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Investigating the Link between Cell Cytoskeleton, P-Bodies and Ageing in *Caenorhabditis elegans*

MASTER'S THESIS

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Μελέτη της Πιθανής Σχέσης του Κυτταροσκελετού με τα P-Bodies και τη Γήρανση στο Νηματώδη *Caenorhabditis elegans*

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

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This work is dedicated to my family,

my friends and especially to Nikolas

Abstract

Ageing is the biological phenomenon by which a fit and healthy organism changes into a less healthy and fit one for its environment. It is accompanied by specific age-related characteristics, like the inability to maintain tissue homoeostasis [19]. In humans, ageing is associated with a subset of diseases including neurodegeneration, cardiovascular disorders and others. As the human life expectancy has been increased, more people live long enough to suffer from age-related loss of function and disease. Unveiling the mechanisms of ageing, will hopefully give answers about how science could deal with the age-related diseases. In this work, we aim to investigate the relationship between ageing, microtubules and P-bodies. Microtubules provide critical functions in cells, including mechanical support, intracellular trafficking and others [31]. P-bodies are dynamic, non-membrane structures that host different types of mRNA processing [40]. In this work, we show that ageing in *C. elegans* could be accompanied by P-body induction and that microtubule disruption maybe leads to the opposite effect. Hopefully, research in this field, will further shed light on the mechanisms of ageing, and the ageing-related diseases.

Keywords

ageing, *C. elegans*, microtubules, P-bodies

Περίληψη

Η γήρανση, είναι το βιολογικό φαινόμενο κατά το οποίο ένας υγιής, εύρωστος για το περιβάλλον του οργανισμός, σταδιακά χάνει αυτόν τον χαρακτήρα. Η γήρανση συνοδεύεται από συγκεκριμένα χαρακτηριστικά, όπως την αδυναμία διατήρησης της ομοιόστασης στους ιστούς [19]. Μερικά ανθρώπινα νοσήματα, νευροεκφυλιστικά, καρδιαγγειακά και άλλα έχουν συσχετιστεί με τη γήρανση. Η αύξηση του προσδόκιμου ζωής, έχει αυξήσει τα τελευταία χρόνια τον αριθμό των ανθρώπων που είναι πιθανό να τα αντιμετωπίσουν και έτσι για τις σύγχρονες κοινωνίες, η έρευνα γύρω από τους μηχανισμούς της γήρανσης είναι σημαντική. Στην παρούσα εργασία, μελετήθηκε η πιθανή σχέση μεταξύ του κυτταροσκελετού, των P-bodies και του φαινομένου της γήρανσης. Ο κυτταροσκελετός, υποστηρίζει δομικά τα κύτταρα και επιπλέον σχετίζεται με άλλες σημαντικές λειτουργίες, όπως η ενδοκυτταρική μεταφορά των μακρομορίων [31]. Τα P-bodies είναι ευμετάβλητες μη μεμβρανικές δομές και αποτελούν κέντρα επεξεργασίας του mRNA. Στην παρούσα εργασία, φαίνεται ότι στον νηματώδη *C. elegans*, η γήρανση σχετίζεται με την επαγωγή αυτών των δομών και ότι η διατάραξη των μικροσωληνίσκων πιθανόν προκαλεί το αντίθετο αποτέλεσμα. Η έρευνα σχετικά με το φαινόμενο της γήρανσης, θα μπορέσει ίσως να οδηγήσει στην πληρέστερη κατανόηση των μηχανισμών που την διέπουν και να δώσει λύση στις ασθένειες που έχουν συσχετιστεί με αυτήν.

Λέξεις Κλειδιά

γήρανση, μικροσωληνίσκοι, *C. Elegans*, P-bodies

Ευχαριστίες

Θα ήθελα πολύ να ευχαριστήσω τον καθηγητή μου Νεκτάριο Ταβερναράκη, καθώς χάρη σε αυτόν είχα την ευκαιρία να συνεργαστώ με μία πολύ αξιόλογη ερευνητική ομάδα, να ασχοληθώ με ένα πολύ ενδιαφέρον για μένα κομμάτι της έρευνας και να αποκομίσω πολλές γνώσεις.

Επίσης, ιδιαίτερα ευχαριστώ τον Matthias και τη Μαίρη για την πολύτιμη φιλία, βοήθεια και στήριξη τους. Δεν θα μπορούσα να μην ευχαριστήσω επίσης την Αγγέλα, καθώς χωρίς αυτήν τα πειράματα (ιδιαίτερα αυτά στα οποία χρειάστηκε DMSO) δεν θα υπήρχαν…

Τέλος, θα ήθελα να ευχαριστήσω τον Νίκο, τη Νάντια, τον Μάνο, την Άρτεμις, την Ευγενία, την Μαρκέλλα, την Κατερίνα και τον Κωνσταντίνο, γιατί χωρίς αυτούς το εργαστήριο δεν θα έμοιαζε με αυτό που εγώ γνώρισα.

Table of Contents

List of Figures

List of Pictures

1. Introduction

1.1. Ageing as a Biological Process

Ageing, is a complex process of damage accumulation that gradually changes a fit and healthy organism into a less healthy and fit one. This biological phenomenon is characterized by a variety of disorders that are associated with general organism decline and inability to maintain tissue homoeostasis [19].

Recent discoveries have shown that mutations in single genes can extend lifespan of laboratory model organisms and that the mechanisms involved in this process are conserved. It is impressive that most life-forms share common weak spots that become increasingly susceptible to failure over time. One of the main "ageing targets" is the genome itself, a fragile and highly conserved structure that can accumulate a variety of damaging alterations with age, despite its mechanisms for continuous surveillance and repair [18]. Additionally, it has been found that mutations in specific genes, can keep animals functional and pathology free to later ages, and they can also protect them against many ageing-related, neurodegenerative diseases, and cancer.

These discoveries could lead to the development of new drugs and promote the preventative medicine. Such a progress would be really significant for human populations. Human life expectancy in developed countries has been increased due to improvements in public health and lifestyle (clean water, immunization, antibiotics, better housing etc.), so more people live long enough to suffer from age-related loss of function and disease. Dealing with the effects of the ageing process is predicted to be globally one of the most important health care challenges for the next years.

In humans, ageing is associated with a subset of diseases including cardiovascular disorders, diabetes, cancer and others which result in low life quality. Both for the health benefits to ageing individuals and the economic benefits to the societies where they live, the ageing research is quite promising. Today, specific

ageing related diseases are generally viewed as medically tractable, however this is not the case for the ageing process itself [16, 17].

One of the most important advances in ageing research has been the discovery of mutations in single genes that extend the lifespan of laboratory animals. They first came as lifespan-extending mutations in *C. elegans*. Subsequent work with these mutations revealed that it is possible to double the lifespan of this worm with a mutation in a single gene. Furthermore, the mutations cause the worms to remain healthy and youthful. It has been found that those genes encode components of an invertebrate insulin/insulin-like growth-factor-like signaling (IIS) pathway.

Mutations with similar effects were soon discovered in other model organisms too. In yeast, it was discovered that over-expression of a protein deacetylase, SIR2, could extend replicative lifespan, while mutations in Methuselah gene in Drosophila increased fly lifespan. Additionally, in the mouse, mutations in genes encoding transcription factors resulted in long-lived dwarf mice. It was also discovered that dietary restriction can produce substantial increases in lifespan too.

Humans are longer lived than any of the laboratory model organisms and much more complicated. This could mean that human lifespan is not as plastic as that of laboratory animals, that it doesn't permit similar interventions and that such dramatic effects in lifespan with no other unwanted ones, is impossible. However, the same may not be true for the ageing-related diseases. After all, the aim of the ageing research is to improve life quality during ageing and not to extend people's lifespan. Research in this field, seems to be really promising [16].

1.1.1. The Role of the Insulin Signaling Pathway in Ageing

The IIS pathway was first linked to ageing in *C. elegans*, where mutations of some of its key gene components were found to dramatically extend the lifespan of this nematode. The Insulin/IGF-1 pathway is required for both reproductive growth and metabolism and also for the regulation of normal lifespan. Importantly, the IIS pathway has an evolutionarily conserved role in determining longevity using

methods that are –at least to some extent- universal. For example, mutations in the single drosophila insulin receptor has been proved to extend lifespan in the fly and also mutations in the genes encoding both the insulin and Igf-1 receptor can extend lifespan in the mouse.

In the case of *C. elegans*, insulin-like ligands that are expressed in response to nutrition or other stimuli, mediate input to the DAF-2 insulin receptor kinase acting as agonists or antagonists. Then, a molecular cascade including AKT-1 and AKT-2 protein kinases, regulates the forkhead (FOXO) transcription factor DAF-16, which translocates to the nucleus where it is phosphorylated, or stays inactive in the cytoplasm upon dephosphorylation. It has been shown, that reduced Insulin/IGF-1 or stress conditions can result in the nuclear localization of DAF-16/FOXO where it regulates the expression of metabolic, immune, autophagic, and stress resistance genes.

Interestingly, recent work with *C. elegans* has also revealed that mutations in IIS that increase lifespan can additionally reduce the pathology associated with genetic models of cancer and neurodegeneration. Furthermore, mutations in IIS in mice seem to protect against the pathology associated with specific genetic models of Alzheimer's disease.

It is promising that these interventions can produce an improvement in health and function in diverse tissue systems. Furthermore, early evidence from population–genetic association studies has also started to implicate the insulin pathway in determination of human lifespan [10,16].

1.1.2. Protein Synthesis and Ageing

Proper accuracy and control of protein synthesis is essential. For example, the accumulation of mistranslated and potentially misfolded proteins can lead to neurodegeneration. Furthermore, protein synthesis is also a costly process, as it consumes amino acids and energy. Also, it promotes the production of ATP in mitochondria which is associated with the generation of reactive oxygen species

(ROS) which may in turn have damaging effects on a variety of different cellular components.

As it has been found, protein synthesis and protein degradation decline during ageing. Additionally, in a variety of different organisms, lowering the rate of protein synthesis also lowers the rate of ageing and increases lifespan. During ageing but also during embryonic development, cell growth and cell differentiation, many alterations that are sometimes irreversible can happen to proteins. Oxidation is one of the most common factors that damage proteins. Also glycation, deamination and other protein modifications can contribute to the formation of a non-functional protein background that gradually brings the cell to senescence.

Normally, both general and specific degradation pathways, such as the proteasome system, the process of autophagy and lysosomal degradation can remove damaged, non-functional proteins. The decline in those procedures has been also related to ageing and senescence. Furthermore, mutating specific protein synthesis regulators can reduce protein synthesis and extend lifespan. An example comes from the *C. elegans*. This worm has five eIF4E (eukaryotic translation initiation factors) isoforms (IFE-1–IFE-5). IFE-2 mutations have been found to lower protein synthesis in somatic cells and prolong lifespan. Depletion of IFE-1 during adulthood can also extend lifespan. It is noteworthy that the mutated animals are more resistant than the wild type ones to stress, such as heat shock, oxidative stress, UV irradiation or other.

The finding that reduction of mRNA translation rates extends lifespan establishes a direct link between protein synthesis and ageing. It is possible, that lowering protein synthesis in somatic tissues generates energy available for cellular repair and maintenance, with the final outcome of the lifespan extension. Of course there should be a precise balance between the protein metabolism and a cell's wellbeing, as protein synthesis and degradation are vital for a cell [20, 21].

1.1.3. The Role of the TOR Pathway in Ageing

The nutrient sensing TOR pathway (named after the protein kinase 'target of rapamycin') is a key regulator of ageing, affecting several crucial cellular functions. The mammalian mTOR, is a multidomain protein kinase that interacts with other proteins to form two main types of complex, mTOR complexes 1 and 2 (mTORC1 and mTORC2). The mTORC1 is an important cell regulator and it seems to be related to ageing. It is activated by various hormones, mitogens and growth factors and it is negatively regulated by stress. Deregulation of the mTORC1 signaling, has been found to contribute to several human diseases like cancer, cardiac hypertrophy, tuberous sclerosis and others. Rapamycin, a substance that inhibits TOR it is used for the treatment of some of them.

In general, mTORC1 regulates many cellular functions like mRNA translation, while it impairs autophagy. Also, mTORC1 signaling can regulate mitochondrial function. Interestingly, it has been shown experimentally that inhibiting TOR signaling can extend life span. This could be due to the fact that TOR is related to both autophagy and protein synthesis, mechanisms that play critical roles in an organism's lifespan [21].

1.1.4. Dietary Restriction and Ageing

Dietary restriction (DR), a reduction in food calories without affecting the balance of the nutrients that an organism needs, has been found to prolong lifespan in yeast, nematodes, flies, rodents, and probably primates. Interestingly, those happen while most of the physiological processes are kept in a youthful state and many ageing-associated diseases are prevented. Various mediators of this way of lifespan extension have been proposed. For example, DR positive effects on yeast replicative lifespan, requires the activity of the SIR2 histone deacetlyase and NPT1, a gene required for the production of NAD (the oxidized form of the nicotinamide adenine dinucleotide).

In *C. elegans*, (DR) can be achieved through mutations in genes regulating feeding with some of them lowering the food intake due to defects of the

pharyngeal function. Alternatively, the worms' bacterial food can be diluted in order to achieve DR. In both cases, dietary restriction significantly prolongs the worms' lifespan. The beneficial effects of dietary restriction on lifespan may depend on its ability to minimize the oxidative stress, reducing protein oxidation. Furthermore, dietary restriction has been associated with elevated protein turnover which could also be the case.

Research in *C. elegans* has revealed two transcription factors that are required for the lifespan extension under dietary restriction; PHA-4 and SKN-1. PHA-4 is a member of the forkhead family of transcription factors, and it is similar to mammalian FOXA proteins. SKN-1 is related to mammalian NRF2 transcription factors. Also findings in other organisms, have confirmed the relationship of low calorie uptake and longevity. As it has been observed, DR extends life in Drosophila by reducing the flies' initial mortality rate and this is also the case for DR in mice [10, 16].

1.1.5. Mitochondrial Dynamics and Ageing

Mitochondria are involved in important processes, such as cellular metabolism and ATP synthesis and as a result, their damages could influence aging. Although someone would expect that disrupting such an important organelle could lead to shorter lifespan, this is not always the case. Sometimes, the opposite effect is observed.

As mitochondria are the major source of ROS and they do not have the enzymes necessary for protection, it has long been suspected that mtDNA is the prime and vulnerable target of ROS attack. Also the observation that mtDNA damage increases with age and that mitochondrial dysfunction is a common aetiology of many age-associated neurodegenerative diseases created a link between mtDNA mutations and premature ageing. However, more studies showed that mitochondrial mutations do not limit the lifespan of wild-type mice, so the first indications that mitochondrial malfunctions do not always lead to normal ageing were presented.

The first long-lived mitochondrial mutant that was found carried lesions in the nuclear gene clk-1 which encodes a mitochondrial protein involved in ubiquinone biosynthesis. Also, a mutation in the isp-1 gene, which encodes the Rieske iron-sulphur subunit of complex III of the electron transport chain (ETC), was also found to increase lifespan. Moreover, other experiments suggested that cell cycle checkpoint control plays an important role in specifying longevity of mitochondrial mutants.

It is possible that alteration of mitochondrial function affects longevity, in part through components of the IIS pathway, however there is still no clear mechanism for the observed increased longevity of some mitochondrial mutants. Because of the significance of these organelles, exploring the mechanisms that govern the mitochondrial-mediated life extension could lead to a better understanding of how lifespan of organisms is regulated [10, 22].

1.1.6. The Role of DNA Damage and Cellular Senescence to Ageing

Unlike most other types of age-related damage, which can theoretically be reversed, mutations in DNA are permanent. Such errors are tightly linked to cellular senescence and overall organ dysfunction. DNA damage represents a critical threat to cell function. Furthermore, damage and the concomitant repair process can induce genome-wide epigenetic changes, which may finally promote a variety of age-related changes.

There is a wide range of threats, including replication errors, DNA repeat instability, telomere shortening and double-strand breaks (DSBs). During mitosis, DSBs can cause chromosomal translocations and other chromosomal rearrangements that may result in cancer and have further been linked to the ageing process. Also, of the major threats seem to be the reactive oxygen species (ROS). ROS can target a variety of macromolecules including lipids and proteins, but it is mostly their effect on DNA that is often considered as the most harmful and results in permanent changes to gene sequences. If the DNA damage is severe or its

accumulation exceeds its elimination by the DNA repair mechanisms, cellular senescence or apoptosis will occur and these may contribute to the ageing process.

Additionally, many DNA repair pathways have been linked to the ageing process. Two main pathways are the BER (base excision repair) and the NER (nucleotide excision repair) pathway. BER and NER represent two multi-step processes during which a variety of repair factors recognize and repair the sites of damaged bases or nucleotides. Malfunctions in those pathways, seem to contribute to cell ageing, promoting the gradual accumulation of lethal genomic catastrophe.

As it has been proposed, the telomeric DNA seems to be more prominent to DNA damage and breakage. Given the impact of telomere maintenance on cell proliferation and genomic stability, telomeres play a central role in both tumor formation and ageing. Due to their continued shortening, telomeres are particularly susceptible to DNA damage and, consequently, to defects in DNA repair that can lead to various abnormalities. There have been efforts to increase the telomerase activity (the enzyme that is responsible for the lengthening of telomeres) as a way to promote longevity. However, the tricky part of this is that such an intervention can lead to cellular immortalization and cancer.

In general, many findings suggest a causal link between genomic instability, senescence and ageing. A large body of evidence has demonstrated beyond doubt that DNA damage is a crucial mediator for various stresses during cellular senescence regardless of whether they are telomere dependent or independent and that oxidative DNA damage accumulates with age.

Ageing has been associated with major alterations in gene expression patterns, which have been observed in a variety of different tissues and species. For example, in the Hutchinson-Gilford Progeria Syndrome (HGPS), a mutation disrupts a critical component of the nuclear lamina, lamin A, it changes the nuclear architecture and it finally leads to symptoms that resemble normal human ageing such as loss of hair, restricted joint mobility, and atherosclerosis.

Furthermore, there is evidence that epigenetic deregulation may not be limited to specific gene subsets but rather reflect a genome-wide phenomenon. Recent findings have shown for example that global transcriptional deregulation can modulate neuron function in a mouse model of Alzheimer's disease. Different DNA repair pathways may promote ageing independently. DNA damage and chromatin modifications, affecting genome universally can be responsible for a variety of ageing phenotypes, including senescence, loss of proper cell function and other ageing related processes [18, 22].

1.2. *C. elegans* as a Model Organism for Ageing Studies

While originally *C. elegans* was introduced as a model organism to study development and neurobiology, today, it is used to study a much larger variety of biological processes including apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism, ageing and sex determination.

C. elegans is a free living nematode. In nature, it lives in soil and feeds on bacteria. It is an attractive model for longevity and ageing, and it is used extensively for that purpose. Its many advantages have brought this organism in the front line of the ageing research. Its short lifespan which is about 3 weeks (picture 1.), its small size that is about 1mm, the easy reproduction and the low cost of its maintenance are some of its significant advantages. Its species is consisted of both hermaphrodite and male animals. The hermaphrodites cover the greatest part of this worm's populations, while the males arise less often in a physical population with this possibility getting higher the more stressful and harsh the environment is. The ability to use both sexes in a lab taking advantage of their different characteristics, combined with the fact that hermaphrodites can asexually give a progeny genetically identical to the parental worm, have made *C. elegans* a classic organism for genetic studies.

Furthermore, some of the advantages of this organism are that it is transparent, that is has a well characterized cell population with known location and fate and that although the adult hermaphrodite has only 959 somatic cells, these

form many different organs and tissues including muscle, hypodermis (skin), intestine, reproductive system, glands, and a nervous system containing 302 neurons. Additionally the whole genome's sequence of this worm its known and a powerful genetic toolkit for this worm is available to scientists.

Importantly, the somatic cells of the adults are post-mitotic, so it is an excellent model to study aging of non-dividing cells. Also, ageing in C. elegans shares many characteristics with ageing in humans, such as muscle atrophy (sarcopenia), reduced skin elasticity and increased susceptibility to infection. This fact, helps in the immediate connection and study of many conserved molecular pathways of ageing that are found in both organisms. Additionally, the development of methods for gene downregulation based on RNAi (RNA interference) in the case of *C. elegans* is possible for almost any gene, making genetics at this worm quite easy and giving the opportunity for high-throughput functional genomics [5].

The ageing process still seems a mystery. Even in the extreme case of fully homozygous, genetically identical *C. elegans* there can be differences. We don't know the reason. Epigenetic variation in gene expression or chromatin could be a cause of this phenotypic heterogeneity and furthermore, microenvironmental fluctuations or stochastic damage accumulation, could also be the case. There is growing evidence that both stochastic and genetic factors are significant in *C. elegans* ageing, with their combination giving extensive variability between animals of the same age and between cells of the same type within individuals [8].

Further research is needed, and *C. elegans* is a quite convenient model. The extensive knowledge of the biology of this worm, the ability to control the culture conditions to a high degree, and the large inter-individual differences in longevity, make this nematode an ideal system for the development of specific 'biomarkers of ageing'. Biomarkers of ageing are specific parameters that a scientist can follow, that can predict the age of death of an individual assisting the discovery of the molecular mechanisms behind it [6].

An important ageing biomarker of *C. elegans* is the concentration of problems in macromolecular homeostasis. Abnormal degradation and synthesis of

protein products for example, can lead to lipofuscin accumulation and muscle decline. Also, genes that change their expression over time, especially those that have longevity phenotypes on knockout or overexpression, may have a role in inducing or limiting senescence, and thus, stand as transcriptional biomarkers of ageing.

Additionally, a common characteristic that comes with ageing in *C. elegans* is the high accumulation of its feeding bacteria in the pharynx and the intestine. It is speculated that this comes due to the decreased bacterial grinding and disruption in the pharynx of the old worms and it is a feature that can also stand as an ageing biomarker in the case of this nematode [6,7].

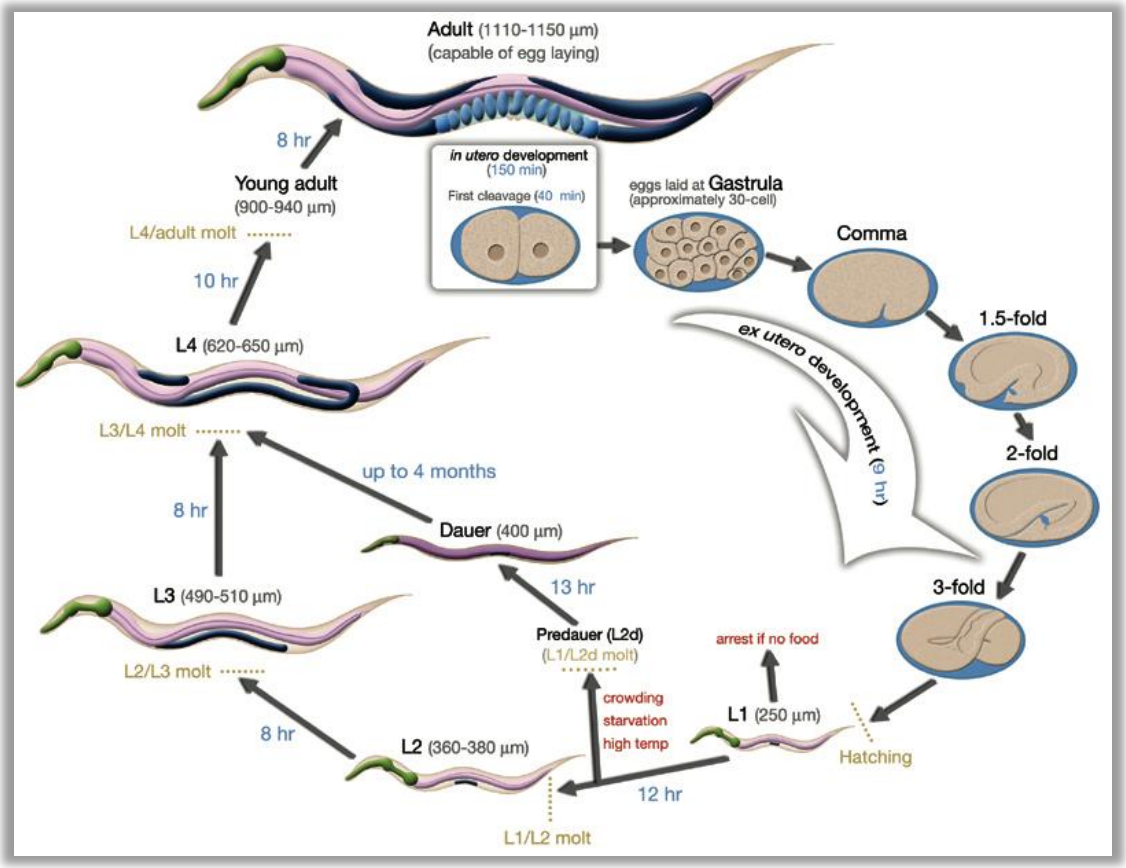
As it has been mentioned before, ageing in *C. elegans* shares many characteristics with ageing in humans. The comparison of the human and the *C. elegans* genomes has confirmed that many of human disease genes and disease pathways can be also found in *C. elegans*. In most cases, there is not a direct link between human and *C. elegans* phenotypes. Even mammalian models are often not reliable so, expecting an invertebrate system to be able to predict for example a drug's action in humans is unrealistic. However, *C. elegans* is a valuable disease model if the disease can be defined on a molecular basis and it can be used taking advantage of its simplicity and in parallel its availability in pathways conserved between nematodes and humans in order to unveil the basic mechanism behind a normal or pathologic phenotype.

Furthermore, an important aspect of the use of this worm in ageing research is that every treatment can be studied and examined in the context of a whole living organism and not separately. Whole-animal assays are of great importance as they can increase the chance of identifying synergistic or off-target effects.

There are different ways to generate a *C. elegans* disease model. For example it is possible to knock out or downregulate the *C. elegans* homologue of the human disease gene, choose a process in *C. elegans* with certain molecular or cellular aspects of the disease or express the human gene to produce a similar phenotype in

C. elegans and be able to study for example a neurodegenerative disorder such as Huntington's disease [5].

Interestingly, up to now, research on ageing of *C. elegans* has given important results about the mechanisms that regulate this complicated process. Many of its mutations increase longevity and much evidence suggests that they do so due to changes in metabolism [9]. Also, other pathways that regulate growth, reproduction, stress response, mitochondria and other physiological parameters seem to somehow be connected to lifespan [10]. Additionally, protein aggregation seems to happen in parallel with ageing in the worm and autophagy may also contribute to lifespan regulation [11, 12].



Pic. 1: The *Caenorhabditis elegans* life-cycle

1.3. The Cell Cytoskeleton

The cytoskeleton is a dynamic and adaptive network of different structures that support cell shape, compartmentalization, intracellular trafficking and even whole-cell movement [24]. The ability of a eukaryotic cell to resist deformation, to transport the intracellular cargo and to change shape during movement, depends on this interconnected network of filamentous polymers and regulatory proteins [25]. The cytoskeleton carries out important functions. It organizes the contents of the cell, it connects the cell physically and biochemically to the external environment and it mediates a variety of biochemical events.

To achieve these functions, the cytoskeleton collaborates with many cytoplasmic proteins and organelles. There are three main types of cytoskeletal polymers: actin filaments, microtubules and intermediate filaments. These polymers control the shape and the mechanics of eukaryotic cells. They are organized into networks that have specific formation but in parallel, they can reorganize in response to external forces. Their role is really important as they arrange and maintain the shape and place of all the intracellular compartments.

Microtubules are the stiffest of the three polymers and have the most complex assembly and disassembly dynamics. The persistence length of microtubules, a measure of filament flexibility that increases with stiffness, is so large (~5 mm) that single microtubules can form tracks that are almost linear and span the length of a typical animal cell. A microtubule can switch between two states: stably growing and rapidly shrinking. This 'dynamic instability' enables the microtubule cytoskeleton to reorganize rapidly and allows individual microtubules to search the cellular space quickly, up to 1,000-fold faster than a polymer that is sensitive only to changes in the cellular concentration of its constituent subunits or to the actions of regulatory proteins.

Actin filaments are much less rigid than microtubules. Unlike them, they do not switch between different states of polymerization, instead, they elongate steadily in the presence of nucleotide-bound monomers. This steady elongation is perfect for the sustained forces required at the leading edge of a migrating cell. Also

unlike the microtubule cytoskeleton, the actin cytoskeleton is continually assembled and disassembled in response to the local activity of signaling systems, not following a specific organizing center.

Intermediate filaments are the least stiff polymers. They can be cross linked to each other, as well as to actin and microtubules, by proteins called plectins. Many cell types assemble intermediate filaments in response to mechanical stresses. Also they have been found to contribute to the mechanical integrity of the eukaryotic nucleus. Unlike microtubules and actin filaments, intermediate filaments are not polarized and cannot support directional movement of molecular motors (see the "focusing on microtubules" part).

The cytoskeleton, in order to perform its main functions, relies not only on its main polymers but also on a variety of other factors. For example, nucleation-promoting factors which initiate filament formation, capping proteins which terminate filament growth, polymerases which promote faster or more sustained filament growth, depolymerizing factors, severing factors which disassemble filaments, crosslinkers and stabilizing proteins are only some of them. In cooperation, all these factors guarantee the maintenance of this complicated and sophisticated functional network [25].

Until recently, the cell cytoskeleton was thought to have been evolved as a structure originated from the eukaryotes. However, recent findings have demonstrated that cytoskeletal homologs existed not only in prokaryotes and archaea but also possibly in the even more ancient common ancestor of all three. Specifically, research on some bacteria revealed the proteins FtsZ and MreB. FtsZ is a cell division related protein that resembles to tubulin, while MreB can assemble into cytoskeletal filaments that are clear analogs of the eukaryotic actin. These findings and others show that cytoskeleton is an extremely conserved structure and confirm its importance for a cell's proper function [26].

The importance of the proper cytoskeleton function is also shown by the fact that mutations which contribute to many diseases are associated with dysfunction of cytoskeleton components. These mutations often influence vesicular biogenesis,

vesicle/organelle trafficking, synaptic signaling and other vital functions. It has been found that in many cases of neurodegenerative disorders, dysfunction of the cytoskeleton results from mutations that alter the conformation and result in the accumulation of the affected gene product. It is also possible, that cytoskeleton disruption initiates a cascade of events including mitochondrial dysfunction and oxidative stress that, ultimately, activates the DNA damage response.

As the cell cytoskeleton is responsible for molecules' proper cytoplasmic trafficking, defects mostly in microtubules, or actin filaments, cause the aggregation and finally the malfunction of many proteins that are involved to known neurological defects such as Alzheimer's disease, Tauopathies, Parkinson's disease, and others. Furthermore, disruption of the cytoskeleton may lead to cell death by impairing the function of mitochondria and energy depletion in the cell because part of the cytoskeleton's cargo has to do with these organelles [27].

Furthermore, it has been found that the communication between the nuclear envelope and the cytoskeleton is the main determinant of nuclear position, migration and anchorage. All three components of the cytoskeleton are involved in nuclear positioning to varying degrees in different cell types. It has been proposed that KASH proteins that exist on the outer nuclear membrane and that are connected to SUN proteins on the inner nuclear membrane, transfer forces between the cytoskeleton and the nuclear lamina. Nuclear migrations are a component of many cellular migration events and defects in nuclear positioning can lead to a variety of diseases such as lissencephaly [28].

Importantly, mRNAs and polysomes have been found to be associated to the cytoskeleton. The cell cytoskeleton seems to have a role in the anisotropic spatial distribution of mRNAs and ribosomes observed in certain specific situations such as amphibian oocytes and skeletal muscle. It is also interesting that the extent of polysome interaction with microfilaments has been found to change under conditions where it is known that there are alterations in protein synthesis [29].

Moreover, some recent studies have revealed the importance of cytoskeletal elements such as actin microfilaments and microtubules in specific aspects of

autophagy. Despite the different hypotheses about the exact role of microtubules in autophagy, it is clear that they facilitate autophagosome trafficking. Autophagosomes are formed at the periphery of the cell and move along microtubule tracks toward the lysosomes. Dynein seems to be involved in this movement with a mechanism that is still not well understood [24].

The cytoskeleton is a complicated system of protein polymers extending throughout the cytoplasm. It not only provides structural support for the cell but also plays a central role in a range of dynamic processes from signaling to endocytosis and intracellular trafficking. Its structures determine not only the cell shape, but also the cell to cell interactions and the variety of cytoplasmic molecular events, the force accompanying many types of cellular movements, and are significant for the general cell homeostasis. Research on its multiple functions can reveal a variety of vital cell processes [30].

1.3.1. Focusing on Microtubules

Microtubules are hollow tubes of about 25 nm diameter and they participate in the eukaryotic cytoskeleton. They provide critical functions in cells, forming the mitotic spindle of dividing cells, the tracks for polarized vesicle and organelle movements, and the core structure of cilia and flagella. Microtubule assembly and turnover are essential for maintenance of the genome over generations and for the cell shape changes and movements necessary for development.

Microtubules consist of alfa and beta tubulin heterodimers that associate to form protofilaments running lengthwise along the microtubule wall with the b-tubulin subunit facing the microtubule plus end conferring a structural polarity. The a- and b-tubulins are highly conserved. Both monomers have similar masses of about 55 kDa, and they both have about 450 amino acid residues. The monomers interact non-covalently to form the very stable tubulin heterodimer, which is the functional form of the protein. Between different organisms, the expression of multiple genes leads to several coexisting, and slightly different, a- and b-tubulins, commonly called

isotypes. Apart from the existence of its genetic isotypes, tubulin is also subject to many post-translational modifications.

There is also a third member of the tubulin family, c-tubulin which plays a role in microtubule nucleation and assembly. Microtubule assembly is accompanied by hydrolysis of GTP molecules that are associated to b-tubulin. An important property of microtubules is dynamic instability which is characterized by growth randomly interrupted by shrinkage.

Many proteins interact with microtubules within the cell and are involved in essential functions such as microtubule growth, stabilization, destabilization, and interactions with chromosomes during cell division. Of the most important proteins are the motor proteins kinesin and dynein, which use microtubules as pathways for transport and are also involved in cell division [31].

The rigidity of microtubules is very important for their biological functions. They need to be stiff in order to be able to create cell shape, especially for the extended morphologies and to enable efficient, long-range transport, since the microtubule network makes up the tracks for cargo-carrying motor proteins in the cell. Moreover, microtubules form a rigid structural network to which actin and myosin attach to create tensile forces during cell motility and membrane rearrangements [32].

From the one hand, during interphase, the part of the cell cycle during which cells prepare for division, many cells take advantage of the microtubule stiffness, in order to assemble radial arrays of microtubules that function as central hubs and 'highways' for intracellular traffic. From the other hand, during mitosis, the part of the cell cycle during which cells separate chromosomes into two identical sets, the microtubule cytoskeleton rearranges itself into a high-fidelity DNA-segregating machine called the mitotic spindle. The ability of the mitotic spindle to find and align chromosomes depends, in part, on the complex assembly dynamics of individual microtubules [25]. When microtubules assemble, the plus end usually grows more quickly than the minus end. There is a region called the centrosome or 'microtubule organizing center' (MTOC) at which microtubules are nucleated and grow outwards

toward the cell membrane with the plus end leading. The microtubule minus end often remains attached to the MTOC and the resulting microtubule networks can mediate the intracellular transport.

There are other important structures too. In most vertebrate cells, except for the higher plants, there is a specific microtubule-based 'organelle' called the centriole, which is embedded within the MTOC. Another structure called axoneme, comes within motile cilia and flagella and is organized as a ring-like array of nine microtubule doublets around a central microtubule [31]. Importantly, another structure, radial arrays of microtubules called asters, has been found to be able to determine the intracellular localization of organelles. Asters can be generated through either the microtubule organizing center (MTOC) or through a self-organization process [33].

Moreover, basal bodies, centrioles and other structures, performing different functions, contribute to the valuable microtubule network system. A large number of proteins interact with microtubules including the conventional microtubule associated proteins (MAPs), the plus-end tracking proteins, and the motor proteins dynein and kinesin.

Conventional kinesins are heterotetrameric proteins consisting of two identical heavy chains and two light chains with molecular weights of 110–130 and 60–80 kDa respectively. They are present in all eukaryotes and are involved in fast axonal transport in nerve cells, in the organization of the cytoplasm during interphase, in directional vesicle movement within the cell and in cell division.

Dyneins are microtubule motors that use the energy of ATP hydrolysis in order to move along microtubules. Cytoplasmic dyneins usually move toward the minus end but they can also move laterally across the microtubule surface lattice and even in reverse direction. They consist of one to three heavy chains of 500 kDa, various intermediate chains, and light chains. They perform important roles in the orientation of the mitotic spindle and in nuclear migration. Also, other cytoplasmic dyneins are involved in the retrograde transport of vesicles and organelles, in mitotic

spindle organization and in chromosome segregation. Importantly, dynein mutants have been also implicated in motor neuron degeneration.

It is of great interest that also other molecules that can come from a variety of different sources, strongly influence microtubule assembly and stability and are widely used as specific antimitotic drugs with important medical applications –often in cancer therapy- and usage in research. Colchicine, vinblastine and taxol are some of them [31]. Benzimidazoles, another important category are analyzed after few lines.

1.4. P-bodies

Exported mRNAs are targeted for translation or can undergo degradation by several decay mechanisms. The 5' to 3' degradation machinery localizes to cytoplasmic P bodies (PBs). P-bodies are cytoplasmic foci found in somatic cells of vertebrate and invertebrate origin as well as in yeast, plants and trypanosomes [40]. They are dynamic aggregates of specific mRNAs and proteins [34, 35]. The complete protein composition of P bodies is not yet determined. However, a conserved core of proteins has been found in P bodies and consists of the mRNA decapping machinery, the decapping enzyme Dcp1p/Dcp2p, the activators of decapping Dhh1p/RCK/p54, Pat1p, Scd6p/RAP55, Edc3p, the Lsm1p-7p complex, and the 5' to 3' exonuclease, Xrn1p [37].

P bodies serve a dual function: first, they harbour mRNAs that are translationally silenced. Such mRNAs can exit again from P-bodies to re-engage in translation. Secondly, P-bodies recruit mRNAs that are targeted for deadenylation and degradation by the decapping/Xrn1 pathway [41]. In eukaryotes, mRNA degradation typically begins with deadenylation, and then either of two major pathways is used. The exosome protein complex degrades mRNAs in the 3' to 5' direction, whereas the 5' to 3' direction involves other factors, including a decapping enzyme followed by the Xrn1 exonuclease. The second procedure seems to be hosted by the P-bodies.

PBs are dynamic structures. They are observed in a variety of different sizes and numbers in the cell. However this variability hasn't yet been estimated and it isn't known if functional differences are hidden behind it. Nevertheless, changes in PB number and size per cell have been correlated to the cell cycle, to the proliferation status of the cell, and to the availability of nutrients [34, 35].

During stress, PBs can transiently associate with stress granules (SGs), structures that accumulate stalled translational preinitiation complexes [42]. Stress granules are large aggregates of untranslated mRNAs, translation initiation factors and 40S small ribosomal subunits that accumulate in the cytoplasm of cells undergoing translational arrest. They are formed in cells that have been exposed to stresses such as heat shock, or energy deprivation. Although P-bodies and stress granules are distinct structures, they could be functionally linked, as transient contacts between them are frequently observed. There is the hypothesis that stress granules and P-bodies can merge under certain conditions, and that cells make use of those two structures in order to regulate the flux of their mRNAs in the cytoplasm [34, 35].

P-bodies are associated with multiple mRNA processes. It has been found that all the enzymes required for mRNA degradation via the deadenylation–decapping 5'–3' pathway, exist in P-bodies. So, P-bodies act as factories equipped with the full machinery that allows for the processive, highly efficient degradation of mRNAs. It has been shown that this process has as a prerequisite the deadenylation step that provides the "entering" signal for an mRNA molecule and happens outside P-bodies [38]. It is interesting, that once they are into P-bodies, mRNAs are not always degraded; some of them exit P-bodies and can re-initiate translation.

Furthermore, many protein factors required for NMD (nonsense-mediated mRNA decay), the pathway responsible for the degradation of short-lived mRNAs, have been localized to P-bodies. Another process happening into P-bodies, is AMD [ARE (AU-rich element)-mediated mRNA decay]. AREs are found in many cytokine and proto oncogene mRNAs, and they mediate rapid mRNA degradation, inhibiting

gene expression at the post-transcriptional level. It has been found that both AMD and NMD factors are associated to the P-bodies transiently.

Another important process that has been found to happen into P-bodies is miRNA (microRNA)-induced mRNA silencing [39]. The presence of miRNAs can silence gene expression by either repressing translation or by inducing decay of the mRNAs to which they are bound. Important step of this process is the recruitment of RISC (RNA-induced silencing complex). Many proteins that are associated with RISC and are needed for miRNA-mediated suppression have been found to be concentrated in P bodies.

Additionally, it has been proposed, that as in the case of AMD and NMD, the pool of silenced mRNAs is even by itself, a determinant of P-body formation. P-bodies seem to be formed and resolved according to the amount of the mRNA that is subject to silencing, translational arrest, or rapid decay [34].

As it was mentioned before, P-bodies are functionally dynamic structures. This is also the case for their locomotion. They are not static foci. It has been found that most PB movements are confined to a certain cytoplasmic region giving an impression of constrained movement, while other PBs move extensively. Only few PBs per cell seem to be relatively stationary. Also, less frequently, directional movements of PBs have been reported.

PBs, rely much on the cytoskeleton for their locomotion and their normal function. It has been proposed that stationary PBs are associated with actin bundles, whereas others that move rapidly, performing saltatory movements, are on microtubules. Other studies have demonstrated that PBs can even move along the microtubules, arrive at microtubule intersections and then there change direction in order to travel from one microtubule to another. PBs in this way could encounter mRNAs, as we know that they also use the microtubules to move across the cytoplasm. However, it is not known yet whether all mRNAs encountering PBs on microtubules functionally interact, because it is possible that some mRNAs are protected, for example by the granule complexes containing them.

Importantly, some series of experiments have shown that when different inhibitors of microtubule assembly are used, for example nocodazole or vinblastine that lead to microtubule destabilization, the mobility of PBs, is significantly reduced. This showed that PBs require an intact microtubule network for anchoring and moving within the cytoplasm. Probably, in that way, the cytoplasmic volume that PBs can access is bigger in comparison to non-anchored PBs which can only diffuse over short distances. Furthermore, it has been found that microtubule disruption leads to increased PB assembly. However, there are also other experiments that have shown the exactly opposite phenomenon, with the PB assembly to become lower.

It has been hypothesized after these observations, that under conditions of microtubule disruption, the assembly of PBs depends only on the availability of free cytoplasmic RNA and therefore, the size of transcriptional/translational activity in each time [35] and the specific situation or cell type, can alter the formation levels of these "structures". In this case the inhibition of transcription or protein synthesis would not allow PB assembly even when the microtubule network is disassembled. Many parts of the story are still missing. In general, it seems that the microtubule network is necessary for increasing the volumes that PBs probe and the targets that they encounter, having an important role in their proper function and a significant contribution to the whole mRNA "life-cycle".

Several observations, propose that cytoplasmic mRNAs, cycle between polysomes, P bodies and stress granules. For example, it has been found that inhibition of translation initiation by drugs, stresses, or mutations leads to loss of mRNAs from polysomes and a corresponding increase of mRNAs in both P bodies and stress granules. Of course, the movement of mRNAs between polysomes, stress granules and P bodies implies transitions between different mRNP states through specific re-arrangements and exchanges of proteins on individual mRNAs. This could be facilitated by RNA helicases as well as being influenced by post-translational modifications of the key RNA binding proteins.

Once into PBs, mRNAs complexed with the decapping machinery could be degraded, aggregated in there, or undergo an mRNP rearrangement wherein the

degradation machinery is exchanged for translation initiation factors. Such mRNAs could then go on to initiate translation and enter polysomes. However, if steps in initiation are limiting, these mRNPs could accumulate in a stress granule state before eventually entering polysomes. It has also been proposed that the composition of the stress granule might vary in different organisms or in response to different stresses. Specific mRNAs may preferentially accumulate in stress granules, P bodies, or polysomes depending on their relative rates of transitions between these different biochemical states [36].

The work which is presented here, will hopefully give some first hinds for the relationship between the cell cytoskeleton and the PBs, expanding the knowledge on the functions they perform.

1.5. Benomyl

Benomyl, a benzimidazole systemic agricultural pesticide, is extensively used against a wide range of fungal diseases. The proposed mechanism of its action is the disruption of microtubule turn-over. Binding to tubulin, it inhibits polymerization of heterodimer subunits into microtubules. It is known to exert selective toxicity against fungal cells due to its higher affinity for fungal tubulin than mammalian tubulin and it is selectively toxic to microorganisms and to invertebrates, especially earthworms.

In *C. elegans*, benomyl, in a dosage dependent manner, causes paralysis, severely uncoordinated locomotion and can slow the growth like other benzimidazoles such as mebendazole, nocodazole and thiabendazole. More specifically, larvae freshly hatched in the presence of benomyl look normal, probably because the drug does not enter the eggshell. Later, from the L2 stage and later, the worms are severely paralyzed. Usually, head movement seems normal, while the rest of the body towards the end cannot move.

Benomyl sensitivity depends on temperature. As the temperature gets higher, it seems that the dose of benomyl needed in order to have the paralyzing

effect is higher too. Also, at higher temperatures the drug destabilizes and it is less effective at microtubule disruption [4]. Benomyl binds strongly to soil and does not dissolve in water to any great extent. It is quite unstable and it decomposes in aqueous solutions into more stable compounds like carbendazim. The conversion of benomyl to carbendazim which is its main metabolite is slow in aqueous environment and it could take several days [2].

Benomyl has a half-life in turf of three to six months, and in bare soil, a half-life of six months to one year. It differs structurally from carbendazim by the butylcarbamoyl side chain, whereas they have the benzimidazole ring in common. The side chain is known to produce the active metabolite n-butyl-isocyanate, which is conjugated to intermediaries that are then bioactivated and form thiocarbamates. In general, benomyl and carbendazim, are thought to have similar functions, however in many cases, those actions are mediated differently.

Tubulin is believed to be the target of benomyl in yeast cells according to specific mutation studies in the b tubulin gene, which altered the resistance or sensitivity of yeast cells to benomyl. Originally, benomyl was thought to bind at or near the colchicine binding site based on the structural similarities of these two substances. However, recent studies have suggested that benomyl and colchicine bind at distinct sites on tubulin.

An important action of benomyl is that it can inhibit the proliferation of both fungal and mammalian cells by arresting the cells at mitosis. Furthermore, benomyl has been shown to be able to inhibit the polymerization of brain microtubules in vitro. In addition, it has been shown that it suppresses the assembly of cold-depolymerized spindle microtubules in HeLa cells by perturbing the microtubule–kinetochore interactions and activating the mitotic checkpoint protein BubR1 [1].

Apart from its activity in microtubules, benomyl and possibly also carbendazim might also act on the DNA, inducing oxidative stress as dominant mechanism of action. In addition, other studies have proposed that it can cause chemical hypoxia in various tissues, strengthening the idea of the generation of

oxidative stress. Furthermore, benzimidazoles have been recently proposed to be histone deacetylase inhibitors [3].

Benomyl is widely used in research as microtubule inhibitor and it is a promising substance that could lead to the development of new anticancer drugs, as disruption of normal polymerisation and depolymerisation is already the target of many known cancer therapeutics [1].

1.6. The *ben-1* Gene

Molecular cloning and DNA sequence analysis performed by Driscoll et al, [4] established that *ben-1* encodes a beta tubulin. *Ben-1* was the first gene found to confer resistance in benomyl. Some of its resistant alleles were complete deletions of the gene, and even those mutants, apart from the resistance to benomyl were otherwise wild type. This led to the hypothesis of functional redundancy between the *ben-1* and the other members of the beta-tubulin family which allows the mutants to be viable and coordinated.

The benomyl resistant alleles were mapped between daf-2 and unc-93 and apart from benomyl, they were found to confer resistance to a variety of benzimidazoles such as carbendazim, nocodazole and others. However *ben-1* mutations confer specifically resistance to those drugs and not other anti-microtubule agents like colchicine, something that implies specificity for this function. The *ben-1* gene contains four introns from which two are short (52 and 57 bases for introns II and III, respectively), a feature common for *C. elegans* introns and it encodes a 444-amino acid beta-tubulin.

About the mechanism of action of those mutations, it has been proposed that the dominant effects of the *ben-1* deletion mutations exist due to the fact that they lower the intracellular amount of the only one or most sensitive to those drugs tubulin, rendering the drugs ineffective. Also it has been proposed that the alleles which retain partial sensitivity are mutations that affect negatively the benomyl binding. Additionally, the fact that *ben-1* mutations don't seem to affect the wild

type phenotype of the worms, could be explained if its function is replaced by other members of the beta tubulin family. These genes could be silent in *ben-l*-expressing cells and become transcriptionally active as a consequence of the *ben-1* deletion or they could be co expressed with *ben-1* and upregulated when the activity of *ben-1* is reduced [4, 13, 14 and 15].

1.7. Aim of this Work

The work presented here aims to discover the possible relationship between a common biological phenomenon, -ageing-, a basic polymer of the cytoskeleton, -microtubules- and the P-bodies.

P-bodies, as critical regulators of mRNA metabolism and effectors of the cell balance between creation and degradation could affect biological ageing. Ageing, as mentioned before, has been linked to various conditions. Oxidative stress, accumulation of genomic catastrophe, mitochondrial damage and other factors, seem to complement the series of events that lead to ageing and finally death of a living system. Understanding the mechanisms that support this process, could give answers about the origin of many age-related diseases and support the development of new therapies that will hopefully offer relief to the aged human societies.

As P-bodies are physically and functionally linked to microtubules, and as it has been demonstrated that specific antimicrotubule drugs or gene knockouts affect them, we thought that it would be interesting to use this relationship as a means to see phenotypes related to P-body function alterations, possibly including ageing.

Additionally, as cell cytoskeleton mediates a variety of important cellular functions (see the introductory part), we thought that disruption of it may itself give an interesting ageing phenotype, and that is what we also aim to check with this work. Hopefully, research in those fields will produce significant knowledge on the mechanisms that govern the ageing process and its relationship with many cell functions.

2. Materials and Methods

2.1. *C. elegans* mutants and lines

- N2 bristol: wild type worms.
- N2 Ex [P*dcap-1* DCAP-1::DsRED]: line in which red fluorescence protein is expressed under the promoter and gene of *dcap1*, marking specifically the p-bodies. Often referred as dcap1::dsred worms in the text.
- Ife-2: long lived worm mutant that carries mutation on the second *C.elegans* isomorph of eIF4E.

2.2. *E. coli* bacterial strains

- OP50: used for normal worm maintenance.
- HT115 (DE3) (CGC): used for the RNAi experiments.

2.3. Preparation of NGM (Nematode Growth Media) plates for worm maintenance

In about 1 L of nanopure water we add

- 6g NaCl
- 5g bactopeptone
- 0.4g streptomycin
- 34g agar

Sterilization for 20 min and then we add

- 2ml $CaCl_2$ 1M
- 2ml $MgSO_4$ 1M
- 2ml cholesterol
- 2ml nystatin
- 50ml KPO_4^* 1M, pH 6

We fill up to 2 L with sterilized nanopure water

2.4. Preparation of NGM (Nematode Growth Media) for RNAi plates

In about 1 L of nanopure water we add

- 6 g NaCl
- 5 g bactopeptone
- 34 g agar

Sterilization for 20 min and then we add

- 2 ml $CaCl_2$ 1M
- 2 ml $MgSO_4$ 1M
- 2 ml cholesterol
- 2 ml nystatin
- 50 ml KPO_4* 1M, pH 6
- 1 ml ampicillin (stock: 100mg/ml)
- 15 ml IPTG (the substance that induces the T7 polymerase of the HT115 bacteria so that the appropriate dsRNA will be produced by them and downregulate the corresponding gene of interest in *C. elegans*).

2.5. M9 buffer

- 3g KH_2PO_4
- 6g Na_2HPO_4
- 5g NaCl
- 1ml $MgSO_4$ 1M

2.6. Freezing solution for worm storage in -80° C

- 5,85g NaCl
- 50ml KPO_4* 1M, pH 6
- ≥200ml glycerol

Sterilization

- 3 ml $MgSO_4$ 1M

* Contains 204.4 g KH_2PO_4, 114.12 g K_2HPO_4 and water in 2L volume

2.7. LB medium for the bacteria (1 L)

- 10 g bacto-tryptone
- 5 g yeast extract
- 10 g NaCl
- 15 g bacto-agar

LB + ampicillin

Ampicillin added in LB in a total concentration of 50μg/ml.

LB + tetracycline

Tetracycline added in LB in a total concentration of 10μg/ml.

2.8. PCR (Polymerase Chain Reaction)

1. 50ng-100 ng genomic DNA or 10ng-50ng plasmid DNA
2. 1μM for each primer
3. 0.25mM dNTPs
4. 1-5 units of Taq DNA polymerase
5. Buffer for Taq (1.5 mM $MgCl_2$, 50 mM KCl) and distilled water up to 20 μl
6. 3min at 94 °C
7. 40sec at 92 °C
8. 30sec - 1 min at 2-5 °C lower than the primers' Tm
9. 71 °C for 1min per 1000bp
10. Repetition of the above (6-9) for 5 circles
11. 40sec at 92 °C
12. 30sec- 1 min at 2-5 °C higher than the primers' Tm
13. 71 °C for 1min/ 1000bp
14. Repetition of the 11-13 for 6-8 circles
15. 15min at 70 °C
16. 4-10°C for ever

2.9. RNAi experiments

A variety of species exhibit a defense response in which a double stranded RNA (dsRNA) `trigger' produces a premature loss of endogenous RNAs with extended

regions of sequence identity to the trigger. In the case of *C. elegans*, this ability is one of the most important genetic tools. Bacteria that carry those dsRNAs and feed *C. elegans* can silence the worm's corresponding sequence in its every cell genetic material with the only exception of the nervous system cells where the method is not so effective. In our experiment, HT115 bacteria were transformed with the plasmid L4440 (pPD129.36) cloning vector. This plasmid contains two convergent T7 polymerase promoters in opposite orientation separated by a multicloning site in which we have added the sequence of interest for each case [23]. The activation of the T7 polymerase needs the IPTG which we had in the nematode growth medium in the case of the RNAi experiments.

The RNAi experiments were performed in 15˚ C. Young adults of the worm strain or line of interest were isolated on simple NGM containing plates with OP-50 bacterial lawn to lay eggs for 4-6 hours and then removed. After 3 days approximately, the young adults of the new generation were harvested and isolated on NGM-RNAi containing plates, with HT115 bacteria transformed with the plasmid with the gene of interest, to lay eggs for 4-6 hours and then removed.

The new generation was that of the experiment. The first transfer to fresh RNAi plates was taking place when the worms reached young adult phase, and from then on transferred every 1-2 days according to the needs, with measurements taken every 1-2 days, and microscopy taking place when needed with worms from specific plates for that purpose, however grown in parallel and exactly the same way as the ones for the ageing experiment measurements. For each situation studied at the ageing experiments, a worm population of no less than 100 worms was tested.

2.10.Benomyl experiments

The benomyl (Sigma Aldrich) experiments were also performed in 15˚ C. This substance is quite unstable and better dissolved in DMSO. We diluted it in DMSO/water dilutions in order to keep the proper for each benomyl concentration balance between the substance's solubility and the lower possible DMSO concentration. In all experiments, the final concentration of DMSO had been kept in 1% of the final plate where worms were growing, while a plate with only 1% DMSO,

was for each case the control situation. Young adults of the worm strain or line of interest were isolated on simple NGM containing plates with OP-50 bacterial lawn to lay eggs for 4-6 hours and then removed. After 3 days approximately, the young adults of the new generation were harvested and isolated on NGM-OP-50 containing plates, after the adding of the appropriate concentration of benomyl diluted to DMSO-water solution and few hours more so the plates were dry. The benomyl containing plates weren't used for more than 3 days before the next worm transfer, in order to avoid possible substance deactivation. Also, in most experiments before the addition of the drug bacteria had been UV-killed in order to exclude the possible interference between them and the drug that could change its characteristics or action. In any other case the method followed is explained. For each situation studied at the ageing experiments, a worm population of no less than 100 worms was tested.

2.11.Observing P-body intensities

P-bodies studies had been performed in dcap1::dsred worms in which those structures are marked red under an epifluorescence microscope. Worms of the desired age and condition were put into a NaN3 drop of 10mM concentration that had been placed onto microscopy glass slide. Usually, for each preparation, 15-18 worms were put into 16 μl of NaN3. Afterwards, the drop was covered with a cover slip and fixed around with nail lacquer. The worms were in general intact by this procedure, however anaesthetised by the NaN3, immobile and flattened. For each treatment, a number of 25-35 worms was tested. Images of the pharynx region of the worms were obtained with the use of an epifluorescence microscope.

2.12.Software used for statistics

- GraphPad Prism: the software used for the statistical analysis and graph preparation of the imaging and the lifespan values.
- Image J: the software with which the fluorescence images were analyzed. It was used to obtain a mean intensity of the P-bodies of a specific head region, common for all worms. The value obtained each time, represents the total mean intensity of the area. It is not indicative separately of the size, the number or the possible aggregation of the P-bodies.
- Microsoft Excel: The program used to organize the arithmetic values.

3. Results

3.1. Destabilization of microtubules using a drug. Effects on P-bodies and total lifespan

In this series of experiments, we studied the effect of microtubule disruption in P-body formation and ageing, using the Sigma Aldrich benzimidazol "Benomyl". This drug is known to confer microtubule disruption (see introductory part), and other observable characteristics in nematodes, such as paralysis, usually in a dose dependent manner as it has been established [4]. The aim was first to establish the proper set of benomyl concentrations that would be studied. Trying different concentrations, we focused on those that did not cause severe phenotypic movement disability at the worms. The worms used for both the ageing and the P-body experiments belong to the line dcap1::dsred (see materials and methods part). Each experiment presented here has both studies (a lifespan study and the corresponding P-body intensity study). The two worm populations for each experiment were of equal type, age and treatment. The results of the experiments follow in this part.

3.1.1. Alterations of Lifespan and P-bodies Intensity, of Benomyl Treated *C. elegans* Populations

In a first approach, benomyl concentrations of 0,1 μg/ml, 0,5 μg/ml and 1μg/ml were tested in comparison to the 1% DMSO control worms that were affected only by the solvent. In the case of the lifespan study (figure 1), the median population survival for each situation was 15, 15, 14 and 13,5 days respectively. These values do not represent the real survival of the population but the one according to the termination of the measurements that happened before the death of the whole population. This happened due to the quick death of the worms that belong to the high concentration, which cancelled the trial. However, for the comparison between the different concentrations, the results are reliable and informative.

This experiment showed that as the benomyl concentration gets higher, the worms' lifespan is affected negatively. Furthermore, in figure 1 is shown that the 0.1 μg/ml benomyl concentration keeps the worms healthier even from the control situation. Their health span (the appearance of the curves at the first days of the experiment, which is indicative of how sick could a worm population be in comparison to others) is better. This could be interesting, and remains to be further validated. Also, severe paralysis effect was obvious in the case of the 1 μg/ml concentration, which was the reason to omit it, while in the other concentrations the phenomenon was mild. The results of the P-body intensity measurements are shown in figure 2. In this trial, there was relatively big variation between the values. Interestingly, there was a first indication that ageing is accompanied by higher P-body intensities.

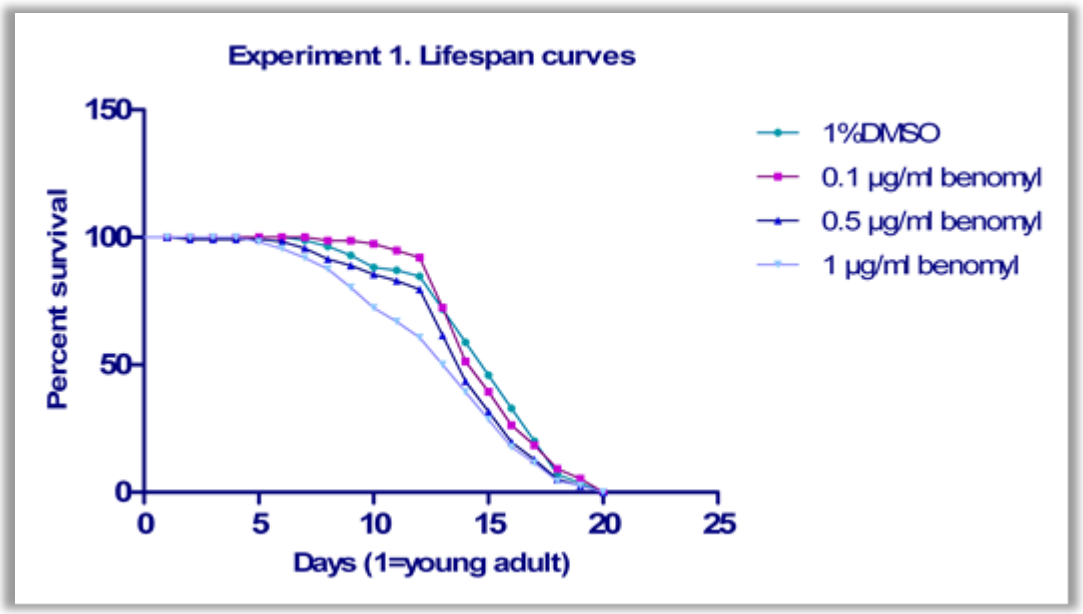


Fig. 1: Lifespan curves of benomyl and DMSO treated dcap1::dsred line worms. The median survival was 15, 15, 14 and 13,5 days for the 1% DMSO, 0,1 μg/ml, 0,5 μg/ml and 1μg/ml treatments respectively.

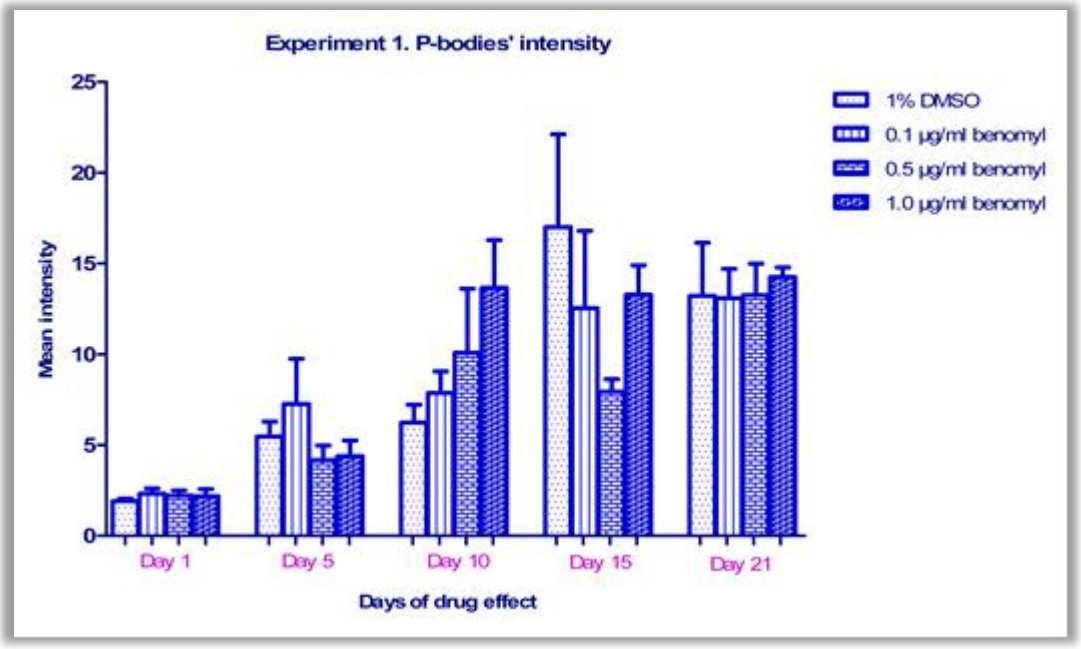


Fig. 2: P-body intensity as it was count in benomyl and DMSO treated dcap1::dsred worms. Error bars represent SEM.

3.1.2. Alterations on Lifespan and P-bodies Intensity, of Benomyl Treated *C. elegans* Populations

At a next trial, the concentrations of 1% DMSO, 0,1 μg/ml, 0,3 μg/ml and 0,5 μg/ml benomyl, were used, and characterized as optimum for the purposes of these experiments. Worms at these concentrations seem to have paralysis effect that varies from almost non-detectable (1% DMSO, 0,1 μg/ml benomyl) to mild (0,3 μg/ml and 0,5 μg/ml benomyl). In this case, no significant differences between the different drug values on lifespan were detected. Interestingly however, according to the curves appearance (figure 3) from about the day 17 of the worms, the high benomyl concentration shifts is action to protection, following the fate of the control. The P-bodies' intensities were really interesting (figure 4). With the results being statistical significant, it was shown that P-body intensity becomes greater with time, a characteristic observed in most of the situations.

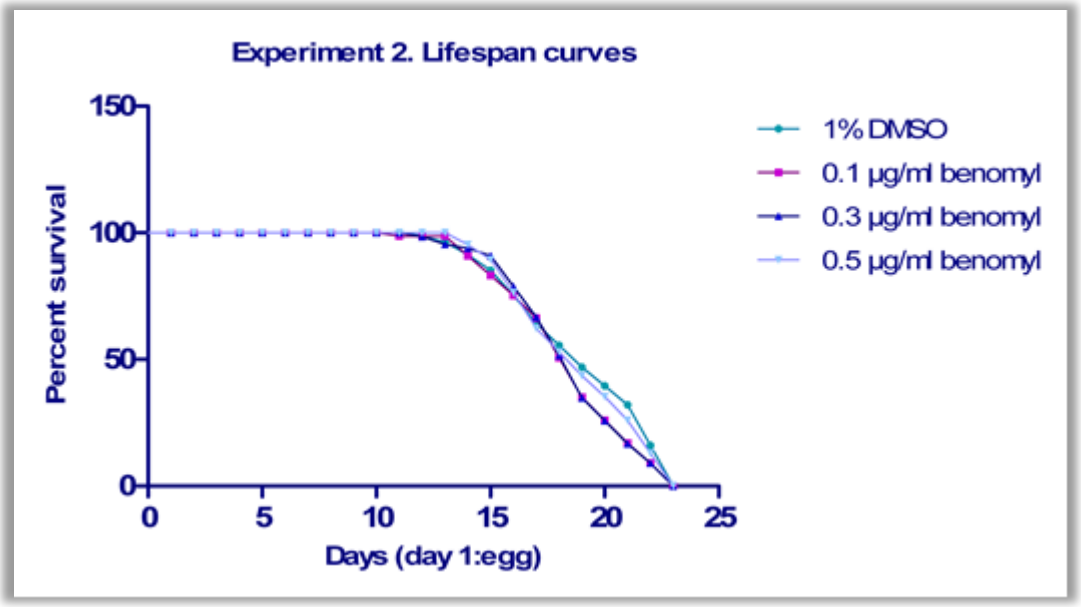


Fig. 3: Lifespan curves of benomyl and DMSO treated dcap1::dsred line worms. The median survival was 19 days in all cases.

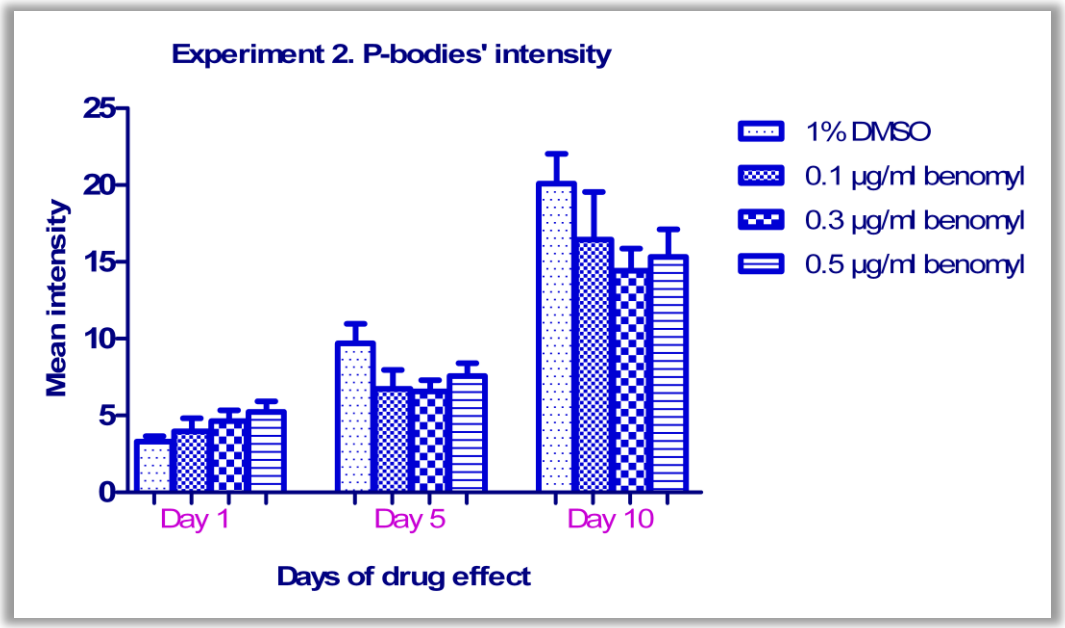


Fig. 4: P-body intensity as it was count in benomyl and DMSO treated dcap1::dsred worms. Error bars represent SEM.

3.1.3. Alterations on Lifespan and P-bodies Intensity, of Benomyl Treated *C. elegans* Populations

A next experiment, showed again that the health span of 0,1 μg/ml benomyl situation is better than the control, strengthening this possibility, while the same seems to hold truth for the 0,3μg/ml benomyl concentration. The higher concentration, as also previously noticed, gives the shortest lifespan (figure 5). The P-bodies appearance in this experiment was again more intense with ageing but this was the case for certain time points and situations in a non-continuous manner. Additionally, on day 5, all the benomyl treatments gave lower P-bodies' intensities in comparison to the control (figure 6). This was the case for the previous P-body intensity measurements too, with an exception only for the 0,1 μg/ml benomyl concentration on day 5 (figure 2).

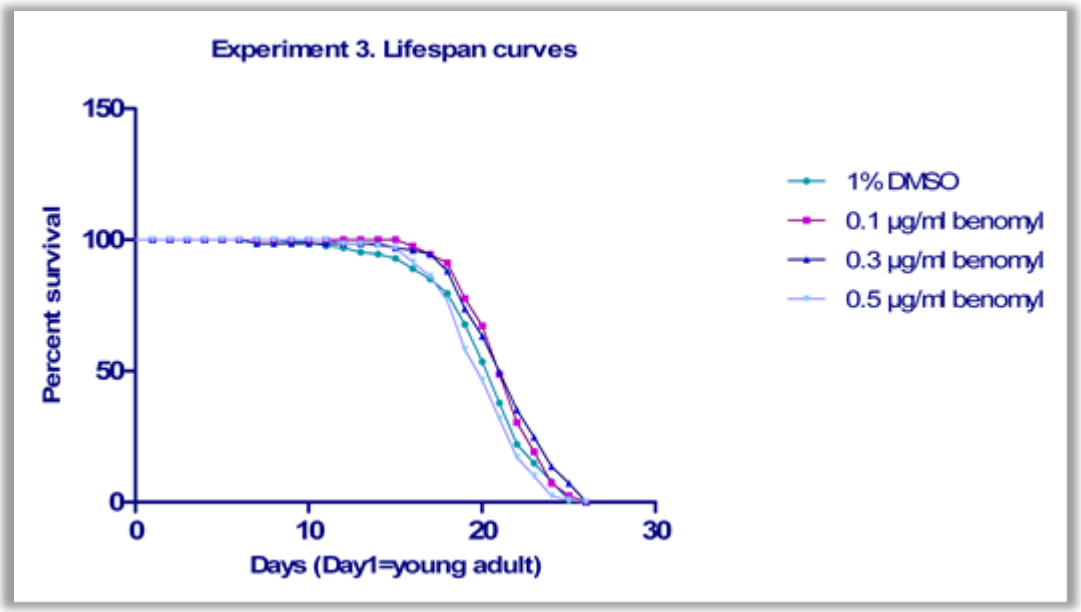


Fig. 5: Lifespan curves of benomyl and DMSO treated dcap1::dsred line worms. The median survival was 21, 21, 21 and 20 days for the 1% DMSO, 0,1 μg/ml, 0,3 μg/ml and 0,5 μg/ml treatments respectively.

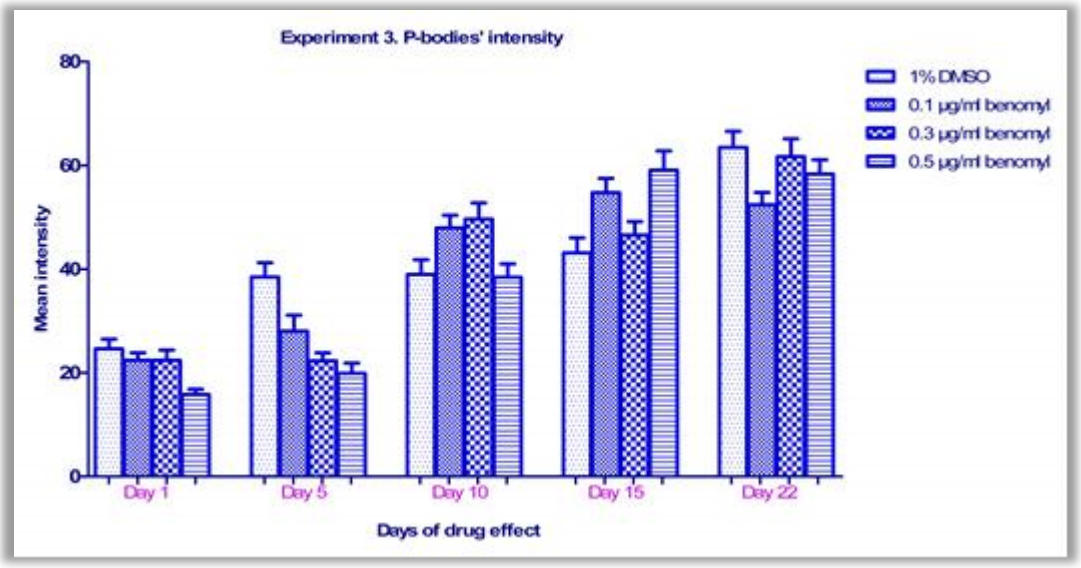


Fig. 6: P-body intensity as it was count in benomyl and DMSO treated dcap1::dsred worms. Error bars represent SEM.

3.1.4. Alterations on Lifespan of two Benomyl Treated *C. elegans* Populations

In this experiment, the relationship between benomyl induced cytoskeleton disruption and ageing was tested in both worms of the line N2 Ex [P*dcap-1* DCAP-1::DsRED], (referred as dcap1::dsred) and wild type N2 bristol ones (figures 7, 8). In the case of the N2 worms, the healthspan of 0,1 μg/ml concentration seems to offer a better healthspan once again, while the highest concentration a statistically significant result of 1 day longer lifespan. In the case of dcap1::dsred worms, no statistically significant difference was observed.

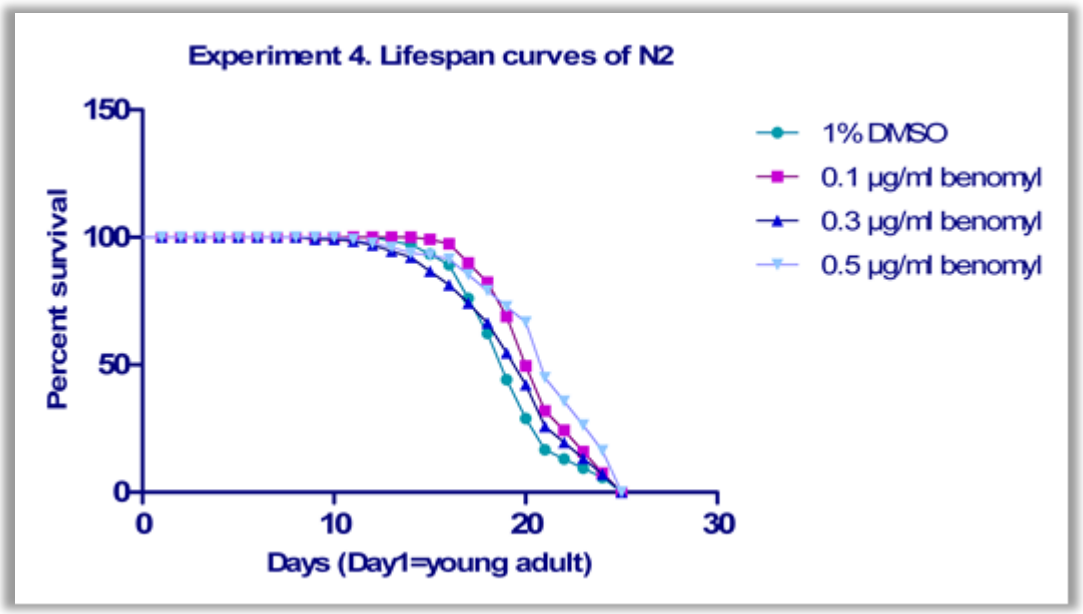


Fig. 7: Lifespan curves of benomyl and DMSO treated N2 worms. The median survival was 19, 20, 20, and 21 days for the 1% DMSO, 0,1 µg/ml, 0,3 µg/ml and 0,5 µg/ml treatments respectively.

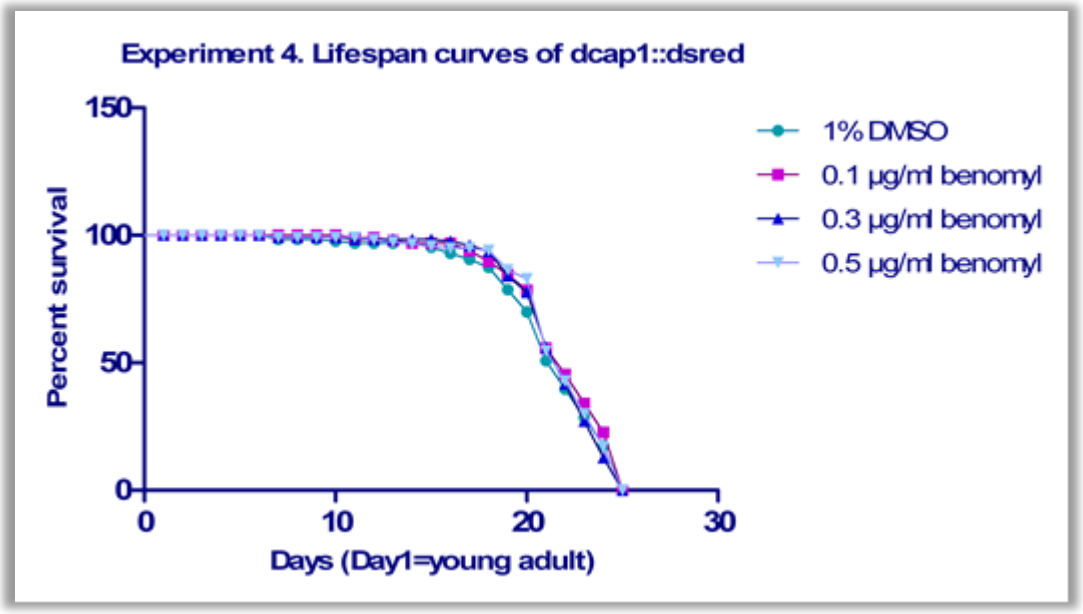


Fig. 8: Lifespan curves of benomyl and DMSO treated N2 worms. The median survival was 22 days for all the treatments.

3.2. Destabilization of microtubules using RNAi for a beta-tubulin gene. Effects on P-bodies and total lifespan

In this series of experiments, we studied the effect of microtubule disruption in P-body formation and ageing, using the RNAi method (see materials and methods part). We downregulated the gene *ben-1*, which encodes a beta-tubulin. (see introductory part). As previously, in this case, both ageing experiments and P-body intensity measurements were perfomed. The Pl4440, is the vector used for the bacteria transformation, and carries each time a different sequence according to the RNAi used each time. Empty, without any sequence for RNAi, it is used as a control of the method. So "Pl4440" represents the control situation where no worm gene is under downregulation, "*ben-1*" the RNAi downregulation of *ben-1* gene and "ife-2" the downregulation in some cases of the *ife-2* gene, known to confer longevity.

3.2.1. RNAi on the genes *ben-1* and *ife-2*. Comparison of P-body intensities

In this experiment, the P-bodies appearance was compared between two different RNAi interventions. In the one case, the beta-tubulin gene *ben-1*, while in the other, the *ife-2* gene which corresponds to a worm eIF4E factor was downregulated. The results of P-body appearance showed no significant difference in this case (figure 9).

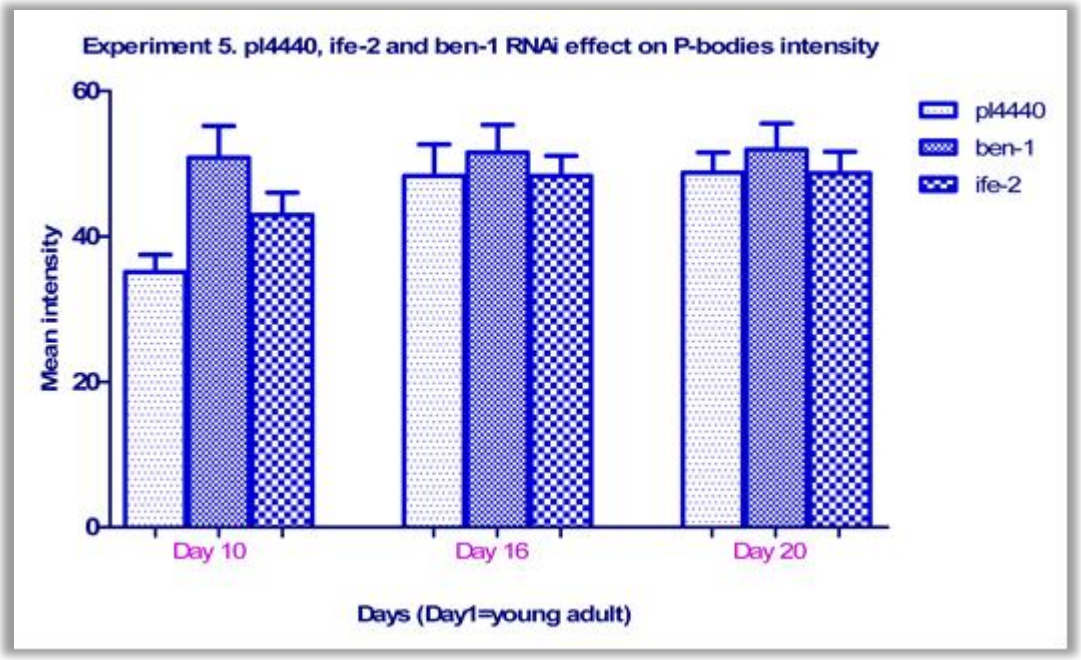


Fig. 9: Comparison of the P-bodies' intensities between RNAi on *ben -1* and *ife-2* genes of dcap1::dsred worms

3.2.2. RNAi on the gene *ben-1* in N2 and dcap1::dsred worms. Effect on worm lifespan and P-bodies

Asking what could be the effect on worm lifespan when a beta-tubulin is downregulated, aging experiments on both N2 bristol and dcap1::dsred worms were performed. Interestingly, in the case of the N2 worms, *ben-1* downregulation, showed a trend towards better healthspan (figure 10). This remains to be confirmed with more experiments. In the case of the dcap1::dsred worms, no significant difference was observed (figure 11). The P-bodies' appearance confirmed the observed trend that ageing is accompanied by higher intensities. This seems to be true for both the control and the RNAi affected worms. Furthermore, in all time points, the downregulation of the tubulin makes the intensity weaker, proposing that there is a relationship between normal P-body general distribution and the existence of functional beta tubulins (figure 12, picture 2.).

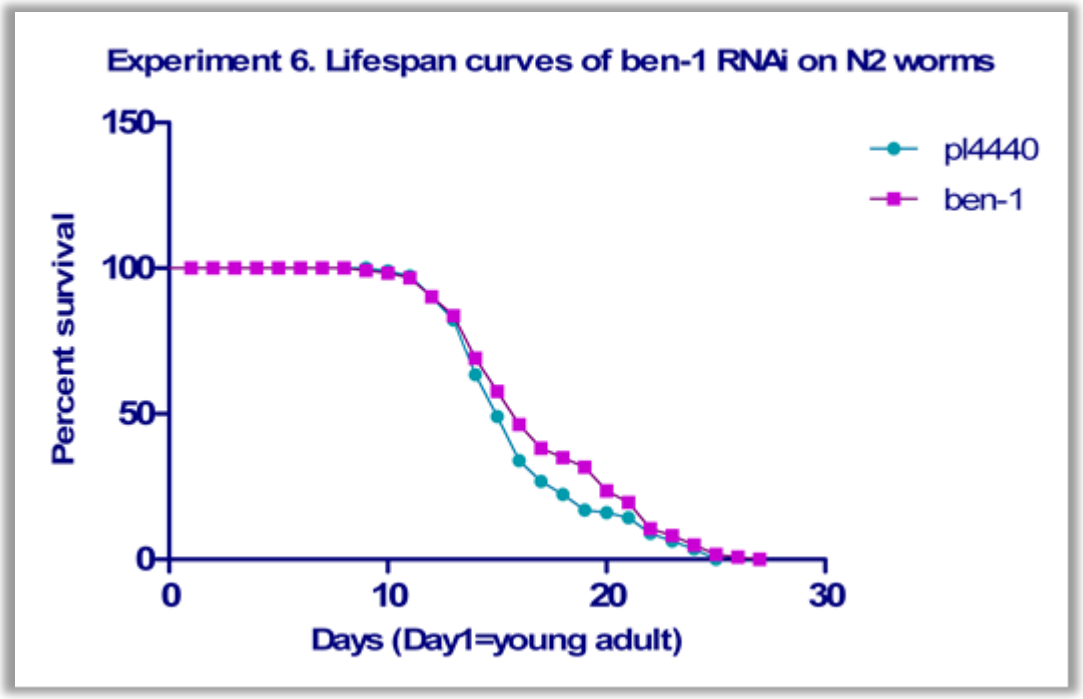


Fig. 10: Lifespan curves of *ben-1* RNAi affected N2 bristol worms. The median survival was 20 days in both control and *ben-1* downregulated worms.

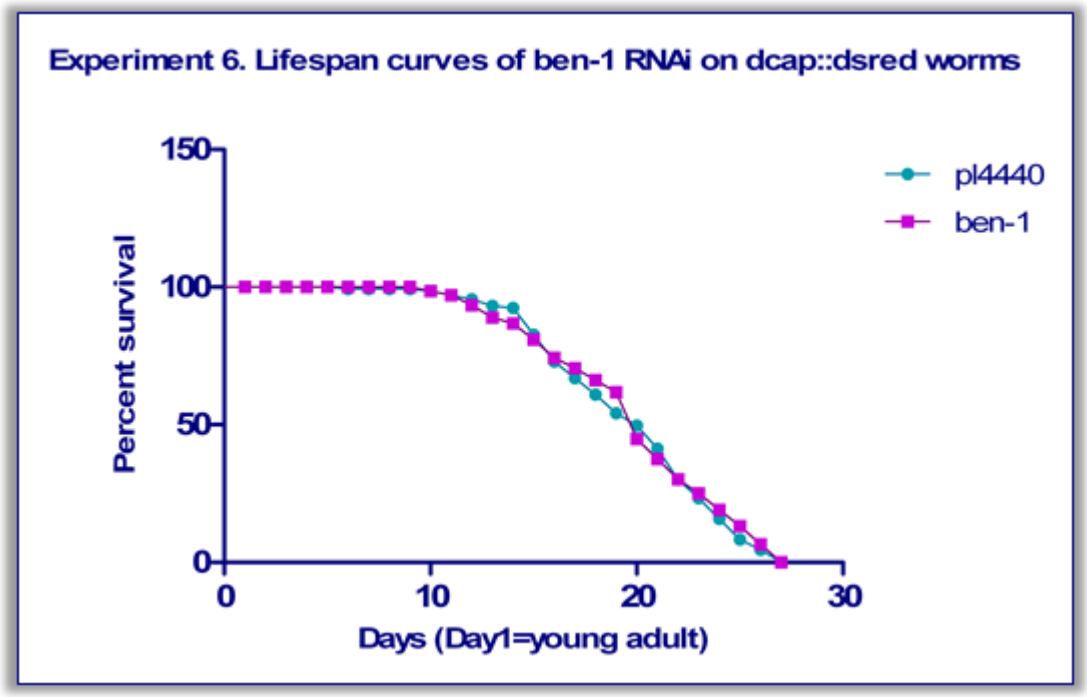


Fig. 11: Lifespan curves of *ben-1* RNAi affected dcap1::dsred line worms. The median survival was 20 days in both control and *ben-1* downregulated worms.

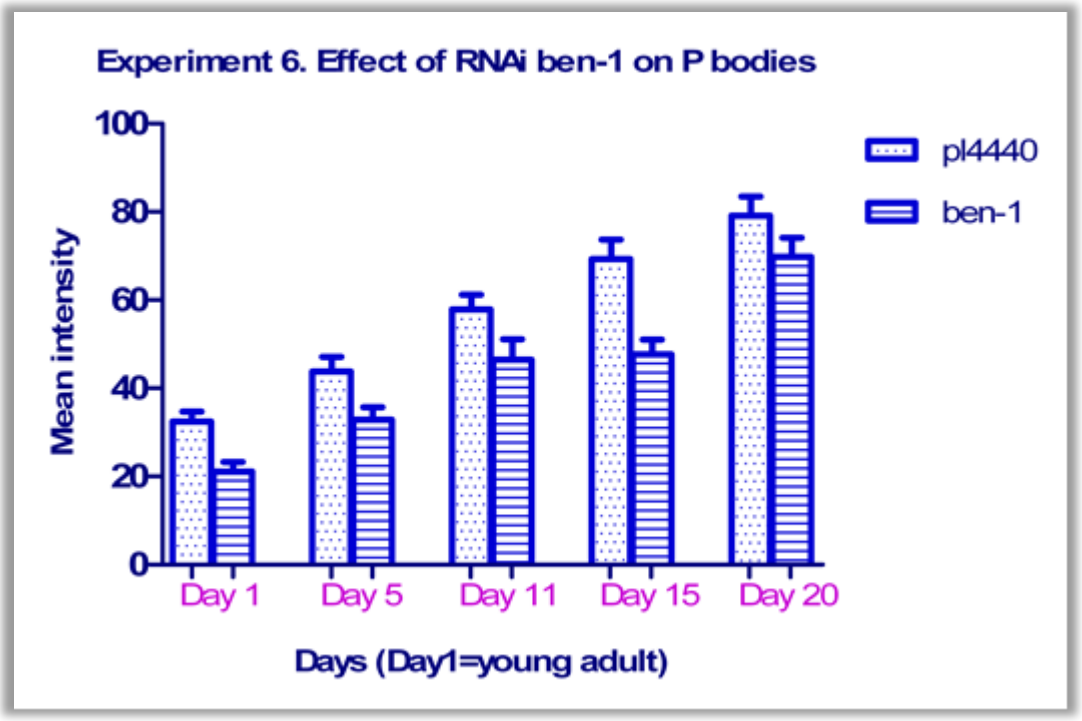
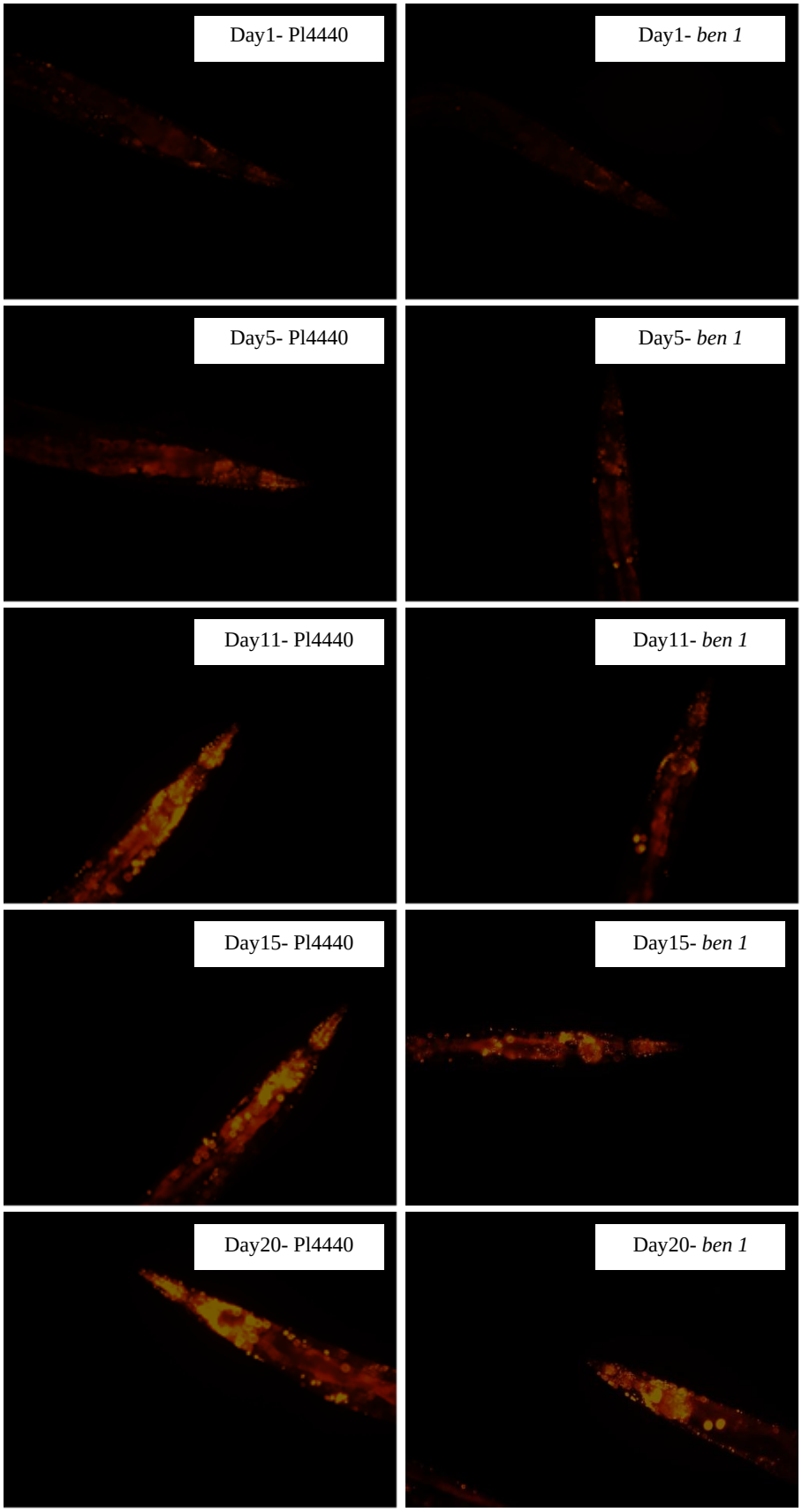


Fig. 12: P-body appearance of worms subjected to RNAi of the *ben-1* gene, in different time points.



Pic. 2: How P-bodies look under an epifluorescence microscope. The images correspond to the dcap1::dsred worms of the experiment 6. The gradually stronger intensity with ageing is observable and also the weaker intensity of *ben-1* downregulated animals in comparison to the control

3.2.3. RNAi on the gene *ben-1*, in N2 and dcap1::dsred worms. Studying the effect on worm lifespan and P-bodies

In this assay, a repetition of the *ben-1* RNAi in N2 and dcap1::dsred worms was perfomed. The lifespan curves' differences were not statistically significant (figures 13, 14). Interesting is however, that in the case of the P-bodies appearance, the effect of the lower intensities in *ben-1* RNAi in comparison to the control that was observed previously, seems to hold true for the first days, however not the later. Further experiments will confirm the existence of a significant trend (figure 15).

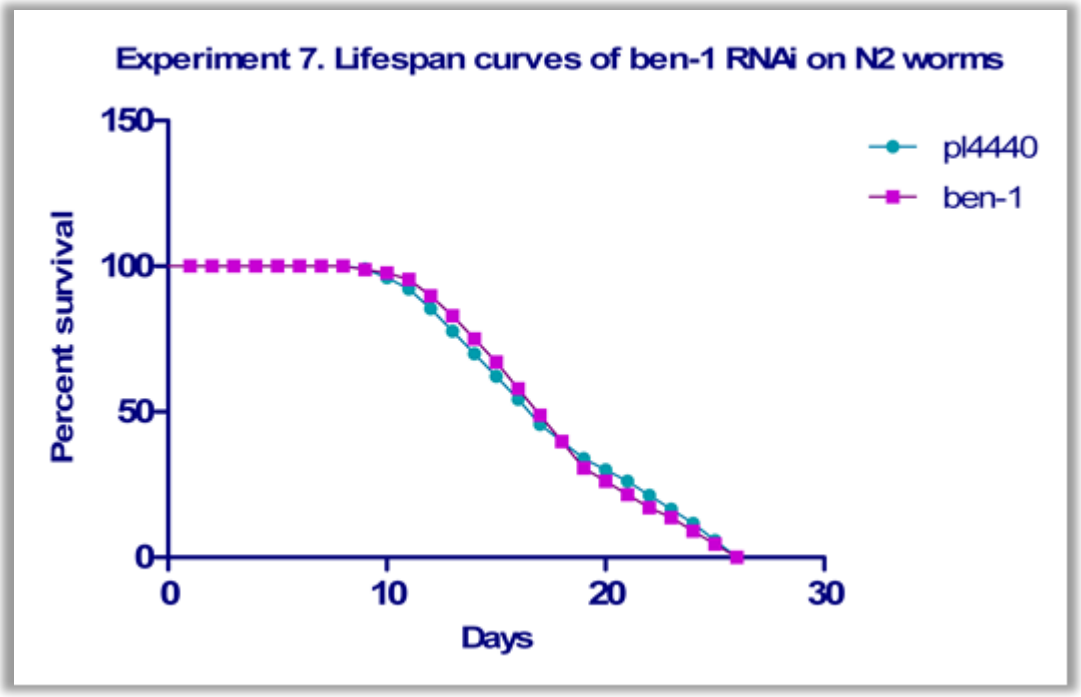


Fig. 13: Lifespan curves of *ben-1* RNAi affected N2 bristol worms. The median survival was 17 days in both control and *ben-1* downregulated worms.

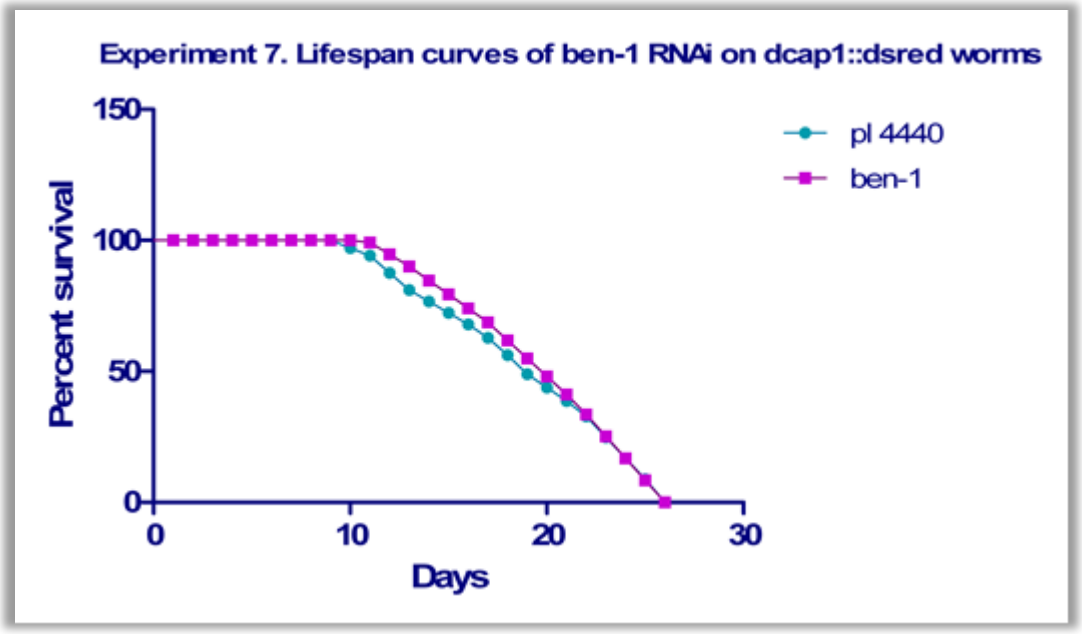


Fig. 14: Lifespan curves of *ben-1* RNAi affected dcap1::dsred line worms. The median survival was 19 and 20 days for the control and *ben-1* downregulated worms respectively.

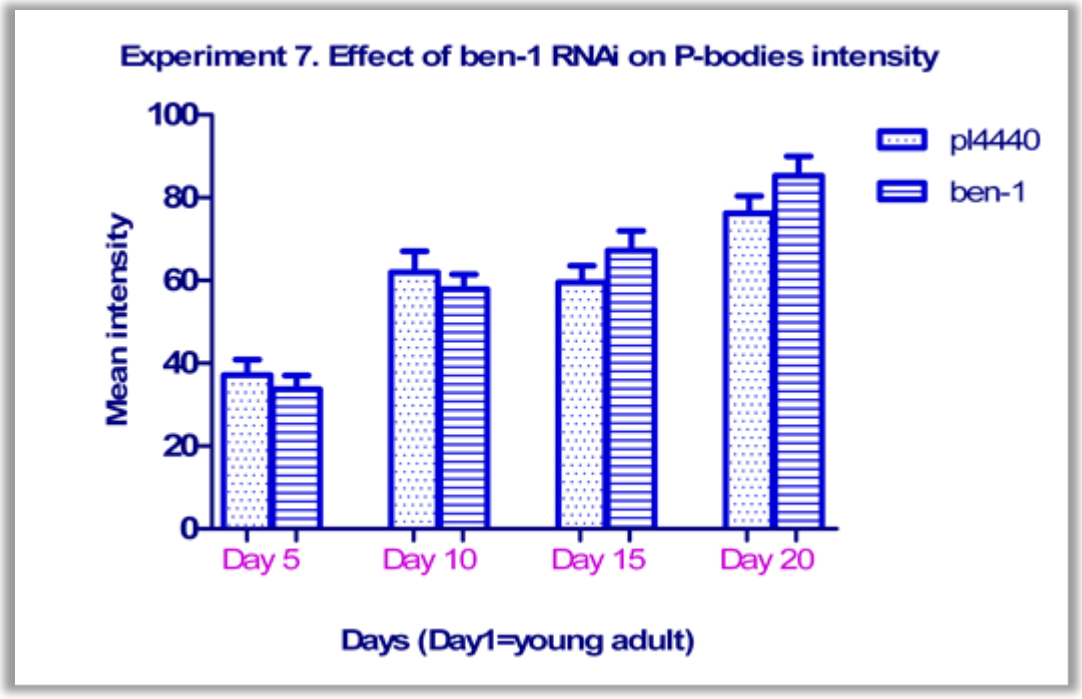


Fig. 15: P-body appearance of worms subjected to RNAi of the *ben-1* gene, in different time points.

4. Discussion

During this work, we investigated the relationship that ageing could have with the function of the cell cytoskeleton and the P-bodies. The developmental stage of the organism, the environmental influences and possible stresses, and the need for a general maintenance of homeostasis are only some of the things that in a specific time-point guide an organism's actions and are able to determine its fate. Establishing a relationship between ageing, cytoskeleton and P-bodies, could lead to a better understanding of the complex and sophisticated network to where these processes belong. This work represents a first approach towards this direction.

Interestingly, we found that ageing, causes P-bodies to appear more intense. This held truth for most of the times, however not always. It is possible, that this relationship lasts for a specific time period or developmental stage in a worm's life, and afterwards either is lost or masked. For example, it is possible that during ageing, the accumulation of cellular damage, triggers an elevated P-body function, in order to deal with the changes in mRNA production and degradation, but maybe there is a threshold for the P-body appearance and as a result, there are no more differences on it when one compares two already old animals, even if they are not of the exactly same age. Further experiments remain in order to confirm this hypothesis.

Additionally, in some cases we noticed that when the beta tubulin *ben-1* is downregulated, the P-body signal appears weaker. The existing literature is contradictory on whether disruption of proper microtubule formation can lead to this or to the exactly opposite, meaning higher intensities. Both results have been observed before, and this is also the case here. As it has been phrased before, it is possible, that when the microtubule network is destroyed, P-bodies are no longer anchored on them and they don't have the option to travel along them in order to incorporate basic components of their machinery and mRNAs, resulting to move mainly with diffusion. In this case, the incorporation of their "material", which is synonymous to their formation, will depend only in the availability of this "material" in the free cytoplasm, this specific period. And this means that in that case, chance

has the leading role. Further experiments could confirm the trend and this hypothesis.

Furthermore, we found that in some cases, two different interventions that disrupt microtubules, the RNAi method on the *ben-1* gene and also a specific concentration of benomyl (0,1 μg/ml) applied to the worms, showed in the lifespan experiments, a trend towards a better healthspan in comparison to the control worms. Taking into account that the *ben-1* RNAi keeps the worms with lower P-body intensities and that according to our results, this is indicative of a younger phenotype, it would be interesting this to hold true also in the animals' fitness. However, these trends have to be confirmed.

Concluding, it is possible, that P-bodies, and microtubules, mediating vital cellular processes could additionally have an impact on ageing. Investigating the link between those, could lead to a better understanding of the mechanisms behind them. Translating these discoveries into medical treatments, could advance the preventative medicine and create novel routes to drug development. Hopefully, many of the unwanted situations related to the ageing process, and ageing-related diseases, could then be solved.

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