CHARACTERISATION OF RICTOR'S ROLE IN *Caenorhabditis elegans*

A DISSERTATION

Submitted in partial fulfilment of the requirements for the award of the Degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Submitted by

SMRITI RAINA

2K22/MSCBIO/48

Under the supervision of

Prof. JAI GOPAL SHARMA

Department of Biotechnology

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Shahbad Daulatpur, Main Bawana Road, Delhi- 10042, India

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Smriti Raina

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Shahbad Daulatpur, Main Bawana Road, Delhi- 10042

CANDIDATE'S DECLARATION

I, Smriti Raina, 2K22/MSCBIO/48 hereby certify that the work which I presented in the Major Project titled **"Characterisation of RICTOR's role in *Caenorhabditis elegans*"** in fulfilment of the award of the Degree of Masters of Science in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own work.

The work presented in this thesis has not been submitted by me for the award of any other degree of this or any other University. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated any idea/data/fact/ in my submission.

Smriti Raina
2K22/MSCBIO/48

NATIONAL INSTITUTE OF IMMUNOLOGY

Aruna Asaf Ali Marg, New Delhi, Delhi 110067

CERTIFICATE

I hereby certify that the Dissertation Project titled **"Characterisation of RICTOR's role in *Caenorhabditis elegans*"** which is submitted by Smriti Raina, 2K22/MSCBIO/48, Department of Biotechnology, Delhi Technological University, New Delhi in partial fulfilment of the requirement for the award of degree of Master of Science, is a record for the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

Place: New Delhi

Date: May, 2024

Dr Arnab Mukhopadhyay
Staff Scientist VI
Molecular Aging Laboratory
National Institute of Immunology
New Delhi-110067

National Institute of Immunology
राष्ट्रीय प्रतिरक्षाविज्ञान संस्थान

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Shahbad Daulatpur, Main Bawana Road, Delhi- 10042

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Place: New Delhi

Date: May, 2024

Prof. Yasha Hasija
Head of Department
Department of Biotechnology
Delhi Technological University

Prof. Jai Gopal Sharma
(Supervisor)
Department of Biotechnology
Delhi Technological University

ABSTRACT

Dietary flexibility is crucial for an organism's survival, yet the molecular mechanisms governing this adaptation and its impact on aging remain poorly understood. Our findings shed light on the multifaceted role of RICTOR, a key component of the mTOR signaling pathway, in modulating stress responses, mitophagy, and longevity using *C. elegans* as a model organism.

We first demonstrate that *rict-1* mutants exhibit enhanced osmotic stress tolerance, particularly when fed an HT115 diet, implicating mTORC2 signaling in stress response regulation. Then we looked at the role of RICTOR in mitochondrial homeostasis. The lack of significant changes in *hsp-60* expression challenges us to understand mitochondrial unfolded protein response (UPRmt) as a cellular mechanism underlying stress responses.

Moreover, investigation of mitophagy markers including PINK-1 expression reveals intriguing insights into mitochondrial quality control mechanisms. Further, we got interested in understanding the relation between lipid metabolism and collagen biosynthesis. We observe elevated lipid accumulation in *rict-1(ft7)*, particularly on an OP50 diet. These findings underscore the importance of RICTOR in regulating fat metabolism and ECM dynamics.

Lastly, our lifespan analysis highlights the critical roles of *pink-1* and *pdr-1*, in regulating longevity in *rict-1* mutants, further emphasizing the importance of mitochondrial quality control pathways in aging.

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LIST OF SYMBOLS OR ABBREVIATIONS

AMPK: Adenosine monophosphate-activated protein kinase

ATP: Adenosine triphosphate

cAMP: Cyclic-adenosine monophosphate

DR: Dietary Restriction

dsRNA: Double-stranded Ribonucleic acid

FOXO: Forkhead box class O

GFP: Green fluorescent protein

IGF: Insulin/insulin-like growth factor

IIS: Insulin/IGF-1 signaling

IPTG: Isopropyl β-D-1-thiogalactopyranoside

Kb: Kilo base pairs

kDa: Kilo Dalton

kg: Kilogram

LB Broth: Luria-Bertani broth

LDR: liquid DR

M: Molarity

mg: Milligrams

ml: Millilitres

mM: Millimolar

MRC: mitochondrial respiratory chain

mRNA: Messenger-Ribonucleic acid

mtDNA: mitochondrial DNA

mTOR: mammalian, or mechanistic, target of rapamycin

NADPH: Nicotinamide adenine dinucleotide phosphate

ng: Nanogram

NGM: Nutrient growth medium

NHR: Nuclear hormone receptor

RFP: Red Fluorescence Protein

RICT: Rictor (Rapamycin-insensitive companion of mammalian target of rapamycin)

RTK: Receptor tyrosine kinase

RNAi: RNA interference

ROS: Reactive Oxygen Species

rpm: Revolution per minute

rRNA: Ribosomal-Ribonucleic acid

RT: Room temperature

SIRTs : Sirtuins

SGK: Serum and glucocorticoid-regulated kinase

TOR: Target Of Rapamycin

CHAPTER 1

INTRODUCTION

Nutritional inputs play a crucial role in maintaining cellular functions and sustaining life-history traits such as development, aging and reproduction. Nutritional availability significantly impacts organism's health and aging. Dietary composition, including macronutrients and micronutrients like vitamins, serves as a vital source of metabolic cofactors. Animals have evolved various mechanisms to maintain homeostasis and regulate life-history traits in response to diverse dietary cues, although the underlying molecular mechanisms are not yet fully understood (Figure 1).

The nematode *Caenorhabditis elegans*, has been extensively utilized to study the role of nutrient signaling in aging. Studies have shown that dietary restriction can promote longevity in *C. elegans*, highlighting the importance of nutrient availability in regulating lifespan. The metabolically active intestinal microbiota of *C. elegans* serves as a major source of micronutrients, providing a genetically tractable model to investigate the effects of diet on lifespan.

C. elegans displays remarkable adaptive capacity to different bacterial diets, primarily regulated by genes identified through serendipitous discoveries. Disruption of these "gene-diet pairs" results in impaired homeostasis and altered life-history traits, emphasizing the importance of dietary quality on lifespan regulation.

Even though diet significantly impacts health, genetics also plays a significant role. Dietary choices determine the nutritional quality an individual receives, but genetics influence processes such as metabolism and protein homeostasis. Thus, both diet and genetics collectively contribute to an organism's overall health and lifespan (Yen CA *et al*., 2016).

Recent research in *C. elegans* has uncovered diet-gene pairs, showing that the effect of a gene mutation depends on the specific diet. These findings are significant because many metabolic pathways are conserved from worms to humans. Although only a few diet-gene pairs have been studied so far, it is possible that there are hundreds or even thousands of such interactions. These interactions could help explain the variability in aging rates and the prevalence and severity of age-related diseases in humans (Mullins *et al.*, 2020).

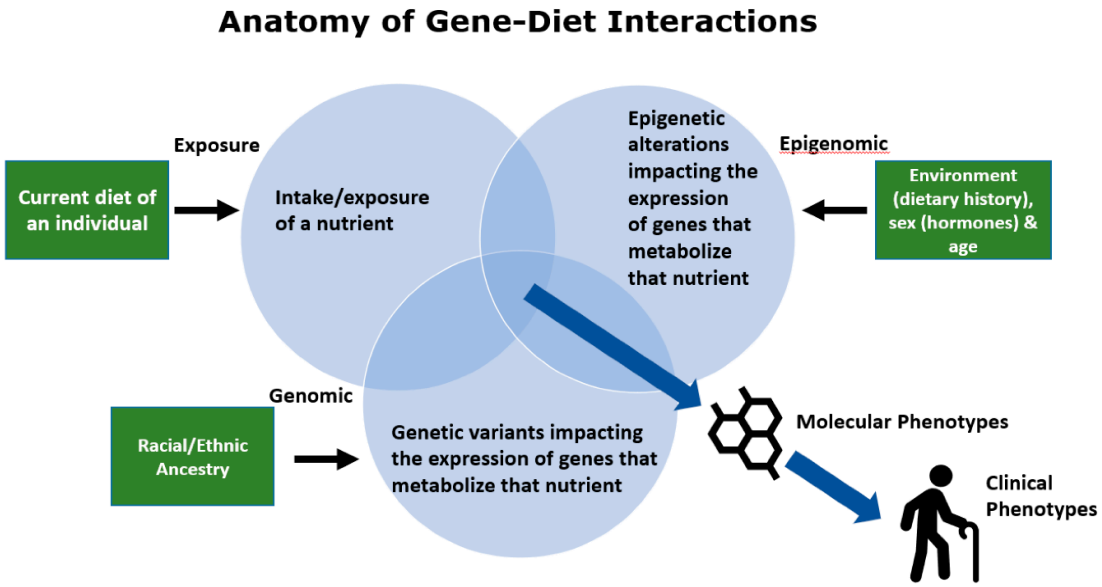

Figure 1: Anatomy of gene–diet interactions giving rise to molecular and clinical phenotypes (Mullins *et al.*, 2020)

1.1 DIET- GENE PAIR

The interaction between genes and diet significantly influences an organism's lifespan and healthspan, highlighting the importance of gene-diet pairs. In response to various dietary conditions, organisms employ adaptive strategies to maintain physiological balance. These nutrient-responsive mechanisms are elucidated through the study of mutants exhibiting diet-specific physiological differences, revealing critical gene-diet interactions that impact life-history traits. In the lab, the two most commonly used diets include, *E. coli* OP50 and HT115 diets, differing in their nutritional content.

Several gene-diet pairs have been identified in *C. elegans*, shedding light on the relationship between genetics and diet (Table 1). Examples include:

alh-6: Encodes ALH-6, an ortholog of Aldehyde dehydrogenase in humans involved in proline catabolism. Mutants of alh-6 show suppressed lifespan on the OP50 diet, but this effect is rescued when supplemented with antioxidants like ascorbate and N-acetylcysteine (Pang and Curran, 2014)

flr-4: It is a serine-threonine kinase gene that displays food-type-dependent life-span extension, where the mutants show a longer lifespan on HT115 diet in comparison to OP50 (Verma, Sonia, *et al*., 2018).

osm-3: This gene encodes a kinesin motor protein crucial for cilia formation in sensory neurons. Mutants of *osm-3* exhibit extended lifespan on the OP50 diet but not on the HT115 diet (Maier *et al*., 2010).

rict-1: A key component of mTORC2, RICTOR regulates fat metabolism and lifespan in a diet-dependent manner. Studies reveal that *rict-1(-)* mutants exhibit lean body structure when fed HT115 or HB101 compared to OP50. Despite similar protein and fat levels across bacteria, variations in carbohydrate content account for differential fat metabolism (Soukas *et al*., 2009).

It has also been revealed that *rict-1* plays a crucial role in regulating feeding behavior, with mutant worms consuming less when fed HB101. Moreover, there have been studies that highlight the involvement of insulin signaling pathways mediated by *akt-1, daf-2,* and *daf-16* in the regulation of lifespan in *rict-1* mutants. These findings suggest the significance of diet quality and genetic factors in modulating an organism's adaptive responses to dietary variations (Soukas *et al*., 2009).

Table 1: Diet-gene pair induced physiological changes in *C. elegans* (Chia-An Yen *et al.*, 2016)

Gene	Description	Diet	Physiological Response
rict-1	component of the Target of Rapamycin complex 2 (TORC2)	HB101	less fat, lengthened lifespan
		HT115	less fat, lengthened lifespan
		OP50	increased fat, shortened lifespan
skn-1	transcription factor orthologous to mammalian Nuclear factor erythroid- related factor (NRF)	OP50	Asdf (+) (Age-dependent Somatic Depletion of Fat)
		HT115	Asdf (-) (Age-dependent Somatic Depletion of Fat)
nmur-1	mammalian homolog of the neuromedin U receptor	OP50	longer lifespan
		HT115	normal lifespan
pept-1	intestinal peptide transporter	OP50	normal brood size

		HB101	reduced brood size
alh-6	proline metabolism gene: 1-pyrroline-5-carboxylate dehydrogenase (P5CDH)	OP50	shortened lifespan, rapid lipid depletion under acute starvation
		HT115	normal lifespan, WT level of lipid depletion under acute starvation

1.2 RICTOR: an essential subunit of TORC2 Complex

Rictor, a component of the target of rapamycin complex 2 (TORC2), plays a crucial role in signaling pathways related to insulin and growth factors. However, the precise functions of TORC2 and its interactions remain unknown. It is expressed in various tissues throughout the worm body, including head neurons, intestine, pharynx, and ventral nerve cord, suggesting its involvement in multiple physiological processes.

Mutations in the *rictor* homolog have been found responsible for increased body fat in *C. elegans*. Despite exhibiting high body fat, *rictor* mutants display developmental delay, reduced energy expenditure, reduced body size, diminished brood size, and significantly extended lifespan compared to wild-type worms when fed nutrient-rich bacterial strains..

These findings suggest that RICTOR is essential for balancing energy between long-term storage and essential physiological processes. RICTOR is vital for maintaining normal feeding behavior on nutrient-rich diets (Blackwell TK *et al.,* 2009).

RICTOR functions directly in the intestine to regulate fat mass and overall growth. Moreover, the high-fat phenotype of *rict-1* mutants is dependent on the genes *akt-1, akt-2*, and serum and glucocorticoid-induced kinase-1 (*sgk-1*).

However, the effects on lifespan, growth, and reproduction are primarily mediated by *sgk-1*. These studies highlight the role of RICTOR/TORC2 as a nutrient-sensitive complex that modulates various physiological processes, including fat metabolism, growth, feeding behavior, reproduction, and lifespan, via the AKT and SGK signaling pathways (Blackwell TK *et al.,* 2009).

Different mutant alleles of the *rict-1* gene in *C. elegans*:

***rict-1(mg360), rict-1(mg451), rict-1(ok386)*:** Each of these alleles is a type of deletion mutation that removes a different segment of the *rict-1* gene, leading to a loss of function. This can be used to study the effects of *rict-1* loss on metabolism, growth, and development in *C. elegans*.

***rict-1(ft7)*:** This allele is a point mutation that causes an amino acid substitution in the RICTOR protein, affecting its function. The *ft7* mutation helps researchers explore the impact of specific protein alterations on the mTORC2 complex and its downstream effects.

TOR (Target Of Rapamycin)

TOR, initially identified as a Target Of Rapamycin in yeast, and later in mammalian cells, is commonly known as mTOR. It is a protein kinase that belongs to the phosphatidylinositol 3-kinase-related kinase family.

This serine/threonine kinase forms two distinct complexes: TOR Complex 1 (TORC1) containing TOR and RAPTOR, and TOR Complex 2 (TORC2) containing TOR and RICTOR, along with other accessory proteins.

These play crucial roles in sensing nutrient levels and regulating downstream activities related to development, reproduction, metabolism, behavior, stress responses, and aging. While TOR complexes drive growth and anabolic metabolism, reduced TOR activity is known to activate protective mechanisms against stress.

Understanding the role of the TOR complex is essential for exploring the minute details of the process of aging and various human diseases. Investigations using model organisms, particularly *C. elegans*, are increasingly important for studying TOR complex functions *in vivo* under specific physiological conditions (Soukas AA *et al* ., 2009).

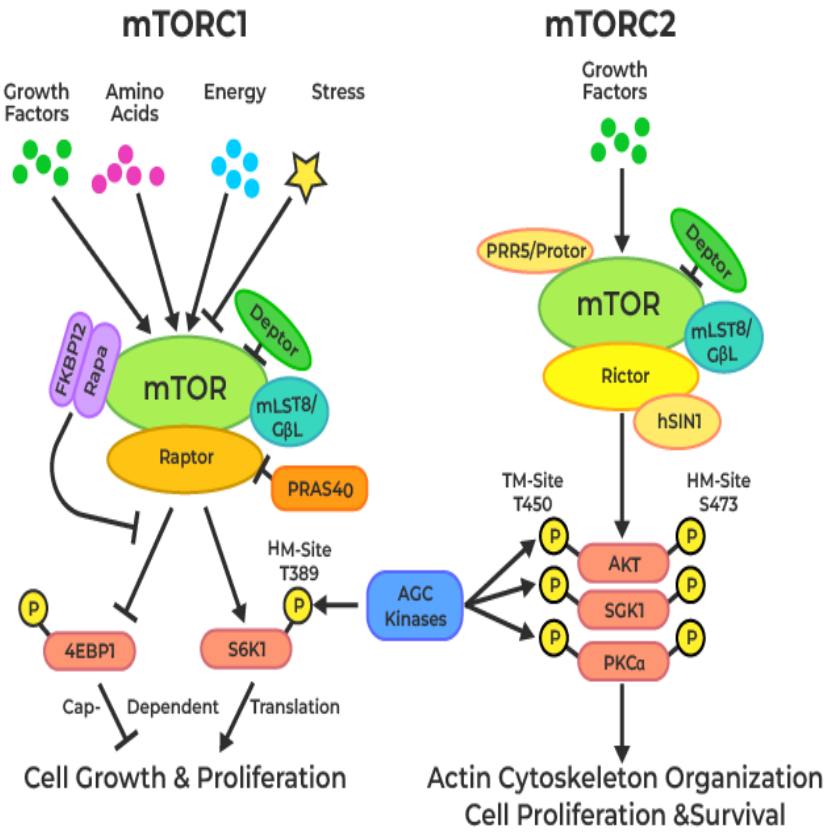

Figure 2: mTOR associated signalling pathways (Adapted from geeksforgeeks.org).

mTOR Signaling

The mTOR signaling pathway involves two key complexes, mTORC1 and mTORC2, each with distinct components and functions. The three main components of mTORC1 are mTOR, Raptor, and mLST8 (mammalian lethal with Sec13 protein 8, also known as GL). Raptor, a regulatory protein associated with mTOR, enhances substrate recruitment by binding to the TOR signaling (TOS) motif on many mTORC1 substrates and is essential for mTORC1's subcellular localization. mLST8 interacts with the catalytic domain of mTORC1, maintaining kinase activation loop, which is crucial for proper mTORC1 function.

mTORC2 is mainly involved in regulating cell survival and proliferation by phosphorylating members of the protein kinase family (PKA/PKG/PKC). It controls the actin cytoskeleton through PKC, and can phosphorylate other PKC family members, affecting cell migration and cytoskeletal remodeling. Additionally, mTORC2 phosphorylates and activates Akt, a critical component in the PI3K signaling pathway, as well as SGK1, PKC, and HDACs (Figure 2).

1.3 OBJECTIVES

1. Generation of tissue specific RNAi lines in the background of *rict-1* mutation

2. Screening of mitochondrial stress markers on knockdown of *rict-1* in the OP50 and HT115 backgrounds

3. Evaluation of mitophagy levels in *rict-1* mutant fed on *E. coli* OP50 and HT115 diets

4. Effect of bacterial genes on lifespan of host.

CHAPTER 2

REVIEW OF LITERATURE

2.1.1 AGING

For decades, scientists have delved into the phenomenon of aging, a natural process marked by the gradual loss of intrinsic physiological function due to various cellular and molecular damages accumulated over time through metabolic processes. Aging, essentially, is a time-related dysfunction which results in a progressive decline in physical and mental health, ultimately resulting in death. This phenomenon exhibits no consistent pattern concerning an organism's size, species, or metabolic function, leading to the wide variability of lifespans observed across different species.

The underlying mechanisms of aging remain largely unknown despite extensive research efforts. Multiple potential contributors to aging have been identified, including a complex interplay of biological, psychological, and ecological factors that shape an individual's aging trajectory throughout their lifetime. Aging manifests as a multifaceted phenomenon involving cellular, tissue, and organ-level changes that contribute to morbidity and mortality within the aging population.

Carlos López-Otín and Blasco *et al*., (2013) gave the nine hallmarks of aging. These hallmarks collectively drive the aging phenotype and increase the risk of chronic illnesses such as cancer, cardiovascular disease, neurodegenerative disorders, and metabolic dysfunction.

Early studies on aging identified calorie restriction in rodents as a factor extending lifespan (McCay *et al.,* 1935). Subsequently, specific genes affecting longevity were discovered, including mutations in the insulin-IGF-1-like receptor, *daf-2*, doubling the lifespan of *C. elegans* (Kenyon *et al*., 1993).

The histone deacetylase RPD3 and the SIR-2 protein were found to impact lifespan in yeast (*Saccharomyces cerevisiae*) (Kaeberlein *et al.,* 1999). These findings suggest a similar regulatory role of these genes in mammalian lifespan and aging processes.

Researchers continue to explore aging mechanisms to develop strategies for enhancing healthspan in the aging population.

2.1.2 The Hallmarks of Aging

Aging and cellular damage can result from various factors, making it complex to understand. To simplify this, researchers have categorized the contributing factors into nine broad categories known as the hallmarks of aging (Figure 3) (López Otín Carlos *et al*., 2023).

For a factor to qualify as a hallmark of aging, it ideally should meet three criteria:

1) The hallmark should be observable during the natural aging process.

2) Intentionally exacerbating the hallmark in experiments should accelerate the aging process.

3) Conversely, intentionally improving the hallmark in experiments should decelerate the natural aging process and enhance the duration of a healthy lifespan (Gems and Partridge, 2013).

Figure 3: The Hallmarks of Aging **(**López-Otín, Carlos *et al.,* 2013)

Genomic Instability

Genomic instability is a fundamental contributor to aging, characterized by the accumulation of damage to the genomic content. Increased aggregation of such

damage can lead to disorders like Werner or Bloom's syndrome (Burtner and Kennedy, 2010). Both external factors such as physical, chemical, and biological threats, as well as internal factors like DNA replication errors, spontaneous hydrolytic reactions, and reactive oxygen species (ROS), constantly disrupts the stability and integrity of DNA. These ultimately lead to abnormal protein translation or disruption of homeostasis, contributing to diseases and aging (Hoeijmakers, 2009).

Telomere Attrition

Telomeres, consisting of non-coding repeats and associated proteins, are situated at the ends of chromosomes, serving as protective caps critical for maintaining genomic stability. With each round of replication, telomeres naturally shorten until they reach a critical length, triggering a state of replicative senescence. However, this enzyme is typically absent in somatic cells, preventing uncontrolled cellular replication and the development of cancer. The shortening and eventual exhaustion of telomeres in somatic cells thus act as a molecular clock contributing to mammalian aging (Blackburn *et al*., 2006).

Epigenetic Alterations

Epigenetic alterations, referring to changes in the regulation of gene expression without alterations to the underlying DNA sequence, play a significant role in aging. These alterations encompass changes in DNA methylation patterns, modifications to histones, and remodeling of chromatin structure, resulting in differential gene expression and cellular homeostasis across different age groups.

Histone methylation, a crucial aspect of epigenetic regulation, has been implicated in aging in various organisms. Deletion of components involved in histone methylation complexes, such as H3K4 and H3K27, has been associated with lifespan extension in organisms like *C. elegans* and *D. melanogaster*, respectively. Additionally, inhibiting histone demethylases, such as H3K27, has been shown to extend lifespan in worms by modulating the insulin/IGF-1 pathway (Han and Brunet, 2012).

Loss of Proteostasis

Proteostasis refers to the intricate regulation of functional proteins within a cell, encompassing processes such as protein biogenesis, folding, and degradation. This dynamic system involves a network of biological pathways that ensure the proper synthesis and maintenance of the proteome. When proteins unfold due to endogenous or exogenous stress, mechanisms such as heat-shock proteins (HSP) assist in refolding them, or they undergo degradation via the ubiquitin-proteasome or lysosomal pathways.

However, disruptions in proteostasis can lead to cellular dysfunction and contribute to various conditions such as neurodegenerative diseases like tauopathies. Studies show that as organisms age, the efficiency of proteostasis mechanisms diminishes, leading to an imbalance in protein homeostasis and contributing to age-related cellular dysfunction and degeneration (Koga *et al.,* 2011).

Deregulated Nutrient Sensing

Nutrient sensing is a fundamental process by which cells detect and respond to changes in nutrient availability in their environment. This mechanism ensures adaptive responses for energy homeostasis and metabolism. Various signaling pathways are involved in nutrient sensing, which regulate cellular functions such as metabolism, growth, and proliferation in response to different nutrients like glucose, amino acids, and fatty acids.

Deregulated nutrient sensing, leads to alterations in these signaling pathways. With aging, these pathways become less responsive to nutrient signals, leading to metabolic imbalances and dysfunctions. This dysregulation can result in chronic inflammation, insulin resistance, impaired glucose tolerance, and increased susceptibility to age-related diseases such as diabetes, cardiovascular disease, and neurodegeneration (Barzilai *et al*.,2012, Fontana *et al*., 2010).

Mitochondrial Dysfunction

As cells and organisms age, there is decline in the efficiency of the mitochondrial respiratory chain that leads to increased electron leakage and reduced ATP generation. This mitochondrial dysfunction contributes to the accumulation of dysfunctional mitochondria, which produce reactive oxygen species, that were previously believed to be a primary cause of cellular damage and aging.

Mitochondrial dysfunction may directly impact cellular signaling and interorganellar crosstalk by affecting the interface between the outer mitochondrial membrane and the endoplasmic reticulum (Calabrese *et al*., 2011).

Cellular Senescence

Cellular senescence can be characterized by cell cycle arrest, with some associated phenotypic changes. During aging, cell cycle arrest may occur due to telomere shortening, or by derepression of the INK4/ARF locus (Collado *et al.,* 2007). Senescent cells have a characteristic secretory phenotype known as “senescence-associated secretory phenotype (SASP)”; their secretome contains proinflammatory cytokines matrixmettalo-proteinases.

Stem Cell Exhaustion

The regenerative potential of tissues decreases dramatically with age, which leads to multiple age-related diseases; for example, the decline in haematopoiesis causes a decrease in adaptive immune cells that lead to immunosenescence, a common phenotype in old individuals. Stem cell rejuvenation may be a step toward reversing the aging process (Shaw *et al*., 2010). As a result of various types of damage associated with aging, stem cell exhaustion is believed to be a significant contributor to the decline in tissue and organismal function with age (Rando and Chang, 2012).

Altered Intercellular Communication

Proper intercellular communication is necessary for the appropriate functioning of different cells, but with age, intercellular communication tends to decrease. Many neurohormonal signaling pathways, like adrenergic or insulin-IGF1, tend to get downregulated with aging due to increased inflammatory reactions.
One of the age-related defects in intercellular communication is “inflammaging”. Aging not only causes "inflammaging,” but studies have found that age-related defects in one tissue cause defects in other tissues through inter-organ coordination of the aging phenotype (Salminen *et al*., 2012).

2.1.3 Why should we study aging?

Studying aging is crucial due to its close association with a number of age-related diseases, which challenges the healthcare systems worldwide. As individuals age, they become more susceptible to various health conditions, including neurodegenerative disorders like Parkinson's diseases, cardiovascular diseases such as hypertension, type 2 diabetes, and certain types of cancers.

Understanding the biological processes underlying aging provides insights into the mechanisms that drive these age-related diseases. By studying these mechanisms, potential targets for therapeutic interventions can be identified that aim at delaying the onset or progression of age-related diseases.

Hence by investigating the molecular pathways involved in these processes, scientists can develop personalized interventions, and lifestyle recommendations to optimize healthspan and reduce the burden of age-related diseases on individuals and healthcare systems.

2.2 *C. ELEGANS* AS A MODEL ORGANISM TO STUDY AGING

Sydney Brenner's foresight in the development and establishment of the nematode or roundworm Caenorhabditis elegans as a genetic model organism is hailed to be one of the best. Brenner brought in *C. elegans* almost 50 years ago as a model to understand questions pertaining to developmental biology and neurobiology.

Advantages of using *C. elegans* as a model organism include features like full genome characterization with relatively few genes compared to higher organisms, making genetic manipulation and functional studies more feasible.

Secondly, *C. elegans* have a relatively short lifespan, with a generation time of about three days. This enables fast experimentation and observation of multiple generations within a short period of time, facilitating studies on developmental biology and aging. Their transparent body allows visualization of internal organs, tissues, and cellular processes, without the need for invasive procedures. This makes it well-suited for studying developmental processes, neuronal circuits, and cellular dynamics *in vivo*.

In *C. elegans,* many biological pathways and processes are evolutionarily conserved across species, including humans. Therefore, findings from studies in *C. elegans* often have relevance to human biology, and can provide insights into the molecular mechanisms underlying human diseases and other physiological processes (Wormbook).

A number of mutant strains, transgenic lines, and RNA interference techniques are available for manipulating gene expression and studying gene function in *C. elegans*. Additionally, comprehensive databases and resources, such as WormBase, provide valuable information on the genome, gene function, and experimental protocols for researchers.

C. elegans is a tiny, free-living nematode found worldwide. Newly hatched larvae are 0.25 millimeters long, and adults are a millimeter long (Figure 4). They are self-fertilizing hermaphrodites (XX), with a rare occurrence of males (XO). It contains only about 1000 cells, however it has well-defined tissues and neurons, making it easy to study neural networks. The ability of an adult worm to produce up to 300 eggs makes it ideal for keeping them in large numbers so that we can conduct widespread drug testing and other experiments.

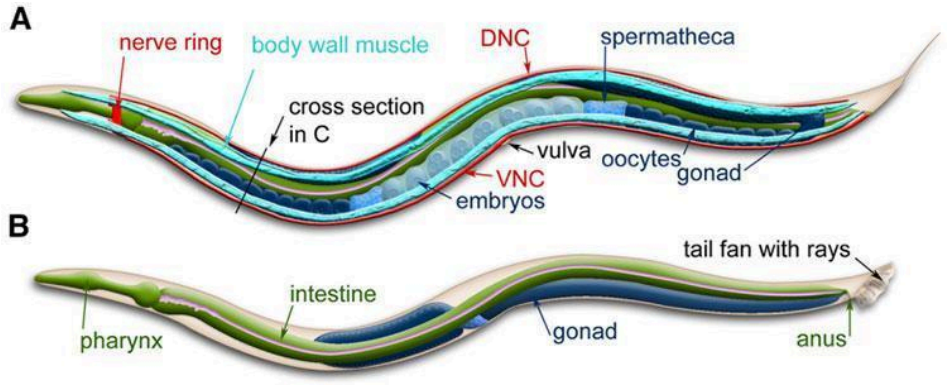

Figure 4: Anatomy of *C. elegans* : Anatomical features of a hermaphrodite (A) and male (B) viewed laterally (Corsi *et al*., 2015)

Genetic interventions like RNA interference (RNAi) are very easy to conduct in worms. C. *elegans* express the *sid* family of genes, *sid-1 and sid-2*, which are transmembrane proteins involved in the uptake of dsRNA from the intestine, making RNAi through feeding bacteria expressing the double-stranded RNA.

Worms, when fed bacteria that express double-stranded RNA, work in tandem with the RNA interference machinery to silence the corresponding genes. RNAi is also done by soaking the worms in bacterial culture and microinjecting the plasmids into the worm germline. Feeding RNAi, however, is the most simple of them, and has moderate to high efficiency. This technique gives researchers the power to screen for a large number of genes (Rankin, 2002).

Life Cycle of *C. elegans*

The life cycle of *C. elegans* proceeds through four distinct larval stages: L1 to L4 (Figure 5). Each cycle ends with moulting, in which the worm sheds its cuticle and grows a new one. All four larval stages exhibit an increase in body size. The sexes of the worms can be clearly distinguished only after the L3 stage.

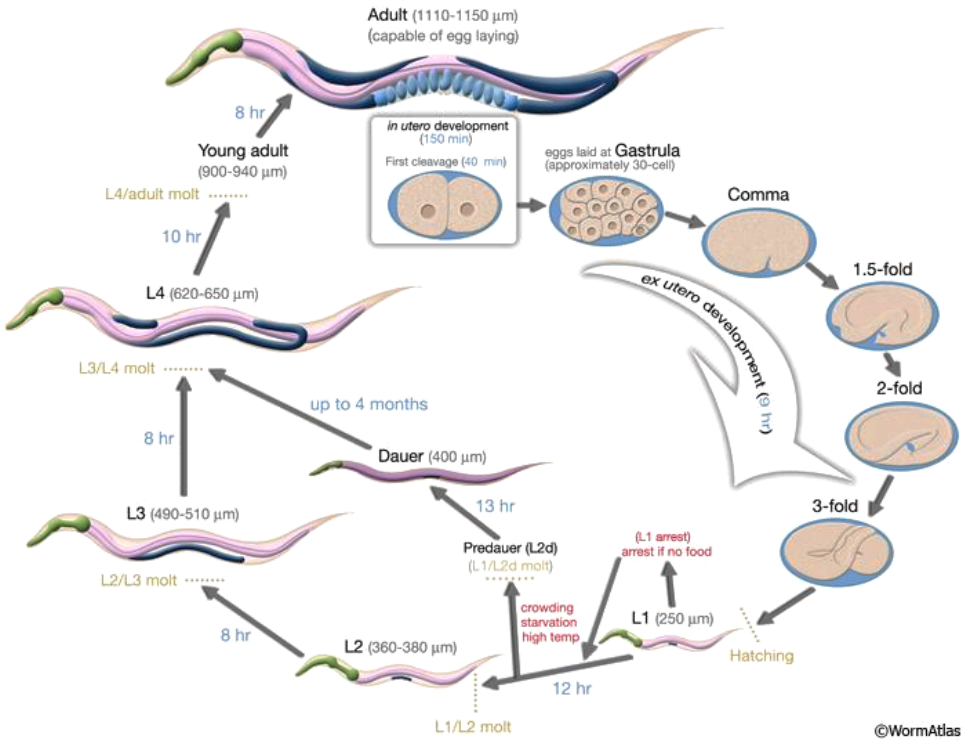

Figure 5: A diagrammatic representation of developmental stages of *C. elegans* (WormAtlas)

At the L4 stage, hermaphrodites have a tapered tail, and a developing vulva can be seen as a clear half circle in the center of the ventral side. The wider girth and tapered tail of the hermaphrodite and the slimmer girth and fan-shaped tail of the male allow adults to distinguish between the two sexes. Hermaphrodite sperm, or male sperm obtained through mating, can fertilize oocytes. L4 larvae molt into adults that survive for approximately 3 weeks under normal laboratory conditions.

Apart from the minute size differences, L1 and L2 are phenotypically similar, and the formation of the vulva crescent starts to develop at the L3 stage. At L4, hermaphrodites develop a clear tapered tail (fan-shaped in males), and the developing vulva can be seen as a clear half circle on the ventral side (Adapted from WormAtlas).

Aging in *C. elegans*

In *C. elegans,* certain established markers for aging include the buildup of gut granules in the intestine that are made up of lipofuscin or advanced glycation end products. Hermaphrodites' limited capacity for sperm storage implies that after days

3-5 of adulthood, procreation begins to slow down and eventually stops unless they mate with males who could provide extra sperm.

Oocyte quality starts to decline with age as well (Luo *et al.,* 2011). Like any other organism, *C. elegans* shows a slower locomotory capability in older age as movement gradually declines. The appearance of *C. elegans* also changes with age as they start to shrink, and the mid body appears swollen. The pharynx weakens, the intestine atrophies, the cuticles begin to wrinkle, and the lumen also begins to bloat as a result of bacterial growth. (Herndon *et al*., 2017).

2.3: Signaling Pathways Regulating Aging

In the complex process of aging, multiple signaling pathways intersect and regulate its systemic effects. These pathways include nutrient and energy-sensing pathways like insulin/IGF-1 and mTOR, as well as cell proliferation or regulation pathways like TGF-β pathways.

2.3.1 Insulin/ IGF-1 Signaling

The insulin/IGF-1 signaling (IIS) pathway is used to sense nutrient levels, and is responsible for metabolism, growth, development, and longevity in *C. elegans* as well as various other model organisms.

In worms, insulin-like peptides bind the DAF-2 receptor (the worm ortholog of IGFR), which controls the activity of its downstream phosphoinositide 3-kinase cascade. Mutations that reduce PI3K signalling show an increase in lifespan, by nuclear localization and activation of the transcription factor DAF-16/FOXO (McCormick MA *et al.*,2012).

2.3.2 mTOR signaling

mTOR belongs to the phosphoinositide 3-kinase family with its downstream targets being AKT. It is essential for several processes like apoptosis, cell proliferation, and inflammation. TOR inhibition stimulates autophagy, which leads to lifespan extension, and has been found to confer resistance to environmental stresses. It is transcriptionally regulated by PHA-4, FOXA transcription factor.

Inhibition of S6 kinase has shown to increase lifespan in worms, yeast, and mice. TOR also upregulates translation by activating ribosomal subunit S6, and inhibiting 4E-BP, the inhibition of TOR by some drugs like rapamycin leads to longevity (Selman, C. *et al,*. 2009).

2.3.3 TGF-β signaling

Transforming growth factor-β (TGF-β) signaling plays an important role in cell growth, differentiation, and death. In *C. elegans*, five TGF-β-related genes have been identified, of which *dbl-1*, *daf-7*, and *unc-129* are important. As DBL-1 can be identified by the mutant phenotypes of small body size (Sma) and mail tail abnormalities (Mab), the pathway is also known as the sma/mab pathway (Padgett *et al.,* 1998).

The DBL-1 pathway is involved in innate immunity and reproductive aging (Almedom *et al.,* 2009), and the ligand DBL-1 and its receptors SMA-6 and The lifespan benefits of TGF-β mutants are suppressed by the loss of function of DAF-16 mutants, showing that there is a crosstalk between TGF-β and the IIS pathway (Sun, X., *et al.,* 2017).

2.3.4 AMP Kinase Signaling

AMP kinase is a nutrient and energy sensor that is known to activate pathways like glycolysis, and represses anabolic pathways (gluconeogenesis) when the cell's AMP/ATP ratio goes up (Tom, R. Z., 2013).

In *C. elegans*, the overexpression of AAK-2 by AMPK has shown to extend its lifespan by integrating energy levels and IIS signaling to extend lifespan, as AMPK and DAF-16/FOXO play a vital role in lifespan extension in the *daf-2* mutants (Kruempel, J., 2018).

2.3.5 JNK Signaling Pathway

The c-Jun N-terminal kinase (JNK) acts parallel to the IIS pathway and positively regulates DAF-16 in order to provide lifespan extension and stress resistance. JNK-1 phosphorylates DAF-16 which leads to the expression of various stress response genes responsible for mitigating damage from harmful stresses (Neumann-Haefelin, E. *et al*., 2008).

In *C. elegans*, the JNK homolog KGB-1 plays an important role in germline proliferation and protein folding stress (Twumasi‐Boateng, K., *et al.* 2012), but with age, the activity of KGB-1 declines, making the adult worms more prone to protein folding stress, heavy metals, and infection, leading to a short lifespan.

2.3.6 Steroid Hormone Signaling

The steroid hormone nuclear receptor, such as the bile-like steroid hormone Dafachronic acid (Daf), affects lifespan by binding to DAF-12 and DAF-2. DAF-12 works as a switch between the L2 and L3 stages of the worm's life cycle by helping in the L2 to L3 transition via microRNAs mir-84 and mir-214. Under

environmentally stressed conditions, however, the worms form dauer. Moreover, in the absence of germline, DAF-12 signaling increases with the help of DAF-9, DAF-36, and DAF-16 (Kathleen J. Dumas *et al*., 2013).

2.4 Role of innate immunity in longevity and lifespan

C. elegans lack adaptive immunity and rely solely on its innate immune system to fight pathogens and various environmental stresses.

The p38-MAPK pathway in *C. elegans* is a key regulator of innate immunity that functions as a crucial modulator of antimicrobial, antifungal, and stress response pathways. PMK-1, the *C. elegans* ortholog of mammalian p38-MAPK is the main component of this pathway. Upstream of PMK-1 are the kinases SEK-1 and NSY-1, which correspond to mammalian MKK3/6 and MKK5/15, respectively (Soo *et al.,* 2023). Loss-of-function mutations in any of these proteins leads to effective immune response via the PMK-1 pathway. UNC-43, a Ca2+/calmodulin-dependent protein kinase II, plays a critical role upstream of NSY-1 and SEK-1, influencing neuronal fate in addition to immune responses.

SKN-1, a *C. elegans* ortholog of the mammalian Nrf-2 protein contains a phosphorylation site for PMK-1, and phosphorylation by PMK-1 triggers its nuclear localization (Figure 6). Once in the nucleus, SKN-1 regulates the expression of genes involved in xenobiotic detoxification (Matthew G *et al*., 2016).

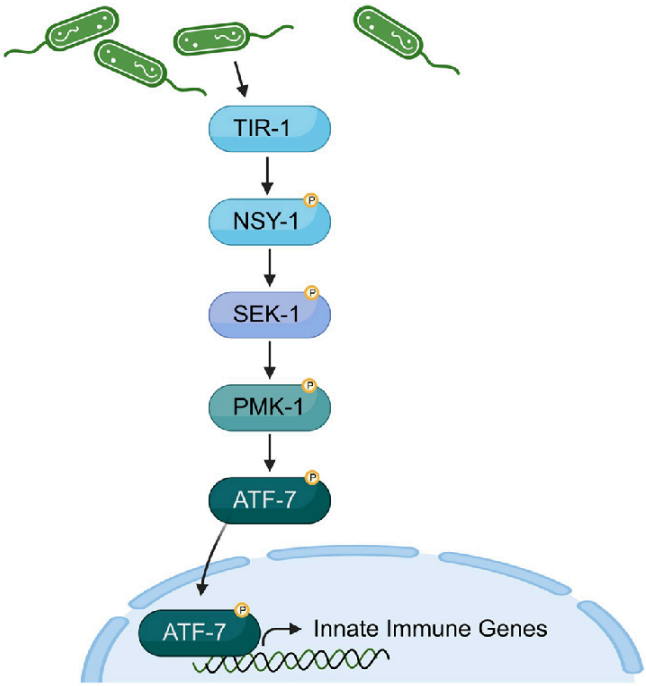

Figure 6: The p38-mediated innate immune signaling pathway (Soo *et al.,* 2023).

2.5 The Role of Mitochondria in the life of *C. elegans*

Mitochondria are essential organelles involved in energy metabolism via oxidative phosphorylation and play key roles in biological processes such as aging and apoptosis.
Deficiencies in the mitochondrial respiratory chain (MRC) are linked to a wide range of diseases. Using *C. elegans* as a model, significant advancements are made in the area of mitochondrial dynamics and the impact of mutations in both nuclear and mitochondrial DNA (mtDNA).

The primary function of mitochondria is to generate ATP through oxidative phosphorylation, which is the main source of cellular energy.

Mutations particularly affecting the nuclear or mtDNA genes can lead to diseases including symptoms related to aging, neurodegenerative conditions like Alzheimer's, Parkinson's, and Huntington's diseases, as well as cancer, such as hereditary paraganglioma.

The presence of mitochondrial DNA (mtDNA) mutations has been linked to the aging process, and oxidative stress. It has also been reported that there are deletions in the mitochondrial genome of *C. elegans* during aging. These deletions were first identified in an aging wild-type population of nematodes. These deletions were found to arise spontaneously within the mitochondrial genome, and their frequency was observed to increase with age, hence suggesting a correlation between the accumulation of mtDNA deletions and the aging process (William Y. Tsang. *et al.,* 2003).

Mitochondria and Aging

The connection between mitochondria and the aging process, as proposed by Harman (1983) with the mitochondrial free radical theory of aging, has been widely acknowledged for decades. Mitochondrial function typically declines with age across various tissues and organisms, accompanied by morphological changes and reduced respiration (Figure 7).

Theories suggest that the accumulation of mutations in mitochondrial DNA and decreased mitochondrial biogenesis may lead to loss of mitochondrial function during aging.

Recent studies in yeast, *C. elegans*, and mice indicate that disruptions in the removal of damaged mitochondria, known as mitophagy, contribute to the accumulation of dysfunctional mitochondria during aging. Downregulation of proteins involved in

mitophagy leads to mitochondrial damage and a shortened lifespan, while activating them enhances mitochondrial health and lifespan (Machiela *et al.,* 2020).

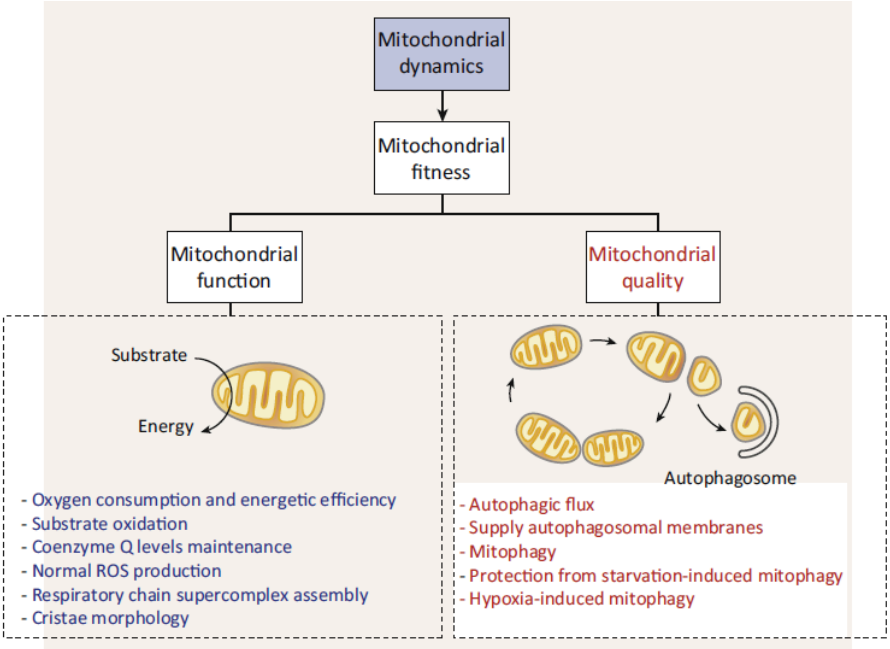

Figure 7: Mitochondrial Dynamics: A Key Process in the Preservation of Mitochondrial Fitness (Sebastián D *et al.,* 2017)

Increased production of ROS has been linked to various metabolic diseases. The induction of ER stress, cellular stress response, or unfolded protein response occurs when ER homeostasis is disrupted, and this activation has been associated with the pathogenic mechanisms underlying obesity and type 2 diabetes mellitus, resulting in metabolic disorders.

The induction of apoptosis can contribute to cardiomyopathy and neurodegeneration. Instability in mitochondrial DNA (mtDNA) has been correlated with cardiomyopathy, neurodegeneration, and muscle atrophy, while a decrease in mitophagy has been associated with cardiomyopathy and sarcopenia.

Thus, alterations in mitochondrial dynamics serve a dual role, influencing both mitochondrial health and quality control mechanisms. Dysregulated mitochondrial dynamics can disrupt autophagy and mitophagy processes. Consequently, this imbalance in mitochondrial dynamics may lead to the accumulation of dysfunctional mitochondria over time (Sebastián D *et al*., 2017).

2.6 TOR Complexes

mTORC1 is shown to induce phosphorylation of several factors involved in autophagy regulation, including the MiT-TFE factors, ATG13, and ULK1. Phosphorylation by mTORC1 renders these factors unable to positively regulate autophagy (Figure 8).

Similarly, mTORC2 induces phosphorylation of Beclin-1, GFAP, and VDAC1, inhibiting their ability to activate autophagy. Additionally, mTORC2-mediated phosphorylation of FOXO proteins is shown to modulate their subcellular localization, resulting in autophagy regulation (Ballesteros J. O *et al.,* 2021).

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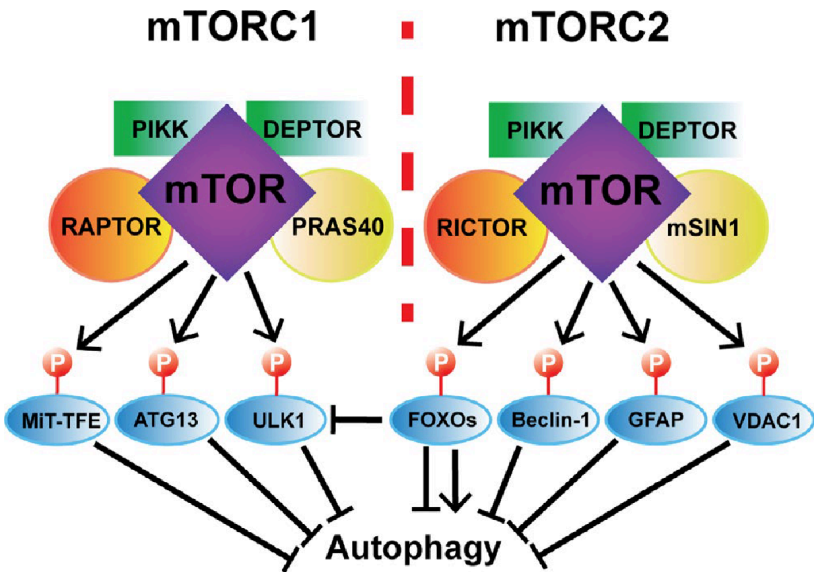


Figure 8: Regulatory mechanisms by which mTOR complexes modulate autophagy, influencing cellular homeostasis (Ballesteros J. O *et al.,* 2021)

mTORC2 has shown to respond to growth factors such as insulin, and participate in the regulation of cell metabolism and survival, mostly through AKT/protein kinase B (PKB) as its main downstream effector (Figure 9).

AKT/PKB is the best described substrate of mTORC2 phosphorylation. It is a member of the family of AGC kinases that includes PKC and serum and glucocorticoid regulated kinase (SGK). Several of the AGC kinases contain a turn motif adjacent to hydrophobic residues that is subject to phosphorylation by mTORC2 (Dai & Thomson, 2019; Pearce *et al*., 2010).

Multi-step activation of AKT involves the phosphorylation of Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and Ser473, one of the targets of mTOR kinase activity that requires the presence of RICTOR, others being Thr450, Ser477, and Thr479.

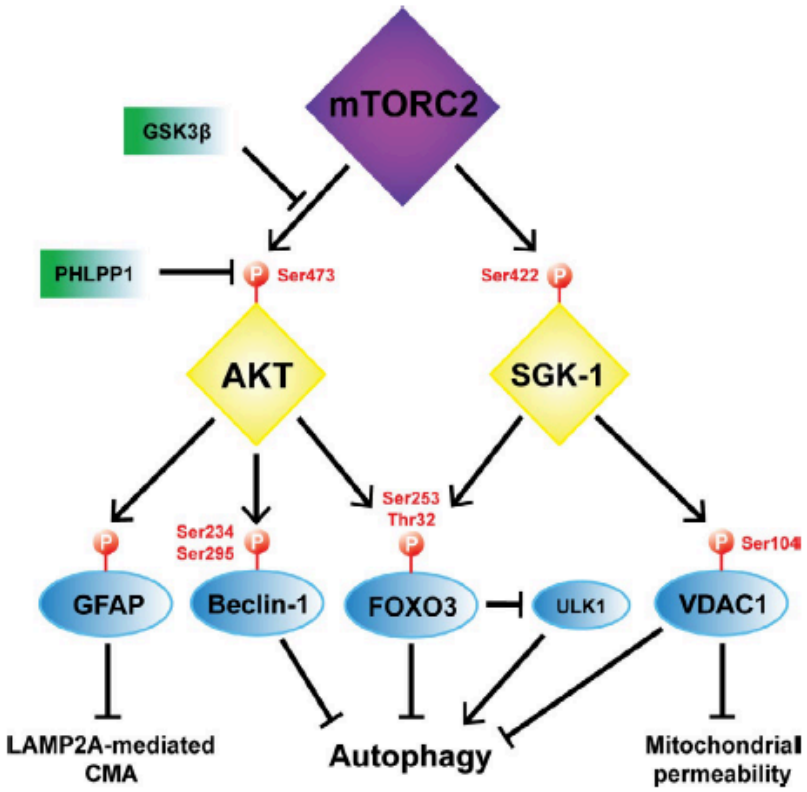


Figure 9: The mTORC2-AKT-SGK axis in autophagy (Ballesteros J. O *et al.,* 2021)

CHAPTER 3

MATERIALS AND METHODS

3.1 C. elegans Strains and Maintenance

The *C. elegans* strains were obtained from the Caenorhabditis Genetics Center and maintained at 20^0C on 90mm Nematode growth media (NGM) agar plates (Appendix-I.1), having OP50 (*Escherichia coli*) bacterial lawn on it. Worms were synchronised by the process of bleaching, and L1 worms after hatching were used during all experiments.

Strains used during experiments were, N2 Bristol as wild type; *rict-1(ft-7)*; *hsp-60p::GFP*, *cyp14A4P::GFP*, *col-19::GFP*

3.2 Bacterial Growth

Bacteria from glycerol/live stocks were streaked on Luria-Bertani plates and incubated overnight (16 h) at 37^0C, till single colonies were visible. For primary culture: OP50 and HT115, single colonies were inoculated in Luria-Bertani broth with tetracycline and grown overnight (12h). Next day, secondary cultures were inoculated with 1/100th volume of primary culture without any antibiotic in media for HT115 and OP50, and were incubated at 37^0C till the optical density (OD600) reached 0.6.

The secondary cultures were then pelleted down at 6000 RPM for 5 minutes at 4^0C, and supernatant was discarded. The pellet was then resuspended in M9 buffer (1/10th volume of original secondary culture), and mixed well. Then, 350ul or 1 ml of secondary cultures was plated on 60mm and 90mm NGM plates respectively (Plates were pre-dried in the hood for approximately 2 hours before seeding), the bacterial lawn was grown for 2 days at 20^0C before worm pouring.

3.3 Feeding RNAi

Bacteria from glycerol/live stocks were streaked on Luria-Bertani plates and incubated overnight (16 h) at 37^0C, till single colonies were visible. For primary culture: OP50 and HT115, single colonies were inoculated in Luria-Bertani broth having Ampicillin and tetracycline and grown overnight (12h).

Next day, secondary cultures were inoculated with 1/100th volume of primary culture in Luria-Bertani broth containing ampicillin and were incubated at 37^0C till the optical density (OD600) reached 0.6. The secondary cultures were then pelleted down at 6000 RPM for 5 minutes at 4^0C and supernatant was discarded. The pellet

was then resuspended in M9 buffer (1/10th volume of original secondary culture), and mixed well with Ampicillin and IPTG. Then, 350µl or 1 ml of secondary cultures were plated on 60mm and 90mm NGM plates respectively (Plates were pre-dried in the hood for approximately 2 hours before seeding), the bacterial lawn was grown for 2 days at 20^0C before worm pouring.

3.4 Synchronisation of worms

Gravid worms maintained on *E. coli* OP50, were bleached and kept for egg hatching for 16 hours in an M9 buffer (Appendix-I.4) at 20^0C. The hatched L1 worms were then centrifuged at 3000 RPM for 1 minute at 20^0C, and the extra M9 buffer was aspirated out leaving the dense L1 Pellet, then L1 worms were poured on respective plates for experiments or maintenance.

3.5 Lifespan analysis

Once the worms reached the late L4 stage and were about to lay eggs, 5-fluorodeoxyuridine (FUDR, final concentration 0.1 mg/ml of media, 120 µl) was overlaid on them and kept for drying for 20 minutes. The lifespan scoring started on the 7th day of adulthood, and continued alternate days till all the worms were dead. OASIS software was used for all the statistical analyses of survival rates. Statistical analyses were performed using Graphpad 9.0.

3.6 Osmotic stress assay

Two different salt concentration plates were used for this assay, 50mM NaCl NGM (Appendix-I.3) plates were used till the worms reached the L4 stage, after that approx. 40 worms were transferred to unseeded NGM plates containing high salt concentration i.e. 350mM for 10 minutes.

After 10 minutes, worms were transferred back to normal, 50mM NaCl NGM plates and checked for a fraction of motile worms over 15 minutes with an interval of 3 minutes to determine the percentage recovery.

3.7 Gene expression study

For the expression of the cytoprotective gene in *rict-1(ft7)* worms, worms were subjected to different diets, or RNAi. Imaging was done at L4 and day1 stage.

hsp-60p::GFP strain was used to study the expression of *hsp60* (mitochondrial stress marker) while *cyp14A4p::GFP* was used to study expression of *cyp14* (innate immunity marker).

col-19::GFP was used to study the expression of col-19 (to check for collagen levels). Imaging was done at Young adult and Day1 stage to check the expression on OP50 and HT115 diets.

For all the above studies respective worms were mounted on glass slides (~50 worms, 3-4 stacks), using 10mM tetramisole . The GFP fluorescence of worms was captured at a suitable exposure time, with the help of an AxioImager M2 microscope (Carl Zeiss, Germany) fitted with Axiocam MRc at 10X magnification (Excitation 488 nm and Absorbance at 520 nm). The photos of stacks from an experimental condition were stitched and quantified using Fiji (Image J2). Statistical analysis was done using GraphPad Prism 9.0.

3.8 Genetic Crosses

First of all, males were generated by using the heat shock method where 10-15 L4 worms were put on OP50 seeded plates and incubated for 4-6 hours at 30^0C and then placed back at 20^0C. Once males were generated, males of one strain were put with hermaphrodites of the other, in a 7:3 ratio, on 35mm NGM plates seeded with a drop of OP50 in the middle, and kept for mating for 2 days at 20^0 C. After 2 days, the F1 progenies were segregated out on 60mm NGM plates. F1 progenies were then left to grow and lay eggs, for 2 days.

Then the mother worm was lysed from individual plates and genotyping was performed using PCR-based methods. Once the heterozygosity was found for the desired genotype in the F1 generation, then those plates were separated out and F2 worms from those plates were segregated. F2 worms were then left to grow and lay eggs, for 2 days, after that the mother worm was lysed from individual plates and genotyping was performed using PCR-based method to obtain homozygous of the desired genotypes.

3.9 Oil Red O' Staining

Oil-Red-O staining was conducted by washing around 100 day 1/ young adult worms with M9. The worms were resuspended in 120ul of 1X PBS. Then an equal volume of 2X MRWB buffer (Appendix-I.5) was added and incubated by shaking for 45 minutes. The mix was pelleted down at 2000 rpm for 1 minute at 20^0C. After removing the supernatant, the pellet was again washed with 1X PBS (200ul) for three times. Followed by addition of 200ul of working oil red O solution and incubated it for around 45 minutes on a shaker at RT.

Then we again washed the worms with 1X PBS for around three times and mounted on 2% agarose pad slides, and observed them under the microscope.

3.10 Western Blotting

For sample preparation 100 synchronized L4 worms were collected in M9 and washed multiple times to get rid of bacteria. followed by addition of 1X SDS laemmli buffer (Appendix- I.6).

Proteins were separated by SDS gel electrophoresis, and transferred to nitrocellulose membranes. The transfer was set for around 1.5 hours at 250mA. After the transfer, ponceau staining was done to confirm successful transfer of proteins onto the nitrocellulose membrane. Then blocking was done with 5% BSA for about an hour.

Then primary antibody was added against pink-1(1:2000) and blot was kept overnight at 4^0C. After washing with TBST, secondary antibody was added (anti-rabbit-HRP) at 1: 5000 dilution, and incubated for an hour. The blot was developed by adding substrate and visualised in chemi-doc.

Generation Of Mutant Worms

1. rict-1(ft7);rde-1(ne219)

rict-1(ft7)

The *ft7* allele is a point mutation in the *rict-1* gene, which encodes the RICTOR protein. This mutation results in a specific amino acid substitution in the RICTOR protein. For PCR validation of *rict-1(ft7)*, we have designed two primers forward and reverse in such a way that they amplify fragments both in the wild type and *rict-1(ft7)*. The *ft7* amplified DNA fragment has a restriction site that is recognized, and digested by the EcoR1-HF restriction enzyme giving digested bands of **180 and 50** bp in length in a 3% agarose gel.

Meanwhile, absence of the restriction site in the WT renders it undigested by the enzyme thereby showing a band size of **210 bp.**

rde-1(ne219)

Genetic screens aimed at identifying mutants resistant to RNA interference (RNAi) are crucial for understanding the intricate mechanism behind processing exogenously introduced double-stranded RNAs (dsRNAs) and triggering gene silencing. In *C. elegans*, RDE-1 serves as the principal Argonaute component within the RNA-induced silencing complex (RISC).

This complex functions by degrading the passenger strand of the small interfering RNA (siRNA) and utilizing the remaining strand to direct mRNA targeting (Zhang, C *et al.,* 2012).

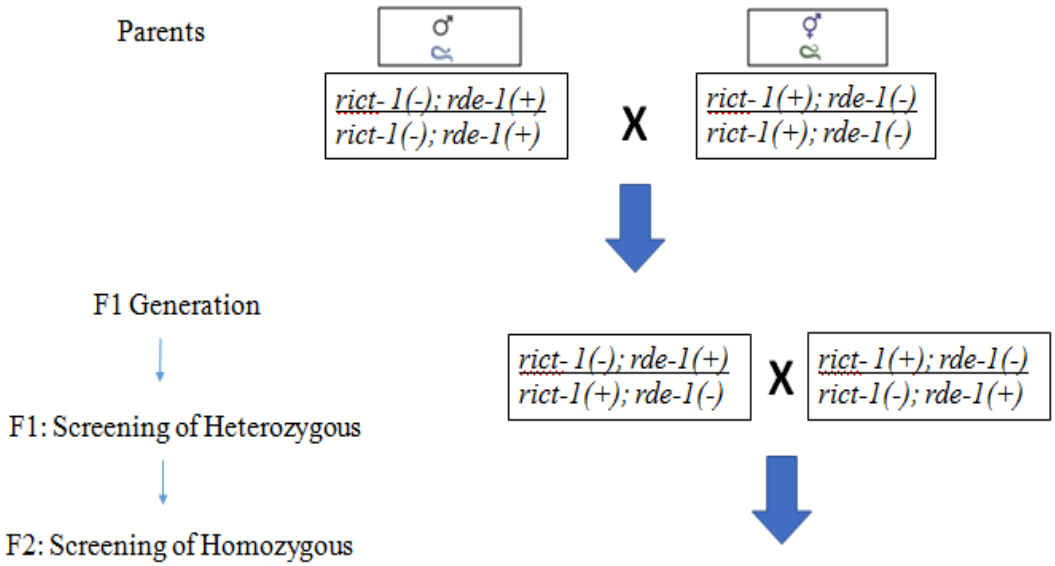
rde-1 is a commonly used dsRNA induced RNAi-resistant mutant strain that retains considerable RNAi capacity against RNAi, and thus is used for construction of a robust tissue-specific RNAi strain.

For PCR validation of *rde-1(ne219)*, we have designed two primers forward and reverse in such a way that they amplify the fragment both in the wild type and *rde-1(ne219)*. The *rde-1(ne219)* amplified DNA fragment has a restriction site that is recognized and digested by the Nco1-HF restriction enzyme giving digested bands of **280 and 30** bp in length in a 3% agarose gel.

Meanwhile, absence of the restriction site in the WT renders it undigested by the enzyme thereby showing a band size of **210bp.**

To generate *rict-1(ft7);rde-1(ne219)* double mutant, *rict-1(ft7)* males mated with *rde-1(ne219)* hermaphrodite. Worms homozygous for each of these mutated genes are selected for further experiments. Generally, heterozygous mutants are found in F1 generation and homozygous mutants found in F2 generation.

Crossing *rict-1(-)* X *rde-1(-)*



F2 Generation	rict − 1(+); rde − 1(−)	rict − 1(−); rde − 1(+)	rict − 1(−);rde − 1(−)	rict − 1(−); rde − 1(−)
rict − 1(+); rde − 1(−)	$\frac{rict-1(+);rde-1(-)}{rict-1(+);rde-1(-)}$	$\frac{rict-1(-);rde-1(+)}{rict-1(+);rde-1(-)}$	$\frac{rict-1(+);rde-1(+)}{rict-1(+);rde-1(-)}$	$\frac{rict-1(-);rde-1(-)}{rict-1(+);rde-1(-)}$
rict − 1(−); rde − 1(+)	$\frac{rict-1(+);rde-1(-)}{rict-1(-);rde-1(+)}$	$\frac{rict-1(-);rde-1(+)}{rict-1(-);rde-1(+)}$	$\frac{rict-1(+);rde-1(+)}{rict-1(-);rde-1(+)}$	$\frac{rict-1(-);rde-1(-)}{rict-1(-);rde-1(+)}$
rict − 1(+); rde − 1(+)	$\frac{rict-1(+);rde-1(-)}{rict-1(+);rde-1(+)}$	$\frac{rict-1(-);rde-1(+)}{rict-1(+);rde-1(+)}$	$\frac{rict-1(-);rde-1(+)}{rict-1(+);rde-1(+)}$	$\frac{rict-1(-);rde-1(-)}{rict-1(+);rde-1(+)}$
rict − 1(−); rde − 1(−)	$\frac{rict-1(+);rde-1(-)}{rict-1(-);rde-1(-)}$	$\frac{rict-1(-);rde-1(+)}{rict-1(-);rde-1(-)}$	$\frac{rict-1(+);rde-1(+)}{rict-1(-);rde-1(-)}$	$\frac{rict-1(-);rde-1(-)}{rict-1(-);rde-1(-)}$

Figure 11: Genetic cross: A representation of *rict-1(-);rde-1(-)*

F1 Screening:

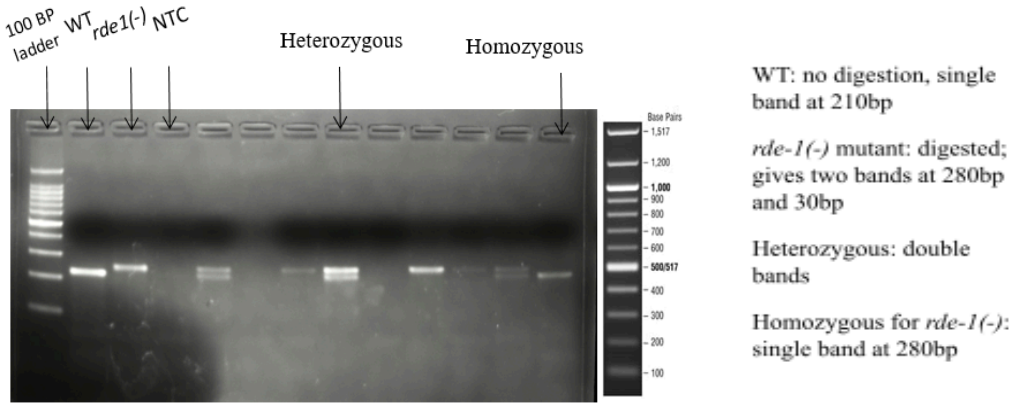


Figure 12: Digestion by Nco1-HF for *rde-1(-)* in F1 generation; screening for *rict-1(-) ;rde-1(-)* cross

F2 Screening:

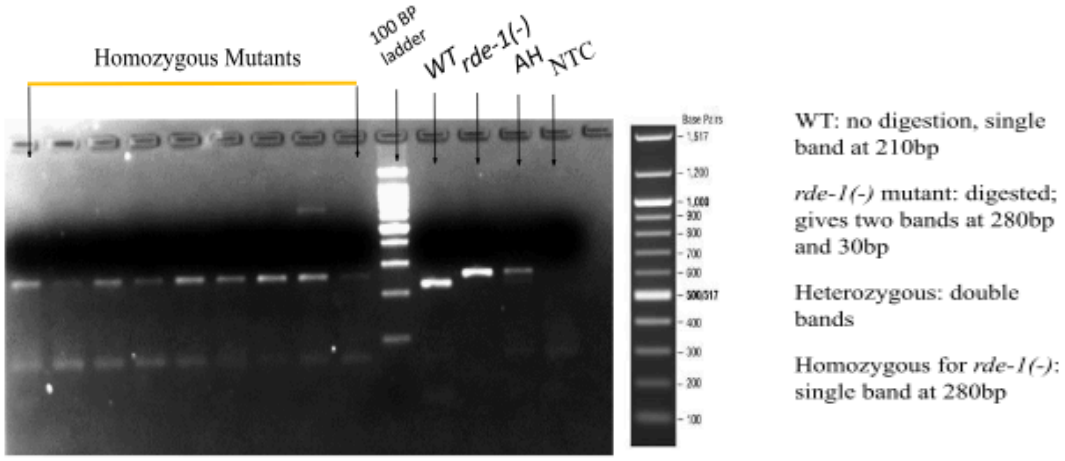


Figure 13: Digestion by Nco1-HF for *rde-1(-)* in F2 generation*;* screening for *rict-1(-);rde-1(-)* cross

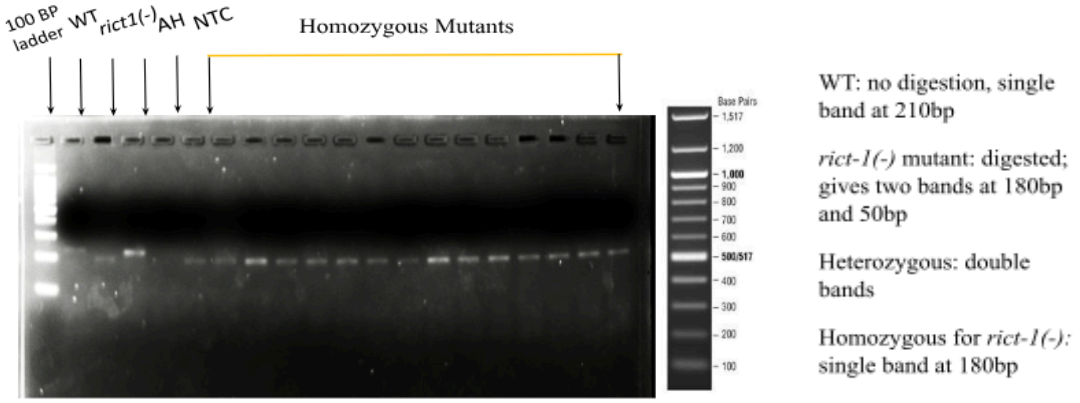


Figure 14: Digestion by EcoR1-HF for *rict-1(-)* in F2 generation*;* screening for *rict-1(-);rde-1(-)* cross

Tissue specific RNAi line

♂ *rict-1(ft7);rde-1(ne219)* x NR350 (Muscles) ♀

NR350

In *C. elegans*, NR350 strain has been used to rescue RNAi in Muscles. These are used to study the pathways involved specifically in the muscles. These are GFP tagged and are screened under Leica microscope.

In this experiment, *rict-1(ft7)*;*rde-1(ne219)* males mated with NR350 hermaphrodite.

Worms homozygous for each of these mutated genes are selected for further experiments.

Generally, heterozygous mutants are found in F1 generation and homozygous mutants are found in F2 generation.

F1 Screening :

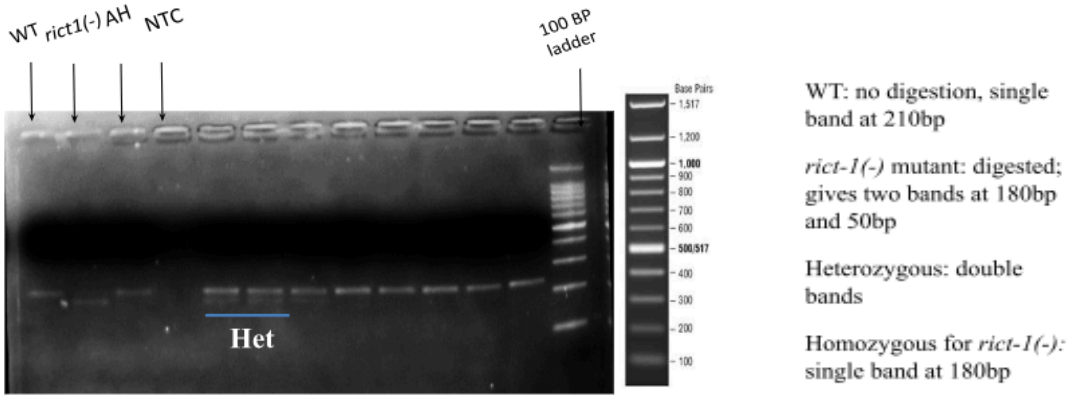
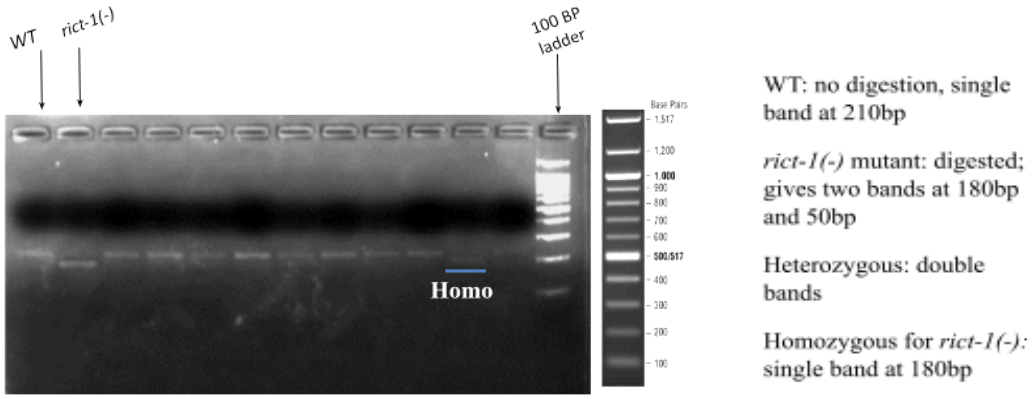
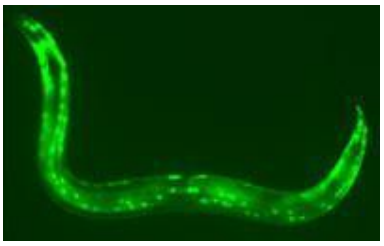


Figure 15: Digestion by EcoR1-HF for screening for *rict-1(ft7)* mutant in F1 generation

F2 Screening:





GFP+ (Screening for NR350)

Figure 16: Digestion by EcoR1-HF for F2 screening of *rict-1(ft7)* mutant and GFP expression under Leica microscope for NR350 positive strains.

2. rict-1(ft7);sid-1(pk3321)

rict-1(ft7)

The *ft7* allele is a point mutation in the *rict-1* gene, which encodes the RICTOR protein. This mutation results in a specific amino acid substitution in the RICTOR protein. For PCR validation of *rict-1(ft7)*, we have designed two primers forward and reverse in such a way that they amplify fragments both in the wild type and *rict-1(ft7)*. The *ft7* amplified DNA fragment has a restriction site that is recognized and digested by the EcoR1-HF restriction enzyme giving digested bands of **180 and 50** bp in length in a 3% agarose gel.

Meanwhile, absence of the restriction site in the WT renders it undigested by the enzyme thereby showing a band size of **210bp.**

sid-1(pk3321)

SID-1 is a multi-pass transmembrane protein that acts as a channel to transport dsRNA across cellular membranes in *C. elegans*. Its expression increases the response of neurons to dsRNA delivered by feeding.

Sid-1 mutants exhibit no impairment in RNA interference (RNAi) itself but specifically lack the ability to spread silencing signals between cells. While feeding RNAi is effective in nearly all cells except neurons, RNAi in neurons occurs only when double-stranded RNA (dsRNA) is generated within the neurons themselves.

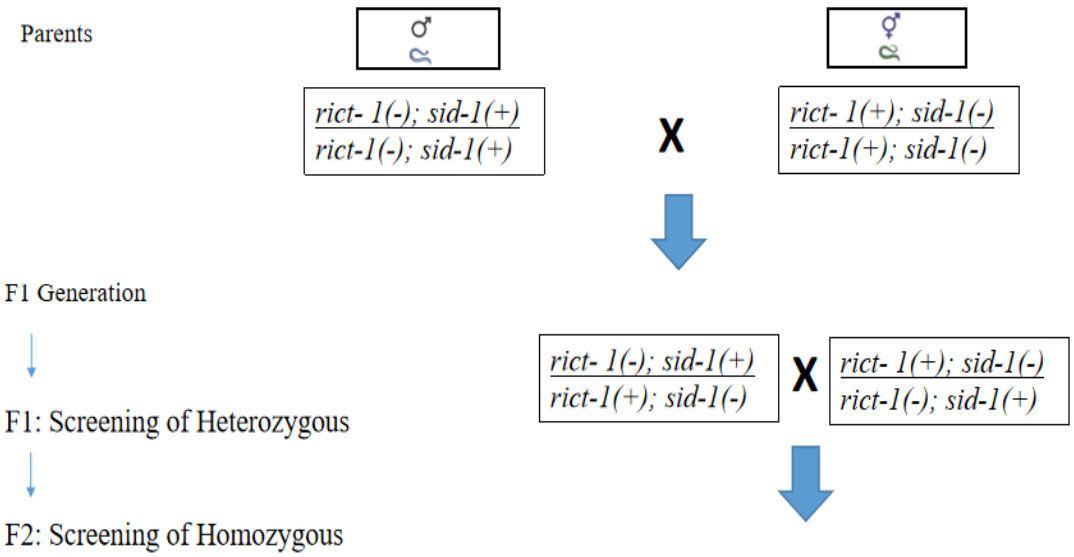
SID-1 is present in all cells outside the nervous system but is found in very few cells within it. Neurons expressing SID-1 display efficient responses to feeding RNAi (Calixto, A *et al.,* 2010).

For PCR validation of *sid-1(pk3321)*, we have designed two primers forward and reverse in such a way that they amplify fragments both in the wild type and *sid-1(pk3321)*. The *sid-1(pk3321)* amplified DNA fragment has a restriction site that is recognized and digested by the EcoR1-HF restriction enzyme giving digested bands of **280 and 30** bp in length in a 3% agarose gel.

Meanwhile, absence of the restriction site in the WT renders it undigested by the enzyme thereby showing a band size of **210bp.**

To generate *rict-1(ft7);sid-1(pk3321)* double mutant, *rict-1(ft7)* male was mated with *sid-1(pk3321)* hermaphrodite. Worms homozygous for each of these mutated genes are selected for further experiments. Generally, heterozygous mutants are found in F1 generation and homozygous mutants found in F2 generation.

Crossing *rict-1(-)* X *sid-1(-)*



F2 Generation	*rict* − 1(+); *sid* − 1(−)	*rict* − 1(−); *sid* − 1(+)	*rict* − 1(+); *sid* − 1(+)	*rict* − 1(−); *sid* − 1(−)
rict − 1(+); *sid* − 1(−)	$\frac{rict-1(+);sid-1(-)}{rict-1(+);sid-1(-)}$	$\frac{rict-1(-);sid-1(+)}{rict-1(+);sid-1(-)}$	$\frac{rict-1(+);sid-1(+)}{rict-1(+);sid-1(-)}$	$\frac{rict-1(-);sid-1(-)}{rict-1(+);sid-1(-)}$
rict − 1(−); *sid* − 1(+)	$\frac{rict-1(+);sid-1(-)}{rict-1(-);sid-1(+)}$	$\frac{rict-1(-);sid-1(+)}{rict-1(-);sid-1(+)}$	$\frac{rict-1(+);sid-1(+)}{rict-1(-);sid-1(+)}$	$\frac{rict-1(-);sid-1(-)}{rict-1(-);sid-1(+)}$
rict − 1(+); *sid* − 1(+)	$\frac{rict-1(+);sid-1(-)}{rict-1(+);sid-1(+)}$	$\frac{rict-1(-);sid-1(+)}{rict-1(+);sid-1(+)}$	$\frac{rict-1(-);sid-1(+)}{rict-1(+);sid-1(+)}$	$\frac{rict-1(-);sid-1(-)}{rict-1(+);sid-1(+)}$
rict − 1(−); *sid* − 1(−)	$\frac{rict-1(+);sid-1(-)}{rict-1(-);sid-1(-)}$	$\frac{rict-1(-);sid-1(+)}{rict-1(-);sid-1(-)}$	$\frac{rict-1(+);sid-1(+)}{rict-1(-);sid-1(-)}$	$\frac{rict-1(-);sid-1(-)}{rict-1(-);sid-1(-)}$

Figure 17: Genetic cross: A representation of *rict-1(-);sid-1(-)*

F1 Screening:

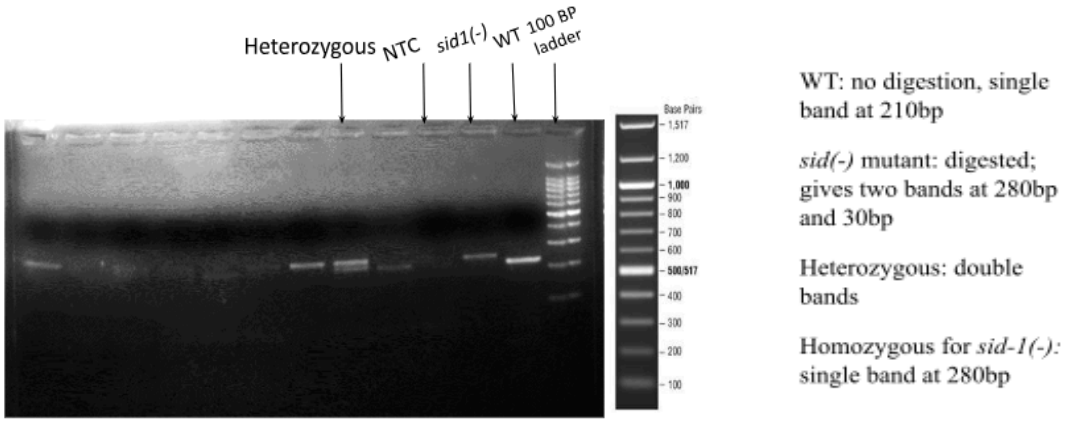


Figure 18: Digestion by EcoR1-HF for *sid-1(-);* screening for *rict-1(-);sid-1(-)* cross

F2 Screening:

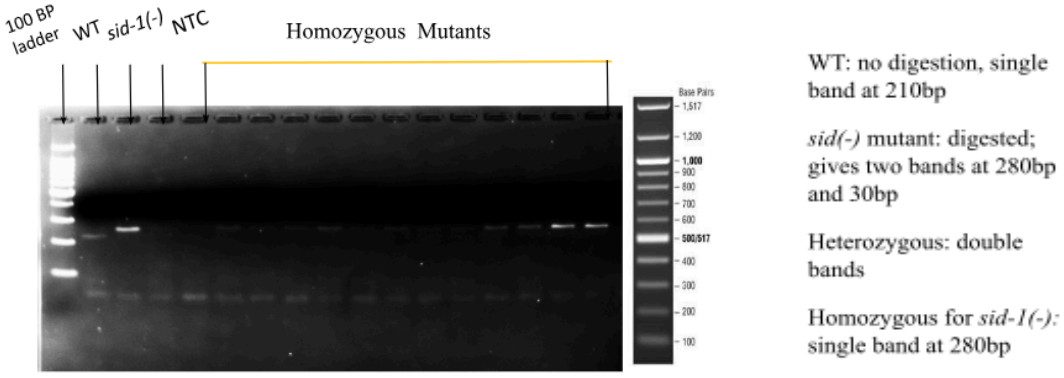


Figure 19: Digestion by EcoR1-HF for *sid-1(-)* in F2 generation*;* screening for *rict-1(-);sid-1(-)* cross

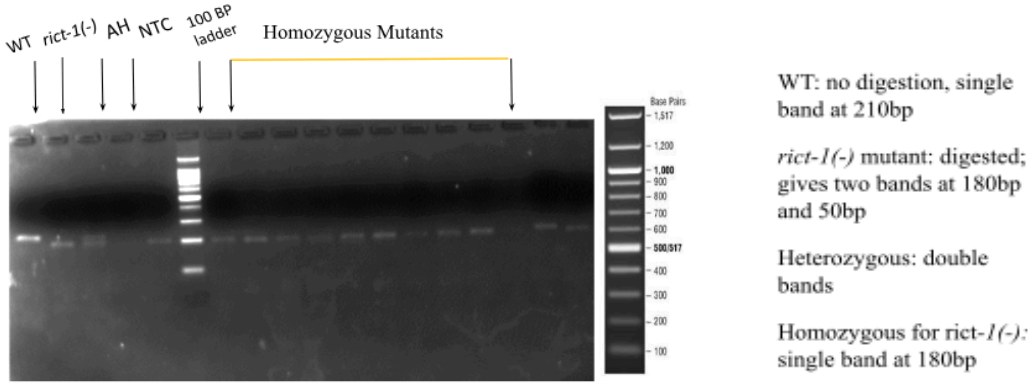


Figure 20: Digestion by EcoR1-HF for *rict-1(-)* in F2 generation*;* screening for *rict-1(-);sid-1(-)* cross

Tissue specific RNAi line

♂ *rict-1(ft7);sid-1(pk3321)* x Tu3401 (Neuronal) ♀

Tu3401

In *C. elegans*, Tu3401 strain has been used to rescue RNAi in Neurons. These are used to study the pathways involved specifically in the neurons. These are RFP tagged and are screened under Leica microscope.

In this experiment, *rict-1(ft7)*;*sid-1(pk3321)* males mated with Tu3401 hermaphrodite. Worms homozygous for each of these mutated genes are selected for further experiments.

Generally, heterozygous mutants are found in F1 generation and homozygous mutants are found in F2 generation.

F1 Screening:

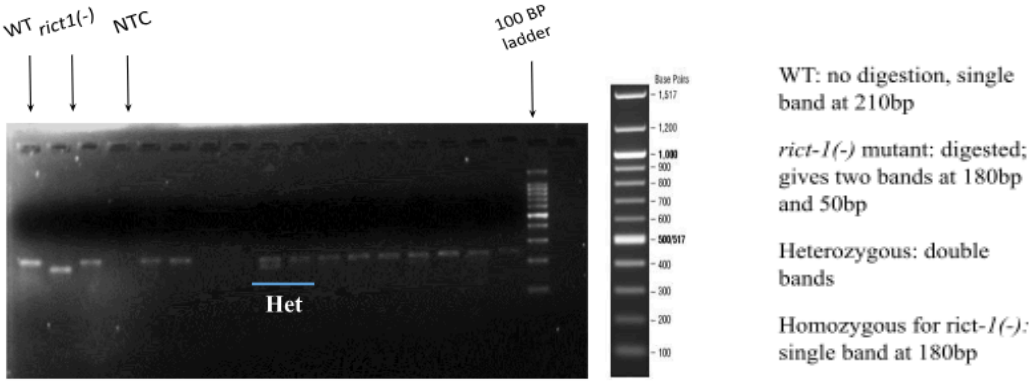
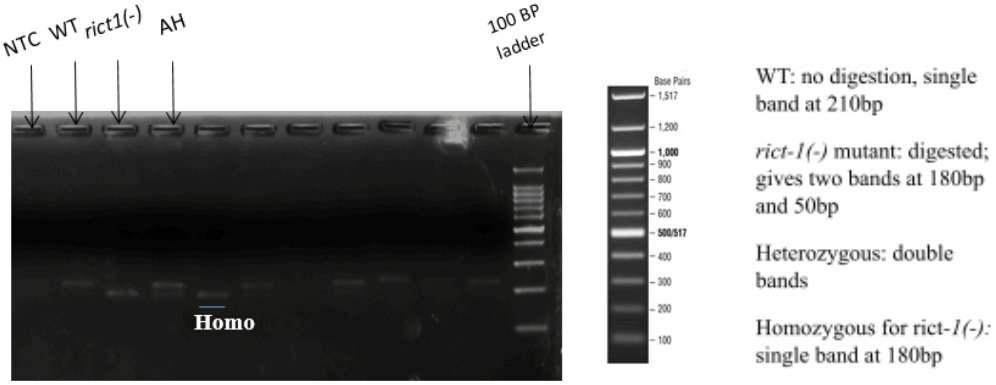
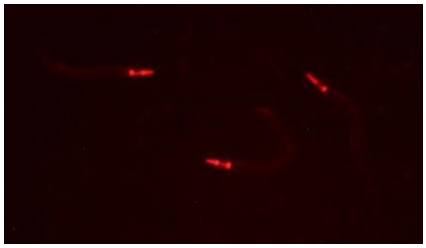


Figure 21: Digestion by EcoR1-HF for screening for *rict-1(ft7)* mutant in F1 generation

F2 Screening:





RFP+ (Screening for Tu3401)

Figure 22: Digestion by EcoR1-HF for F2 screening of *rict-1(ft7)* mutant and RFP expression under Leica microscope for Tu3401 positive strains.

CHAPTER 4

RESULTS

1. Osmotic stress tolerance of *rict-1* mutants on different diets.

It has already been seen in our lab that osmotic stress tolerance of *rict-1* mutant is enhanced on the HT115 diet as compared to the wild-type. To replicate the same, osmotic stress tolerance was performed. Here, we again found that *rict-1(ft7)* mutants on HT115 diet showed an increased osmotic stress tolerance as compared to wild-type worms. These findings confirm that *rict-1(ft7)* mutants exhibit enhanced osmotic stress tolerance compared to wild-type worms when fed an HT115 diet, replicating previously observed results.

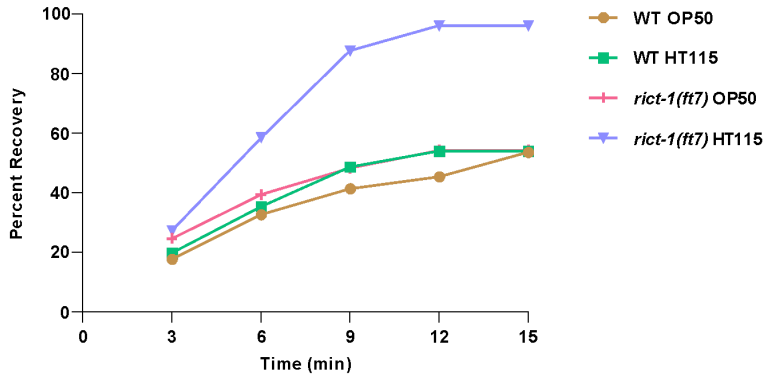


Figure 23: Osmotic Stress Tolerance Assay performed using WT and *rict-1(-)* mutant worms fed on *E. coli* OP50 and HT115 diet.

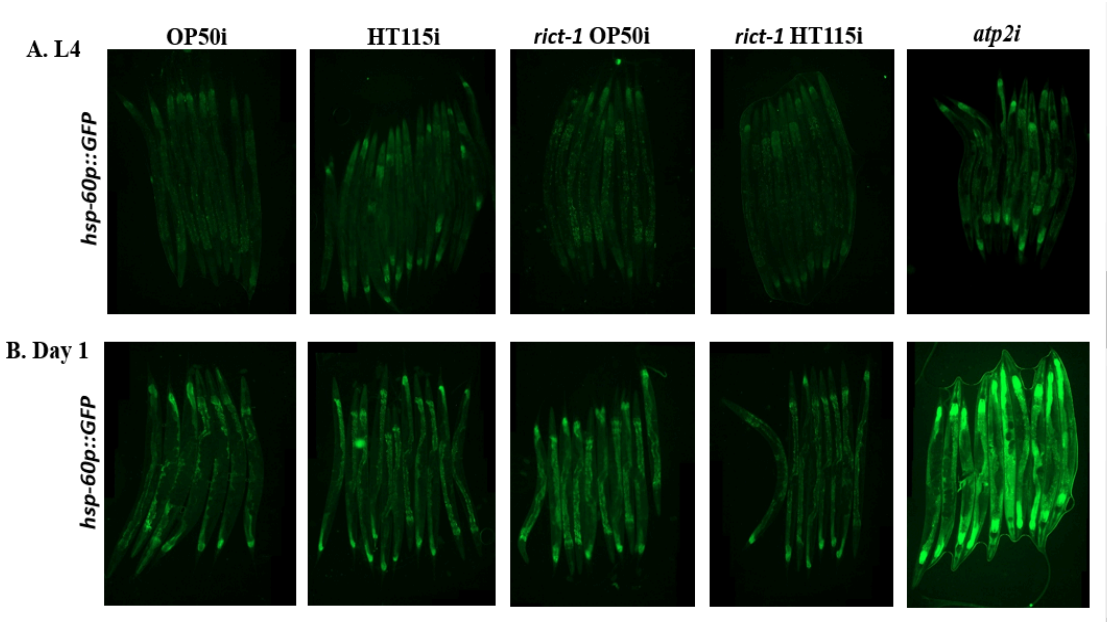
2. Expression levels of HSP-60 on knockdown of *rict-1* in the OP50 and HT115 backgrounds.

Osmotic stress can lead to increased production of ROS, which in turn can cause mitochondrial damage. This can trigger protective mechanisms that support mitochondrial function, such as upregulation of osmoprotective genes that also have roles in maintaining mitochondrial integrity.

Here, we have checked for mitochondrial stress markers to understand the crosstalk between osmotic and mitochondrial stress. For this, we used *hsp-60p::GFP* strain to check for *hsp-60* expression levels, which is involved in mitochondrial unfolded protein response. We saw a slight increase in the expression of HSP-60 protein in *rict-1* mutants fed on OP50 diet.

However, the lack of significant changes in HSP-60 expression implies that the osmotic stress may not strongly induce the mitochondrial unfolded protein response (UPRmt). Although this needs to be repeated a few more times to clarify the relationship between RICTOR, mitochondrial stress, and HSP-60 expression.

The UPRmt is a protective mechanism activated in response to mitochondrial stress, characterized by the upregulation of chaperones such as HSP-60. The slight increase in *hsp-60* expression observed in *rict-1* mutants suggests some level of mitochondrial stress. The UPRmt can be influenced by various factors, including mitochondrial DNA mutations, and disruptions in mitochondrial protein import.



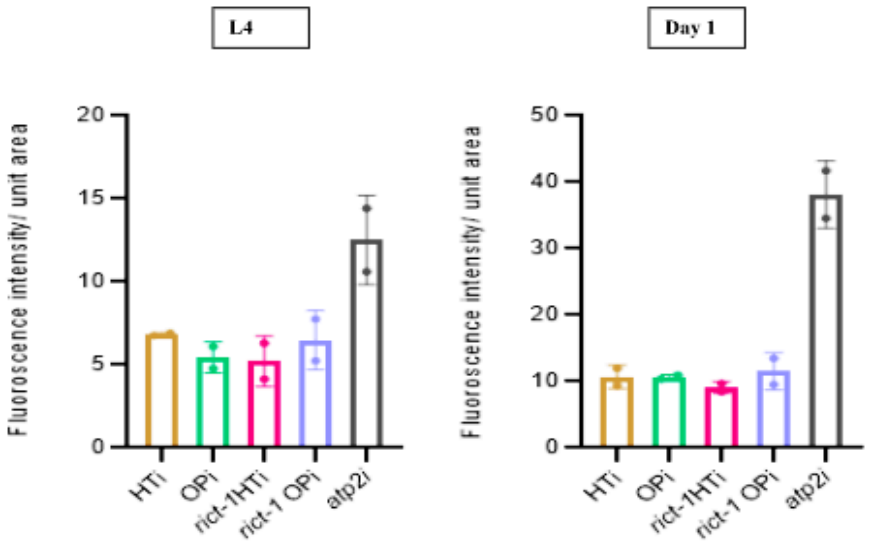


Figure 24: Expression and quantification of HSP-60 on knockdown of *rict-1* in OP50 and HT115 backgrounds at L4 and Day 1

3. Expression levels of CYP14A4 on knockdown of *rict-1* in the OP50 and HT115 backgrounds.

We then looked for the expression levels of CYP-14, which are members of the *C. elegans* Cytochrome P450 enzyme family, known to play important roles in mitochondrial dysfunction, lipid metabolism, and lifespan regulation.

The CYP-14 members share homology with human CYP families, and have shown complex crosstalk between mitochondrial stress, detoxification mechanisms, and lifespan regulation, emphasizing the complexity of these interconnected pathways (Lim SYM *et al*., 2024). We used *cyp14A4P::GFP* strain to check for CYP14A4 expression levels in *rict-1* mutants fed on different diets.

However, since the increase was not statistically significant, we cannot definitively conclude any significant relation between RICTOR and CYP-14A4 expression. Further repeats with larger sample sizes, and additional controls are needed to clarify its effects.

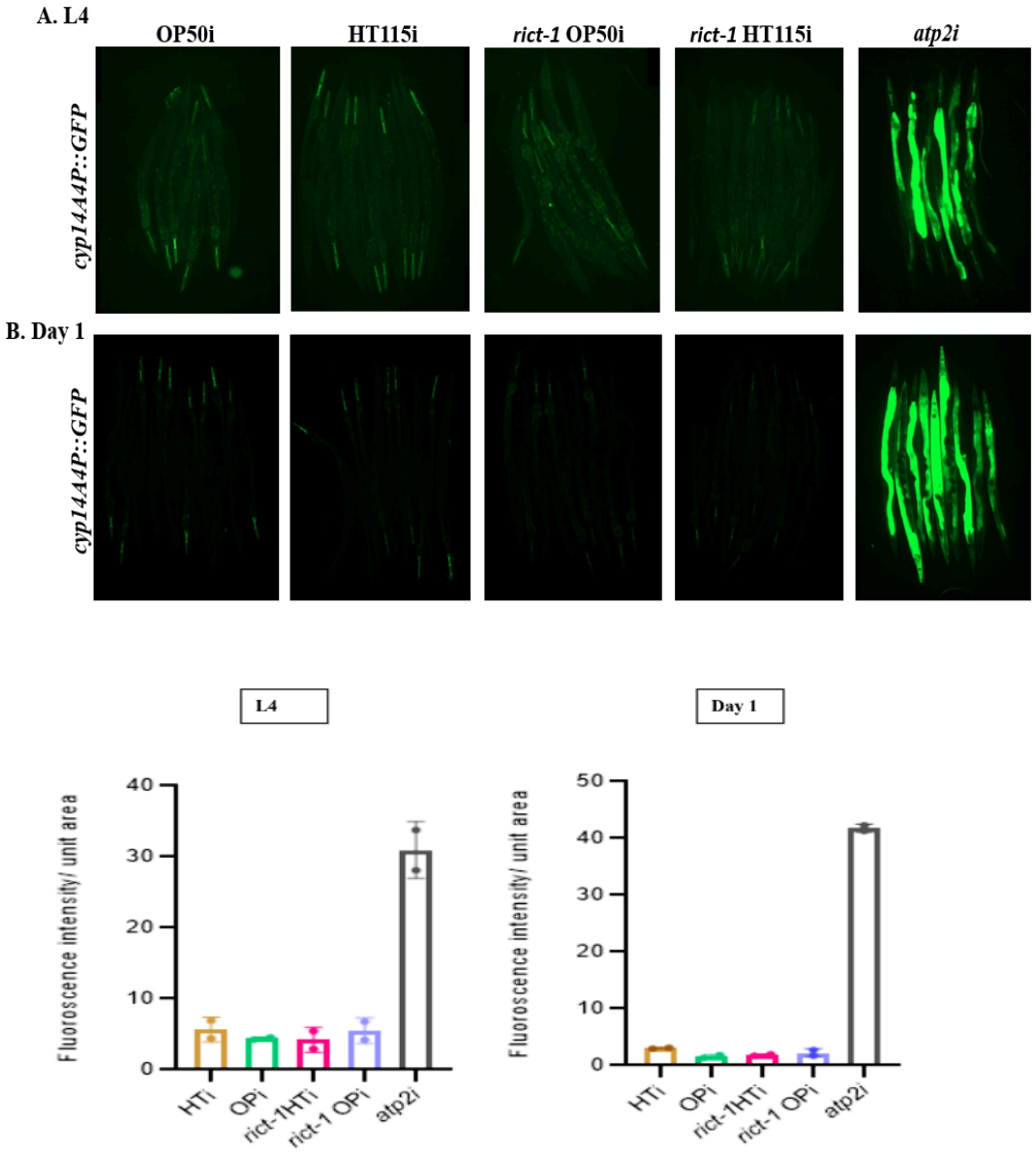


Figure 25: Expression and quantification of *cyp14* on knockdown of *rict-1* in OP50 and HT115 backgrounds at L4 and Day 1

4. To estimate the levels of PINK-1 in *rict-1* mutant

A) Supplementation of vitamin B12 and methionine

Cellular adaptive responses to osmotic stress, including the activation of autophagy and the mitochondrial unfolded protein response (mitoUPR), are crucial for

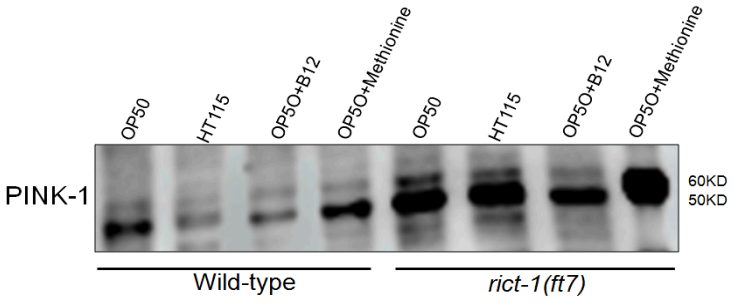
maintaining mitochondrial function and overall cellular homeostasis. The induction of mitophagy, a selective degradation process for damaged mitochondria, is particularly important in mitigating mitochondrial stress under osmotic stress conditions.

Consequently, our subsequent objective was to examine the role of mitophagy. Parkin is a cytosolic E3-ubiquitin ligase, and PINK is a mitochondrial serine/threonine-protein kinase and both are known to function in mitophagy (Matsuda *et al.,* 2010).

One of the studies has shown the activation of the mitochondrial unfolded protein response (mitoUPR) to riboflavin depletion and its role in lifespan extension (Kimura *et al.,* 2007), hence here we looked at the effects of Vitamin B12, belonging to the same vitamin B group and tried to understand the relation between mitophagy and vitamin B12 and methionine supplementation.

Western Blot Analysis showed that the PINK-1 levels were significantly increased in the *rict-1(ft7)* mutants fed on both OP50 and HT115 diets as compared to the wild-type. Further interesting analysis showed that there was an increase in the *pink-1* levels on supplementation of Methionine but not vitamin B12. This suggests the possible role of RICTOR in the process of mitophagy. But since the blot was run only once, thus we could not quantify using the loading control, and cannot accurately conclude from the same.

A.



B) Inhibition of bacterial genes

Western Blot Analysis showed that the PINK-1 levels were significantly increased in the *rict-1(ft7)* mutants grown on *E.coli* parent strain, BW25113 which has inherently high levels of vitamin B12 which contradicts with the previous result where we didn't find an increase on supplementation of vitamin B12 in OP50.

This indicates involvement of other metabolites in increasing the expression of PINK-1 in *rict-1* mutant. Also there was a decrease in the *pink-1* levels on knockdown of *pfs* (*pfs* is considered an integral component of the methyl cycle) and *tonB* (bacterial outer membrane proteins that help in transporting Vitamin B12) in the *rict-1* mutants. Since the blot was run only once and further quantification using the loading control is needed, we can only then conclude accurately from the same .

The significant increase in PINK-1 levels in *rict-1(ft7)* mutants grown on *Δpfs* strain upon supplementation of Methionine suggests a potential role of Methionine in modulating PINK-1 expression, and hence influencing cellular processes, like mitochondrial quality.

The decrease in PINK-1 levels upon knockdown of *Δpfs*, suggests a potential link between the bacterial metabolic processes and PINK-1 expression. Dysregulation of the bacterial genes could influence the host physiological processes, which may in turn affect mitochondrial function and PINK-1-mediated pathways.

The decrease in PINK-1 levels upon knockdown of *ΔtonB* in *rict-1* mutant indicates a potential role of Vitamin B12 availability in modulating PINK-1 expression.

C. elegans and bacteria have a complex relationship in the gut microbiome, where bacterial metabolism can impact host physiology and vice versa. Therefore, the observed effects on PINK-1 levels may involve cross-species interactions between *C. elegans* and its bacterial diet.

B.

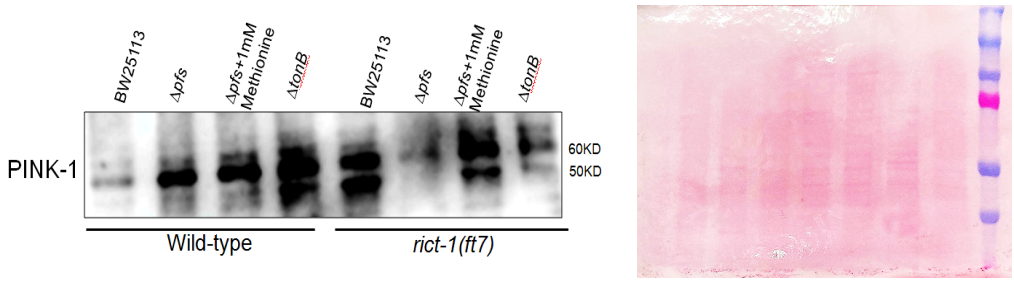


Figure 26: Estimation of PINK-1 levels in *rict-1* mutants on: A) supplementation of vitamin B12 and methionine in the OP50 diet, B) inhibition of bacterial genes

Lifespan Analysis:

After checking for the PINK-1 levels using western blot, we also wanted to see what effect would knockdown of *pink-1* and *pdr-1* have on the life span of *rict-1* mutants. *pdr-1* encodes an ortholog of human parkin (PARK2) and enables ubiquitin conjugating enzyme binding activity. Here we observed that *rict-1* mutants showed a slight decrease in lifespan on knockdown of *pink-1*(involved in mitophagy) and *pdr-1* as compared to the wild type. These results suggest the important roles of *pink-1* and *pdr-1* in regulating the lifespan.

The observed effects of *pink-1* and *pdr-1* knockdown on the lifespan of *rict-1* mutants highlight the intricate interplay between mitochondrial quality control pathways and aging. Proper functioning of genes involved in mitophagy and protein ubiquitination, such as *pink-1* and *pdr-1*, is crucial for maintaining mitochondrial health and promoting longevity. Dysregulation of these genes can lead to mitochondrial dysfunction, cellular damage, and premature aging.

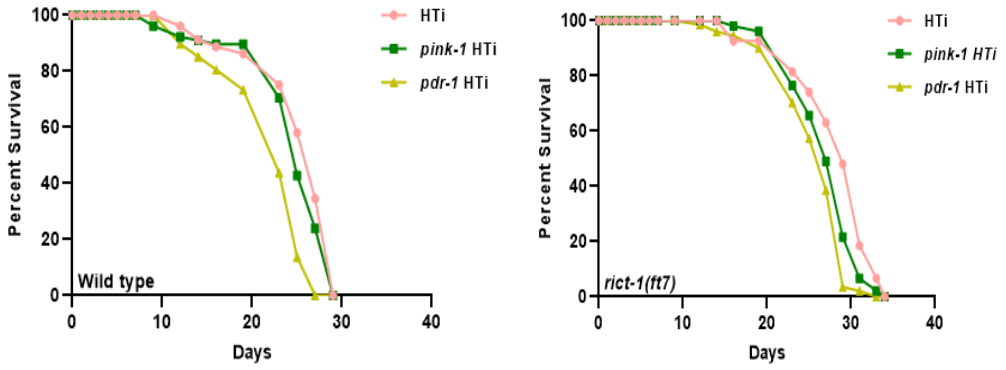


Figure 27: Life span of WT and *rict-1(ft7)* on knockdown of *pink-1* and *pdr-1:* WT and *rict-1* mutant worms show a decrease in the life span on knockdown of *pink-1* and *pdr-1*as compared to the controls, indicating relation between mitochondrial quality control and aging.

5. Deducing the role of RICTOR in lipid metabolism and collagen biosynthesis

It has earlier been shown that *rict-1(mg360)* mutants show an increase in the lipid content on OP50 diet as compared to HT115 diet, on performing Oil Red O' staining (Sakai, N.,*et al*., 2017). Hence we got interested to understand the status of lipid metabolism in null mutant i.e, *rict-1(ft7)* on different diets.

On performing Oil Red O' staining, we found an elevated fat storage in *rict-1(ft7)* fed *E.coli* OP50 as compared to those fed on *E.coli* HT115. It is evident from the increased red droplets as observed under the microscope. Further quantification of the data revealed that there was a significant increase in the lipid content observed on OP50 diet as compared to the HT115 diet in both *rict-1(ft7)* and *rict-1(mg360)* mutants as compared to the wild type.

This is in relation with the earlier shown role of *rict-1* in regulation of fat via insulin-like signaling through the AKT pathway (Soukas *et al*., 2009).

Lipid reserves have been implicated in *C. elegans* lifespan and innate immunity. Several long-lived mutant strains have been found to have markedly reduced lipid content (Nandakumar M *et al*., 2008).

Since both *rict-1(ft7)* and *rict-1(mg360)* mutants show a significant increase in lipid content on OP50 diet compared to HT115, we can say that the observed effect is not specific to a particular *rict-1* mutant allele but is a general phenomenon associated with loss of RICT-1 function.

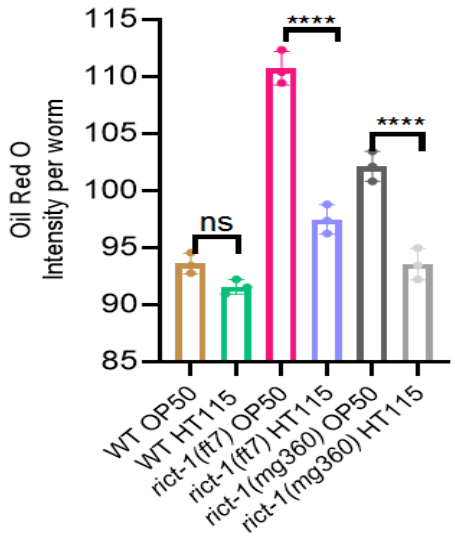


Figure 28: Quantification of Oil Red O' staining of WT and *rict-1* mutant worms on OP50 and HT115 diets. (*) $P < 0.05$

Lipid metabolism is closely linked to mitochondrial function, as mitochondria play a central role in lipid metabolism through processes such as β-oxidation and lipid synthesis. Dysfunctional mitochondria can lead to metabolic imbalances and

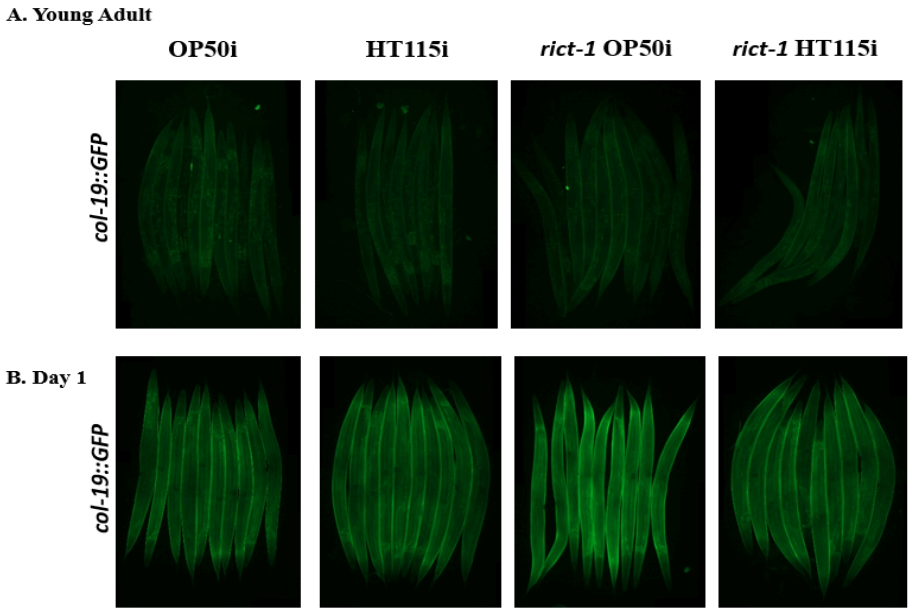
alterations in lipid metabolism, which may impact extracellular matrix (ECM) dynamics and collagen synthesis (Teuscher, A. (2021).

Hence after checking at the lipid levels, we looked at the expression of collagen in *rict-1* mutants on different diets. For this study, we used *col-19::GFP* strain that has a green fluorescent protein–collagen fusion, constructed by using the *C. elegans* adult-specific, hypodermally synthesized collagen COL-19 (Thein C Melanie *et al.,* 2003).

We observed significant increase in the collagen levels in *rict-1* mutants on OP50 diet at both Young Adult and Day 1 stage, as compared to that of wild-type. These results suggest the possible role of RICTOR in lipid metabolism and collagen biosynthesis.

Relating lipid levels and collagen levels in *C. elegans* involves understanding the interconnectedness of lipid metabolism and extracellular matrix (ECM) dynamics, where collagen is a major component.

Lipid metabolism is closely linked to mitochondrial function, as mitochondria play a central role in lipid metabolism through processes such as β-oxidation and lipid synthesis. Dysfunctional mitochondria can lead to metabolic imbalances and alterations in lipid metabolism, which may impact ECM dynamics and collagen synthesis.



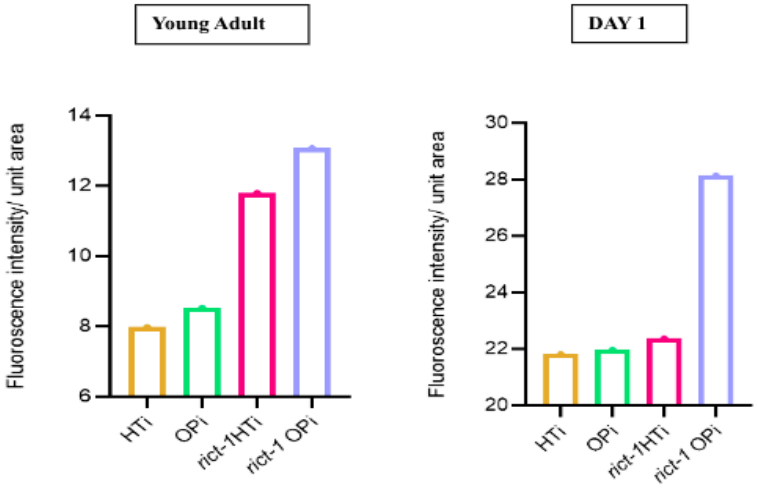


Figure 29: Expression and quantification of WT and *rict-1* mutants on OP50 and HT115 diets at Young Adult and Day 1 stage

CHAPTER 5

DISCUSSION AND CONCLUSION

Aging is linked with a variety of variables, including decreased mitochondrial function and the formation of aberrant mitochondria. However, the specific methods by which aging induces these mitochondrial changes, as well as their involvement in aging, remain unknown. Mitochondrial dynamics is a critical process that controls mitochondrial function and quality. Hence, we try to find the link between alterations in mitochondrial genes, aging, and age-related impairment (Byrne, J. J *et al.,* 2019).

Our study was aimed to investigate the osmotic stress tolerance, mitochondrial genes, lipid metabolism, collagen levels and mitophagy regulation in *rict-1* mutants of *C. elegans*, particularly focusing on the effects of different diets. Here, we found that *rict-1(ft7)* mutants exhibited enhanced osmotic stress tolerance compared to wild-type worms when fed an HT115 diet. This enhancement in osmotic stress tolerance may be linked to altered mTORC2 signaling, affecting downstream targets involved in cellular stress responses.

Next, we tried to find a potential link between osmotic stress and mitochondrial stress response pathways, although the relationship with mitochondrial stress, as indicated by *hsp-60* expression levels, remains inconclusive. Further investigation is needed to clarify the relationship between RICTOR, mitochondrial stress, and stress response pathways.

RICTOR appears to play a role in lipid metabolism, as indicated by the increased lipid content observed in *rict-1* mutants on the OP50 diet compared to HT115. This finding aligns with previous research findings that suggest the involvement of RICTOR in regulating fat via insulin-like signaling through the AKT pathway.

Study of mitophagy regulation via PINK-1 showed an increase in PINK-1 levels in the *rict-1* mutants, suggesting a potential role of RICTOR in mitophagy regulation. Further investigation is needed to elucidate the specific mechanisms by which RICTOR influences mitophagy pathways.

Our study also found a diet-dependent increase in collagen levels in *rict-1* mutants on the OP50 diet, suggesting a possible role of RICTOR in collagen biosynthesis. This finding underscores the interconnectedness of lipid metabolism and extracellular matrix dynamics, where collagen is a major component.

Hence, this study provides valuable insights into the multifaceted roles of RICTOR in regulating various cellular processes, including stress responses, lipid metabolism, collagen biosynthesis, and mitophagy. These findings highlight the importance of diet in modulating the effects of RICTOR on cellular pathways, emphasizing the need for further research to understand the underlying mechanisms. Future studies, particularly involving tissue-specific crosses, are needed to elucidate the specific roles of RICTOR in muscles and neurons and to further dissect the pathways involved.

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APPENDIX- I

I.1 Preparation of 1 litre (Nematode Growth Media) NGM Agar

Components	Amount/ Conc. /Volume
NaCl	3 gm
Agar	17 gm
Peptone	2.5 gm
H_2O	975 ml

Sterilize above solution by autoclaving at 15 psi for 30 minutes and allow it to cool up to 50°C. After cooling the medium, add following pre-sterilized components:

1M $CaCl_2$	1 ml
1M $MgSO_4$	1ml
1M PPB	25 ml
Cholesterol (10 mg/ml)	0.5 ml

I.2 Preparation of 1 litre (Nematode Growth Media) NGM Agar RNAi

Components	Amount/ Conc. /Volume
NaCl	3 gm
Agar	17 gm
Peptone	2.5 gm
H_2O	975 ml

Sterilize above solution by autoclaving at 15 psi for 30 minutes and allow it to cool up to 50°C. After cooling the medium, add following pre-sterilized components:

1M $CaCl_2$	1 ml

1M $MgSO_4$	1ml
1M PPB	25 ml
Cholesterol (10 mg/ml)	0.5 ml
Ampicillin (100 mg/ml) IPTG (1M)	1 ml
IPTG (1M)	2 ml

I.3 Preparation of 100 ml Osmotic stress media

Components	Amount/ Conc. /Volume
NaCl	2.1 gm
Agar	1.7 gm
Peptone	0.25 gm
H_2O	97.5 ml

Sterilize above solution by autoclaving at 15 psi for 30 minutes and allow it to cool up to 50°C. After cooling the medium, add following pre-sterilized components:

1M $CaCl_2$	0.1 ml
1M $MgSO_4$	0.1ml
1M PPB	2.5 ml
Cholesterol (10 mg/ml)	0.05 ml

I.4 M9 Buffer

Components	Amount/ Conc. /Volume
Na_2HPO_4	60 gm
KH_2PO_4	30 gm
NaCl	50 gm

1 M $MgSO_4$	10 ml

Adjust volume to 1 litre with Milli-Q water. Sterilize above solution by autoclaving at 15 psi for 20 minutes and allow it to cool up to 50°C before addingpre-sterilized 1 M $MgSO_4$.

I.5 MRWB

Components	Amount/ Conc. /Volume (for 1ml)
1M KCl	160 µl
0.1M Na_2 EGTA	140 µl
1M NaCl	40 µl
300MM Na PIPES	100 µl
4mM spermine	100 µl
100mM spermidine HCl	100 µl
10% Paraformaldehyde	200 µl
2% β-mercaptoethanol	100 µl
MQ	60 µl

I.6 4X Laemmli Buffer

Components	Amount/ Conc. /Volume
Tris (1M, pH 6.8)	10 ml
SDS	4 gm
Glycerol	20 ml
β-mercaptoethanol	10 ml
MQ	make upto 50 ml
Add β-mercaptoethanol before use (200 µl/ 1 ml buffer)	

PUBLICATION

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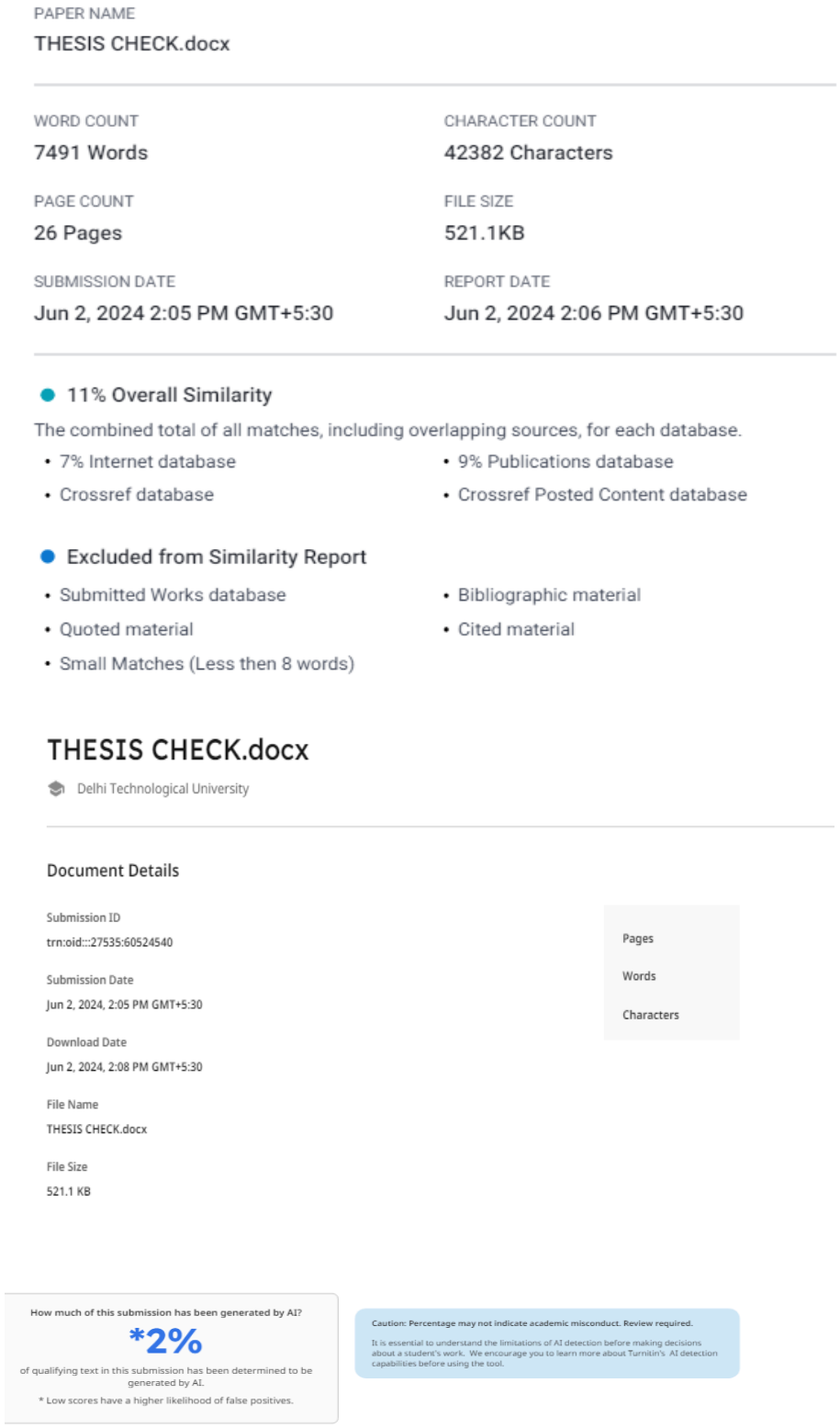

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CURRICULUM VITAE

SMRITI RAINA

EDUCATION

M.Sc.| Biotechnology — 2022 - present
Delhi Technological University, New Delhi, India — CGPA: 8.32/10

B.Sc. (Hons) | Biochemistry — 2019 - 2022
University Of Delhi — CGPA: 8.56/10
Sri Venkateswara College, New Delhi, India

Grade 12 | Subjects: Physics, Chemistry, Biology — March 2018-May 2019
St Francis De Sales School, New Delhi, India — 94/100

Grade 10 — April 2016-May 2017
St Francis De Sales School, New Delhi, India — 10/10

RESEARCH INTERNSHIP & PROJECTS

Project | Genetics | *C.elegans* | Longevity — December 2023 – May, 2024
National Institute Of Immunology (NII) — Supervisor: Dr. Arnab Mukhopadyay

- Six months M.Sc. dissertation project titled **"Characterisation of RICTOR's role in *Caenorhabditis elegans*"**

Project | Cloning | *Plasmodium vivax* | Malaria — 15th May – 31st July, 2023
National Institute Of Malaria Research (ICMR-NIMR) — Supervisor: Dr. Kailash C Pandey

- Three months Summer Internship on Project ***"Cloning and expression of Plasmodium vivax MSP-119 in order to evaluate its serological response in malaria patients living in different epidemiological settings"***

Project | Molecular Docking | Dengue — 3rd July – 31st Aug, 2021
University Of Delhi (DU) — Supervisor: Dr. Nimisha Sinha

- Two month Summer Research Project on ***"Identification and characterization of drug targets in infectious disease causing organisms and screening of ligands against them"*** under Sri Venkateswara Internship Program.

Project | Meta-analysis | Pulmonary Health — 20th June - 15th July, 2020
University Of Delhi (DU) — Supervisor: Dr. Nandita Narayanasamy

- Two month Summer Research Project on ***"Association between Occupational Exposure to Air Pollutants and Pulmonary Health in Construction Workers"*** under Sri Venkateswara Internship Program.

NATIONAL INSTITUTE OF IMMUNOLOGY

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CERTIFICATE

I hereby certify that the Dissertation Project titled **"Characterisation of RICTOR's role in *Caenorhabditis elegans*"** which is submitted by Smriti Raina, 2K22/MSCBIO/48, Department of Biotechnology, Delhi Technological University, New Delhi in partial fulfilment of the requirement for the award of degree of Master of Science, is a record for the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

Place: New Delhi

Date: May, 2024

Dr Arnab Mukhopadhyay
Staff Scientist VI
Molecular Aging Laboratory
National Institute of Immunology
New Delhi-110067

डा. अर्नब मुखोपाध्याय / Dr. Arnab Mukhopadhyay
स्टाफ वैज्ञानिक-VI /Staff Scientist-VI
राष्ट्रीय प्रतिरक्षाविज्ञान संस्थान
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अरुणा आसफ अली मार्ग/Aruna Asaf Ali Marg
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Place: New Delhi

Date: May, 2024

Prof. Yasha Hasija
Head of Department
Department of Biotechnology
Delhi Technological University

Prof. Jai Gopal Sharma
(Supervisor)
Department of Biotechnology
Delhi Technological University