Do You Really Want to Live Forever? - Forever Young: A Multifaceted Analysis of Molecular, Environmental and Genetic Factors that Contribute to Healthy Aging and Exceptional Longevity

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ABSTRACT

The area of longevity research has skyrocketed within the past few decades, thereby creating an unprecedented narrative for the process of aging, and science's ability to impact this process. With regard to the field of longevity, it is important to analyze the distinction between lifespan, which is the amount of time one lives, and healthspan, which is the amount of time that one lives while remaining relatively healthy. Throughout previous centuries these two aspects of the human condition developed alongside one another, as the relatively healthy individuals within a society were able to outlive the less healthy individuals. More recently a disparity between the two has developed, causing an increase in global lifespan without the respective increase in population healthspan. This can be due to medical intervention delaying morbidity of many chronic aging related diseases such as heart disease and cancer. The field of longevity is attempting to resolve this lagging behind of the global healthspan by addressing the multiple aspects that affect the healthy aging process, including the role that genetic and environmental factors contribute to senescent changes.

This paper attempts to analyze genetic and environmental factors that have been previously correlated to healthy aging, as well as understand how the interplay between the two leads to gene expression, and thereby the molecular impact of aging. Furthermore, NAD+ dependent deacetylases have been previously shown to have a strong impact on the healthy aging process, and when environmental factors influence the genetic expression of these molecules, individuals may present with exceptional cases of longevity. The molecular research discussed in this paper is studying the protein Sirtuin 6, which has been previously correlated with longevity. By further characterizing this protein, we can gain more insight into the role not only of sirtuin in the aging process but more broadly the overall impact of molecular expression on aging.

Another vital aspect of aging research is the study of centenarians, or individuals who have lived past 100 years of age. This study attempts to incorporate anecdotal evidence into the understanding of various environmental factors that may contribute to the development of cognitive "super-agers," who are individuals who maintain exceptional cognitive abilities. Anecdotally it is evident that these individuals maintain significant mobility and cognitive activity into their old age, which can be further understood as a contributing factor to their exceptional longevity and overall healthy aging.

Additional research is necessary in order to bridge the gap between the molecular research being done in the field of longevity and the clinical research being done. This critical connection can allow for a greater understanding of the interplay of genetics and environmental factors that contribute to their exceptional longevity. This connection can allow for greater insight into the components of healthy aging, thereby opening up a field of possibilities for manipulation and downstream implications of potentially raising the upper limit of a human's lifespan and healthspan.

BACKGROUND

Introduction

Mankind has always been fascinated by death and the process of aging, but until recently aging has been viewed as a stagnant and irreversible progression by the scientific community (Johnson 2002). Over the past two centuries, scientific understanding has progressed at record rates. This, along with the many advancements in our changed perception and knowledge of the world has caused lifespan to undergo a dramatic increase, with the average global lifespan doubling from approximately 30 to 40 years to close to 80 years of age, as depicted in Figure 1 (Crimmins, 2015).

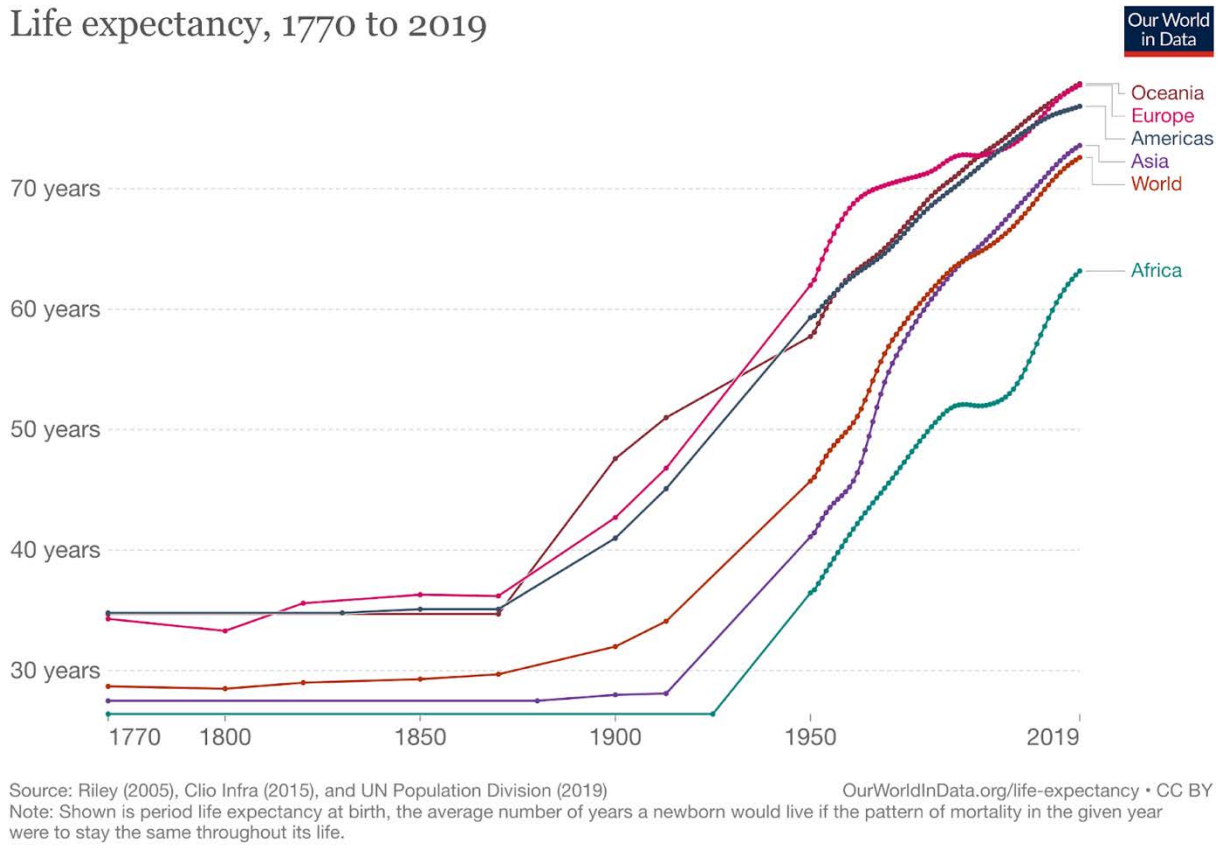

Figure 1. Graph depicting lifespan trends rising from under 30 years in 1770 to a global life expectancy of over 70 years in 2019. (Courtesy of Our World In Data, Life Expectancy)

This dramatic increase can be due to multiple overlapping factors. One of the most significant contributing factors has been the advancement of medical assistance and intervention as well as drastic improvements of the environment (Baumann, 2017). Infrastructure such as clean water systems, food safety standards and an overall increase in the understanding of hygiene techniques such as hand washing have contributed to a dramatic reduction in diseases and thereby decreased the infant mortality rates and increased the overall life expectancy of the population (Olshansky, 2019). The developments of vaccinations and sewage systems throughout the 19th century are some examples of innovations that generated the rise in lifespan throughout the 19th and 20th centuries.

In addition to people beginning to live longer over the past few centuries, the global population has been living healthier for longer as well. This important aspect of the rise in health along with the increase in longevity is mainly due to the reduction of communicable diseases (Baumann, 2017). These developments thereby allow individuals to achieve the lifespan "potential" that is already encoded in their genome by maintaining relative overall health throughout their lifetime by avoiding infectious diseases (Falah, 2020). An example of this can be seen from the number of centenarians, or individuals who surpass 100 years of age rising in Italy from 165 individuals in 1951 to over 1,500 Italian centenarians in 2011 (Caselli, 2020). This could potentially demonstrate the genetic predispositions of these individuals that now had the capabilities to achieve their potential.

Over the past few decades, there has been an approximately five-year global lifespan increase (Westendorp, 2006; Caselli, 2020) . Unfortunately, this extension of lifespan in contrast to the previous prolongation of lifespan over the past few centuries has not been correlated with a respective overall increase in the healthspan. Many of these individuals, while living longer, are

spending their final years extremely dependent on medical intervention and care. These developments coincide with the epidemiological transition theory which explains that as the prevalence of communicable diseases decrease within a society, there is an increase in noncommunicable diseases such as cancer or cardiovascular disease (Geidl 2020). These diseases, in contrast to infectious acute diseases, are often chronic diseases and are known as age-related disease or geriatric diseases, which are diseases that correlate with an increase in senescence. People begin to live long enough to develop these age-related diseases, which impede the correlated increase in healthy aging of the overall population (Klenk, 2016).

Oftentimes when people think of longevity research, dystopian popular culture comes to mind. Many are fearful of outliving society, being dependent on others, or even being "hooked up" to various machines that would enable them to live a long but fruitless life. Fortunately, the area of longevity research more recently is pivoting with one critical distinction in mind: the difference between healthspan and lifespan. Not only is this area of research interested in understanding what genetic or environmental factors allow for these select outliers of a population to live longer, but in addition to live healthier as well, creating a new field of interest known as healthspan.

Healthspan: Definition and Affecting Factors

Healthspan, which is defined as the general duration of a person's life in which they are healthy, relying on relatively little medical intervention, is considered a critical aspect of aging by the National Institute of Health. Healthspan focuses on extending the overall length of relative health of individuals in a population in contrast to simply an increase or prolongation of lifespan due to medical intervention and assistance, specifically with regards to age related disease such as cardiovascular diseases and cancer.

Environmental factors have been shown to play a crucial role in lifespan as well as healthspan. Arguably, these environmental factors may be the current limiting factors in the overall aging process, thereby causing health decline faster than our genetic programing is predisposed to deteriorate. This explains why current research focuses on the intersection of both the genetic and environmental components that may be regulating various metabolic pathways that effect both lifespan and healthspan (Passarino, 2016).

Conversely, other competing literature has speculated that the healthspan of a population may be more strongly impacted by the genome of the population rather than the environment (Levine, 2018). This opens a whole new understanding of aging research, focusing on genetic manipulation and expression, in addition to environmental factors, to increase the heathspan of individuals.

Research is now focusing on a greater understanding of the interplay of these two aspects of longevity, genetic predisposition as well as environmental impact (Passarino, 2016). Understanding how the environment may impact genes by regulating their expression and in turn the process of aging may be the crucial missing piece. Understanding the interconnection of the two, and its impact on molecular expression within our cells, may allow for the potential manipulation in order to increase lifespan. Furthermore, this intersection could explain an interesting recent phenomenon of the increase in extreme longevity, along with preservation of overall health.

GENETIC FACTORS OF AGING

It is evident that genetics play a critical role within the field of longevity. On a most basic anecdotal level, this can be seen by overall health and lifespan trends that are often preserved

within a family line. The Leiden Longevity study compared the offspring of the siblings of nonagerians, which are individuals who live to over 90 years of age. The siblinngs performed better on various cognitive tasks at middle aged compared to control subject with no history of familial longevity (Tanprasertsuk, 2019). The study concluded that the offspring of the siblings of nonagerians preformed significantly better on various attention, speed, and memory tasks, in contrast to their control counterparts (Tanprasertsuk, 2019). These differences in performance can contribute an overall average difference of three cognitive aging years, displaying that even distant relatives of super agers can correlate to lengthened cognitive health, showing the impact of genetics on lifespan.

Current research within the field of epigenetics is attempting to identify biomarkers which can be correlated with an increase in both lifespan and healthspan. A recent study identified a novel biomarker that has been correlated with an increase of proto-inflammatory response within cells and a decrease in the function of DNA repair mechanisms (Levine, 2018). This marker can therefore correlate with an increase in the "cellular age" of an individual and thereby the decrease in health. Downstream applications can then manipulate this biomarker expression or potentially slow the aging process.

These discoveries lead to a question in the field of longevity and genetics whether or not there is a natural upper limit on human lifespan or if the aging process is something that can be manipulated and potentially slowed, halted or in the most extreme case, even reversed (Barbi, 2018). As this is a new field of study, there is an unclear consensus as to whether the aging process is reversible, but it is already evident that the answer is not clear cut (Dolgin, 2018).

Research in the field of epigenetics has shown that cellular genes can be induced to turn on various genes that reduce chemical markers of aging, and repair damaged tissue. One study of

this was done in mouse models to target the expression of four genes that are connected to the aging process. When these genes were manipulated in mouse models, the mice appeared younger and some of their cells reverted to pre-differentiated stem cells. This epigenetic manipulation of the organisms' cells allowed for the organism to repair damaged cells or organs(Sebastiani, 2021). One example from this study involved manipulating the corneal genes thereby causing the eyes of older mice to have decreased signs of macular degeneration, and appear as eyes of younger mice (Sebastiani, 2021). Additionally, these manipulations change the overall metabolic fingerprint of the mice, causing various molecular markers of aging to decline or in some cases even vanish.

ENVIRONMENTAL FACTORS OF AGING

Previous research has demonstrated that in model organisms, such as mice, environmental factors can also have a drastic impact on healthspan and lifespan. A recent study examined the effect of diet on longevity, showing that healthy dieting such as calorie restriction allow the organisms to live healthier for longer. Additionally, these organisms maintain overall physical health for longer as well, often maintaining greater muscle mass and abilities to participate in strenuous activity even into old age, in contrast to the control mice fed regular diets (Taormina, 2019). Furthermore, these organisms have been shown to have different genome expression patterns, supporting the hypothesis that the environmental factors in conjunction with genomic factors increase the health of the mice. This evidence not only shows that both aspects of longevity are vital but raises the potential of a cause-and-effect relationship. Meaning that the diet affects the expression of the mice's genome causing them to express and repress various genes, some of which may be genetic markers for healthy aging.

Interestingly, centenarians often escape many age-related diseases. This can potentially follow the same trend of the synergistic relationship between one's genetics and environment being the strongest contributing factor to lifespan and healthspan. These individuals have been seen to have decreased rates of heart disease, cancer, and Alzheimer's disease, just to name a few (Rogalski, 2015). These select few, are not simply living longer, but unlike the rest of the population- due to various fascinating interconnected factors that scientists are still attempting to understand- they are living healthier as well.

While we have come to better understand the process of aging and factors that contribute to longer health from a scientific perspective, throughout human history, man has always been interested in aging and factors that allow for the select few outliers of the population to surpass the rest of society. In records dating as far back as ancient Egypt, society has been fascinated with methods that could prevent the process of aging. An ancient record known as the "Sir Edwin Smith Surgical Papyrus" which dates to 2800 B.C., is one such example. The document is a detailed description of various ointments and treatments that can be used to "remove signs of old age, and weakness from the flesh." This is just one of many examples that document various remedies people have used to combat aging and death. Society has come to understand that the environment and how one treats their body, can impact their lifespan.

Conversely, following the development of scientific inquiry, until relatively recently, the area of aging research and longevity has been viewed quite negatively from the scientific community. Oftentimes lifespan was viewed as something inevitable, and thereby unpreventable and, because of this, research in this area was often quite "unscientific in its approach." Dr. Danica Chen, a Professor of Metabolic Biology at the University of California at Berkeley, explains that the area of longevity research has skyrocketed since 1980, thereby allowing our

understanding of factors that contribute to lifespan and health span to dramatically increase as well. Dr. Chen continues to explain that only recently did factors including environment, metabolism, and genetics obtain a strong correlation with our understanding of aging cycle (De Winter, 2015). Additionally, more recently it has become more widely accepted that aging is a fluid and potentially even reversible process, as opposed to the previous scientific consensus that it was a stagnant and inescapable cycle. Moreover, the scientific community is now even going as far as to understand the process of aging through the lens of disease, rather than a predestined natural life cycle (De Winter, 2015).

Additionally, among these centenarians there is often, although not always, a trend of longevity that runs in their family history as well. This interesting commonality may point to various genetic markers that allow for this subset of the population to age so well, but it is important to note that longevity can also be due to other familial factors such as lifestyle and culture, which can play a critical role in the healthy aging of a population.

CENTENARIAN ANALYSIS

Introduction to "Cognitive Super-agers"

Amongst the population of centenarians, and those who live to an exceptionally old age, there is also a subpopulation known as "cognitive super-agers." This subpopulation is of significant interest due to their unique and exceptional cognitive abilities even at extreme old age. Understanding the factors that allow for this population not only to live longer and healthier, but to maintain many of their cognitive abilities such as fluid intelligence, which is often understood to decline with aging, is of incredible interest (Geidl, 2020).

According to the National Health Institute of Aging, cognitive super-agers are the select few that preform significantly better on memory tests, compared to peers of similar age (NIA, 2020). Dr. Molly Wagster, the chief of the Behavioral and Systems Neuroscience Branch in NIA's Division of Neuroscience explains that "there is a tendency to equate [age] with a decline in mental function." The goal of research on this subpopulation is to understand how these "cognitive super-agers… break the expectations of cognitive decline.(NIA, 2020)" Dr. Claudia Kawas attributes physical factors as one of the most prominent determinants of aging related disease. Dr. Kawas explains that "atrophy is the strongest correlate of age." Kawas concluded, based on a longitudinal study with close to two thousand participants, that the typical brain of a 90-year-old weighs approximately as least 100 grams less than the average brain of a 40-year-old (Giorgio, 2010). This decline can be due to a declining level of grey matter and has the greatest impact on the prefrontal cortex, hippocampus, and cerebral cortex, which can thereby impair cognitive abilities.

Anecdotal Experience and Analysis of "Cognitive Super-agers"

I currently work at The Integrative Longevity Omics Study at the Albert Einstein College of Medicine in the Centenarian Study. Throughout the screening process of centenarians, I have seen first handed the distinction between these select "cognitive super-agers" and the rest of the centenarian population. I will now discuss a few individuals whom I have conversed with throughout the course of this study. Many of these unique individuals share many commonalities, including familial histories of longevity and a determination to occupy themselves with various therapeutic activities. These commonalties, although anecdotal, draw on the same themes of the interplay of genetic and environmental factors.

Meet Barbra

One individual I had recruited for the study was a 103-year-old woman named Barbra*. Barbra explained that until her birthday this past year she had been driving, living on her own and running errands on a regular basis. She proclaimed to me how much she enjoys going grocery shopping and the various lunches that she continues to cook for herself each day. She explained that having a very regimented schedule was critical for the maintenance of her health especially during the outbreak of the pandemic, which had restricted an already vulnerable population to further isolation and vulnerability. Barbra continued to explain that her children became fearful of her driving once she turned 103, and she has not been driving since then. She did confide that she feels she lost some of her independence because of this but maintains every effort to keep busy including taking daily walks in her garden outside. Barbra spoke eloquently and clearly and sounded over the telephone as if she had been in her late 70s or early 80s, with a strong voice.

It was evident from our conversation that Barbra likes to keep both her mind and her body active, likely a contributing factor to her exceptional longevity.

Meet Robert "Bob"

Bob* immediately began by introducing himself to me as a proud centenarian, having just turned 100 in the previous month. He explained to me how he lives in an assisted living facility and is one of the oldest residents in the facility. He described many things he likes to busy his day with including having lunch with a few of his friends in the facility, having "zoom parties" with his children, grandchildren, and great-grandchildren through his "Grand-pad" and lastly by reading, especially non-fiction, his favorite.

Throughout our conversation, I began learning more about Bob, who is a veteran who served in World War II. He explained that because of this, he was fortunate to receive better healthcare than many of his peers. He explained to me that throughout his life, he was always relatively healthy and has never had any hospitalizations or surgeries. He attributed a significant portion of his health to his diet, explaining that he enjoys fruits and vegetables which often made up a large portion of his diet.

This conversation, like the one with Barbra, drew on genetic predispositions to Bob's expectational age, such as his relative health throughout his lifetime. Additionally, it is also evident that the maintenance of his health may in part be due to his continuous activity and the overall healthy lifestyle that he leads.

Conclusion of Cognitive Super-agers

It is evident that for these cognitive super-agers both genetic and environmental factors have contributed to their expectational health and age. Based on conversations with these individuals, it is evident that maintaining activity and exercising their cognitive abilities has allowed for the preservation of their cognition (Geidl, 2020). Previous literature has correlated continual cognitive tasks such as reading and crossword puzzle solving with the slowing or halting of the otherwise "natural" deterioration of cognition through the process of aging (Hötting, 2013).

Genetic factors likely also play a key role in cognitive super-agers likely allowing for them to achieve their long lifespan (Dong, 2016). Further research connecting the potential biomedical markers of these centenarians along with their environmental history could potentially allow for further understanding of on the causal effect of environment and gene expression.

MOLECULAR AGING FACTORS

Introduction

It is evident that both environmental and genetic factors may affect the overall lifespan and healthspan of individuals, since the environment can affect gene expression and in turn, have either a positive or negative impact on health. The intersection between the two can be most evidentially seen within the area of molecular aging factors (Passarino, 2016). This area of research attempts to understand how the regulation of gene expression and thereby protein expression can impact one's health and lifespan. Biomarkers for longevity, and proteins connected to longevity, are constantly being discovered, opening an area of research that can allow for the synthesis of genetic and environmental impact. This field can bring future hope of understanding how the two connect and discover ways to manipulate this connection in order to increase both the lifespan and healthspan of the population.

Introduction to Sirtuin Proteins

In addition to the previously mentioned aspects of lifespan and healthspan, in recent years there has been a significant correlation between NAD+ dependent protein deacetylases and longevity, specifically the sirtuin proteins (Gertler, 2013). Sirtuin 6, or SIRT6, is one of these sirtuin proteins that has been significantly correlated to lifespan and healthspan. Recent literature has shown that the overexpression of SIRT6 lengthened the lifespan of mice by 23%, while also maintaining their healthspan (Roichman, 2021). In addition to the catalytic pocket, SIRT6, like many other proteins, contains a zinc-finger, a structural motif on the Sirtuin. While little is currently known about the function of the zinc-finger on SIRT6, it may play a crucial role in the proper folding of the enzyme despite its large distance from the catalytic site of SIRT6. The novel research being presented in this paper attempts to analyze and explore the potential

allosteric effects of the zinc-finger via thermal analysis and activity assays. Through various molecular manipulations of the SIRT6 zinc-finger, it is evident that it plays a critical role in the function of the protein. As SIRT6 is a protein with a strong connection to a lengthened lifespan, the analysis of its zinc-finger as well as its overall structure can contribute to further breakthroughs in the study of human aging.

The effects of overexpression of SIRT6 have been connected to extending healthy lifespan through maintaining and restoring energy homeostasis. It plays a vital role in various metabolic processes as depicted in Figure 2. Its various catalytic abilities explain why it has such a strong impact on healthspan. Most notably SIRT6 has been connected to DNA repair mechanisms, such as telomerase regulation which can play a vital aspect in aging (Naiman, 2019). Additionally, SIRT6 enzymatically regulates gluconeogenesis, which is an aspect of metabolism that has been strongly correlated with healthspan. Gluconeogenesis is the process responsible for glucose synthesis increasing the glucose serum concentration, which can be strongly correlated with an increased risk for stroke, heart disease and kidney disease. By regulating gluconeogenesis SIRT6 can lower the level of blood glucose levels and thereby decrease the long-term effects of elevated glucose serum levels.

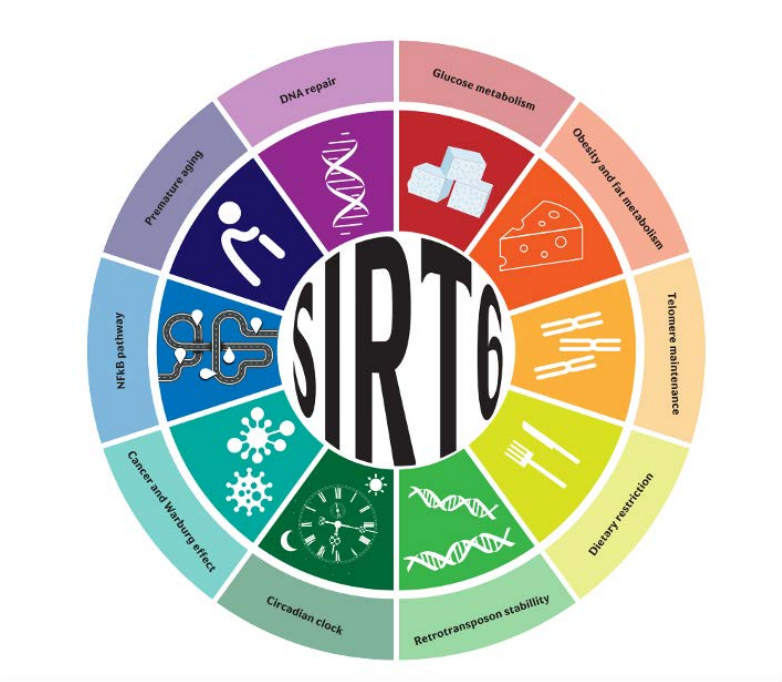

Figure 2. Metabolic activities of SIRT6. (Courtesy of Kanfi, 2012)

Previous literature has shown the vitality of SIRT6 in healthy aging specifically within mice models (Kanfi, 2012). Mice were used as model organisms due to the genetic similarity between humans and mice. Additionally, the lifespan of mice is approximately two years, making them a feasible model organism for a lifespan study. Mice also share approximately 85% of the protein coding region of the genome with humans and 99% of the proteins encoded have human orthologs, including SIRT6, allowing for them to be an ideal model organism for this study (Kanfi, 2012). Most significantly, a recent study has also shown that the overexpression of SIRT6 can extend the lifespan of mice by up to 27%, as displayed below in Figure 3 (Roichman, 2021).

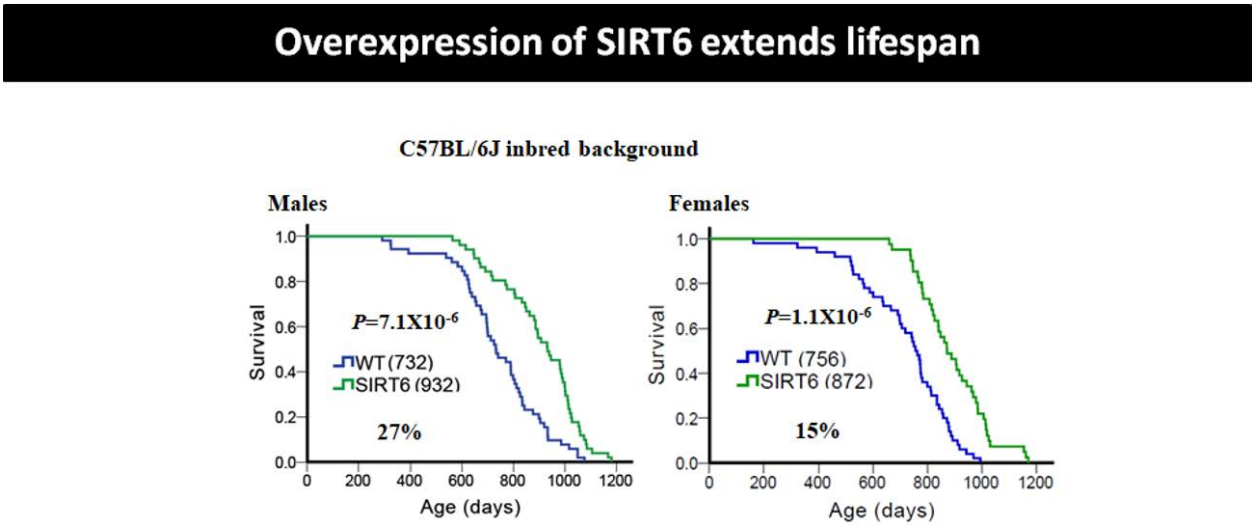

Figure 3. Overexpression of SIRT6 in C57BL mice extends the lifespan of mice on average by 23%. The survival rate of the mice is graphed against the age of the mice. (Courtesy of Roichman, 2021)

On a molecular level, SIRT6 can act both as an ADP-ribosyl transferase and histone deacetylase, playing a critical role in various metabolic cycles previously mentioned including gluconeogenesis, DNA repair, and lipid metabolism. As depicted in Figure 4, the zinc-finger is a significant distance from the catalytic pocket which is why the potential allosteric effects of this structural motif are unusual. Four cysteine residues hold the zinc-finger in place as seen on the right image in Figure 4. Cysteine has a strong affinity for the Zn2+, via the electrostatic interactions with the zinc-and the sulfur atom of the residue (Pace, 2014). Manipulations of this zinc-finger on a molecular level can provide significant insight into the function of the zinc-finger within the broader picture of Sirtuin as well as its effect on lifespan and healthspan.

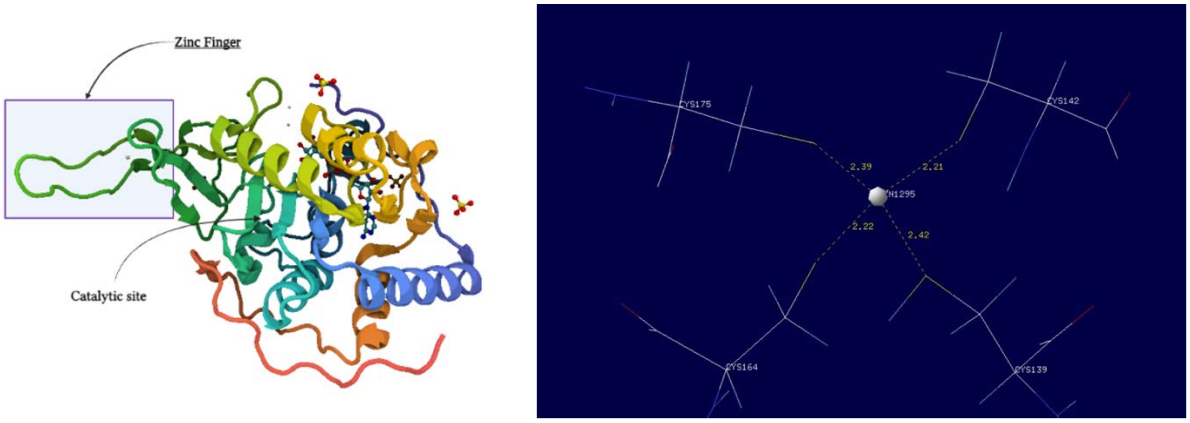

Figure 4 Left. Depiction of the SIRT6 protein. The zinc-finger is highlighted in blue. *Right.* Molecular depiction of the cysteine residues interacting with the zinc-finger.

In the novel study presented in this paper, a single cysteine residue was replaced with an arginine residue in order to attempt to remove the interactions between the sulfur atom in the cysteine residue and the Zn^{2+} atom. Additionally, arginine was selected due to it containing a positive charge at neutral pH. It was hypothesized that this positively charged residue may affect the interaction between the molecules of the zinc-finger, potentially affecting the overall proteins catalytic abilities. It is important to note that only a single residue, out of the four possible cysteine residues, were mutated at a time, thereby leaving the other three intact, in order to interact normally with the zinc molecule. Depicted below is the cysteine residue as well as the arginine residue, in order to display the differences in the side chain of the residues that were exchanged as displayed in Figure 5 below.

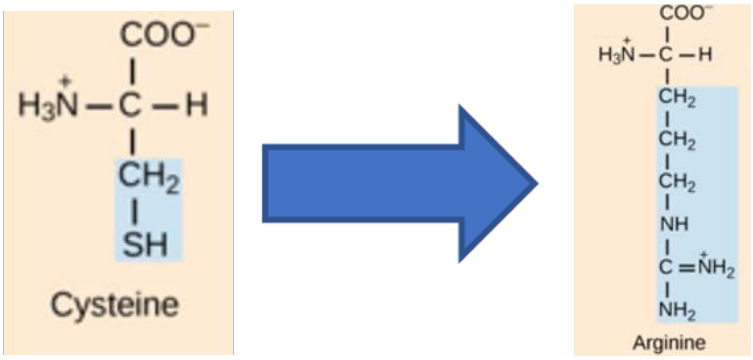

Figure 5. Structural representation of cysteine and the arginine residue that was used for the mutation.

Upon further analysis an additional mutagenesis exchanged the cysteine residue for tyrosine, a larger aromatic amino acid, that has been found in various forms of thyroid cancer and the mutation may affect its native folding (Mutation Overview Page SIRT6, 2021*)*. This specific mutation in SIRT6 has been connected to thyroid cancer and was therefore of significant interest to understand its effect on a molecular level (Qu, 2017). A third mutation of interest was the removal of the thiol group from the cysteine residue, which is understood to have the strongest electrostatic interactions with the zinc molecule. This mutation was accomplished by substituting a cysteine residue for an alanine residue. As displayed in Figure 6 below these two amino acids are identical excluding the -SH side chain present on the cysteine residue. It has been hypothesized that various electrostatic interactions between the thiol and the zinc hold the zinc in place. This mutation was of interest, to understand if the absence of a single thiol group may affect the overall enzymatic function of the protein.

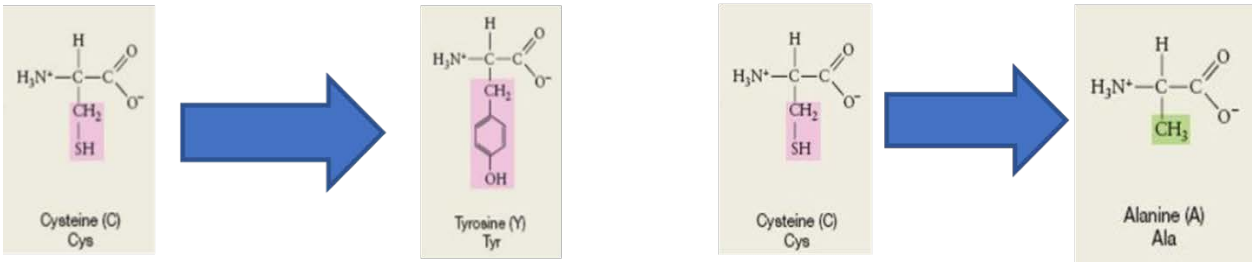

Figure 6. Structural representation of cysteine and the tyrosine and alanine residues that was used for the mutations.

Introduction to Sirtuin Research

Throughout the summer of 2021, I worked in The Longevity Laboratory at Bar Ilan university under the guidance of Professor Haim Cohen on a molecular project in attempts to understand this crucial connection in the field of longevity. The research discussion that I will now present on the molecular manipulation of the Sirtuin 6 protein was work that I did in this lab in collaboration with Michael Akhavan. I conducted thermal assays, as well as enzymic activity

assays, on sirtuin proteins with various molecular mutations and manipulations. The purpose of this study was to further characterize the zinc-finger of SIRT6 within the broader picture of longevity.

Research Discussion of the Molecular Components of Aging

To explore the allosteric effects of the SIRT6 zinc-finger, mutagenesis was used in order to exchange one of the cysteine residues for an arginine residue. Mutant protein samples were obtained from BL21 Transformed E. Coli, with a mutation of the cysteine residue of the zinc-finger. After protein isolation and purification, multiple assays were performed to gain insight to the stability and enzymatic activity of the mutant proteins. This would allow for an understanding as to whether this mutation, and thereby the zinc-finger are essential to the overall protein. This research can have further downstream and broader implication due to the metabolic impact of this protein on the process of aging. By further characterizing the protein, it can allow for insight as to how to maximize the benefits of the SIRT6 protein, while reducing any deleterious effects of it.

Thermal Analysis Assay

A thermal analysis was conducted to determine the overall stability of both the wildtype and mutant proteins. This was accomplished by incubating the samples at varying temperatures and then running both the soluble protein present in the supernatant and the aggregated protein levels present in the pellet of the samples, after which the membrane was then imaged for analysis.

As depicted in Figure 7 below, the thermal analysis displayed that the wildtype protein was significantly more stable than the mutant, as it only began to degrade at 42˚C, which is the expected temperature for protein degradation. In contrast, the mutant C→R protein of SIRT6 had

a much more significant protein degradation than the wildtypes depicted in the gradient of the soluble protein on the right side of Figure 7. In addition to having less soluble protein present, the mutant samples also appeared to have significantly more protein aggregate present than the control, even at temperatures that the protein would normally be expected to be stable at, such as 37˚C. These results indicate the important role of the cysteine residue in the native protein folding, as it may have inhibited proper secondary structures from forming, thereby decreasing the overall thermostability of the protein.

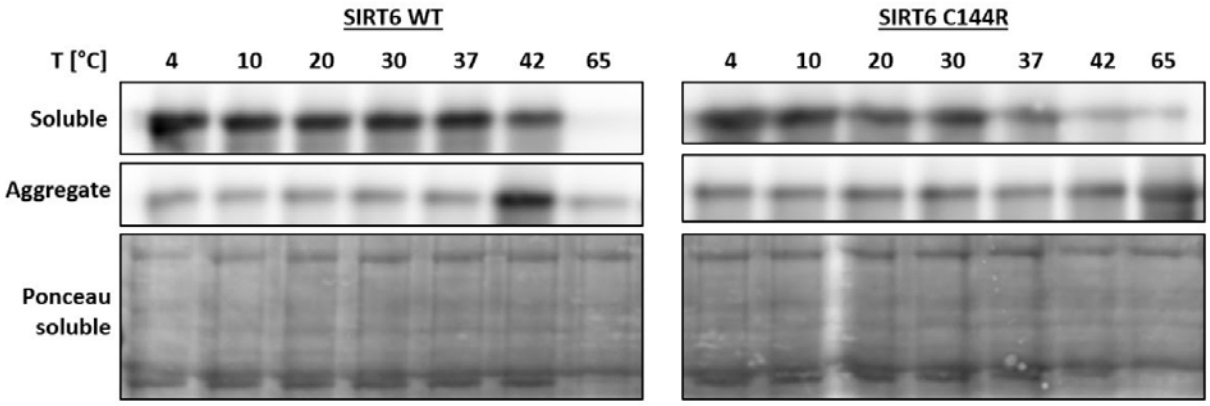

Figure 7. Western Blot imaging of the thermal analysis of SIRT6 compared to that of the mutant.

In addition to the thermal stability assay giving insight into the overall stability of the mutant protein, it also allows for the determination of the most suitable temperature for all further analysis. Based on the assay it was determined that the mutant protein was most stable at 30˚C and all further experimentation was conducted at this temperature due to it being the highest, and thereby most realistic and feasible temperature to work at that still had protein in the native, non- aggregated form.

Activity Assay

An activity assay was then conducted upon successful quantification of the mutant protein. This assay was significant to determine whether the enzymatic activity was affected by

the point mutation. The activity assay was conducted at 30˚C due to the stability of the mutant protein based on the previous thermal analysis. As SIRT6 acts as a histone deacetylase, the acetylation of H3 K9a, a critical histone that contributes to chromatin structure, was monitored in the presence of SIRT6 as well as NAD+. The NAD+ was included in the sample as well, due to SIRT6, like many other aging related proteins being regulated by the coenzyme. H4, which has not been shown to be affected by SIRT6, was used as a negative control to ensure that the enzymatic activity was specific. Additionally, it allowed for the further confirmation that an equal amount of the enzyme and substrate were placed in each sample. Upon analysis, compared to the wildtype, which had increasing deacetylation activity over time, the mutant protein appeared to have almost no catalytic activity at the site of the histone as depicted below in Figure 8. The gradation of the bands present at the site of H3 acK9 indicates the successful deacetylation of H3 via the SIRT6 wildtype over time. Conversely, the C→R SIRT6 mutant contained a strong dark band at all time increments for both the H3 and H4 histone substrates. This indicates that the mutant protein did not catalyze the deacetylation of the H3, thereby potentially indicating the inactivation of the catalytic abilities of the protein due to the missense mutation.

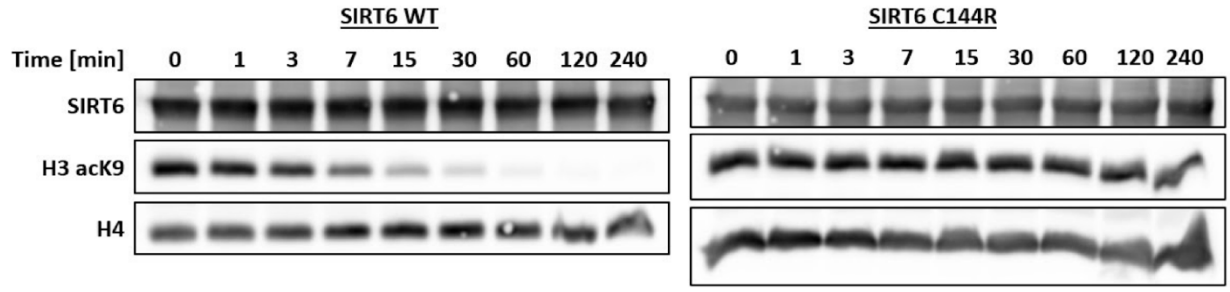

Figure 8. Analysis of the SIRT6 wild type compared to the SIRT6 mutation using Image J.

The results were then further analyzed using the ImageJ software, which allows for a relative quantification of the pixels present in the western blot images. The quantified bands

were then normalized based on previous protein concentration and displayed in graph in Figure 9 below. Upon analysis, compared to the wildtype there was almost no catalytic activity of the mutant SIRT6 protein. As displayed, the wildtype protein had close to 90% normalized activity as would be expected, while the C→R mutant had less than 5% activity even at 4 hours, which was determined as a more than sufficient duration of time for the catalytic abilities of the enzyme.

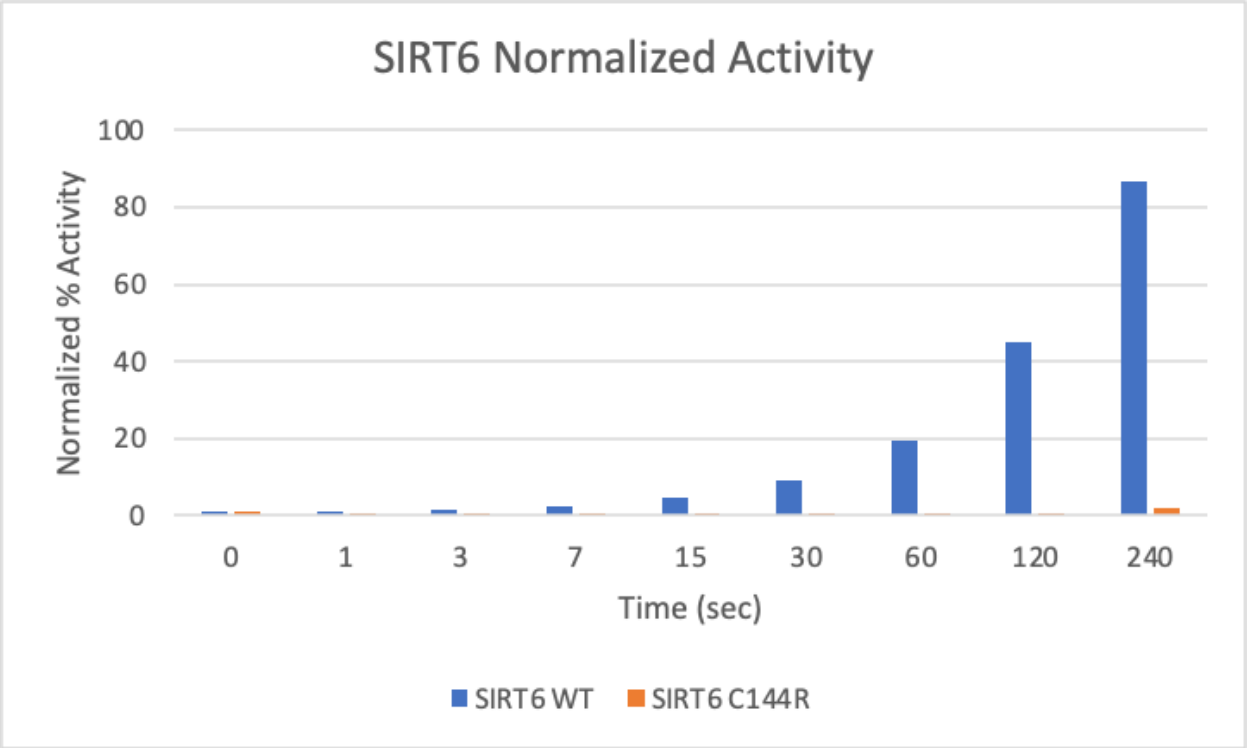

Figure 9. Graphical display of the normalized activity SIRT6 WT (blue) compared to the C—> R mutant over time.

Further Mutagenesis and Future Research

Additional mutagenesis was performed using primers obtained to mutate the cysteine residue into tyrosine and alanine residues. Obtained primers were then transformed into H2ka bacterial cells, using a heat shock technique. To ensure the successful presence of the transformation and SIRT6 insert, a western blot assay was performed on multiple colonies.

Restriction enzymes were used to test for the successful replication and transformation of the plasma inset which was present at approximately 1kb. As depicted in Figure 10 below all the selected colonies had the insert present at 1kb indicating successful transfections for all four colonies selected from the HEK 2ka colonies.

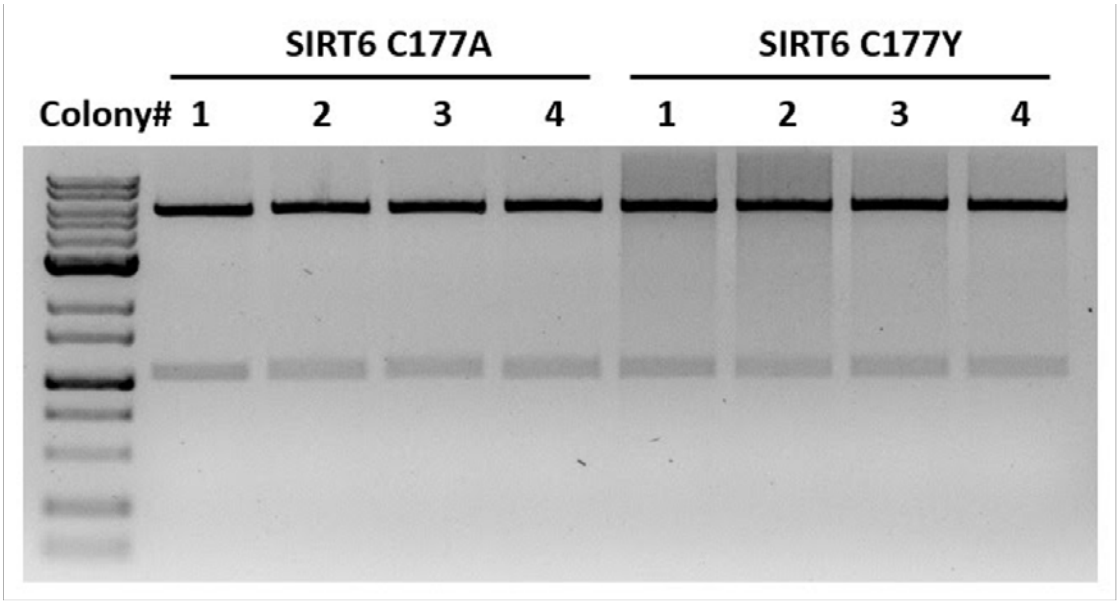

Figure 10. Top bands indicate the presence of the vector while the second row of bands at ~1kb indicate the presence of the plasma insert in both mutations in four different colonies.

Lastly, genomic sequencing was used to verify the successful mutagenesis. This would not only ensure that the plasmids were present in the isolated colonies but additionally ensured that it was present in both the correct location with the correct mutation present. The DNA sequencing was done to confirm that no other mutations were induced in the process, to make sure that the mutant protein was only different by the single residue of interest. Mini-Prep was used to obtain DNA samples from the mutagens. The Pet 28a. plasmid samples were then sent to a third-party laboratory for sequencing.

The sequencing results indicated that successful C→Y and C→A mutations were performed. Both the mutant colonies contained the desired point mutations that are indicated in Figure 11, thereby confirming successful mutagenesis.

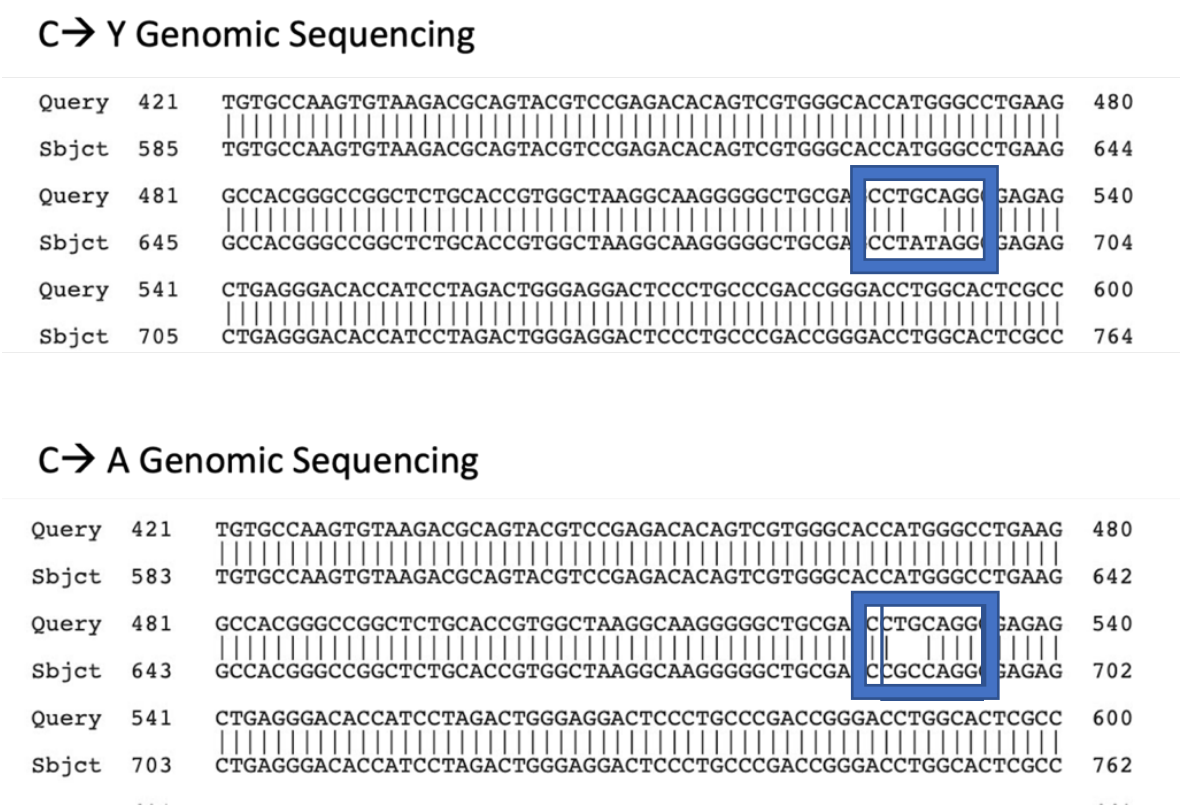

Figure 11. Genomic sequencing results of the mutagenesis from cysteine to tyrosine (above) and cysteine to alanine (below). The two-point mutations are highlighted above.

Further analysis of mutant proteins is necessary to obtain a more sufficient understanding of the role of the cysteine residues in the zinc-finger, as well as the overall role of the zinc-finger in the enzymatic activities of the sirtuin proteins. A thermal analysis, activity analysis as well x-ray crystallography of the mutant proteins can further the understanding of the role of the zinc-finger. The tyrosine missense is of interest due to its broader potentially therapeutic implications, due to its correlation with papillary thyroid cancer *in vivo.* This finding on the one hand is unusual, because upregulation of sirtuins has also been linked to various forms of cancers (Roth, 2014). While in contrast, functional loss of sirtuin integrity compromises the maintenance of genome integrity and DNA repair thereby leaving the cells more susceptible to tumorigenesis. This discrepancy further highlights the multifaceted implications of the sirtuin proteins. Both extreme overexpression as well as underexposing can have dramatic implications into an

organism's DNA stability further impacting the overall health and well-being of the organism (Qu, 2017).

In addition to further analysis of this mutation due to their potential therapeutic impact, another mutation of interest would be the replacement of a cysteine residue of the zinc-finger with a serine residue. In contrast to the tyrosine mutation, this mutation would potentially allow for a greater understanding of the structural components of the SIRT6 protein, by strengthening the role of the zinc-finger in the protein. These two residues as depicted in Figure 12 below have almost identical structures except for the substitution of the thiol group in the cysteine for a hydroxyl group in the serine.

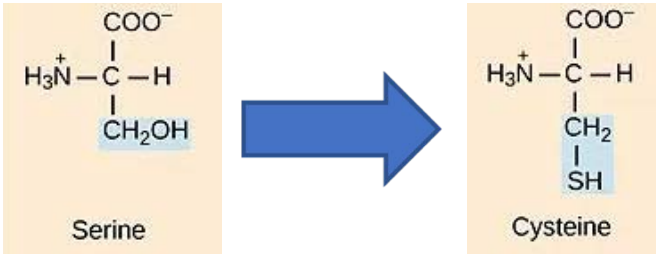

Figure 12. Structural representation of cysteine and the serine residue that can be used for future research.

It is hypothesized that this mutation may not have as drastic an impact on the protein as the other mutations if the interaction of the radius is due to electrostatic interactions. Like sulfur, oxygen is also a significantly electronegative species and therefore may be able to successfully interact with the positively charged zinc-molecule. If the enzymatic activity of the mutant protein is not significantly affected by the replacement for the serine residue this may allow for a greater understanding of the residues of the zinc-finger both for the SIRT6 protein as well as all other proteins that contain this structural motif. The effects of understanding the overall structure and enzymatic function of the protein are twofold. Firstly, as this protein has previously been proven to have a dramatic impact on the lifespan, albeit in mouse models, understanding the structure and function may give impact to potential therapeutic abilities in situations when this protein

may be mutated and have deleterious effects. Secondly, as overexpression of this protein has been linked to both an increase in lifespan and healthspan as well as in some cases cancer, understanding how to best manipulate the protein to maximize its beneficial effects while minimizing its detrimental effects will have broad and long-term downstream implications.

Conclusion of Molecular Analysis of Aging

Based on these results, it is evident that the zinc-finger has a significant effect on the catalysis effect on the SIRT6 molecule. The cysteine residue that was replaced with an arginine residue appeared to have an impact on both the protein's stability as well as activity. Mutagenesis of the cysteine residues that interact with the zinc-finger showed significant importance in the catalytic and folding abilities of the protein, despite the residues significant distance from the catalytic site. One potential justification for these results may be due to the zinc-finger playing a critical role in the binding and stabilization of the protein to the DNA substrate. Other possible effects of this single residue substitutions impact on the enzymatic activity could be due to a disruption of the precise, accurate and efficient folding of the protein into its native structure, either due to electrostatic repulsion due to the positively charged arginine or due to steric hindrance. Further characterization is necessary in order to obtain a complete picture of the effect of the zinc-finger structural motif on the SIRT6 protein.

Further analysis of SIRT6 and its structural motifs is imperative in the understanding of SIRT6 as well as its connection to both lifespan and healthspan. Successful mutagenesis of the C→Y and C→A mutants can allow for further study of its impact on protein stability and activity. Furthermore, as these mutations within SIRT6 have been strongly connected to cancer, further investigation into this mutation can provide insight into potential therapeutic avenues in the future.

CONCLUSION

There are many aspects that effect both lifespan and healthspan, that can potentially contribute to why a select few in a population are able to surpass the typical age boundaries of a population. Throughout history, humanity has attempted to determine some of these links or factors that may contribute to an increase in lifespan. Interestingly, while lifespan has undergone a dramatic increase within the past few centuries, the scientific community has only begun to methodically analyze factors that can contribute to this increase in lifespan.

Both biological and environmental aspects appear to play a critical role in both the health and lifespan of a person. Biological factors such as genetic markers, and overexpression of NAD+ dependent deacetylases are correlated with an increase in lifespan. Additionally, physical factors such as the degradation of brain matter have been correlated to directly impact cognitive abilities as one ages.

Analysis of molecular factors that contribute to aging can allow for a greater understanding of the intersection between environmental and genetic factors that affect lifespan and healthspan. Further research is necessary in order to strengthen the interconnection between the two and determine further biomarkers of longevity. This can allow for potential manipulation of these various biomarkers and molecular products, thereby allowing for the potential to lengthen both the lifespan and healthspan of a population. Regardless of whether aging is seen as a disease, that can otherwise have no upper limit, or if there is a genetic predisposition to the natural process of aging, it is evident that manipulation of these factors can have potential therapeutic downstream affects.

Furthermore, through the analysis of the health and family history of centenarians, as well as their daily activities, more can be understood as to what may allow for these outliers of

society to age so uniquely. By analyzing this along with their genetic biomarkers, further conclusions can be drawn as to what may allow for some individuals to live to such a old age while maintaining their overall health.

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It should be noted that any references to conversations with centenarians have had details changed in order to preserve the privacy of the participants. Furthermore, rather than specific individual profiles, unless previously publicly published, many anecdotal cases discussed are composites of multiple stories to ensure maximal confidentiality. Lastly all names and

identifiable information throughout this paper have been changed in order to preserve the identity of the participants and have been noted by the indication of an asterisk.

Additionally, minute details of the molecular research have been omitted in order to conserve the privacy of the work that is still undergoing further research and analysis.

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