Studies of Changes in Histone Posttranslational Modifications as Possible Markers of Aging

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Bina Rosenblatt

Mentor: Dr. Vigodner, Dept. of Biology

Table of Contents

I. ABSTRACT:

Longevity research has experienced a remarkable surge in recent decades, prompting an unprecedented discourse on the process of aging and the potential for scientific intervention to ameliorate age-related diseases. A key issue of this field is the distinction between lifespan, representing the duration of one's life, and healthspan, which represents the length of time spent in a healthy state. Historically, individuals in better states of health generally experienced extended life expectancies in comparison to people who were less healthy. However, in recent times, a divergence has emerged that has resulted in an increase in global lifespan without the corresponding increase in healthspan. Multiple elements have contributed to this disparity including the advancement of science and medical interventions as well as improved public health, living standards and education. The field of longevity aims to overcome this discrepancy by addressing the multifaceted elements that influence healthy aging. Using the knowledge of how molecular and cellular changes impact aging, interventions can be implemented to combat age related diseases, thereby lengthening healthy lifespan and ultimately improving the quality of life for individuals and society.

The objective of this paper is to explore the molecular effects of aging. It is focused on the implications of the enzyme sirtuin 6, SIRT6, one of the seven mammalian sirtuins that acts as an NAD+ dependent protein deacetylase. This enzyme has previously proved to play a vital role in controlling cellular aging and metabolism. Using SIRT6 deficient mice as a model, the research aims to examine if the modifications of lamin proteins, major architectural proteins of the nucleus, are correlated with signs of aging. Furthermore, the research attempts to analyze the methylation patterns of histone H3K9me3 since the changes in the localization may indicate changes in lamin expression and could potentially be associated with aging processes.

II. INTRODUCTION

1. Changes in Human Longevity Over the Years:

Humans have the longest lifespan of all primates. In recent times, the human life expectancy has dramatically increased, and continues to increase at a rapid rate (as seen in figure 1). In fact, since the 1800s, the human lifespan has doubled (Finch, 2009). The reasons for this are multifaceted and are a result of the advancements of science, modern medicine and the improvements of environmental factors. Modern medicine has had a profound impact on life expectancy. The implementation of vaccines, antibiotics and surgery have played a major role in the protection against diseases, which previously had been a major cause of premature mortality. In addition, other factors such as improved public health, living standards, lifestyle and education have played a large role in the increased life expectancy (Rodrigues, *et al*, 2020).

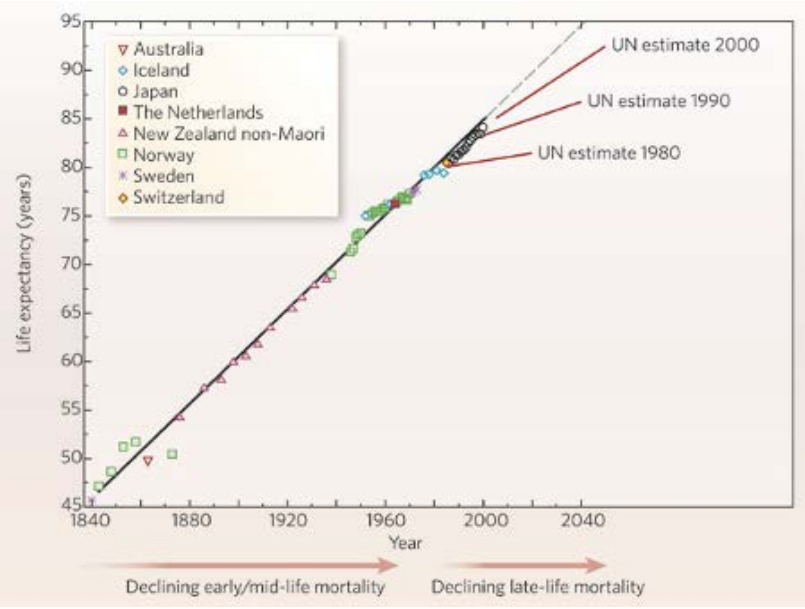

Figure 1. The dramatic increase in life expectancy that has increased steadily for nearly 200 years https://www.nature.com/scitable/content/life-expectancy-around-the-world-has-increased-19786/

Vaccines have aided in preventing the spread of many infectious diseases, and have significantly decreased illnesses that were once common, such as polio, measles and smallpox (Greenwood, 2014). Antibiotics treat and prevent the further spread of bacterial infections. As a result of antibiotics, conditions that were once deadly such as strep throat, urinary tract infections and pneumonia can now be easily treated (Doron, et al, 2008). Surgery has also dramatically improved in recent times; procedures including organ transplants are now more common and less invasive. Cancer treatments, such as chemotherapy and radiation therapy in addition to early detection screening have significantly improved the survival rates of many types of cancers.

In addition to the improvements in medicine, other factors such as the improved living standards have also drastically increased life expectancy. Public health measures including regulations in sanitation, pure water and nutrition mandates have both improved health and prevented the onset of infectious diseases. Superior access to healthcare and enhanced education systems have also increased life expectancy. Teaching and spreading knowledge regarding health and fitness has enabled this generation to gain a better understanding of the influence that lifestyle choices have on health (Reimers *et al*, 2012). This includes the implementation of healthier diets, increased exercise, decrease in smoking and the use of harmful chemicals.

Research using twin studies has indicated that around 20-30% of a person's lifespan is related to genetics while the rest is influenced by an individual's behaviors and environmental factors. That notwithstanding, the studies also determined that regarding longevity, after the age of around 80, almost everything in advanced aging is a factor of genetics rather than environmental and lifestyle matters (Disabled World, 2022).

While the recent extension of life expectancy is a major advancement for society, longevity alone is not sufficient. We are confronted by the issue that the rise in life expectancy is

not correlated by a proportionate increase in the quality of life in elderly people. Rather, it is largely associated with a surge in the risk of disease, disability, dementia and progressive aging (Brown, 2014) as seen in figure 2. “Lifespan” describes the total period of time a person lives, solely representing the quantity. In contrast, “healthspan” constitutes both the quantity and quality of a person’s life, representing the period a person lives free from disease. It is critical for lifespan increase to be paralleled by an increase in healthspan in order to improve the quality of life for the growing population of older individuals, and society as a whole (Garmany *et al*, 2021).

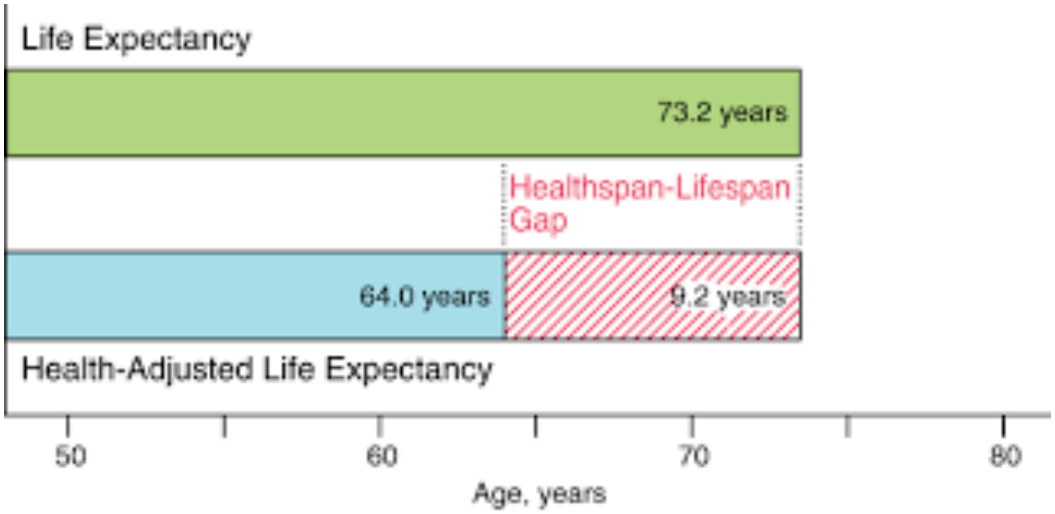

Figure 2. Comparing lifespan vs. healthspan demonstrates a 9.2 year gap
https://www.nature.com/articles/s41536-021-00169-5

When considering both elements of lifespan and healthspan, it is important to recognize the distinctions in the goals of each type of medical research. Research in lifespan prioritizes the reduction of mortality, thereby increasing life expectancy. In contrast, research in healthspan, aims to reduce aging in addition to combating age related diseases, disability and other detrimental components of aging (Brown, 2015).

Longevity research incorporates a large variety of disciplines including genetics and biochemistry and it uses many techniques to explore aging including animal models, cell cultures as well as clinical trials. The goal of research in longevity is to ultimately seek a larger understanding of the biological mechanisms behind aging. With the knowledge of how molecular and cellular changes affect aging, interventions can be implemented to combat age related diseases, thereby lengthening healthy lifespan and ultimately improving the quality of life for individuals and society.

2. Cell Nucleus and Aging:

Cellular aging is a natural and inevitable process that takes place in cells as they divide over time. As the cells age, they experience a progressive decline and reduced cellular function in addition to increased susceptibility to age-related diseases. The eukaryotic nucleus is the epicenter of most cellular processes. The nuclear envelope is the dynamic structure that plays a role in nuclear organization, chromatin arrangement, DNA replication, regulation of gene expression as well as signal transduction. Many proteins interact with the nuclear envelope in association with these important functions. Additionally, inside the nucleus, DNA in conjunction with both histone and non-histone proteins are condensed into chromatin and are allocated as either heterochromatin or euchromatin.

The structure of the nucleus is critical to the proper functioning of the cell. Stress and aging can drastically alter the nuclear composition. Every organism is exposed to many stresses, ranging from environmental stresses (which can include temperature, pathogens and nutrients) to internal stresses (including mutations). These factors can lead to the epigenetic changes in

histone modifications, which ultimately regulates the patterns of gene expression (Romero-Bueno, 2019).

The effects of aging can be visualized through the deterioration of the cell nucleus. In young cells, the nucleus is typically rigid and round, containing a defined membrane. The chromatin is generally uncondensed and spread uniformly throughout the nucleus. In contrast, old nuclei appear smaller, disorganized and unstructured. They possess a greater amount of DNA damage, and less heterochromatin.

As seen in figure 2, the young cell on the left has a structured and rigid nucleus in comparison to the older nucleus which is unstructured and irregularly shaped. The nucleolus in the young nucleus remains intact whereas the nucleolus in the old nucleus has fallen apart. The nuclear membrane in the old nucleus has lost its organized structure, and there is a lot more DNA damage and a reduction of heterochromatin in the old nucleus in comparison to the young nucleus (Madhavi *et al*, 2022).

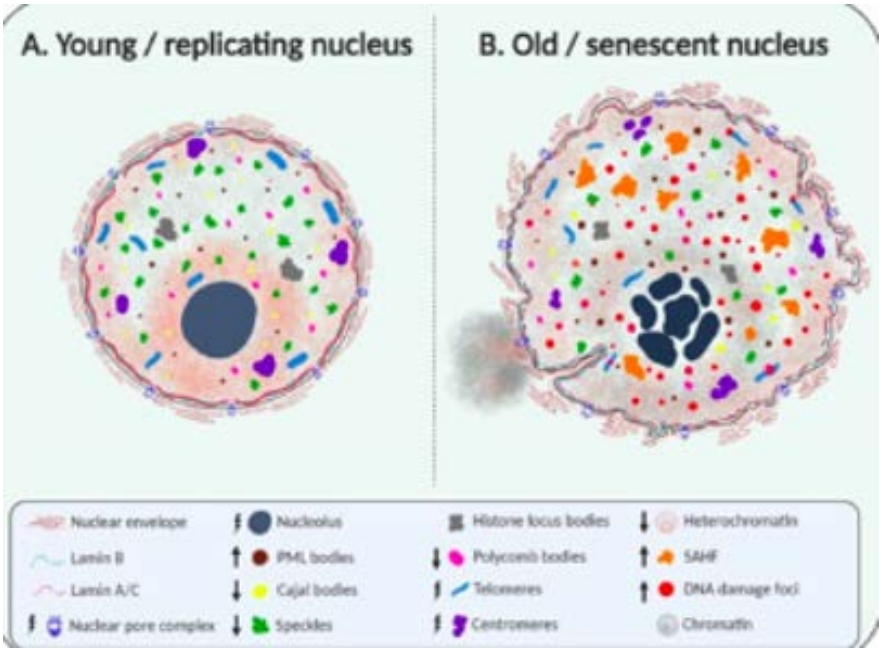

Figure 2: A representation of a young vs. old nucleus
https://www.sciencedirect.com/science/article/abs/pii/S1568163721000118

3. Lamins:

Some of the nuclear membrane proteins that can be seen in figure 2 are the lamin A/C and B proteins denoted by the red and blue lines around the membrane. Lamins are a family of intermediate filaments that are critical architectural proteins in the animal cell nucleus. The inner nuclear envelope is lined by a mesh-like network of lamin proteins that provide a foundation for the binding of proteins and chromatin, creating mechanical stability (as seen in figure 3).

Lamins are involved in a large range of nuclear functions. They are responsible for the nuclear membrane structure and they interact with the lamin-associated domains (LAD) of the chromatin. They also play a role in regulating gene expression, genome organization, chromatin regulation, DNA replication and DNA repair (Misteli, *et al*, 2011). Defects in the expression of lamins can cause premature aging and increase the predisposition for aging related diseases.

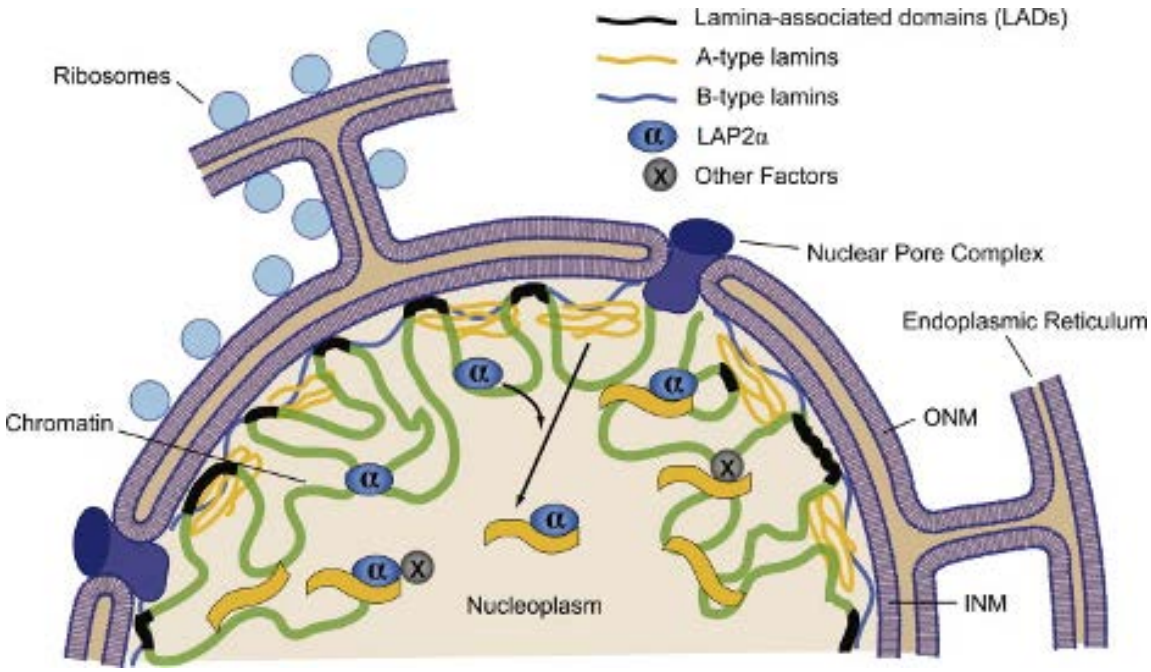

Figure 3. Lamins and their interactions with the nuclear membrane structure. https://www.sciencedirect.com/science/article/pii/S1084952113001389

Lamin proteins have also been shown to impact cellular senescence, a process where the cell enters a condition of permanent growth arrest, ceasing to divide, yet not dying[10]. Lamin proteins have been shown to control the expression of genes involved with senescence associated secretory phenotype (SASP), a process whereby senescent cells emit inflammatory factors involved in tissue damage and age-related diseases (NIH, 2023). Senescent cells possess unique morphological changes and may acquire changes in posttranslational modifications on lamins that eventually can result in age-related diseases.

4. Age Accelerated Diseases:

Progeria, also known as Hutchinson-Gilford Progeria syndrome (HGPS), is a rare and fatal disease that produces symptoms of premature aging in children. This disease is the result of a mutation in LMNA, the gene that encodes lamin A/C, which are alternatively spliced isoforms (Freund *et al*, 2012). Normally the LMNA creates the prelamin A protein that is then further processed; however, in individuals with HGPS, there is a disruption in the prelamin A processing that leads to a form of protein called progerin. This toxic form of the protein cannot be incorporated suitably into the nuclear lamina (Noda *et al*, 2015). The vital nuclear structure and function therefore become disrupted (as seen in figure 4), inducing premature aging. Children with HGPS generally appear normal at birth, yet later display growth retardation prior to the age of two. Other symptoms include hair loss, lipodystrophy, sclerodermatous skin, osteolysis, and progressive atherosclerosis. The condition manifests in death at the average age of 13, usually as a result of myocardial infarctions and strokes (Taimen *et al*, 2009).

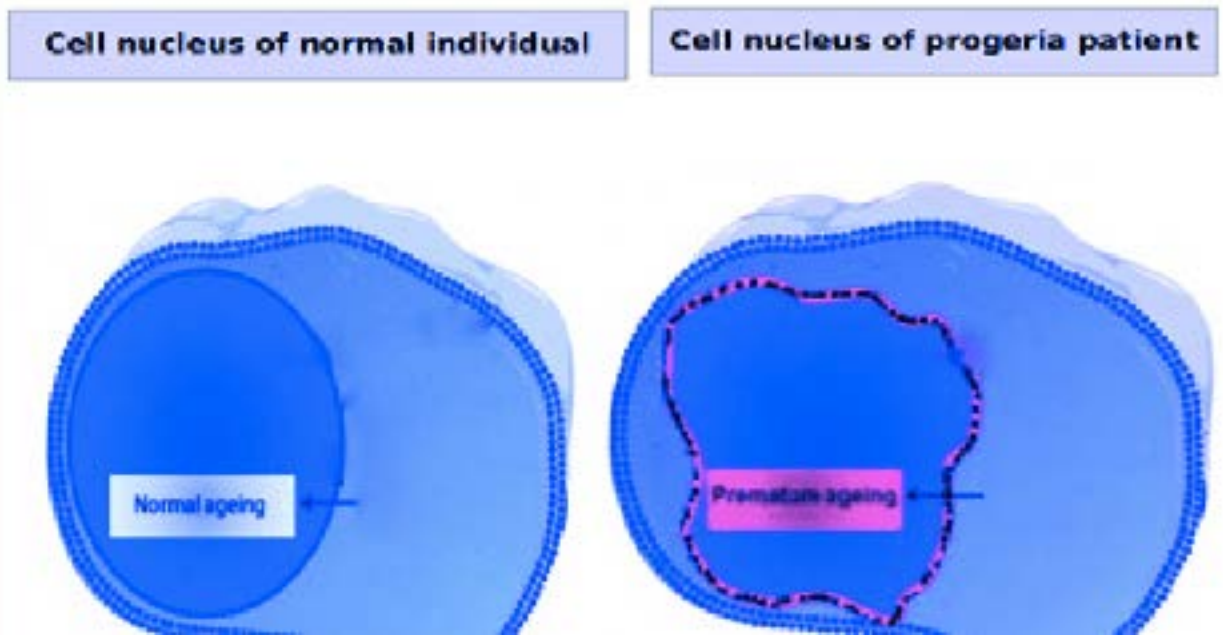

Figure 4. Healthy vs. Progeria cell nucleus
https://www.researchgate.net/figure/Healthy-and-Progeria-Cell-Nucleus_fig1_323695640

Alzheimer's and Parkinson's diseases are both neurodegenerative disorders that are associated with abnormal function of lamins. Lamin B dysfunction has been associated with mediating neuronal death and eventually leading to Alzheimer's. Werner syndrome, also called adult progeria, is another age related disease that increases the susceptibility of aging since it disrupts critical cellular functions including DNA replication and repair. This disease may be caused by multiple factors, including genetic mutations (specifically in the WRN gene), environmental factors as well as other lifestyle elements, including diet and exercise. Some symptoms of this disease include short height, thinning and graying hair, unusual facial features and thin arms and legs (NIH, 2023).

Gaining an understanding of the molecular mechanisms of cellular aging is crucial for understanding age-related pathologies. Such an understanding can serve as a valuable foundation to facilitate the development of novel therapeutic interventions, aimed at enhancing the longevity and vitality of cells, thereby improving the overall health of society.

5. Histones and Post Translational Modifications on Histones (Histone Code):

Histones are a family of small, basic proteins found within the nucleus of eukaryotic cells. They are vital in providing structural support for chromosomes. Histones aid in organizing and packaging DNA inside the nucleus and also aid in gene expression in addition to other nuclear functions. They accomplish this by binding to DNA, which provides the chromosomes with its shape, controlling the gene activity (NIH, 2023).

After proteins are synthesized, different groups and peptides can be added to them. These modifications are called post-translational modifications, also referred to as “PTMs”. PTMs on histones can ultimately change the structure of the chromatin that is associated with the histones, thereby regulating and influencing the transcriptional activity of associated genes.

Various post-translational modifications on histones include acetylation, methylation, phosphorylation, sumoylation as well as ubiquitination (Bowman *et al*, 2015). Acetylation is a chemical reaction that involves the addition of an acetyl group onto lysine residues on the histones. This neutralizes the positive charge of the lysine residue which ultimately activates gene expression by loosening the DNA and enabling easier access of transcription factors to the gene promoter region. Methylation is another chemical modification on lysine or arginine residues that, depending on the residue, has the ability to either activate or repress gene expression. Phosphorylation involves the covalent addition of a phosphate group onto the serine, threonine or tyrosine residues of the histone. Such modifications can either repress or activate genes (Gujral *et al*, 2020). Sumoylation and ubiquitination on histones involves the covalent attachment of a small ubiquitin-like modifier (also known as a SUMO protein) to the lysine

residues of the protein, changing the chromatin structure as well as gene expression (Madhavi, 2022).

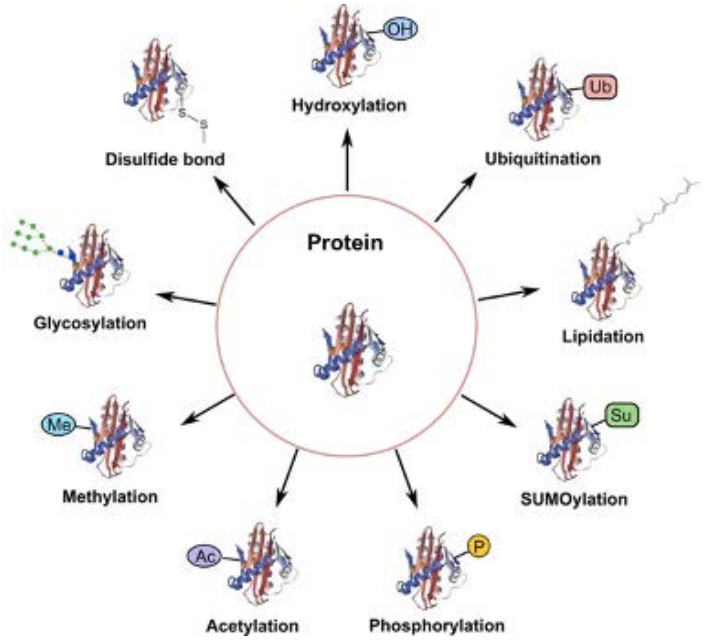

Figure 5. Post-translational modifications of proteins (although for this research, it is relevant specifically for histones).
https://www.creative-proteomics.com/blog/index.php/strategies-for-post-translational-modifications-ptms/

6. Aging and Posttranslational Modifications on Histones:

The deterioration of the cell nucleus is an indicator that aging has occurred. Changes in the chromatin structure, which is influenced by post-translational modifications on histones, is associated with aging. For example, studies have shown that levels of histone acetylation on lysine residues of histones H3, H4 and H2B decrease as a person ages. This causes chromatin structure to be more compact, and less accessible, thereby decreasing gene expression and causing age-related diseases including cancer and neurodegeneration (Santos, 2018).

Changes in methylation and phosphorylation are also associated with age-related diseases. For example, changes in the histone phosphorylation have been associated with the development of cancer. Specifically, the phosphorylation of histone H3 at serine 10 has been implicated in the poor prognosis in several cancers such as melanoma, breast, gastric, and lung cancer (Komar, 2020). Post-translational modifications on histones are essential for the maintenance of cellular function and structure; thus, defects in these important processes are implicated in many aging related problems.

III. SUMMER RESEARCH AT BAR ILAN

1. Role of SIRT 6 (deacetylase) in Posttranslational Modifications of Lamins and its Correlation with Aging:

In the summer of 2022, I researched in the lab of Dr. Haim Cohen at Bar Ilan University. Dr. Haim Cohen and his team are conducting research in the field of molecular biology of aging. The lab uses mice as a mammalian model for the experiments due to the similarities between the mouse and the human genome and the short lifespan in mice which allows for the studies of aging in controlled environments. The team is specifically focused on the implications of the enzyme sirtuin 6, SIRT6, one of the seven mammalian sirtuins that acts as an NAD+ dependent protein deacetylase. They have proved that SIRT6 plays a vital role in controlling cellular aging as well as metabolism through creating transgenic mice.

Specifically, an overexpression of SIRT6 in mice (SIRT6+) has been shown to increase mouse lifespan and decrease some effects of aging such as genomic instability, inflammation, cancer and cognitive decline. In addition, the SIRT6+ mice also experienced improved glucose

tolerance, younger hormone profiles, a decrease in age-related adipose inflammation and increased physical activity (Cohen *et al*, 2017). In contrast, SIRT6-deficient mice (SIRT6-) exhibit an accelerated aging phenotype that is manifested at around two weeks. SIRT6- mice have reduced body weight, increased glucose uptake and demonstrate an age-dependent deterioration in their retinal function. SIRT6- mice have a lifespan of less than a month (Peshti, 2017).

Previous analysis, executed by PhD student, Noga Touitou, found protein acetylation in the livers of SIRT6+ mice at four lysine residues on the lamin A/C protein. Specifically, K97, K114, K171, and K180 had statistically significant higher acetylation rates. While there has been research conducted on other lamin protein PTMs, there is limited published research on the effects of acetylation.

SIRT6, however, is primarily known to be associated with deacetylation, so this discovery presents the questions: why do SIRT6+ mice have increased lamin A/C acetylation? How does this affect the cell phenotype and what might be its connection to aging related disorders?

2. Goals of Research Projects:

The aim of the first project was to investigate the possible relationship between modifications on lamins and aging-related changes, using SIRT6- mice as a model. Specifically, the study focused on understanding if the modifications seen in the lamins of SIRT6- mice were correlated with signs of aging in the nucleus. To accomplish this, we used site directed mutagenesis to create the mutant isoforms of lamins where acetylation on the lysine (K) was either constant or totally removed.

Since acetylation on lamins was implicated in aging, another goal of the research in the lab was to test the effect of acetylation on the four specific lysine residues (K97, K114, K171, and K180) of lamins on cell activity and aging. As previously discussed, lamin A/C acetylation is associated with SIRT6 overexpression. To understand how lamin A/C acetylation might affect cell phenotype and aging, we mutated the lamin A/C protein in the four residues to be either constitutively acetylated or permanently deacetylated.

After mutating the lamin A protein, we planned to transfect lamin A deficient cells with these mutated lamin A proteins. We hoped to visualize the resulting phenotypes and find the exact location of protein modification. Ultimately, this knowledge would deepen our understanding of the relationship between lamin modifications and age-related changes in the nucleus.

We mimicked permanent acetylation by replacing the lysine with glutamine (Q), an amide-containing amino acid. We initiated deacetylation by replacing lysine with arginine (R), the other positively charged amino acid (seen in figure 6). We confirmed a successful mutagenesis with sanger sequencing and then transfected the cells with each of these plasmids containing a fluorescent marker to confirm successful transfection.

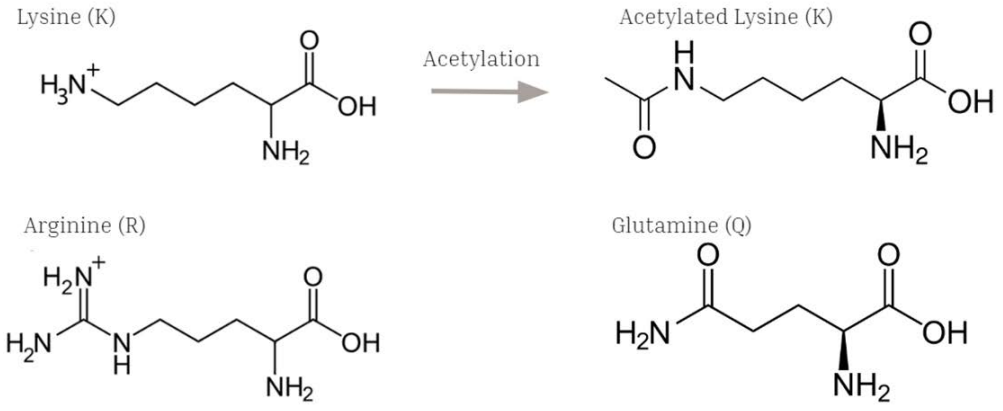

Figure 6. Lysine acetylation and replacement amino acids arginine and glutamine (courtesy of my lab partner, Yonit Krebs)

The second goal of the research was to analyze the pattern of methylation in Histone 3 (H3K9me3) at young, aged and SIRT6 cells since prior research had demonstrated a correlation between changes in H3K9me3 and aging in other tissues (Cohen, 2021). H3K9me3 is associated with the highly condensed sections of chromatin that make up the lamin-associated domains (LAD) (as seen in figure 7). Therefore, changes in the localization of this histone mark could be indicative of changes in lamin expression and could be potentially related to the aging process.

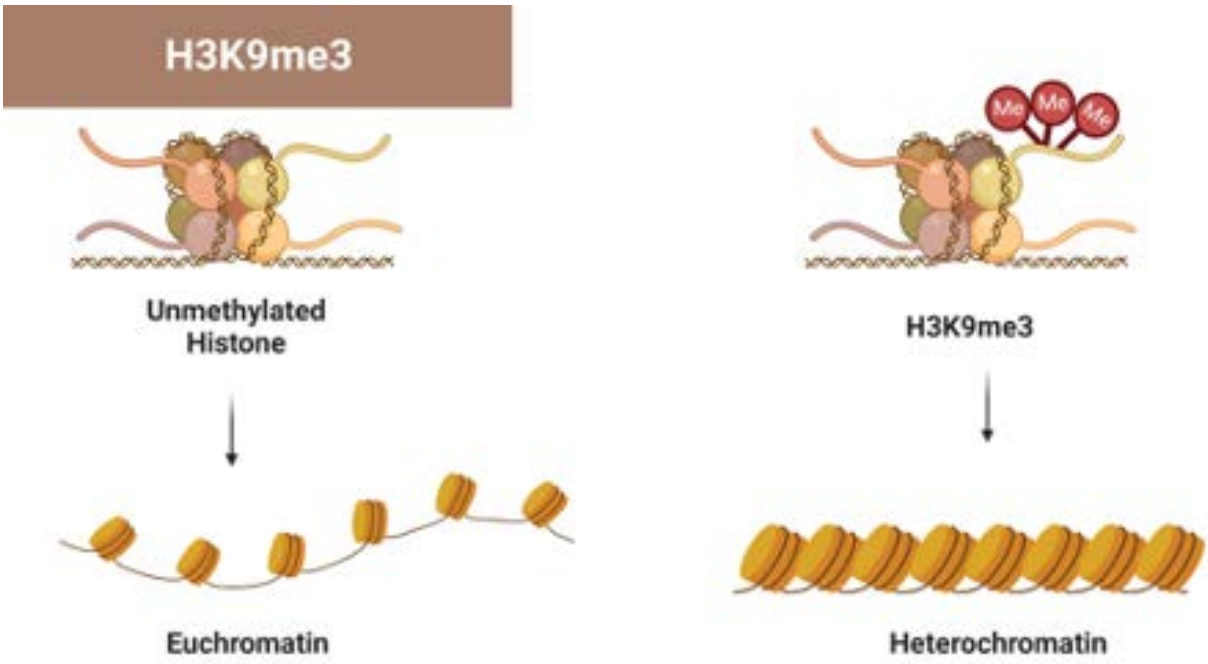

Figure 7. H3K9me3 and its association with heterochromatin, highly condensed sections of chromatin (courtesy of the Haim Cohen lab).

3. Methods:

We preserved liver tissues in paraformaldehyde (PFA) and then used paraffin blocking and the microtome apparatus to cut the tissue samples into 8 μm slices. We placed these slices

onto positively charged slides. The purpose of the slides' positive charge is to help the negatively charged tissue sample adhere to the slide. We then dissolved the paraffin using xylene and rehydrated the tissue sample with ethanol and water.

We calibrated an antigen retrieval method (TRIS buffer, 5 minutes) to break the covalent bonds between proteins formed during PFA fixation and retrieved the epitope. We then blocked the samples in Bovine Serum Albumin (BSA) with triton to prevent all nonspecific protein binding and permeabilize the nuclear membrane. We incubated the samples with the primary and then the secondary antibody (conjugated to a fluorophore, Alexa) and stained the DNA with Hoescht (361/497 nm) as well. We used the Leica Stellaris confocal microscope to view the results.

4. Results:

H3K9me3 shows a different distribution of nuclear staining in SIRT6- mice as compared to the control.

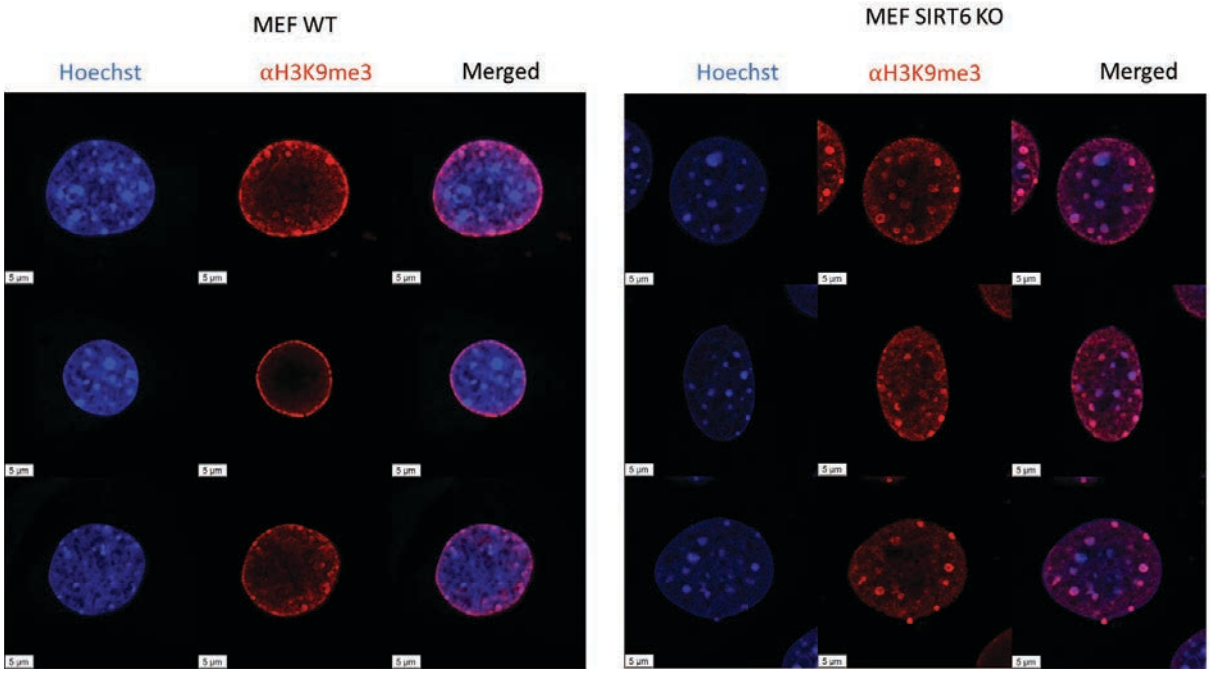

Figure 8. Relationship between H3K9me3 and SIRT6 (courtesy of *M.Sc. student Ron Nagar*)

The staining was less intense around the nuclear envelope while more foci were observed within the nucleus. However, no significant changes were observed in the localization of lamin acetylation (as seen in figure 8).

Our next experiments focused on more detailed analysis of H3K9me3 distribution in control and transgenic SIRT6- mice of different ages. We preserved livers from young and old wild-type mice as well as young and old transgenic mice.

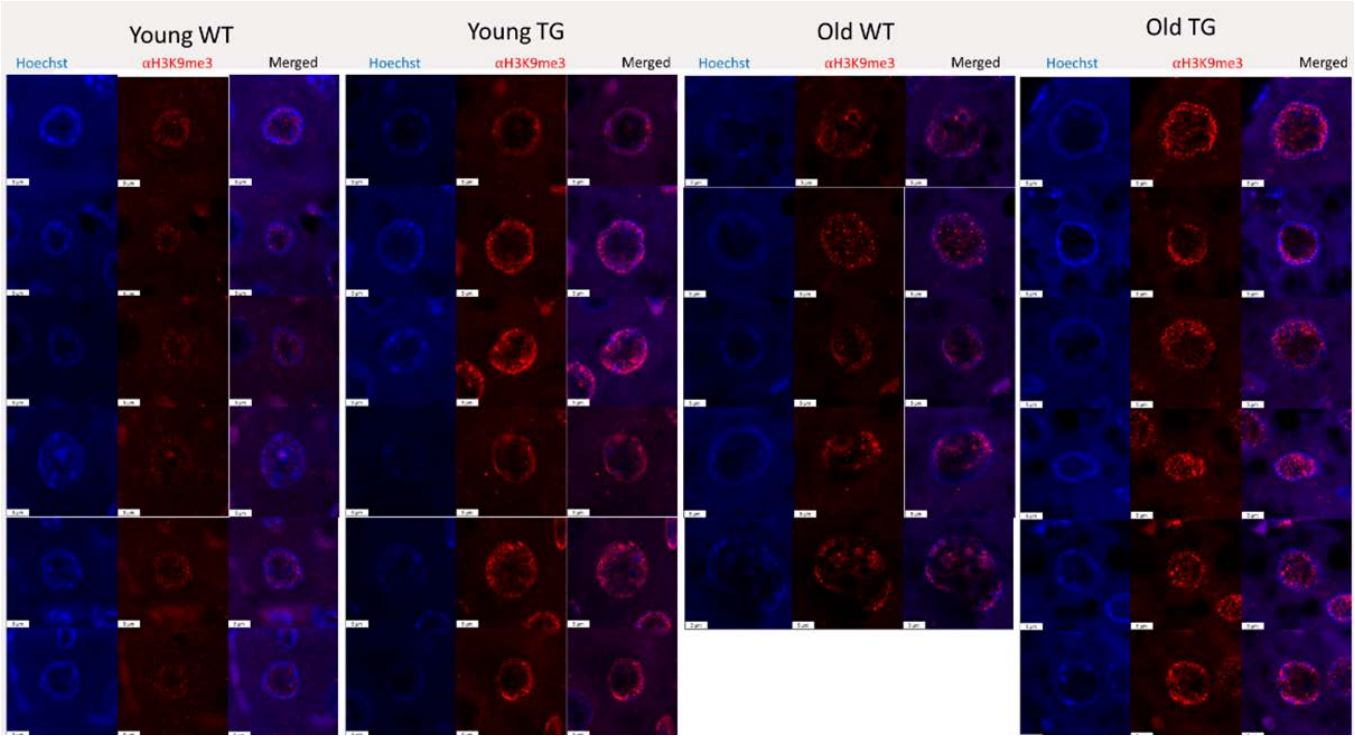

Figure 9. H3K9me3 immunofluorescence on liver tissue sample results. (courtesy of *M.Sc. student Ron Nagar*)

Figure 9 shows the results of this experiment. We compared four different groups of mice. Young wild type (WT) mice, old WT mice, young TG mice and old TG mice. Although,

due to the limitations of our project, we were not able to fully analyze the data and quantify the results seen in these images, we were able to observe promising phenotypic differences among the four groups. First, the nuclei in the old groups were larger and less structured than those in the young groups, an expected result of aging.

Second, the H3K9me3 is localized to the nuclear membrane in the young WT and TG groups, whereas the H3K9me3 in the old WT sample is more dispersed and looks somewhat similar to the SIRT6 KO phenotype. In contrast to the old WT cells, the old TG group seems to have preserved some of the nuclear membrane localization of the H3K9me3, indicating that SIRT6 might play a role in this process.

The transfection was successful as seen in figure 10. Unfortunately, due to a contamination in the laboratory, the results of this experiment were unable to be attained. However, studies are on the way to understand how acetylation on each residue might impact the cell phenotype.

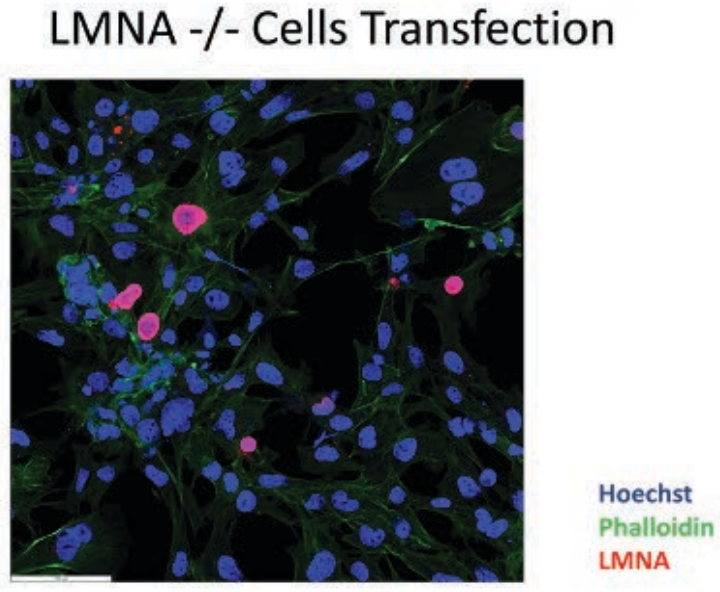

Figure 10. The transfection of LMNA knockout cells (courtesy of *M.Sc. student Ron Nagar*)

III. Conclusion:

The study of longevity has gained a lot of interest in recent times, giving rise to the discussion about the aging process and the ability of scientific intervention to mitigate age-related diseases. When studying longevity, it is crucial to understand the difference between lifespan and healthspan. Lifespan represents the duration of a person's life, whereas healthspan, represents the duration of one's life spent in a healthy state. Recently, the human life expectancy has dramatically increased and continues to increase at a rapid rate. Many elements have influenced this including the advancements of science, modern medicine, the improvements of environmental factors and public health initiatives.

Despite the recent expansion of life expectancy, we are faced with an issue that the rise in life expectancy is not correlated by a proportionate increase in the quality of life in elderly people. Although historically, healthier individuals typically experienced heightened lifespans in comparison to less healthy individuals, today, longer lifespans have been associated with a surge in the risk of age related diseases. It is crucial for lifespan increase to be paralleled by an increase in healthspan.

The study of longevity and healthspan ultimately serves to reduce aging by combating age related diseases and disabilities. The aim of research in longevity is to ultimately gain a better understanding of the biological mechanisms behind aging. By uncovering the intricacies behind how the molecular and cellular changes affect aging, interventions can be implemented to counter age related diseases, thereby increasing healthy lifespan.

The organization and composition of the nucleus play a vital role in ensuring that the cell functions properly. Stress and aging can dramatically alter the nuclear composition. These effects

can be seen through analyzing the cell nucleus. Young cells generally contain a rigid and round nucleus with a defined membrane. The chromatin is typically uncondensed and spread uniformly throughout the nucleus. In contrast, old nuclei appear smaller, disorganized and unstructured. They possess a greater amount of DNA damage, and less heterochromatin.

Post-translational modifications on histones are essential for the maintenance of cellular function and structure; thus, defects in these important processes are implicated in many aging related problems. Studies have shown that levels of histone acetylation on lysine residues of histones H3, H4 and H2B decrease as a person ages. This causes chromatin structure to be more compact, and less accessible, thereby disrupting the gene expression and causing age-related diseases including cancer and neurodegeneration.

The enzyme SIRT6 is known to play a major role in controlling cellular aging and metabolism. An overexpression of SIRT6 has proven to increase mouse lifespan and decrease some aging related health issues. The first project used SIRT6- mice as a model to explore if modifications seen in the lamins of SIRT6- mice were correlated with signs of aging in the nucleus. To execute this, we mutated the lamin A protein to be either acetylated or deacetylated at four different lysine residues. We aimed to find the exact location of protein modification. We mimicked permanent acetylation by replacing the lysine with glutamine and imitated deacetylation by replacing lysine with arginine. The next goal was to study the pattern of methylation in Histone 3 (H3K9me3) in different types of cells, young, aged and SIRT6 cells.

A body of evidence had been collected suggesting an association between changes in histone posttranslational modifications and aging. Our data supported this hypothesis. Ultimately, H3K9me3 showed a different distribution of nuclear staining in SIRT6- mice as compared to the control. The staining in the SIRT6- cells was slightly less intense around the

nuclear envelope with more foci within the nucleus. Despite these differences, no significant changes were seen in the localization of lamin acetylation. When comparing the four groups, young WT mice, old WT mice, young TG mice and old TG mice, we found significant phenotypic differences. The nuclei in the old groups were larger and less structured than those in the young groups, an expected result of aging.

Furthermore, the H3K9me3 was localized to the nuclear membrane in the young WT and TG groups, whereas the H3K9me3 in the old WT sample was more dispersed and looked similar to the SIRT6- phenotype. The old WT cells and the old TG group, seem to have preserved some of the nuclear membrane localization of the H3K9me3. This ultimately indicates that SIRT6 might play a role in this process.

The transfection was successful, however, due to a contamination in the laboratory, the results of this experiment were unable to be attained. Studies are continuing to try to uncover how acetylation on each residue might impact the cell phenotype.

IV. Acknowledgements:

The research discussed in this paper was conducted at Bar Ilan University in the lab of Dr. Haim Cohen under the guidance of M.Sc. student Ron Nagar. I would like to thank Dr. Haim Cohen for welcoming me and my lab partner so graciously into his lab. I would like to thank Ron Nagar for being incredibly patient, kind, and for taking so much time to teach us, making sure we understood the complex concepts and for always encouraging questions.

I would like to take this opportunity to express my sincere gratitude and appreciation to Dr. Vigodner for her invaluable guidance and mentorship throughout my senior project. I appreciate all of the time you put in to help me organize and construct this project.

I would also like to thank Dr. Wachtell for her outstanding leadership, sincere enthusiasm and guidance as the head of the Stern Honors Program. Thank you for the countless hours that you spent, ensuring that all of our programming was interesting and relevant.

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