

**INVESTIGATING THE EFFECTS OF LITHIUM
CHLORIDE TREATMENT AND
AGE-ACCUMULATED CIRCULAR RNA
DELETIONS ON *Caenorhabditis elegans*
LONGEVITY**

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN
MATERIALS SCIENCE AND NANOTECHNOLOGY

BY
UMUTCAN KAAAN BOZAN
DECEMBER 2025

INVESTIGATING THE EFFECTS OF LITHIUM CHLORIDE TREATMENT AND
AGE-ACCUMULATED CIRCULAR RNA DELETIONS ON *Caenorhabditis*
elegans LONGEVITY

By Umutcan Kaan Bozan
December 2025

We certify that we have read this thesis and that in our opinion it is fully adequate, in
scope and in quality, as a thesis for the degree of Master of Science.

Michelle Marie Adams (Advisor)

Ayça Arslan (Co-advisor)

Gürkan Yeşilöz

Bala Gür Dedeoğlu

Approved for the Graduate School of Engineering and Science:

Orhan Arıkan
Director of Graduate School

ABSTRACT

INVESTIGATING THE EFFECTS OF LITHIUM CHLORIDE TREATMENT AND AGE-ACCUMULATED CIRCULAR RNA DELETIONS ON *Caenorhabditis elegans* LONGEVITY

Umutcan Kaan Bozan

M. S. in MATERIALS SCIENCE AND NANOTECHNOLOGY

Advisor: Michelle Marie Adams

Co-Advisor: Ayça Arslan

December 2025

Aging is defined as the defects that occur over time in the tissues and cells of the organism. These defects constitute the primary risk for neurodegenerative and cardiovascular diseases, cancer, diabetes, and many other diseases. Even though some common hallmarks are defined in aging organisms such as genomic instability, loss of proteostasis, cellular senescence and more, the reason behind aging is not completely understood. The aging field, therefore, research how and why do organisms age, and tries to delay or possibly revert this process. This is mostly done on model organisms, and the roundworm *Caenorhabditis elegans* (*C. elegans*) is one of the best models for aging research due to its short life cycle.

In the first part of this study, the correlation between aging and lithium chloride treatment was aimed to be understood. This substance is commonly used as a drug in bipolar disorder, and an extension of lifespan upon lithium chloride treatment has been explored in multiple studies on different models. A pathway analysis was performed to determine what could be an unknown regulator of this process, and the worm ortholog of the human protein Rev-erb- α , NHR-85, was identified as a possible contributor. A loss-of-function mutation of this protein was found to extend lifespan in *C. elegans* when compared to its wild type, and lifespan was further extended by treatment with lithium chloride. A decay in brood size and locomotion functions was also observed upon treatment, but it is suggested that this may contribute to the

extension of lifespan. Furthermore, it is suggested that this extension in lifespan could be caused by a developmental delay.

In the second part of this study, it was aimed to understand whether some age-accumulated circular RNAs of *C. elegans* could be contributing to the aging process. Circular RNAs are covalently linked single-stranded RNA molecules joined at their ends, resulting in a loop shape. This covalent link is considered to contribute significantly to their stability, as most RNA-degrading enzymes require an open end to initiate degradation. Upon analysis of a circular RNA-specific RNA-seq study that sequenced RNA across different life stages until aging, four circular RNAs of two different genes were identified to accumulate extensively during aging. CRISPR/Cas9 was then utilized to specifically silence selected circular RNAs by targeting their circularization, and the effects of these deletions on *C. elegans* longevity were investigated.

Keywords: C. elegans, Aging, Longevity, Lithium, NRDR1, Rev-erb- α , Circular RNA, CRISPR/Cas9

ÖZET

LİTYUM Klorür ve DAİRESEL RNA DELESYONLARININ *Caenorhabditis elegans* YAŞAM ÖMRÜNE ETKİSİNİN ARAŞTIRILMASI

Umutcan Kaan Bozan

MALZEME BİLİMİ VE NANOTEKNOLOJİ, Y. L.

Tez Danışmanı: Michelle Marie Adams

İkinci Tez Danışmanı: Ayça Arslan

Aralık 2025

Yaşlanma, organizmanın doku ve hücrelerinde zamanla meydana gelen bozukluklar olarak tanımlanır. Bu bozukluklar, nörodejeneratif ve kardiyovasküler hastalıklar, kanser, diyabet ve diğer birçok hastalığın başlıca risk faktörlerini oluşturur. Genomik instabilite, proteostaz kaybı, hücresel yaşlanma ve daha fazlası gibi yaşlanan organizmalarda bazı ortak özellikler tanımlanmış olsa da yaşlanmanın ardındaki neden tam olarak anlaşılamamıştır. Bu nedenle yaşlanma bilimi, organizmaların nasıl ve neden yaşlandığını araştırır ve bu süreci geciktirmeye veya mümkünse tersine çevirmeye çalışır. Bu araştırmalar çoğunlukla model organizmalar üzerinde yapılır ve iplik kurdu *Caenorhabditis elegans* (*C. elegans*), kısa yaşam döngüsü nedeniyle yaşlanma araştırmaları için en ideal modellerden biridir.

Bu çalışmanın ilk bölümünde, yaşlanma ve lityum klorür tedavisi arasındaki ilişkinin anlaşılması amaçlanmıştır. Bu madde genellikle bipolar bozuklukta ilaç olarak kullanılır ve lityum klorür tedavisiyle ömür uzamasının farklı modeller üzerinde birçok çalışmada bildirilmiştir. Bu yolağın bilinmeyen bir düzenleyicisinin ne olabileceğini belirlemek amacıyla bir yolak analizi yapılmış ve insan proteini Rev-erb- α 'nın kurtçuk ortologu NHR-85'in bir katkısının muhtemel olduğu tespit edilmiştir. Bu çalışmada, bahsi geçen proteinin işlev kaybı mutasyonunun, *C. elegans*'ın yabanıl türüne kıyasla ömrü uzattığı ve lityum klorür tedavisiyle ömrün daha da uzadığı bulunmuştur. Tedavi sırasında yavru sayısı ve hareket fonksiyonlarında bir azalma da

gözlemlenmiştir, ancak bunun ömür uzatmaya katkıda bulunabileceği öne sürülmektedir. Ayrıca, ömür uzamasının gelişimsel bir gecikmeden kaynaklanabileceği öne sürülmektedir.

Bu çalışmanın ikinci bölümünde, *C. elegans*'ın yaşla biriken bazı dairesel RNA'larının yaşlanma sürecine katkıda bulunup bulunmadığının anlaşılması amaçlanmıştır. Dairesel RNA'lar, uçlarından birleşen kovalent bağlı tek iplikli RNA molekülleridir ve bu birleşim bir halka şekli oluşturur. Çoğu RNA parçalayıcı enzim, parçalanmayı başlatmak için açık bir uca ihtiyaç duyduğundan, bu kovalent bağın stabilitelerine önemli ölçüde katkıda bulunduğu düşünülmektedir. *C. elegans*'ta yaşlanmaya kadar farklı yaşam aşamalarında dairesel RNA'ya özgü bir RNA sekanslama çalışmasının analizi sonucunda, iki farklı genin dört dairesel RNA'sının yaşlanma sürecinde yoğun bir şekilde biriktiği tespit edilmiştir. Daha sonra CRISPR/Cas9 sistemi kullanılarak, seçilen dairesel RNA'ların daireselleşmesini hedef alarak bu RNA'ları spesifik olarak susturmuş ve bu silmelerin *C. elegans*'ın ömrü üzerindeki etkileri araştırılmıştır.

Anahtar kelimeler: C. elegans, Yaşlanma, Uzun ömür, Lityum, NRDR1, Rev-erb-α, Dairesel RNA, CRISPR/Cas9

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Ayça Arslan, for believing in a young undergraduate student who simply wanted to follow his scientific passion. From the moment I joined her lab about 4.5 years ago, I felt supported in both my ideas and my enthusiasm, and that support played a defining role in my development as a scientist. Her kindness, friendly attitude, and fun personality made these years pass like days. Through all the ups and downs, I feel very proud of what we have achieved. I would also like to thank Dr. Michelle Adams for agreeing to be my supervisor.

I would like to express my gratitude to Dr. Sebiha Çevik Kaplan and Dr. Oktay İsmail Kaplan, the co-PIs of the Kaplan Lab at Kayseri AGÜ, for generously opening their laboratory to me for my CRISPR/Cas9 experiments. They were wonderful supervisors and made my time there truly enjoyable. I am especially thankful to Ferhan Yeniser, whose expertise, dedication, and ability to teach made her one of the best people I have ever worked with. The two weeks we spent collaborating were incredibly productive and genuinely fun, and I am very grateful for everything we accomplished together.

I would also like to thank the former members of our lab, who shaped my early years here in ways that continue to stay with me. I am grateful to Elif Sena Temirci, the first person I worked with on *C. elegans*, for her early guidance. My thanks also go to Elif Akış, whose warmth and steady support made my transition into the group much easier. I want to thank Tuba Sena Oğurlu for her sharp humor and insight, and Ayşe Reyhan Kutan Başçı for the smart perspective and positive energy she brought to the lab. I am also grateful to the members of the Bahar Değirmenci Lab and the İnci Lab for their fun conversations.

I am grateful to Volkan Demir for his remarkable attention to detail and his constant willingness to help in every situation. I would also like to thank Esra Arman Karaarslan for the enjoyable conversations and the coffees we shared; I always appreciated her friendliness.

I would like to thank Mariya Tulkibayeva, who helped me tremendously with both my experiments and the day-to-day maintenance of the lab. Her dedication and constant willingness to help made a real difference. I am also grateful to Elif Akgüloğlu, Nehir Yılmaz, and Neslihan Şengül, who joined the lab as interns during my final three

months; they were incredibly helpful and brought great energy as I wrapped up my experiments.

I am especially grateful to my closest friends in the lab, Ceren Aydoğan, Bora Deniz Yılmaz, and Almina Düzen. Ceren was the first new friend I made at the beginning of my master's, and from the very start I felt that we would get along incredibly well. As we spent time together, that feeling proved right, and we built a wonderful friendship. When Almina joined the lab a year later, I felt that same instant connection. She turned out to be incredibly funny, brilliant, and truly enjoyable to spend time with. Bora, one of my oldest friends since my bachelor's years, joined the lab one semester after me, and it was always a pleasure to reminisce about old times while creating new memories together. He is also one of the most brilliant people I know, and I deeply enjoyed discussing experiments and ideas with him. With the three of you, I shared daily conversations about everything happening in our lives, and your presence made my time here immeasurably better. Finally, I would like to thank Tuğrul Orak, who felt like a brother to me from his very first day as an intern.

A special thanks goes to Deniz, the love of my life. I am so grateful to have my best friend to grow old with. I am excited to see what the next chapters of our lives will bring, and whatever they are, I am so lucky to be able to experience them with you. Thank you for supporting me through every up and down of this scientific journey.

My final and deepest appreciation goes to my family. To my mother, the strongest woman I know, who somehow always found a way to get me up whenever I feel down or stressed. To my father, who was always behind my back with whatever decision I make. Their pride in me always fueled my motivation to do better. Thank you so much for believing in me and my dream of becoming a "prösörer" one day. I feel so lucky to have you as my parents. My brother and my sisters, my "core friends". I feel incredibly grateful to have siblings with whom I can share everything and have conversations that truly matter. You are the best siblings anyone could ever imagine.

I would like to thank the TÜBİTAK 2210-E scholarship program for supporting my Master's education. Part of this thesis was funded by the TÜBİTAK 1002-A (224Z104) grant.

Contents

Chapter 1: Investigating the effects of lithium chloride treatment on <i>Caenorhabditis elegans</i> longevity	1
1.1 Introduction	1
1.1.1 The roundworm <i>Caenorhabditis elegans</i>	1
1.1.2 Aging and Longevity Research	5
1.1.3 <i>C. elegans</i> as a Model for Aging and Longevity.....	7
1.1.4 Lithium and Longevity.....	9
1.1.5 Lithium Studies on <i>C. elegans</i>	10
1.1.6 A Circadian Rhythm Related Target.....	11
1.2 Aim of the Study	11
1.3 Materials & Methods.....	12
1.3.1 Materials.....	12
1.3.1.1 <i>C. elegans</i> Strains.....	12
1.3.1.2 Bacterial Strain.....	12
1.3.1.3 Equipment and Tools.....	12
1.3.1.4 Chemicals & Reagents	13
1.3.1.5 Enzymes & Kits	13
1.3.2 Buffer and Media Preparations	14
1.3.3 Methods.....	17
1.3.3.1 <i>C. elegans</i> maintenance.....	17
1.3.3.2 Age synchronization via NaClO treatment.	17

1.3.3.3 Lifespan Assay	18
1.3.3.4 Thrashing Assay	19
1.3.3.5 Brood Size Assay	19
1.3.3.6 RNA isolation.....	19
1.3.3.7 cDNA synthesis.....	20
1.3.3.8 qRT-PCR	20
1.3.3.9 Confocal Imaging.....	22
1.4 Results.....	22
1.4.1 Lifespan Assays.....	22
1.4.2 Thrashing Assay	26
1.4.3 Brood Size Assay	28
1.4.4 qRT-PCR Assay.....	30
1.4.4.1 Stage Based Gene Expression – L4	31
1.4.4.2 Stage Based Gene Expression – D1	32
1.4.4.3 Stage Based Gene Expression – D3	33
1.4.4.4 Stage Based Gene Expression – D7	34
1.4.4.5 Stage Based Gene Expression – D10	36
1.4.5 Confocal Microscopy Imaging.....	37
1.5 Conclusion	38
Chapter 2: Investigating the effects of age-accumulated circular RNA deletions on <i>Caenorhabditis elegans</i> longevity.....	40
2.1 Introduction	40

2.1.1 Circular RNAs.....	40
2.1.2 Biogenesis of Circular RNAs.....	41
2.1.3 Circular RNAs and Aging	42
2.1.4 Circular RNAs of <i>C. elegans</i>	42
2.2 Aim of This Study	43
2.3 Materials & Methods.....	44
2.3.1 Materials.....	44
2.3.1.1 <i>C. elegans</i> Strains.....	44
2.3.1.2 Bacterial Strains	44
2.3.1.3 Equipment and Tools.....	44
2.3.1.4 Chemicals and Reagents	44
2.3.1.5 Enzymes and Kits.....	45
2.3.2 Buffer and Media Preparations	45
2.3.3 Methods.....	46
2.3.3.1 Circular RNA Filtering.....	46
2.3.3.2 RCM Detection	47
2.3.3.3 CRISPR/Cas9 gRNA Designs.....	49
2.3.3.4 gRNA Cloning.....	50
2.3.3.5 DH5 α Transformation of pRB1017	50
2.3.3.6 Colony PCR	51
2.3.3.7 Microinjections	51
2.3.3.8 Mutant selection and deletion validation	52

2.3.3.9 Sanger Sequencing of the Deletion Regions.....	53
2.3.3.10 Divergent qRT-PCR	53
2.3.3.11 qPCR for Aging Markers.....	54
2.3.3.12 Lifespan Assays.....	54
2.3.3.13 Brood Size Assay	54
2.3.3.14 Thrashing Assay	54
2.4 Results	55
2.4.1 Circular RNA Filtering.....	55
2.4.2 RCM Detection	55
2.4.3 gRNA Cloning & Colony PCR	55
2.4.4 Microinjections	56
2.4.5 Deletion Validations	57
2.4.6 Sanger Sequencing.....	59
2.4.7 qRT-PCR Assay.....	59
2.4.7.1.1 Stage Based Expression – L4.....	61
2.4.7.1.2 Stage Based Expression – D1	62
2.4.7.1.3 Stage Based Expression – D3	63
2.4.7.1.4 Stage Based Expression – D7	64
2.4.7.1.5 Stage Based Expression – D10	65
2.4.7.2 Timelapse Expression	66
2.4.8 Lifespan Assay	68
2.4.9 Brood Size Assay	69

2.4.10 Thrashing Assay	70
2.5 Conclusion	72
References	74
Appendix A – RCM Detection Script	84



List of Figures

Figure 1: <i>C. elegans</i> life cycle.....	1
Figure 2: <i>C. elegans</i> anatomy, illustrated. [2] A) Hermaphrodite worm B) Male worm	3
Figure 3: <i>C. elegans</i> life stages under a stereo microscope [2]......	4
Figure 4: <i>C. elegans</i> DAF-2 (Insulin) Signaling. Left panel depicts the state before activation, right panel depicts upon activation.....	8
Figure 5: Kaplan-Meier survival plots of lithium-based lifespan assay. Lithium treatment started from L1.	24
Figure 6: Kaplan-Meier survival plots of lithium-based lifespan assay. Lithium treatment started from L4.	26
Figure 7: Video screenshots of a thrashing worm. A full bend is observed in a cycle from left panel to right.	27
Figure 8: WrmTrck identification of thrashing.	27
Figure 9: Thrashing assay results. Error bars represent mean with SEM. ns: non- significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001	27
Figure 10: Total brood size assay results. Viable progeny were counted, unhatched eggs were not analyzed. Error bars represent mean with SEM. ns: non-significant, p- values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001	28
Figure 11: Individual brood size assay results. The same results were used for total brood size assay. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001	29
Figure 12: L4 N2 vs L4 RB1661 qRT-PCR results. Relative gene expressions were calculated by using L4 N2 control Ct values. Error bars represent mean with SEM. ns:	

<i>non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001.</i>	32
<i>Figure 13: D1 N2 vs D1 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D1 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001.</i>	33
<i>Figure 14: D3 N2 vs D3 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D3 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001.</i>	34
<i>Figure 15: D7 N2 vs D7 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D7 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001.</i>	36
<i>Figure 16: D10 N2 vs D10 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D10 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001.</i>	36
<i>Figure 17: D1 JDW628(nhr-85::GFP) worm grown on control NGM. GFP was targeted with Alexa488 preset. Scale bar: 50μm.</i>	37
<i>Figure 18: D1 JDW628(nrh85::GFP) worm grown on control NGM, observed under DIC mode. Scale bar: 50 μm.</i>	37
<i>Figure 19: D1 JDW628(nhr-85::GFP) worm grown on 10 mM LiCl NGM. GFP was targeted with Alexa488 preset. Scale bar: 50μm.</i>	37

<i>Figure 20: Integrated fluorescence density and corrected total fluorescence calculated for the JDW628 worms</i>	38
Figure 21: Circular RNA Biogenesis [103].....	41
Figure 22: Supplementary table S3 of [91].	46
Figure 23: Filtered data from supplementary table S3 of [91].....	47
Figure 24: Input and output examples from RCM detection code. See Appendix A.	48
Figure 25: Representative image for RCM identification. RCM regions from <i>afd-1</i> introns were annotated on benchling.com.....	48
Figure 26: Representative image for CRISPR/Cas9 deletions.....	49
Figure 27: Colony PCR agarose gel result for <i>pqn-41</i> , <i>afd-1</i> and <i>afd-1</i> gRNAs. Expected band size was 260 bps.	56
Figure 28: Roller phenotype worms observed under stereo microscope. Roller worms leave a circle-like bacterial path as they move around the plate.....	57
Figure 29: Gel electrophoresis images of validation PCRs for <i>pqn-41</i> , <i>afd-1</i> and <i>afd-1</i> RCM deletions.	58
Figure 30: Sequencing result annotated with intended deletion on <i>afd-1</i> intron 6. ...	59
<i>Figure 31: Stage based qRT-PCR results for L4 N2 vs L4 TUR015. Relative gene expressions were calculated by using L4 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	61
<i>Figure 32: Stage based qRT-PCR results for L4 N2 vs L4 TUR016. Relative gene expressions were calculated by using L4 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	62

<i>Figure 33: Stage based qRT-PCR results for D1 N2 vs D1 TUR015. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>62</i>
<i>Figure 34: Stage based qRT-PCR results for D1 N2 vs D1 TUR016. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>63</i>
<i>Figure 35: Stage based qRT-PCR results for D3 N2 vs D3 TUR015. Relative gene expressions were calculated by using D3 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>63</i>
<i>Figure 36: Stage based qRT-PCR results for D3 N2 vs D3 TUR016. Relative gene expressions were calculated by using D3 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>64</i>
<i>Figure 37: Stage based qRT-PCR results for D7 N2 vs D7 TUR015. Relative gene expressions were calculated by using D7 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>64</i>
<i>Figure 38: Stage based qRT-PCR results for D7 N2 vs D7 TUR016. Relative gene expressions were calculated by using D7 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>65</i>

<i>Figure 39: Stage based qRT-PCR results for D10 N2 vs D10 TUR015. Relative gene expressions were calculated by using D10 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>66</i>
<i>Figure 40: Stage based qRT-PCR results for D10 N2 vs D10 TUR016. Relative gene expressions were calculated by using D10 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>66</i>
<i>Figure 41: Time-lapse qRT-PCR results for N2 vs TUR015. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>67</i>
<i>Figure 42: Time-lapse qRT-PCR results for N2 vs TUR016. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>68</i>
<i>Figure 43:Kaplan-Meier survival plot for RCM deletion based lifespan assay.</i>	<i>69</i>
<i>Figure 44: Total brood size assay results. Viable progeny were counted, unhatched eggs were not analyzed. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>70</i>
<i>Figure 45:Individual brood size assay results. The same results were used for total brood size assay. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as *<0.05, **<0.001, ***<0.0001, ****<0.00001</i>	<i>70</i>

*Figure 46: Thrashing assay results. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001*

..... 71



List of Tables

<i>Table 1: Strains used in chapter 1 of this study. All the strains were donated by CGC.</i>	12
<i>Table 2: Equipment used in chapter 1 of this study.</i>	12
<i>Table 3: Chemicals & reagents used in chapter 1 of this study.</i>	13
<i>Table 4: Enzymes and kits used in chapter 1 of this study.</i>	13
<i>Table 5: qRT-PCR Primers used in chapter 1.</i>	21
<i>Table 6: Median lifespans, sample sizes, and statistical analysis results of lithium-based lifespan assay. Lithium treatment started from L1.</i>	23
<i>Table 7: Median lifespans, sample sizes, and statistical analysis results of lithium-based lifespan assay. Lithium treatment started from L4.</i>	25
<i>Table 8: qRT-PCR targets and their human orthologs.</i>	30
<i>Table 9: Strains used in chapter 2 of this study. N2 & BB2 strains were donated by CGC, TM4439 strain was purchased from NBRP Japan, TUR015 and TUR016 strains are created on this study.</i>	44
<i>Table 10: Equipment used in chapter 2 of this study.</i>	44
<i>Table 11: Chemicals and reagents used in chapter 2 of this study.</i>	44
<i>Table 12: Enzymes and kits used in chapter 2 of this study.</i>	45
<i>Table 13: Optimized input parameters for RCM detection code.</i>	48
<i>Table 14: gRNA ssDNA oligo sequences.</i>	49
<i>Table 15: Contents of CRISPR/Cas9 microinjection mix.</i>	52
<i>Table 16: Deletion validation primers.</i>	53
<i>Table 17: BSJ specific divergent primers for circular RNA detection.</i>	54
<i>Table 18: qRT-PCR Primers for aging markers.</i>	54

Table 19: Filtered circular RNAs, their corresponding genes, exons and RCM regions..... 55

Table 20: Genomic locations of detected RCM regions..... 55

Table 21: Expected amplicon sizes of the targets after RCM deletion..... 57

Table 22: qRT-PCR targets for RCM deletion strains..... 60

Table 23: Median lifespans, sample sizes, and statistical analysis results of RCM deletion based lifespan assay. 68



Chapter 1: Investigating the effects of lithium chloride treatment on *Caenorhabditis elegans* longevity

1.1 Introduction

1.1.1 The roundworm *Caenorhabditis elegans*

Caenorhabditis elegans, or shortly *C. elegans*, is a nematode that primarily inhabits soil in nature [1]. It is a relatively small animal that is around 1 mm in length, making it barely visible in its adult stage without a microscope. The worm has a transparent yet quite resistant cuticle and a fixed number of cells [2]. The primary food source of the worms are bacteria, such as *E. coli*, and it can survive in a wide range of ambient temperatures from 15 to 25°C, making it resistant to varying conditions. They have a short life cycle of about 15 days. At lower temperatures such as 15°C, the life cycle is slower than it is at high temperatures like 25 °C [3].

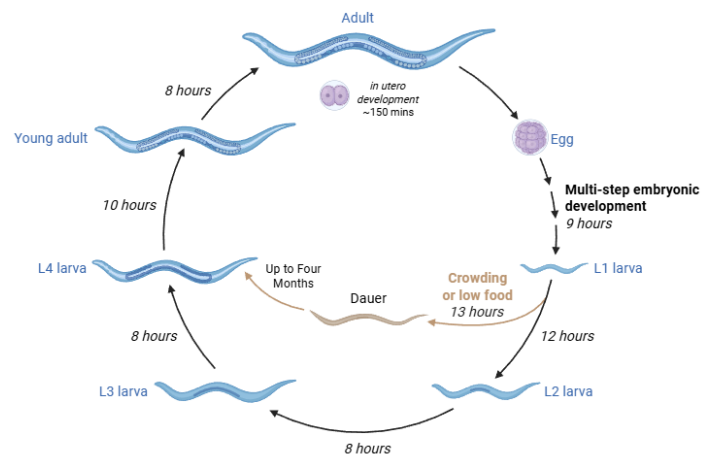


Figure 1: *C. elegans* life cycle. Figure created on BioRender

The roundworm can be found as two different sexes, hermaphrodite or male, yet the males are extremely rare in the nature with an abundance of around 0.2% [5]. Starting from fertilization, the eggs develop *in utero* for ~150 minutes [6]. At the gastrula stage of development, hermaphrodite mother lays the eggs to the environment, where they

need ~9 hours of *ex utero* development before hatching. When hatched, the worms are classified as larvae until adulthood, and the larval period is divided into four stages that are named L1, L2, L3 and L4, separated by brief “lethargus” or a “sleep state” where the animal sheds the previous cuticle and synthesizes a new one, leading to growth in size (See: Figure 1.1) [6]. The hermaphrodite worm reaches adulthood in approximately 3 days after fertilization and lays eggs for 5-6 days, creating a brood size of about 325 [7].

In extreme conditions such as high or low temperatures, starvation, lack of oxygen, the worm can develop into a special developmental stage called “dauer”. The decision to develop into dauer is made right before the first lethargus where the L1 larva usually develops into L2. The dauer worm can adapt to harsh conditions and can survive for up to four months. If the conditions become favorable for development, dauer larva can continue its normal development into adulthood, resuming from the L4 stage [8].

Anatomically, worms are quite simple. They have the essential internal systems for feeding, reproduction and signal transduction [2]. The feeding system starts with the pharynx, where food is taken in and sent to the intestine, colon and finally the rectum and anus for disposal [9]. The hermaphrodite worms have a symmetrical reproductive system where there are two gonads that are connected to the uterus through the spermatheca. After oogenesis, premature eggs move through the gonads and once they are mature enough, they are fertilized in the spermatheca. After fertilization and a short *in utero* development, the eggs are laid out via the vulva [10]. The nervous system of the worm takes the majority of the cells, that is 302 of 959 in hermaphrodites. This system is divided into two major categories: pharyngeal nervous system with 20 neurons and the resting 282 belongs to the somatic nervous system [11] (See: Figure 1.2).

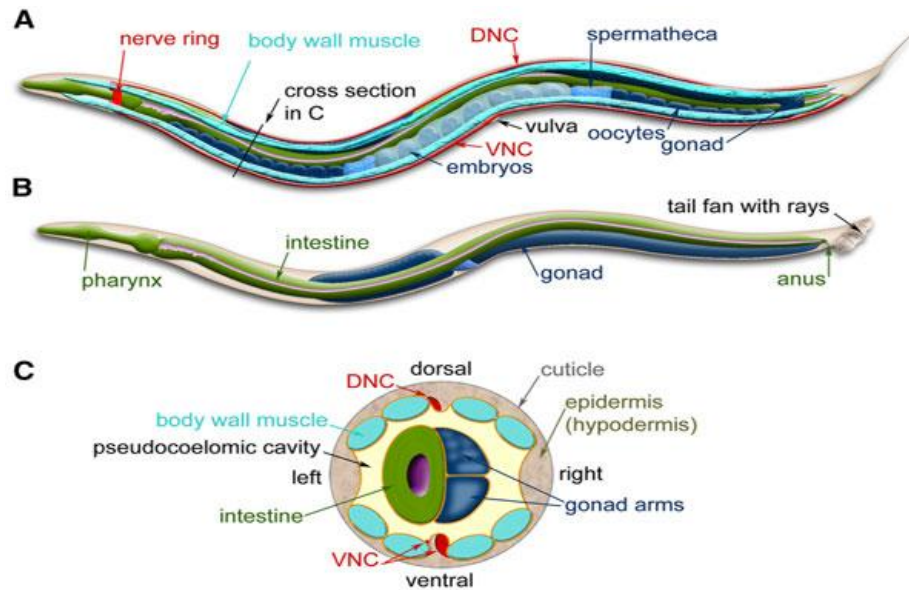


Figure 2: *C. elegans* anatomy, illustrated. [2] A) Hermaphrodite worm
B) Male worm

With great efforts since its establishment as a nervous system model by Sydney Brenner in 1960s [12], the worm was investigated thoroughly. With its fixed number of cells, it was one of the first models of developmental biology. Every cell of the worm was given a unique identifier name, and the complete lineage and fate of each is known [13]. It is also that had its whole genome sequenced, which consists of five autosomal pairs and a pair of sex chromosomes [14]. Finally, the worm was the first model that had its neural connectome identified, so every single synapse is identified [15].

After its successful establishment as a model organism for neurobiology, the worm was then adapted to other fields of biology including molecular biology, aging research [16], toxicology [17] and more. This led to discoveries of gene manipulation methods that allowed researchers to manipulate the worm according to their experimental needs. RNA interference (RNAi) is a great example of such process, which utilizes small RNA molecules to transiently silence mRNA translation [18], and its discovery even led to leaps in research in many other models, resulting in a Nobel Prize being awarded to its discovery. In 2003, a library of RNAi that covered 86% of the 19000

predicted genes (at the time) was constructed and it was later completed to cover all the genes of the worm [19]. Worldwide availability of this library allowed researchers to easily manipulate protein translation. Furthermore, a consortium named the “*C. elegans* gene knockout consortium” had created more than 6000 knockout mutants, which were distributed around the world [20]. The distribution was made through a worldwide hub that was established at the University of Minnesota in 1978, named the *Caenorhabditis* Genetics Center (CGC). This center hosts more than 20000 different strains, which creates a great selection for many labs, allowing faster research [21].

The ease of handling, the ability to rapidly grow large populations, transparent nature, short life cycle, low cost, years of accumulated research experience and worldwide availability of cheap resources made *C. elegans* one of the most used model organisms in various fields [2].

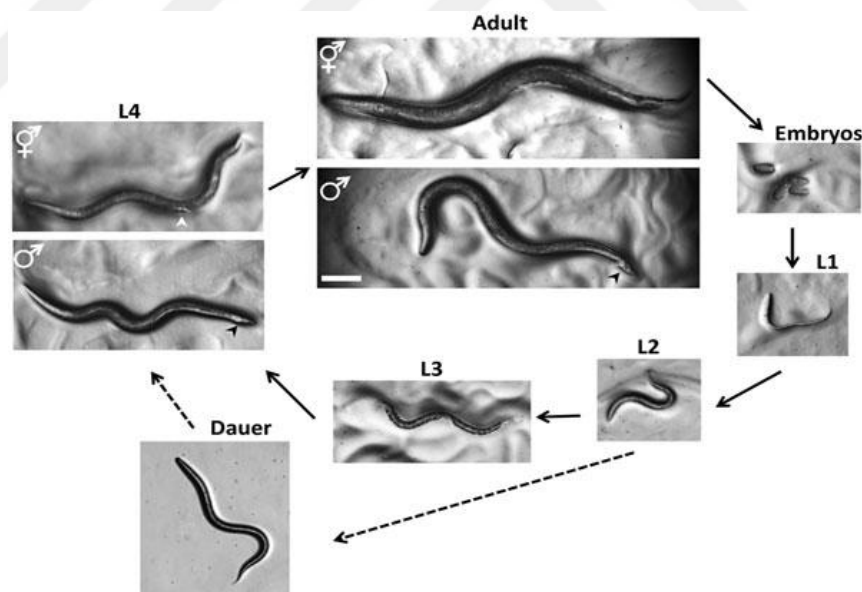


Figure 3: *C. elegans* life stages under a stereo microscope [2].

Even though the worms are invertebrates that are evolutionarily distanced to humans, homologs for two thirds of the human disease genes are found in their genome [22]. This makes them a good starting point to model diseases and understand the basics of many mechanisms. Research done on these worms was highly impactful, and as of

2025, four Nobel Prizes were awarded to such research. These include the 2002 Nobel Prize in Physiology or Medicine, awarded to Sydney Brenner, Robert Horwitz and John Sulston for their discovery of the programmed cell death mechanism [23]; 2006 Nobel Prize in Physiology or Medicine, awarded to Andrew Fire and Craig Mello for their discovery of RNA interference [24]; 2008 Nobel Prize in Chemistry to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien for their discovery of green fluorescent protein (GFP) [25]; and 2024 Nobel Prize in Physiology or Medicine to Victor Ambros and Gary Ravkun for their discovery of the microRNAs and their roles in gene regulation [26].

1.1.2 Aging and Longevity Research

Aging is defined as the deterioration that occurs over time in the tissues and cells of an organism. These abnormalities constitute the primary risk factors for neurodegenerative and cardiovascular diseases, cancer, diabetes, and many other diseases [27]. The distinguishing features, or “hallmarks”, of aging were defined in 2013 by Carlos López-Otín and colleagues as nine items. These are, in order: impaired intercellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of protein homeostasis, dysregulation of nutrient sensing, mitochondrial dysfunction, cellular senescence, and stem cell exhaustion (López-Otín et al., 2013). These items were revised in 2023; chronic inflammation, dysbiosis, and the disengagement of the macro-autophagy mechanism were added to the list. These characteristics were essentially evaluated as common features of aging cells and began to be used as markers of aging [28].

Although the common characteristics of aging have been defined as described above, the triggering factors behind these characteristics are not fully understood. For this reason, aging research continues rapidly worldwide. These studies are primarily

conducted through lifespan experiments, which involve altering different factors and tracking changes in median lifespan, or by monitoring established aging markers in the literature [29]. Different models are used in these experiments to understand aging, including *in vitro* and *in vivo* models. While cell experiments take a backseat because they do not represent the entire organism, experiments conducted on model organisms have set the gold standard in the field of aging. The preferred factors in these organisms are determined around simplifying the experimental design and not losing human homology in the process. For this reason, the most used model organisms are mice (*Mus musculus*), fish (zebrafish – *Danio rerio*), and worms [29].

Mice have been one of the most widely used model organisms in basic biology for many years. The greatest advantage of mice is that they are vertebrates with the most scientific knowledge available. Specifically in aging research, mice are an ideal model for long-term experiments due to their relatively long lifespan (varying by strain, averaging 300-900 days). However, the difficulty and high cost of their care, coupled with the difficulty of increasing the total number of animals, make them less than ideal for pioneering studies that have not previously been conducted in other organisms [30]

Zebrafish have established a strong presence in literature as a widely used model organism for aging research. High protein similarity to humans, external fertilization, the ability to produce multiple offspring at once, and the prevalence of mutant strains have made zebrafish a powerful aging model [31] [32]. However, their relatively long average lifespan leads to long waiting times, making them less preferred for pioneering studies.

1.1.3 *C. elegans* as a Model for Aging and Longevity

The worm's short life cycle and high brood size make it an excellent model for aging research. With the median lifespan being around 15 days, it is very convenient to monitor the entire lifespan in a controlled experiment [33].

One problem with the worm as an aging model is the fact that they reach adulthood in a relatively short time. After they reach adulthood, they immediately start laying eggs to the environment, which again, reach adulthood in about three days. With both the mother and the progeny looking the same, it can be problematic to separate the subject worms from the progeny [34]. There are essentially two methods to overcome this. First way is to take the worms into new plates until their reproductive period is over. This can work most of the time, but with experiments that have multiple strains with high number of worms, this can be time consuming, and worms can be harmed during the transfer process, resulting in loss of animals. Second method is the use of a non-functional thymidine analog 5-Fluorodeoxyuridine (FUDR). Worms stop cell division except for reproduction after they reach adulthood, so transferring the worms to FUDR containing plates as they reach adulthood results in a shutdown in their reproductive systems [35]. The problem with this method is the possibility of this treatment to be determinant of lifespan by itself, but as most experiments done on the field take advantage of this method, results are mostly comparable [36].

Practicality aside, *C. elegans* have been used as an aging model for decades, and many aspects of worm aging have been well documented. With age, worms become less motile [37], less reproductive [38] and overall, less healthy [39]. These phenotypes are all common with most animals including humans [40], and the identified pathways responsible for these phenotypes on worms are found to be conserved through human

evolution including the insulin signaling [41], which is one of the main pathways for human aging [42].

Master regulator of lifespan for worms is the insulin/IGF signaling [43]. *C. elegans* have 40 insulin like peptides (ILP), and a single receptor for these peptides named DAF-2 [44]. This receptor is the ortholog for two human insulin receptors, INSR and INSRR, and the insulin-like growth factor receptor, IGFR1. Human insulin is also an antagonist for this receptor [45]. Upon activation via ILPs, DAF-2 phosphorylates its own, resulting in an active state of the receptor kinase [44]. In its active form, it activates the human PI3K ortholog AGE-1, which then activates the human AKT-1 ortholog with the same name [44]. This final activation leads to phosphorylation of the human FOXO4 ortholog DAF-16 [44]. Phosphorylated DAF-16 cannot access the nucleus, which prevents the expression of lifespan extending genes, along with other genes such as dauer formation genes [46]. AKT-1 also regulates GSK-3 β , which can regulate the worm's energy levels by inhibiting glycogen synthesis [47] (See: Figure 1.4). With great conservation of this crucial pathway and the availability of the wide

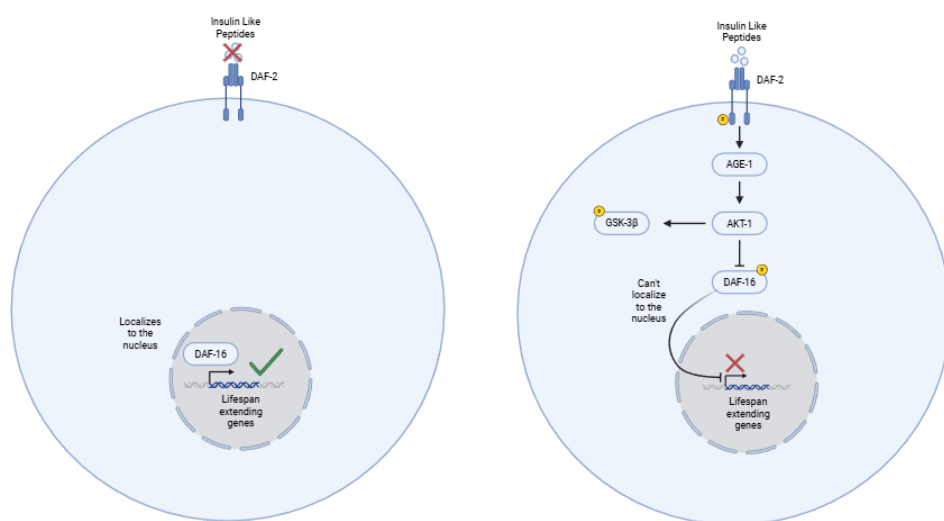


Figure 4: *C. elegans* DAF-2 (Insulin) Signaling. Left panel depicts the state before activation, right panel depicts upon activation. Figure created on BioRender.

genetic toolbox, aging research with *C. elegans* led to many pioneering discoveries on how human insulin signaling works [43].

1.1.4 Lithium and Longevity

Lithium, in biological context, is an important ion with various functions. Even though it is found in trace amounts under normal circumstances [48], when provided from outside the body, lithium can have effects on various parts of the body, yet the most prominent functions are found to be related to neurons [49]. These effects include modulation of neurotransmitter levels [50], neuroprotection [51], and regulation of circadian rhythm [52]. These effects result in various phenotypes, most prominently mood stabilization [53]. This effect is taken advantage of for stabilizing bipolar disorder, and lithium is the main prescription for many bipolar patients [54]. Currently, most prescribed form of lithium salts are lithium citrate, lithium carbonate and lithium orate [55]. However, in previous years, lithium chloride was also used as a pharmaceutical [56].

Even though the mechanism of action is not completely understood, lithium was found to be inhibiting functions of various proteins. GSK-3 β [57] and Inositol monophosphatase (IMPase) [58], are two human proteins that are found to be directly and indirectly inhibited by lithium ions, possibly leading to previously described phenotypes. GSK-3 β inhibition occurs via direct competition of lithium ions with magnesium ions [57].

Given its wide use on bipolar patients, lithium was extensively researched in different contexts. One of these was longevity, and some models were found to have extended lifespan upon lithium treatment, such as the fission yeast *Schizosaccharomyces pombe* [59], the fruit fly *Drosophila melanogaster* [59], and the roundworm *C. elegans* [60]. In mice, however, lithium treatment was not effective in lifespan extension, but it was

found to be effective in improved motor performance and kidney function [61]. Human studies also show similar effects. One correlative study showed that in Japan, higher lithium content on drinking water resulted in less overall deaths [62]. Another study done with the data from the UK Biobank showed that people prescribed with lithium drugs showed longer lifespan [63].

1.1.5 Lithium Studies on *C. elegans*

Understanding the mechanism of action of lithium on longevity is an important challenge that remains to be tackled completely. As discussed before, *C. elegans* is a great model for this type of research, and there are some studies that focus on how lithium works inside the worm.

First paper on lithium's effect on worms were published back in 2007, and the paper focused on lithium's effect on Huntington's Disease model. It was shown that increased concentration of lithium (from 10 to 100 mM) increased neuronal survival, without which was about 15-20%. This effect was also shown to be occurring regardless of presence of DAF-16 protein [64]. In 2008, a conceptual study by Gawain McColl was. In that study, it was shown that lithium treatment induces a delay in aging, resulting in changes in lifespan of different mutant worm strains. Most importantly, lithium treatment resulted in a 36% median lifespan extension on wild type worms, 21% increase on *daf-2* mutants, 50% increase on *daf-16* mutants, and interestingly, 7% decrease on *gsk-3 β* mutants. The same study suggested that lithium decreases the expression of a histone demethylase called LSD-1 [60]. Another study, importantly, showed that lithium prevents the nuclear localization of DAF-16, likely caused by GSK-3 β inhibition. Same study also showed that SGK-1, DAF-2 and AKT-1 are also required for prevention of DAF-16 nuclear localization via lithium, and that their gene expression is effected after treatment. B-arrestin ortholog ARR-1 is also required, yet

its expression remains stable with lithium treatment [65]. In another study, lithium treatment was shown to induce non-hatching eggs, increased autophagy, and increase of ATP production at later life stages [66]. One other study showed that the phosphatase named BPNT-1 was inhibited by lithium. Both the inhibition and mutation of BPNT-1 was shown to cause neuronal dysfunction in two ASJ neurons [67].

1.1.6 A Circadian Rhythm Related Target

NRDR1 or Rev-erb- α , is a highly conserved gene that encodes a nuclear receptor in humans [68]. It is associated with circadian rhythm as it was found that its expression oscillates [69] [70]. Its expression is in a feedback loop with BMAL/CLOCK, which are the master regulators of human circadian rhythm [71]. Importantly, it was reported to be a lithium sensitive component, and its expression and activation were inhibited via lithium treatment [72]. This was related to lithium's inhibition of GSK-3 β , as it usually activates Rev-Erb- α via phosphorylation [72].

C. elegans genome also encodes an ortholog of Rev-Erb- α , named NHR-85 [73]. This relatively under-researched protein, is only known to regulate mitochondrial and lipid homeostasis [74], enhance autophagy and mitophagy in Alzheimer's Disease models [75], and regulate timed gene expression [76]. Given what was previously known with GSK-3 β – PI3k – IIS signaling plane, NHR-85 becomes a prominent target to investigate on the effects of lithium on lifespan extension.

1.2 Aim of the Study

The aim of this part of the study is to investigate the effects of lithium treatment on the *C. elegans* Rev-Erb- α ortholog NHR-85, and more broadly, to investigate how lithium effects *C. elegans* lifespan. The hypothesis of this study is that lithium treatment will

extend the *C. elegans* lifespan, as GSK-3 β mutant worms show decreased lifespan, and GSK-3 β inhibits the function of human Rev-Erb- α .

To test this, a missense mutation strain of *nhr-85* is ordered, together with supporting mutants about the discussed pathways. Then the lifespan analyses, behavioral tests, and qRT-PCR assays are performed for gene expression studies.

1.3 Materials & Methods

1.3.1 Materials

1.3.1.1 *C. elegans* Strains

Table 1: Strains used in chapter 1 of this study. All the strains were donated by CGC.

Strain Name	Genotype (allele)	Mutant Type
N2	Wild Type (Bristol)	-
CF1038	<i>daf-16 (mu86)</i>	Deletion
DA465	<i>eat-2 (ad465)</i>	Missense
GR1310	<i>akt-1 (mg144)</i>	Missense
JDW628	<i>nhr-85 (wrd29)</i>	GFP Labeled
RB1661	<i>nhr-85 (ok2051)</i>	Splice Acceptor
RB660	<i>arr-1 (ok401)</i>	Splice Acceptor
TJ1052	<i>age-1 (hx546)</i>	Missense

1.3.1.2 Bacterial Strain

All worms were fed with streptomycin resistant *E. coli* strain OP-50.1.

1.3.1.3 Equipment and Tools

Table 2: Equipment used in chapter 1 of this study.

Equipment	Brand	Model Number
20°C Incubator	Memmert	IPP55
37°C Incubator	Memmert	IN55
Incubator	Sartorius Stedim	Certomat BS-1
Shaker Incubator	New Brunswick	Innova44
Thermal Cycler	Bio Rad	C1000
Real Time System	Bio Rad	CFX96
Spectrometer	ThermoFisher	NanoDrop2000
Scale	Precisa	BJ410C
pH Meter	Mettler Toledo	SevenCompact
Autoclave	Nüve	SteamArt OT 90L
Oven	Binder	ED400
Stereo Microscope	Optika	SLX-3
Stereo Microscope	Leica	S8 APO

Microscope Camera	Optika	C-B5
Microscope Camera	Leica	MC170 HD
Benchtop Centrifuge	Eppendorf	Centrifuge 5418
Mini Centrifuge	WVR	Galaxy MiniStar
Confocal Microscope	Leica	SP8
Vortex	WVR	-
Glass Cell Spreader	-	-

1.3.1.4 Chemicals & Reagents

Table 3: Chemicals & reagents used in chapter 1 of this study.

Chemical/Reagent	Brand	Catalog Number
American Bacteriological Agar	Condalab	1802
Bacteriological Peptone	Condalab	1616
Sodium Chloride	Merck	106404
Potassium Hydroxide	Aropi	-
NaClO	ERLAB	ERL.HIPO1
KH ₂ PO ₄	Merck	104873
K ₂ HPO ₄	Merck	105104
LiCl	ThermoFisher	AC449040000
Cholesterol	Merck	228111-5G
Nystatin	Merck	475914-1GM
Absolute Ethanol	Isolab	920.026.2500
Nuclease Free Water	Ambion	AM9937
MgSO ₄ .7H ₂ O	Sigma-Aldrich	M1880
CaCl ₂ .2H ₂ O	Merck	102382
Glycerol	Isolab	927.023.1000
Sodium Azide	Sigma-Aldrich	S2002
EDTA	Sigma-Aldrich	E4884
Tris Base	Sigma-Aldrich	T1503
Triton-X-100	Sigma-Aldrich	T8787
5'-fluorodeoxyuridine	Cayman	14154

1.3.1.5 Enzymes & Kits

Table 4: Enzymes and kits used in chapter 1 of this study.

Enzyme/Kit	Brand	Catalog Number
2xTaq DNA Polymerase RED	Ampliqon	A200003
2X RealQ Plus Master Mix Green	Ampliqon	A323402
iScript cDNA Synthesis Kit	Bio Rad	1708891
iScript cDNA Synthesis Supermix	Bio Rad	1708841
Proteinase K	Sigma	1.24568

1.3.2 Buffer and Media Preparations

M9 Buffer

1.5 g KH_2PO_4

3 g Na_2HPO_4

2.5 g NaCl

Dissolved in 500 mL ddH₂O, autoclaved at 121°C, 15 psi for 15 minutes, stored at room temperature.

1M LiCl

0.43g LiCl was dissolved in 10 mL ddH₂O, autoclaved at 121°C, 15 psi for 15 minutes, stored at +4°C.

KPO₄ Buffer

9.5g KH_2PO_4

5.25g K_2HPO_4

Dissolved in a total volume of 100 mL autoclaved ddH₂O, followed by filter sterilization. Stored at room temperature.

1M MgSO₄

2,46 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in a total volume of 10 mL autoclaved ddH₂O, followed by filter sterilization. Stored at +4°C.

1M CaCl₂

1,47 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was dissolved in a total volume of 10 mL autoclaved ddH₂O, followed by filter sterilization. Stored at +4°C.

Cholesterol Solution

50 mg of cholesterol was dissolved in a total volume of 10 mL absolute ethanol. The mixture was thoroughly mixed until cholesterol dissolved, followed by filter sterilization. Stored at +4°C.

Nystatin

100 mg of Nystatin was suspended in a total volume of 10 mL 70% ethanol. Stored at -20°C, re-suspended before each use.

150 mM FUDR

100 mg FUDR was dissolved in 2.7 mL nuclease free water, and the solution was aliquoted into 100 µL tubes, which were then stored in -20°C.

Bleach Solution

200 µL 15% NaClO

100 µL 10M KOH

700 µL ddH₂O

Were mixed per tube of bleaching. This solution was prepared fresh before each experiment, and NaClO was stored away from light to prevent its degradation.

LB Broth

5g Tryptone

2.5g Yeast extract

5 g NaCl

Were mixed with 1L water, autoclaved at 121°C, 15 psi for 15 minutes, stored at +4°C.

OP50.1 Liquid Culture

A single colony of OP50.1 bacteria was selected from LB Agar streak plate and grown in 25 mL LB broth in a 50 mL falcon tube overnight. Mixed thoroughly before use.

Nematode Growth Medium (NGM)

17g Agar

2.5g Peptone

3g NaCl

Were added to 969 mL of ddH₂O. The mixture was autoclaved at 121°C, 15 psi for 15 minutes, and cooled down to 55°C in a water bath for 20 minutes. Afterwards; 25 mL KPO₄ buffer, 1 mL 1M MgSO₄, 1 mL CaCl₂, 1 mL cholesterol solution, 1 mL nystatin suspension were added, and the bottle was shaken vigorously. 6 cm petri plates were seeded with 8 mL NGM, and 10 cm petri plates were seeded with 25 mL NGM.

For LiCl treatment plates, 80 µL of LiCl was seeded with to the plates before the addition of NGM, to obtain a final concentration of 10 mM. For lifespan experiment plates with FUDR, 33 µL of 150 mM FUDR was added per 100 mL NGM before plate seeding.

After seeding the plates with NGM, they were left for drying in a dark and cool environment overnight. Next day, 6 cm plates were seeded with 80 µL and 10 cm plates were seeded with 230 µL OP50.1 liquid culture. The seeded bacteria was carefully spread with ethanol sterilized glass cell spreader, and left for drying at least one day overnight.

1.3.3 Methods

1.3.3.1 *C. elegans* maintenance

C. elegans maintenance was performed as described [2]. In brief, the worms mentioned on section 1.2.1.1 were grown at 20°C unless otherwise stated, on standard 6 cm NGM plates seeded with OP50.1 bacteria. They were maintained via picking weekly to prevent starvation and contamination, and aseptic techniques were applied to prevent possible contamination. The NGM contained antifungal agent nystatin as an extra preventative measure against fungal contamination.

1.3.3.2 Age synchronization via NaClO treatment.

C. elegans cuticles are very resistant exoskeletons, yet their eggshells are even more resistant to chemical stress. Taking advantage of this, worms can be treated with a bleach solution to eliminate every hatched worm, and as a result, obtain only the eggs of gravid adults [99]. This is a useful method since the obtained eggs can be allowed to hatch in the absence of OP50.1 bacteria, which results in an L1 stage arrest. This gets a population age synchronized, which is very useful in experimental setups. As an addition, this method helps eliminating bacterial and fungal contamination as bleach is detrimental to those organisms.

To age synchronize a plate of worms, an NGM plate with gravid adults is washed with M9 buffer into a 1.5 mL conical tube. To make sure worms are not lost in this process, glass Pasteur pipettes are used instead of plastic pipette tips. After washing, the tube of worms are centrifuged at a benchtop centrifuge at 2000 RPM for 1 minute. This gentle centrifuge step pellets the worms but leaves the bacteria on the supernatant. The supernatant is then pipetted out as presence of bacteria lowers the yield of NaClO treatment. After the tube is washed until the supernatant is clear, pellet is then added with 1 mL bleach solution, and the tube is vigorously shaken for three minutes. At the

end of shaking, the tube is observed under the microscope to confirm the elimination of intact worms. If there are worms present, the tubes are shaken until the worms are eliminated. After confirming that there are no more intact worms, the tube is centrifuged at 4000g for 1 minute. The supernatant is carefully discarded, and the pellet is washed with 1 mL M9 buffer. This process is repeated 4-5 times. After the final wash, 1 mL M9 buffer is added to the tube, and the cap is wrapped with parafilm. Sealed tube is then placed into a rotator inside a 20°C incubator to allow L1 arrest overnight.

1.3.3.3 Lifespan Assay

Lifespan assay is the gold standard for measuring the median lifespan of *C. elegans*. In essence, a set number of worms are counted until their deaths. To prevent their starvation, the worms are transferred to a fresh NGM plate every 2 days. As *C. elegans* embryos grow quickly into adults that are indistinguishable from their mothers, egg production is altered in the experiment worms with a non-functional thymidine analog called FUDR. As *C. elegans* only produce DNA for their progeny upon reaching adulthood, FUDR does not change the rest of their life cycles and it is widely used in *C. elegans* aging research [100].

After age synchronization of the desired strain, 100 L1 worms are seeded into NGM plates. After around 45 hours (when they reach adulthood, which differs from strain to strain), the worms are transferred on FUDR NGM plates and counted every two days. After counting, the worms are transferred to new plates to prevent starvation.

Worms that do not respond to a gentle touch with the worm pick are considered dead. Worms are considered censored if they crawl into NGM, crawl out towards the side of plates, become internally hatched (bagged phenotype) or they lose their anatomic integrity. Counts are analyzed with Kaplan-Meier survival curves on Graphpad Prism.

1.3.3.4 Thrashing Assay

Thrashing or body bending is a behavior that worms show when they are submerged in liquids. One thrash is considered as a worm bending to its two sides. This behavior is representative of *C. elegans* motility, which is an indicator of overall health.

For thrashing assay, 2% Agar is melted in a microwave and poured into a petri dish. Once it is solidified, 80 μ L M9 buffer is dropped into the middle of the plate, and a worm is placed into the drop. After 30 seconds of initial adaptation, two 30 second videos of the thrashing worm is recorded. The recorded video is then analyzed, either manually or with the help of ImageJ plugin WrmTrck [101]. Analyzed thrashing counts are then analyzed on Graphpad Prism.

1.3.3.5 Brood Size Assay

Brood size of a worm is an indicator of reproductive capacity, and overall health [101]. For this analysis, fifteen individual worms are grown upon age synchronization. On D1 of adulthood, they are transferred to individual plates for egg laying. Next day, the adult worms are transferred into new individual plates, and the previous plates are kept for 2 more days at 20 °C for the progeny to grow. This process is continued for five days and on day six, the subject worms are sacrificed. The progeny plates are counted before they reach adulthood (so at L4 stage) for easier counting. Total brood size and brood size each day is then analyzed on Graphpad Prism.

1.3.3.6 RNA isolation

For gene expression analysis via qRT-PCR, RNA isolation is required. For this, a protocol that allows for rapid RNA isolation is followed [102]. This protocol allows total RNA isolation from even a single worm, which reduces the required number of worms.

For isolation, an RNA lysis buffer is required with the following content: 5 mM Tris (pH: 8.0), 0.5% Triton-X-100, 0.25 mM EDTA and 1.5 mg/mL Proteinase K. Proteinase K is added freshly before use, and the prepared lysis buffer can be stored in +4°C. For each six qPCR wells, 3 µL of isolated RNA is required. For each 3 µL of required RNA, 3 µL of RNA lysis buffer with Proteinase K is pipetted on the bottom of 0.2 mL PCR tubes, and the tubes were then briefly centrifuged to get all the buffer to the bottom of the tube. 5 worms are then picked inside of the buffer per tube. Release of the worms from the pick is observed carefully under microscope to prevent worms sticking to the sides of the tube. Tubes are then centrifuged for 5 seconds, then they are placed to thermal cycler for proteinase K activation. Conditions are: 10 minutes at 65 °C, 1 minute at 85 °C and cooling of at 12 °C for 1 minute. The isolated RNA can be stored at -80 °C.

1.3.3.7 cDNA synthesis

cDNA synthesis done with iScript Reverse Transcription kit (Biorad). In brief, a 1X master mix of 17 µL is prepared for each RNA isolation tube. The master mix contains 12 µL of nuclease free water, 4 µL of iScript reaction buffer and 1 µL of iScript reverse transcriptase enzyme. Each tube of RNA was directly converted into cDNA, so the reverse transcription master mix was directly pipetted onto the isolated RNA. The tubes were centrifuged before the thermal cycler program, which is 5 minutes at 25°C for priming, followed by a 20 minute reverse transcription step at 46°C and 1 minute heat inactivation at 95°C. The samples were then cooled on ice and were stored at -20 °C until use.

1.3.3.8 qRT-PCR

Ampliqon RealQ 2X Master Mix Green kit was used for qRT-PCR assay. For each target, 3 µL of synthesized cDNA was mixed with 1 µL forward and 1 µL reverse

primers, 5 µL nuclease free water and 10 µL enzyme master mix, yielding a 20 µL reaction mix. Each qRT-PCR target was amplified as two technical replicates of two biological replicates. Tubulin- α was used as the housekeeping gene for normalization. PCR conditions were 15 minutes at 95°C, then a 40 cycle of 15 seconds at 95°C, 15 seconds at 55°C and 30 seconds at 70°C. Plate readings were done with SYBR/FAM only mode at the end of each cycle. At the end of 40th cycle, a melt curve analysis was conducted with 0.5°C increments from 65°C to 95°C, 5 seconds at each step and a plate read after each step. After verification of melt curves, gene expression analysis was done with $2^{-(\Delta\Delta Ct)}$ method. Ct values were calculated on Microsoft Excel, and statistical analysis was done on Graphpad Prism.

Primers used in this assay are listed on the table below.

Table 5: qRT-PCR Primers used in chapter 1.

Name	Sequence (5-3)
age-1_f	GTGACGAGCTTCGATCGATTAG
age-1_r	GGACGATGGCTTTGTCGATTA
akt-1_f	CCGTTTCCTGAGCCATTGA
akt-1_r	TGTCCACTGAAGGCATCTTAC
arr-1_f	TCTCATCCAACCTACACGAAGAATTA
arr-1_r	CGATGGCTGGTCTTCACTATC
bpnt-1_f	CTTGCCCCGAAATCGAAATG
bpnt-1_r	GATCCACCCAGACTACAACATC
daf-2_f	GAGCTTCGGAGTTGTTCTCTATG
daf-2_r	CTTCCGGGCCATTCCAATATAA
daf-16_f	TTGCTCCACCACCATCATAAC
daf-16_r	GTGGCATTGGCTTGAAGTTAG
gsk-3_f	TCGTACATTGCTCGCGATATT
gsk-3_r	CGAGAAGAAGCTCTGCCATTAC
lsd-1_f	CAGCTGATAGACCAACGGAAA
lsd-1_r	GGCTAGCCAGATACTCAAAGTC
nhr-85_f	GTCAGCAGCCAATGCTACTA
nhr-85_r	CGAACATCAGCCAACAGAGA
ssu-1_f	CAGCTGGTTACGGTAGTGTATG
ssu-1_r	CTACTCAGCGAAAGTGGACAA
hsp-16.2_f	TACGCTATCAATCCAAGGAGAAC

hsp-16.2_r	GAAGCAACTGCACCAACATC
sod-3_f	TGGTGGTGGACACATCAATC
sod-3_r	ACCGAAGTCGCGCTTAATAG
tba-1_f	CCTCTCGGTTGACTATGGAAAG
tba-1_r	GTGGTGTGAGTCGTCAGTATAG

1.3.3.9 Confocal Imaging

The JDW628 strain has a GFP label attached to its NHR-85 proteins. To analyze the GFP intensity, Leica SP8 confocal microscope was utilized. To analyze worms under the confocal microscope, 2% agar pads were prepared on microscope slides by melting agar and placing a drop on a microscope slide, and perpendicularly closing another slide to solidify the agar, resulting in a pad shape. To anesthetize the worm on the agar pad, 3 μ L 50 mM NaN₃ was carefully pipetted and the worm to be imaged was carefully placed on the drop. After a minute of waiting for anesthesia to act, a cover slip is carefully placed on top of the agar pad. The slide is then imaged with the confocal microscope at 40x lens with immersion oil. To observe GFP, Alexa488 preset of the LasX software was used. To analyze the whole worm in three dimensions, 35 Z-stack images were taken with 1 μ m increments. The images were then analyzed on ImageJ for signal intensity. For that, images were converted to 8-bit greyscale, then “mean grey value” was utilized on “set measurements” tab. Worms were selected by hand to cover for all their bodies, and the raw values were obtained. To calculate for background, three random selections were measured from the non-grey background.

1.4 Results

1.4.1 Lifespan Assays

In the beginning of the study, the *C. elegans* strains mentioned on chapter 1.3.1.1 (except JDW628) were subjected to lifespan analysis upon 10 mM lithium chloride treatment. The worms were age synchronized as described on 1.3.2.2, L1 arrested, and

transferred to FUDR plates at their beginning of adulthood. Each strain was subjected to lifespan analysis both in the presence (treatment) and absence (control) of LiCl.

Lithium treatment groups reached adulthood about two days after the control groups on DA465 strain. Rest of the strains reached adulthood about one day after their control group. The analysis counted for days of adulthood, which corrected for this variation. Two replicates were analyzed with 100 worms per replicate, resulting in a sample size of N=200. The worms were counted every 2-3 days, and the data was plotted into a Kaplan-Meier survival plot. Median lifespans were compared with two statistical analyses. Log-rank (Mantel-Cox) test counts every death event with equal weight, and the Gehan-Breslow test counts later deaths with higher weight.

Median lifespan results of the strains were as shown on table 6.

Table 6: Median lifespans, sample sizes, and statistical analysis results of lithium-based lifespan assay. Lithium treatment started from L1.

Strain	Lithium Treatment	Median Lifespan (days)	Median Lifespan Change (%)	Sample Size (censored)	Mantel-Cox p-value	Gehan-Breslow p-value
N2	-	18	+5.6%	200(2)	0.0003	0.8669
	+	19		200(9)		
TJ1052	-	20	+20%	200(6)	0.001	0.0096
	+	24		199(31)		
CF1038	-	14	+7.1%	195(0)	<0.0001	0.0084
	+	15		175(0)		
RB660	-	23	-21.1%	200(2)	<0.0001	<0.0001
	+	19		200(13)		
RB1661	-	18	+16.7%	200(1)	<0.0001	<0.0001
	+	21		190(4)		
GR1310	-	16	-18.8%	200(14)	<0.0001	<0.0001
	+	13		200(0)		
DA465	-	25	-24.0%	200(2)	<0.0001	<0.0001
	+	19		200(1)		

Kaplan-Meier survival plots of the lifespan assays were as follows:

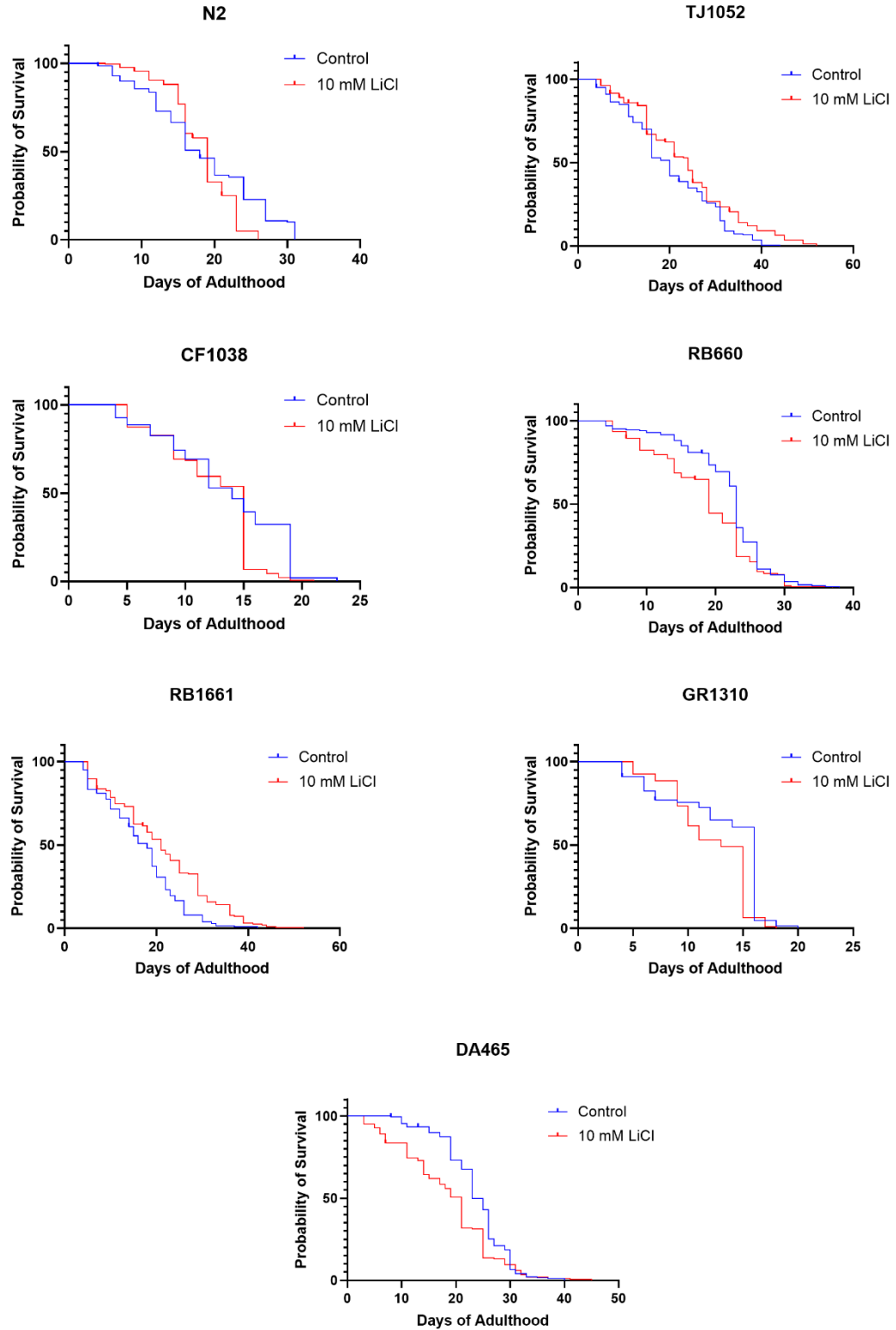


Figure 5: Kaplan-Meier survival plots of lithium-based lifespan assay. Lithium treatment started from L1.

Lithium treatment extended the median lifespans of N2, TJ1052, CF1038, and RB1661 mutant strains. All survival curves were significantly different within their own graphs,

except for N2 according to Gehsan-Breslow test. This explained that the difference in lifespan occurred later in the life stages, but not on the early stages. On the other hand, lithium treatment shortened the median lifespan of RB660, DA465 and GR1310, again with significant p-values according to two different tests.

Upon analysis of these lifespan curves, the rest of the experimental design was focused on NHR-85 mutant RB1661, as it was a target of interest and it was not investigated before.

Stemming from previous results and the fact that all the strains reached adulthood slower on lithium treatment, another lifespan assay was designed to start the treatment at L4 stages of the worms. N2 and RB1661 worms were age synchronized, L1 arrested, and grown on regular NGM plates until their L4 stage, then they were transferred to LiCl plates. Median lifespan results of this experiment were as follows:

Table 7: Median lifespans, sample sizes, and statistical analysis results of lithium-based lifespan assay. Lithium treatment started from L4.

Strain	Lithium Treatment	Median Lifespan (days)	Median Lifespan Change (%)	Sample Size (censored)	Mantel-Cox p-value	Gehan-Breslow p-value
N2	-	14	0	200(28)	0.6503	0.2402
	+	14		200(4)		
RB1661	-	16	+12.5%	200(12)	<0.0001	0.0057
	+	18		199(6)		

Kaplan-Meier survival plots were as follows:

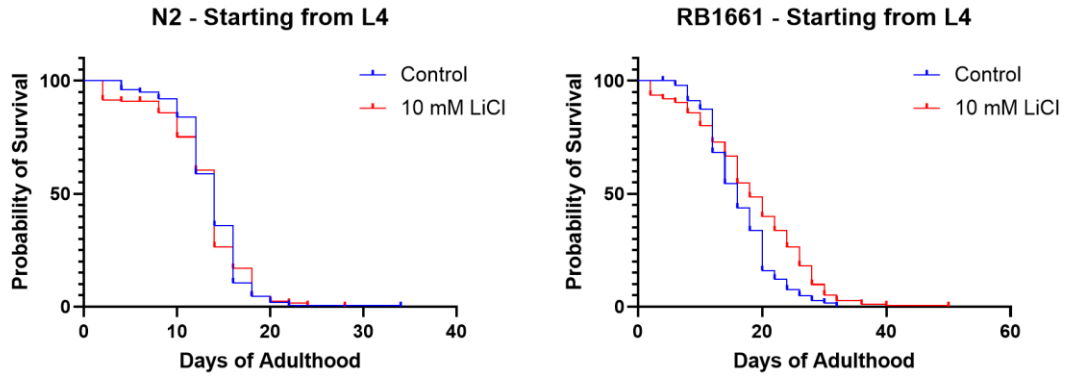


Figure 6: Kaplan-Meier survival plots of lithium-based lifespan assay. Lithium treatment started from L4.

Starting the lithium treatment at a later life stage reverted the lifespan extension effects on N2, and significantly decreased the lifespan extension on RB1661.

1.4.2 Thrashing Assay

For thrashing assays, four experiment groups were prepared: N2 control, N2 lithium treatment, RB1661 control, RB1661 lithium treatment. For all the groups, 40 worms were grown in two separate plates per condition, until first day of adulthood. 10 worms from each plate, as two biological replicates, were then placed on a drop of M9 as explained on chapter 1.3.3.4, and two 30 second videos were recorded for each worm as technical replicates. The videos were then batch-analyzed on ImageJ plugin WrmTrck. For this analysis, videos were first batch-edited into 8-bit greyscale format, then they were passed from a deflicker plugin to eliminate flickering that happens due to the light source of the microscope. The videos were then thresholded to be able to count the bends with Otsu mode, and WrmTrck plugin was run. The analysis looked like the following screenshots:



Figure 7: Video screenshots of a thrashing worm. A full bend is observed in a cycle from left panel to right.

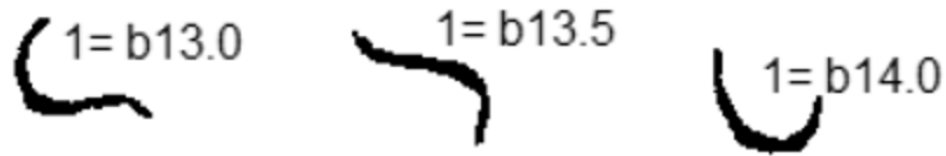


Figure 8: WrmTrck identification of thrashing.

Thrashing counts analyzed by the WrmTrck plugin was analyzed statistically on Graphpad Prism using one way ANOVA with Tukey's multiple comparisons test.

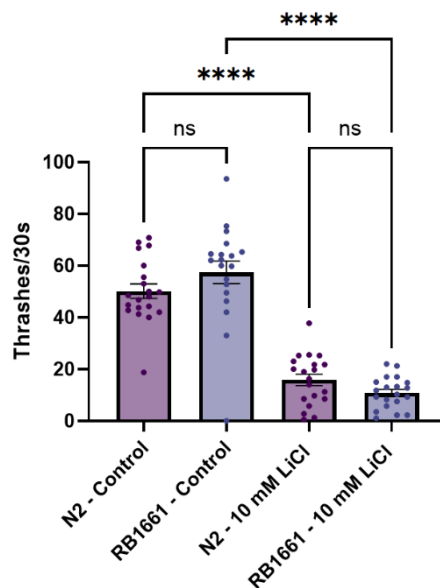


Figure 9: Thrashing assay results. Error bars represent mean with SEM. ns: non-significant, p -values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

Thrash counts were not significantly different between N2 and RB1661, both in control and treatment groups. However, thrash counts significantly decreased upon LiCl treatment, both on N2 and RB1661 groups.

1.4.3 Brood Size Assay

For brood size assay, four experiment groups were prepared: N2 control, N2 lithium treatment, RB1661 control, RB1661 lithium treatment. For all the groups, 15 worms were age synchronized and their brood size were monitored for 5 egg laying days, as described on chapter 1.3.3.5. Monitored brood size was analyzed on Graphpad Prism using one way ANOVA with Tukey's multiple comparisons test.

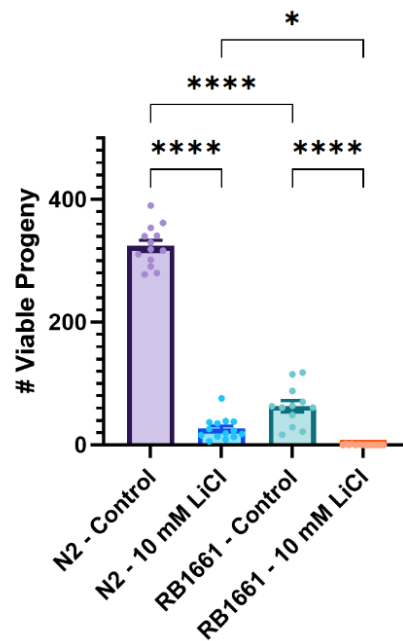


Figure 10: Total brood size assay results. Viable progeny were counted, unhatched eggs were not analyzed. Error bars represent mean with SEM. ns: non-significant, p -values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

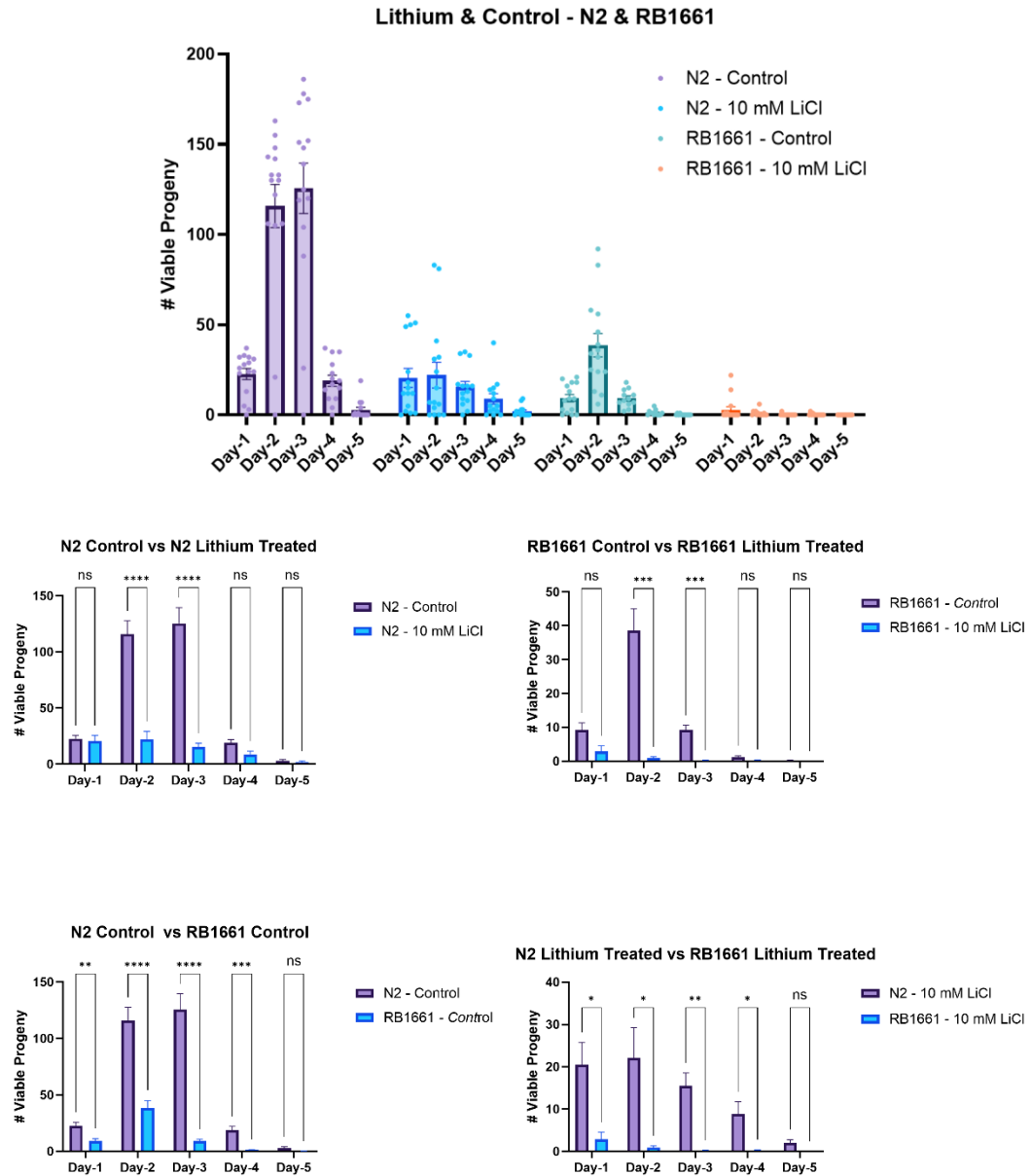


Figure 11: Individual brood size assay results. The same results were used for total brood size assay. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * < 0.05, ** < 0.001, *** < 0.0001, **** < 0.00001

Total brood size of both N2 and RB1661 were significantly decreased upon lithium treatment. However, RB1661 brood size was already significantly lower than N2 without the treatment, and it was almost completely diminished upon treatment.

1.4.4 qRT-PCR Assay

For qRT-PCR assay, four experiment groups were prepared: N2 control, N2 lithium treatment, RB1661 control, RB1661 lithium treatment. For all the groups, 5 different time points were set for L4, Day 1 of adulthood (D1), Day 3 of adulthood (D3), Day 7 of adulthood (D7) and Day 10 of adulthood (D10). For each group and time point, 2 plates with 50 worms were prepared upon age synchronization. 2 Biological replicates were repeated as 2 technical replicates. Lithium treatment was started from L1 stage. Targets were as follows:

Table 8: qRT-PCR targets and their human orthologs.

Target Gene	Human Ortholog
<i>age-1</i>	PI3K
<i>daf-16</i>	FOXO4
<i>daf-2</i>	IGF1R/INSRR
<i>gsk-3</i>	GSK3B
<i>arr-1</i>	ARR1B
<i>bpnt-1</i>	BPNT-1
<i>ssu-1</i>	SULT2B1
<i>lsd-1</i>	KDM1A
<i>nhr-85</i>	NRDR1/Rev-Erb- α
<i>akt-1</i>	AKT-1
<i>hsp-16.2</i>	HSPB9
<i>sod-3</i>	SOD-2

Obtained Ct values were normalized with housekeeping gene *tba-1*, and relative gene expression was calculated as described on chapter 1.3.3.8. The analysis was focused on gene expression analysis within days and strain, which was based on the condition's N2 control Ct values. Statistical analysis was done on Graphpad Prism, using two way ANOVA with Tukey's multiple comparisons test.

1.4.4.1 Stage Based Gene Expression – L4

At L4 stage, gene expression of *age-1* was significantly increased in lithium treated RB1661, and there occurred a significant difference between treated N2 and treated RB1661, which did not occur without the treatment. Expression of *daf-2*, *gsk-3* and *arr-1* was detected to be significantly less on control RB1661 when compared to control N2, and this was observed to be the opposite on treated groups. Expression of *bpnt-1*, *ssu-1*, *lsd-1* and *akt-1* were significantly higher on control N2 groups compared to control RB1661 and treated N2 groups. Rest of the data remained non-significant.

N2 vs RB1661 - Control & 10 mM Lithium Treatment - L4

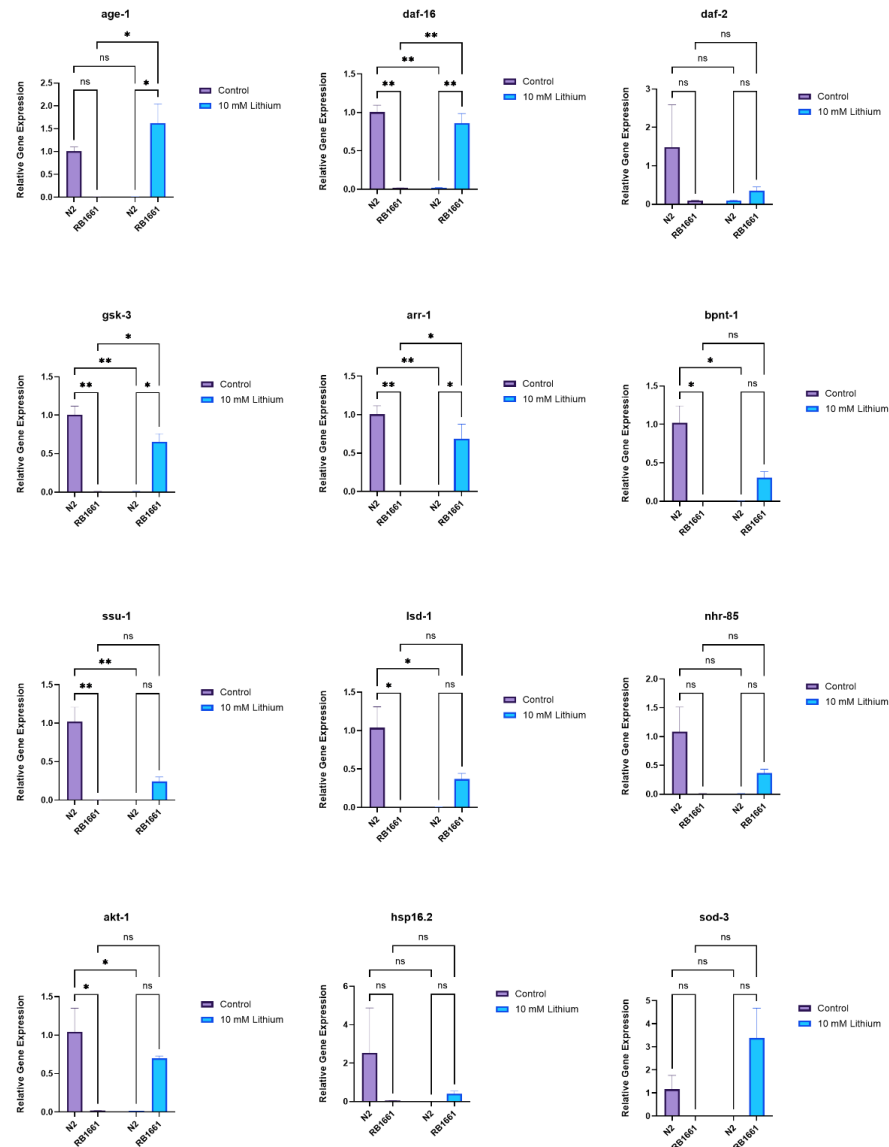


Figure 12: L4 N2 vs L4 RB1661 qRT-PCR results. Relative gene expressions were calculated by using L4 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001 .

1.4.4.2 Stage Based Gene Expression – D1

Only significant differences at D1 stage were observed on *age-1* gene, where the data showed increased expression on control RB1661 compared to control N2. Lithium treatment decreased *age-1* expression on RB1661 but not on N2.

N2 vs RB1661 - Control & 10 mM Lithium Treatment - D1

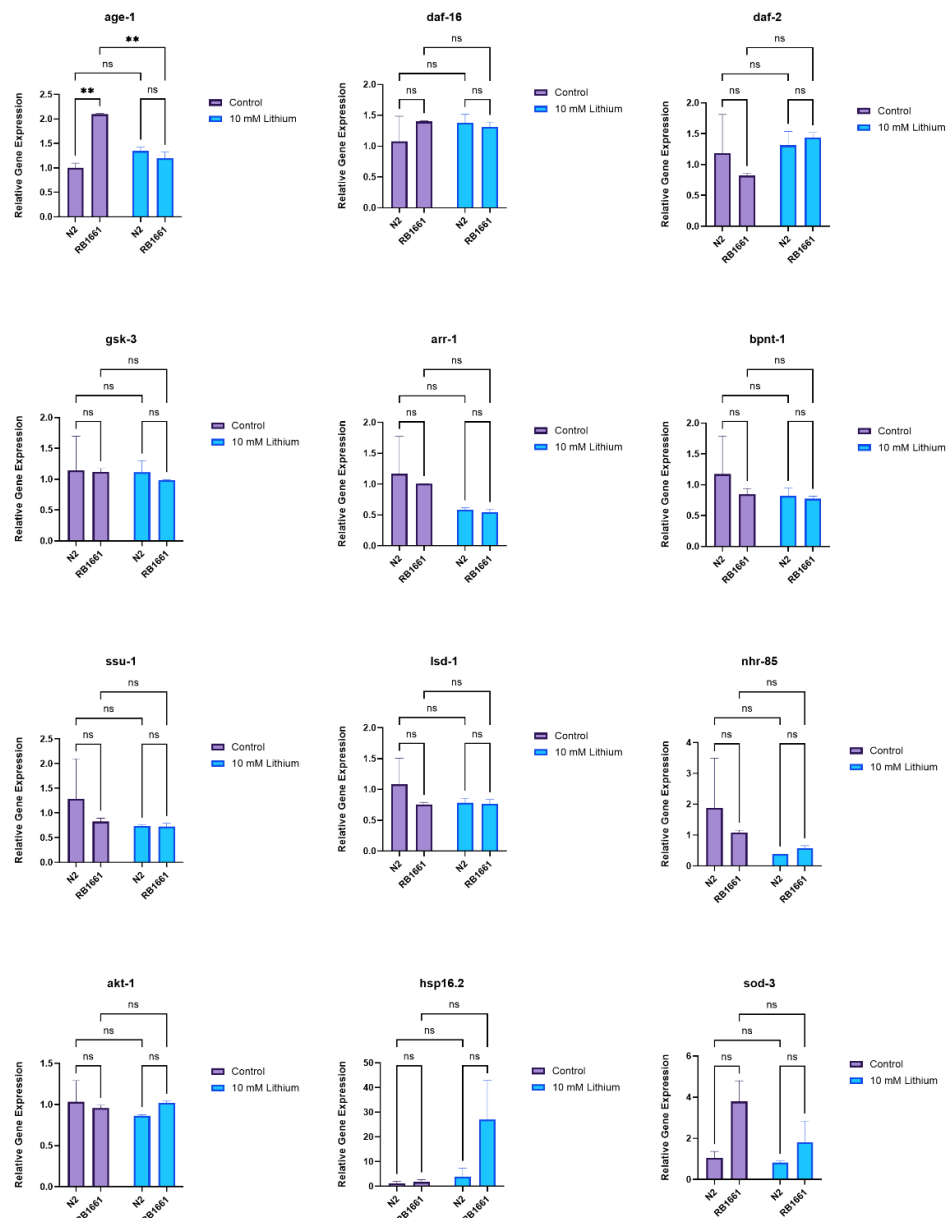


*Figure 13: D1 N2 vs D1 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D1 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001*

1.4.4.3 Stage Based Gene Expression – D3

On D3, *nhr-85* expression was significantly higher on control N2 than control RB1661 and control N2 groups. Expression of *akt-1* was significantly higher on control RB1661 when compared to control N2. This was also the case with lithium treated groups, and the lithium treatment increased the *akt-1* expression on N2. *hsp16.2* expression was significantly higher on control RB1661 when compared to control N2, and lithium treatment decreased the expression on RB1661. Finally, *sod-3* expression was significantly lower on N2 than RB1661, in both control and lithium treatment groups.

N2 vs RB1661 - Control & 10 mM Lithium Treatment - D3

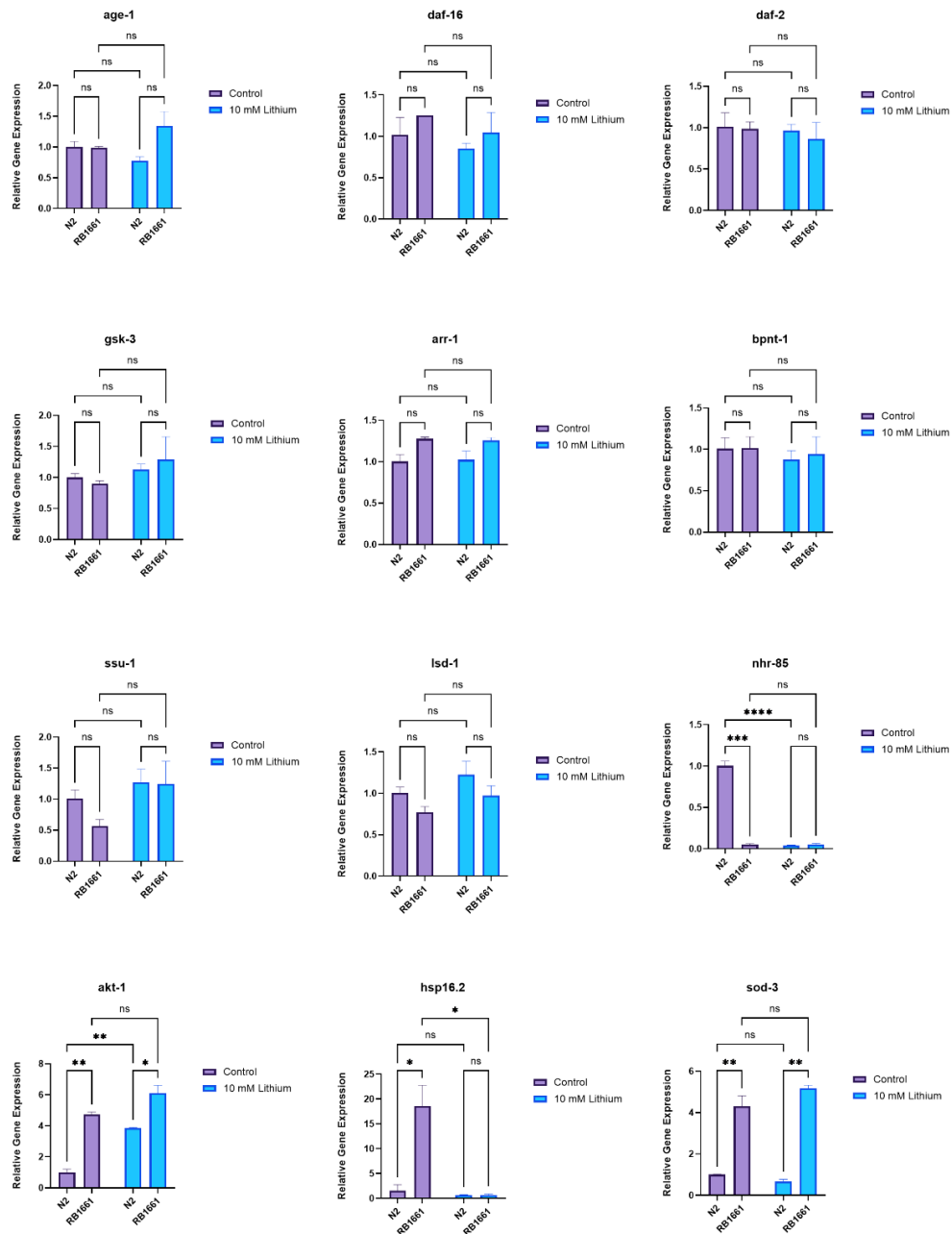


Figure 14: D3 N2 vs D3 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D3 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

1.4.4.4 Stage Based Gene Expression – D7

At D7, *daf-16* expression was significantly decreased upon lithium treatment on RB1661. Expressions of *daf-2*, *bpnt-1* and *lsd-1* were significantly decreased upon

lithium treatment on N2. Expression of *arr-1* was significantly decreased upon lithium treatment on both N2 and RB1661; it was expressed higher on treated N2 when compared to treated RB1661. Expression of *sod-3* was significantly higher on both control and treatment N2 groups when compared to control and treatment RB1661 groups, respectively. Lithium treatment also decreased *sod-3* expression on RB1661.

N2 vs RB1661 - Control & 10 mM Lithium Treatment - D7

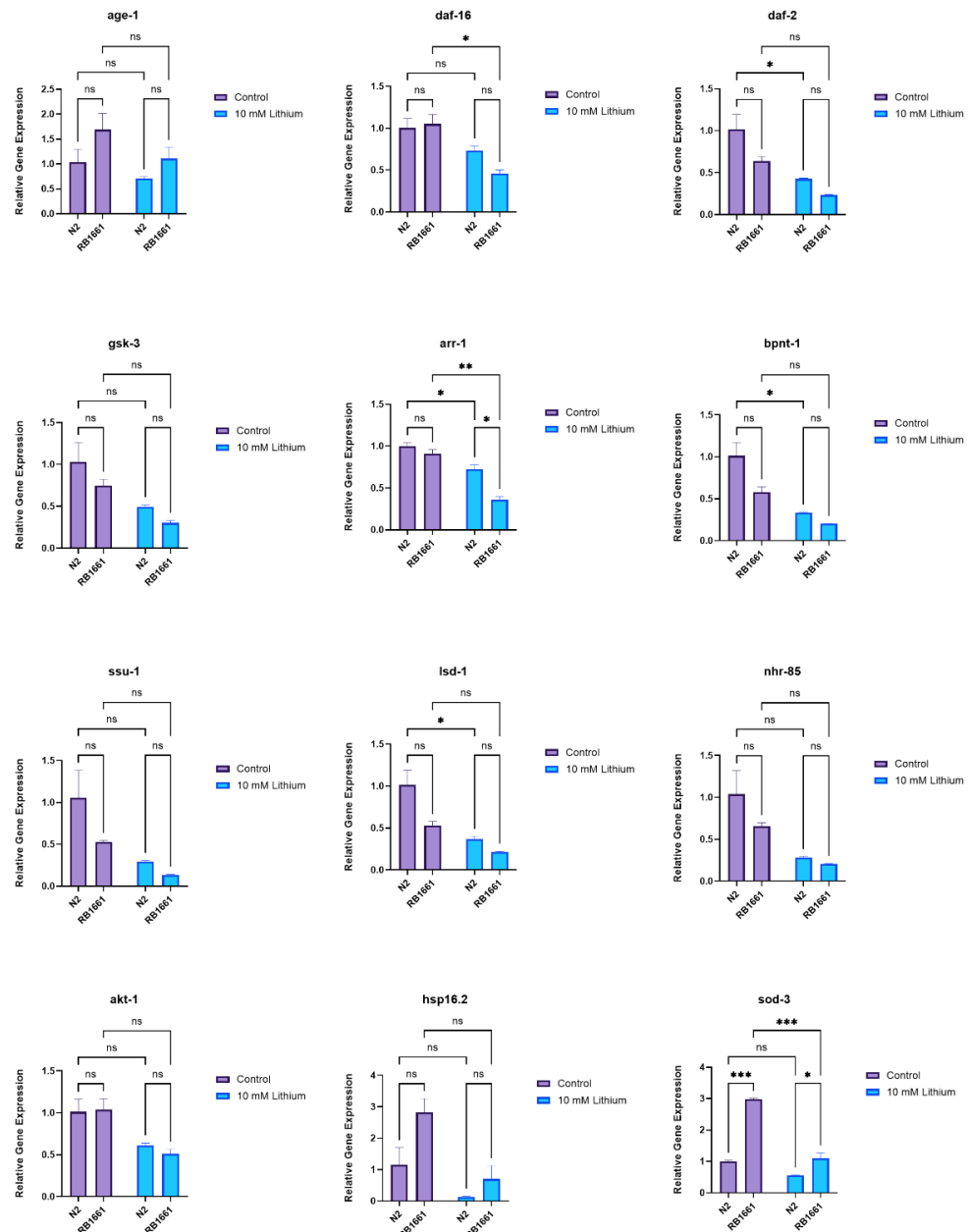


Figure 15: D7 N2 vs D7 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D7 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

1.4.4.5 Stage Based Gene Expression – D10

At D10, no significant changes were detected on the expressions of targeted genes.

N2 vs RB1661 - Control & 10 mM Lithium Treatment - D10

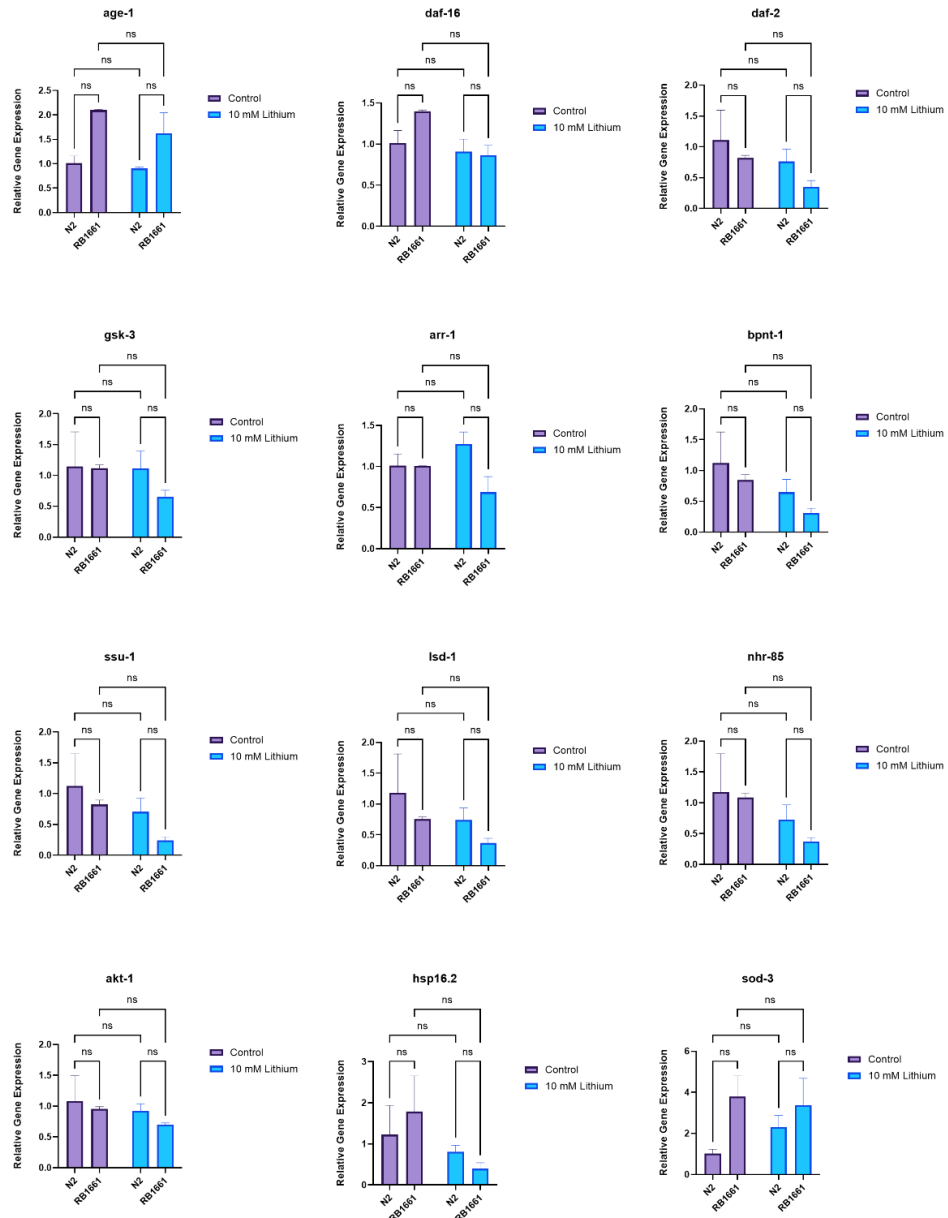


Figure 16: D10 N2 vs D10 RB1661 qRT-PCR results. Relative gene expressions were calculated by using D10 N2 control Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

1.4.5 Confocal Microscopy Imaging

JDW628 strain was grown on lithium and control plates until first day of adulthood and imaged with Leica SP8 confocal microscope as explained on chapter 1.3.3.9. In addition to fluorescent imaging, the worms were also imaged with DIC mode. White light laser was utilized at 45% power for fluorescent imaging, and the following images were acquired.

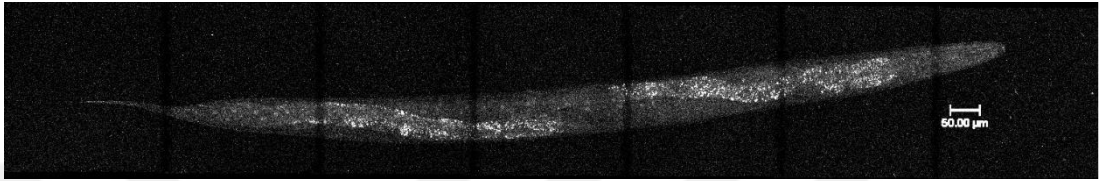


Figure 17: D1 JDW628(nhr-85::GFP) worm grown on control NGM. GFP was targeted with Alexa488 preset. Scale bar: 50μm.

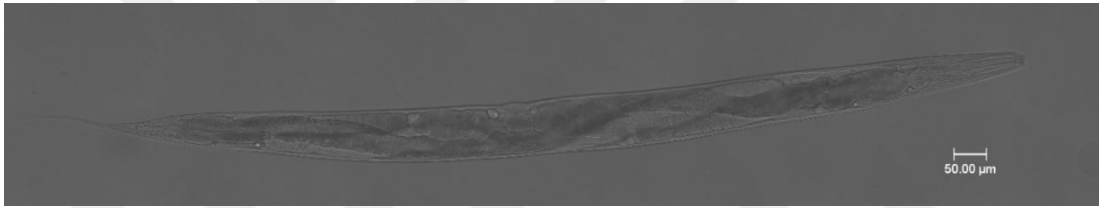


Figure 18: D1 JDW628(nrh85::GFP) worm grown on control NGM, observed under DIC mode. Scale bar: 50 μm.

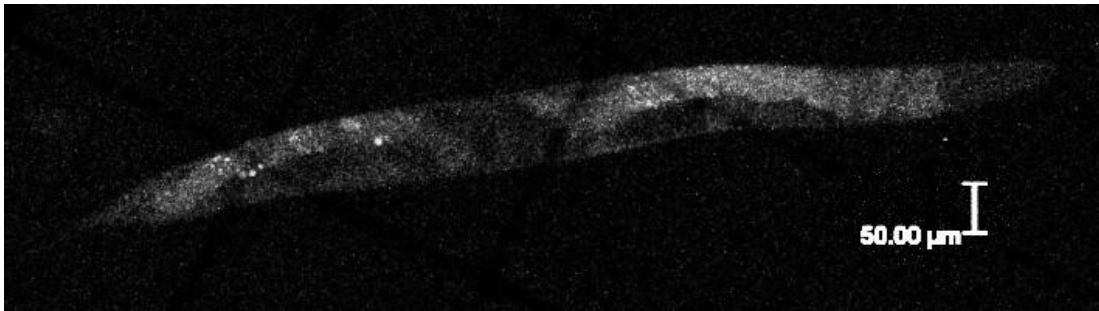


Figure 19: D1 JDW628(nhr-85::GFP) worm grown on 10 mM LiCl NGM. GFP was targeted with Alexa488 preset. Scale bar: 50μm.

Total fluorescence intensity was measured via ImageJ. Intensity of 3 background samples were averaged, multiplied with the total area of the subject and subtracted from the total intensity of the subject to obtain corrected total fluorescence.

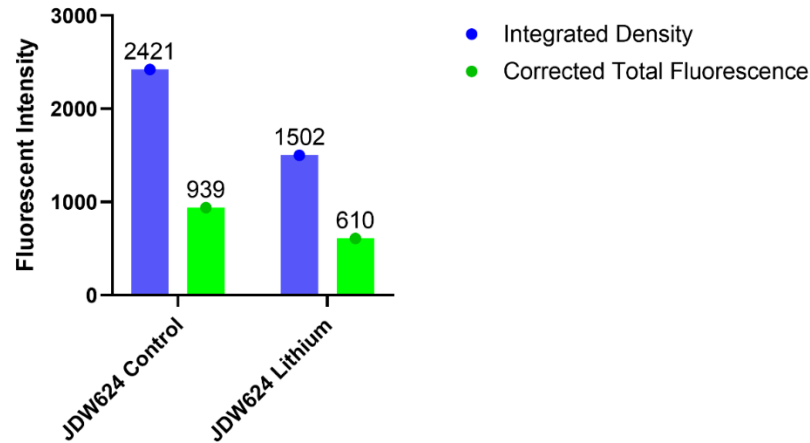


Figure 20: Integrated fluorescence density and corrected total fluorescence calculated for the JDW628 worms

1.5 Conclusion

The aim of this chapter was to investigate the effects of lithium chloride treatment on an understudied component of the *C. elegans* GSK-3 – DAF-2 pathway, which is NHR-85. The hypothesis was that lithium treatment would extend the NHR-85 mutant worm's life span.

In this context, a lifespan experiment was designed with multiple mutant strains that is focused on GSK-3 – DAF-2 pathway. The lifespan experiment revealed that lithium treatment on NHR-85 mutation indeed extended the worm's median lifespan by 16.7%. Additionally, age-1 mutant's median lifespan was also found to be increased by 20% upon lithium treatment. Interestingly, *akt-1* mutants showed a decrease in median lifespan by 18.75%.

Upon observing that lithium treatment resulted in slower growth into adulthood, an assay was designed to test whether the lifespan extension effect of lithium would decrease if the treatment was started at L4 stage. As a result, lithium's lifespan extension decreased on both WT and NHR-85 mutants, suggesting that lithium may be slowing down the growth instead of extending lifespan.

To test this further, brood size and thrashing assays were done on both WT and NHR-85 mutants. NHR-85 mutants' brood size were already significantly lower than WT before lithium treatment. Still, the lithium treatment wiped out the fertility of NHR-85 worms and significantly decreased the brood size of the wild type. Thrashing assays also showed the decreasing effect of lithium on motility.

To observe how the gene expression was affected upon lithium treatment on both WT and NHR-85 mutants, a time lapse qRT-PCR assay was designed. Lithium treatment was shown to decrease *age-1*, *daf-16* and *gsk-3* expressions on N2 L4. This effect was the exact opposite on NHR-85 mutants, which showed significant increase of the same genes' expression upon treatment at L4 life stage. Expression of *nhr-85* gene was shown to be decreased upon lithium treatment on L4, D3 and D7 WT worms, suggesting that lithium downregulates the expression. Rest of the relative gene expression data was not pointing out to a clear expression pattern.

Finally, GFP labelled NHR-85 protein containing mutant worms were subjected to lithium treatment, and the fluorescent signal was shown to be decreased. As the NHR-85 mutant was not a deletion, qRT-PCR was not able to pinpoint decreased protein levels. Therefore, this decrease was a clear identifier that lithium somehow inhibits NHR-85.

In conclusion, the hypothesis was confirmed as lithium treatment extended the median life span of NHR-85 mutant worms. However, rest of the experiments pointed out to the fact that in this case, lifespan extension may be costly as two essential functions of the worm, reproductivity and motility, were significantly decreased upon treatment.

Chapter 2: Investigating the effects of age-accumulated circular RNA deletions on *Caenorhabditis elegans* longevity

2.1 Introduction

2.1.1 Circular RNAs

With the completion of the Human Genome Project, it was determined that only about 2% of DNA consists of protein-coding genes, while the remaining 98% comprises various regions such as intron regions and non-coding RNAs [77]. Although it was thought for many years that non-coding RNAs had no function, it has been discovered that microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) perform various tasks within organisms. For example, miRNAs affect many transcription and translation processes [78], lncRNAs act as scaffolds in protein interactions [79], and circRNAs function in transcription by acting as miRNA sponges [80] (Xing et al., 2017; Soudeh Ghafouri-Fard et al., 2021; Knupp and Miura, 2018).

Among the non-canonical RNAs circular RNAs are a relatively new class of RNAs with only a few known functions [80]. They were initially thought as transcriptional byproducts [81], and with more research, more functions were understood. Their primary specification is their shape, as their name suggests. They have a covalently closed loop shape, yet they are different than a plasmid as they are single stranded RNA molecules [82]. Their unique shape makes them quite hard to degrade with RNase enzymes, as most of them require an open ending to start degradation. As a result, they are highly stable molecules, and this makes them accumulate with aging in many organisms including mice and worms [83].

Among the known functions of circRNAs, whose full range of functions has not yet been fully elucidated, are serving as miRNA sponges (binding to miRNAs through base pairing and transporting and silencing them within the cell), acting as protein

scaffolds (forming proteins around them), and functioning as transcriptional regulators [84]. In addition to these functions, protein-coding circular RNAs have recently been discovered, and number of research focusing on this area is increasing [85].

2.1.2 Biogenesis of Circular RNAs

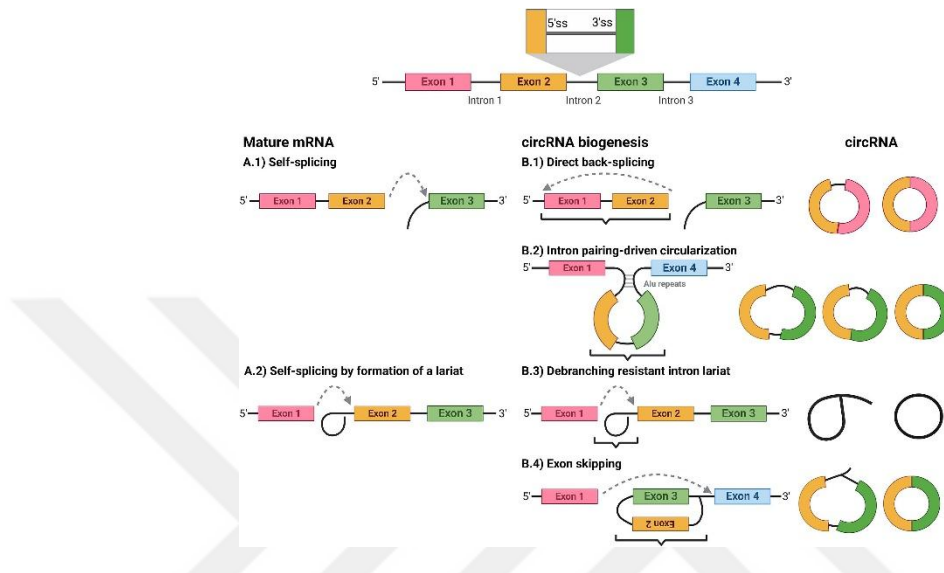


Figure 21: Circular RNA Biogenesis [103].

Circular RNA biogenesis mainly stems from an event called back-splicing [82]. This event can be where a part of a pre-mRNA folds towards its 5' side, and matches with itself, then the formed circle gets cleaved and released as a circular RNA. This event can occur in various ways. First of these is via an intronic region that reversely complements its matching region on the back folded site, which is usually another intron. These regions are called “reverse complementary matches” or RCMs, and by reverse complementary base pairing, they form a transient loop structure, which is then spliced into a covalent loop [86]. Another way of back-splicing is through direct back-splicing, which is not exactly understood. Another way of circular RNA formation is through splicing lariats. Lariats can prevent some exons to be included in the mature mRNA and keep them in their looped structures, which is called “exon skipping”. Subsequent splicing of introns from the lariats can result in a circular RNA [87]. In

some cases, introns can stay within the circular RNA structure, and even some circular RNAs can be completely formed of intronic regions [88].

2.1.3 Circular RNAs and Aging

CircRNAs are thought to have a clear connection with aging, as various RNA sequencing studies have observed that circular RNAs accumulate with aging in different organisms including mice [89], *D. melanogaster* [90], and *C. elegans* [91]. This accumulation could possibly arise from two different reasons. First possibility is that, with aging, as alternative splicing starts occurring more often, number of back splicing events could be increasing, resulting in higher number of new circular RNAs [92]. The second possibility is relying on the fact that circular RNAs are very difficult to degrade, so the organisms can't find a way to digest these RNAs, leading to their accumulation [93]. The accumulation could result in increased function of circular RNAs, for example, increased inhibition of small RNA mediated translational regulation [94]. Finally, this stability of circular RNAs could be extremely useful for treatment of genetic diseases, if possible translation mechanisms are optimized and these molecules can be turned into targeted vaccines [95].

2.1.4 Circular RNAs of *C. elegans*

C. elegans is a widely used model for RNA research. There are many small RNA related pathways were discovered in worm research, and as previously mentioned, RNAi was discovered on *C. elegans* [18]. Consequently, there are many available methods and databases for *C. elegans* RNA research, creating ways of researching new types of RNA. Circular RNAs, though, were not as investigated as other types of RNAs on *C. elegans*. Although there are more than 1000 identified worm circular RNAs [86], their biogenesis and functions were not as investigated as other models such as mice.

One study on *C. elegans* performed a circular RNA specific RNA-sequencing and showed that from L4 stage to D10 of adulthood, circular RNAs accumulate through the worm [91]. The circular RNAs found to accumulate in this study have been linked to *C. elegans* aging in different studies. For example, the accumulation of *crh-1* circRNAs shortens the lifespan of the organism [96], the accumulation of *cpsf-1* circRNAs exacerbates radiation-induced stress damage [97], and inhibiting the accumulation of *zip-2* gene circRNAs extends the lifespan of a Parkinson's model *C. elegans* strain and reduces α -syn accumulation [98]. Many circular RNAs observed to accumulate at much higher rates in the aforementioned RNA sequencing data have not been studied in *C. elegans*.

2.2 Aim of This Study

The initial aim of this study was to identify the highest accumulating circular RNAs of the *C. elegans*. After identification, it was aimed to silence these RNAs without diminishing their linear mRNA expressions. To do this, it was aimed to identify these circular RNAs' RCM regions that promote their back splicing and target those regions via CRISPR/Cas9. After deletion, it was aimed to see whether the accumulation of these circular RNAs were contributing to worm aging, so a lifespan analysis was designed, combined with behavior assays and a qRT-PCR assay to analyze aging markers.

The hypothesis of this experiment was that the deletion of the most accumulated circular RNAs would result in an extension of lifespan.

2.3 Materials & Methods

Only the materials and the methods that were not related to and not mentioned in Chapter 1 will be discussed in this part.

2.3.1 Materials

2.3.1.1 *C. elegans* Strains

Table 9: Strains used in chapter 2 of this study. N2 & BB2 strains were donated by CGC, TM4439 strain was purchased from NBRP Japan, TUR015 and TUR016 strains are created on this study.

Strain Name	Genotype (allele)	Mutant Type
N2	Wild Type (Bristol)	-
BB2	<i>adr-1 (gv6)</i>	Deletion
TM4439	<i>fust-1(tm4439)</i>	Deletion
TUR015	<i>pqn-41(tur015)</i>	Deletion
TUR016	<i>afd-1(tur016)</i>	Deletion

2.3.1.2 Bacterial Strains

Competent *E. coli* strain DH5 α was used in this part of the study for transfection.

2.3.1.3 Equipment and Tools

Table 10: Equipment used in chapter 2 of this study.

Equipment/Tool	Brand	Model Number
Imaging system	Bio rad	Chemidoc Universal Hood III
Gel electrophoresis tank	E-C	EC370M
Power supply	Bio Rad	PowerPac Basic
Inverted microscope	Zeiss	Axio Vert.A1
Micromanipulator	Narishige	MMO-4

2.3.1.4 Chemicals and Reagents

Table 11: Chemicals and reagents used in chapter 2 of this study.

Chemical/Reagent	Brand	Catalog Number
Halocarbon oil	Sigma-Aldrich	9002-83-9
Potassium Chloride	Merck	104936
Glacial Acetic Acid	Merck	100063
20000X SAFE DNA Stain	Eco Tech	SDGS1
Agarose	Biomax	HS-8000
Kanamycin	Gibco	15160047

2.3.1.5 Enzymes and Kits

Table 12: Enzymes and kits used in chapter 2 of this study.

Chemical/Reagent	Brand	Catalog Number
EasyPure Plasmid Miniprep Kit	TransGen	EM101-02
2x Taq DNA Polymerase RED	Ampliqon	A180301
EasyTaq DNA Polymerase	TransGen	AP111-01
High Pure dNTPs	TransGen	AD101-01
BsaI-HF [®] v2	NEB	R3733S

2.3.2 Buffer and Media Preparations

10X TAE Buffer

48.5g Tris

11.4 mL Glacial Acetic Acid

20 mL 0.5M EDTA (pH: 8)

Dissolved in 1L of total ddH₂O.

1% Agarose Gel

0.33g Agarose

33 mL 1X TAE

Are mixed thoroughly and microwaved to obtain a clear solution. 1.67 µL 20000X

SAFE DNA Stain is added to the solution and it is poured into a gel cast.

DNA Lysis Buffer

4 mL 500 mM KCl

4 mL 100 mM Tris

2.4 mL 25 mM MgCl₂

Dissolved in 40 mL of total ddH₂O. Add 20 mg/mL of proteinase K before use.

2.3.3 Methods

2.3.3.1 Circular RNA Filtering

C. elegans circular RNAs were shown to be accumulating with aging via circular RNA specific RNA sequencing. This data was publicly available, so the data was filtered to identify the highest accumulating circular RNAs. The data before filtering looked like the following:

circID	TOTAL_REA	L4_TPM	D-1_TPM	D-7_TPM	D-10_TPM	gene_name	chr11_loci	stranc	WBID	L4_FPKM	D-1_FPKM	D-7_FPKM	D-10_FPKM
cel_circ_0000001	21	0.039948	0.174954	0.109221	0.109434	Y48G1C.5	chr1:55889-56293	-	WBGene0002167E	2.974760	4.821910	0.848557	1.157450
cel_circ_0000002	92	0.219568	0.096511	0.463135	0.570651	Y48G1C.5	chr1:59044-60236	-	WBGene0002167E	2.974760	4.821910	0.848557	1.157450
cel_circ_0000010	17	0.023327	0.089629	0.055238	0.197619	Y95B8A.8	chr1:865730-866858	+	WBGene0002238E	19.386000	23.641900	35.647200	35.213800
cel_circ_0000014	96	0.233562	0.304165	0.702127	0.807460	mlg-1	chr1:952676-953092	-	WBGene0000323E	3.964210	3.722440	3.129120	3.194450
cel_circ_0000016	20	0.051757	0.066726	0.141932	0.193167	haf-6	chr1:1167651-1167976	-	WBGene0000181E	38.247100	27.301400	15.402200	14.128800
cel_circ_0000017	18	0.028429	0.066726	0.139278	0.155306	Y48G8A.13	chr1:1225175-1225535	-	WBGene0002169E	29.855800	26.278700	41.579100	48.873800
cel_circ_0000018	44	0.267093	0.238379	0.332970	0.094866	K12C11.3	chr1:1335995-1336299	+	WBGene0001967A	7.990290	6.174010	2.781130	3.527440
cel_circ_0000022	228	0.530110	0.411922	1.581734	2.308619	Y92H12B.3	chr1:1420210-1421308	+	WBGene0002236E	33.327400	34.880500	47.453500	54.068600
cel_circ_0000027	509	2.281528	2.540134	2.938282	3.330891	Y92H12A.4	chr1:1524297-1524643	+	WBGene0002236E	25.966400	24.787300	27.760600	25.402600
cel_circ_0000028	12	0.016621	0.021927	0.056637	0.165298	Y92H12A.4	chr1:1525844-1532397	+	WBGene0002236E	25.966400	24.787300	27.760600	25.402600
cel_circ_0000029	136	0.228750	0.349909	0.964760	1.374989	Y92H12A.2	chr1:1544284-1544490	+	WBGene0002235E	27.235900	23.999100	29.606500	34.362800
cel_circ_0000030	22	0.075084	0.083473	0.183755	0.112744	mppa-1	chr1:1748286-1748695	-	WBGene0002215E	21.095000	11.449900	4.324080	3.198710
cel_circ_0000031	30	0.000000	0.110113	0.213812	0.310674	lin-65	chr1:1763588-1764896	+	WBGene0002214E	36.850600	48.078100	41.033600	39.604800
cel_circ_0000032	172	0.307041	0.611160	1.298161	1.406168	Y71G12B.2	chr1:1817300-1817612	+	WBGene0002214E	4.398380	5.864780	7.580310	7.585410
cel_circ_0000035	318	0.290131	0.607386	2.573997	3.185693	gap-3	chr1:1999952-2001193	-	WBGene0002123E	9.306990	9.332980	16.704600	21.335900
cel_circ_0000037	31	0.039948	0.065314	0.230456	0.313673	ari-13	chr1:2066703-2067238	+	WBGene0002134E	2.062000	2.949530	5.914900	6.496570
cel_circ_0000038	1218	1.830150	3.418721	9.282171	11.203063	afu-1	chr1:2173753-2174984	+	WBGene0002100E	21.034500	18.352000	31.433300	36.240500
cel_circ_0000040	354	0.853772	1.062689	2.333361	3.305501	abtm-1	chr1:2478227-2478800	-	WBGene0002228E	18.622500	17.842400	12.644100	13.040900
cel_circ_0000046	37	0.080186	0.154439	0.342330	0.197681	cogc-1	chr1:3253862-3254447	+	WBGene0000325E	16.475600	13.538400	28.869300	33.539600
cel_circ_0000048	82	0.135150	0.174954	0.644091	0.775970	lpd-3	chr1:3598619-3598974	-	WBGene0000306E	20.080800	17.134900	25.569600	28.409100
cel_circ_0000053	47	0.337368	0.350376	0.197745	0.165235	nab-1	chr1:4231142-4232703	+	WBGene0000351E	22.733000	15.669500	16.907300	20.672300
cel_circ_0000054	13	0.000000	0.000000	0.111874	0.158678	eri-6	chr1:4465737-4466008	+	WBGene0001656E	10.964800	13.639400	14.795200	10.322900
cel_circ_0000045	36	0.028429	0.066726	0.139278	0.155306	Y48G8A.13	chr1:1225175-1225535	-	WBGene0002169E	29.855800	26.278700	41.579100	48.873800

Figure 22: Supplementary table S3 of [91].

Since the aim was to look for accumulating circular RNAs, three filters were applied to the data. Three filters were:

1 – L4_circ – D10_circ – Smaller values mean more circular RNA accumulation with aging

2 – D10_linear / D10_circ – Smaller values mean higher relative circRNA expression in aged worm

3 – Total_linear / Total_circ – Smaller values mean higher relative circRNA expression in total

Filter 1 was the most important filter for the research question, so when by applying it and reordering the data in descending order, the following was obtained:

Supplementary Table S3 of Cortés-López et. Al

circID	TOTAL_RI	L4_TPM	D-1_TPM	D-7_TPM	D-10_TPM	gene_name	L4_circ - D10_circ	D10_linear/D10_circ	Total Linear / Total Circ
cel_circ_0000038	1218	1.830150	3.418721	9.282171	11.203063	afd-1	-9.372913	3.234874	4.160250
cel_circ_0000333	859	3.053512	3.424778	5.225377	6.904483	pqn-41	-3.850971	4.960487	5.556458
cel_circ_new_0001917	463	0.482140	1.318026	3.930198	4.021155	zip-3	-3.539015	4.278522	12.003074
cel_circ_new_0000735	364	1.011517	1.320182	2.393040	3.099988	afd-1	-2.088471	11.690530	13.682304
cel_circ_0000334	510	1.657529	2.160977	3.443427	3.699007	pqn-41	-2.041478	9.259134	9.433077
cel_circ_0000764	191	0.178598	0.280354	1.748949	1.798927	pqn-41	-1.620329	19.038907	25.804800
cel_circ_new_0000732	127	0.120134	0.194525	1.095890	1.301186	afd-1	-1.181051	27.851907	39.480371
cel_circ_0000747	112	0.023327	0.128744	1.040260	1.145381	afd-1	-1.122054	31.640560	45.797041
cel_circ_0000335	146	0.268698	0.529099	1.082190	1.283121	pqn-41	-1.014423	26.692419	32.687914
cel_circ_0001294	86	0.193614	0.129211	0.713173	0.782589	pqn-41	-0.588975	43.764466	56.854786
cel_circ_new_0003180	39	0.039948	0.084885	0.294375	0.380794	pqn-41	-0.340846	89.942525	129.243875
cel_circ_0001353	33	0.000000	0.044326	0.332394	0.303494	pqn-41	-0.303494	112.850872	152.004094
cel_circ_new_0000737	29	0.028429	0.020515	0.248354	0.317045	afd-1	-0.288615	114.307206	174.267961
cel_circ_new_0000742	21	0.000000	0.044799	0.162628	0.250172	afd-1	-0.250172	144.862117	233.960862
cel_circ_0000955	41	0.016621	0.195937	0.367656	0.250235	pqn-41	-0.233614	136.869920	124.505616
cel_circ_new_0003178	54	0.169996	0.172126	0.397423	0.370554	pqn-41	-0.200557	92.428193	93.140748
cel_circ_0000908	14	0.016621	0.042442	0.079987	0.158554	pqn-41	-0.141933	216.012856	347.426607
cel_circ_new_0003177	23	0.023327	0.043854	0.267220	0.155306	pqn-41	-0.131979	220.529664	211.137093
cel_circ_new_0000736	13	0.028429	0.000000	0.101938	0.155119	afd-1	-0.126690	233.630013	375.009873
cel_circ_new_0003183	13	0.000000	0.020515	0.157176	0.088247	pqn-41	-0.088247	388.111438	388.795662
cel_circ_0000932	20	0.056859	0.089125	0.181925	0.109434	afd-1	-0.052576	331.161963	244.796826

Figure 23: Filtered data from supplementary table S3 of [91].

This data suggested that the circular RNAs of *afd-1*, *pqn-41*, and *afd-1* were the highest accumulating with circular RNAs of *C. elegans* during aging. The other two filters were also supportive of this claim. Therefore, these 3 genes and their circular RNAs were selected as the targets.

2.3.3.2 RCM Detection

These circular RNAs were not studied before, therefore the potential RCM regions for back-splicing for these circular RNAs were not identified. Previous research papers utilized a publicly available software to select introns for potential back splicing events and RCM detection, yet these algorithms were not supported anymore. Therefore, intron regions were manually selected by examining the exons of these circular RNAs, and then, a python script was written to determine potential RCM regions (See: Appendix A). An example input and output of the code are as follows:

2.3.3.3 CRISPR/Cas9 gRNA Designs

Once the RCM regions were identified, the intronic regions that covered the RCM regions were uploaded to the online gRNA design tool CHOPCHOP. This tool analyzes the nGG sites for gRNA designs and assigns a quality score to the potential gRNAs. By filtering the CHOPCHOP results, gRNA couples that targeted the RCM regions were selected for deletion.

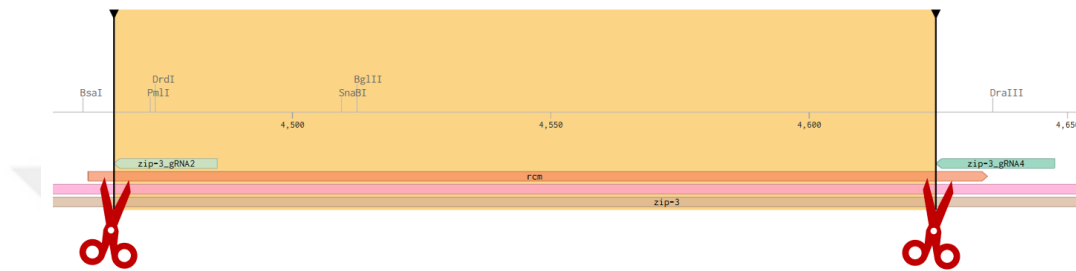


Figure 26: Representative image for CRISPR/Cas9 deletions.

CRISPR/Cas9 deletion on *C. elegans* is based on gRNA cloned tracrRNA coupled plasmid injections to its germline, together with Cas9 enzyme coding plasmid and an injection marker. The worm then translates the Cas9 enzyme and transcribes the gRNA, which can lead to CRISPR/Cas9 deletions on the next generation. Cas9 plasmids, injection markers and tracrRNA coupled empty gRNA vectors are commercially available, so the next step is to clone a double stranded DNA of the designed gRNAs into the gRNA vector. To do that, the following gRNA oligomers were ordered:

Table 14: gRNA ssDNA oligo sequences.

gRNA	5'-3' DNA sequence
afd-1_gRNA1-sense	TCTTGGTTGTATTGTCTAAATGATG
afd-1_gRNA1-antisense	AAACCATCATTAGACAATACAACC
afd-1_gRNA2-sense	TCTTGCCATTTTAGCACAAAATAAA
afd-1_gRNA2-antisense	AAACTTTATTTTGTGCTAAAATGGC
pqn-41_gRNA1-sense	TCTTGCTATCTGGATGGTTTTCGCA
pqn-41_gRNA1-antisense	AAACTGCGAAAACCATCCAGATAGC
pqn-41_gRNA2-sense	TCTTGGAGAGAATCGACACAGCGCC
pqn-41_gRNA2-antisense	AAACGGCGCTGTGTCGATTCTCTCC

zip-3_gRNA1-sense	TCTTGGATTCAATCAGTCTCACACA
zip-3_gRNA1-antisense	AAACTGTGTGAGACTGATTGAATCC
zip-3_gRNA2-sense	TCTTGGCTGACTGACCACGTGGTCT
zip-3_gRNA2-antisense	AAACAGACCACGTGGTCAGTCAGCC

These oligos contained a cleaved site for BsaI restriction enzyme, as the aim was to extend them to double strand DNA molecules and insert into the gRNA vector.

2.3.3.4 gRNA Cloning

The ordered ssDNA gRNA oligo sequences were required to anneal. So; 1 μ L sense, 1 μ L antisense oligo sequences were combined with 2 μ L DNA ligase buffer and 6 μ L nuclease free water. The 10 μ L test tubes were then placed into a thermal cycler that starts at 95°C, then gradually cools down to 4°C with cooling 1 degree per 10 seconds. This results in annealed dsDNA gRNA molecules with flanking sites for BsaI.

Next step is to insert the annealed oligos into the gRNA vector. To do that, the plasmid vector is also required to be digested with BsaI, to create an entry site for gRNA oligos to the vector. To do so, the following ingredients were collected in a test tube: 1 μ g (3 μ L) pRB1017 vector, 1 μ L aligned oligos, 2 μ L T4 DNA ligase buffer, 0.5 μ L T4 DNA ligase, and 13.5 μ L nuclease free water. The test tubes were then placed into a thermal cycler for both *BsaI* digestion and oligo ligation. Conditions were 60 minutes at 37°C, 5 minutes at 50°C and 20 minutes at 65°C, followed by a cool down to 4°C.

2.3.3.5 DH5 α Transformation of pRB1017

Now that the gRNA cassette was cloned into the gRNA expression plasmid, the plasmid was needed to be produced in mass amounts for microinjection. For this, the plasmid was needed to be transformed into competent E. coli strain DH5 α . 1 μ L the cloned vectors were combined with 100 μ L competent DH5 α . A control tube with only the bacteria was also prepared. The tubes were then incubated on ice for 30 minutes,

followed by a 2-minute heat shock at 42°C on a water bath. After two minutes, the tubes are immediately placed back on ice, and the tubes were added with 900 µL LB Broth and placed on 37°C with shaking for initial growth for 30 minutes. After 30 minutes, the cells were spread on kanamycin LB agar plates. Since pRB1017 contained kanamycin resistance, but DH5α did not, a growth on the plates would mean successful transformation. Plates were left overnight for incubation.

2.3.3.6 Colony PCR

After confirming growth on kanamycin LB agar plates, to validate the insertion of the gRNA cassette, colony PCR was required. For this, firstly, a colony for each gRNA was needed to be grown in LB. For this, single colonies were picked and grown on LB broth for at least 4 hours. Afterwards, by using TransGen EasyPure plasmid miniprep kit, plasmids were isolated from the bacteria. The isolated plasmids were then subjected to PCR with a stable reverse primer designed for the plasmid backbone, and the sense gRNA oligo as the forward primer. For this, 10 ng plasmids were added with 2.5 µL Taq Polymerase Buffer, 0.5 µL dNTPs, 0.5 µL M13 reverse primer, 0.5 µL forward gRNA oligo (as a primer), 0.125 µL Taq Polymerase and completed to 25 µL with nuclease free water. The tubes were then placed into a thermal cycler, with standard PCR conditions: 10 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 50°C, 30 seconds at 72°C, and a final extension for 10 minutes at 72°C. The samples were then ran on a 1% agarose gel stained with SAFE DNA stain. Expected band size was ~260 bps.

2.3.3.7 Microinjections

After validation of the gRNA plasmids, CRISPR/Cas9 injection mix was prepared. The mix contained the following:

Table 15: Contents of CRISPR/Cas9 microinjection mix.

Plasmid	Content	Amount
gRNA inserted pRB1017	gRNA+tracrRNA	50 ng
Peft-3::cas9+empty sgRNA	Cas9 protein sequence +empty sgRNA	15 ng
pAD67 pRF4	Roller injection marker	50 ng

Total injection mix volume was prepared as 20 μ L. At least 50 young adult worms were selected for microinjections. 3-5 worms were picked onto a glass slide with a drop of halocarbon oil for temporary immobilization. 10 pL of the plasmid mix was injected into the gonad arms of the worms. Injected worms were placed back on plates with bacteria for recovery, and if recovered, they are placed in individual NGM plates to follow their progeny. The presence of an injection marker made tracking of the successful injections on the next generation of the injected worms easier.

2.3.3.8 Mutant selection and deletion validation

The next generation was waited for 2-3 days to observe the injection marker. Roller phenotypes were observed in the successfully injected worms' progeny, which is a phenotype where the worm has a moving defect that causes a helical twist on their body.

Random roller worms were selected to lay eggs for at least a day on individual plates, and upon egg laying, the rollers were lysed to isolate their DNA. For this, 7 μ L of DNA lysis buffer with proteinase K was placed on the caps of PCR tubes, and single worms were picked onto the drops of lysis buffer, which were then centrifuged to the bottom of the tube. The tubes were then kept in -80°C freezer for 30 minutes, and then placed on a thermal cycler with the following program: 1 hour at 65°C, followed by 15 minutes at 95°C and cooling off.

Obtained genomic DNA was then used for PCR validation of CRISPR/Cas9 deletions. The PCR conditions were specific to primer pairs and the amplicon size, but

essentially, it was the same program with the colony PCR conditions. The PCR products were then again ran on a 1% agarose gel and the gel was then imaged with ChemiDoc, validating deletions. Following primers were used for deletion validation:

Table 16: Deletion validation primers.

Primer code	5'-3' sequence
afd-1_val_F2	GTAGGTATCCAACGACGATGAC
afd-1_val2_R2	GACATTGGTAGGGTGACTGA
pqn-41_val_f	ACCGTCCTCTTCTCTAGCACAC
pqn-41_val_R2	CGTTCCTCGAGATGTGTAAGAC
zip-3_val_F2	CCATTACCTCAACAACCACC
zip-3_val_R2	GGTTGTTGCTGAAGCATTCC

2.3.3.9 Sanger Sequencing of the Deletion Regions

As an extra validation of deletions, the PCR products were sent out for sanger sequencing. The sequencing was made from two ends, as sanger sequencing efficiency significantly drops on longer products. Both ends of the results were aligned with the genome to confirm the deletions.

2.3.3.10 Divergent qRT-PCR

There are many ways of detecting circular RNA expression, such as northern blot or RNA sequencing. One other way of detecting circular RNAs is a specially designed qRT-PCR called divergent PCR. This PCR aims for the back splice junction (BSJ) of the circular RNAs. Forward and reverse primers are designed to cover for BSJ, which would, in the linear form of the same RNA, diverge from each other. This way, the linear transcript is not amplified, and the circular target is specifically targeted. To measure the hypothesized decrease on specific circular RNAs, following divergent primers were designed:

Table 17: BSJ specific divergent primers for circular RNA detection.

Primer code	5'-3' sequence
pqn-41_div_f	TCATCATCTCCAGCAGCAACAT
pqn-41_div_r	GTTGACGGAGCTGATTTTCACG
pqn-41_div2_f	CAGTGAAACACCGCCAAATG
afd-1_div_f	TCAGCTCGATGGTCCGGATA
afd-1_div_r	GGAGACATTGGTAGGGTGACTGA
afd-1_div2_f	CATCAGTCACCCTACCAATGTC
zip-3_div_f	GCACCCGATTCCAGTTGATACG
zip-3_div_r	TGGGAGCGGGGTGTAGTAGT

2.3.3.11 qPCR for Aging Markers

Aging markers and linear forms of targeted genes were analyzed as described on chapters 1.3.3.6 – 1.3.3.8. Primers for these analyses were as follows:

Table 18: qRT-PCR Primers for aging markers.

Primer code	5'-3' sequence
afd-1_qF	CAGCCTCCAAGTGGTTCATATC
afd-1_qR	CATCCTCCAACGTCAGCTTATC
pqn-41_qF	GGCACAGGCACAACAAATG
pqn-41_qR	GCCTGAAGCAGACGAATAAGA
hsp-16.2_f	TACGCTATCAATCCAAGGAGAAC
hsp-16.2_r	GAAGCAACTGCACCAACATC
sod-3_f	TGGTGGTGGACACATCAATC
sod-3_r	ACCGAAGTCGCGCTTAATAG
vit-2_f	TTTCACCGCTCATACCTTCTC
vit-2_r	CTCCTCGTCTCTCTCGTACTT
sip-1_f	CACACCGTCATCAACAAGGA
sip-1_r	TCCAGCAGAAGTGTGGATTG
spe-8_f	GCACGGAAAGCAGGTTATTC
spe-8_r	CCTCCCTTTCGCTCCTTTAT
tba-1_f	CCTCTCGGTTGACTATGGAAAG
tba-1_r	GTGGTGTGAGTCGTCAGTATAG

2.3.3.12 Lifespan Assays

Lifespan assays were done as described on chapter 1.3.3.3.

2.3.3.13 Brood Size Assay

Brood size assays were done as described on chapter 1.3.3.4.

2.3.3.14 Thrashing Assay

Thrashing assays were done as described on chapter 1.3.3.5.

2.4 Results

2.4.1 Circular RNA Filtering

Filtered circular RNAs were:

Table 19: Filtered circular RNAs, their corresponding genes, exons and RCM regions.

Circular RNA	Corresponding Gene	circRNA Exons	RCM Introns
cel_circ_0000038	<i>afd-1</i>	Exon 7,8,9	Intron 6
cel_circ_0000333	<i>pqn-41</i>	Exon 15	Intron 14
cel_circ_new_0001917	<i>zip-3</i>	Exon 4	Intron 4
cel_circ_new_0000735	<i>afd-1</i>	Exon 8	Intron 6
cel_circ_0000334	<i>pqn-41</i>	Exon 15, 16	Intron 14

Initially, *cel_circ_0000038*, *cel_circ_new_0000333* and *cel_circ_new_0001917* were identified as main targets. Upon RCM analysis, *cel_circ_0000735* and *cel_circ_0000334* were realized to share the targeted RCMs, so they were added to qRT-PCR analyses.

2.4.2 RCM Detection

Detected RCM regions of the targeted circular RNAs were marked on the genome.

Regions were as follows:

Table 20: Genomic locations of detected RCM regions.

Circular RNA	RCM Genomic Location
<i>cel_circ_0000038</i>	I:2172394:2173573
<i>cel_circ_0000333</i>	III:1926912:1927533
<i>cel_circ_new_0001917</i>	II:4461:4634
<i>cel_circ_new_0000735</i>	I:2172394:2173573
<i>cel_circ_0000334</i>	III:1926912:1927533

2.4.3 gRNA Cloning & Colony PCR

Plasmid transformation was confirmed upon observation of colonies on kanamycin LB

Agar plates, and observation of no colonies on control plates. Colony PCR was subsequently performed, and the PCR products were run on 1% agarose gel. Expected band size was 260 bps.

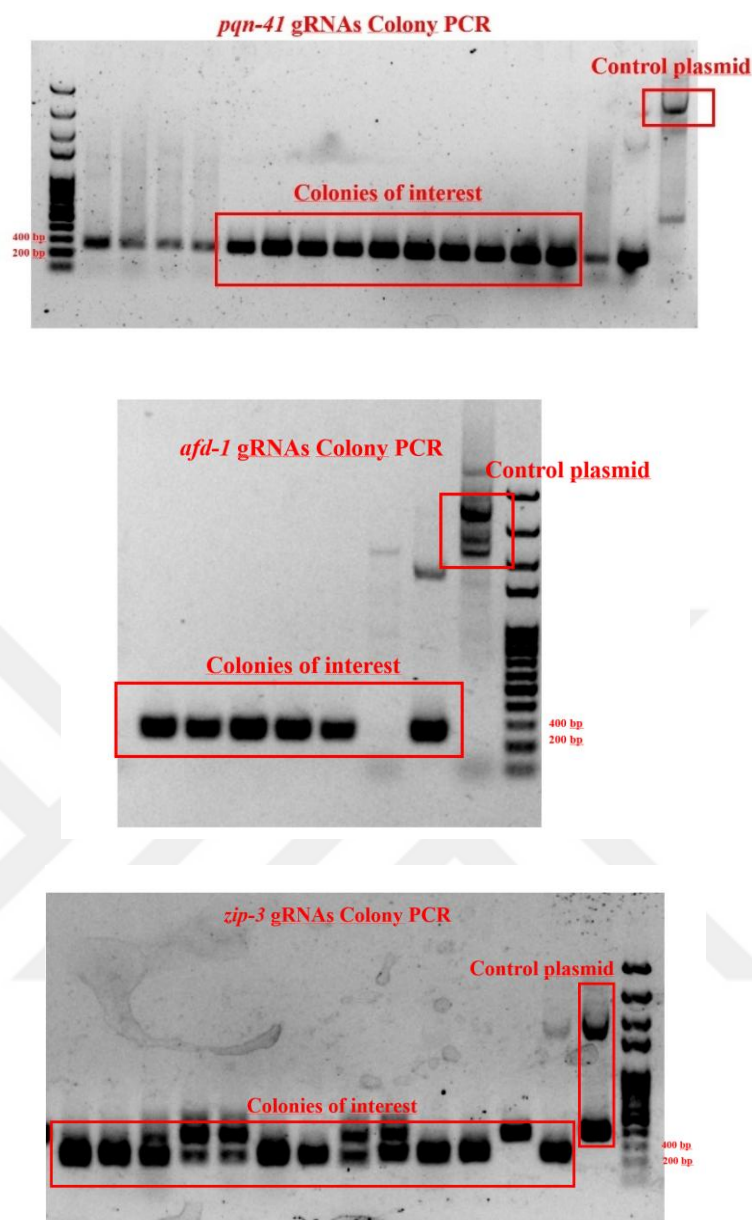


Figure 27: Colony PCR agarose gel result for *pqn-41*, *afd-1* and *zip-3* gRNAs. Expected band size was 260 bps.

All colonies were verified, and their miniprep isolates were then stored for microinjection purposes.

2.4.4 Microinjections

After microinjections were done, and worms were singled out to individual plates as described on chapter 2.3.3.7, roller phenotype was searched under stereo microscope on the progeny of injected worms. As high number of possible rollers were singled to individual plates for egg laying.



Figure 28: Roller phenotype worms observed under stereo microscope. Roller worms leave a circle-like bacterial path as they move around the plate.

2.4.5 Deletion Validations

After roller worms lay enough progeny (at least 5-10 eggs), their genomic DNA was isolated, and validation PCR was done in each singled-out worm. Expected amplicon sizes were as follows:

Table 21: Expected amplicon sizes of the targets after RCM deletion.

Gene Name	Normal Amplicon Sizes	Expected Amplicon Size After Deletion	Deleted DNA Size
<i>afd-1</i>	1805	502	1303
<i>pqn-41</i>	1254	446	808
<i>zip-3</i>	867	714	153

Validation PCR results were as follows:

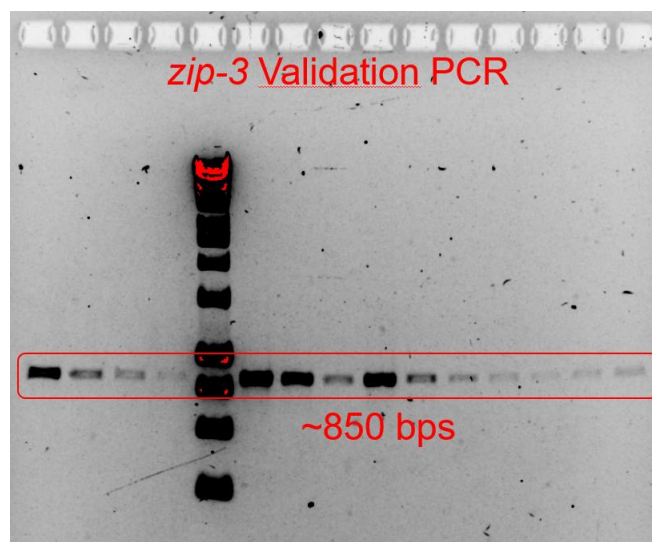
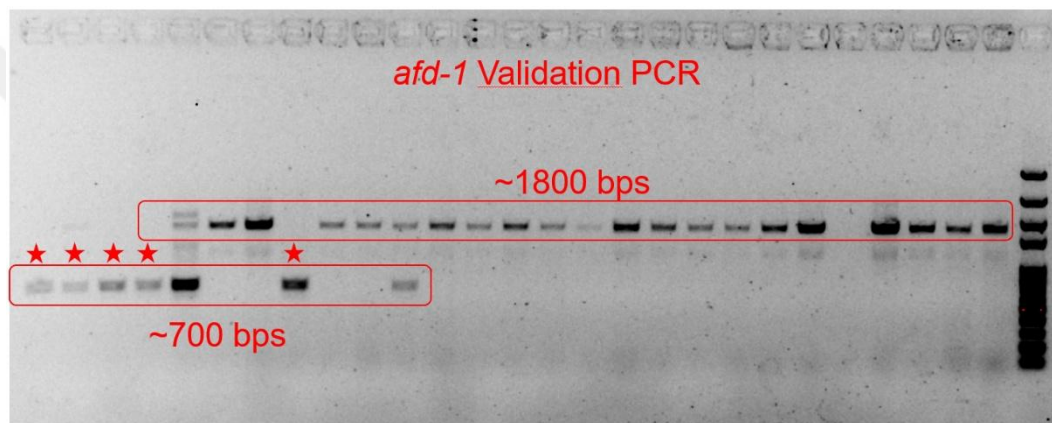
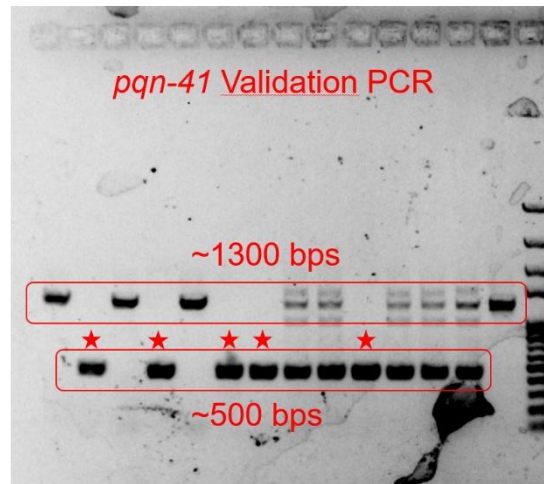


Figure 29: Gel electrophoresis images of validation PCRs for *pqn-41*, *afd-1* and *zip-3* RCM deletions.

Homozygous deletions were marked with red stars. Deletions for *pqn-41* and *afd-1* were confirmed, but not for *zip-3*. Band size for *afd-1* was ~200 more than expected

but it was in an acceptable range. Progeny of the deletion confirmed worms were identified as new strains, named TUR015 for *pqn-41* deletion and TUR016 for *afd-1* deletion. Injection for *zip-3* was repeated 3 more times, yet deletion was not successful. Since its RCM region was in a relatively smaller intronic segment, there were no more available gRNAs to re-design, so *zip-3* deletion was considered failed.

2.4.6 Sanger Sequencing

For further validation of CRISPR/Cas9 deletions, PCR amplified products of the validation regions were sent out for sequencing to BM Labosis Biyoteknoloji. Sequencing was done from both ends.

Sequencing result validated that CRISPR/Cas9 deletions on *pqn-41* were as designed. However, as observed on validation PCRs, *afd-1* deletions were not as efficient. Sequencing showed that deletions were inside the intended region, but they started from unintended regions of the intron.

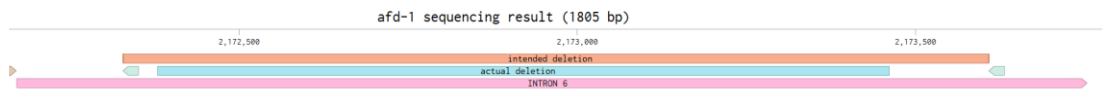


Figure 30: Sequencing result annotated with intended deletion on *afd-1* intron 6.

When designing the deletion region for RCMs, gRNAs were selected to be distanced from the actual region for error proofing. Even though the deletion was not as wide as intended, it covered most of the RCM region. Therefore, TUR016 strain was also kept for further experiments.

2.4.7 qRT-PCR Assay

For validation of circular RNA specific silencing of the target genes and to test for various aging markers, three experiment groups were prepared: N2, TUR015 and TUR016. For all the groups, 5 different time points were set for L4, Day 1 of adulthood (D1), Day 3 of adulthood (D3), Day 7 of adulthood (D7) and Day 10 of adulthood

(D10). For each group and time point, 2 plates with 50 worms were prepared upon age synchronization. 2 Biological replicates were repeated as 2 technical replicates. Targets were as follows:

Table 22: qRT-PCR targets for RCM deletion strains.

Target Gene	Tested Strains	PCR Type	Purpose	Primer Code
<i>cel_circ_0000038 (afd-1)</i>	N2, TUR016	Divergent qRT-PCR	Deletion identification	afd-div1
<i>cel_circ_0000333 (pqn-41)</i>	N2, TUR015	Divergent qRT-PCR	Deletion identification	pqn-div1
<i>cel_circ_new_0000735 (afd-1)</i>	N2, TUR016	Divergent qRT-PCR	Deletion identification	afd-div2
<i>cel_circ_0000334 (pqn-41)</i>	N2, TUR015	Divergent qRT-PCR	Deletion identification	pqn-div2
<i>afd-1</i>	N2, TUR016	qRT-PCR	Deletion identification	afd-q
<i>pqn-41</i>	N2, TUR015	qRT-PCR	Deletion identification	pqn-q
<i>hsp-16.2</i>	All	qRT-PCR	Aging marker	hsp-16.2
<i>sod-3</i>	All	qRT-PCR	Aging marker	sod-3
<i>vit-2</i>	All	qRT-PCR	Aging marker	vit-2
<i>sip-1</i>	All	qRT-PCR	Aging marker	sip-1
<i>spe-8</i>	All	qRT-PCR	Aging marker	spe-8
<i>tba-1</i>	All	qRT-PCR	Housekeeping	tba-1

Obtained Ct values were normalized with housekeeping gene *tba-1*, and relative gene expression was calculated as described on chapter 1.3.3.8. There were two separate analyses made. First analysis was focused on gene expression analysis within days and strain, which was based on the condition's N2 control Ct values. Second analysis was to observe expression with elapsed time, so all calculations were made based on D1 control N2 Ct values. For time lapse analysis, data from L4 stage was excluded. Statistical analysis was done on Graphpad Prism, using two way ANOVA with Tukey's multiple comparisons test for time lapse analysis and unpaired t-test for analysis within

days. Primer codes are used for analysis explanations as RNA names are longer. L4 stage is excluded from time lapse analysis.

2.4.7.1.1 Stage Based Expression – L4

No significantly different expression was observed on TUR015 L4s compared to N2.

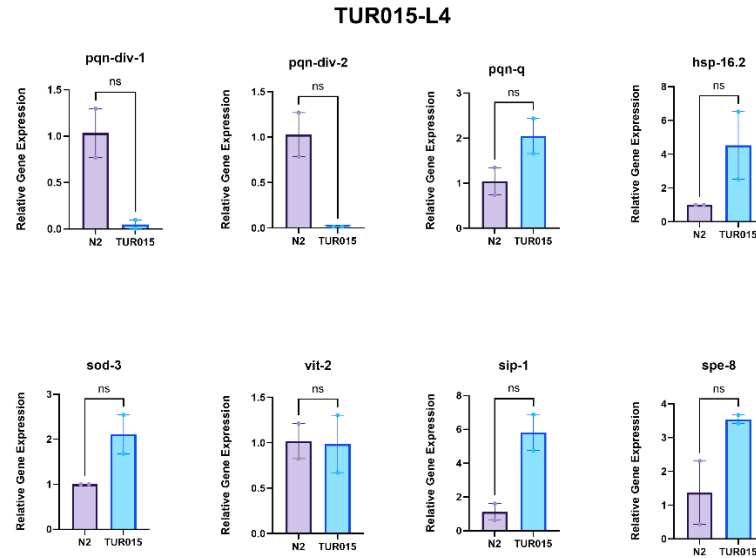


*Figure 31: Stage based qRT-PCR results for L4 N2 vs L4 TUR015. Relative gene expressions were calculated by using L4 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001*

On TUR016 L4s, *afd-div1* expression was significantly lower than N2. Expression levels of *sod-3* and *sip-1* were significantly higher than N2.

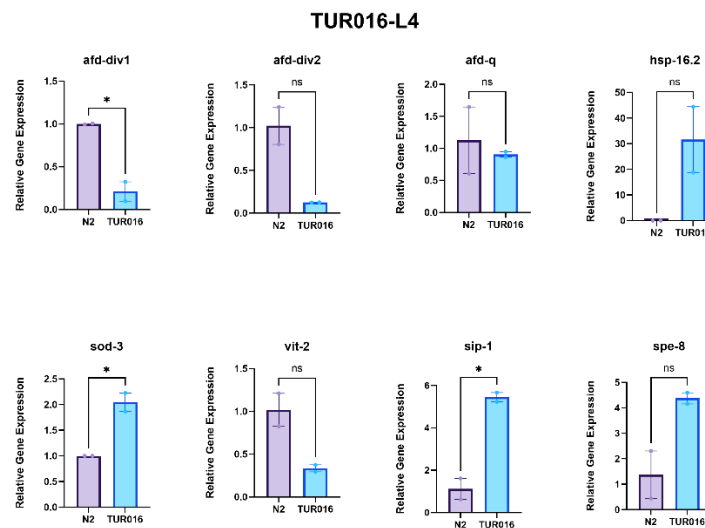


Figure 32: Stage based qRT-PCR results for L4 N2 vs L4 TUR016. Relative gene expressions were calculated by using L4 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

2.4.7.1.2 Stage Based Expression – D1

On TUR015 D1 worms, *sip-1* expression was significantly higher than N2 whereas *spe-8* expression was significantly lower.

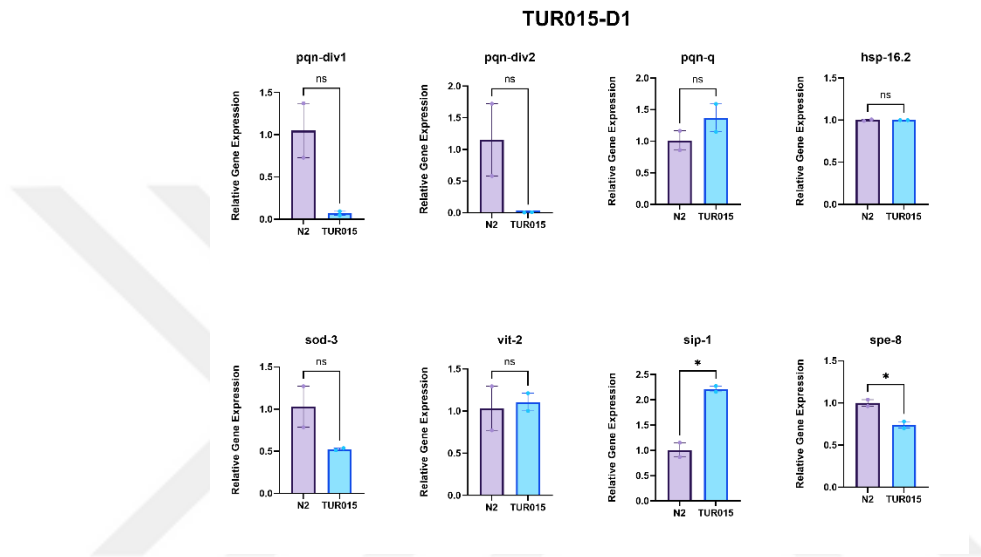


Figure 33: Stage based qRT-PCR results for D1 N2 vs D1 TUR015. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

On TUR016 D1 worms, *sip-1* expression was significantly higher than N2.

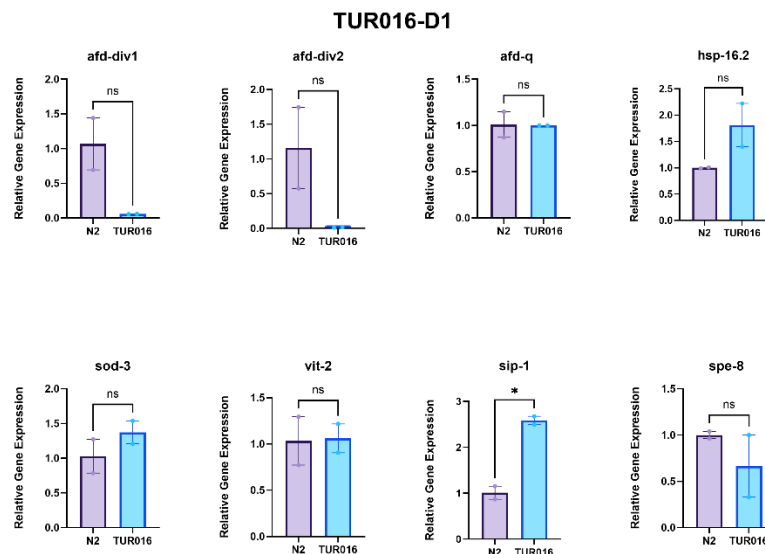


Figure 34: Stage based qRT-PCR results for D1 N2 vs D1 TUR016. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

2.4.7.1.3 Stage Based Expression – D3

No significantly different expression was observed on TUR015 D3 worms compared to N2.

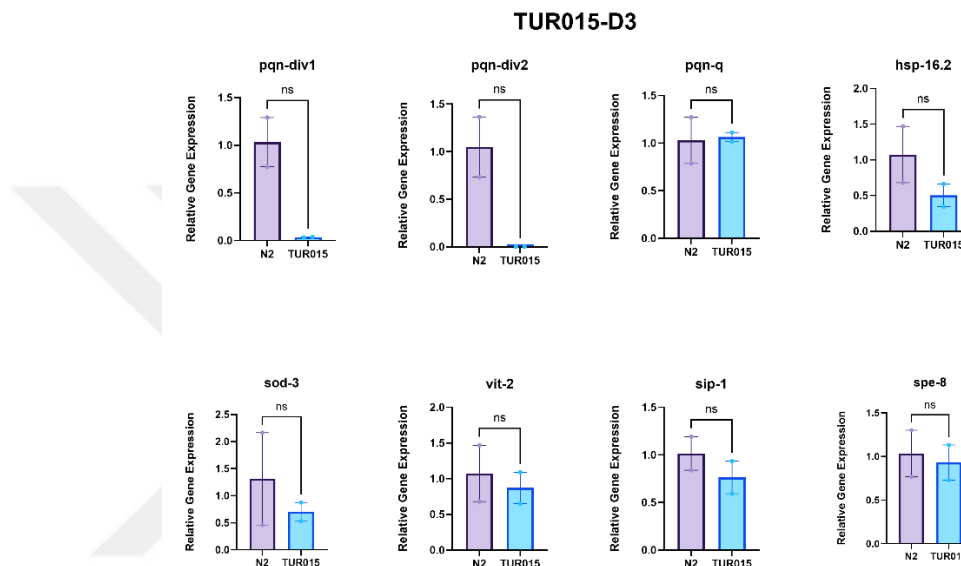


Figure 35: Stage based qRT-PCR results for D3 N2 vs D3 TUR015. Relative gene expressions were calculated by using D3 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

On TUR016 D3 worms, *afd-div1* and *afd-div2* expressions were significantly lower than N2.

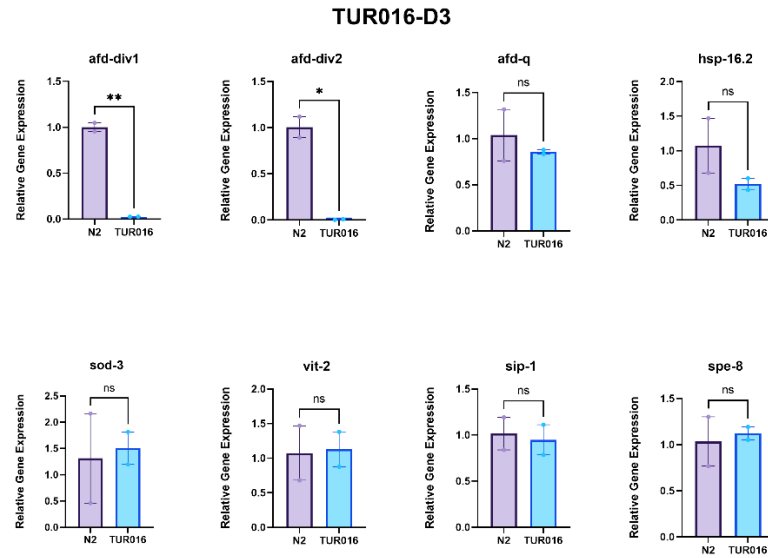


Figure 36: Stage based qRT-PCR results for D3 N2 vs D3 TUR016. Relative gene expressions were calculated by using D3 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

2.4.7.1.4 Stage Based Expression – D7

On TUR015 D7 worms; *pqn-div1*, *pqn-div2*, *sod-3* and *vit-2* expressions were significantly lower than N2 D7 worms.

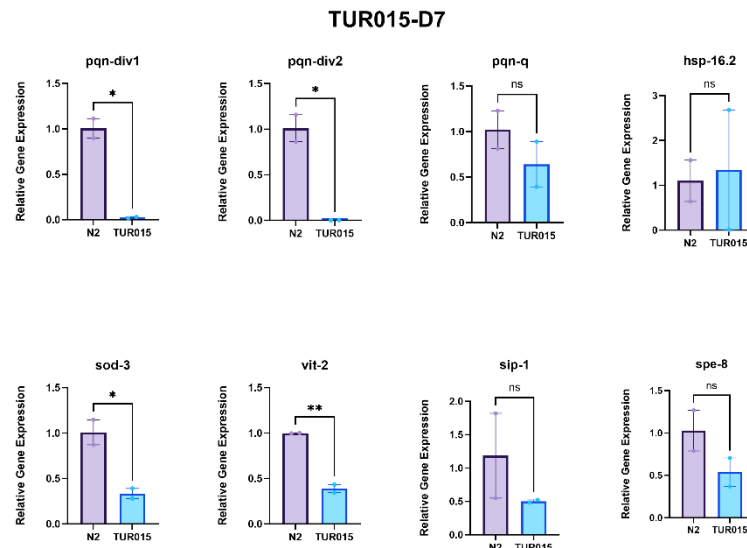


Figure 37: Stage based qRT-PCR results for D7 N2 vs D7 TUR015. Relative gene expressions were calculated by using D7 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

On TUR016 D7 worms, *afd-div1* and *vit-2* expressions were significantly lower than N2 D7 worms, whereas *hsp-16.2* expression was significantly higher.

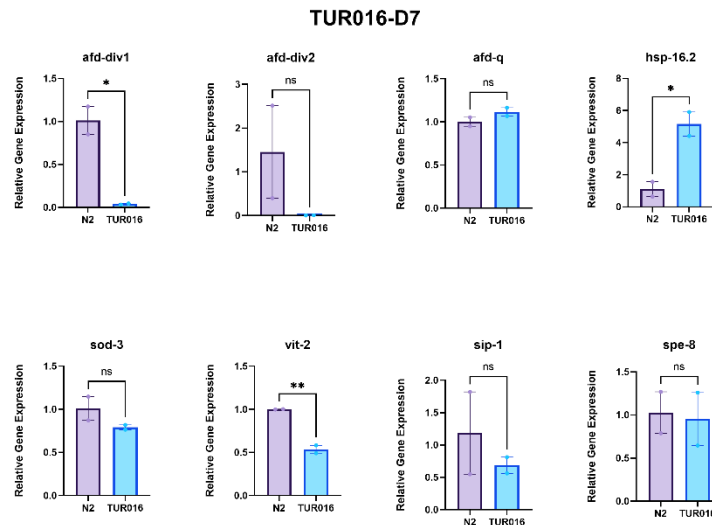


Figure 38: Stage based qRT-PCR results for D7 N2 vs D7 TUR016. Relative gene expressions were calculated by using D7 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p -values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

2.4.7.1.5 Stage Based Expression – D10

No significantly different expression was observed on TUR015 D10 worms compared to N2.

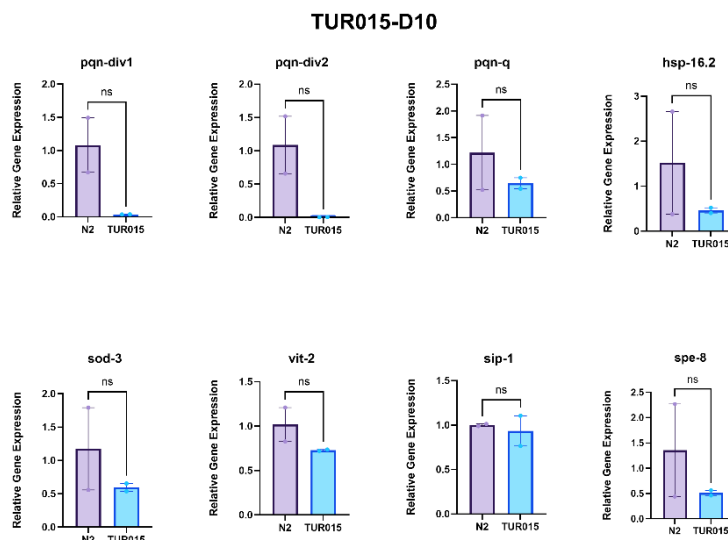


Figure 39: Stage based qRT-PCR results for D10 N2 vs D10 TUR015. Relative gene expressions were calculated by using D10 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

On TUR016 D10 worms, *afd-div2* expression was significantly lower than N2 D10 worms, whereas *sip-1* expression was significantly higher.

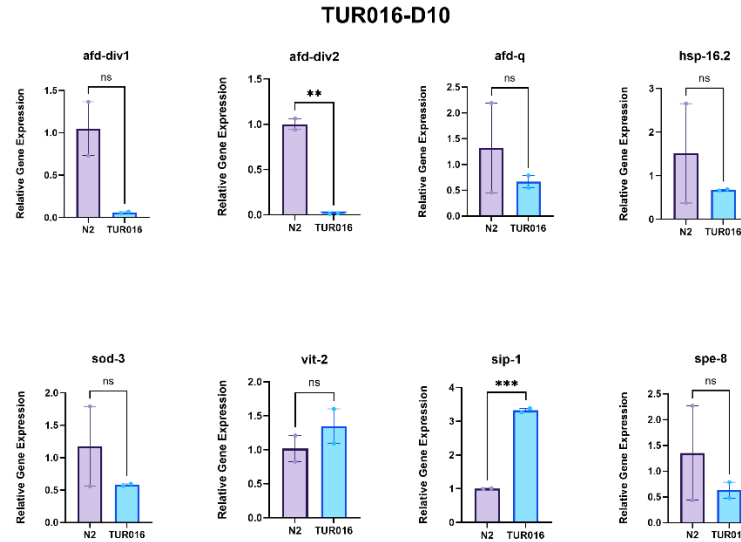


Figure 40: Stage based qRT-PCR results for D10 N2 vs D10 TUR016. Relative gene expressions were calculated by using D10 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

2.4.7.2 Timelapse Expression

When investigated on a timelapse expression that is normalized to the relative gene's D1 Ct values, expression of both targeted *pqn-41* circular RNAs on TUR015 is significantly decreased with aging, whereas the linear mRNA target remained unchanged. This verifies the circular RNA specific deletion of the targets.

Aging markers, on the other hand, were not significantly affected by this deletion as there is not a clear increase or decrease on most of the markers.

TUR015 qRT-PCRs - by Days

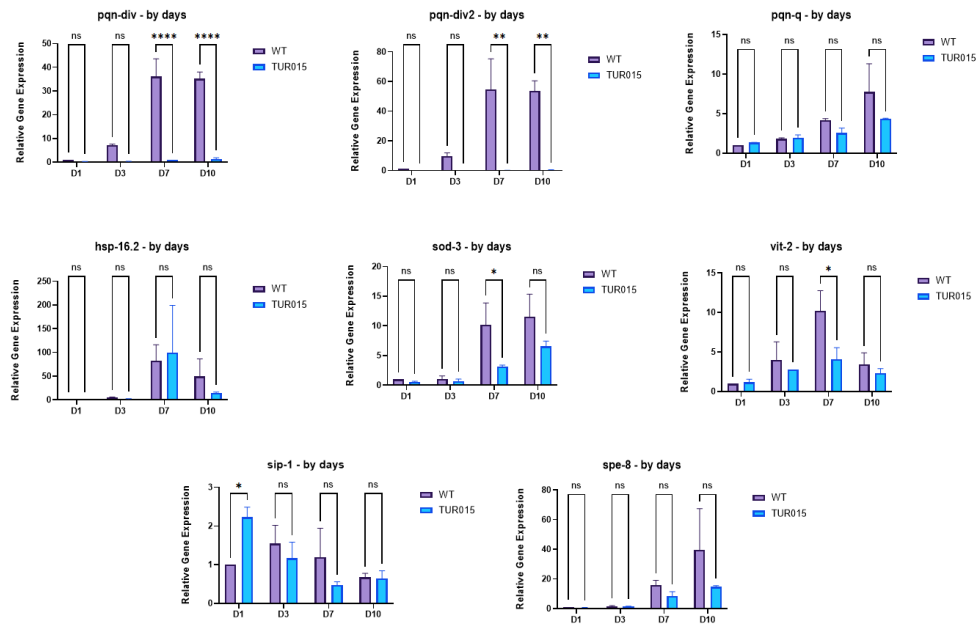


Figure 41: Time-lapse qRT-PCR results for N2 vs TUR015. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

The same can be said for TUR016 strain, where the deletions silenced the targeted circular RNAs without disturbing the linear mRNA expression of the targeted *afd-1* gene.

TUR016 qRT-PCRs - by Days

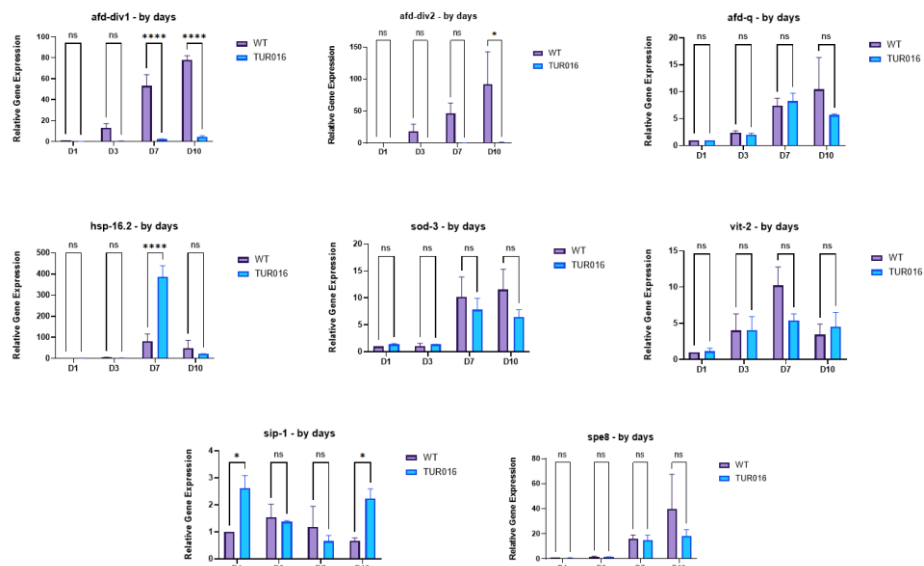


Figure 42: Time-lapse qRT-PCR results for N2 vs TUR016. Relative gene expressions were calculated by using D1 N2 Ct values. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as * <0.05 , ** <0.001 , *** <0.0001 , **** <0.00001

2.4.8 Lifespan Assay

To observe the hypothesized lifespan extension of CRISPR/Cas9 mutated the *C. elegans* strains; N2, TUR015 and TUR016 strains were subjected to lifespan analysis. The worms were age synchronized as described on 1.3.2.2, L1 arrested, and transferred to FUDR plates at their beginning of adulthood.

Two biological replicates were subjected to analysis. The worms were counted every 2-3 days, and the data was plotted into a Kaplan-Meier survival plot. Median lifespans were compared with two statistical analyses. Log-rank (Mantel-Cox) test counts every death event with equal weight, and the Gehan-Breslow test counts later deaths with higher weight.

Table 23: Median lifespans, sample sizes, and statistical analysis results of RCM deletion based lifespan assay.

Strain	Median Lifespan	N2 Median Lifespan (N= 293(2))	Median Lifespan Change (%)	Sample Size (censored)	Mantel-Cox p-value	Gehan-Breslow p-value
TUR015	16	16	0	176(1)	0,0002	0,0522
TUR016	16	16	0	180(0)	0,0004	0,0424

Kaplan-Meier survival plots of the lifespan assays were as follows:

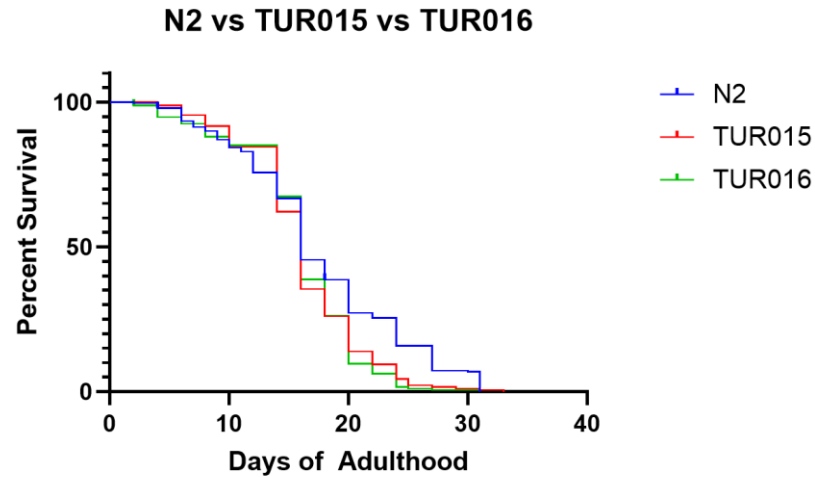


Figure 43: Kaplan-Meier survival plot for RCM deletion based lifespan assay.

Median survival rates, together with Kaplan-Meier survival curves suggested that deletion of the *afd-1* and *pqn-41* circular RNAs did not result in a lifespan extension.

2.4.9 Brood Size Assay

To investigate possible effects of the deletions on brood size, the assay with N2, TUR015 and TUR016 worms. For all the groups, 15 worms were age synchronized and their brood size were monitored for 5 egg laying days, as described on chapter 1.3.3.5. Monitored brood size was analyzed on Graphpad Prism using one way ANOVA with Tukey's multiple comparisons test.

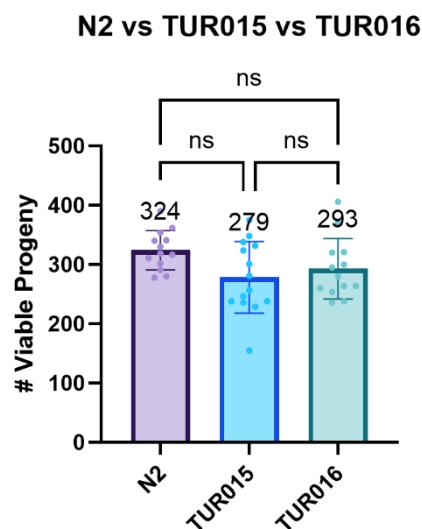


Figure 44: Total brood size assay results. Viable progeny were counted, unhatched eggs were not analyzed. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

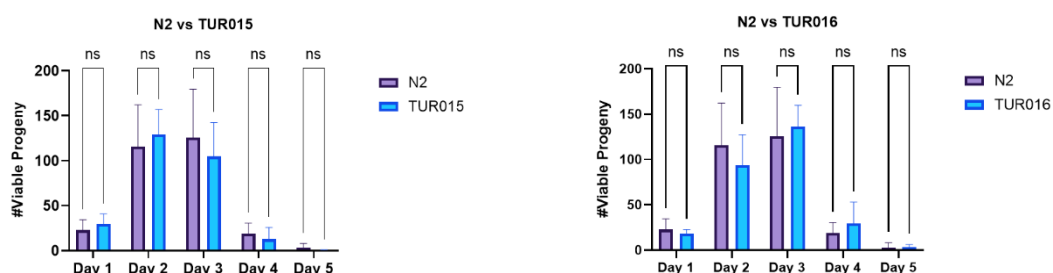


Figure 45: Individual brood size assay results. The same results were used for total brood size assay. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

Brood size was not affected by the CRISPR/Cas9 deletions of targeted circular RNAs.

2.4.10 Thrashing Assay

For all three groups, 40 worms were grown in two separate plates per condition, until first day of adulthood. 10 worms from each plate, as two biological replicates, were then placed on a drop of M9 as explained on chapter 1.3.3.4, and two 30 second videos were recorded for each worm as technical replicates. The videos were then batch-analyzed on ImageJ plugin WrmTrck. For this analysis, videos were first batch-edited into 8-bit greyscale format, then they were passed from a deflicker plugin to eliminate flickering that happens due to the light source of the microscope. The videos were then thresholded to be able to count the bends with Otsu mode, and WrmTrck plugin was run.

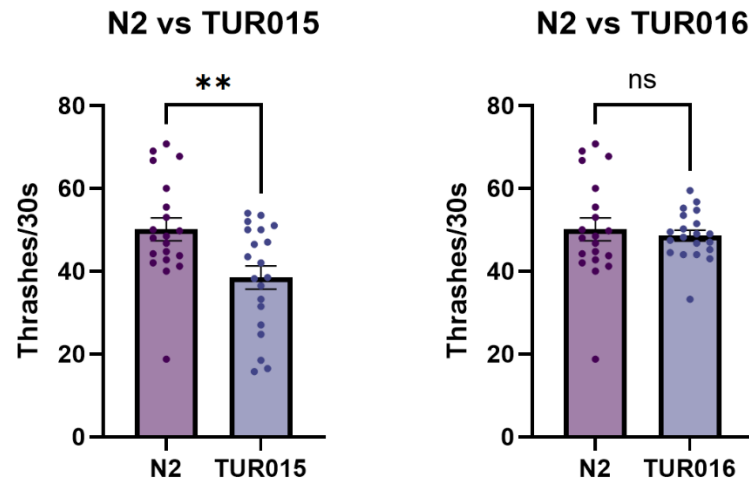


Figure 46: Thrashing assay results. Error bars represent mean with SEM. ns: non-significant, p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$, $**** < 0.00001$

Thrashing rates of N2 was significantly higher than TUR015, but not significantly different from TUR016.

2.5 Conclusion

The aim of this chapter was to investigate the effects of deletion of the age-accumulated circular RNAs on *C. elegans* lifespan. Since circular RNAs were shown to be accumulating with aging, and some deletion of some circular RNAs were shown to be extending life span, the hypothesis was that the deletions would extend worm lifespan.

In this context, the most age-accumulated circular RNAs of *C. elegans* were filtered and identified. Circular RNAs of *afd-1*, *pqn-41* and *afd-1* were selected as the targets.

To specifically eliminate the circular form instead of complete removal of the gene from the genome, reverse complementary matching (RCM) regions of the targeted circular RNAs were identified through a python code. To delete the RCM regions to obtain circular RNA mutants, a CRISPR/Cas9 deletion was planned. For each RCM region that was identified, two gRNAs were designed to cleave out the sequence from the worm genome.

The designed gRNAs were then cloned into plasmids, which were then isolated from competent bacteria to inject into the worm, together with another plasmid for Cas9 enzyme and another one for the injection marker. Upon phenotypic confirmation of injection on the progeny owing to the injection marker, deletions were validated on F2 generation. Deletions were successful for *pqn-41* and *afd-1* RCMs, however, *afd-1* RCM deletions were unsuccessful despite multiple repeats. No other gRNA couples were available for deletion on *afd-1* RCMs as the gene was relatively short (5500 bps). The reason *afd-1* deletions did not work is not obvious, but it is quite likely about CRISPR efficiency.

Confirmed deletions of *pqn-41* and *afd-1* were then validated with Sanger sequencing, which shed light on the unexpected smaller deletion of *afd-1* RCM. Cas9 unexpectedly

cut from about 50 bps downstream and 150 bps upstream from the NGG regions. This did not effect the deletion efficiency as circular RNA silencing was successful after both *pqn-41* and *afd-1* RCM deletion, and these deletions did not significantly alter the mRNA expression for the target genes.

After confirmation of deletion, the hypothesis was tested with longevity studies including lifespan experiments, qRT-PCR assays for aging markers, brood size and thrashing assays. Lifespan assays resulted in equal median lifespans with WT worms. Brood sizes of both deletion mutants were not significantly different from WT worms as well. Thrashing assay between N2 and *afd-1* deletion mutants were also insignificant, but *pqn-41* deletion resulted in significantly lower thrashing rate when compared to N2. This data could be meaningful if it was supported with any other type of marker, but this was not the case. There were not any significant patterns of increase or decrease on none of the aging markers tested on qRT-PCR.

These results denied the hypothesis that *pqn-41* or *afd-1* circular RNAs, which are the highest accumulated circular RNAs during worm aging, were contributing to the aging process.

References

- [1] C. Kenyon, "The Nematode *Caenorhabditis elegans*," *Science*, vol. 240, no. 4858, pp. 1448–1453, Jun. 1988, doi: <https://doi.org/10.1126/science.3287621>.
- [2] A. K. Corsi, B. Wightman, and M. Chalfie, "A Transparent window into biology: A primer on *Caenorhabditis elegans*," in *WormBook*, WormBook: The C. elegans Research Community, 2015. Accessed: Nov. 01, 2025. [Online]. Available: https://www.wormbook.org/chapters/www_celegansintro/celegansintro.html
- [3] Z. F. Altun and D. D. Hall, "Introduction," *Wormatlas*, 2009. <https://www.wormatlas.org/hermaphrodite/introduction/Introframeset.html> (accessed Nov. 01, 2025).
- [4] D. L. Riddle, T. Blumenthal, B. J. Meyer, and J. R. Priess, "Sexual Dimorphism," *Nih.gov*, 1997. <https://www.ncbi.nlm.nih.gov/books/NBK20094/#:~:text=ISexual%20Dimorphism-,C.,solely%20for%20internal%20self%2Dfertilization.> (accessed Nov. 01, 2025).
- [5] S. Ward and J. S. Carrel, "Fertilization and sperm competition in the nematode *Caenorhabditis elegans*," *Developmental Biology*, vol. 73, no. 2, pp. 304–321, Dec. 1979, doi: [https://doi.org/10.1016/0012-1606\(79\)90069-1](https://doi.org/10.1016/0012-1606(79)90069-1).
- [6] D. H. Hall, L. A. Herndon, and Z. K. Altun, "Introduction to C. elegans Embryo Anatomy," *Wormatlas*, 2017. <https://www.wormatlas.org/embryo/introduction/mainframe.htm> (accessed Nov. 01, 2025).
- [7] J. Hodgkin and T. M. Barnes, "More is Not Better: Brood Size and Population Growth in a SelfFertilizing Nematode," *Proceedings: Biological Sciences*, vol. 246, no. 1315, pp. 19–24, 1991, Accessed: Nov. 29, 2025. [Online]. Available: <http://www.jstor.org/stable/76629>
- [8] D. L. Riddle, M. M. Swanson, and P. S. Albert, "Interacting genes in nematode dauer larva formation," *Nature*, vol. 290, no. 5808, pp. 668–671, 1981, doi: <https://doi.org/10.1038/290668a0>.
- [9] J. D. Kormish, J. Gaudet, and J. D. McGhee, "Development of the C. elegans digestive tract," *Current Opinion in Genetics & Development*, vol. 20, no. 4, pp. 346–354, Jun. 2010, doi: <https://doi.org/10.1016/j.gde.2010.04.012>.
- [10] D. Hirsh, D. Oppenheim, and M. Klass, "Development of the reproductive system of *Caenorhabditis elegans*," *Developmental Biology*, vol. 49, no. 1, pp. 200–219, 1976, doi: [https://doi.org/10.1016/00121606\(76\)902670](https://doi.org/10.1016/00121606(76)902670).
- [11] O. Hobert, "Specification of the nervous system," in *WormBook*, WormBook: The C. elegans Research Community, 2005. Accessed: Nov. 01, 2025. [Online]. Available: https://www.wormbook.org/chapters/www_specnervsys/specnervsys.html

- [12]S. Brenner, “The Genetics of *Caenorhabditis elegans*,” *Genetics*, vol. 77, no. 1, pp. 71–94, May 1974, doi: <https://doi.org/10.1093/genetics/77.1.71>.
- [13]J.E. Sulston, E. Schierenberg, J. G. White, and J. N. Thomson, “The embryonic cell lineage of the nematode *Caenorhabditis elegans*,” *Developmental Biology*, vol. 100, no. 1, pp. 64–119, Nov. 1983, doi: [https://doi.org/10.1016/0012-1606\(83\)90201-4](https://doi.org/10.1016/0012-1606(83)90201-4).
- [14]The, “Genome Sequence of the Nematode *C. elegans*: A Platform for Investigating Biology,” *Science*, vol. 282, no. 5396, pp. 2012–2018, 1998, doi: <https://doi.org/10.1126/science.282.5396.2012>.
- [15]S. J. Cook *et al.*, “Whole-animal connectomes of both *Caenorhabditis elegans* sexes,” *Nature*, vol. 571, no. 7763, pp. 63–71, Jul. 2019, doi: <https://doi.org/10.1038/s41586-019-1352-7>.
- [16]S. Zhang, F. Li, T. Zhou, G. Wang, and Z. Li, “*Caenorhabditis elegans* as a Useful Model for Studying Aging Mutations,” *Frontiers in Endocrinology*, vol. 11, pp. 554994–554994, Oct. 2020, doi: <https://doi.org/10.3389/fendo.2020.554994>.
- [17]P. R. Hunt, “The *C. elegans* model in toxicity testing,” *Journal of Applied Toxicology*, vol. 37, no. 1, pp. 50–59, Jul. 2016, doi: <https://doi.org/10.1002/jat.3357>.
- [18]D. Conte, L. T. MacNeil, A. J. M. Walhout, and C. C. Mello, “RNA Interference in *Caenorhabditis elegans*,” *Current Protocols in Molecular Biology*, vol. 109, no. 1, pp. 26.3.1–26.3.30, Jan. 2015, doi: <https://doi.org/10.1002/0471142727.mb2603s109>.
- [19]R. Kamath, “Genome-wide RNAi screening in *Caenorhabditis elegans*,” *Methods*, vol. 30, no. 4, pp. 313–321, Jun. 2003, doi: [https://doi.org/10.1016/s1046-2023\(03\)00050-1](https://doi.org/10.1016/s1046-2023(03)00050-1).
- [20]The, “Large-Scale Screening for Targeted Knockouts in the *Caenorhabditis elegans* Genome,” *G3 Genes|Genomes|Genetics*, vol. 2, no. 11, pp. 1415–1425, Nov. 2012, doi: <https://doi.org/10.1534/g3.112.003830>.
- [21]A. L. Daul, E. C. Andersen, and A. E. Rougvie, “The *Caenorhabditis* Genetics Center (CGC) and the *Caenorhabditis elegans* Natural Diversity Resource,” in *The Biological Resources of Model Organisms*, CRC Press, 2019, pp. 69–94. doi: <https://doi.org/10.1201/9781315100999-5>.
- [22]A. E. Sluder and R. Baumeister, “From genes to drugs: target validation in *Caenorhabditis elegans*,” *Drug Discovery Today: Technologies*, vol. 1, no. 2, pp. 171–177, Oct. 2004, doi: <https://doi.org/10.1016/j.ddtec.2004.09.007>.
- [23]“Nobel Prize in Physiology or Medicine 2002,” *NobelPrize.org*, 2018. <https://www.nobelprize.org/prizes/medicine/2002/summary/> (accessed Nov. 01, 2025).

- [24]“Nobel Prize in Physiology or Medicine 2006,” *NobelPrize.org*, 2018.
<https://www.nobelprize.org/prizes/medicine/2006/summary/> (accessed Nov. 01, 2025).
- [25]“Nobel Prize in Chemistry 2008,” *NobelPrize.org*, 2018.
<https://www.nobelprize.org/prizes/chemistry/2008/summary/> (accessed Nov. 01, 2025).
- [26]“Nobel Prize in Physiology or Medicine 2024,” *NobelPrize.org*, 2018.
<https://www.nobelprize.org/prizes/medicine/2024/summary/> (accessed Nov. 01, 2025).
- [27]C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, “The Hallmarks of Aging,” *Cell*, vol. 153, no. 6, pp. 1194–1217, Jun. 2013, doi: <https://doi.org/10.1016/j.cell.2013.05.039>.
- [28]C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, “Hallmarks of aging: An expanding universe,” *Cell*, vol. 186, no. 2, pp. 243–278, Jan. 2023, doi: <https://doi.org/10.1016/j.cell.2022.11.001>.
- [29]M. Keshavarz, K. Xie, D. Bano, and D. Ehninger, “Aging – What it is and how to measure it,” *Mechanisms of Ageing and Development*, vol. 213, pp. 111837–111837, Jun. 2023, doi: <https://doi.org/10.1016/j.mad.2023.111837>.
- [30]R. Yuan, L. L. Peters, and B. Paigen, “Mice as a Mammalian Model for Research on the Genetics of Aging,” *ILAR Journal*, vol. 52, no. 1, pp. 4–15, Jan. 2011, doi: <https://doi.org/10.1093/ilar.52.1.4>.
- [31]M. J. H. Gilbert, T. C. Zerulla, and K. B. Tierney, “Zebrafish (*Danio rerio*) as a model for the study of aging and exercise: Physical ability and trainability decrease with age,” *Experimental Gerontology*, vol. 50, pp. 106–113, Dec. 2013, doi: <https://doi.org/10.1016/j.exger.2013.11.013>.
- [32]M. S. Abou-Dahech and F. E. Williams, “Aging, Age-Related Diseases, and the Zebrafish Model,” *Journal of Dementia and Alzheimer’s Disease*, vol. 1, no. 1, pp. 48–71, Jul. 2024, doi: <https://doi.org/10.3390/jdad1010004>.
- [33]P. Shen, Y. Yue, and Y. Park, “A living model for obesity and aging research: *Caenorhabditis elegans*,” *Critical Reviews in Food Science and Nutrition*, vol. 58, no. 5, pp. 741–754, 2018, doi: <https://doi.org/10.1080/10408398.2016.1220914>.
- [34]C. J. Kenyon, “The genetics of ageing,” *Nature*, vol. 464, no. 7288, pp. 504–512, Mar. 2010, doi: <https://doi.org/10.1038/nature08980>.
- [35]S. Gandhi, J. Santelli, D. H. Mitchell, J. W. Stiles, and D. Rao Sanadi, “A simple method for maintaining large, aging populations of *Caenorhabditis elegans*,” *Mechanisms of Ageing and Development*, vol. 12, no. 2, pp. 137–150, Feb. 1980, doi: [https://doi.org/10.1016/0047-6374\(80\)90090-1](https://doi.org/10.1016/0047-6374(80)90090-1).

- [36]J. Gruber, L. F. Ng, S. K. Poovathingal, and B. Halliwell, “Deceptively simple but simply deceptive – *Caenorhabditis elegans* lifespan studies: Considerations for aging and antioxidant effects,” *FEBS Letters*, vol. 583, no. 21, pp. 3377–3387, Oct. 2009, doi: <https://doi.org/10.1016/j.febslet.2009.09.051>.
- [37]O. Trader, D. Tzepos, H. N. Reyes Cardozo, and J. Magnes, “Age-dependent Dynamics of Locomotion in *Caenorhabditis elegans*: A Lyapunov Exponent Analysis,” *Journal of Visualized Experiments*, no. 223, Sep. 2025, doi: <https://doi.org/10.3791/68955>.
- [38]C. C. Kern *et al.*, “*C. elegans* ageing is accelerated by a self-destructive reproductive programme,” *Nature Communications*, vol. 14, no. 1, Jul. 2023, doi: <https://doi.org/10.1038/s41467-023-40088-1>.
- [39]J. Cho, J. Lu, D. Kim, and Y. Park, “Determination of health status during aging using bending and pumping rates at various survival rates in *Caenorhabditis elegans*,” *Scientific Reports*, vol. 15, no. 1, pp. 9057–9057, Mar. 2025, doi: <https://doi.org/10.1038/s41598-025-93876-8>.
- [40]S. N. Andreassen, M. Ben Ezra, and M. Scheibye-Knudsen, “A defined human aging phenome,” *Aging*, vol. 11, no. 15, pp. 5786–5806, Aug. 2019, doi: <https://doi.org/10.18632/aging.102166>.
- [41]R. Zhu and I. D. Chin-Sang, “*C. elegans* insulin-like peptides,” *Molecular and Cellular Endocrinology*, vol. 585, p. 112173, May 2024, doi: <https://doi.org/10.1016/j.mce.2024.112173>.
- [42]A. A. Akintola and D. van Heemst, “Insulin, Aging, and the Brain: Mechanisms and Implications,” *Frontiers in Endocrinology*, vol. 6, Feb. 2015, doi: <https://doi.org/10.3389/fendo.2015.00013>.
- [43]H. Lee and S.-J. V. Lee, “Recent Progress in Regulation of Aging by Insulin/IGF-1 Signaling in *Caenorhabditis elegans*,” *Molecules and Cells*, vol. 45, no. 11, pp. 763–770, Nov. 2022, doi: <https://doi.org/10.14348/molcells.2022.0097>.
- [44]R. Zhu and I. D. Chin-Sang, “*C. elegans* insulin-like peptides,” *Molecular and Cellular Endocrinology*, vol. 585, pp. 112173–112173, Feb. 2024, doi: <https://doi.org/10.1016/j.mce.2024.112173>.
- [45]R. Haque *et al.*, “Human insulin modulates α -synuclein aggregation via DAF-2/DAF-16 signalling pathway by antagonising DAF-2 receptor in *C. elegans* model of Parkinson’s disease,” *Oncotarget*, vol. 11, no. 6, pp. 634–649, Feb. 2020, doi: <https://doi.org/10.18632/oncotarget.27366>.
- [46]I. Sen *et al.*, “DAF-16/FOXO requires Protein Phosphatase 4 to initiate transcription of stress resistance and longevity promoting genes,” *Nature Communications*, vol. 11, no. 1, pp. 138–138, Jan. 2020, doi: <https://doi.org/10.1038/s41467-019-13931-7>.

- [47]R. S. Jope and G. V. W. Johnson, “The glamour and gloom of glycogen synthase kinase-3,” *Trends in Biochemical Sciences*, vol. 29, no. 2, pp. 95–102, Jan. 2004, doi: <https://doi.org/10.1016/j.tibs.2003.12.004>.
- [48]Amdi Amdisen, “Clinical and Serum-Level Monitoring in Lithium Therapy and Lithium Intoxication,” *Journal of Analytical Toxicology*, vol. 2, no. 5, pp. 193–202, Sep. 1978, doi: <https://doi.org/10.1093/jat/2.5.193>.
- [49]Young, Wise, “Review of Lithium Effects on Brain and Blood,” *Cell Transplant*, vol. 18, no. 9, pp. 951–975, 2009, doi: <https://doi.org/10.3727/096368909X471251>.
- [50]K. Sakrajda and Rybakowski, Janusz K, “The Mechanisms of Lithium Action: The Old and New Findings,” *Pharmaceuticals*, vol. 18, no. 4, p. 467, 2025, doi: <https://doi.org/10.3390/ph18040467>.
- [51]S. Nonaka, C. J. Hough, and D.-M. Chuang, “Chronic lithium treatment robustly protects neurons in the central nervous system against excitotoxicity by inhibiting *N*-methyl-D-aspartate receptor-mediated calcium influx,” *Proceedings of the National Academy of Sciences*, vol. 95, no. 5, pp. 2642–2647, Mar. 1998, doi: <https://doi.org/10.1073/pnas.95.5.2642>.
- [52]K. E. Rohr and M. J. McCarthy, “The impact of lithium on circadian rhythms and implications for bipolar disorder pharmacotherapy,” *Neuroscience Letters*, vol. 786, p. 136772, Aug. 2022, doi: <https://doi.org/10.1016/j.neulet.2022.136772>.
- [53]L. Shao, J. Cui, L. T. Young, and J. F. Wang, “The effect of mood stabilizer lithium on expression and activity of glutathione transferase isoenzymes,” *Neuroscience*, vol. 151, no. 2, pp. 518–524, 2008, doi: <https://doi.org/10.1016/j.neuroscience.2007.10.041>.
- [54]I. Grande, M. Berk, B. Birmaher, and E. Vieta, “Bipolar disorder,” *The Lancet*, vol. 387, no. 10027, pp. 1561–1572, Sep. 2015, doi: [https://doi.org/10.1016/s0140-6736\(15\)00241-x](https://doi.org/10.1016/s0140-6736(15)00241-x).
- [55]K. Chokhawala, S. Lee, and A. Saadabadi, “StatPearls: Lithium,” *Statpearls*, Jan. 14, 2024. <https://www.ncbi.nlm.nih.gov/books/NBK519062/> (accessed Nov. 29, 2025).
- [56]G. Curran and A. Ravindran, “Lithium for bipolar disorder: a review of the recent literature,” *Expert Review of Neurotherapeutics*, vol. 14, no. 9, pp. 1079–1098, Aug. 2014, doi: <https://doi.org/10.1586/14737175.2014.947965>.
- [57]W. Jonathan Ryves and A. J. Harwood, “Lithium Inhibits Glycogen Synthase Kinase-3 by Competition for Magnesium,” *Biochemical and Biophysical Research*

Communications, vol. 280, no. 3, pp. 720–725, Jan. 2001, doi: <https://doi.org/10.1006/bbrc.2000.4169>.

[58]S. Sarkar *et al.*, “Lithium induces autophagy by inhibiting inositol monophosphatase,” *The Journal of Cell Biology*, vol. 170, no. 7, pp. 1101–1111, Sep. 2005, doi: <https://doi.org/10.1083/jcb.200504035>.

[59]O. SofolaAdesakin *et al.*, “Lithium suppresses A β pathology by inhibiting translation in an adult *Drosophila* model of Alzheimer’s disease,” *Frontiers in Aging Neuroscience*, vol. Volume 62014, 2014, Available: <https://www.frontiersin.org/journals/agingneuroscience/articles/10.3389/fnagi.2014.00190>

[60]G. McColl, D. W. Killilea, A. E. Hubbard, M. C. Vantipalli, S. Melov, and G. J. Lithgow, “Pharmacogenetic Analysis of Lithium-induced Delayed Aging in *Caenorhabditis elegans*,” *Journal of Biological Chemistry*, vol. 283, no. 1, pp. 350–357, Jan. 2008, doi: <https://doi.org/10.1074/jbc.m705028200>.

[61]T. Nespital, B. Neuhaus, A. Mesaros, A. Pahl, and L. Partridge, “Lithium can mildly increase health during ageing but not lifespan in mice,” *Aging Cell*, vol. 20, no. 10, pp. e13479–e13479, Sep. 2021, doi: <https://doi.org/10.1111/accel.13479>.

[62]K. Zarse, T. Terao, J. Tian, N. Iwata, N. Ishii, and M. Ristow, “Low-dose lithium uptake promotes longevity in humans and metazoans,” *European Journal of Nutrition*, vol. 50, no. 5, pp. 387–389, Feb. 2011, doi: <https://doi.org/10.1007/s00394-011-0171-x>.

[63]E. Araldi, C. R. Jutzeler, and M. Ristow, “Lithium treatment extends human lifespan: findings from the UK Biobank,” *Aging*, vol. 15, no. 2, pp. 421–440, Jan. 2023, doi: <https://doi.org/10.18632/aging.204476>.

[64]C. Voisine, H. Varma, N. Walker, E. A. Bates, B. R. Stockwell, and A. C. Hart, “Identification of Potential Therapeutic Drugs for Huntington’s Disease using *Caenorhabditis elegans*,” *PLoS ONE*, vol. 2, no. 6, pp. e504–e504, Jun. 2007, doi: <https://doi.org/10.1371/journal.pone.0000504>.

[65]K. R. Weeks, D. S. Dwyer, and E. J. Aamodt, “Clozapine and lithium require *Caenorhabditis elegans* β -arrestin and serum- and glucocorticoid-inducible kinase to affect Daf-16 (Foxo) localization,” *Journal of Neuroscience Research*, vol. 89, no. 10, pp. 1658–1665, Jul. 2011, doi: <https://doi.org/10.1002/jnr.22705>.

[66]Zhi Yang Tam, J. Gruber, Li Fang Ng, B. Halliwell, and R. Gunawan, “Effects of Lithium on Age-related Decline in Mitochondrial Turnover and Function in *Caenorhabditis elegans*,” *The Journals of Gerontology*, vol. 69, no. 7, pp. 810–820, Jan. 2014, doi: <https://doi.org/10.1093/gerona/glt210>.

- [67]J. Meisel and D. Kim, “Inhibition of Lithium-Sensitive Phosphatase BPNT-1 Causes Selective Neuronal Dysfunction in *C. elegans*,” *Current Biology*, vol. 26, no. 14, pp. 1922–1928, Jul. 2016, doi: <https://doi.org/10.1016/j.cub.2016.05.050>.
- [68]Y.-S. Koh and D. D. Moore, “Linkage of the Nuclear Hormone Receptor Genes NR1D2, THRB, and RARB: Evidence for an Ancient, Large-Scale Duplication,” *Genomics*, vol. 57, no. 2, pp. 289–292, Apr. 1999, doi: <https://doi.org/10.1006/geno.1998.5683>.
- [69]T. P. Burris, “Nuclear Hormone Receptors for Heme: REV-ERB α and REV-ERB β Are Ligand-Regulated Components of the Mammalian Clock,” *Molecular Endocrinology*, vol. 22, no. 7, pp. 1509–1520, Jan. 2008, doi: <https://doi.org/10.1210/me.2007-0519>.
- [70]L. Yin, N. Wu, and M. A. Lazar, “Nuclear Receptor Rev-Erb α : A Heme Receptor that Coordinates Circadian Rhythm and Metabolism,” *Nuclear Receptor Signaling*, vol. 8, no. 1, pp. e001–e001, Jan. 2010, doi: <https://doi.org/10.1621/nrs.08001>.
- [71]N. Preitner *et al.*, “The Orphan Nuclear Receptor REV-ERB α Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator,” *Cell*, vol. 110, no. 2, pp. 251–260, Jul. 2002, doi: [https://doi.org/10.1016/s0092-8674\(02\)00825-5](https://doi.org/10.1016/s0092-8674(02)00825-5).
- [72]L. Yin, J. Wang, P. S. Klein, and M. A. Lazar, “Nuclear Receptor Rev-erb α Is a Critical Lithium-Sensitive Component of the Circadian Clock,” *Science*, vol. 311, no. 5763, pp. 1002–1005, Feb. 2006, doi: <https://doi.org/10.1126/science.1121613>.
- [73]A. Antebi, “Nuclear hormone receptors in *C. elegans*,” in *WormBook*, WormBook: The *C. elegans* Research Community, 2015. Accessed: Nov. 01, 2025. [Online]. Available: https://www.wormbook.org/chapters/www_nuclearhormonerecep/nuclearhormonerecep.html
- [74]D. Tsagkari, M. Markaki, and N. Tavernarakis, “NHR-85 modulates mitochondrial and lipid homeostasis to protect against α -synuclein aggregation in *C. elegans*,” *Journal of Cell Science*, vol. 138, no. 9, May 2025, doi: <https://doi.org/10.1242/jcs.263651>.
- [75]S. Zhang *et al.*, “NR1D1 Inhibition Enhances Autophagy and Mitophagy in Alzheimer’s Disease Models,” *Aging and Disease*, 2025, doi: <https://doi.org/10.14336/ad.2024.1654>.
- [76]B. Kinney *et al.*, “A circadian-like gene network programs the timing and dosage of heterochronic miRNA transcription during *C. elegans* development,” *Developmental Cell*, vol. 58, no. 22, pp. 2563–2579.e8, Nov. 2023, doi: <https://doi.org/10.1016/j.devcel.2023.08.006>.

- [77]J. S. Mattick and I. V. Makunin, “Non-coding RNA,” *Human Molecular Genetics*, vol. 15, no. suppl_1, pp. R17–R29, Apr. 2006, doi: <https://doi.org/10.1093/hmg/ddl046>.
- [78]Soudeh Ghafouri-Fard *et al.*, “Role of miRNA and lncRNAs in organ fibrosis and aging,” *Biomedicine & Pharmacotherapy*, vol. 143, pp. 112132–112132, Aug. 2021, doi: <https://doi.org/10.1016/j.biopha.2021.112132>.
- [79]W. Xing *et al.*, “Long non-coding RNAs in aging organs and tissues,” *Clinical and Experimental Pharmacology and Physiology*, vol. 44, no. S1, pp. 30–37, Oct. 2017, doi: <https://doi.org/10.1111/1440-1681.12795>.
- [80]D. Knupp and P. Miura, “CircRNA accumulation: A new hallmark of aging?,” *Mechanisms of Ageing and Development*, vol. 173, pp. 71–79, Jul. 2018, doi: <https://doi.org/10.1016/j.mad.2018.05.001>.
- [81]J. M. Nigro *et al.*, “Scrambled exons,” *Cell*, vol. 64, no. 3, pp. 607–613, Feb. 1991, doi: [https://doi.org/10.1016/0092-8674\(91\)90244-s](https://doi.org/10.1016/0092-8674(91)90244-s).
- [82]C.-Y. Yu and H.-C. Kuo, “The emerging roles and functions of circular RNAs and their generation,” *Journal of Biomedical Science*, vol. 26, no. 1, pp. 29–29, Apr. 2019, doi: <https://doi.org/10.1186/s12929-019-0523-z>.
- [83]L. Liu, J. Wang, Ramin Khanabdali, B. Kalionis, X. Tai, and S. Xia, “Circular RNAs: Isolation, characterization and their potential role in diseases,” *RNA Biology*, vol. 14, no. 12, pp. 1715–1721, Aug. 2017, doi: <https://doi.org/10.1080/15476286.2017.1367886>.
- [84]W.-Y. Zhou, Z.-R. Cai, J. Liu, D.-S. Wang, H.-Q. Ju, and R.-H. Xu, “Circular RNA: metabolism, functions and interactions with proteins,” *Molecular Cancer*, vol. 19, no. 1, Dec. 2020, doi: <https://doi.org/10.1186/s12943-020-01286-3>.
- [85]H. J. Hwang and Y. K. Kim, “Molecular mechanisms of circular RNA translation,” *Experimental & Molecular Medicine*, vol. 56, no. 6, pp. 1272–1280, Jun. 2024, doi: <https://doi.org/10.1038/s12276-024-01220-3>.
- [86]D. Cao, “Reverse complementary matches simultaneously promote both back-splicing and exon-skipping,” *BMC Genomics*, vol. 22, no. 1, Aug. 2021, doi: <https://doi.org/10.1186/s12864-021-07910-w>.
- [87]L.-L. Chen and L. Yang, “Regulation of circRNA biogenesis,” *RNA Biology*, vol. 12, no. 4, pp. 381–388, Mar. 2015, doi: <https://doi.org/10.1080/15476286.2015.1020271>.
- [88]A. Rasmussen *et al.*, “Circular stable intronic RNAs possess distinct biological features and are deregulated in bladder cancer,” *NAR Cancer*, vol. 5, no. 3, pp. zcad041–zcad041, Jun. 2023, doi: <https://doi.org/10.1093/narcan/zcad041>.

- [89]H. Gruner, M. Cortés-López, D. A. Cooper, M. Bauer, and P. Miura, “CircRNA accumulation in the aging mouse brain,” *Scientific Reports*, vol. 6, no. 1, Dec. 2016, doi: <https://doi.org/10.1038/srep38907>.
- [90]Jakub O. Westholm *et al.*, “Genome-wide Analysis of *Drosophila* Circular RNAs Reveals Their Structural and Sequence Properties and Age-Dependent Neural Accumulation,” *Cell Reports*, vol. 9, no. 5, pp. 1966–1980, Nov. 2014, doi: <https://doi.org/10.1016/j.celrep.2014.10.062>.
- [91]M. Cortés-López *et al.*, “Global accumulation of circRNAs during aging in *Caenorhabditis elegans*,” *BMC Genomics*, vol. 19, no. 1, pp. 8–8, Jan. 2018, doi: <https://doi.org/10.1186/s12864-017-4386-y>.
- [92]M. Baralle and M. Romano, “Age-Related Alternative Splicing: Driver or Passenger in the Aging Process?,” *Cells*, vol. 12, no. 24, p. 2819, Dec. 2023, doi: <https://doi.org/10.3390/cells12242819>.
- [93]S. Memczak *et al.*, “Circular RNAs are a large class of animal RNAs with regulatory potency,” *Nature*, vol. 495, no. 7441, pp. 333–338, Feb. 2013, doi: <https://doi.org/10.1038/nature11928>.
- [94]Thalyana Smith-Vikos and F. J. Slack, “MicroRNAs and their roles in aging,” *Journal of Cell Science*, vol. 125, no. 1, pp. 7–17, Jan. 2012, doi: <https://doi.org/10.1242/jcs.099200>.
- [95]Z. Gong, W. Hu, C. Zhou, J. Guo, L. Yang, and B. Wang, “Recent advances and perspectives on the development of circular RNA cancer vaccines,” *npj Vaccines*, vol. 10, no. 1, Mar. 2025, doi: <https://doi.org/10.1038/s41541-025-01097-x>.
- [96]D. Knupp *et al.*, “Loss of circRNAs from the *crh-1* gene extends the mean lifespan in *Caenorhabditis elegans*,” *Aging Cell*, vol. 21, no. 2, Jan. 2022, doi: <https://doi.org/10.1111/accel.13560>.
- [97]J. Yuan *et al.*, “Circ-CPSF1 Worsens Radiation-Induced Oxidative Stress Injury in *Caenorhabditis elegans*,” *Biomolecules*, vol. 13, no. 1, pp. 102–102, Jan. 2023, doi: <https://doi.org/10.3390/biom13010102>.
- [98]L. Kumar, Shamsuzzama, P. Jadiya, R. Haque, S. Shukla, and A. Nazir, “Functional Characterization of Novel Circular RNA Molecule, circzip-2 and Its Synthesizing Gene zip-2 in *C. elegans* Model of Parkinson’s Disease,” *Molecular Neurobiology*, vol. 55, no. 8, pp. 6914–6926, Jan. 2018, doi: <https://doi.org/10.1007/s12035-018-0903-5>.
- [99]M. Porta-de-la-Riva, L. Fontrodona, A. Villanueva, and J. Cerón, “Basic *Caenorhabditis elegans* Methods: Synchronization and Observation,” *Journal of Visualized Experiments*, no. 64, Jun. 2012, doi: <https://doi.org/10.3791/4019>.

- [100]H.-E. H. Park, Y. Jung, and S.-J. V. Lee, “Survival assays using *Caenorhabditis elegans*,” *Molecules and Cells*, vol. 40, no. 2, pp. 90–99, Feb. 2017, doi: <https://doi.org/10.14348/molcells.2017.0017>.
- [101]C. I. Nussbaum-Krammer, M. F. Neto, R. M. Brielmann, J. S. Pedersen, and R. I. Morimoto, “Investigating the Spreading and Toxicity of Prion-like Proteins Using the Metazoan Model Organism *C. elegans*,” *Journal of Visualized Experiments*, no. 95, Jan. 2015, doi: <https://doi.org/10.3791/52321>.
- [102]K. Ly, S. J. Reid, and R. G. Snell, “Rapid RNA analysis of individual *Caenorhabditis elegans*,” *MethodsX*, vol. 2, pp. 59–63, 2015, doi: <https://doi.org/10.1016/j.mex.2015.02.002>.
- [103]A. Dawoud, Z. I. Zakaria, H. H. Rashwan, M. Braoudaki, and R. A. Youness, “Circular RNAs: New layer of complexity evading breast cancer heterogeneity,” *Non-coding RNA Research*, vol. 8, no. 1, pp. 60–74, Oct. 2022, doi: <https://doi.org/10.1016/j.ncrna.2022.09.011>.

Appendix A – RCM Detection Script

Written in Python 3.12

```
from tqdm import tqdm

def calculate_gc_content(sequence):
    """Calculate the GC content of a sequence."""
    gc_count = sequence.count("G") + sequence.count("C")
    return (gc_count / len(sequence)) * 100 if sequence else 0

def find_base_pairing_regions(seq1, seq2, min_length=20, max_gaps=1, gap_size=1,
                             max_alignments=None, is_rna=True):
    """
    Find regions of base pairing between two RNA/DNA sequences.

    Args:
        seq1 (str): First sequence.
        seq2 (str): Second sequence.
        min_length (int): Minimum length of a pairing region.
        max_gaps (int): Maximum number of gaps allowed in an alignment.
        gap_size (int): Maximum size of a single allowed gap.
        max_alignments (int): Maximum number of alignments to return for display.
        is_rna (bool): Whether the sequences are RNA (if False, assumes DNA).

    Returns:
        list: List of tuples containing aligned regions and their positions, sorted by
        pairing count and GC content.
    """
    if is_rna:
        pairing = {"A": "U", "U": "A", "G": "C", "C": "G"}
    else:
        pairing = {"A": "T", "T": "A", "G": "C", "C": "G"}

    results = []
    len1, len2 = len(seq1), len(seq2)

    total_iterations = len1 * len2
    progress_bar = tqdm(total=total_iterations, desc="Processing alignments",
                        unit="alignment")

    for start1 in range(len1):
        for start2 in range(len2):
            aligned1, aligned2, pairing_map = [], [], []
            gaps_used = 0
            current_gap_length = 0
            pairing_count = 0

            i, j = start1, start2
            started = False
            while i < len1 and j < len2:
```

```

if seq2[j] == pairing.get(seq1[i], None):
    if current_gap_length > 0:
        if gaps_used >= max_gaps:
            break
        gaps_used += 1
        current_gap_length = 0

    aligned1.append(seq1[i])
    aligned2.append(seq2[j])
    pairing_map.append("|")
    pairing_count += 1
    started = True
else:
    if started and current_gap_length < gap_size:
        aligned1.append(seq1[i])
        aligned2.append(seq2[j])
        pairing_map.append(" ")
        current_gap_length += 1
    else:
        break

i += 1
j += 1

while aligned1 and (aligned1[0] not in pairing or seq2[start2] !=
pairing.get(seq1[start1], None)):
    aligned1.pop(0)
    aligned2.pop(0)
    pairing_map.pop(0)
    start1 += 1
    start2 += 1

while aligned1 and (aligned1[-1] not in pairing or seq2[j-1] !=
pairing.get(seq1[i-1], None)):
    aligned1.pop()
    aligned2.pop()
    pairing_map.pop()
    i -= 1
    j -= 1

total_length = i - start1

if total_length >= min_length:
    gc_content = calculate_gc_content(aligned1)
    results.append((pairing_count, start1, start2, gc_content, "".join(aligned1),
"".join(pairing_map), "".join(aligned2)))

progress_bar.update(1)

progress_bar.close()

```

```

# Sort primarily by pairing_count, then by GC content, both in descending order
results.sort(key=lambda x: (x[0], x[3]), reverse=True)

return results

def save_results_to_file(results, file_name):
    """Save alignment results to a text file."""
    with open(file_name, "w") as file:
        for idx, (pairing_count, matching_percentage, start1, start2, gc_content,
aligned1, pairing_map, aligned2) in enumerate(results, 1):
            alignment_info = (f'Alignment {idx} (start1={start1}, start2={start2},
pairing_count={pairing_count}, "
f'Matching %={matching_percentage:.2f}%%, GC
Content={gc_content:.2f}%):\n'
f'{aligned1}\n{pairing_map}\n{aligned2}\n\n')
            file.write(alignment_info)
    print(f'Results saved to {file_name}')

def main():
    print("Sequence Base Pairing Finder")
    seq1 = input("Enter the first sequence: ").upper()
    seq2 = input("Enter the second sequence: ").upper()
    sequence_type = input("Is this RNA or DNA? (Enter 'RNA' or 'DNA'):
").strip().upper()

    is_rna = sequence_type == 'RNA'

    min_length = int(input("Enter the minimum length of alignments to consider: "))
    max_gaps = int(input("Enter the maximum number of gaps allowed: "))
    gap_size = int(input("Enter the maximum size of a single gap allowed: "))
    max_alignments_display = int(input("Enter the maximum number of alignments to
display: "))

    min_pairing_count = int(input("Enter the minimum number of matches required:
")) # Threshold for minimum matches
    min_match_percentage = float(input("Enter the minimum matching percentage
required: ")) # New threshold for match percentage

    results = find_base_pairing_regions(seq1, seq2, min_length, max_gaps, gap_size,
None, is_rna)

    # Calculate matching percentage and filter results based on thresholds
    filtered_results = []
    for res in results:
        pairing_count, start1, start2, gc_content, aligned1, pairing_map, aligned2 = res
        total_alignment_length = len(aligned1) # Total length of the matched sequence
        matching_percentage = (pairing_count / total_alignment_length) * 100 if
total_alignment_length > 0 else 0

```

```

        if pairing_count >= min_pairing_count and matching_percentage >=
min_match_percentage:
            filtered_results.append((pairing_count, matching_percentage, start1, start2,
gc_content, aligned1, pairing_map, aligned2))

    # Sort results first by pairing count, then by matching percentage (both
descending)
    filtered_results.sort(key=lambda x: (x[0], x[1]), reverse=True)

    if not filtered_results:
        print("No alignments found that meet the criteria.")
    else:
        print("\nAlignments:")
        for idx, (pairing_count, matching_percentage, start1, start2, gc_content,
aligned1, pairing_map, aligned2) in
enumerate(filtered_results[:max_alignments_display], 1):
            alignment_info = (f'Alignment {idx} (start1={start1}, start2={start2},
pairing_count={pairing_count}, "
                f'Matching %={matching_percentage:.2f}% , GC
Content={gc_content:.2f}%):\n'
                f'{aligned1}\n{pairing_map}\n{aligned2}\n')
            print(alignment_info)

        save_option = input("Do you want to save the results to a file? (yes/no):
").strip().lower()
        if save_option == 'yes':
            file_name = input("Enter the file name (with .txt extension): ").strip()
            save_results_to_file(filtered_results, file_name)

    print(f"\nTotal alignments meeting the criteria: {len(filtered_results)}")

if __name__ == "__main__":
    main()

```