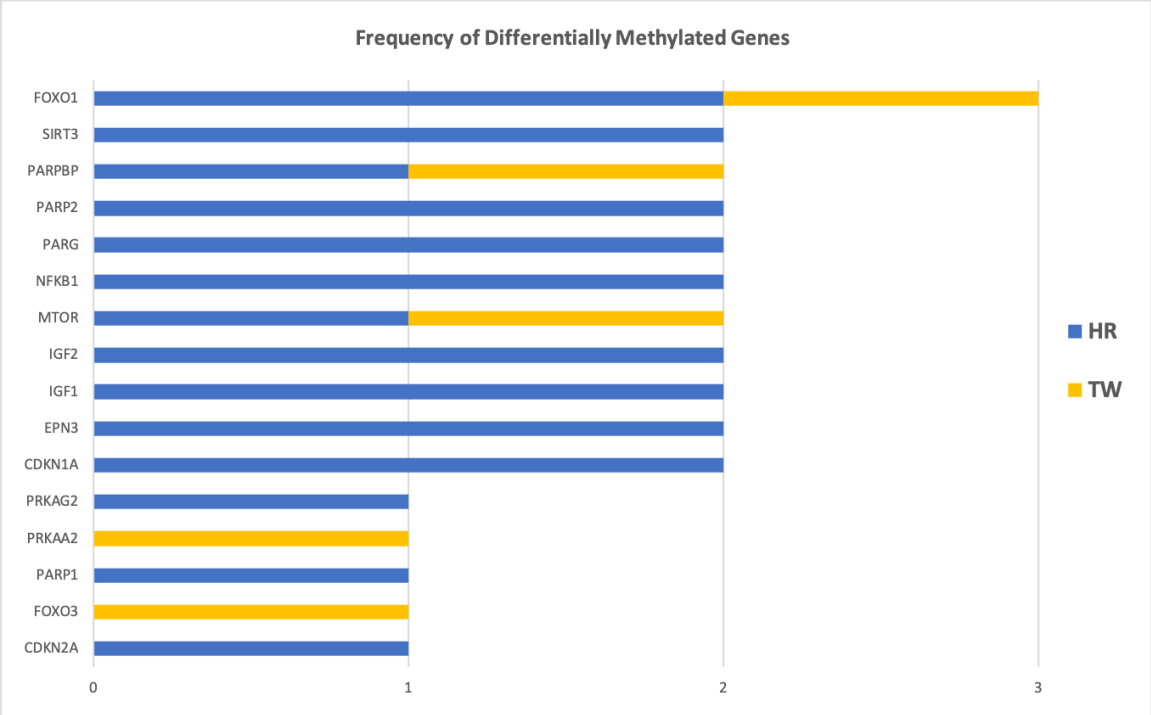
Figure 5. Statistically significant differential methylation results for TW.



Statistically significant differential methylation (methDiff) results for TW illustrate the percentage of mean methDiff per gene for the postflight vs. inflight period in CD8+ cells.

The mean was derived from all individually changed CpGs (i.e., differentially methylated cytosines, aka DMCs) in each gene. Each gene had multiple DMCs, and the mean of those DMCs was calculated to determine the net direction and amplitude of the change. Only those genes with statistically significant mean methDiff were included—importantly, for TW's results, the mean methDiff cutoff was relaxed to ≥ +15% or ≤ -15% with a p-value and q-value of < 0.05. The relaxed threshold was applied because these were the only statistically significant methylation differences observed in TW in any mission period or cell type among the candidate genes included in this investigation. These five differentially methylated genes are key longevity regulators involved in cell growth and proliferation as described in Table 1. methDiff, the percentage of differential methylation per gene between mission periods; TW, astronaut twin Scott Kelly.

Figure 6. Frequency of genes with statistically significant differential methylation.



A total of 16 genes were significantly differentially methylated across all mission periods, cell types, and subjects combined. Genes are listed on the y-axis. The x-axis represents the frequency (number of times) a gene was significantly differentially methylated. The FOXO1 gene was the most frequently differentially methylated, with three occurrences across combined results for HR and TW. FOXO1, PARPBP, and MTOR genes were differentially methylated in both HR and TW. methDiff, percentage of differential methylation per gene between mission periods; HR, earthbound twin Mark Kelly; TW, astronaut twin Scott Kelly.

Table 3. Summary of differentially methylated genes and direction of change.

Sample Period	Number of genes with significant mean methDiff	% increased methylation	% decreased methylation
CD4+ HR Inflight vs Preflight	2	0	100
CD4+ HR Postflight vs Inflight	9	22.22	77.78
CD4+ HR Postflight vs. Preflight	9	11.11	88.89
CD4+ TW Inflight vs Preflight	0	0	0
CD4+ TW Postflight vs Inflight	0	0	0
CD4+ TW Postflight vs. Preflight	0	0	0
CD8+ HR Inflight vs Preflight	3	33.33	66.67
CD8+ HR Postflight vs Inflight	0	0	0
CD8+ HR Postflight vs. Preflight	0	0	0
CD8+ TW Inflight vs Preflight	0	0	0
CD8+ TW Postflight vs Inflight*	5	0	100
CD8+ TW Postflight vs. Preflight	0	0	0

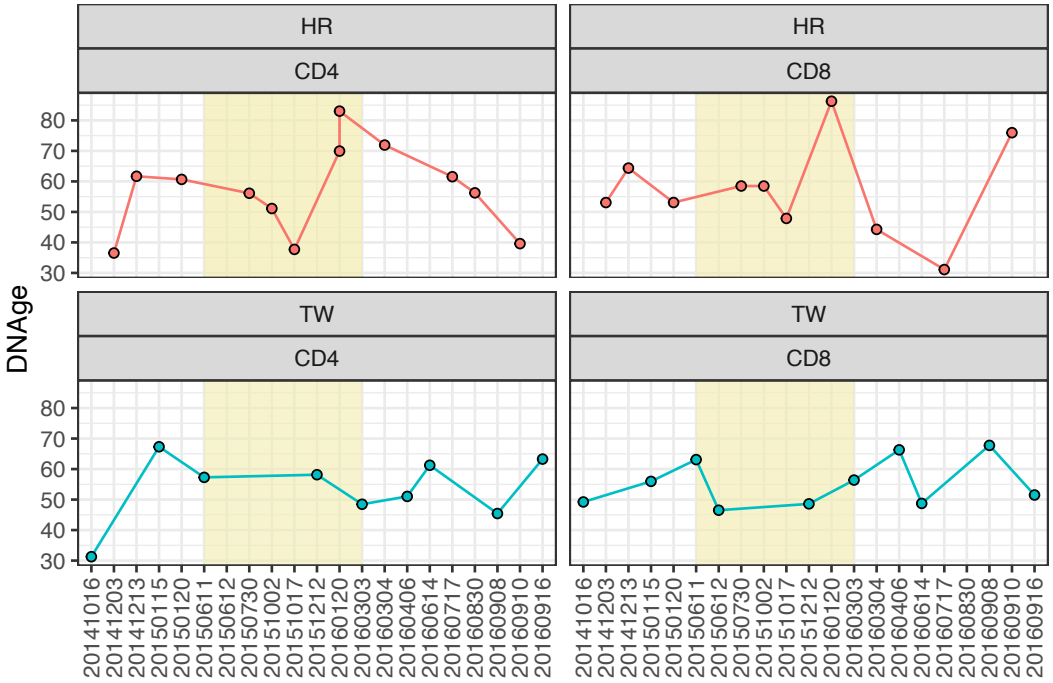
**methDiff threshold was relaxed to ≥ +15% or ≤ -15% as these were the only genes for TW showing any amount of differential methylation at a q-value of < 0.05. Otherwise, column header "% increased methylation" reflects the proportion of genes in the period with a mean methDiff ≥ +25% and "% decreased methylation" reflects the proportion of genes in the period with a mean methDiff ≤ -25%. methDiff, percentage of differential methylation per gene between mission periods; HR, earthbound twin Mark Kelly; TW, astronaut twin Scott Kelly.*

DNA Methylation Age Calculations

Plots showing variation in DNAmAge for HR and TW across longitudinal timepoints and cell types were generated using the Horvath DNAm age calculator (2013) as described in the Materials and Methods section. HR had more overall variation in magnitude and direction of DNAmAge than TW, both overall and in the inflight portion of the mission. The average Δ between DNAmAge and chronological age (ChronAge) across preflight, inflight, and postflight periods for HR and TW in both CD4+ and CD8+

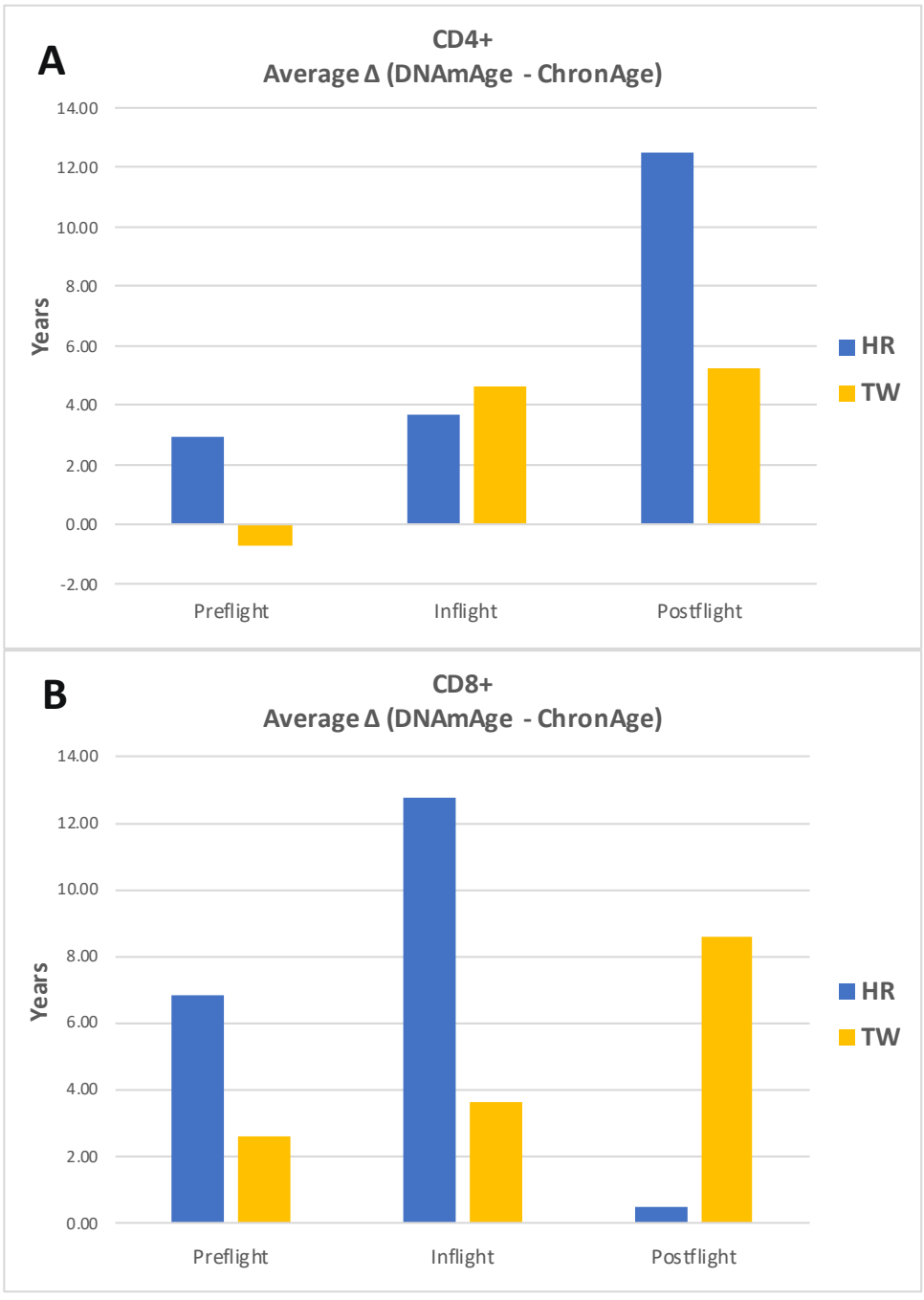
T cells was calculated using the data in Table 4. Consistent with the methDiff results for candidate genes (Figures 4 and 5), TW had less variation in epigenetic age than HR across all mission time points. Overall minimum, maximum, and average DNAmAge was calculated for HR and TW in CD4+ and CD8+ cells (Table 4). Across both cell types and across time points, HR reached an older maximum DNAmAge and an older average DNAmAge than TW. The oldest DNAmAge reached by HR at any time point was 86.28 years, while the oldest DNAmAge reached by TW at any time point was 67.76 years. On average, HR was 3.45 years (CD4+) and 1.89 years older (CD8+) in DNAmAge than TW.

Figure 7. DNA methylation age (DNAmAge) results.



DNA methylation age (DNAmAge) results for HR and TW across longitudinal time points, in CD4+ and CD8+ T cells, revealed slightly more variation in age in HR than in TW, with less variation in TW during the inflight period. The y-axis represents DNAmAge. The x-axis represents the time points at which samples were collected. The light-yellow shaded area indicates the inflight period of the mission. DNAmAge, an estimate of biological age using methylation data and an epigenetic clock calculator as described by Horvath (2013); HR, earthbound twin Mark Kelly; TW, astronaut twin Scott Kelly.

Figure 8. Average Δ between DNAmAge and chronological age.



These standardized results illustrate the relative magnitude and directionality of variation in epigenetic age for HR and TW during the preflight, inflight, and postflight periods in CD4+ and CD8+ cells. The results were derived by subtracting each subject's chronological age (ChronAge) from their DNAmAge at each sample time point and then

calculating the average delta for each of the three longitudinal time periods. The x-axis represents the mission periods. The y-axis represents number of years. ***(a)*** *Average Δ between DNAmAge and ChronAge during preflight, inflight, and postflight periods for HR and TW in CD4+ T cells, revealed greater variation in HR.* ***(b)*** *Average Δ between DNAmAge and ChronAge during preflight, inflight, and postflight periods for HR and TW in CD8+ T cells, revealed greater variation in HR. DNAmAge, an estimate of biological age using methylation data and an epigenetic clock calculator as described by Horvath (2013); ChronAge, chronological age at the time of sample collection as calculated using the subject's birth date; HR, earthbound twin Mark Kelly; TW, astronaut twin Scott Kelly.*

Table 4. Summary of DNAmAge results.

	HR CD4+	**TW CD4+**	**HR CD8+**	**TW CD8+**
Minimum DNAmAge	36.53	31.27	31.11	46.51
Maximum DNAmAge	83.04	67.31	86.28	67.76
Average DNAmAge	57.17	53.72	57.30	55.41

Using the longitudinal data in Table 5 (Appendix A), the overall minimum, maximum, and average DNAmAge was calculated for HR and TW in CD4+ and CD8+ cells. DNAmAge, an estimate of biological age using methylation data and an epigenetic clock calculator as described by Horvath (2013); HR, earthbound twin Mark Kelly; TW, astronaut twin Scott Kelly.

Chapter IV.

Discussion

Collectively, the data resulting from this investigation reveal negligible significant methylation differences in candidate genes and no significant acceleration in epigenetic age in astronaut subject TW during the One-Year Mission (Figure 5). Significant differences in methylation of key longevity-related genes were observed in ground control subject HR (Figure 4). These results defy the expectation that a long-duration spaceflight mission > 6 months would induce changes in the methylation of key genes associated with DDR and senescence in CD4+ and CD8+ T cells, and that these changes would contribute to accelerated epigenetic aging in astronaut TW. The results are more remarkable considering the fact that the One-Year Mission study found deleterious effects due to spaceflight persisted in TW for up to 6 months post-mission, including increased DNA damage due to chromosomal inversions and a greater number of short telomeres. Chromosomal inversions and shortened telomeres are strongly associated with biological aging and persistent, increased inversions post-mission may indicate damage to stem cell compartments (Garrett-Bakelman et al., 2019).

30 candidate genes were selected for the roles they play in longevity, particularly in processes of DDR and cell-cycle regulation. 16 genes in total showed significantly differential methylation between mission time periods. Of those genes, all 16 were differentially methylated in HR, while only 5 of the 16 genes showed differential methylation in TW at a relaxed methDiff threshold (Figure 5). The longevity-related roles of each of these genes are described in Table 1. The methylation changes observed in HR are not unexpected; life on Earth is highly variable and both internal and external

stressors are to some extent unpredictable and uncontrolled. As described by the ITA, as an individual ages over time, damage is accrued and epigenetic information is lost—methylation changes in key longevity-associated genes such as those in this investigation would thus be expected. The majority of differential methylation was observed in CD4+ cell samples. This is consistent with the One-Year Mission findings of greater differences in mean global methylation levels and stochasticity in CD4+ cells than in CD8+ cells.

The paradoxical aspect of this investigation's results is the overall lack of differential methylation in the candidate genes for TW throughout the mission, except for five genes during the postflight vs. inflight period in the CD8+ cell population, all of which showed a mean decrease in methylation. This result was not replicated in TW's CD4+ cell population during the same period. It is also worth revisiting the fact that although negligible differences in candidate gene methylation were observed in TW in this analysis, 27 of the 30 candidate genes were differentially expressed in TW across time periods in the One-Year Mission study results obtained by Garrett-Bakelman et al. (2019). Of the candidate genes that were differentially methylated in TW (Figure 5), all but one gene (*PARBP*) were also differentially expressed. This adds to the paradox, but as previously discussed, DEG and DMGs are not always correlated and this could be due to biological reasons, sample size, or poor coverage of CpG sites in the bisulfite libraries used.

It is important to reiterate that the effects of methylation on aging cannot be classified in a binary manner—that is, while aging is generally associated with an overall global reduction in DNA methylation, this does not translate directly to individual CpG sites (Jung & Pfeifer, 2015). For example, increased methylation can silence genes

involved in senescence or tumor suppression, inducing a disease state and accelerating aging, while demethylation can result in a loss of cellular identity. Therefore, while we can speculate about the effects of the differentially methylated genes, it should be with the caveat that these systems do not work in isolation and it is not accurate to classify a directional shift in methylation of an individual gene as *de facto* beneficial or deleterious to aging on its own. Rather, taking a holistic view of methylation changes together with other indicators of biological aging in the context of a subject's environment is prudent.

The few genes exhibiting decreased mean methylation in TW are highly evolutionarily conserved genes that play key roles in lifespan regulation by mediating processes like DDR, inflammation, cell growth, and metabolic stress response: *FOXO1, FOXO3, MTOR, PARPBP, PRKAA2*. As shown in Figure 5, the mean methDiff for these genes in the postflight vs. inflight period ranged from -16.9% to -20.3%. This means that compared to the inflight period, the mean methylation signal in TW's CD8+ cell population during the postflight period decreased by -16.9% to -20.3% in those genes. A reduction in methylation could indicate increased gene activity, though there is not always a strong association between methylation and transcriptional expression in peripheral blood cells. Additionally, it is important to point out that if differences were seen in the postflight vs. inflight period, then differences should also have been seen in at least one of the other comparison periods and this was not observed. This could mean the results are an artifact, or that differences were not captured in the other periods. The inferences that follow regarding the reduced mean methDiff in each of TW's differentially methylated genes are speculative and provide cues for future study.

FOXO1 (-17.86% postflight vs. inflight) and *FOXO3* (-17.4% postflight vs. inflight): The *FOXO* gene family is of particular note, as *FOXO* transcription factors play critical roles in DNA damage and oxidative stress responses and increased *FOXO* activity would be expected in a high-radiation environment. Ionizing radiation has been shown to activate *FOXO3a* (Yang, Xia, & Hu, 2006). Additionally, *FOXO* factors are central to T cell differentiation and may program T cells to respond to their environment (Michelini et al., 2013). This is important when we consider the fact that the methylation observations in this investigation are limited to CD4+ and CD8+ T cells. Indeed, gene expression was altered in many immune-related pathways and gene ontology analysis ranked by epigenetic discordance in the One-Year Mission study revealed CD8+ cell enrichment in the regulation of neutrophil activation in TW in both the inflight vs. preflight and postflight vs. preflight periods, resulting in a markedly increased number of new lymphocytes in TW (Garrett-Bakelman et al., 2019). The significant effects of spaceflight on the immune system have been previously described and include increased cytokine levels and altered inflammation signatures that mediate cell growth and proliferation, which were also observed in TW during flight and continued to be elevated 6 months after returning from space. Interestingly, a study of immunodeficient individuals found significantly decreased mean methylation at the CpG site of the *FOXO1* gene in naive B lymphocytes (Pino-Molina et al., 2019). This may be of relevance considering that the number of naive T cells in TW was significantly increased during spaceflight while the immune system was highly stressed, and thus in the naive lymphocyte population we might expect to see a decreased mean methylation in *FOXO1*.

MTOR (-19.4% postflight vs. inflight): In all eukaryotes, *MTOR* is a central regulator of fundamental cellular metabolic processes such as autophagy and protein synthesis, and it is widely understood that excessive *MTOR* activity is associated with deleterious effects such as tumor proliferation via inhibition of tumor-suppressor genes like *CDKN1A* and various other pathways (Saxton & Sabatini, 2017). Importantly, the mTOR (mechanistic target of rapamycin) signaling pathway is closely related to the DNA damage response pathway and it has been shown that these two pathways communicate and coordinate in response to internal and external stressors (Ma, Vassetzky, & Dokudovskaya, 2018). Decreased methylation of *MTOR* may be associated with increased gene expression and could indicate dysfunction that contributes to deleterious cell proliferation, or it could indicate mTOR signaling pathway activation in coordination with the DDR—both scenarios seem likely in the context of spaceflight and the outcome could be positive, negative, or both. Additionally, an increase in *MTOR* activity would be expected when considering the significant proliferation of T cells in TW during spaceflight, as *MTOR* also plays a key role in mediating T cell function and differentiation by integrating signals from the immune system (Waickman & Powell, 2012).

PARPBP (-20.3% postflight vs. inflight): Playing a key role in DDR, *PARBP* is specifically necessary for the suppression of inappropriate homologous recombination during the DNA damage repair process. However, there is evidence that overexpression of *PARPBP* in various types of cancer correlates with tumor progression and poorer outcomes, and that *PARPBP* may be a prognostic marker in some cancers (Chen et al., 2019; Yu et al., 2019). Decreased methylation of *PARPBP* could be indicative of active

DDR in the context of spaceflight, considering the observation of significant and persistent DNA damage in TW. There is a strong correlation between DNA damage and the development of cancer, and if overexpression of PARBP serves as a prognostic cancer marker, this may be because the presence of persistent damage induces *PARBP* activity.

PRKAA2 (-16.9% postflight vs. inflight): The genes encoding subunits of AMPK (AMP-activated protein kinase), like *PRKAA2*, are important to the maintenance of cellular energy balance, especially during starvation, autophagy, stimulation of mitochondrial biogenesis, and antioxidant activity. As such, AMPK has emerged as a target of longevity interventions. *PRKAA2* is involved in insulin sensitivity and secretion, and is required for the maintenance of energy homeostasis in response to metabolic stress (Viollet et al., 2003; Dasgupta et al., 2012). Mitochondrial dysfunction is a hallmark health risk of spaceflight, and reactive oxygen species (ROS)-induced mitochondrial dysfunction was observed in TW. Increased mitochondrial DNA (mtDNA) has been observed during spaceflight, including in the One-Year Mission, which is consistent with the suggestion that mtDNA induces signaling to support nuclear DNA repair (Luxton et al., 2020). Further, it is well-established that the effects of microgravity contribute to insulin resistance during spaceflight, and insulin resistance has been observed in both male and female astronauts on 6-month long missions (Hughson et al., 2016). If a reduction in mean methylation of *PRKAA2* is associated with increased activity of the gene, it would be consistent with these results.

DNAmAge results for HR and TW are consistent with the paradoxical methylation results, as illustrated in Figures 7 and 8. While this assessment has

limitations, it serves as a relative comparison that shows a lack of epigenetic aging in TW over the 25-months, and also shows a lack of dynamic variation in biological age. Given the stressors of space, the expectation would be evidence of either increased biological age or significant variation in biological age before, during, and after spaceflight and neither were observed. This is, however, consistent with the lack of overall methylation dynamics in TW. Additionally, in the One-Year Mission it was observed that there was more genome-wide epigenetic variance in HR than there was in TW throughout the study (Garrett-Bakelman et al., 2019). Taken together, all of these results beg the question: Is spaceflight an antidote to aging?

Explaining the Paradox

Though the negligible evidence of differential methylation in TW is remarkable given the extreme hazards of spaceflight, there are several possible explanations to explore. First, an anti-aging effect of the space environment has previously been suggested by researchers, based on observations in *Caenorhabditis elegans* worms flown on the ISS. Compared to ground-control worms, *C. elegans* flown in space appeared to age slower due to the down-regulation of genes that enhance longevity when their expression is restricted—however, the same researchers noted that in similar experiments, male *Drosophila melanogaster* experienced a shortening of lifespan upon return to Earth as compared to ground controls (Honda et al., 2012). This is analogous to the appearance of longer telomeres in TW during spaceflight, followed by the rapid shortening of telomeres upon return to Earth. Ultimately, TW ended up with more short telomeres after the mission than at baseline, as well as more DNA damage, both of which accelerate biological age and increase disease risk. Suffice to say, while spaceflight

appears to temporarily suspend the progression of some markers of aging, it likely does not slow aging and in the long term may accelerate it.

Another potential explanation emerges from the evidence of elongated telomeres in TW. During the One-Year Mission, a significant upregulation in neutrophil activation and the release of an increased number of new white blood cells in TW was observed. In a recent paper, Luxton et al. (2020) suggest that this likely resulted in a marked increase in stem-like progenitor cells with longer telomeres. In space, peripheral blood is continually being exposed to ionizing radiation which may cause lymphocytes to die and repopulate at a much higher rate than normal; naive cells inherently possess properties like longer telomeres that are not found in normally circulating mature cells. I hypothesize that this may be one explanation for the lack of observed methylation differences in TW, especially in genes related to senescence. That is, the results are simply a function of shifting cell population dynamics; looking for methylation signals in a naive T cell population might be one reason why there are few observable changes. While the CD4+ and CD8+ T cells are sorted, they are still T cells, and we are not observing methylation dynamics in any other cell type that might offer another picture of the epigenetic landscape. Indeed, these results may be a reflection of the cell type and population of the cells sampled, rather than indicators of any real functional difference in longevity-related mechanisms induced by spaceflight. In fact, spaceflight may actually be causing the expected effect—inducing damage and causing turnover in lymphocytes. Just because we cannot observe the signals we are expecting, does not mean that accelerated aging is not occurring.

Yet another explanation related to the T cell population is based on the finding that T cells with dysfunctional mitochondria activate premature cellular senescence (Desdín-Micó et al., 2020). It was observed that in TW there was significant ROS-induced mitochondrial dysfunction. However, it has also been suggested that during spaceflight, a cell moves telomerase out of its nucleus and into the mitochondria to combat this damage. This is one explanation proposed by researchers to account for the increase in telomere length (Luxton et al., 2020). If T cell mitochondrial damage was being mitigated by the reallocation of telomerase, combined with the increased number of naive T cells, the resulting cell sample population may not have reflected significant changes in methylation of these genes.

In a simulated 520-day ground simulation space travel experiment, Nwanaji-Enwerem et al. (2020) demonstrated that mission duration was associated with significant decreases in epigenetic aging, contradicting their hypothesis that such a long-duration spaceflight mission should result in accelerated epigenetic aging. They conclude that their paradoxical findings are in line with the telomere length results from the One-Year Mission, suggesting that this may indicate an acute initial stress response followed by adaptation (Nwanaji-Enwerem et al., 2020). Such adaptation likely has a cost, as was observed with the immediate shortening of TW's telomeres within 48-hours of returning to Earth. Indeed, it appears that the results of this investigation and others may reveal an adaptive yet temporary response to the extreme conditions of space in which human beings have not evolved to thrive, let alone survive.

Finally, there are some simple explanations that can stand on their own or in combination with the more complex possibilities described in this discussion. First, the

space station is a stable, highly controlled environment. It is fraught with hazards, but it is also defined by a lack of variability—ISS temperature, humidity, and pressure are tightly regulated, and astronaut nutrition, sleep, and exercise are all highly controlled, from timing of meals, workouts, and sleep schedules, to the prescription of macronutrient ratios and precise strength training and cardiovascular activities. Research on Earth has demonstrated the longevity-enhancing effects of a consistent sleep-wake schedule (Mander, Winer, & Walker, 2017). Because circadian rhythm is disrupted in space due to fluctuations in light, astronauts maintain tightly regulated sleep schedules. Astronauts also exercise routinely during spaceflight to combat microgravity-associated muscle and bone loss, and AMPK is activated by exercise. As discussed earlier, AMPK is associated with mitochondrial biogenesis and, potentially, telomere lengthening (Luxton et al., 2020). One-Year Mission fitness logs for TW reveal precisely prescribed conditioning exercises scheduled at recurring intervals inflight and postflight; due to confidentiality, the details of these records cannot be disclosed.

It is possible that the combination of regular, regimented aerobic exercise and tightly controlled sleep and diet confers longevity-enhancing effects. Ultimately, the methylome may be significantly less perturbed in a highly controlled environment in the short term. While it is recognized that intermittent stressors are beneficial for aging—the hormesis effect—exposure to chronic stress over time and a lack of variation in stressors negatively impact aging. Thus, it stands to reason that exposure to the spaceflight environment over a longer period of time will ultimately elicit an undesirable effect on biological age even if its effects on methylation are not immediately observable. Of course, it is also possible that the methylome is simply not ideal for observing the effects

of spaceflight on aging in real-time. Telomere length, DNA damage, and other physiological effects may ultimately prove to be better measures and future studies with larger groups of astronauts and different cell types are required. Additionally, it is possible that this dataset simply did not obtain sufficient coverage of the candidate genes during bisulfite sequencing.

Study Limitations

The greatest limitation of the One-Year Mission is that it is an N of 1 study. Recent findings from other studies demonstrate significant inter-individual differences in astronauts with regard to telomere length and DNA damage responses to spaceflight, prompting researchers to call for a personalized medicine approach to future long-duration missions (Luxton et al., 2020). It is certainly possible that although TW did not exhibit significant changes in methylation in this investigation's candidate genes, another astronaut may have. The opportunity to include a genetically matched ground control subject makes this dataset unique, but does not nullify its major flaw.

On a related note, a limitation shared by all astronaut studies to date is a lack of demographic diversity. The historical pool of subjects from which to draw is small—only 562 humans have been to space as of 2020, and only 8 of them have spent > 300 days in space. The average first-time astronaut is a 40-year-old Caucasian male and the historical astronaut gender ratio is 88.6% male to 11.4% female (Smith, Kelley, & Basner, 2020). The lack of gender diversity is particularly important when considering risks of spaceflight that may disproportionately impact females, such as bone loss and anemia. There is no existing data for astronauts on long-duration (≥12 month) missions beyond the protection of our planet's magnetic field where exposure to damaging space radiation

far exceeds that in lower-Earth orbit, such as missions to Mars or sustained exploration and development on the Moon. Plans for both such missions have been publicly announced by SpaceX and NASA. The dataset used in this investigation may provide clues to mine for those future endeavors, but does not reflect all of the same hazards.

There are a number of additional limitations to address, including cell types used for analysis, lack of tissue samples for senescence observations, transcription regions in candidate genes, epigenetic clock limitations, and restricted astronaut data disclosure. As previously discussed, observing differential methylation in T cell populations that include a larger proportion of stem-like cells may confound results. It is worth noting, however, that in a recent publication, researchers were able to mirror the DNA damage and telomere alterations observed in TW's T cells obtained via urine samples instead of plasma (Luxton et al., 2020). Ideally, methylation data could have been obtained from a diverse set of cell types, especially highly-proliferative radiation-sensitive tissues like the intestinal epithelium. There is no published data from the One-Year Mission on cellular senescence, which is typically determined by beta-galactosidase staining of tissue cell samples. The obvious constraints of the space environment limit collection of diverse sample types in humans. Mouse model studies have shown that IR exposure in spaceflight increases senescence signaling, especially in tissues with highly proliferative cells that are vulnerable to radiation, and upregulation of senescence markers in hematopoietic stem cells has been previously reported in mice, suggesting that observations in cell types other than T cells are necessary (Blaber, Pecaut, & Jonscher, 2017; Kumar et al., 2018). The human body is composed of complex and highly specialized systems, and the mechanistic drivers of aging differ in scope and effect by

organ system and cell type. Whether cellular senescence shares common pathways across all immune cells and all mammalian cell types remains to be demonstrated. While this investigation reports results based on mean differential methylation levels and weighs differences at all CpG sites equally, there are multiple transcription start sites (TSSs) in human genes and it remains unclear whether all sites are truly functional. This is a limitation that broadly affects epigenetic research and there is still debate around whether or not alternative TSSs are molecular errors (Xu, Park, & Zhang, 2019). As such, a full list of all significantly differentially methylated (≥ +25% or ≤ -25%) individual CpGs per gene, organized by time period and cell type, is included in Table 6 (Appendix B) so that as future research elucidates the relevance of TSSs per gene, mean methylation results can be revisited. Limitations of using this investigation's EM-seq WGBS library data with the Horvath DNAm age clock have been addressed in the Results section—the clock uses microarray data and thus the results are a directional approximation rather than a direct comparison. Lastly, there is a great deal of informative astronaut data that cannot be disclosed in this investigation, such as detailed sleep, exercise, and nutrition logs, as well as related blood biochemistry. This data could guide new directions of study and should be mined in subsequent investigations.

Future Directions

The results of this investigation underscore the need for personalized medicine in spaceflight. At a minimum, future studies should follow larger groups of astronauts on long-duration missions for at least one year after landing to observe differential methylation and other markers of aging over time. A limitation of this study is that data for TW was only collected for up to 6 months postflight, and it is possible that the rate of

biological aging in astronauts post-mission may accelerate over a longer period compared to ground-controls. Repeating the study in larger, more diverse groups of astronauts is of utmost importance, given the vast number of covariates that can influence results and need to be accounted for. Another avenue of exploration is to intersect astronaut exercise, diet, and sleep data logs with longitudinal measures of epigenetic aging. As described earlier in this discussion, exercise induces activation of AMPK, a pro-longevity target that declines with age. Indeed, in TW one of the few genes with significant differential methylation was *PRKAA2*, which encodes a subunit of AMPK. Does the increased volume and frequency of exercise in spaceflight mediate expression of AMPK and act on longevity? Do strict sleep-wake and nutrient intake schedules exert pro-longevity effects in space? These are questions future studies might address. The resulting answers may yield novel preventative and therapeutic approaches to longevity and its associated disease states.

The fact that diseases behave and develop differently in microgravity makes the ISS the most unique research lab in human history. Indeed, the study of biology in space has resulted in breakthrough discoveries and advancements in medicine and technology back on Earth (Prasad et al., 2020). If aging can be classified as a treatable disease, then what insights might we draw on this investigation to lead us in new directions? The ITA proposes that an accumulation of epigenetic noise over time alters DNA methylation patterns and induces aging (Sinclair & LaPlante, 2019). While spaceflight exposes astronauts to damaging forms of noise like radiation and physical stress, TW's methylome was not significantly more altered than HR's—neither globally, nor in the genomic regions of interest interrogated here. In fact, TW's methylome was overall

slightly less changed than the ground-control (Garrett-Bakelman et al., 2019). One possible explanation for this paradoxical result is that the mechanisms normally functioning to address DNA and mitochondrial damage may be silenced due to changes in biochemistry induced by spaceflight. For example, in the presence of DNA damage, sirtuins are called into action; when sirtuin activity is impaired, the organism runs into problems. It is especially surprising that there was no significant differential methylation in any of the *SIRT* genes in TW, given the number of chromosomal inversions accrued. Sirtuins only function in the presence of the substrate nicotinamide adenine dinucleotide (NAD+) and NAD+ declines over time with age (Imai and Guarente, 2014). Additionally, a decrease in NAD+ is associated with cellular senescence and NAD+ has been shown to prevent senescence in mesenchymal stem cells via *SIRT1* signaling (Wang et al., 2021). Thus, future studies could aim to track astronaut NAD+ levels longitudinally and intersect that data with transcriptional expression and methylation of *SIRT* genes and the *NAMPT* gene, which is responsible for generating NAD+. If spaceflight impairs the activity of sirtuins by altering levels of NAD+, then elucidating the underlying mechanisms may yield novel insights applicable to longevity on Earth. Supplementation with NAD+ precursors could be a preventative intervention for astronauts.

The mechanisms by which cellular processes are altered by space are poorly understood (Prasad et al., 2020). It is known that significant epigenetic alterations are induced by microgravity in animal models, in plants, and in human stem cells which are actively being studied both aboard the ISS and in simulated environments (Imura et al., 2019; Johnson, 2020). Microgravity also causes significant changes in cell growth behavior and differentiation and cancer cells flown aboard the ISS have been shown to

re-differentiate (Grimm et al., 2020). Exactly why and how these alterations occur remain unclear and is a ripe area for future study. A simple explanation is that organisms may alter only what is deemed most essential for short-term survival in an extreme environment, deprioritizing longevity; cellular programming for immediate survival may override that of long-term stability. When it comes to lifespan, there are always evolutionary trade-offs made between maintenance and reproduction (Kirkwood & Austad, 2000). Additionally, cellular machinery required to address critical matters like DNA repair may simply be impaired by microgravity or radiation. In either case, the longer-lasting results of spaceflight would prove to be damaging over time.

Where DNA methylation is concerned, gaining a better understanding of how the epigenetic programming mechanism is altered by spaceflight is possibly the most critical piece of the biological puzzle, with the broadest implications for longevity research. The epigenetic programming mechanism remains elusive and is actively being studied. Indeed, if spaceflight research has taught us anything, it is that there are very few natural laws by which life abides, and life itself is a dynamic example of infinite possibilities (Prasad et al., 2020). A groundbreaking study published in December 2020 by the Sinclair Lab at Harvard Medical School demonstrated, for the first time, the successful epigenetic reprogramming of retinal ganglion cells (RGCs) to recover lost epigenetic information and restore vision in mice (Lu et al., 2020). To do this, Lu et al. expressed three of the four Yamanaka factors, OSK (the genes *Oct4*, *Sox2*, *Klf4*), ectopically on mouse RGCs. Yamanaka transcription factors are critical regulators of embryonic development and can be overexpressed in mature differentiated cells to reprogram them into induced pluripotent stem cells, acting as a reset button for DNA methylation age

(Takahashi & Yamanaka, 2006). The conclusion reached by Lu et al. is that aging is not unidirectional and that cells retain a backup copy of youthful epigenetic information that is encoded by DNA methylation and can be restored via the application of the OSK factors. Importantly, DNA demethylases TET1 and TET2 are required to induce epigenetic reprogramming by OSK expression. Mice lacking TET did not experience a restoration of vision or a youthful epigenome. Exactly how the TET enzymes know which DNA methyl tags to reverse in order to turn back the aging clock and restore vision, and where this "backup copy" of the epigenome resides, remains a great mystery.

This mystery begs many questions. What if those methyl tags are recognizable only to TET enzymes and under certain conditions? Perhaps additional information is stored within the genome, and perhaps a communication system exists that allows for the access of differential information states stored within a cell, like a memory bank. Explaining this requires a literal quantum leap in scientific thinking, one that integrates Newtonian physics and quantum physics and may be elucidated via the space environment. Classical physics explains the conservation of energy via Newton's first law of thermodynamics—the Law of Conservation of Energy holds that energy (matter) is transferred or transformed but never lost. Quantum physics explains the conservation of information—if information is lost in one subsystem, it simply relocates to another subsystem. Specifically, the No-Hiding Theorem describes the conservation of quantum information and has been experimentally proven (Samal, Pati, & Kumar, 2011).

I propose that this may explain how epigenetic information can be restored with such precision via OSK expression; analogous to the CD that is buffed back to its gleaming finish without erasing all of its song data. Consider the principle of quantum

superposition, which states that objects can exist in two places simultaneously as wave functions and collapse or decohere upon observation—theoretically, epigenomic information could exist in more than one place at one time in a cell, with TET acting as the observer. In other words, a cell's backup restoration program is retained in a quantum state of superposition (where all subatomic particles exist in two places simultaneously until observation) and the interaction of TET enzymes allows that program to be accessed. Importantly, according to the widely-accepted Copenhagen Interpretation, the observer does not need to be conscious; it can be a tool, a living being, or in this case, an enzyme (Heisenberg & Northrop, 1958). We might imagine the entire genome itself as a quantum computer with superpositions of base pairs and methyl groups as qubits. Evidence of quantum features has appeared in living cells before, in the photosynthetic process in plants, and consciousness in the human brain has been speculatively connected to quantum theory (Engel et al., 2007; Fisher, 2015). It is not out of the realm of possibility for quantum features to be at work in epigenetic programming. Where does spaceflight factor into this equation? Matter behaves differently in microgravity; this extends to all subatomic particles or quantum objects.

Here, I propose a radical hypothesis: that a backup copy of the youthful epigenome exists in a state of superposition and that the microgravity environment in spaceflight can elucidate the epigenetic reprogramming mechanism via alteration of quantum object activity. This hypothesis is highly theoretical, but it can be interrogated in the spaceflight environment. Space could provide an opportunity to observe quantum states not observable on Earth, elucidating key epigenetic information retention and transfer patterns. An important proof-of-principle was demonstrated last year by NASA's

Cold Atom Lab operating aboard the ISS. For the first time, physicists were able to create a Bose-Einstein condensate (BEC) that was stable for multiple seconds, proving that the microgravity of space can be leveraged in ways that allow scientists to create and observe exotic phenomena that would be impossible on Earth (Gibney, 2020). To test my hypothesis, I would need to measure the possible quantum effect of the interaction of TET, which is far beyond the scope of most biological investigations. A more feasible first step would be to repeat the vision restoration experiment by Lu et al. in microgravity. Such observations could help explain the paradox of the lack of differential DNA methylation observed in TW in spaceflight. Incidentally, it is also known that spaceflight adversely affects eye health in more than 30% of astronauts (Mader et al., 2019). Repeating the experiment to restore vision in space would thus serve a dual purpose. Elucidating quantum behavior in epigenetics marks a new frontier of scientific investigation and has the potential to shift the paradigm of biology as we know it.

Conclusions

It is clear that the study of DNA methylation in spaceflight can yield intriguing results and that great potential for novel discoveries lies ahead. This investigation hypothesized that long-duration spaceflight would result in significant changes in methylation of longevity-related genes involved in DNA damage repair and cellular senescence, two key hallmarks of aging. Negligible differential methylation was observed in astronaut TW in the 30 candidate genes interrogated, while more significant methylation changes were observed in ground-control HR. The paradoxical results of this investigation underscore the need for additional research to better understand the underlying mechanisms and to address the limitations of an N of 1 study given inter-

astronaut differences in molecular responses to spaceflight. These results corroborate the conclusions of other recent studies which call for a personalized medicine approach to spaceflight. It is also clear that while life in space is hard, so is life on Earth. Earth is a dynamic environment where environmental variables are less controlled, as evidenced by the changes in HR's methylome over time. If additional studies confirm that DNA methylation in longevity-associated genes is less perturbed in a highly-controlled, extreme environment and that temporary periods of stability are beneficial to aging, then perhaps the future holds the promise of simple therapeutic interventions akin to a pause button. We might imagine a "vacation without variation" taken regularly in a spartan biosphere in microgravity where diet, sleep, and fitness are tightly regulated and protect against DNA damage and senescence. Minus the radiation, of course. It may be an unenjoyable holiday but one with long-term benefits.

Ultimately, whether humanity remains earth-bound or departs in search of a new home, we face a reckoning of epic proportions when it comes to lifespan and planetary health. Climate disaster does not await us, it is already here; up to 37% of the species on Earth may be lost by 2050 (Thomas et al., 2004). The planet's natural resources are being depleted and scientists' warnings of breaching the climate cliff—a 2°C rise in temperature—are increasingly repeated. Recently, researchers have suggested that the hotter the environment, the more rapidly an organism ages (Stark, Pincheira-Donoso, & Meiri, 2020). Human longevity is not uncoupled from ecological longevity. Perhaps the greatest result to be gained from our pursuit to reverse aging will be the means by which to improve the vitality of our shared home so that we do not need to leave it.

Appendix A.

Candidate Genes

Table 1. Candidate genes used in this investigation and descriptions of their longevity-related functions.

Gene	Alias	Gene function
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A	Also known as tumor suppressor gene p21, this gene is a key regulator of the cell cycle and plays roles in both cancer and senescence. Its activity is tightly controlled by major tumor suppressor gene p53 and it has been directly linked to cellular aging. Expression of p21 induces senescence while repression may elicit multiple outcomes including an anticancer effect, as p21 also inhibits apoptosis (Gartel and Radhakrishnan, 2005).
CDKN1C	Cyclin-Dependent Kinase Inhibitor 1C	This gene is a negative regulator of the cell cycle and can induce senescence and increase the activity of tumor suppressor gene p16 (Giovanni et al., 2012).
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A	Also known as tumor suppressor gene p16, this gene can induce senescence. Senescence triggered by DNA damage response or stress signaling is executed by the p16 pathway. Hypermethylation of p16 thus has significant implications in multiple cancer types (He and Sharpless, 2017).
DAO	D-Amino Acid Oxidase	This gene can induce detoxification activity which removes D-amino acids accumulated during aging and promotes senescence by producing reactive oxygen species. It is downstream of tumor suppressor gene p53 and is directly regulated by it (Nagano et al., 2019).
EPN3	Espin 3	This gene is a tumor angiogenesis modifier and promotes senescence. It is downstream of tumor suppressor gene p53 (Nagano et al., 2016)

FOXO1	Forkhead Box Protein O1	A member of the Forkhead transcription factor family subgroup, *FOXO1* plays a key role in critical cellular processes such as growth signaling, apoptosis, inflammation, and immune response. The *FOXO* factors are highly conserved regulators of longevity, involved in DNA damage and oxidative stress responses, and are implicated in autophagy across cell types. They are also central to T-cell differentiation and may program T cells to respond to the environment (Hedrick et al., 2013; Webb and Brunet, 2014).
FOXO3	Forkhead Box Protein O3	A member of the Forkhead transcription factor family subgroup, *FOXO3* plays a key role in critical cellular processes such as growth signaling, apoptosis, inflammation, and immune response. The *FOXO* factors are highly conserved regulators of longevity, involved in DNA damage and oxidative stress responses, and are implicated in autophagy across cell types. They are also central to T-cell differentiation and may program T cells to respond to the environment (Hedrick et al., 2013; Webb and Brunet, 2014).
FOXO4	Forkhead Box Protein O4	A member of the Forkhead transcription factor family subgroup, *FOXO4* plays a key role in critical cellular processes such as growth signaling, apoptosis, inflammation, and immune response. The *FOXO* transcription factors are highly conserved regulators of longevity, involved in DNA damage and oxidative stress responses. They are also central to T-cell differentiation and may program T cells to respond to the environment. Cellular senescence may be regulated by the *FOXO4*-p53 axis (Bourgeois and Madl, 2018; Webb and Brunet, 2014; Hedrick et al., 2013).
IGF1	Insulin-Like Growth Factor 1	This gene plays a key role in mediating mammalian growth and development, as well as longevity. *IGF1* both promotes cell proliferation and induces senescence. Downregulation of IGF-1 pathway is associated with extension of lifespan in mice (Tran et al., 2014; Mao et al. 2018).

IGF2	Insulin-Like Growth Factor 2	This gene is involved in mammalian growth and development, as well as longevity. It promotes cell proliferation and although its activity appears limited to early stages of development, epigenetic activation of *IGF2* occurs during cellular senescence. *IGF2* deficiency has been shown to increase hematopoietic stem cell renewal in adulthood (Barocca et al., 2017; Zhang et al., 2010).
IGFBP5	Insulin-Like Growth Factor Binding Protein 5	This gene is involved in the control of cellular senescence and cell inflammation, and can induce senescence independent of *IGF1* (Sanada et al., 2018).
MTOR	Mechanistic Target Of Rapamycin	This gene is a key mediator of cellular responses to stressors like DNA damage and nutrient-sensing, and is thus considered a central regulator of lifespan as it is implicated in aging-associated processes such as mitochondrial function, proteostasis, immune response, autophagy, stem cell proliferation, and cellular senescence. *MTOR* also plays a critical role in mediating T cell function and differentiation by integrating signals from the immune system. Repression of *MTOR* may extend lifespan but results in humans remain inconclusive (Papadopoli et al., 2019).
NFKB1	Nuclear Factor Kappa B Subunit 1	Also known as p50, this gene is a key regulator of transcription in response to stimulation by cytokines, reactive oxygen species, radiation, and pathogens. When appropriately expressed *NFKB1* reduces inflammation and slows aging, while a loss of *NFKB1* results in accelerated aging via increased senescence, reduced apoptosis, as well as dysfunctional immune cell development (Bernal et al., 2014).
PARG	Poly(ADP-ribose) glycohydrolase	This gene encodes the major enzyme responsible for the cleavage of poly (ADP-ribose), a chromosomal protein modifier that is synthesized by the *PARP* family of genes. *PARG* thus plays a role in the mediation of chromatin, transcription, apoptosis, and DNA damage repair (Masutani, Nakagama, and Sugimura, 2005; Lüscher et al., 2018).

PARP1	Poly(ADP-Ribose) Polymerase 1	This is a key longevity gene that plays a major role in maintaining genomic stability by mediating DNA damage repair, chromatin remodeling, telomere dynamics, senescence, inflammatory response, apoptosis. It is also a regulator of the NF-κB protein complex which controls DNA transcription, inflammation, and apoptosis (Mangerich and Bürkle, 2012).
PARP2	Poly(ADP-Ribose) Polymerase 2	A member of the *PARP* family of protein-coding genes that plays a key role in DNA damage repair. May play a more specific role in inflammatory response and T cell development (Yélamos, Schreiber, and Dantzer, 2008).
PARP3	Poly(ADP-Ribose) Polymerase	A member of the *PARP* family of protein-coding genes that plays a key role in DNA damage repair. Specifically, *PARP3* is shown to selectively respond to double-stranded DNA breaks relative to other *PARP* family genes (De Vos, Schreiber, and Dantzer, 2012).
PARPBP	PARP1 Binding Protein	A member of the *PARP* family of protein-coding genes that plays a key role in DNA damage repair. Specifically, *PARPBP* is necessary for the suppression of inappropriate homologous recombination in the process of DNA damage repair (Chen et al., 2020).
PRKAA1	Protein Kinase AMP-Activated Catalytic Subunit Alpha 1	An evolutionarily conserved gene that encodes a protein subunit of AMP-activated protein kinase (AMPK). AMPK plays a central role in cellular energy balance especially during starvation, induces autophagy, stimulates mitochondrial biogenesis and antioxidant activity. This gene is an emerging pro-longevity target as AMPK declines with age and is activated by stimuli like exercise (Jeon, 2016).
PRKAA2	Protein Kinase AMP-Activated Catalytic Subunit Alpha 2	(Protein Kinase AMP-Activated Catalytic Subunit Alpha 2) An evolutionarily conserved gene that encodes a protein subunit of AMP-activated protein kinase (AMPK). Plays a role in insulin sensitivity and secretion and is required for the maintenance of energy homeostasis in response to metabolic stress (Viollet et al., 2003; Dasgupta et al., 2012).

PRKAG1	Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 1	Encodes a regulatory subunit of AMPK and is involved in the longevity regulating pathway of multiple species (Swindell et al., 2018).
PRKAG2	Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 2	An important paralog of *PRKAG1*. *PRKAG2* abnormalities are recently found to be associated with cardiac syndrome with the basis for pathology being mediated by glycogen storage and cell growth (Hu et al., 2020).
PRODH	Proline Dehydrogenase 1	Encodes a mitochondrial protein involved in proline degradation. Overexpression of *PRODH* promotes senescence in response to DNA damage induced by ROS (Nagano et al., 2017).
SIRT1	Sirtuin 1	A member of the sirtuin family of NAD+-dependent protein-coding genes that play key roles in epigenetic expression and regulation of myriad processes associated with lifespan such as telomere length, genomic stability, and DNA damage repair, inflammation, and insulin sensitivity. *SIRT1* is localized in the nucleus and cytoplasm and is critical in mediating DNA damage repair and cellular senescence, and is associated with overall lifespan extension (Haigis and Guarente, 2006; Lee et al., 2019).
SIRT2	Sirtuin 2	A member of the sirtuin family, *SIRT2* is localized in the cytoplasm and functions in aging by regulating the cell cycle (Lee et al., 2019).
SIRT3	Sirtuin 3	A member of the sirtuin family, *SIRT3* is localized in the mitochondria and plays roles in aging by regulating mitochondrial function and oxidative stress; it is also associated with centenarian-linked SNPs (Lee et al., 2019).
SIRT4	Sirtuin 4	A member of the sirtuin family, *SIRT4* is localized in the mitochondria and plays roles in aging by regulating fatty acid oxidation and apoptosis (Lee et al., 2019).

SIRT6	Sirtuin 6	A member of the sirtuin family, *SIRT6* is localized in the nucleus/chromatin and plays roles in aging by mediating DNA damage repair, telomere dynamics, and is associated with overall lifespan extension (Lee et al., 2019).
SIRT7	Sirtuin 7	A member of the sirtuin family, SIRT7 is localized in the nucleolus and plays roles in aging by mediating epigenetic regulation, stress resistance, and apoptosis (Lee et al., 2019).
TP53	Tumor protein 53	Also known as p53, this is a key tumor suppressor gene that regulates target gene transcription and induces apoptosis, DNA repair, cell cycle arrest, and metabolic changes in response to stressors. p53 mediates both aging and senescence. Transcription of p53 is critical for senescence initiation and it is commonly used as a marker of senescence, with multiple downstream genes dependent upon it for senescence induction (Rufini et al., 2013).

Table 2. CD4+ and CD8+ T cell sample collection dates.

CD4+					CD8+				
			EM-Seq	WGBS				EM-Seq	WGBS
TW	Pre	10/16/14	0	1	TW	Pre	10/16/14	1	1
		01/15/15	3	0			01/15/15	3	0
	Flight	06/12/15	0	1		Flight	06/12/15	2	1
		12/12/15	1	1			12/12/15	2	1
		03/03/16	1	0			03/03/16	1	0
	Post	04/06/16	1	0		Post	04/06/16	1	0
		6/14/16	0	1			09/08/16	1	0
		09/08/16	1	0			06/14/16	0	1
		09/16/16	1	0			09/16/16	1	0
HR	Pre	12/03/14	1	1	HR	Pre	12/03/14	1	1
		12/13/14	1	0			12/13/14	1	0
		01/20/15	3	0			01/20/15	3	0
	Flight	07/30/15	2	1		Flight	07/30/15	1	1
		10/02/15	3	0			10/02/15	2	0
		10/17/15	2	0			10/17/15	2	0
		1/20/16	2	1			1/20/16	1	1
	Post	3/4/16	1	0		Post	3/4/16	1	0
		7/17/16	0	1			07/17/16	0	1
		08/30/16	1	0			9/10/16	1	0
		9/10/16	1	0					

This table shows sample collection dates and sequencing employed (WGBS and/or EM-seq). Note that sample collection dates are roughly equivalent between subjects but not exact. The discrepancies in collection dates do not confer a significant effect and are still considered simultaneous.

Table 5. DNAmAge vs. chronological age across all sample collection dates for both subjects.

HR CD4+				
Period	**Date**	**DNAmAge**	**ChronAge**	**Δ (DNAmAge - ChronAge)**
Preflight	2014-12-03	36.53	50.77	-13.47
Preflight	2014-12-13	61.68	50.80	11.68
Preflight	2015-01-20	60.66	50.90	10.66
Inflight	2015-07-30	56.11	51.43	6.11
Inflight	2015-10-02	51.11	51.60	1.11
Inflight	2015-10-17	37.71	51.64	-12.29
Inflight	2016-01-20	69.94	51.90	19.94
Postflight	2016-01-20	83.04	51.90	33.04
Postflight	2016-03-04	71.91	52.02	21.91
Postflight	2016-07-17	61.54	52.39	11.54
Postflight	2016-08-30	56.25	52.51	6.25
Postflight	2016-09-10	39.61	52.54	-10.39

TW CD4+				
Period	**Date**	**DNAmAge**	**ChronAge**	**Δ (DNAmAge - ChronAge)**
Preflight	2014-10-16	31.27	50.64	-18.73
Preflight	2015-01-15	67.31	50.89	17.31
Inflight	2015-06-11	57.28	51.29	7.28

Inflight	2015-12-12	58.15	51.80	8.15
Inflight	2016-03-03	48.46	52.02	-1.54
Postflight	2016-04-06	51.03	52.11	1.03
Postflight	2016-06-14	61.25	52.30	11.25
Postflight	2016-09-08	45.42	52.54	-4.58
Postflight	2016-09-16	63.27	52.56	13.27

HR CD8+				
Period	**Date**	**DNAmAge**	**ChronAge**	**Δ (DNAmAge - ChronAge)**
Preflight	2014-12-03	53.08	50.77	3.08
Preflight	2014-12-13	64.37	50.80	14.37
Preflight	2015-01-20	53.09	50.90	3.09
Inflight	2015-07-30	58.50	51.43	8.50
Inflight	2015-10-02	58.48	51.60	8.48
Inflight	2015-10-17	47.87	51.64	-2.13
Inflight	2016-01-20	86.28	51.90	36.28
Postflight	2016-03-04	44.28	52.02	-5.72
Postflight	2016-07-17	31.11	52.39	-18.89
Postflight	2016-09-10	75.97	52.54	25.97

TW CD8+				
Period	**Date**	**DNAmAge**	**ChronAge**	**Δ (DNAmAge - ChronAge)**
Preflight	2014-10-16	49.21	50.64	-0.79
Preflight	2015-01-15	55.97	50.89	5.97
Inflight	2015-06-11	63.10	51.29	13.10
Inflight	2015-06-12	46.51	51.30	-3.49
Inflight	2015-12-12	48.59	51.80	-1.41
Inflight	2016-03-03	56.39	52.02	6.39
Postflight	2016-04-06	66.29	52.11	16.29
Postflight	2016-06-14	48.73	52.30	-1.27
Postflight	2016-09-08	67.76	52.54	17.76
Postflight	2016-09-16	51.50	52.56	1.50

The difference between DNAmAge and chronological age (Δ DNAmAge - ChronAge) for each collection time point was obtained to provide a more detailed view of biological age dynamics. DNAmAge was derived from the Horvath clock output. Chronological age (ChronAge) was derived using a formula to obtain HR and TW's exact chronological ages at the time of each sample collection. This calculation does not take into account the time dilation derived from Einstein's theory of special relativity, wherein an individual in space would age more slowly than their counterpart on Earth. Given the relatively brief time period and distance, this effect would not confer a biologically significant age difference. ChronAge is chronological age at the time of sample collection as calculated using the subject's birth date. DNAmAge is an estimate of biological age using methylation data and an epigenetic clock calculator as described by Horvath (2013); HR is earthbound twin Mark Kelly; TW is astronaut twin Scott Kelly.

Appendix B.

All Significant Differentially Methylated CpGs

Table 6. Significant methDiff across individual CpGs.

HR					
CD4+					
Time Period	**CpG**	**p-value**	**q-value**	**methDiff**	**Gene**
Inflight vs. Preflight	36682963	9.73E-07	0.0405	35.63	CDKN1A
Inflight vs. Preflight	20354050	3.18E-08	0.0051	-26.43	PARP2
Postflight vs. Inflight	21975059	1.3E-05	4.2E-02	32.77	CDKN2A
Postflight vs. Inflight	21975077	1.6E-06	1.2E-02	34.60	CDKN2A
Postflight vs. Inflight	50541607	9.0E-06	3.0E-02	-27.77	EPN3
Postflight vs. Inflight	50539925	1.3E-05	3.7E-02	-25.06	EPN3
Postflight vs. Inflight	40615818	2.3E-05	3.8E-02	-26.71	FOXO1
Postflight vs. Inflight	40603994	8.9E-07	1.2E-02	-24.66	FOXO1
Postflight vs. Inflight	40613208	1.4E-05	3.5E-02	19.76	FOXO1
Postflight vs. Inflight	40613042	9.6E-06	2.8E-02	20.10	FOXO1
Postflight vs. Inflight	40605747	4.7E-06	1.9E-02	20.29	FOXO1
Postflight vs. Inflight	40564775	1.1E-08	7.7E-04	21.05	FOXO1
Postflight vs. Inflight	40612946	2.4E-05	4.8E-02	22.53	FOXO1
Postflight vs. Inflight	40608203	2.5E-05	4.9E-02	23.10	FOXO1
Postflight vs. Inflight	40608212	3.9E-07	4.5E-03	26.10	FOXO1
Postflight vs. Inflight	40589807	6.0E-06	3.4E-02	26.91	FOXO1
Postflight vs. Inflight	40581847	1.1E-05	4.9E-02	28.54	FOXO1
Postflight vs. Inflight	40559546	3.3E-06	1.7E-02	31.15	FOXO1
Postflight vs. Inflight	40640519	4.7E-06	2.0E-02	36.58	FOXO1
Postflight vs. Inflight	40629965	3.4E-07	4.1E-03	39.77	FOXO1
Postflight vs. Inflight	40589734	8.1E-08	1.5E-03	45.74	FOXO1
Postflight vs. Inflight	40589767	3.6E-09	1.3E-04	46.08	FOXO1
Postflight vs. Inflight	40630049	7.6E-06	2.7E-02	47.73	FOXO1
Postflight vs. Inflight	108609597	2.4E-07	4.0E-03	-34.44	FOXO3
Postflight vs. Inflight	108561900	1.0E-06	9.0E-03	-34.09	FOXO3
Postflight vs. Inflight	108609614	7.8E-06	2.9E-02	-29.98	FOXO3
Postflight vs. Inflight	108629209	1.4E-05	4.0E-02	16.28	FOXO3

Postflight vs. Inflight	108629817	4.5E-06	2.1E-02	17.89	FOXO3
Postflight vs. Inflight	102445016	9.5E-06	3.7E-02	-32.64	IGF1
Postflight vs. Inflight	102442069	2.0E-07	4.1E-03	-27.20	IGF1
Postflight vs. Inflight	102441972	1.2E-05	4.3E-02	-26.47	IGF1
Postflight vs. Inflight	102404862	4.1E-06	2.9E-02	-25.39	IGF1
Postflight vs. Inflight	2138343	3.8E-06	1.7E-02	-27.39	IGF2
Postflight vs. Inflight	2138320	1.4E-05	3.8E-02	-26.34	MTOR
Postflight vs. Inflight	11245522	8.3E-06	3.2E-02	-27.56	MTOR
Postflight vs. Inflight	11243196	4.2E-06	2.2E-02	-24.46	MTOR
Postflight vs. Inflight	11197644	4.5E-06	2.3E-02	-24.28	MTOR
Postflight vs. Inflight	11243141	3.5E-06	2.1E-02	-23.54	MTOR
Postflight vs. Inflight	11243198	1.8E-05	4.8E-02	-21.46	MTOR
Postflight vs. Inflight	11114930	8.6E-07	9.2E-03	19.91	MTOR
Postflight vs. Inflight	11150257	2.1E-09	1.9E-04	32.58	MTOR
Postflight vs. Inflight	102607835	2.5E-06	1.2E-02	-33.16	NFKB1
Postflight vs. Inflight	102535016	2.3E-06	2.1E-02	-31.81	NFKB1
Postflight vs. Inflight	102586202	1.9E-06	2.0E-02	-30.36	NFKB1
Postflight vs. Inflight	102591386	1.5E-06	9.4E-03	-26.54	NFKB1
Postflight vs. Inflight	102547340	1.6E-05	3.8E-02	-25.47	NFKB1
Postflight vs. Inflight	49960741	4.7E-07	5.1E-03	-38.50	PARG
Postflight vs. Inflight	49960802	1.2E-09	2.4E-04	-38.48	PARG
Postflight vs. Inflight	49937673	4.6E-06	1.5E-02	-37.06	PARG
Postflight vs. Inflight	49960773	3.1E-07	4.2E-03	-35.98	PARG
Postflight vs. Inflight	49960804	2.7E-07	4.0E-03	-35.98	PARG
Postflight vs. Inflight	49890025	1.3E-05	3.8E-02	-34.27	PARG
Postflight vs. Inflight	49837531	5.7E-06	2.6E-02	-34.14	PARG
Postflight vs. Inflight	49937581	4.1E-06	1.4E-02	-33.23	PARG
Postflight vs. Inflight	49843478	2.0E-06	1.3E-02	-30.84	PARG
Postflight vs. Inflight	49931689	1.4E-05	2.9E-02	-27.54	PARG
Postflight vs. Inflight	49836249	1.7E-07	2.9E-03	-26.08	PARG
Postflight vs. Inflight	40770119	1.8E-06	1.0E-02	-41.95	PRKAA1
Postflight vs. Inflight	40773022	3.9E-07	4.0E-03	-38.51	PRKAA1
Postflight vs. Inflight	40773028	2.9E-06	1.3E-02	-33.70	PRKAA1
Postflight vs. Inflight	40765933	2.0E-06	1.3E-02	32.03	PRKAA1
Postflight vs. Inflight	40765887	1.0E-06	8.7E-03	32.33	PRKAA1
Postflight vs. Inflight	40765948	1.9E-08	7.4E-04	41.16	PRKAA1
Postflight vs. Inflight	56706620	1.1E-07	2.7E-03	-27.34	PRKAA2
Postflight vs. Inflight	56688755	1.3E-05	3.7E-02	28.20	PRKAA2
Postflight vs. Inflight	151804523	1.3E-06	9.1E-03	-37.33	PRKAG2

Postflight vs. Inflight	151839637	2.3E-06	1.7E-02	-33.26	PRKAG2
Postflight vs. Inflight	151718117	4.6E-06	3.2E-02	-18.97	PRKAG2
Postflight vs. Inflight	151782616	5.0E-06	3.3E-02	21.15	PRKAG2
Postflight vs. Inflight	151826283	1.7E-06	1.1E-02	21.19	PRKAG2
Postflight vs. Inflight	151756406	7.1E-06	3.1E-02	22.29	PRKAG2
Postflight vs. Inflight	151756415	1.0E-05	3.9E-02	32.81	PRKAG2
Postflight vs. Inflight	38892244	2.1E-07	4.0E-03	-28.91	SIRT2
Postflight vs. Inflight	38883199	1.9E-05	3.4E-02	28.67	SIRT2
Postflight vs. Inflight	219908	1.7E-05	4.1E-02	-28.72	SIRT3
Postflight vs. Inflight	223247	5.9E-06	3.6E-02	-26.07	SIRT3
Postflight vs. Preflight	21975077	1.65E-06	0.0157	41.03	CDKN2A
Postflight vs. Preflight	50541643	2.00E-06	0.0143	-42.78	EPN3
Postflight vs. Preflight	50541643	2.00E-06	0.0143	-42.78	EPN3
Postflight vs. Preflight	50542687	2.84E-06	0.0157	-33.10	EPN3
Postflight vs. Preflight	50542687	2.84E-06	0.0157	-33.10	EPN3
Postflight vs. Preflight	50541336	1.67E-05	0.0395	-26.83	EPN3
Postflight vs. Preflight	50541336	1.67E-05	0.0395	-26.83	EPN3
Postflight vs. Preflight	40591478	6.90E-06	0.0281	-27.54	FOXO1
Postflight vs. Preflight	40602708	1.48E-05	0.0406	26.97	FOXO1
Postflight vs. Preflight	40608203	1.89E-05	0.0449	27.67	FOXO1
Postflight vs. Preflight	40608212	3.38E-07	0.0057	30.30	FOXO1
Postflight vs. Preflight	40629965	3.47E-06	0.0205	41.21	FOXO1
Postflight vs. Preflight	40589767	1.33E-06	0.0176	48.28	FOXO1
Postflight vs. Preflight	108609614	1.57E-07	0.0057	-38.60	FOXO3
Postflight vs. Preflight	108561900	2.31E-06	0.0219	-37.14	FOXO3
Postflight vs. Preflight	108629035	1.24E-06	0.0179	25.28	FOXO3
Postflight vs. Preflight	108629097	2.13E-07	0.0072	33.06	FOXO3
Postflight vs. Preflight	102445016	4.95E-06	0.0326	-36.46	IGF1
Postflight vs. Preflight	102445016	4.95E-06	0.0326	-36.46	IGF1
Postflight vs. Preflight	102445000	1.47E-06	0.0189	-29.89	IGF1
Postflight vs. Preflight	102445000	1.47E-06	0.0189	-29.89	IGF1
Postflight vs. Preflight	102442469	2.99E-08	0.0020	-29.32	IGF1
Postflight vs. Preflight	102442469	2.99E-08	0.0020	-29.32	IGF1
Postflight vs. Preflight	2138343	4.77E-07	0.0066	-30.35	IGF2
Postflight vs. Preflight	9000552	8.50E-06	0.0307	-47.01	KLRG1
Postflight vs. Preflight	8989178	7.86E-06	0.0334	-27.55	KLRG1
Postflight vs. Preflight	11196745	3.45E-06	0.0218	-36.28	MTOR
Postflight vs. Preflight	11244011	2.38E-06	0.0215	-29.63	MTOR
Postflight vs. Preflight	11226891	9.18E-06	0.0340	-28.10	MTOR

Postflight vs. Preflight	11150257	3.18E-08	0.0034	33.35	MTOR
Postflight vs. Preflight	102586362	1.08E-06	0.0170	-36.53	NFKB1
Postflight vs. Preflight	102586362	1.08E-06	0.0170	-36.53	NFKB1
Postflight vs. Preflight	102607835	1.01E-05	0.0306	-33.50	NFKB1
Postflight vs. Preflight	102607835	1.01E-05	0.0306	-33.50	NFKB1
Postflight vs. Preflight	49937673	7.39E-09	0.0009	-44.53	PARG
Postflight vs. Preflight	49937673	7.39E-09	0.0009	-44.53	PARG
Postflight vs. Preflight	49960741	1.21E-07	0.0043	-42.89	PARG
Postflight vs. Preflight	49960741	1.21E-07	0.0043	-42.89	PARG
Postflight vs. Preflight	49960804	1.38E-07	0.0046	-41.85	PARG
Postflight vs. Preflight	49960804	1.38E-07	0.0046	-41.85	PARG
Postflight vs. Preflight	49960773	2.82E-06	0.0224	-38.69	PARG
Postflight vs. Preflight	49960773	2.82E-06	0.0224	-38.69	PARG
Postflight vs. Preflight	49837531	1.74E-06	0.0146	-38.19	PARG
Postflight vs. Preflight	49837531	1.74E-06	0.0146	-38.19	PARG
Postflight vs. Preflight	49869464	1.65E-06	0.0157	-34.62	PARG
Postflight vs. Preflight	49869464	1.65E-06	0.0157	-34.62	PARG
Postflight vs. Preflight	49843478	1.72E-05	0.0438	-31.69	PARG
Postflight vs. Preflight	49843478	1.72E-05	0.0438	-31.69	PARG
Postflight vs. Preflight	49884555	6.60E-06	0.0272	-29.81	PARG
Postflight vs. Preflight	49884555	6.60E-06	0.0272	-29.81	PARG
Postflight vs. Preflight	49836249	1.27E-07	0.0041	-29.52	PARG
Postflight vs. Preflight	49836249	1.27E-07	0.0041	-29.52	PARG
Postflight vs. Preflight	49937751	2.48E-07	0.0050	-28.39	PARG
Postflight vs. Preflight	49937751	2.48E-07	0.0050	-28.39	PARG
Postflight vs. Preflight	49843705	1.80E-06	0.0146	-27.30	PARG
Postflight vs. Preflight	49843705	1.80E-06	0.0146	-27.30	PARG
Postflight vs. Preflight	226404722	7.29E-08	0.0030	-33.12	PARP1
Postflight vs. Preflight	226404722	7.29E-08	0.0030	-33.12	PARP1
Postflight vs. Preflight	102144783	3.81E-06	0.0358	-29.79	PARPBP
Postflight vs. Preflight	102144783	3.81E-06	0.0358	-29.79	PARPBP
Postflight vs. Preflight	102130851	1.49E-07	0.0050	-25.14	PARPBP
Postflight vs. Preflight	102130851	1.49E-07	0.0050	-25.14	PARPBP
Postflight vs. Preflight	40773022	1.58E-07	0.0040	-41.66	PRKAA1
Postflight vs. Preflight	40773028	1.37E-05	0.0357	-35.16	PRKAA1
Postflight vs. Preflight	40765933	1.68E-05	0.0431	32.58	PRKAA1
Postflight vs. Preflight	151839637	6.57E-07	0.0113	-36.14	PRKAG2
Postflight vs. Preflight	151766008	1.35E-07	0.0047	-27.80	PRKAG2
Postflight vs. Preflight	151766019	1.68E-07	0.0049	-27.78	PRKAG2

Postflight vs. Preflight	18917416	8.13E-06	0.0298	-43.88	PRODH
Postflight vs. Preflight	18917416	8.13E-06	0.0298	-43.88	PRODH
Postflight vs. Preflight	18933530	2.04E-05	0.0487	25.87	PRODH
Postflight vs. Preflight	18933530	2.04E-05	0.0487	25.87	PRODH
Postflight vs. Preflight	38884258	5.69E-06	0.0441	-27.35	SIRT2
Postflight vs. Preflight	38884258	5.69E-06	0.0441	-27.35	SIRT2
Postflight vs. Preflight	38883199	4.49E-06	0.0200	34.15	SIRT2
Postflight vs. Preflight	38883199	4.49E-06	0.0200	34.15	SIRT2
Postflight vs. Preflight	219908	1.27E-06	0.0127	-34.11	SIRT3
Postflight vs. Preflight	219908	1.27E-06	0.0127	-34.11	SIRT3
Postflight vs. Preflight	223247	4.49E-07	0.0114	-32.17	SIRT3
Postflight vs. Preflight	223247	4.49E-07	0.0114	-32.17	SIRT3

HR					
CD8+					
Time Period	**CpG**	**p-value**	**q-value**	**methDiff**	**Gene**
Inflight vs. Preflight	36682963	9.73E-07	0.0405	35.63	CDKN1A
Inflight vs. Preflight	108630135	7.87E-07	0.0394	-21.41	FOXO3
Inflight vs. Preflight	20354050	3.18E-08	0.0051	-26.43	PARP2
Inflight vs. Preflight	40770159	6.54E-07	0.0198	-34.86	PRKAA1
Inflight vs. Preflight	40764115	1.14E-07	0.0084	-32.31	PRKAA1
Inflight vs. Preflight	40764124	6.76E-07	0.0310	24.03	PRKAA1
Inflight vs. Preflight	151678938	1.01E-08	0.0024	-37.32	PRKAG2

TW					
CD8+					
Time Period	**CpG**	**p-value**	**q-value**	**methDiff**	**Gene**
Postflight vs. Inflight	40617919	1.32E-06	0.0483	-17.86	FOXO1
Postflight vs. Inflight	108598052	2.23E-06	0.0419	-17.40	FOXO3
Postflight vs. Inflight	11240165	8.19E-07	0.0493	-19.39	MTOR
Postflight vs. Inflight	102190256	1.06E-06	0.0478	-20.29	PARPBP
Postflight vs. Inflight	56702942	1.23E-06	0.0408	-16.90	PRKAA2

For the results described in Figures 4 and 5, mean methDiff is summarized per gene. This table above provides a detailed view of the individual CpG results used to obtain the mean methDiff. For most genes, there are multiple CpG sites at which differential methylation is observed, and although CpG methylation is generally bimodal (around 0% and 100%), in some instances, individual CpG methylation levels shift in opposing directions; such shifts are eliminated by taking the mean methDiff.

Appendix C.

Python Script

A Python script was written to parse raw DMC data derived from WGBS results and intersect it with candidate genes. This tool searches a comma-separated value text file of expression comps for a subset specified by a gene file. It works by looping through each line of the comp file. For each line, it searches the gene file to see if the comp line falls within the range specified by each gene line. If there is a match, it outputs the entire comp line to the results file.

Usage: `python parse_comp_txt.py <comp file> <genes file>`

Comp file format (.txt): `chr,start,end,strand,p.value,q.value,meth.diff,gene_name`

Gene file format (.xls): `Chromosome,Transcript start (bp),Transcript end (bp),Gene name,Transcription start site (TSS)`

The output is a file with the same format and name as the comp file, but with "`_out.csv`" appended to the end.

```
#!/usr/bin/python

import argparse
import xlrd
import os
from datetime import datetime

parser = argparse.ArgumentParser(description='This tool searches a csv
of expression comps for a subset specified by an Excel file.')
parser.add_argument('compFile', default=None, help='The comp file')
parser.add_argument('genesFile', default=None, help='The genes file')

args = parser.parse_args()

compFile = args.compFile
genesFile = args.genesFile
outputFile = compFile + "_out.csv"

numLines = 0
```

```
genesBook = xlrd.open_workbook(genesFile)
sheet = genesBook.sheet_by_index(0)

# Initialize variables
geneList = []
previousResultLine = ""

for row in range(1,sheet.nrows): # Chromosome, Tstart, Tend, Gene Name

geneList.append([sheet.row_values(row)[0],int(sheet.row_values(row)[1])
,int(sheet.row_values(row)[2]),sheet.row_values(row)[3]])

outFile = open(outputFile,'w')

print "Input comp file: " + compFile
print "Input genes file: " + genesFile
print "Output file: " + outputFile

# Print the start time of processing to stdout
now = datetime.now()
start_time = now.strftime("%Y-%m-%d %H:%M:%S")
print("Start Time = " + start_time + "\n")

# Write the header to the first line of the output file
header =
["chr","start","end","strand","p.value","q.value","meth.diff","gene_nam
e"]
headerLine = ",".join(header)
outFile.write(headerLine + "\n")

# Open the comp file
with open(compFile) as infile:
    infile.readline()
    for line in infile: # Loops through all lines of the comp file
        splitLine = line.rstrip("\n").split("\t")
        startSite = int(splitLine[1])
        methDiff = splitLine[6]
        for gene in geneList: # Check comp file line against all the
lines in the genes file
            try:
                if gene[1] <= startSite and startSite <= gene[2]: #
Logic to determine whether to write the line to the output file
                    resultLine = ",".join(splitLine + [gene[3]])
                    if resultLine != previousResultLine:
                        print resultLine
                        outFile.write(resultLine + "\n")
                        previousResultLine = resultLine
            except KeyboardInterrupt: # Try to cleanly exit the script
if you hit ctrl-c
                quit = True
                break
        if quit == True:
            break
        numLines = numLines + 1

# Close the comp file
outFile.close()
```

```
# Print a summary of processing
print "\nProcessed {} lines.".format(numLines)
now = datetime.now()
end_time = now.strftime("%Y-%m-%d %H:%M:%S")
print("Start Time = " + start_time)
print("End Time = " + end_time)
```

Appendix D.

Definition of Terms

Cellular senescence: The phenomenon by which cell growth is irreversibly arrested, either by intrinsic factors like age-driven telomere shortening resulting in replicative senescence, or by extrinsic factors like DNA damage resulting in stress-induced senescence (Campisi & di Fagagna, 2007).

CpG: A DNA region where a single cytosine base is followed by a single guanine base in the 5' to 3' direction in a linear sequence. DNA methylation occurs at CpGs. CpGs occur mostly in regions known as CpG islands.

CpG island (CGI): Regions of the genome rich in CpGs, most often occurring in the promoter region of a gene.

Differentially methylated cytosine (DMC): The resulting differential DNA methylation status across cell samples, where the methylation level is calculated based on a single cytosine base (CpG).

DNA damage response (DDR): A group of cellular pathways that respond to DNA damage by sensing, signaling, and repairing dysfunction.

DNA methylation age (DNAm age): Refers to the results of a high-fidelity, multi-tissue biological age clock used to estimate the epigenetic age of a subject using data derived from most tissues and cell types (Horvath, 2013).

Double-strand breaks (DSBs): A detrimental form of DNA damage characterized by breaks in both strands of the double DNA helix, often induced by ionizing radiation or by DNA replication and repair.

EM-seq: NEBNext Enzymatic Methyl-seq (EM-seq) is a newer alternative method to whole-genome bisulfite sequencing (WGBS) for analyzing DNA methylation, resulting in the same converted sequence while allowing for the production of high-quality libraries using fewer sequencing reads.

Epigenome: The collection of heritable chemical marks on an organism's DNA and histone proteins that regulate gene expression, differentiation, and development; epigenetic changes can arise sporadically or in response to the environment (Heard & Martienssen, 2014).

Galactic cosmic radiation (GCR): An ionizing form of radiation comprised of high-energy atomic fragments, it is the main type of radiation affecting spacecraft (Chancellor et al., 2014).

Homologous recombination (HR): An innate mechanism to repair DSBs in which a homologous gene sequence is used to guide repair of the break site.

Information Theory of Aging: A theory proposed by geneticist and longevity researcher David Sinclair, Ph.D., which states aging results from epigenetic noise that causes a loss of epigenetic information over time, driving genomic instability and other hallmarks of aging (Sinclair & LaPlante, 2019).

Ionizing radiation (IR): Radiation consisting of high-speed particles and electromagnetic waves, like X-rays and gamma rays, that carries enough energy to ionize electrons by detaching them from molecules or atoms and ionizing the material through which it passes (Chancellor et al., 2014).

methDiff: A notation used in this investigation to denote the percentage of difference in mean methylation at an individual CpG site as compared between time points (e.g., postflight vs. preflight).

Methylation: In DNA methylation, methyl groups (CH_3) are added to either the cytosine or adenine bases of a DNA molecule and can modify the expression of a gene or gene segment without modifying the underlying DNA sequence. Methylation of a gene in its promoter region results in decreased expression or silencing, while demethylation results in increased expression and transcription (Moore, Le, & Fan, 2012).

NASA Twins Study (One-Year Mission): A 25-month long (2014-2016) NASA-led study of the biological and cognitive differences observed between twin astronauts Mark Kelly (HR) and Scott Kelly (TW), centered on Scott Kelly's 340-day long mission on the International Space Station (Garrett-Bakelman et al., 2019).

NFκB signaling pathway: Stimulates the senescence-associated secretory phenotype (SASP).

Senescence-associated secretory phenotype (SASP): A phenotype that is characteristic of senescent cells in which proinflammatory signals such as cytokines and growth factors are secreted.

Sirtuins: A family of NAD+-dependent histone deacetylases that are involved in almost all cell biological processes, including metabolism, survival, differentiation, and DNA repair, and are believed to play a central role in mammalian lifespan (Imai & Guarente, 2014).

Telomere: Commonly described as the protective "end cap" of a chromosome. Telomeres generally shorten with age and in response to cellular stressors and damage.

Whole-genome bisulfite sequencing (WGBS): The sequencing technology used to analyze DNA methylation of individual cytosine bases, in which sodium bisulfite treatment is applied to DNA in order to convert unmethylated cytosine.

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