Modifiers of extracellular aggregation in *Caenorhabditis elegans.*

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"Once you stop learning you start dying"
-Albert Einstein

-Dedicated to my parents and my sister-

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Summary

Aberrant protein aggregation leads to various age-related disorders. Studies have also shown that several hundred proteins in *Caenorhabditis elegans* aggregate with age. This suggests that there is an age associated change in the protein homeostasis, which causes proteins to misfold and aggregate. Age-dependent misfolding affects both intracellular proteins and extracellular proteins. The various conditions and factors responsible for maintaining protein homeostasis in the intracellular environment are relatively well understood and protein-quality control in the endoplasmic reticulum has been extensively studied. However, not much is known about protein-quality control outside the cell. A few mammalian extracellular proteins i.e. clusterin, haptoglobin and α2-macroglobulin display chaperone activity by stabilizing secreted misfolded and aggregation-prone proteins such as amyloid-β. Therefore, I speculated that there must be some mechanisms that regulate extracellular protein aggregation in *C. elegans*.

In this study, I used *C. elegans* as a model to investigate the age dependent aggregation of the extracellular protein LBP-2. I found out that the rate of extracellular accumulation is significantly slower in the *daf-2* mutants and by suppression of insulin/IGF-1 signalling by RNAi. I also found that higher temperature leads to increased amount of extracellular aggregation in the worms. In this study, I also carried out an RNAi screen to look for factors that can modify extracellular aggregation. I found 14 such potential candidate factors. Additionally, I found that these candidates do not have any significant influence on the lifespan of the organism. But, these candidates do play an important role in the worm's ability to resist heat stress.

Content

Abbreviations

Aβ	β-amyloid
AD	Alzheimer's disease
~	approximately
ALS	amyotrophic lateral sclerosis
AMP kinase	Adenosine monophosphate-activated protein kinase
°C	Degree Celsius
$CaCl_2$	Calcium chloride
C. elegans	*Caenorhabditis elegans*
cm	centimetre
$CuSO_4 * 5 H_2O$	Copper(II) Sulfate Pentahydrate
dsRNA	Double-stranded RNA
daf	Abnormal Dauer Formation
ddH2O	double distilled water
DNA	deoxy-ribose nucleic acid
DR	Dietary restriction
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol tetraacetic acid
Et al	et alii
e.g.	for example (from latin: *exempli gratia*)
FOXO	forkhead box O transcription factor
$FeSO_4 * 7\ H_2O$	Iron(II) Sulfate Heptahydrate
GFP	green fluorescent protein
HD	Huntington's disease
hrs	hours
HSF	Heat shock factor
Hsp	Heat shock proteins
i.e.	that is (from latin *id est*)
IGF-1	Insulin-like growth factor-1
IIS pathway	IGF-1 signaling pathway
IPTG	Isopropyl-beta-D-thiogalactopyranoside
KCl	Potassium Chloride
KH_2PO_4	Monopotassium phosphate
L	Litre
L1, 2, 3, 4	Larval stage 1, 2, 3, 4
LB	Lysogeny broth
LBP	Lipid Binding Protein

LDL	Low-Density Lipoprotein
M	molar
mA	milliAmper
MES	2-(N-morpholino)ethanesulfonic acid
mins	minutes
ml	milliliter (10^{-3} liter)
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium sulphate
$MnCl_2 * 4 H_2O$	Manganese(II) Chloride Tetrahydrate
MOAG	Modifier of protein Aggregation
n	number of animals/ samples uses
NaF	Sodium Fluoride
NaCl	Sodium Chloride
$Na_2HPO_4, 7H_2O$	Sodium phosphate dibasic heptahydrate
NFTs	Neurofibrillary tangles
NGM	Nematode growth medium
%	Percentage
p	probability
PCR	Polymerase chain reaction
PD	Parkinson's disease

PI3K   phosphatidylinositol 3-kinase

PMSF   Phenylmethanesulfonyl fluoride

polyQ   polyglutamine

Proteinase K   Proteinase kinase

PVDF   Polyvinylidene difluoride

RAB   Reassembly

RFP   Red Fluorescent Protein

RIPA   Radioimmunoprecipitation

RNA   Ribonucleic acid

RNAi   RNA interference

rpm   revolution per minute

RT   Room temperature

s   seconds

SID   Systemic RNA Interference Defective

SDO   *S*odium deoxycholate

SDS   Sodium dodecyl sulphate

TBST   Tris-buffered saline and Tween 20

TOR   Target of rapamycin

UPS   Ubiquitin proteasome system

YFP   Yellow fluorescent protein

ZnSO$_4$ * 7 H$_2$O   Zinc Sulfate Heptahydrate

ZnSO$_4$ * 7 H$_2$O   Zinc Sulfate Heptahydrate

Introduction

1.1 Ageing

Ageing is a natural process which is associated with a time dependent functional decline in the organism. This functional decline with ageing is thought to be due to accumulation of cellular damage over time (Gems and Partridge, 2013; Kirkwood, 2005; López-Otín *et al*., 2013; Vijg and Campisi, 2008). However, the process of ageing is very diverse as some organisms live for only a matter of days while other organisms live for years. The mechanism and natural diversity of ageing has fascinated molecular biologists for years. With an increase in the average age of the world population there is an increased interest in the research into the underlying mechanisms of ageing. It is understood that ageing is a complex process mediated by a number of genetic and environmental factors. The modern era of ageing research was kicked off by the isolation of the first long-lived strains in *Caenorhabditis elegans* (*C. elegans*) (Klass, 1983). We now know that ageing is regulated by signalling pathways and transcription factors that have been conserved during evolution. Changing even a single gene from these pathways can lead to a dramatic extension of the life span of the animal and cause the animal to age normally although slower than usual (Kenyon, 2005). With the ever-expanding knowledge of the cellular and molecular bases of ageing, various factors have been shown to have some kind of influence on ageing. In a recent review, nine hallmarks of ageing were highlighted. These were altered intercellular communication, cellular senescence, deregulated nutrient-sensing, epigenetic alterations, genomic instability, loss of proteostasis, mitochondrial dysfunction, and stem cell exhaustion. All these hallmarks are cellular processes that are interconnected (López-Otín *et al*., 2013).
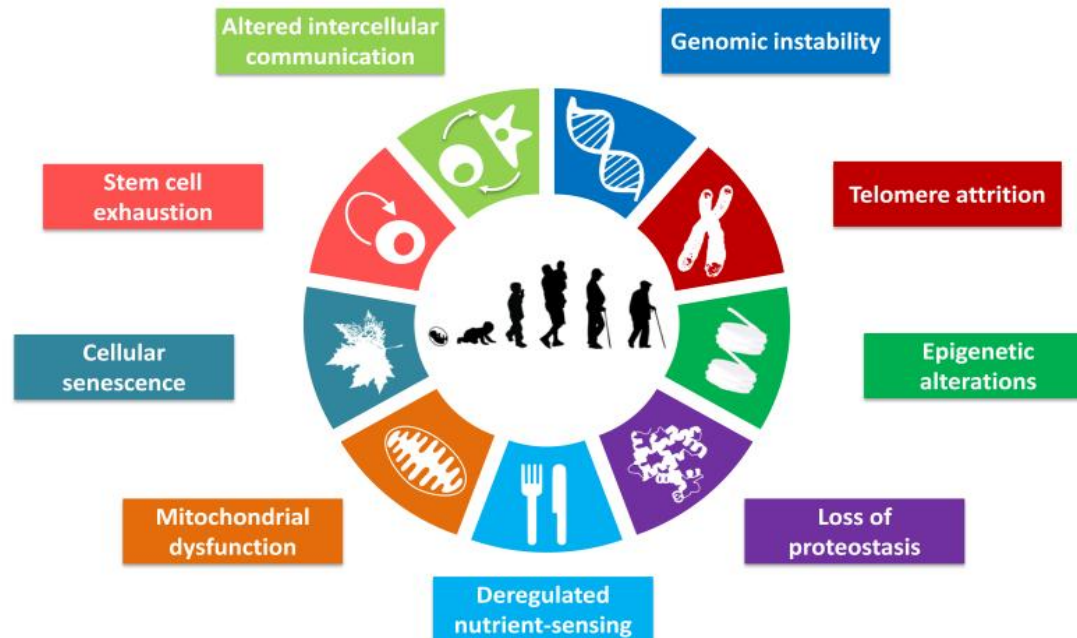
Figure 1: Hallmarks of ageing. The nine cellular and molecular hallmarks of aging contribute to the ageing process in an organism and influence the ageing phenotype. The nine hallmarks are, genomic instability, telomere attrition, Epigenetic alteration, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (source: López-Otín *et al*., 2013).

1.2 Main model organisms used to study ageing

Most of what has been learnt about aging is done using three laboratory model organisms with different lifespans; the nematode worm *C. elegans*, with an average lifespan of 3 weeks, the fruit fly *Drosophila melanogaster* (*D. melanogaster*) with a life span of 3 months and the mouse with an average lifespan of 3 years. One of the main themes that we learnt from these different organisms is the potential commonality of the ageing process in these different organisms (Gems and Partridge, 2013). We have also learnt that contrary to popular assumption that an extension of lifespan by slowing ageing would mean certain death from age related diseases like Alzheimer's disease. However, we are finding that mutations that slow ageing also postpone age-related disease.
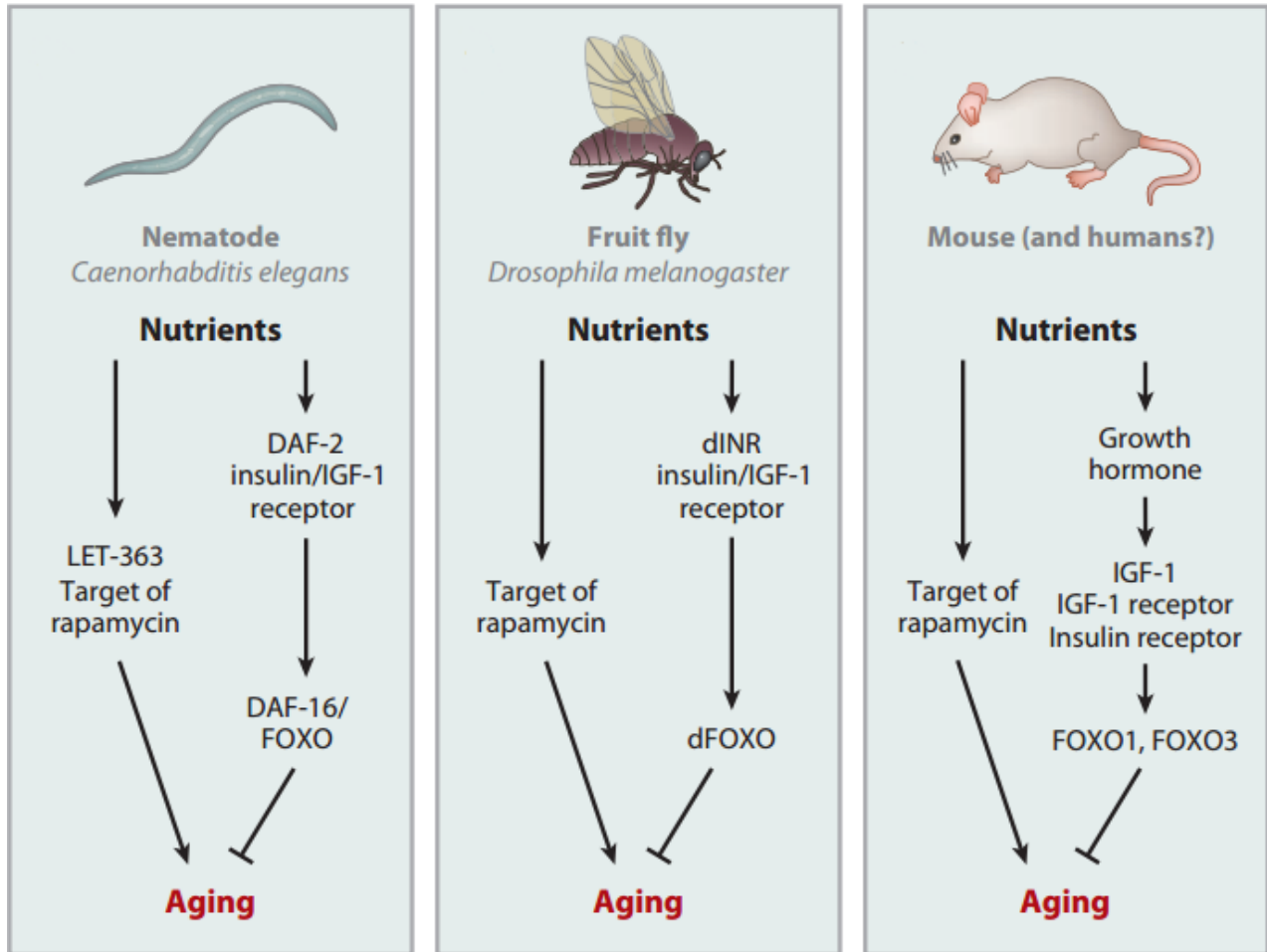
Figure 2: Animal models of ageing. Three most commonly used animal models in ageing research, the nematode *Caenorhabditis elegans*, *Drosophila melanogaster*, Mus musculus. In each of the animal model the nutrient-sensitive signalling network plays an important role in the regulation of the rate of ageing. Modulation of this pathway can slow down aging. Abbreviations: FOXO, forkhead box O transcription factor; IGF-1, insulin-like growth factor 1. (source: Gems and Partridge, 2013).

1.2.1 Caenorhabditis elegans

C. elegans is used in aging research as it has a short lifespan. It is also easy to generate large populations of age synchronized worms. It is easy to culture and maintain *C. elegans* strains as the worms can be kept frozen and then used again after thawing them without damage to the worms. Additionally, various worm strains are available in the worm stock centres. *C. elegans* is a transparent organism which facilitates studies using fluorescent tagged proteins and the worms

can also be used in high-throughput automated experiments. The entire genome of *C. elegans* has been sequenced and there are various molecular and genetic tools which are available. From ageing research using *C. elegans*, *daf-2* and *age-1* have been identified, they are part of the insulin like signalling pathway and they influence the activity of transcription factor *daf-16* (Honda *et al*., 1999; Lin *et al*., 2001; Ogg *et al*., 1997). A number of different factors like high temperature, radiation, oxidative stress have been studied using *C. elegans* to show their effect on ageing. However, *C. elegans* do have some drawbacks as a model organism for ageing research. *C. elegans* are evolutionary distant from humans. In addition, they also lack blood, brain tissue and internal organs. Somatic cells in adult *C. elegans* are post-mitotic and therefore the organism lacks the ability to replace damaged cells.

1.2.2 Drosophila melanogaster

D. melanogaster has a longer lifespan than *C. elegans.* Compared to *C. elegans*, flies have a number of distinct tissues and organs such as eyes, brain, liver and kidney. Since, drosophila shares about 60% of the disease associated genes with humans, it makes flies a highly important model organism. Flies are also easy to handle and have a low cost of maintenance. *D. melanogaster* strains are available at stock centres. Using *D. melanogaster* as a model organism, *mth* gene has been identified to influence ageing and can increase the lifespan of fly by 35%. The *sun-mth* system has also been identified to be in control of ageing of the organism (Cvejic *et al*., 2004). Another fly gene *Indy* has been observed to double the lifespan of the organism without the loss of fertility or physical activity (Knauf *et al*., 2002). However, as *D. melanogaster* cannot be frozen, the maintenance of the transgenic strains is quite labour intensive and costly.

1.2.3 Mus musculus

Mouse is an important model organism used in ageing research as there are approximately 99% of human orthologs in mice (Boguski, 2002). Another advantage of using mouse as a model organism is that a number of genetic engineering technology and genetic resources are available for its genome manipulating (Paigen, 1995). Extensive ageing research has already been carried

out using mouse as a model organism. It has been shown that late age feeding of Rapamycin leads to a lifespan extension. Additionally, effects of caloric restriction, genetic manipulation of Insulin-like growth factor 1, Insulin-like growth factor I receptor, insulin receptor (Bluher *et al.*, 2003; Holzenberger *et al.*, 2003; Li and Ren, 2007; Yuan *et al.*, 2009) and the effects of various other genes on ageing has been studied in mouse. However, one of the major drawbacks of using mouse as a model organism is that its lifespan is about 2-3 years depending on the strain, which makes mouse an expensive tool for ageing research.

1.3 Dietary Restriction

One of the earliest breakthroughs in ageing research was made when it was discovered that rats had extended lifespan if their food intake is reduced to the levels that is just short of malnutrition (McCay *et al.*, 1935). This method of dietary restriction (DR) and its effects on ageing were further reproduced in different organisms like *C. elegans, D. melanogaster* and rhesus monkeys (Colman *et al.*, 2009; Mattison *et al.*, 2012). Additionally, DR was also tested in short-term studies with human volunteers; these studies showed that human volunteers got similar health benefits as observed in non-human primates (Fontana *et al.*, 2008; Meyer *et al.*, 2006).

Studies have shown that the lifespan extension caused by dietary response is regulated by nutrient sensing pathways. In addition, many mutations that cause longevity response also affect stress-response genes. When copious amount of food is available then the stress levels are low, the genes involved promote growth and reproduction. However, when the conditions become hard for survival, then some of these genes become more active while others become less active and hence, the organism experiences a major change in physiological shift to cell protection and cell maintenance (Kenyon, 2010). These changes in the animal, protects it from extensive stress and leads to a lifespan extension. Some of the genes and pathways associated in the lifespan extension involve kinase target of rapamycin (mammalian target of rapamycin) (Hansen *et al.*, 2007; Kaeberlein *et al.*, 2005), AMP kinase (adenosine monophosphate-activated protein kinase)

(Greer *et al*., 2007), sirtuins (Li *et al*., 2008; Rogina and Helfand, 2004), insulin/insulin-like growth factor (IGF-1) signalling (Kenyon, 2005).

1.4 Insulin/IGF-1 pathway

One of the most studied signalling pathways that have been associated with lifespan extension in *C. elegans, D. melanogaster* and mice is insulin/IGF-1 pathway (Slack *et al*., 2011). It was found that reduction in the activity of *daf-2*, which encodes a hormone receptor that is similar to insulin and IGF-1 receptors, leads to double the lifespan of the organism. The DAF-2 receptor activates a conserved phosphatidylinositol 3-kinase (PI3K) signalling pathway which modulates the lifespan of the animal by regulating the nuclear localisation of DAF-16 (Friedman and Johnson, 1988; Hertweck *et al*., 2004; Kenyon, 2005; Lin *et al*., 1997; Ogg and Ruvkun, 1998; Paradis *et al*., 1999; Paradis and Ruvkun, 1998). Other factors such as Nrf-like xenobiotic-response factor (SKN-1) and heat shock transcription factor (HSF-1) also play a role in modulating lifespan in conditions of reduced insulin/IGF-1 signalling (Baumeister *et al*., 2006; Henderson and Johnson, 2001; Lee *et al*., 2001; Lin *et al*., 2001; Kenyon, 2010). These transcriptions factors regulate a diverse set of genes through which they have such a significant effect on the lifespan.

The insulin/IGF-1 pathway was first studied in *C. elegans* (Kenyon *et al*., 1993). It is a widely used organism for ageing (Antebi, 2007; Friedman and Johnson, 1988; Johnson, 2002; Kenyon *et al*., 1993; Kimura *et al*., 1997; Olsen *et al*., 2006) and protein aggregation (Caldwell *et al*., 2003; David *et al*., 2010; Holmberg and Nollen, 2013; Nussbaum-Krammer and Morimoto, 2014). In addition, previous studies with the worms have highlighted a number of genes that are involved in ageing (Lin *et al*., 2001; Lund *et al*., 2002; McElwee *et al*., 2004; Murphy *et al*., 2003; Ogg *et al*., 1997). In numerous studies genome wide RNAi screening has been used in *C. elegans* to discover new genes modulating ageing (Hamilton *et al*., 2005; Poulin *et al*., 2004; Updike and Strome, 2009; Lejeune *et al*., 2012).

Studies have shown that extending lifespan with reduced IIS pathway protects the worms from proteotoxicity caused by aggregation of polyQ peptides associated with Huntington's disease (Morley *et al*., 2002) and also AD linked human Aβ peptide (Cohen *et al*., 2006; Cohen *et al*., 2009). In *C. elegans* Aβ model that expresses human Aβ, they found that reduction of IIS protects from Aβ$_{1-42}$ proteotoxicity without affecting development, reproduction or lifespan. This protection is dependent on HSF-1 and DAF-16. In this process, HSF-1 regulates Aβ disaggregation and DAF-16 facilitates the formation of larger and less toxic Aβ aggregates (Cohen *et al*., 2006). They also found that reduced IGF-1 signalling protects mice from AD associated proteotoxicity. The reduced IGF-1 signalling directed the assembly of Aβ into more densely formed packs forming larger fibrillar structures later in life (Cohen *et al*., 2009). Previous studies have shown that highly aggregated Aβ has lower toxicity than the small oligomers (Haass and Selkoe, 2007; Shankar *et al*., 2008) and in AD murine model high fibrilization can reduce the Aβ toxicity (Cheng *et al*., 2007).

1.5 Protein misfolding and intracellular proteostasis

Proteins are complex biological macromolecules that perform a vast array of biological functions in a living organism. They are synthesized by ribosomes as chains of several hundred of amino acids, and undergo structural changes and modifications to become biologically active. Through highly complex folding reactions, a protein chain can undertake a number of possible conformations. Most of the proteins in an organism fold into three-dimensional structures. It is important for the biological function of the protein for it to fold properly. Abnormally folded proteins are devoid of normal biological activity which often results in loss of critical function. These misfolded proteins have the tendency to aggregate and are potentially harmful for the cell. In a number of protein aggregation associated disorders, certain polypeptides assemble in amyloid-like fibrils which are insoluble. Amyloids are fibrillar aggregates in which β-sheets from misfolded proteins provide hydrogen bonding partners to other β-sheet rich proteins and which allows polypeptides to pack tightly in fibrous protein like structures (Douglas and Dillin,

2010; Nelson *et al*., 2005). β-sheet structures in amyloids are parallel to the fibril axis (Eisenberg and Jucker, 2012). A number of neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's are caused by such protein misfolding prone proteins. The pathogenesis of these different neurodegenerative diseases share many common features. For these diseases, the disease protein that defines the disorder can vary in functionality between different diseases; however, the pathogenesis might occur due to a common mechanism which is the formation of amyloid like structures (Carrell and Lomas, 1997; Douglas and Dillin, 2010).

In the organism, there are many measures that control protein synthesis, protein folding, trafficking and degradation through a process called protein homeostasis or proteostasis. Proteostasis can influence the function of cells, the development and the ageing of the animal, and how well can they adapt to environmental challenges and diseases. The cellular proteostasis has to be adaptable to the regular developmental changes, new proteins and age dependent accumulation of misfolded proteins. Recent experiments have also shown that some cells can increase their capability of proteostasis maintenance when they sense new misfolding-prone proteins (Balch *et al*., 2008; Gidalevitz *et al*., 2006). With age there is a decline in this ability of proteostasis maintenance and an increase of aggregated proteins which leads to a number of age dependent diseases. Age related disruption of protein quality control have been studied in different organisms such a *D. melanogaster*, where age associated protein aggregation was observed in different tissues (Demontis and Perrimon, 2010), in *C. elegans,* where two groups, in two independent studies showed an increase in the insolubility of hundreds of proteins due to the age dependent vulnerability of protein quality control (David *et al*., 2010; Reis-Rodrigues *et al*., 2012) and *Rattus norvegicus*, where the levels of protein hydrophobicity was increased in ageing rats (Dasuri *et al*., 2010).

Another way that the proteostasis can be disrupted in organism is by different types of stress. *hsf-1* gene is critical for the transcriptional activation of *hsp* genes which are associated with thermotolerance in the organism. In addition, it has also been shown that *hsf-1* gene is also essential for the survival of *Saccharomyces cerevisiae* even in the absence of stress (Gallo *et al*., 1993; Imazu and Sakurai, 2005). In *C. elegans*, down-regulation of HSF-1 by RNAi reduced the life span of the worms by 30-40% and it also led to the formation of protein aggregates associated with diseases like Huntington and Alzheimer's (Hsu *et al*., 2003; Morley and Morimoto, 2004) and the worms with additional copies of the *hsf-1* gene are resistant to hyperthermia and oxidative stress and these worms also live longer than wild-types (Anckar and Sistonen, 2011; Morley and Morimoto, 2004). In rats HSF1 activity declines with age, as the stress-induced DNA-binding function of HSF1 is significantly reduced in the older rats (21-16 months old) when compared to other adult rats (5-6 months old) (Fawcett *et al*., 1994; Locke and Tanguay, 1996) even when the amount of HSF1 has not changed in the young adult rats compared to the aged ones (Anckar and Sistonen, 2011). Interestingly, *hsf-1* has been shown to be a part of the IIS/IGF-1 pathway. Recent study has also suggested that hsf-1 is required for longevity caused by down-regulation of components in TOR signalling pathways and that HSF-1 is an essential longevity factor that intersects both IIS and TOR signalling pathways (Seo *et al*., 2013).

Osmotic stress has also been shown to be associated with aberrant folding of proteins. A genome-wide (RNAi) screen in *C. elegans* to identify the genes which are required for survival in hypertonic environments has highlighted 40 genes that when silenced reduce survival of the worms in in hypertonic condition. 20 of these 40 genes have been show to encode proteins associated with detection, transportation and degradation of damaged proteins (Burkewitz *et al*., 2011). In the same study they also observed that hypertonic stress leads to aggregation of aggregation-prone fluorescent reporter protein, Q35::YFP as well as widespread protein aggregation normally associated with ageing in the worms and the knockdown of the hypertonic stress associated genes increased aggregation (Burkewitz *et al*., 2011).

As the aggregation proteins have the tendency to become toxic or damage the protein homeostasis, the cells have methods to degrade aggregation-prone misfolded proteins. Autophagy functions as a degradative pathways and targets proteins associated with neurodegeneration, such as mutant α-synuclein (Parkinson's disease), tau (Alzheimer's disease), mutant huntingtin (that causes Huntington's disease) (Ravikumar *et al*., 2010). Autophagy has been extensively studied in many organisms. Interestingly, in *Saccharomyces cerevisiae* autophagy mutants were shown to be short lived (Matecic *et al*., 2010; Rubinsztein *et al*., 2011). In *D. melanogaster*, reduced expression of *atg-1*, *atg-8* and *sestrin-1* also reduces the lifespan of the fly (Lee *et al*., 2010; Simonsen *et al*., 2008). In *C. elegans*, loss of function mutation of autophagy associated genes like *atg-1*, *atg-7* and *bec-1* resulted in a decreased lifespan (Toth *et al*., 2008). In dietary restriction *eat-2* mutant, a loss of longevity was observed after the worms were exposed to *bec-1* and *atg7* RNAi (Jia and Levine, 2007). In mice, it has been observed that central nervous system specific knockout of Atg5 and Atg7 results in accumulation of ubiquitinylated proteins as inclusion bodies in neurons and cerebellar neuronal loss (Komatsu *et al*., 2006; Hara *et al*., 2006; Rubinsztein *et al*., 2011). Knockout of HDAC6, which controls autophagosome maturation, causes intracellular ubiquitin aggregates in the brain from as early as 6 months (Lee *et al*., 2010), Dynein mutations causes premature aggregate formation of mutant huntingtin and also impaired autophagosome-lysosome fusion (Ravikumar *et al*., 2010; Rubinsztein *et al*., 2011).

In addition to the autophagy system, protein degradation in the cells is regulated by the ubiquitin proteasome system (UPS). The UPS regulates the degradation of various proteins that are involved in signal transduction (Lehner and Cresswell, 1996), cell cycle progression (King *et al*., 1996), gene expression (Palombella *et al*., 1994) and apoptotic pathways (Dubiel *et al*., 1995). The UPS is also responsible for degradation of abnormal and misfolded proteins (Coux *et al*., 1996; Hochstrasser, 1995; Stolzing and Grune, 2001) and the regulated degradation of short-lived proteins. An age-related decrease in proteasome activity has been observed in different tissues (Dahlmann, 2007; Gray *et al*., 2003; Löw, 2011). This decreased proteasome function is primarily due to the reduced rates of proteasome biosynthesis and assembly (Chondrogianni *et al*., 2003). In rats, an age depended decreased proteasome activity was observed in the

hippocampus and cerebral cortex (Keller *et al*., 2000). A dysfunction of the UPS has also been reported in different neurodegenerative disorders such as Parkinson's disease (Furukawa *et al*., 2002; McNaught *et al*., 2001; McNaught *et al*., 2003), Huntington's disease (Bennett *et al*., 2007; Wong *et al*., 2008), Amyotrophic Lateral Sclerosis (Cheroni *et al*., 2009) and Alzheimer's disease (Tseng *et al*., 2008; Lam *et al*., 2000). Recent studies have shown that IGF-I stimulates proteasome activity by the IGF-I receptor, PI3- kinase/mTOR signalling (Crowe *et al*., 2009; Fernandez *et al*., 2007; Löw, 2011).

Another method by which an organism maintains intracellular proteostasis is by chaperones. Chaperones are proteins that interact with other proteins to help them acquire their functionally active conformation (Hartl *et al*., 2011). They have a wide range of functions such as assisting in protein folding (Langer *et al.*, 1992), refolding of misfolded proteins (Voisine *et al.*, 2010) and degradation of misfolded proteins (Kaushik and Cuervo, 2012). Chaperone has been shown to decline in function with age in rats (Nardai *et al*., 2002). A possibility for this decline could be an excessive burden caused by the accumulation of silent mutations in the human genome (Csermely, 2001). In ageing organism, cells with an overload of misfolded proteins the amount of chaperones and misfolded proteins are no longer rescued by the chaperone system (Gidalevitz *et al*., 2006; Soti and Csermely, 2003). This is further supported by studies which shows that mild heat shock leads to increased stress-tolerance and extension of lifespan (Rattan, 2004; Soti and Csermely, 2003), suggesting that an increase in heat shock response and chaperone overexpression aids with maintaining age dependent protein quality control. In addition, studies have also shown that HSF-1 is important for IIS/IGF-1 signalling pathway dependent extension of lifespan (Hsu *et al*., 2003; Morley and Morimoto, 2004).

Specific chaperones such as yeast Hsp104 is a hexameric AAA+ ATPase have been shown to specialise in protein disaggregation and remodelling (Wendler *et al*., 2007). Hsp104 is activated in stressful conditions like heat stress or oxidative stress (Bosl *et al*., 2006). Hsp104 is functionally linked to HSP70 and in response to stress Hsp104 and Hsp70 collaborate to resolve aggregates and enable protein refolding or degradation (Glover and Lindquist, 1998). In the yeast

Aβ42 model (fusion of human amyloid-β (Aβ42) peptide with the C-terminal domain of Sup35), interestingly, deletion of HSP104 decreases the amount of Aβ42 oligomers and Hsp104 is also co-immunoprecipitated with Aβ42 (Bagriantsev and Liebman, 2006). Study showed the lentiviral co-expression of Hsp104 and α-synuclein in rat's *substantia nigra* resulted in reduction of α-synuclein aggregation and additionally, it also protected against α-synuclein-induced loss of dopaminergic neurons in a concentration dependent manner (Bianco *et al.*, 2008; Grimminger-Marquardt and Lashuel, 2010).

The cellular environment is stressed by various factors that can disrupt the homeostasis. Therefore, it is essential for the cell to have programs to protect it against such stresses. The cell responds to stress in various ways ranging from activation of pathways which would promote its survival to programmed cell death that would eliminate the damaged cells. One of the possible stresses that cells undergo is oxidative stress. In cells, oxidative stress is caused by high intracellular levels of reactive oxygen species (ROS) which can cause damage to lipids, proteins and DNA. Cellular ROS is produced by two organelles: the endoplasmic reticulum (ER) and mitochondria (Dröge, 2002). To protect against oxidative stress mediated damage, the cell upregulates the antioxidant proteins, such as glutathione reductase and glutathione peroxidase (Dröge, 2002; Muralidharan and Mandrekar, 2013). Proteins undergo translational modifications and folding in the ER. Stress such as hypoxia, nutrient deprivation can disturb the folding process and cause misfolded proteins in the ER. This causes the activation of unfolded protein response (UPR). UPR is associated with an increased expression of BiP. In the mammalian cells, UPR is regulated by three ER-localized transmembrane proteins which can initiate the transcription of UPR target genes (Chakrabarti *et al.*, 2011; Diehl *et al.*, 2011; Muralidharan and Mandrekar, 2013).

Another cellular stress response is through the mitochondrial UPR. It is a stress response which activates the transcription of nuclear-encoded mitochondrial chaperone genes to promote protein homeostasis within the organelle. Mitochondrial UPR has also been shown to expand the folding capacity of mitochondria during times of stress to maintain proteins and preventing deleterious protein aggregation. (Haynes and Ron, 2010). In *C. elegans* mitochondrial UPR is activated by knockdown of mitochondrial chaperones (Yoneda *et al.*, 2004). It was also observed that knockdown or deletion of UPR components localized in the mitochondrial matrix, cytosol and nucleus alters mitochondrial morphology, reduces mitochondrial function and shortens lifespan of the worm (Benedetti *et al.*, 2006; Haynes and Ron, 2010; Haynes *et al.*, 2007; Haynes *et al.*, 2010). An ATP-dependent protease ClpXP is required for the induction of mitochondrial chaperone genes in response to stress (Haynes *et al.*, 2007). ClpXP is localized within the matrix, where it functions as a potential sensor for degradation of unfolded proteins (Haynes *et al.*, 2010).

1.6 Extracellular regulators of protein homeostasis

Although some human protein aggregation disorders have been associated with extracellular protein deposits, the majority of chaperone research has been focused on intracellular chaperones and how they are released from the cell to interact with extracellular protein deposits. Though intracellular chaperones have been found in the extracellular space, their concentration is low and the different extracellular environment (the low concentration of ATP) suggests that there exists a different system of chaperone working in the extracellular environment. Until now only a few possible systems of extracellular proteostasis has been explored and hence only a few potential extracellular proteins which might have a chaperone like function have been identified. Most of the extracellular chaperones function by keeping misfolded aggregation prone proteins soluble by assisting with their stability and by facilitating their efficient uptake by receptors. The extracellular chaperones known so far do not refold the misfolded proteins, but future studies might reveal some new ones that do.

1.6.1 Clusterin

Clusterin or apolipoprotein J is an extracellular chaperone encoded by the CLU gene in humans and is ubiquitously expressed during developmental and in adults. The CLU gene is highly conserved across mammalian species. Clusterin exhibits high sequence homology and extensive biological distribution, which suggests that it has a fundamentally important function (Jenne and Tschopp, 1992). Intracellular clusterin has been shown to modulate apoptosis (Trougakos *et al.*, 2009), DNA repair (Trougakos and Gonos, 2009) and protease inhibition (Jeong *et al.*, 2012). In the extracellular space, secreted clusterin is known to exhibit, small heat shock proteins like functionality as it inhibits stress induced amorphous aggregation by binding to the exposed hydrophobic regions on partially unfolded intermediates in an ATP-independent manner (Wyatt *et al.*, 2009). For chaperone function, clusterin sequesters its associated proteins into soluble higher molecular weight complexes (Wyatt *et al.*, 2009).

In addition, clusterin also inhibits the fibrillar aggregation of a large number of amyloid forming proteins like Aβ (Oda *et al.*, 1995), α-synuclein and calcitonin by binding to prefibrillar aggregates (Yerbury *et al.*, 2007). In the extracellular environment, clusterin mediates the recognition and disposal of damaged and misfolded proteins via receptor-mediated endocytosis and lysosomal degradation (Wyatt *et al.*, 2011). The cellular uptake of clusterin is mediated via interaction with Low-Density Lipoprotein (*LDL*) receptor, megalin (Kounnas *et al.*, 1995). Clusterin Aβ complex's uptake is also via the same receptor (Hammad *et al.*, 1997). Clusterin has been identified as a significant genetic determinant in Alzheimer's disease (Lambert and Amouyel, 2011), with two genome wide studies having recently implicated certain single nucleotide polymorphisms in the clusterin gene as risk factors for Alzheimer's disease (Harold *et al.*, 2009; Lambert *et al.*, 2009). It has been suggested that at a very low concentration, clusterin stabilizes prefibrillar oligomers that seed fibril growth and are thought to be primarily responsible for amyloid-associated cytotoxicity (Kumita *et al.*, 2007; Yerbury *et al.*, 2007; Dabbs *et al.*, 2013).

With increase of oxidative and proteotoxic stress with age, clusterin has been shown to have increased expression in cellular senescence and during normal ageing of various tissues (Trougakos and Gonos, 2009; Sala *et al*., 2009). Clusterin has been associated with various age-related diseases like cardiovascular diseases, degenerative diseases, cancer and inflammation (Trougakos and Gonos, 2009; Zoubeidi and Gleave, 2012).

1.6.2 α₂-Macroglobulin

α ₂-Macroglobulin or α₂M is a large secreted glycoprotein that is synthesized mainly in the liver. α₂M is secreted from various types of cells and is found in human cerebrospinal fluid and plasma. α₂M is known as a broad spectrum protease inhibitor. α₂M contains a bait region which undergoes proteolysis upon encountering a protease. This results in a conformational change. That change produces a more compact active conformation and inhibits the protease by physically trapping it within a steric cage (Sottrup-Jensen, 1989). α₂M is not just a protease inhibitor, it performs other protective functions as well. Human α₂M binds to and promotes clearance of various endogenous and exogenous molecules.

It has been shown, that reduction of α₂M from human plasma renders proteins in the fluid more susceptible to aggregation and precipitation, even at 37°C (French *et al*., 2008). To suppress the stress-induced aggregation and precipitation of misfolded proteins, α₂M forms stable complexes with misfolded proteins by binding to their exposed hydrophobic domains (French *et al*., 2008). However, it is unable to promote their refolding (French *et al*., 2008). α₂M has been shown to inhibit amyloid fibril formation. α₂M is found co-localized with misfolded protein deposits in many diseases, such as Aβ peptide associated with Alzheimer's disease (Mettenburg *et al*., 2002), β2 microglobulin which forms insoluble deposits in dialysis related amyloidosis (Motomiya *et al*., 2003), and prion protein associated with plaques in Creutzfeldt–Jakob disease (Adler and Kryukov, 2007). α₂M binds to Aβ to form complexes and facilitate its clearance from the extracellular environment via LRP mediated endocytosis (Qiu *et al*., 1999, Narita *et al*., 1997). Several studies have reported a polymorphism in α₂M as a genetic risk factor for

Alzheimer's disease (Flachsbart *et al.*, 2010; Saunders *et al.*, 2003). However, other studies have failed to show this association (Wang *et al.*, 2001; Bruno *et al.*, 2010).

1.6.3 Apolipoprotein E

Apolipoprotein E (ApoE) is a secreted glycoprotein that is synthesized primarily by the liver, however it has also been found expressed by astrocytes, microglia, and oligodendrocytes in the brain. ApoE functions as a ligand in receptor-mediated endocytosis of lipoprotein particles. There are three known isoforms of ApoE: E2, E3 and E4. These isoforms differ only by single amino acid variations. ApoE is known to be associated with transport and clearance of cholesterol and other lipids (Dabbs *et al.*, 2013). ApoE regulates lipid transport by binding to the LDL receptor.

ApoE has the ability to bind to aggregation prone polypeptides, such as tau and Aβ (Strittmatter *et al.*, 1994). In humans, ApoE has been found co-localized with Alzheimer's and CJD plaques (Namba *et al.*, 1991). ApoE ε4/ ε4 homozygotic individuals have a significantly greater risk of developing Alzheimer's disease (Strittmatter *et al.*, 1993). Inheritance of the apoE ε4 allele was found to be the strongest known risk factor for AD besides age, with one copy increasing AD risk 3–5-fold and two copies over 10-fold (Strittmatter and Roses, 1995). On the other hand, the ε2 allele of ApoE is associated with lower risk of AD (Corder *et al.*, 1994; Farrer *et al.*, 1997). In human studies, it has been shown that ApoE ε4 allele dosage is associated with increased neuritic plaques in AD (Tiraboschi *et al.*, 2004). In a large scale study, they examined the CSF of normal middle ages people, they found out that the CSF Aβ42 was ApoE ε4-dose dependently lower (Sunderland *et al.*, 2004) and since the people with brain amyloid deposits have lower levels of CSF Aβ42 (Fagan *et al.*, 2006), this suggest that amyloid deposition begins earlier in subject with higher levels of ApoE ε4 (Kim *et al.*, 2009). This finding has now been confirmed more directly in a study that showed cognitively normal subjects had an *APOE* ε4 dose-dependent increase in fibrillar Aβ burden in brain as detected with an amyloid imaging agent (Reiman *et al.*,

2009). While different studies suggest a potential extracellular chaperone function by ApoE, further research needs to be conducted to ascertain its proper function

The different factors that regulate extracellular proteostasis have only been identified recently. The available evidence suggests that these chaperones have an important functional role to play, whether it is in stabilizing misfolded proteins or the disposal of aggregating proteins from the extracellular space. We lack the proper understanding of the diseases caused by extracellular proteins aggregation. Therefore, it becomes essential to understand the elements and the mechanism by which the organism maintains extracellular proteostasis.

1.6.4 Extracellular proteolysis

One of the well-known extracellular proteolysis enzymes is plasmin. Plasmin is an important enzyme that is found in the blood and it degrades various blood plasma proteins like fibronectin, thrombospodin and fibrin, which are associated with clot formation and reduction of angiogenesis. From these proteins, plasmin's role in dissolving the fibrin and fibronectin blood clots is well established. Plasmin is released as an inactive precursor of an enzyme called plasminogen. The process for the prevention of growth of blood clot starts with the conversion of plasminogen to serine protease plasmin by physiological activators urokinase-type plasminogen activator or tissue-type plasminogen activator (Castellino and Ploplis, 2005). In a study it was shown that this proteolytic system had a relation with Aβ accumulation. The tissue plasminogen activator-plasmin system had a significant effect on the Aβ degradation *in vivo*. The upregulation of plasminogen activator inhibitor and the inhibition of tissue plasminogen activator-plasmin system led to elevated levels of Aβ peptide in the mouse brain (Melchor *et al*., 2003).

Material and Methods

2.1. *C. elegans* maintenance

C. elegans is a transparent organism. It can be observed using a dissecting stereomicroscope with a transmitted light source. I used stereomicroscope Leica S8 APO with maximum magnification at 80x to visualise the worms.

To avoid any contamination, all work with *C. elegans* or preparation of plates and media was carried using ethanol to disinfect the surface and the material. In addition, an ethanol-lamp or Bunsen burner (Flame 100, WLD-TEC) was also used to prevent the contamination from the air.

2.1.1 Bacterial food source preparation

One of the critical factors to culture *C.elegans* (*Caenorhabditis* elegans) in the controlled laboratory condition is food. In laboratory, *C. elegans* is grown monoxenically with the *E. coli* (*Escherichia coli*) strain OP50 (Brenner, 1974). OP50, which is a uracil auxotroph, has a limited growth on the normal growth plates. This limit in the growth of OP50 is important as it makes it easier to observe the worms during the experiments. OP50 from the frozen stock was streaked on LB (Lysogeny broth) agar plate and kept overnight at 37°C in the incubator. The following day, a single colony from the streak plate was picked, LB was inoculated and was allowed to grow overnight at 37°C. 100µL OP50 per plate was poured on the NG (nematode growth) plates using Multipette Stream (Eppendorf, Germany). OP50 was poured in the middle of the plate avoiding the edges. The worms spend most of their time in or around the bacteria, so if the bacteria are around the edges then the worms are more likely to crawl up the side of the plates, dry out and die (Stiernagle, 2006). The OP50 culture was stored at 4°C for future usage.

After incubation overnight at room temperature, the plates were ready to use. Stored in an airtight container, the seeded plates stay usable for 1-2 weeks.

2.1.2 Nematode growth medium plates (NGM)

To maintain worm strains in the laboratory, nematode growth plates are required. For my experiments 60mm petri dish plates (Falcon, USA) were used. To make the nematode growth medium for the plates mix the substances from (table 4). After adding the aforementioned substances, a stirring bar was added to the flask as well. This mixture was autoclaved for 25 minutes at 121°C, followed by cooling it down to 80°C in the autoclave. The media was further cooled down to 55°C while being continuously stirred. Add the chemicals from (table 5) to the media.

Note: Cholesterol should be at the room temperature when it is added to the media.

For the experiments involving RNAi, antibiotics were added to the plates. For such plates, the temperature of the medium was reduced down to 50°C and then I added the antibiotic Carbenicillin to a final concentration of 5mg/ml and 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside). The medium was stirred for 20 minutes at 50°C. Then I poured 10 ml of the media per plate using the plate pourer. The plates were left overnight at room temperature and stored at 4°C until further usage.

2.1.3 *C. elegans* maintenance

For the general upkeep of the different *C. elegans* strains, the worms must be periodically transferred to new plates. The worms on any stock plate multiply very quickly and exhaust the available food. Hence, it is important to make sure the worm stock is not starved. The stocks of each strain were transferred to new seeded NGM-plates every week. Generally, the worm stocks were maintained at 15°C. However, the worms may be moved to 20°C or even a higher temperature depending on the requirements of the experiment. The worms were maintained on a normal growth plate with 100µl of OP50 (*E. coli*) provided as food. The worms were moved to new normal growth plates after a week or when the OP50 available on

the plate was about to be finished; whichever occurred first. To transfer the worms to new plates, either of the two methods was used, i.e., picking and chunking.

2.1.4 Picking

This method is used to transfer single or many (up to 25) worms to a new plate. To construct a picker, the tip of a Pasteur pipet was removed and a 2 cm long piece of platinum wire was melted in its place. One end of this wire was then twisted into a loop like shape. To transfer the worms, the loop of the picker was flamed and after cooling it down. It was loaded with a streak of bacteria from the OP50 lawn. The picker was then used to pick the worm as by touching the worm with the picker, the worm was stuck to the OP50 and the worm was then picked up and placed on a new plate.

2.1.5 Chunking

This method was used to transfer a large number of homozygous worms from one stock plate to a new plate. First a spatula was sterilized by being dipped in 99% ethanol and being flamed. After the spatula has cooled down, chunk of agar containing the worms was cut out of old plate using the spatula and the chunk was transferred to a new plate. The worms from the chunk then crawled to the fresh bacterial lawn on the new plate.

For integrated strains the worms from the older plates were chunked onto new plates. While for the non-integrated strains, ~20 positive worms were picked from the old plate and transferred to the fresh new plate. I kept each stock on multiple plates at one time, in case of contamination on a plate. All transferring was carried out in semi-sterile conditions, next to a flame, and in a room where temperature and humidity were maintained (Temp ~ 21°C, Humidity ~ 60%).
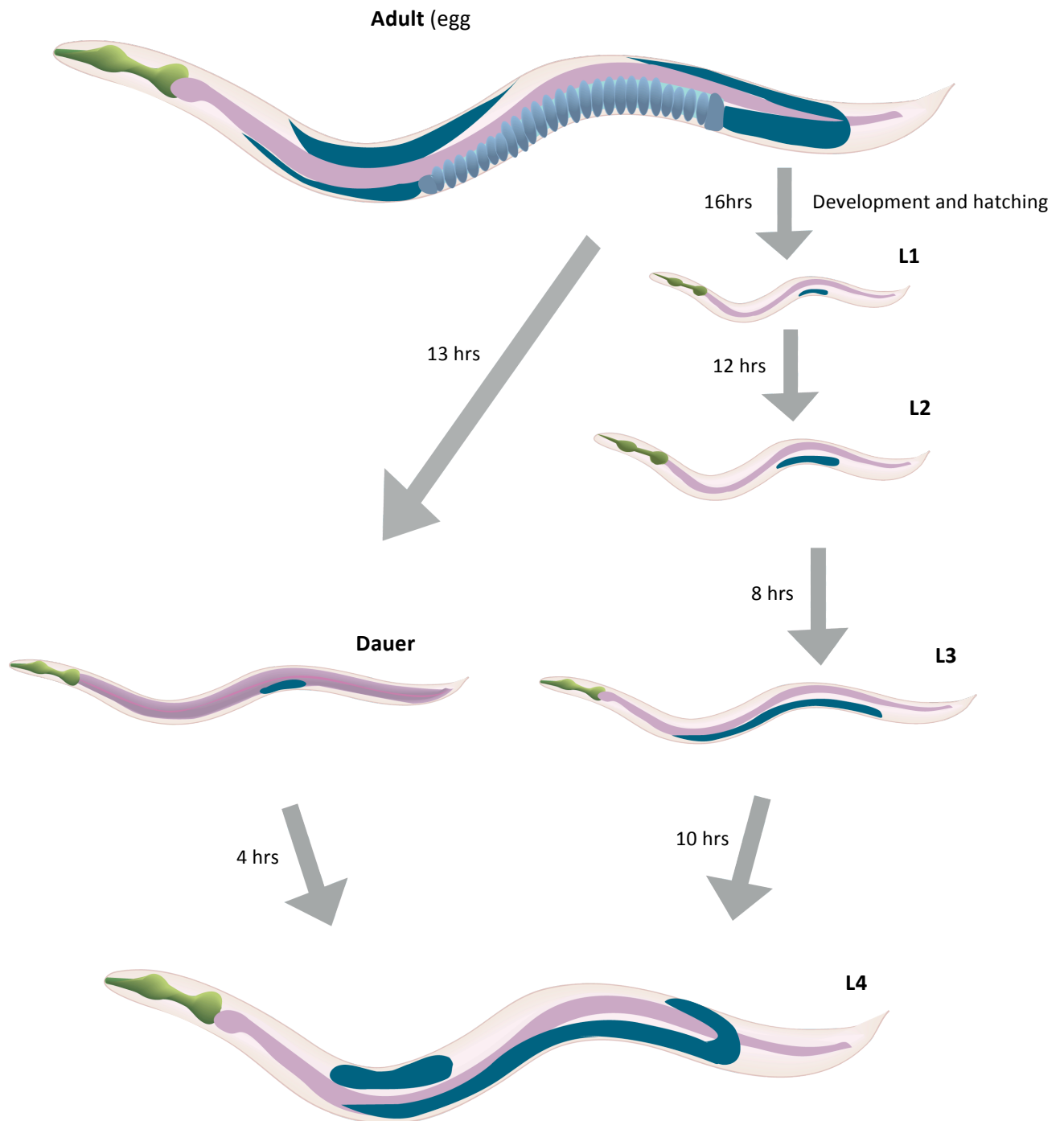
Figure 3: Life cycle of *C. elegans* at 22°C. The adult worm has the ability to lay eggs. The eggs after hatching produce the worms at L1 stage or first larval stage. Under normal conditions the worm grows to the second larval stage, L2. After which the worm matures to L3 or third larval stage and then the L4 or fourth larval stage. If there is lack of food or high temperature, then the worm undergoes L1 arrest and moves to dauer stage at which the wor-m can stay for months before going to the L4 stage (Altun and Hall, 2015).

Table 1: Growth rate of *C. elegans* at different temperature. *C. elegans* grow at different rates at different temperatures (Source: *Caenorhabditis elegans*: Modern Biological Analysis of an Organism).

Temperature °C	Egg Hatching (hours)	Larval Stages (Hours)				Egg laying (First eggs laid after hatch) (hours)
		L1-L2	L2-L3	L3-L4	L4-Adult	
16	29	24	39	54.5	74.5	94-97
20	18	15	24	34	46	59-60
25	14	11.5	18.5	26	35.5	45-46

2.1.6 Freezing and recovery of *C. elegans* stocks

For long-term storage, *C. elegans* were frozen and stored both at -80°C and in liquid nitrogen. Later on, these worms can be thawed and used as per requirement. L1 larval stage worms were used for the storage because the older worms do not survive the freezing. To freeze the worms of a particular strain, I collected the worms from several plates where all OP50 has been finished and the majority of the worms are at the L1 stage. For non-integrated strains, I would pick at least 20 positive animals per plate onto five small nematode growth plates and wait until all OP50 was finished and no more eggs were left on the plates. Plates were incubated at 20°C. To the newly starved plates, 1ml of the freezing buffer was added to wash the worms off. Following which, worms from each plate were pipetted separately into five labelled Nunc Cryovials tubes (neoLab). Then, the tubes are placed on ice for 30 minutes and later, placed in a styropore foam tube holder at -80°C for 24 hours. The next day, one nunc cryovials tube was thawed at room temperature (one extra tube is made to check if the freezing worked) and poured onto two separate seeded NGM plates. After 2-3 days at 20°C the survival rate was scored. Once, it has been confirmed that the freezing worked. The nunc cryovials tubes are transferred to their storage location the next day.

2.2 Generating male worms

To perform crossing in *C. elegans*, male worms are required. However, in wild type hermaphrodite population, the frequency of males is very low (~0.2%). For my study, I generated the N2 (wild-type) males by heat shock. In this method, I heat shocked hermaphrodites at young-adult stage of their lifespan. For which I kept them at 35ºC for 2-3 hours. Afterwards I moved them back to 15ºC. From the progeny of these worms I picked the male worms and then maintained them by regularly crossing them with the N2 hermaphrodites at larval stage, L4.

2.3 Crossing transgenic worms into a mutant background

I crossed different worm strains to get worms with the required mutation and worms expressing a visible phenotypic marker of interest. To cross worms, I would first put 12-15 healthy wild type males on a non-seeded plate and 2-3 L4 hermaphrodites, of the desired transgenic strain with the fluorescent marker were added. It is important to make sure that only the worms at early L4 stage are picked. Then, some OP50 was added to the middle of the plate as it helps the males find the hermaphrodites; a blob of OP50 was also added to the middle of the plate. These plates were incubated at 20°C. After a few days, the F1-generation was heterozygote for the desired transgenes. If the worm crossing worked, then half of the F1 progeny should be males. If this is not the case, then the cross did not work and the process might be repeated again. With the help of the Leica fluorescent dissection scope (Leica Microsystems, Germany), I would pick 12-15 fluorescent young males and put them on a new non-seeded plate. It is preferable to set-up two plates. To the plates, 3 L4 mutants and little amount of OP50 was added, followed by incubation at 20°C. After few days, 12-15 fluorescent young male progeny were picked and placed on a new non-seeded plate. 2 L4 mutants were added, a little amount of OP50 and this step was repeated 3-4 times, for some crosses like the sterile cross where the mutation could not be detected by PCR. It is repeated only if the mutation could not be detected by PCR. Then, 5 early L4 worms, which were

positive for fluorescence, were picked from each crossing plates and put on separate seeded plates. These plates are incubated at 20°C. It is important to keep a track of crossing plates that the L4 were picked from. The goal was to obtain two strains with the mutation from different crosses. On separate seeded plates, 10 virgin F2s (second generation) are picked and allowed to produce progeny. For integrated strains, worms with 100% transmission of fluorescent marker are picked. Approximately, 15-20 F3 worms were picked from each F2 plate into 20µl lysis buffer. Presence of the mutation in the screened worms was evaluated by PCR.

To maintain the male stocks, 12 males and 2 hermaphrodites at L4 stage from the same strain were mated.

2.4 Worm genotyping

To check the worm for the presence of a transgene, the worms first had to be lysed to access their DNA. In a cap of a 1.5ml tube (Eppendorf, Germany) 20µl of worm lysis buffer was pipetted along with 20 worm that were collected in it. The tube was centrifuged shortly using the Ministar Silverline (VWR) and it was frozen for 15 minutes at -80°C. 1µl of Proteinase K was added to the tube and it was incubated for 1 hour at 60°C followed by 15 minutes at 95°C. The lysed worms were stored at 4°C. 1µl of the lysed worms was used in the worm genotyping PCR.

2.5 Polymerase chain reaction

To specifically amplify DNA-sections polymerase chain reaction (PCR) was used. By performing two succeeding PCR (nested PCR) the presence of transgene in the worm can be confirmed. In nested PCR the ingredient-composition and PCR-program were almost similar. The template for the first PCR (External PCR) was the worm lysate (see worm genotyping). The product of the external PCR was used as a template for the second PCR (internal PCR). External primers were used in external PCR and internal primers were used in internal PCR. All PCR was carried out in C1000 touch thermal cycler (BioRad).

2.6 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA-fragments as per their sizes. It also allows for extraction and purification of certain fragments from the gel.

For the agarose gel, 1% agarose in the TAE-buffer was heated until the agarose was completely dissolved in it. The solution was then cooled to approximately 40°C and 3µl of Ethidiumbromide (10mg/ml) was added to it and the gel was poured (PeqLab Biotechnology GmBH). The samples were mixed with a 6x Gel loading Blue dye (New England Biolabs). The size of the DNA fragments was determined by using a 2-log DNA-ladder (New England Biolabs). Gel electrophoresis was performed with 90V and DNA was detected using an UV-transilluminator (Gel Doc[tm] XR+, BioRad).

2.7 Injections to generate transgenic *C. elegans*

To make a transgenic worm containing extrachromosomal arrays of the transgene of interest, microinjection is a very effective method. For injecting the worms, Zeiss inverted microscope (Carl Zeiss AG, Germany) connected to Eppendorf FemtoJet (Eppendorf, Germany) was used. The DNA construct of interest from miniprep and also a co-injection marker was prepared. The DNA concentration was carefully kept between 10ng/µl and 100ng/µl. For the injection, the DNA was diluted to the desired final concentration with ddH$_2$O making a final volume of 10µl. In a 0.5ml Eppendorf tube, the DNA solution was centrifuged for 5 minutes at 4400rpm. This step serves to remove any insoluble material that might be present and any contaminant that might interfere with the injection. 5µl of the supernatant was removed into a new Eppendorf tube and kept on ice. A needle puller was used to prepare needles from glass capillary tubes. After the needles were prepared, they are placed in a box with wet kimwipes (Kimberly-Clark Professional, USA) and 0.3µl of the DNA sample was loaded per needle. Young adults with only a few eggs were picked onto on an agar plate without any bacteria. The needle was inserted into the holder and tube was attached to the Eppendorf pump. In a vertical line, some halocarbon oil was added to a coverslip without an agar pad. Under the flame, the capillary was stretched and the tip was broken into the oil. This step was to obtain a straight glass edge and to break the tip of the needle. Next, we used the lowest

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magnification of the microscope and focused on the stretched capillary. The needle was brought into the same plane and close to the stretched capillary. While keeping both in focus, we sequentially changed to a higher magnification and brought the needle closer without touching. The pump pressure was applied in clean mode to test whether the needle was still intact, if yes, then a very small tap was applied to break the tip. This can be further verified by pressing the right mouse button and making sure that liquid came out of the tip (not too much or too little). The worms were then placed on a 2% agarose pad previously dried and a small drop of halocarbon oil was added and using this halocarbon oil, a worm was picked in a sweeping motion, without any dabbing. The worm was pressed into the oil on the pad. It was made sure that the worm was immobilized. The worm was then quickly placed under the microscope with the eggs and vulva placed away from the needle. The focus of the microscope was changed to the needle by increasing magnification. The needle was brought into the upper gonad arms (where the granulated germ line is present). It is optimal to focus on the liquid inside of the gonad arm. Both the arms were injected for ~10sec with pc = 160 hPa and pi =1200 (up to 1600). For the recovery of the worm, additional M9 was added to the pad so the worm could swim in it. A drop of M9 was added to a new agar plate with OP50 and a hooked stretched capillary was used to rescue the worm. 2-3 injected worms were added per plate and the plates were numbered to keep track of different clones. The injected worms were kept overnight at 15°C and then moved to 20°C the next day. At the F2 generation, about 20 positive worms were picked onto separate plates. Worms from different injection plates were picked. The F2s from different injection plates were considered as different clones. The worms were then checked for transmission and expression pattern. Different transmission rates should be aimed for; keeping clones with transmission rate between 20% and 80%.

2.8 UV integration of transgenes

UV irradiation was used for the integration of extrachromosomal DNA array. For the integration, approximately 100 or so late L4s or young adult worms were picked on a plate without bacteria. Then, I washed these worms twice with 1ml of M9 solution and centrifuged it in a mini-centrifuge. During M9 aspiration it was made sure that all the worms were at the bottom of the tube. Then, I used a Pasteur pipette, to transfer the worms to a plate without bacteria and to dry off the M9 solution. This plate was placed in the centre of the crosslinker, while making sure that the lid was closed. Before using the crosslinker, the settings are adjusted to 275µL. After the worms had been exposed to the UV radiation inside the crosslinker, the worms were transferred to a plate with bacteria and incubated overnight at 15°C. The next day, which was marked as day 1, the adults were separated to new plates with 10 irradiated worms per plate and incubated the plate at 20°C. The following day, I transferred the adults to new plates, this day was marked as day 2 and the progeny from day 1 were kept at 20°C. I repeated this process and transferred the adults to new plates with bacteria and marked it as day 3; the progeny from day 2 was kept at 20°C. The worms were left to starve and I picked about 100 L1 worms from each day, making it around 300 L1s in total. These L1s were then transferred to individual miniplates with one worm per plate and they were allowed to grow and produce progeny. The progeny was checked for transmission. The aim was to pick out progenies with 100% transmission.

Table 2: Worm strain used. The table contains the list if all the worm strain used during the study, their genotype and the strain type.

Strain Name	Strain	Genotype
N2B	Wild Type	
DCD1	N2B; uqEx1[*plbp-2::lbp-2::tagrfp*]	Extrachromosomal array containing *lbp-2::tagrfp*
DCD22	N2B; uqIs4[*plbp-2::lbp-2::tagrfp*]	*lbp-2::tagrfp* integrated in the genome
DCD23	N2B; uqIs5[plbp-2::lbp-2::tagrfp]	*lbp-2::tagrfp* integrated in the genome
DCD40	Y37E3.4 & Y37E3.5(gk513)I; uqIs5[*plbp-2::lbp-2::tagrfp*]	*moag-4(-)* mutant with integrated *lbp-2::tagrfp*
TJ1060	*spe-9(hc88)I; fer-15(b26)II*	Temperature sensitive sterile double mutant
DH26	*fer-15(b26)II*	Temperature sensitive defective sperm
BA671	*spe-9(hc88)I*	Temperature sensitive defective gamete adhesion and recognition
DCD80	*fer-15(b26)II; uqIs5[plbp-2::lbp-2::tagrfp]*	Temperature sensitive sterile mutant with integrated *lbp-2::tagrfp*
CF512	*fer-15(b26)II; fem-1(hc17)IV*	Temperature sensitive defective sperm and sperm substitution
DCD81	*fer-15(b26)II; uqIs5[plbp-2::lbp-2::tagrfp]*	Temperature sensitive sterile mutant with integrated *lbp-2::tagrfp*

Strain Name	Strain	Genotype
DCD82	*fer-15(b26)II; uqIs5[plbp-2::lbp-2::tagrfp]*	Temperature sensitive sterile mutant with integrated *lbp-2::tagrfp*
DCD130	*fer15(b26)II; fem-1(hc17)IV;uqIs[plbp2::lbp-2::tagrfp]*	Temperature sensitive sterile double mutant with integrated *lbp-2::tagrfp*
DCD132	*fer15(b26)II; fem-1(hc17)IV;uqIs[plbp2::lbp-2::tagrfp]*	Temperature sensitive sterile double mutant with integrated *lbp-2::tagrfp*
DCD135	*fer15(b26)II; fem-1(hc17)IV;uqIs[plbp2::lbp-2::tagrfp]*	Temperature sensitive sterile double mutant with integrated *lbp-2::tagrfp*
NL3321	*sid-1(pk3321)V*	Systemic RNAi mutant
DCD132	*fer15(b26)II; fem-1(hc17)IV;uqIs[plbp2::lbp-2::tagrfp]*	Temperature sensitive sterile double mutant with integrated *lbp-2::tagrfp*
DCD120	*sid1(pk3321); uqIs5[plbp-2::lpb-2::tagrfp]*	Systemic RNAi mutant with integrated *lbp-2::tagrfp*
DCD39	*daf-2(e1370); uqIs4[plbp-2::lbp-2::tagrfp]*	*daf-2* mutant with integrated *lbp-2::tagrfp*
DCD43	*daf-2(e1370); uqIs5[plbp-2::lbp-2::tagrfp]*	*daf-2* mutant with integrated *lbp-2::tagrfp*

2.9 Evaluation of aggregation

To evaluate the amount of misfolded proteins accumulated in the worm over time. I developed a scoring system, where I counted the amount of dot-like structures or puncta in the worm (Fig. 5).I decided to look for the accumulation only in the head region of the worm as it is relatively easier to look for puncta in the head region which has less autofluorescence with age and restricting the evaluation to a particular region makes the method less ambiguous and faster. For evaluation, I manually counted the puncta as per different criteria, i.e., worms with no puncta in their head, worms with anywhere between 1 and 10 puncta and worms with more than 10 puncta. Later on in my work I changed the evaluation system and included the worms with any amount between 10 to 20 puncta and further category with more than 20 puncta. This was included to have a better characterization of the aggregation in the worms. For these counts, I had two sets of controls. The first set was the negative control, which should have no effect on the amount of accumulation in the worms as these worms were fed the empty feeding vector L4440 (pPD129.36) (Timmons *et al.*, 2001) which has been transformed into an *E. coli* HT115 strain. The second set of controls used during the evaluation was the positive control, where the worms were fed a bacteria expressing dsRNA (double stranded RNA) (Timmons and Fire, 1998; Timmons *et al.*, 2001). The targets were selected on the basis of literature and multiple rounds of testing that I undertook to evaluate their effectiveness, as shown in the results. Positive controls were selected that led to a significant increase in the amount of puncta in the head region of the worm. The evaluation was done in a blind fashion to the nature and the experiment. For post-blind categorization of the worms the type of bacterial strain fed to the worms was looked into. After all the worms were characterized as per the different conditions and the different number of puncta in their head, the numbers of worms per category were calculated.
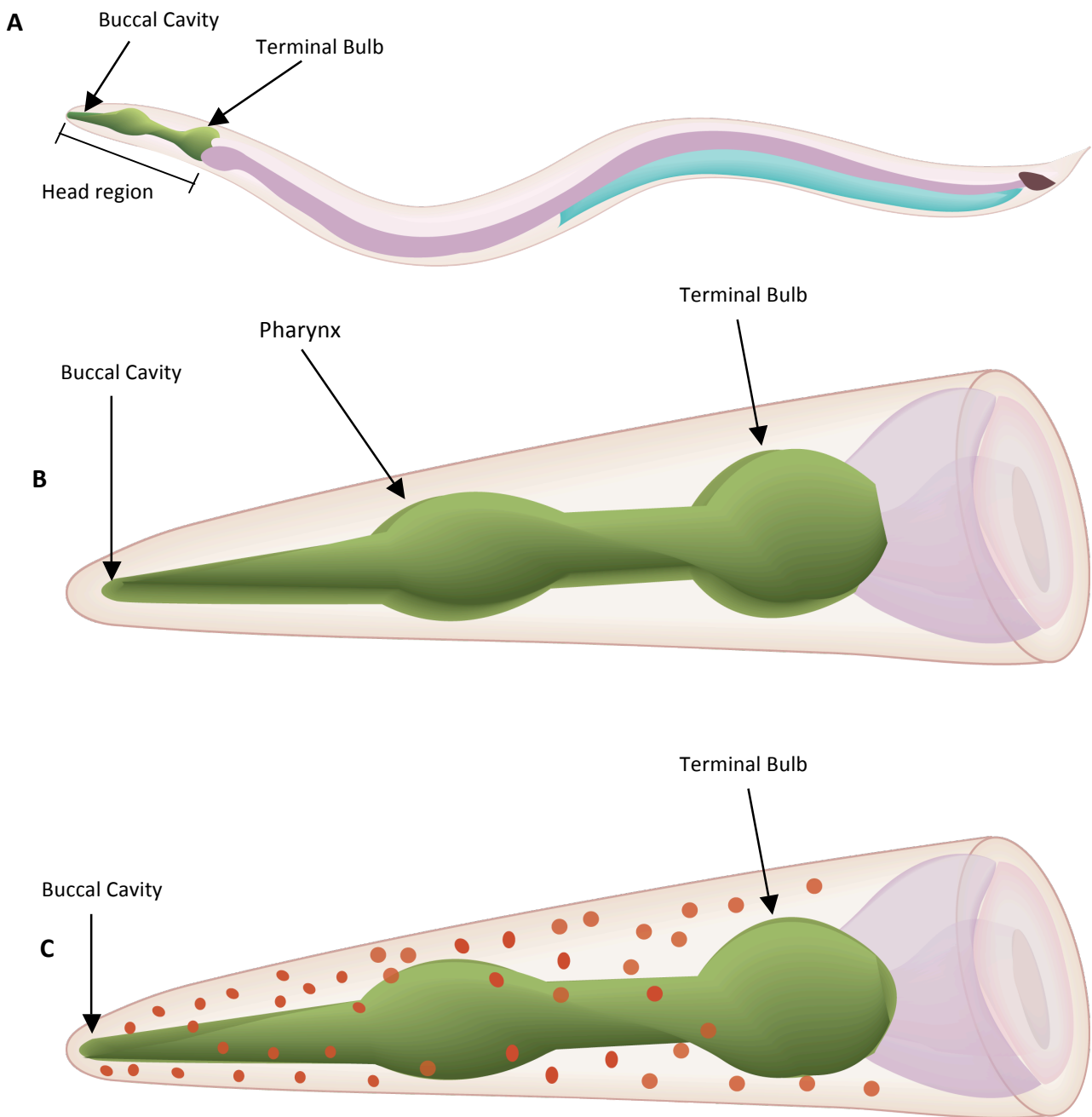
Figure 4: Extracellular accumulation in *C elegans*. A) *C. elegans* with the marked head region. **B)** The head region of *C. elegans* without any extracellular accumulation. **C)** The head region of *C. elegans* with puncta (each red dot represent a puncta).

Table 3: Scoring system to evaluate the extracellular accumulation. To evaluate the rate of extracellular accumulation, the scoring system was used. Worms were counted in different category as per the number of puncta in their head region.

Category Name	The amount of puncta
0	No puncta in the head region. Only diffused pattern of expression of LBP-2::tagRFP.
0 – 10	The amount of puncta in the head region is between 1 puncta to 10 puncta, including 10 puncta.
11 – 20	The amount of puncta in the head region is between 11 puncta to 20 puncta, including 20 puncta.
> 20	The amount of puncta in the head region is more than 20 puncta.

2.10 Bioinformatics analysis for collection of screen candidates

For the RNAi screen, only the genes with transmembrane or signalp domains were required. To get a list of such genes, wormbase database was searched and a list of all such genes was recovered. Following which this list was run against the TMHMM prediction database to verify which genes contain a transmembrane domain. For this I wrote a PHP script and used the scrapping library. On inspecting http://www.cbs.dtu.dk/services/TMHMM/ it was observed it is based on a simple submission protocols to the server http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi. There were two methods using which data could be submitted to the server. In the first method, the sequence is stores in a text file and the file is submitted and the second method it is a direct text submission. With my algorithm I used the text sequence submission option. The code collected each sequence from the file and individually ran it against the server. After I submit data to the server, it responded back with a JavaScript code to wait till the time sequence ran on the server. Once the sequence was processed server responded back with the results. The script keep sending pings to the server until it responds with result and each ping will be of at least in 1 minute interval. Once the server responded, the algorithm stored the results in another file for further analysis.

2.11 RNAi Screen:

2.11.1 High growth plates

High growth plates were required to grow a large number of worms for the purpose of the screen. To make the high growth plates, the chemicals from (table. 6) were mixed in glass flask and a stirring bar was added to the flask. Following which I autoclaved it for 25 minutes at 121°C, and let it cool down to 80°C in autoclave. After the autoclave, the media was cooled down to about 55°C while being stirred. Then, once the media was cooled to about 50°C, and the chemicals from (table. 7) were added.

Note: Cholesterol should be at the room temperature when it is added to the media.

Carbenicillin was added to the media at 5mg/ml. The medium was stirred for 20 minutess at 50°C. 75ml of the media was manually poured per 10cm wide plate.

For seeding high growth plates 0.5-1ml of OP50-1 liquid culture was used and it was spread over the whole plate using a spatula.

2.11.2 Preparation of RNAi

C. elegans has a systemic RNAi response. *E. coli* expressing target – gene dsRNA can be used quite effectively for inactivation of this gene in the whole animal. For smaller experiments, I first needed to check if the *E. coli* clone containing the plasmid of interest was correct. On a medium sized LB plate with carb, the bacterial clone of interest from either one of the RNAi libraries (Ahringer or Vidal) was streaked and was grown overnight at 37°C. A single colony was picked and used to inoculate 5ml of LB with carb. After growing it overnight at 37°C, 3ml of inoculate was used for miniprep and the rest was stored at 4°C. Following the miniprep, I sent it for sequence verification using the M13 forward primer. Then, I froze 0.75ml bacteria suspension with 0.75ml 30% glycerol solution in cryotubes for storage at -80°C. For an experiment, 5ml LB with Carb (50μg/ml) were inoculated with bacteria from the glycerol stock and grown overnight at 37°C. The next day, 5μl 1M IPTG

were added to the culture and it was grown at 37°C until the following day. Approximately 100µl was added to NG plates with Carb and left for two days before usage.

2.11.3 Bleaching of worms

To collect large number of synchronized population of worms which were decontaminated from bacteria or fungi, bleaching was used. As incubation with the bleach for a short time dissolves hermaphrodites and leaves eggs intact, these can be collected afterwards.

First, worms are grown on the medium High growth or NGM plates. Once there were enough number of worms or the food was finished. The bleach solution was made fresh each time before the start of the experiment. The worms were washed with about 5ml of M9 and 0.01% triton per medium plate and the worms were collected into a 15ml falcon tube. The wash was repeated again using 5ml M9 with 0.01% triton to ensure a high yield. Worms were centrifuged in a table-top centrifuge (Centrifuge 5702, Eppendorf) for 30 seconds at 4000rpm and the supernatant was aspirated off (KNFlab Laboport). Then the falcon tube was filled up to 14ml with sterile M9 to wash off some of the bacteria. The tubes were centrifuges again at 4000rpm for 30 seconds. Followed by the addition of 5ml of bleach solution, the tube was inverted twice. The worms were quickly centrifuged in a table-top centrifuge for 30 seconds at 4400rpm. The bleach solution was aspirated off from the falcon tubes and 10ml fresh of bleach solution was added. The worms were shaken and vortex (Vortex-genie 2, Scientific Industries) vigorously for under 4 minutes so as to disrupt all the adult worms in the bleach solution. The falcon tubes were periodically checked under the microscope to check if the adult bodies were broken. When most of the worms were broken up, they were quickly centrifuged for 30 seconds at 4400rpm, following which the eggs formed a white pellet. Then, the bleach solution was aspirated off and 14ml of sterile M9 was added to the falcon tubes. The worms were centrifuged in a table-top centrifuge for 30 seconds at 4400rpm. The last two steps were repeated at least 3 times with sterile M9. In a new falcon tube 3ml of sterile M9 and eggs were rotated overnight on a nutator (VWR Nutading Mixer) at 25°C for an L1 arrest.

2.11.4 Scoring system to evaluate LBP-2::tagRFP accumulation in the RNAi screen

For the RNAi screen, I had to develop a different method for evaluating the amount of LBP-2::tagRFP puncta accumulation in the worms as the total amount of worms to evaluate was too large to be done manually in one sitting. This scoring system was tested and optimized during the pilot screen (Fig. 22). The plates with worms being fed bacteria targeting RNA of interest were compared to controls worms. We used the bacteria with the empty vector as a negative control. As a positive control for increased aggregation, we used RNAi clones which impair endocytosis. All this was done under a fluorescent microscope and only the aggregation in the head region was considered. The worms used for the RNAi screen were either 6 or 7 days into adulthood. The plates where the majority of worms had more aggregation than the negative control, those plates were marked as positive and the plates where the aggregation was lower than the negative control were marked as a negative plate. Later on, the system was further refined, where the worms with considerably more aggregation than the negative control was a single positive (+), and the worms where the amount of aggregation was higher than the positive control, were marked as double positive (++). This progressive scoring system was developed to have a more exact evaluation of the effect of a particular RNAi on the extracellular accumulation.

2.11.5 Worm preparation for RNAi screen

Eggs were collected by bleach treatment from 12-15 number of medium size NG plates with non-starved worms expressing LBP-2::tagRFP. The plates are treated with hypochlorite treatment, following which the eggs were placed overnight on a nutator at 25°C; this day was counted as day 0. On Day 1, the worms at L1 stage were washed once with sterile M9 and centrifuged for 30 seconds at 4400rpm. The washing step was repeated two times. Then, on small cold NG plate, I added three 1μL drops of M9 with L1 suspension, and counted the number of worms at L1 stage. If average number was below 35 worms per μL, then I would centrifuge it for 30 seconds at 4400rpm, remove 2-3ml of M9 and check the number of worms per μL again. I repeated the L1 worm count until the target concentration was reached. Then put six more 1μL drops of M9 with L1, check the consistency of the worm

count per µL. Finally a specific number of worms used to start a liquid culture in a Fernbach flask

To each liquid culture flask, OP50-1 was added proportionally to the number of worms. To prepare OP50-1, OP50-1 from the frozen stock was streaked on LB agar plate with streptomycin and kept overnight at 37°C in the incubator. The following day, a single colony from the streak plate was picked, LB 50ug/ml streptomycin was inoculated and allowed to grow overnight at 37°C. 8 Litres of OP50-1 was grown in LB with 50ug/ml streptomycin. Then, in a Beckman centrifuge flasks with 350ml OP50-1 was poured and centrifuge at 4000rpm for 10 minutes at 4°C. The supernatant was removed and add further 350ml OP50-1 was added and the centrifugation was repeated. Resuspend the double pellet was resuspended in 15ml of S basal. Collect pellets were into 50ml falcon and kept at 4°C until needed. This process was repeated for all of the OP50-1. In the liquid culture, 30ml of OP50-1 was added for 200000 worms with S-basal complete (Table. 10). The final volume of the flask was brought up to 300ml with S basal. Antibiotics were also added to the liquid culture. The worm cultures were completed with S basal to bring the total volume to 300ml. It was then incubated at 25°C, 150rpm in a large refrigerated shaker. At regular intervals, the worms in the liquid culture were checked to see if they have reached L4 larval stage. Once the worms reached the L4 stage, they were collected in 50ml falcons and centrifuge at 4400rpm for 2 minutes. The supernatant was discarded, and the worm pellet was resuspended in sterile M9 and centrifuged for 30 seconds at 4400rpm. This was repeated until the worms are completely washed off any bacteria. Then M9 with 0.01% triton was added. Triton (AppliChem) was used to make sure that worms do not stick to the side of the pipette tips. 5µL drops of the worm suspension were placed on cold NG plates. I counted if the number of worms per 5µL were 30-35 worms. If the number was below desired amount, then I centrifuged 30 seconds at 4400rpm and removed 2-3ml of M9 with triton. The count was repeated until the desired number was reached. Then I added 5µL of L4 worm suspension to each of the seeded NGM plate with Carbenicillin. The plates were checked to make sure that the number of worms per plate is consistent. Plates were kept at 25°C for the experiment and the worms were observed on day 6 of adulthood and the number of LBP-2::tagRFP puncta were evaluated.

2.12 Heat Stress Survival

The heat stress survival assay was developed to see if the worms are exposed to RNAi against selected genes, would be more sensitive to heat induced stress. For the heat stress survival assay, temperature dependent sterile worms were used. First, the worms were grown at 20°C on a large number of NG plates without any antibiotic. Then, the worms were bleached to collect the eggs and synchronise the worms. After the L1 stage arrest, the worms were transferred to the plates with specific RNAi bacteria, approximately 25 worms per plate. These worms were kept at 25°C until they reached L4 stage to induce the temperature dependent sterility and then transferred to 20°C. The worms were then kept at 20°C until they are 8 days into their adult life span. During this period, the worms were checked at regular interval. At day 8 of adulthood, the worms were transferred to 35°C. This step was to induce heat dependent stress. The worms were kept at this temperature until about 50% of the worms in the control plates have died. Then the numbers of alive and dead worms on different plates were counted. The count was also done once at a later time point.

2.13 Lifespan analysis

For lifespan analysis only temperature dependent sterile worms were used. To measure the lifespan of the worms, NGM plates were prepared (details in section 2.3). Depending on the requirement of the experiment, the lifespan can either be done on NGM plates with or without antibiotics. After preparing the plates, they were seeded with the bacteria as per the requirement of the experiment (details in section 2.2). Go get the age synchronised animals, the animals were either collected by bleaching or by manually transferring only the eggs on the NGM plates. For manually transferring the eggs, the hermaphrodite worms were grown on a separately and then 20-30 eggs were transferred to each plate. The lifespan analysis was conducted at 20°C and the worms were counted every other day. To count the worms, the worm population was divided into three categories. The three categories were; alive worms, dead worms and missing or censored. Alive worms were all the worms that were alive at that time point in their lifespan. The dead worms were the worms that are dead at that time point and the missing worms were the ones that have crawled up to the side of the plate and dried out. The count from each time point was noted and one the lifespan analysis was complete

the data was analysed using Online Application for the Survival Analysis of Lifespan Assays Performed in Aging Research (OASIS). OASIS is an online application for survival analysis which will provide various statistical tools to analyse survival data.

2.14 Biochemical method for insoluble protein aggregates

2.14.1 Sucrose separation

Sucrose separation was performed to remove bacteria and dead worms. The worms from day 3 of adulthood and day 10 of adulthood were used for the protocol. The worms were collected from the plates using M9 and then poured into a conical separatory funnel. The worms were allowed to sediment for 10 minutes at room temperature. The worms were poured into a falcon tube, centrifuged, the supernatant was removed and the worms were washed with M9. Then the worms were transferred to a 50ml falcon tube and it was filled to 20ml total volume with ice-cold M9. To the 20ml diluted worm pellet, 20ml of ice-cold 60% sucrose was added. The tube was quickly centrifuged at 3500 rpm for 5minutes with acceleration at 9 and deceleration at 7. The top worm later was removed with a large pipet. Cold M9 was added on top of this layer as it helps pull up the worms. This layer was added to 37ml of ice-cold M9 and 0.01% triton. It was mixed properly and centrifuged at 3500rpm for 3minutes with acceleration at 9 and deceleration at 7. Following which it was washed twice with cold M9 and 0.01% triton and washed once with cold M9. The worms were then transferred to a 15ml falcon and the total volume was brought up to 4ml with M9 and the falcon was rotated on a nutator at 20°C for 40 minutes. The worms were then washed twice with ice-cold M9 and 0.01% triton and twice with ice-cold M9. The worms were washed with 1x RAB buffer without inhibitors. With a Pasteur pipette the worms were sucked up and slowly dripped onto a half filled falcon tube with liquid nitrogen. Once the liquid nitrogen evaporated, the tube was stored at -80°C.

2.14.2 Quick and dirty aggregate separation in *C. elegans*

First ground worms were taken on ice and 50mg of it was measured. 150µl of RIPA buffer was added to the 50mg ground worms. Following which this mixture was vortexed and solubilized. It was then homogenized using a 1ml syringe and centrifuged at 4°C and 14000rpm for 20 minutes. Then 20µl of the supernatant per aliquot was stored at -80°C. The pellet was solubilized in 100µl RIPA buffer and centrifuged at 4°C and 14000rpm for 20 minutes. After the centrifugation, the supernatant was discarded and the pellet was resuspended in 75µl urea/SDS buffer. Then 10µl per aliquot was stored at -80°C.

2.14.3 Western Blotting

After the proteins were separated with SDS-Page the separates proteins were transferred to a methanol activated polyvinylidene difluoride (PVDF) membrane (PVDF Western Blotting Membrane, Roche) using a wet transfer system (Mini-Protean® Tetra System, BioRad). The transfer was done in transfer buffer supplemented with 20% methanol at constant current of 200mA for 2 hours at 4°C.

The membrane was washed with Tris-Buffered Saline and Tween 20 (TBST) for 5 minutes. The membrane was then blocked with 4% non-fat milk extract in TBST for 45 minutes on a nutator to avoid unspecific protein-protein interactions. Incubation with primary antibody diluted with 4% non-fat milk extract in TBST was performed overnight at 4°C on a nutator. To remove unspecific binding, the membrane was washed five times for 5 minutes each with TBST. Afterwards, the membrane was incubated with the secondary antibody diluted in 4% non-fat milk extract in TBS-tween for 1 hour at room temperature. Unspecific binding was avoided by washing the membrane three times for 5 minutes with TBST before detection. The membrane was incubated for 5 minutes in 0.5ml of Solution A and Solution B of the ECLplus[TM] kit (Amersham Hybond-P, GE Healthcare Life Sciences GmBH) each. Luminescence was detected using Stella (Raytest).

2.15 Buffers and Solutions

NGM-Plates

(Nematode Growth Medium)

Table 4: Materials used for the NGM. The following materials were used for the Nematode growth medium.

Name	Amount
Nacl	0.3% (w/v)
Difco Bacto-Agar	2% (w/v)
Bacto-peptone	.25% (w/v)
double distilled water	Upto final amount

Table 5: Materials used for the NGM following the autoclave. The following materials were added to the nematode growth medium after the autoclave and reducing the temperature.

Name	Amount
1M Potassium phosphate Buffer, pH 6.0	2.5% (v/v)
1M MgSO$_4$	0.1% (v/v)
1M CaCl$_2$	0.1% (v/v)
Cholesterol (5µm/ml)	

High Growth Plates

Table 6: Materials used for the high growth plated. The following materials were used for the high growth plates

Name	Amount
Nacl	0.3% (w/v)
Difco Bacto-Agar	3% (w/v)
Bacto-tryptone	2% (w/v)
double distilled water	Upto final amount

Table 7: Materials used for the high growth plates following the autoclave. The following materials were added to the high growth plates after the autoclave and reducing the temperature.

Name	Amount
1M Potassium Phosphate Buffer, pH 6.0	2.5% (v/v)
1M $MgSO_4$	0.1% (v/v)
1M $CaCl_2$	0.1% (v/v)
Cholesterol	20µm/ml

M9 solution

For 1 litre M9 solution

Table 8: Materials used for M9 solution. The following materials were used to make the M9 solution.

Name	Amount
NaCl	0.5% (w/v)
Na_2HPO_4, $7H_2O$	1.13% (w/v)
KH_2PO_4	3% (w/v)

ddH_2O was added to bring to the final amount. The solution was autoclaved, it was then cooled down and 0.02% of 1M $MgSO_4$ was added. For M9+Triton: 0.1% (v/v) Triton was added,

S-Basal

Table 9: Materials used for S-basal. The following materials were used to make S-basal.

Name	Amount
NaCl	0.59% (w/v)
1M Potassium phosphate pH 6	5% (w/v)

ddH_20 was used to bring up to the desired amount. This solution was autoclaved.

S-Basal complete

Table 10: Materials used for S-basal complete. The following materials were used to make S-basal complete.

Name	Amount
Potassium phosphate pH 6	1% (v/v)
Trace metals solution	1% (v/v)
1M MgSO$_4$	0.3% (v/v)
1M CaCl$_2$	0.3% (v/v)
Cholesterol	5µg/ml

1 M Potassium citrate Buffer pH6.0

Table 11: Materials used for potassium citrate buffer. The following materials were used to make potassium citrate buffer

Name	Amount
Citric acid monohydrate	26.2% (w/v)
Tri-potassium citrate monohydrate	268.8% (w/v)

The pH was adjusted using 10N KOH and the total volume was brought up to 500 ml with ddH$_2$O. The solution was then sterilized by autoclaving.

Trace metals solution

Table 12: Materials used for trace metals solution. The following materials were used to make trace metal solution.

Name	Amount
Disodium EDTA (5mM)	1.86g
$FeSO_4 * 7\ H_2O$ (2.5mM)	0.69g
$MnCl_2 * 4\ H_2O$ (1mM)	0.2g
$ZnSO_4 * 7\ H_2O$ (1mM)	0.29g
$CuSO_4 * 5\ H_2O$ (0.1mM)	0.025g
ddH_2O	Fill upto 1 L

It was sterilized by autoclaving and stored in the dark at 4°C.

Worm Lysis Buffer

Table 13: Materials used for worm lysis buffer. The following materials were used to make worm lysis buffer.

Name	Amount
Tris pH8	10mM
KCl	50mM
$MgCl_2$	2.5mM
Tween 20	0.45%
Gelatin	0.05%

Add ddH_2O

TAE Buffer

Table 14: Materials used for TAE buffer. The following materials were used to make the TAE buffer.

Name	Amount
Tris	40mM
Acetic acid	20mM
EDTA	1mM

Add ddH$_2$O to bring up to 100ml.

RAB

Table 16: Materials used for RIPA buffer. The following materials were used to make the RIPA buffer.

Name	Amount
MES	0.1M
EGTA	1mM
EDTA	0.1mM
MgSO$_4$	0.5 mM
NaCl	0.75M
NaF	0.02M

RIPA

Table 16: Materials used for RIPA buffer. The following materials were used to make the RIPA buffer.

Name	Amount
Tris pH 8	50mM
NaCl	150mM
EDTA	5mM
SDS	0.5%
SDO	0.5%
NP-40	1%
PMSF	1mM
Roche Complete Inhibitors	1x

Urea/SDS buffer

Table 16: Materials used for Urea/SDS buffer. The following materials were used to make the Urea/SDS buffer.

Name	Amount
Urea	8M
SDS	2%
DTT	50mM
Tris pH8	50mM

2.16 Material used

All material used are given in the annex section (Table. 25).

<u>Result</u>

Section 1

3.1.1 Evaluation of a model for extracellular aggregation of endogenous protein with age

For the analysis of potential regulators of extracellular accumulation, a worm model with an age-dependent extracellular aggregating protein was required. Lipid binding protein (LBP-2) was chosen as the protein of interest as it was shown in the previous study to aggregate in the extracellular space with age (David *et al*., 2010; unpublished data). In the model used, *lbp-2* was under the expression of its own promoter (*Plbp-2*) and fused to tagRFP to visualize the aggregation in vivo (*Plbp-2::lbp-2::tagrfp*).

A

B

C
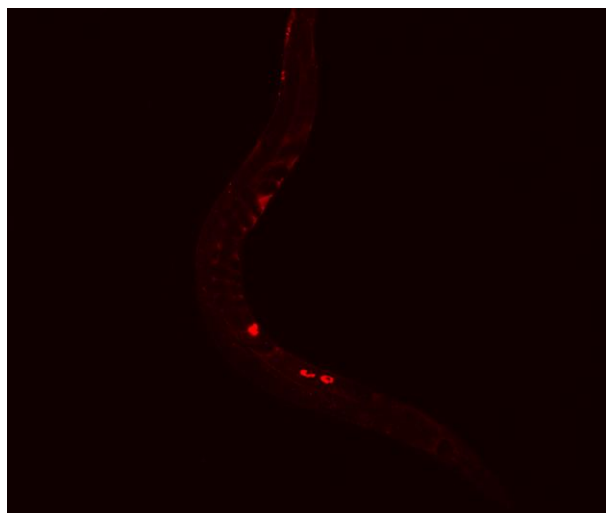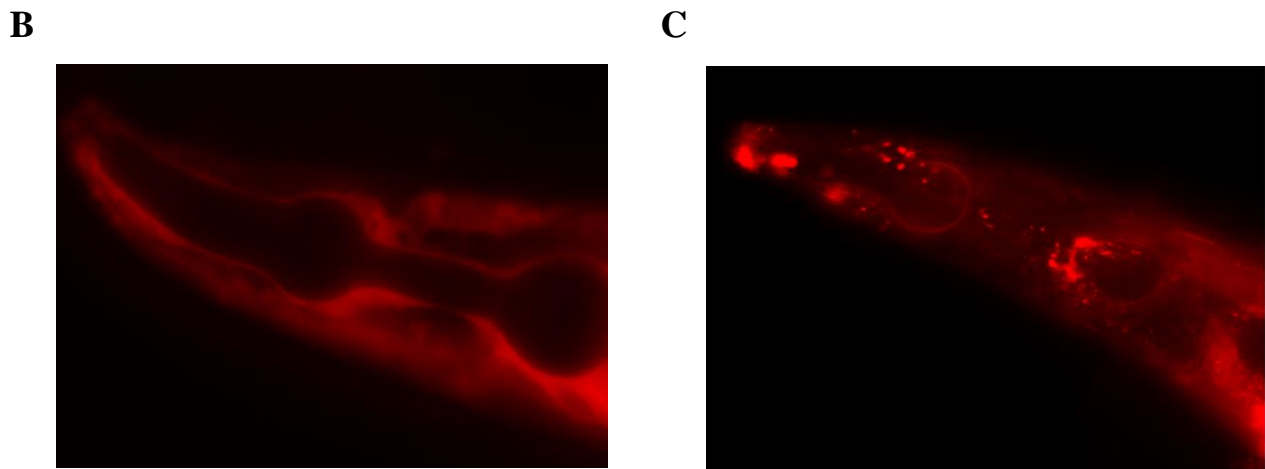
Figure 5: *C. elegans* **expressing** *lbp-2::tagrfp*. **A)** The full worm on day 2 at 25°C. The expression of *Plbp-2::lbp-2::tagrfp* and the coelomocytes are visible. **B)** Head region of the worm on day 2 at 25°C, the anterior bulb and the terminal bulb are visible. No puncta are visible at this time point. **C)** The head region of the worm on day 8 of adulthood, a number of LBP-2 puncta are visible throughout the head region.

3.1.1.1 Evaluation of LBP-2 aggregation in integrated LBP-2 worm strains

To perform a large scale evaluation of aggregation levels in an RNAi screen, we needed to have a stable worm line with LBP-2 integrated into the *C. elegans* genome. For generating such a strain, DCD1 (a worm strain with an extrachromosomal array containing *Plbp-2::lbp-2::tagrfp*) was exposed to UV irradiation and two strains were generated, DCD22 and DCD23. In this section, I determined whether the rate of LBP-2 puncta formation in the integrated strains was similar to that in the non-integrated strain. I first evaluated and compared the rate of LBP-2 puncta formation in both the integrated strains DCD22 and DCD23. About 130 worms at fourth larval stage (L4) from each strain were transferred to 20°C and were evaluated at different time points of their lifespan. A small non-significant difference was observed between the two integrated strains at day 8 of adulthood; DCD23 had more extracellular LBP-2 aggregation when compared to DCD22 worms on the same day (Fig. 6). The rate of extracellular aggregation was similar in later stages of adulthood (Fig. 6) (appendix table 1). Next, I tested whether the rate of extracellular LBP-2 aggregation would

be any different compared to the LBP-2 worms with *Plbp-2::lbp-2::tagrfp* in the extrachromosomal array.
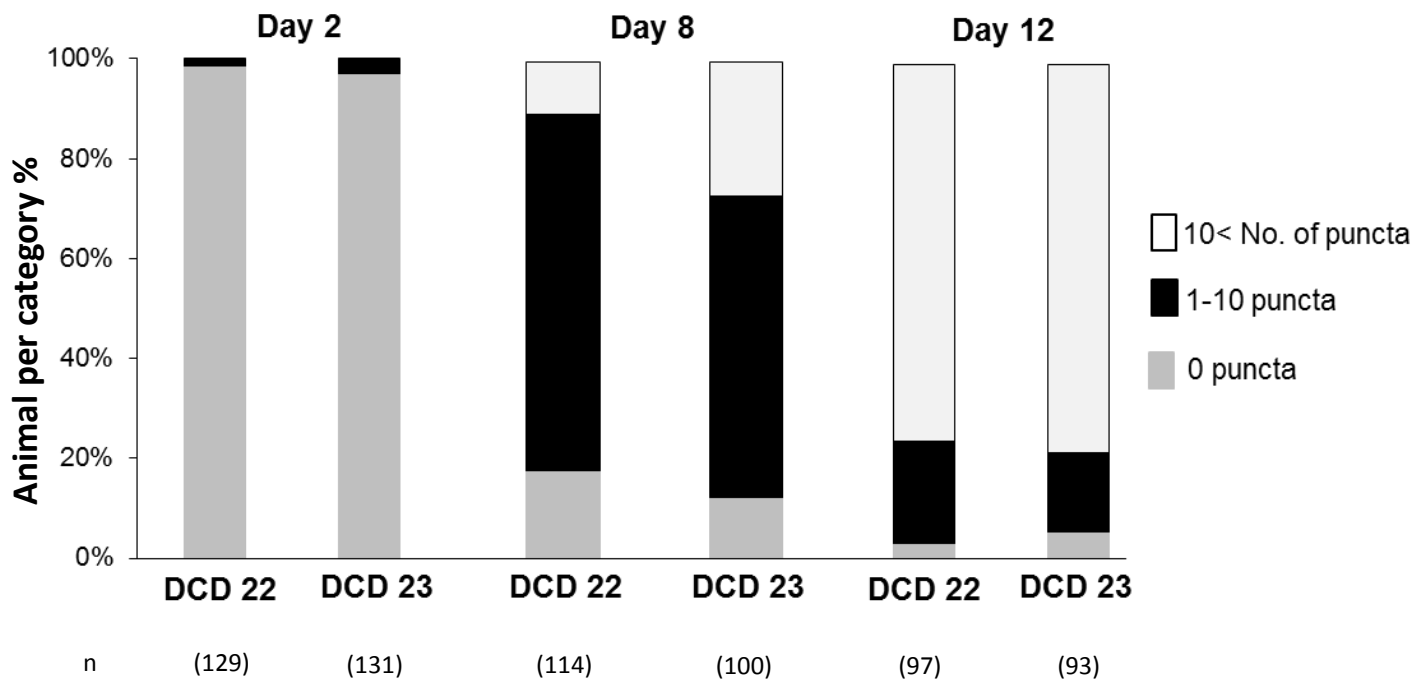
Figure 6: No significant difference in the rate of LBP-2::tagRFP puncta accumulation integrated strains. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between the categories was not significant. Number of worms evaluated is shown at the bottom. Details in appendix table 1.

3.1.1.2 Evaluation of LBP-2 aggregation in integrated LBP-2 worm strains and strain with extrachromosomal LBP-2

Henceforth, I was interested to evaluate the rate of extracellular LBP-2 aggregation in the DCD23, a strain with integrated *lbp-2* and DCD1 (a worm strain containing an extrachromosomal array containing *Plbp-2::lbp-2::tagrfp)*. Worms from L4 larval stage per strain (N=130) were selected, kept at 20°C and observed at different time point of their adult lifespan. No difference in the rate of extracellular LBP-2 aggregation was observed in either DCD1 or DCD23 on day 4. Whereas on day 8, there was a difference between the rates of extracellular aggregation between the two strains i.e., more DCD23 worms showed higher amount of aggregation (Fig. 7). However, this difference was not significant. Similarly, on

day 12 there was no significant difference observed between the two strains in the rates of extracellular LBP-2 aggregation (Fig. 7) (appendix table 2). Therefore, the rate of LBP-2 puncta formation between the integrated worm strains and the non-integrated strain was similar. After developing an extracellular LBP-2 model, I further aimed to understand the effect of the bacterial food source on this worm strain.
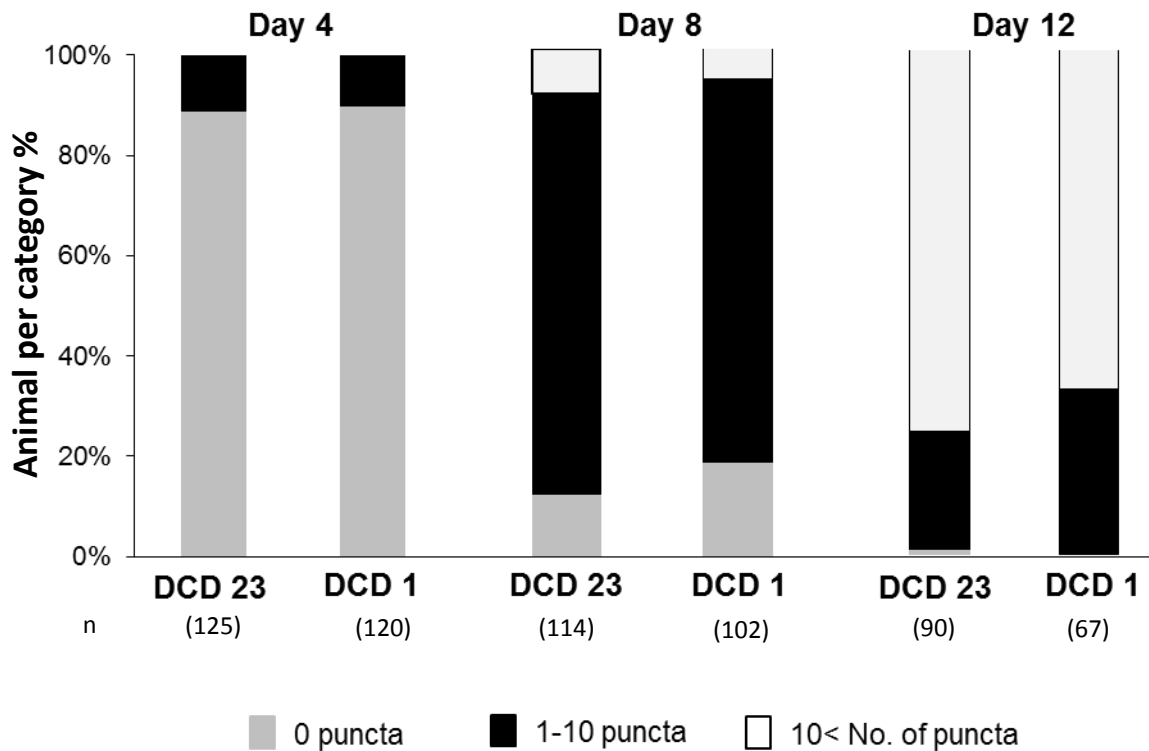
Figure 7: Integrated and non-integrated Lbp-2 worms do not show a difference in the rate of aggregation. The rate LBP-2::tagRFP puncta accumulation is similar in the worms with integrated *Plbp-2::lbp-2::tagrfp* and worms with an extrachromosomal array containing *Plbp-2::lbp-2::tagrfp*. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between the categories was not significant. Number of worms evaluated is shown at the bottom. Details in appendix table 2.

Previous study has shown that proteins aggregate with age (David *et al*., 2010). To evaluate whether there is a change in the amount of strong-detergent insoluble LBP-2 protein with age, a western blot was carried out. For this, DCD130 were grown in liquid culture until 3 days and 10 days of adulthood. After washing the worms from bacteria and removing dead worms by sucrose separation, the proteins were extracted and the amounts of soluble and

insoluble proteins were compared. There was an evident increase in the amount of insoluble proteins in the worms which were 10 days into their adulthood when compared to the levels of insoluble proteins in the worms which were 3 days into their adulthood (Fig. 8). Additionally, a clear change in the amount of soluble proteins can be observed. The amount of soluble proteins tends to decrease with age. So, in the worms that were 3 days into adulthood higher levels of soluble proteins can be observed than in the worms which are 10 days into their adulthood (Fig. 8).
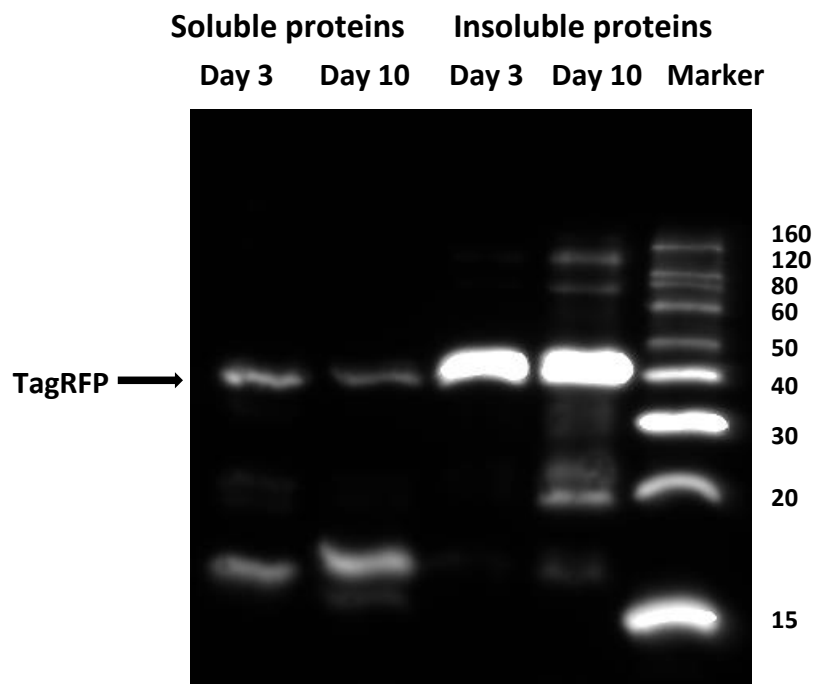
Figure 8: Increase in the levels of insoluble LBP-2 with age. There is a 1.46x change in the levels of insoluble LBP-2 proteins in worms at day 10 when compared to worms at day 3 of adulthood. An age dependent decrease in the amount of soluble LBP-2 proteins was also observed.

3.1.2.1 The type of food does not have an effect on the LBP-2 aggregation at day 8 of adulthood

C. elegans is usually grown in the laboratory with an *E. coli* strain OP50 used as a food source (Brenner, 1974). However, for the RNAi experiments where bacteria expressing dsRNA are fed to *C. elegans, E. coli* strain HT115 is used. Feeding vector L4440 is transformed into HT115; L4440 is an empty vector with two T7 promoters, where the gene of interest is cloned between the T7 promoter and HT115 has IPTG inducible expression of T7 polymerase (Timmons and Fire, 1998). To validate if the food type has any effect on the LBP-2 aggregation, *E. coli* strains HT115 and OP50 were fed to *C. elegans*.

Wild-type worms expressing *Plbp-2::lbp-2::tagrfp* worms at L4 larval stage were kept at 20°C and when they were observed on day 8, in the worms fed 0P50, over half the worm population (59.1%) had LBP-2 puncta. There were even worms with more than 10 LBP-2 puncta (6.8%). In the worms that were fed l4440, 55.9% had between 1-10 LBP-2 puncta. However, there were no worms with more than 10 puncta (Fig. 9). By day 20, a significant change in the rate of LBP-2 puncta accumulation was observed as the worms feeding on OP50 had almost their entire population with more than 10 puncta (93.2%), while 51% worms fed with L4440 showed more than 10 puncta (Fig. 9) (appendix table 3). The different strains of *E. coli* do not have an effect on the rate of LBP-2 accumulation during mid-adulthood, whereas OP50 as a food source leads to higher rate of LBP-2 accumulation in very old age.
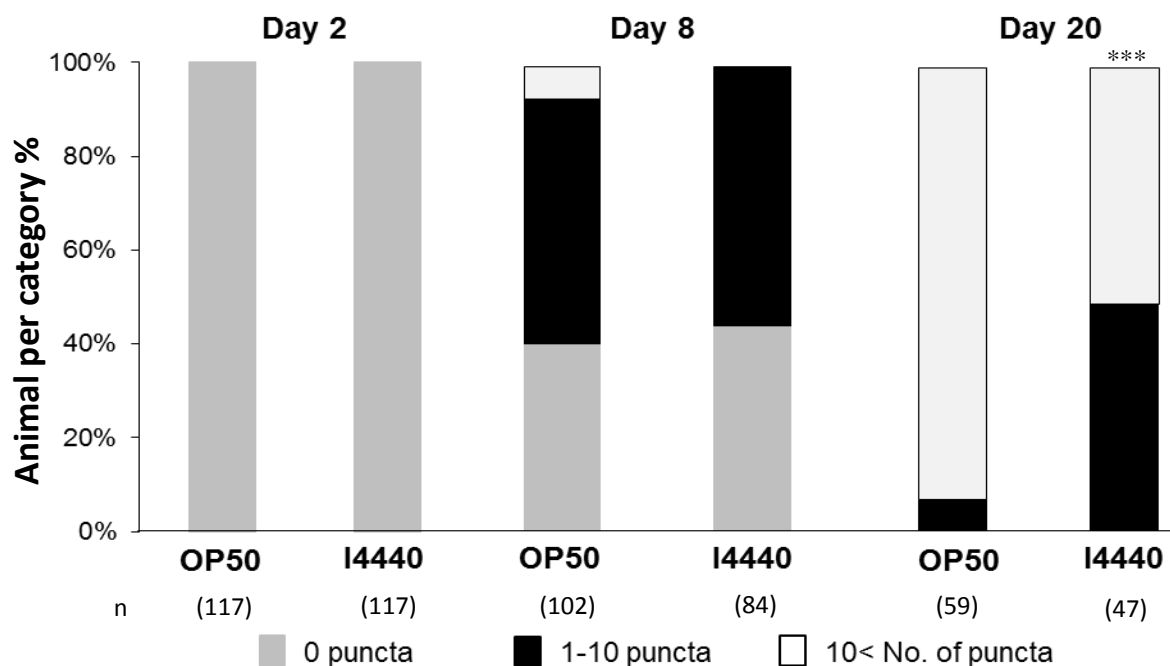
Figure 9: OP50 leads to higher LBP-2 aggregation only at very old age. The rate of LBP-2 puncta accumulation is significantly higher when the worms were 20 days into adulthood. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10 <No. of puncta was significant on day 20, Fisher's exact chi-square test ***p <0.001. Number of worms evaluated is shown at the bottom. Details in appendix table 3.

3.1.2.2 Does the food source have any gene silencing effect that can influence aggregation?

As we observed decreased LBP-2 aggregation in very old animals treated with control RNAi bacteria, we sought to check whether gene silencing could be taking place. Indeed, transgene silencing has been observed previously with l4440 due to common sequences in the backbone vector of the l4440 and transgene constructs (Grishok *et al.*, 2005). To check for potential silencing effects, *sid-1(-)* mutant worms were used. These worms have a mutation in the *sid-1* (*systemic interference defective*) gene. SID-1 is a transmembrane protein that acts as a channel for passive cellular uptake of dsRNA (Winston *et al*., 2002) and hence, plays a critical role in RNA-mediated gene silencing. Therefore, these worms, due to a mutation of

the *sid-1* gene, were a good model to study the effects of any double stranded RNA from the food source. For this, *lbp-2* was crossed into the *sid-1(-)* mutant and hence one can see the effects on LBP-2 accumulation.

During this experiment, *sid-1(-)* L4 worms were fed with both OP50 and L4440 at 20°C and subsequently observed. Worms observed on day 6 showed almost similar levels of LBP-2 puncta in both the worm population (Fig. 10). However on day 14, 6.7% DCD120 worms growing on OP50 have over 10 puncta. Whereas 22.2% of worms fed l4440 have over 10 LBP-2 puncta in their head region (Fig. 10). The difference in the rate of LBP-2 puncta accumulation was significant.
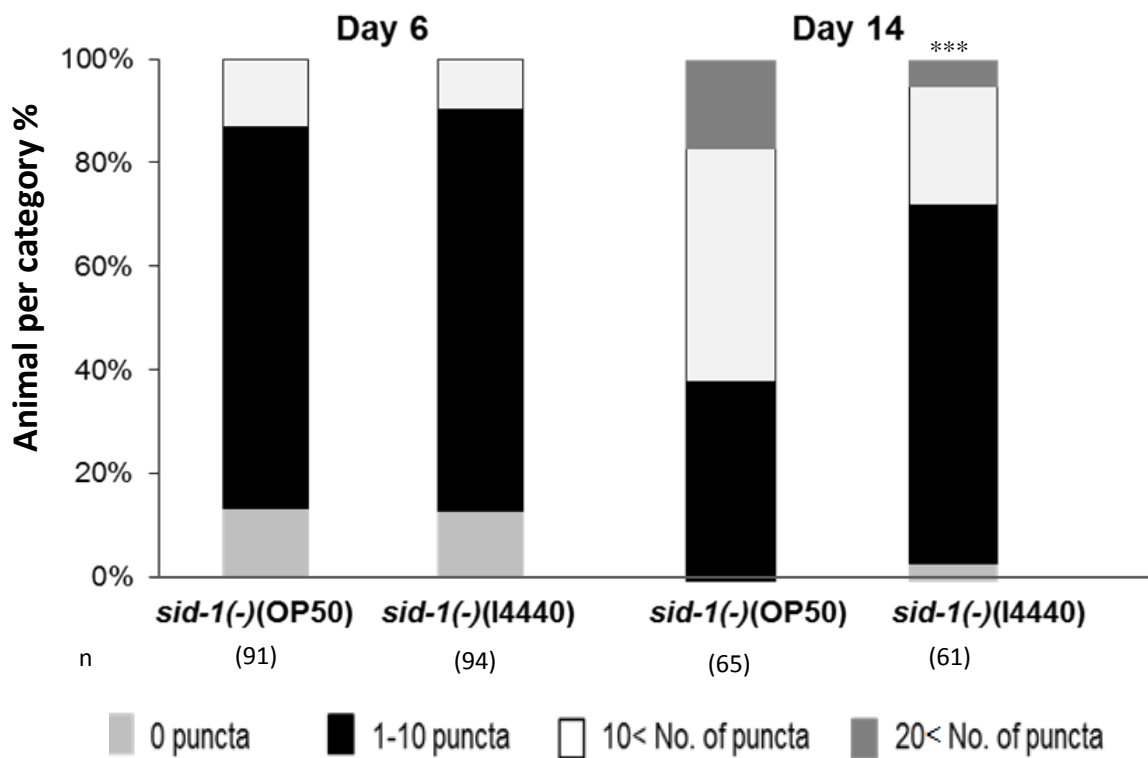
Figure 10: Difference in food does not have a significant effect on LBP-2 accumulation in *sid-1(-)* worms. On day 14, *sid-1(-)* worms with *lbp-2::tagrfp* feeding on OP50 had higher number of LBP-2 puncta than the when they were fed l4440. This difference in the rate of LBP-2 puncta accumulation was insignificant. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between the number of worms with less than 10 puncta and more than 10 puncta was significant on day 14, Fisher's exact chi-square test ***p <0.001. Number of worms evaluated is shown at the bottom. Details in appendix table 4.

3.1.3. Does *daf-2* mutation have any effect on the extracellular aggregation?

daf-2 is a gene which encodes an insulin/IGF-1 like receptor. In *C. elegans daf-2* gene regulates longevity (a *C. elegans* mutant that lives twice as long as wild type) (Kenyon *et al.*, 1993). In *C. elegans*, a *daf-2* mutation leads to extension of adult lifespan and a larval arrest stage called the dauer stage. So far no one has addressed the effect of *daf-2* on extracellular aggregation. Therefore, I studied the effects of *daf-2* mutation on extracellular LBP-2 aggregation and if the lifespan extension caused by *daf-2* mutation could also work though reducing the amount of extracellular aggregation.

daf-2(e1370) mutant worms with a tagged lbp-2 (*plbp-2::lbp-2::tagrfp*) were used in the experiment. Over 128 wild type and *daf-2(-)* L4 worms were transferred to 20°C and observed at different point of their adult lifespan. A significant difference (p<0.0001) was observed in the rate of LBP-2 puncta aggregation between wild type and *daf-2(-)* worms on day 8. 33.3% of wild type worm population had more than 10 LBP-2 puncta in their head region; while none of the *daf-2(-)* worms had more than 10 puncta (Fig. 11). On day 14, the rate of LBP-2 accumulation was significantly higher in the worm population without the *daf-2* mutation (P < 0.0001). 94.6% of wild type worm population had more than 10 LBP-2 puncta in their head, compared to *daf-2(-)* worms where it was observed to be 48% (Fig. 11). Thus suggests a role of *daf-2* mutation in regulating the rate of aggregation of extracellular LBP-2 puncta. Here I showed *daf-2* mutation reduces the rate of LBP-2 aggregation therefore to further understand its role I wanted to validate its effect using another *daf-2* mutant line.
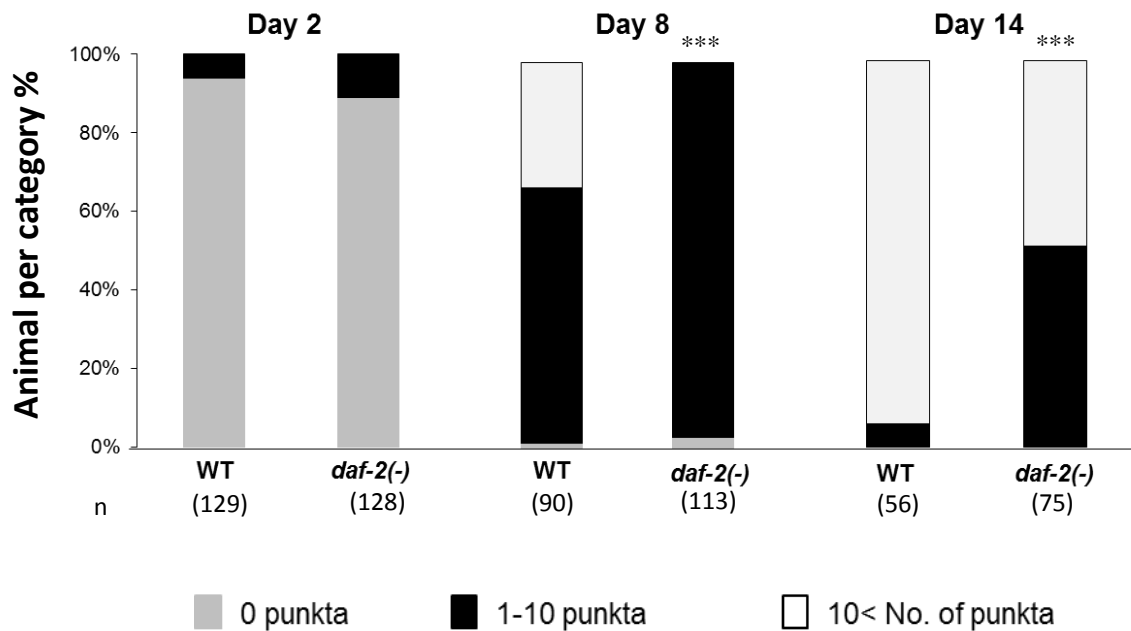
Figure 11: *daf-2* mutation slows down the rate of LBP-2 puncta accumulation. Wild type worms had higher number of LBP-2 puncta than *daf-2(-)* worms during middle and late stages of adulthood. The rate of LBP-2 puncta accumulation is significantly higher when the worms were 8 and 14 days into adulthood. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10< No. of puncta was significant on day 8 and 14, Fisher's exact chi-square test P <0.0001. Number of worms evaluated is shown at the bottom. Details in appendix table 5.

To investigate the effects of the *daf-2* mutation, I crossed *daf-2*(e1370) mutant worms with DCD22, a different integrated LBP-2 line.

The setup of this experiment was similar to the earlier experiment with the *daf-2(-)* mutant. The worms were observed at different time point of their adulthood i.e. day 2, 4, 6, 8, 10, 12, 14. On day 8, Wild type worms showed an increase in puncta number (97.3%) (Fig. 12). 79.4% of *daf-2(-)* worms had 1-10 LBP-2 puncta in the head (Fig. 12). 100% of wild type worms showed more than 10 puncta on day 14, whereas in *daf-2(-)* worms only 29.4% of the worms had more than 10 puncta (Fig. 12) and thus a significant difference in the rate of accumulation between the two worm populations was observed. In this experiments *daf-2* mutants showed slower rate of extracellular accumulation. Henceforth underlying the role of

daf-2 in the regulation of extracellular accumulation rate as verified in the previous experiment.
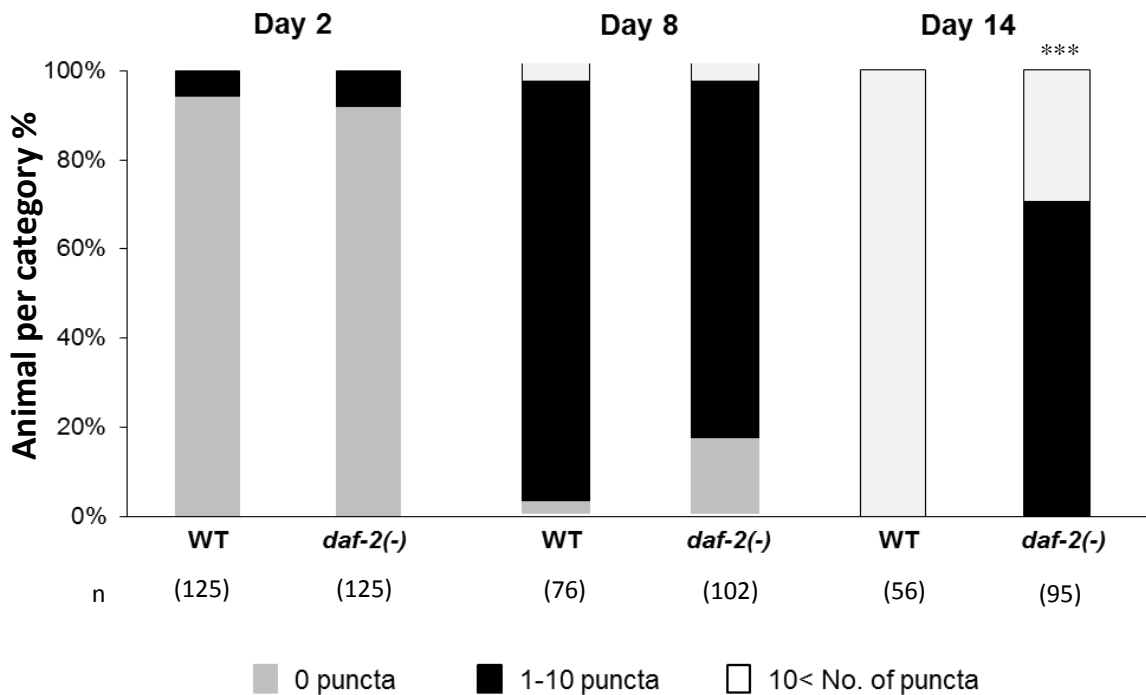
Figure 12: Rate of LBP-2 accumulation is slower in worms with *daf-2* mutation. Wild type worms had significantly higher number of LBP-2 puncta than *daf-2*(e1370) worms on day 14 of adulthood. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10< No. of puncta was significant on day 14, Fisher's exact chi-square test ***p <0.001. Number of worms evaluated is shown at the bottom. Details in appendix table 6.

To further validate the effect of *daf-2* on the extracellular LBP-2 aggregation. I decided to do an RNAi experiment. For this RNAi experiment *gfp* RNAi was used as a negative control as I had tested it earlier and it does not have an effect to increase the extracellular LBP-2 accumulation.

The wild type worms with the LBP-2 transgene were transferred to the respective RNAi seeded plate when they were at the L4 larval stage. The worms growing on either RNAi did not show any difference in aggregation during the early stages of adulthood. Conversely by day 14, worms on *daf-2* RNAi had significantly more puncta than controls (22.6% of the daf-

2 RNAi worms had >10 puncta in the head compared to the *gfp* RNAi control worms (38.3%) (Fig. 13). On day 18, this difference in the rate of LBP-2 accumulation became even more significant (in the *gfp* RNAi worms (P < 0.005) as 88.5% of them had more than 10 LBP-2 puncta in their head compared to only 58.8% of the worms growing on *daf-2* (Fig. 13).
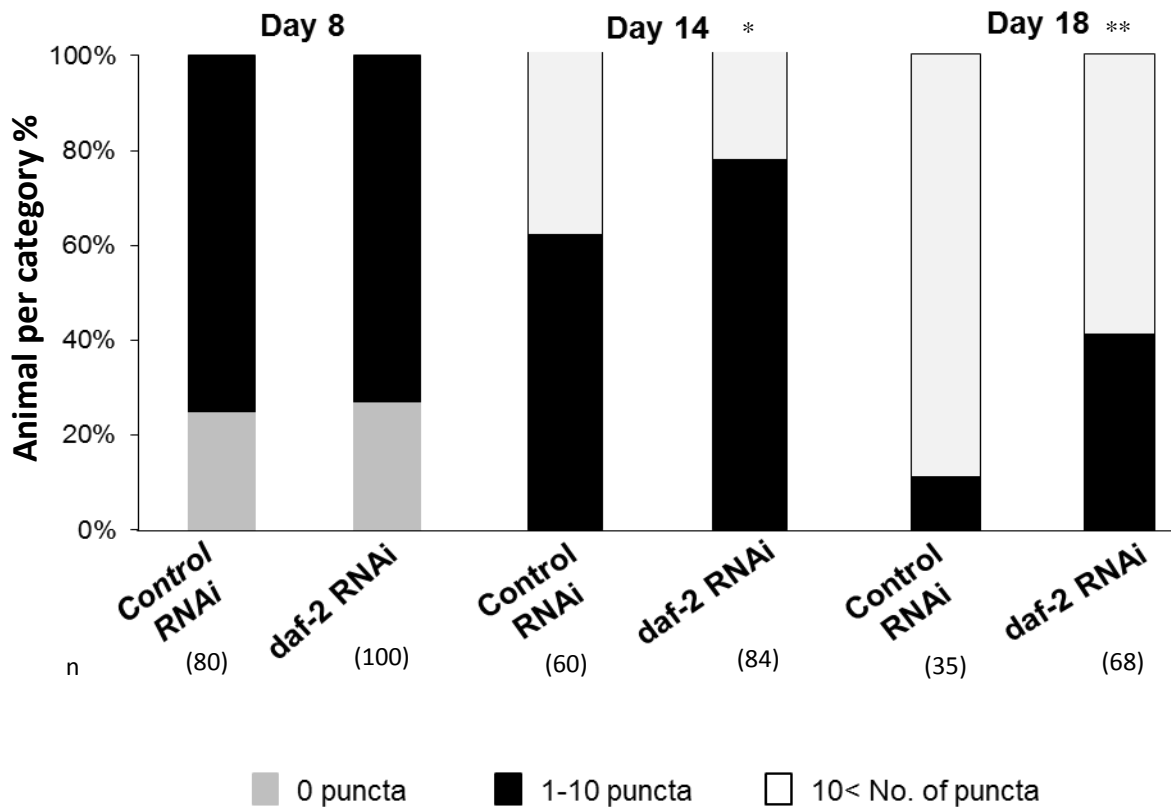
Figure 13: *daf-2* RNAi causes slower rate of LBP-2 accumulation. On day 14 and day 18**,** wild type worms fed control RNAi had significantly higher number of LBP-2 puncta than worms fed *daf-2 RNAi* on day 14 of adulthood. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10 <No. of puncta was significant on day 14 and day 18, Fisher's exact chi-square test * p <0.05 and **p <0.01 respectively. Number of worms evaluated is shown at the bottom. Details in appendix table 7.

3.1.4 Does MOAG-4 regulate the extracellular LBP-2 accumulation?

In a previous study, MOAG-4 has been shown to be a highly conserved modifier of aggregation. In *C. elegans* model for polyglutamine diseases MOAG-4 has functioned as a positive regulator of aggregate formation (van ham *et al*, 2010). Given the effects of MOAG-4 on aggregation, I was interested to understand its effect of extracellular aggregation. For this study, *moag-4(-)* mutant worms were crossed with wild type worms which had integrated *plbp-2::lbp-2::tagrfp* and at L4 larval stage worms from either of the strains were picked and transferred to 20°C. Already at day 2, there was a significant difference between the two backgrounds, with *moag-4(-)* worms showing puncta in the head (~1-10 puncta) (Fig. 14). On day 6, there was a significant increase in the LBP-2 puncta between the two groups with *moag-4*(-) worms (87.3%) compared to DCD23 worms (29.5%) (Fig. 14). Interestingly, on day 10, *moag-4*(-) worms (70.9%) showed significantly higher number of worms with >10 puncta compared to DCD23 worms (23.8%) with a much slower rate of aggregation (Fig. 14). Therefore, the rate of extracellular LBP-2 puncta accumulation in the *moag-4*(-) worms was much faster than in the wild-type background.
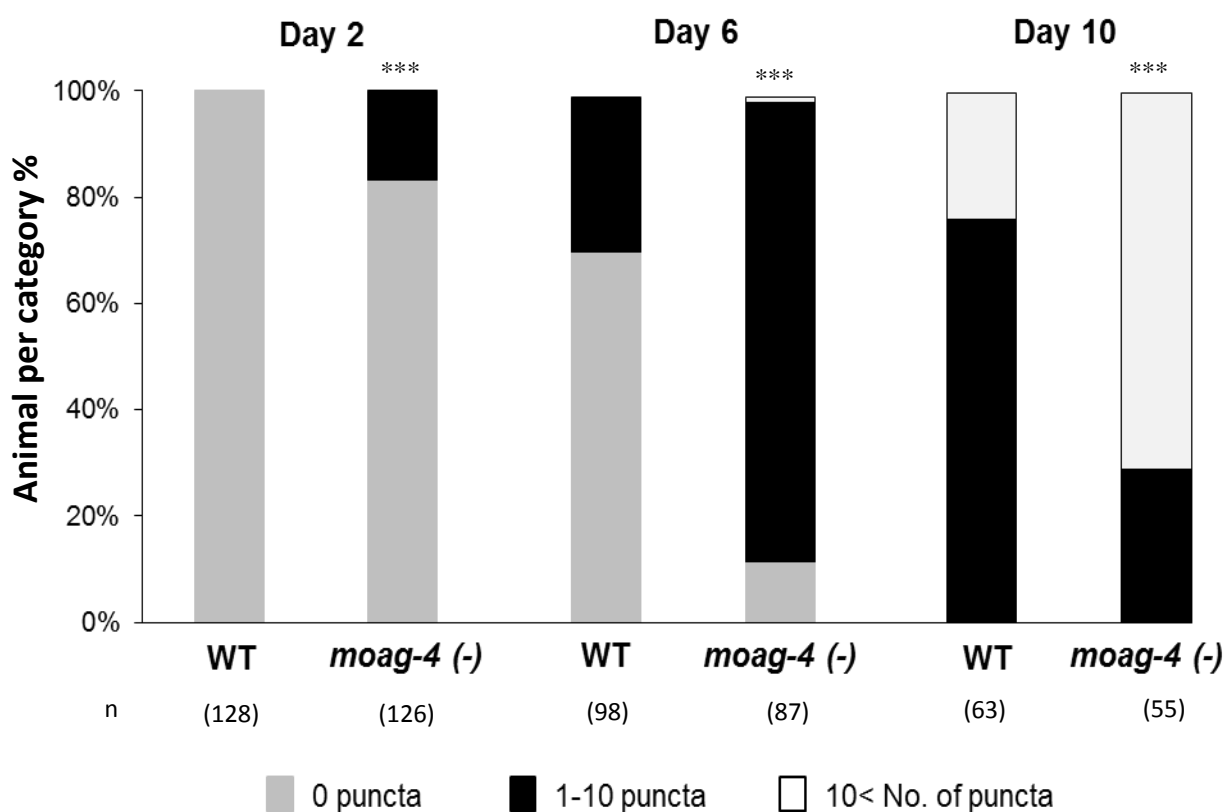
Figure 14: LBP-2 accumulates faster in the *moag-4(-)* mutants. Wild type worms had significantly slower rate of LBP-2 puncta accumulation than *moag-4(-)* worms. This difference in the rate of LBP-2 accumulation was significantly higher in the *moag-4(-)* worms at all different time points. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10 <No. of puncta was significant on day 2, 6 and 14, Fisher's exact chi-square test ***p <0.001 (for each day). Number of worms evaluated is shown at the bottom. Details in appendix table 8.

3.1.5 Evaluation of a temperature sensitive sterile model for extracellular aggregation of endogenous protein with age.

In *C. elegans*, higher temperature is known to decrease the length of the worm's lifespan. For the genome-wide RNAi screen I required temperature sensitive sterile worms which become sterile when they are kept at 25°C. Therefore, I wanted to investigate whether an increase in temperature will modulate extracellular LBP-2 accumulation. Initially, I studied the effect of

temperature on the rate of accumulation of extracellular LBP-2 puncta in wild type LBP-2 worms at L4 larval stage which were kept at 20°C and 25°C.

Two sets of worms kept on different temperature conditions showed protein aggregation on day 6 (25°C=79% worms vs. 20°C=10.4% of worms; P < 0.0001) (Fig. 15). On day 10, there was a significant increase in the rate of extracellular accumulation with more worms having over 10 puncta (temperature: 25°C=50% worms vs. 20°C=6.3% worms) (Fig. 15). Henceforth, increase in temperature leads to faster rate of extracellular LBP-2 accumulation. Next I aimed to screen for potential positive controls of increased accumulation for my RNAi screen.
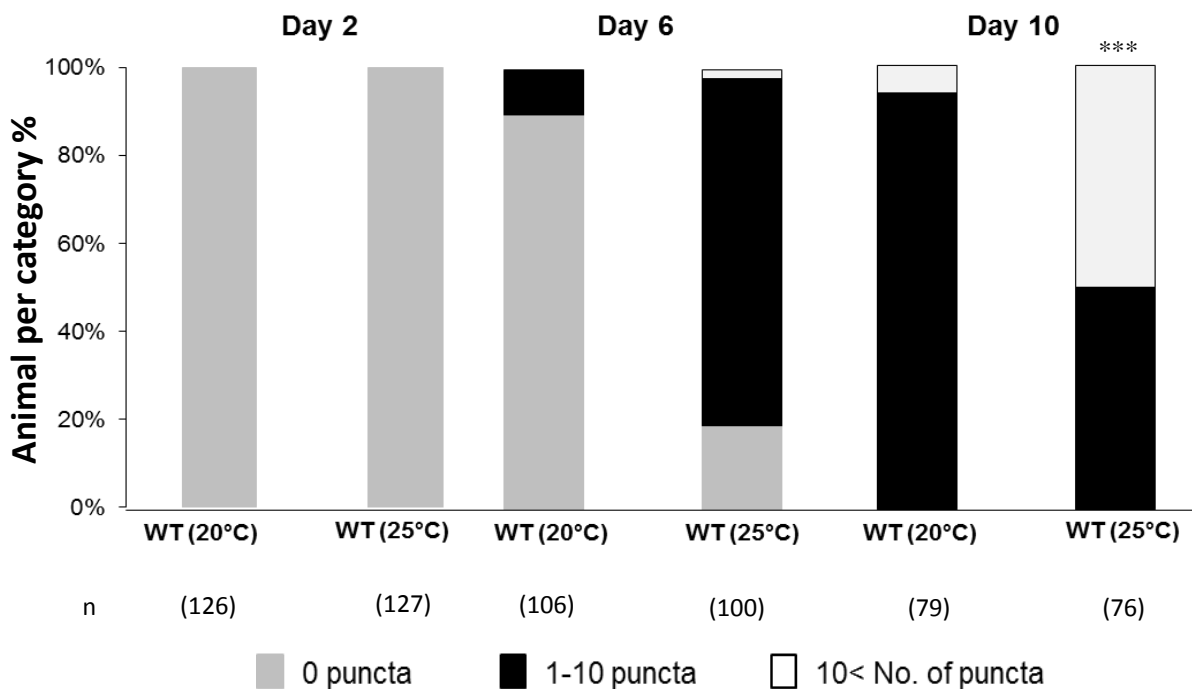
Figure 15: Increased LBP-2 accumulation at higher temperature. Wild type worms at 25°C had significantly faster rate of LBP-2 puncta accumulation than worms at 20°C on day 6 and day 10. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10 <No. of puncta was significant on day 6 and 10, Fisher's exact chi-square test ***p <0.001 (for each day). Number of worms evaluated is shown at the bottom. Details in appendix table 9.

3.1.5.2 Evaluation of a temperature sensitive sterile model for extracellular aggregation of endogenous protein with age

To perform the large-scale RNAi screen to evaluate extracellular LBP-2 aggregation levels, I needed sterile LBP-2 transgenic worms. To make such a worm, first I needed to find a *C. elegans* strain that has the necessary sterility and then cross that strain with the worm line having integrated LBP-2. For this I first started testing different temperature sensitive sterile strains and checking the efficiency of their sterility. I picked three mutant strains that had been used in previous studies and ran a number of sterility tests with them. To make these worms sterile they were transferred as eggs to 25°C, then after the L4 stage they were either moved to 20°C or kept at 25°C and the number of their progeny were counted. The results of one such series of sterility test is shown in table 15. From the table, we can see that TJ1060 the *spe-9*(hc88)I; *fer-15*(b26)II double mutant had complete temperature-dependent sterility induced while the single mutants had some progeny.

Table 15: TJ1060 had complete sterility. Three temperature sensitive sterile strains were tested for their sterility at 25°C.

Strain	No of L4 worms	No of progeny
TJ1060	130	0
BA671	130	155
DH26	130	2

After a series of tests for sterility, TJ1060 was selected and crossed with DCD23, the integrated LBP-2 strain. The progenies were tested for temperature sensitive sterility where two of these crossed worm lines (DCD23 X TJ1060 (12), DCD23 X TJ1060 (9)) had no progeny after first series of test (Table 16).

Table 16: Two of the crossed lines were completely sterile. Two different crossed lines were tested for sterility.

Strain	No. of L4 worms	No. of progeny
TJ1060	192	0
BA671	184	80
DH26	182	4
DCD23 X TJ1060 (12)	180	0
DCD 23 X TJ 1060 (9)	110	0

To validate that the sterile strain were completely functioning sterile worms; I tested the crossed worms a number of times and during one of the later tests I found out progeny in both the crossed strains. During further tests it was confirmed that both the DCD23 crossed strains were not sterile. Therefore, I decided to use a different temperature sensitive sterile strains, test its sterility and if it is sterile, cross it with the integrated LBP-2 worms.

Table 17: Crossed strains not sterile. Further tests showed that the crossed strains were not completely sterile

Strain	No. of l4 worms	No. of progeny
TJ1060	200	0
BA671	200	448
DH26	200	18
DCD23 X TJ1060 (12)	200	40
DCD 23 X TJ 1060 (9)	200	42

I investigated the CF512 strain, a commonly used temperature sensitive sterile strain (Garigan *et al*., 2002). In the tests, CF512 did not have any progeny and hence I decided to cross it with the integrated LBP-2 strain DCD23. Following the cross, I screened the progeny with a series of tests for sterility, and I managed successfully to get a line which had no

progeny in any of the tests. The outcome of the test has been tabulated in the (Table 18). This strain was then used for RNAi screening.

Table 18: Crossed strain is sterile. The new crossed strains were completely sterile.

Strain	No. of l4 worms	No. of progeny
CF512	112	0
DCD23 X CF512 (5)	112	0
DH26	112	9

3.1.6 Positive control for increased extracellular accumulation

To screen for the regulators of extracellular aggregation using RNAi, I first needed to identify positive controls. For this, I looked for the factors that regulate the coelomocyte function. Coelomocytes continously uptake macromolecules from the pseudocoelomic cavity and are known to perform immune and scavenging functions (Fares and Grant, 2002; Yanowitz and Fire, 2005). From a previous study (Fares and Greenwald, 2001a) I picked the most effective inhibitors to be used during the screening. Initially I used RNAi against *rme-1* which is required for modulating endocytotic transport and uptake by the coelomocyte. The wild type worms with integrated LBP-2 were transferred to plates seeded with l4440 and *rme-1* at L4 larval stage. On day 6, a significant difference between the rate of aggregation was observed (worms with >10 LBP-2 puncta, l4440 (9.6%) and *rme-1* (38.2%)) (Fig. 16). On day 8, the rate of LBP-2 accumulation in the worms on *rme-1* was still significantly higher (>10 puncta in the head: 82% DCD23 worms vs. 64.4% *rme-1* worms) (Fig. 16). Therefore, disrupting *rme-1* caused an accumulation of extracellular LBP-2 puncta through reduced endocytotic uptake by the coelomocyte. Henceforth, I tested out other potential regulators.
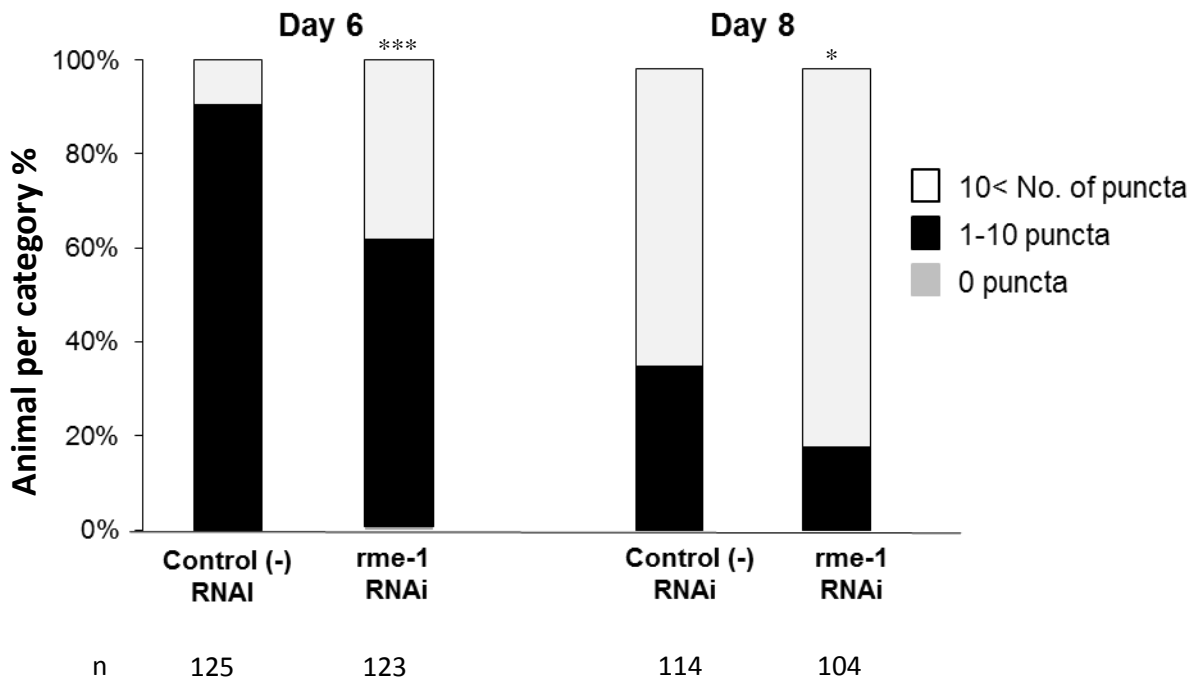
Figure 16: *rme-1* **RNAi increases the rate of LBP-2 accumulation.** Wild type worms that were fed *rme-1* RNAi had significantly higher rates of LBP-2 puncta accumulation than worms that were fed control RNAi. The worms were classified into three groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10 <No. of puncta was significant on day 6 and 8, Fisher's exact chi-square test ***$p<0.001$ (for day 6) and *$p <0.05$ (for day 8). Number of worms evaluated is shown at the bottom. Details in appendix table 10.

Next I evaluate the effect of *sec-6* and *cup-4*, two other regulators of coelomocyte function identified in a study by Fares and Greenwald (Fares and Greenwald, 2001a). I screened for an increase in LBP-2 accumulation by feeding RNAi of these regulators to my LBP-2 worms. No significant difference in the rate of extracellular LBP-2 puncta accumulation was observed either on day 6 or 8 (Fig. 17).
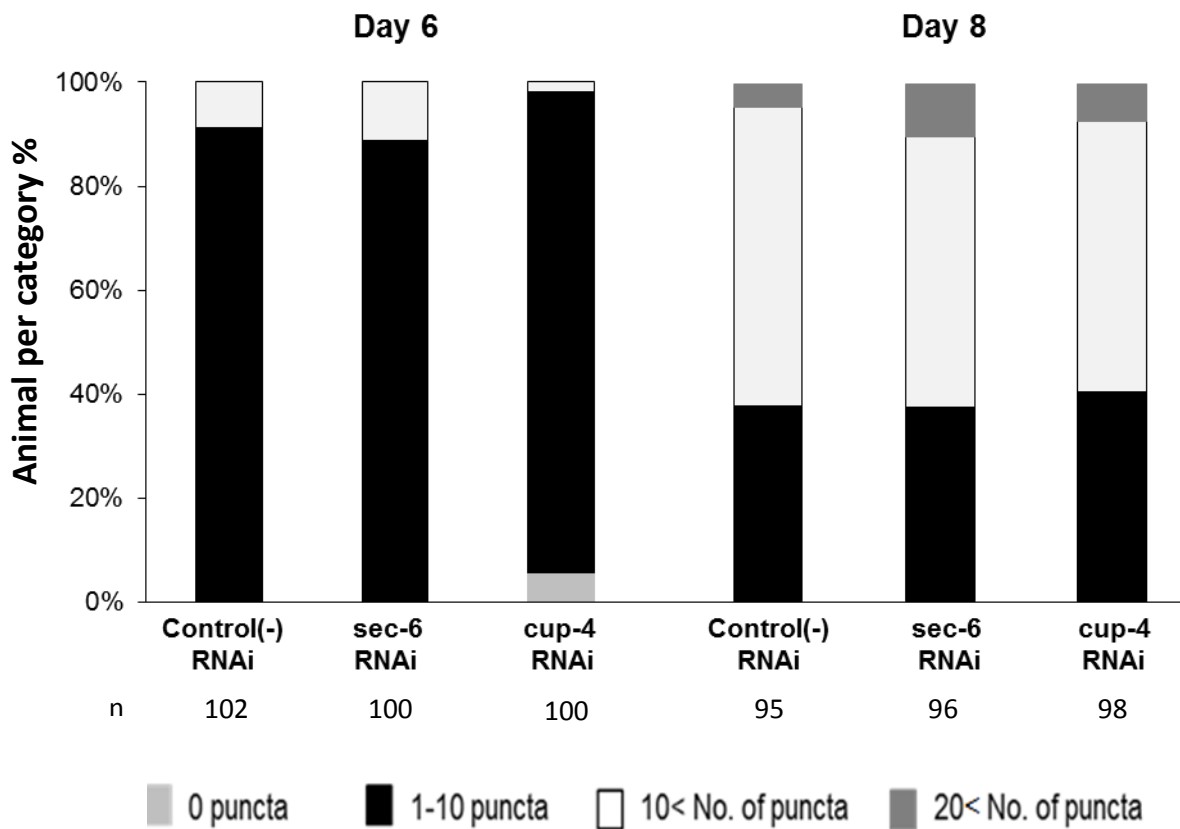
Figure 17: *sec-6* and *cup-4* do not have an effect on the extracellular LBP-2 accumulation. Wild type worms that were fed *sec-6* and *cup-4* RNAi did not had significantly higher rates of LBP-2 puncta accumulation than worms that were fed control RNAi. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. Number of worms evaluated is shown at the bottom. Details in appendix table 11.

To search for additional potential controls, I investigated two proteins TRY-1 and TEP-1; both of them have similarities to human protein regulators. The mammalian extracellular protein chaperone haptoglobin is homologous to *C. elegans try-1* gene (e=0.026). The *tep-1* has a homology to mammalian α2-macroglobulin (e=0.035), which has extracellular chaperone activity (Blandin and Levashina, 2004).
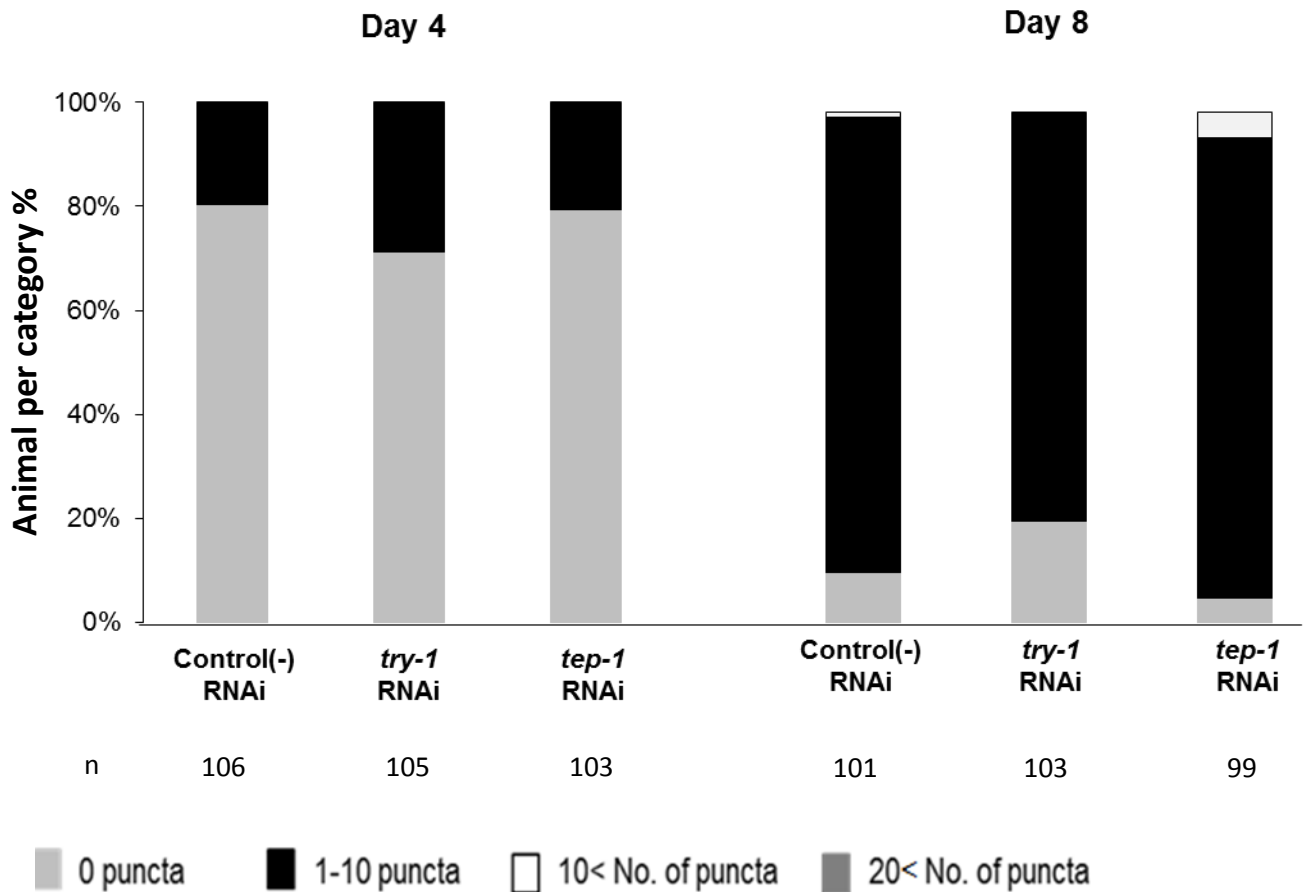
Figure 18: *try*-1 and *tep-1* RNAi does not have an effect on the extracellular LBP-2 accumulation. Wild type worms that were fed *try-1* and *tep-1* RNAi did not had significantly higher rates of LBP-2 puncta accumulation than worms that were fed control RNAi. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. Number of worms evaluated is shown at the bottom. Details in appendix table 12.

From my previous experiments, *rme-1* was the best positive control for increased accumulation that I had (Fig. 16). Therefore, I decided to test it with our temperature sensitive sterile strain expressing LBP-2 i.e., DCD130 (referred to as sterile worm). 100 L4 non-sterile LBP-2 transgenic worms were transferred to plates seeded with l4440 and *rme-1* RNAi and similarly it was done with sterile worms. On day 6, there was a significant difference in the rates of extracellular LBP-2 accumulation aggregation between the wild type worms growing on l4440 and the ones growing on *rme-1* RNAi. When I compared the number of worms on l4440 with 11-20 puncta, there were only 23.6% worms in this category,

while the wild type worms on *rme-1* had 48.2% worms in this category. However, no significant difference was observed in the sterile worm population growing on L4440 and *rme-1* RNAi.
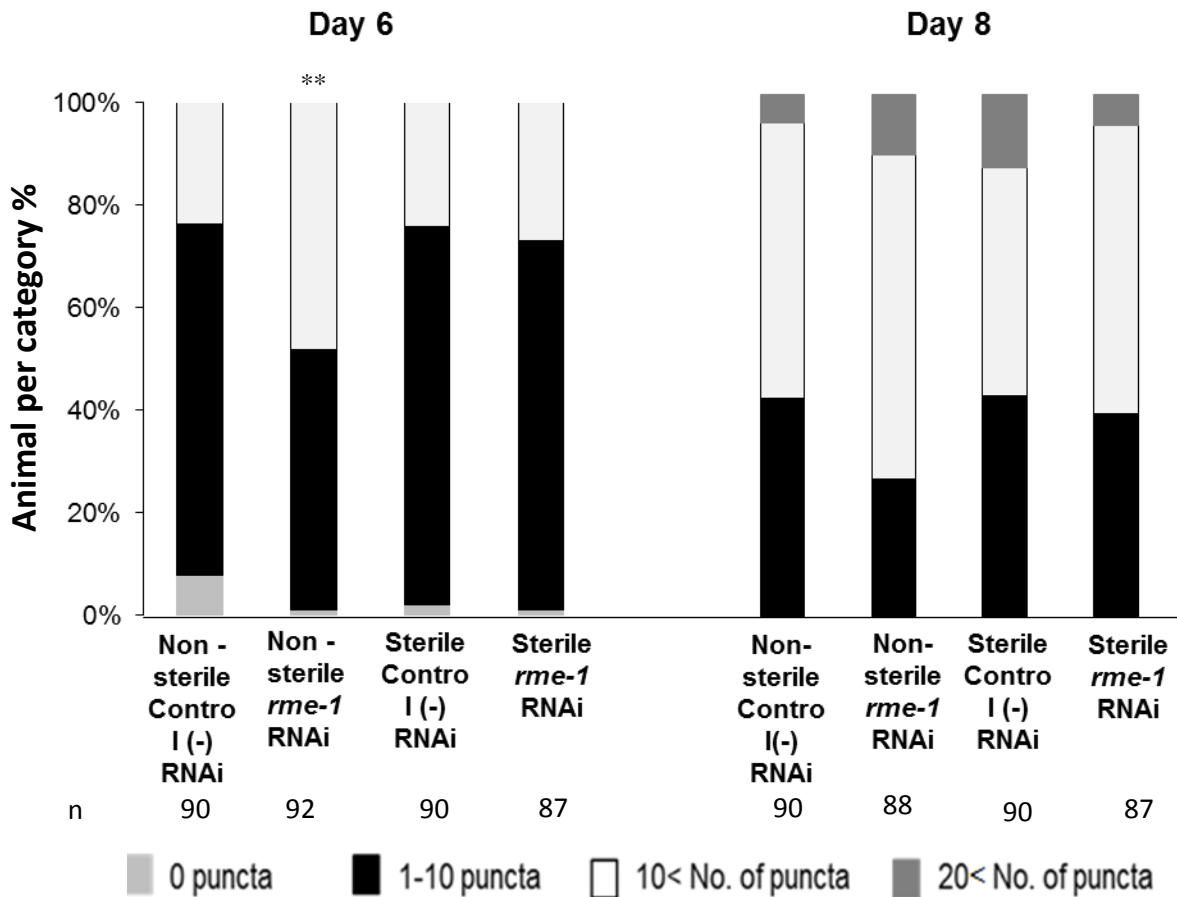
Figure 19: *rme-1* RNAi does not increase the rate of extracellular LBP-2 puncta accumulation in the sterile strain. Wild type worms that were fed *rme-1* RNAi had significantly higher rates of LBP-2 puncta accumulation than sterile worms that were fed *rme-1* RNAi. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 1-10 puncta category and 10 <No. of puncta was significant on day 6 and 8, Fisher's exact chi-square test **p <0.005 (for day 6). Number of worms evaluated is shown at the bottom. Details in appendix table 13.

Since *rme-1* RNAi wasn't working as a positive control with sterile worms. I decided to test the sterile worms with other potential candidates for a positive control. For the first experiment I chose *atg-18*, which plays a role in autophagy and *pbs-3* which plays a role in ubiquitin dependent protein degradation. I tested both non-sterile LBP-2 transgenics and

sterile LBP-2 transgenics with *atg-18* and *pbs-3* RNAi. On day 6, I did not observe higher rates of LBP-2 accumulation in the worms feeding on *atg-18* and *pbs-3* RNAi and on the contrary the rate of aggregation was slower in these worms (Fig. 20). The amount of extracellular LBP-2 accumulation was much lower in these worms when compared to the worms growing on l4440.
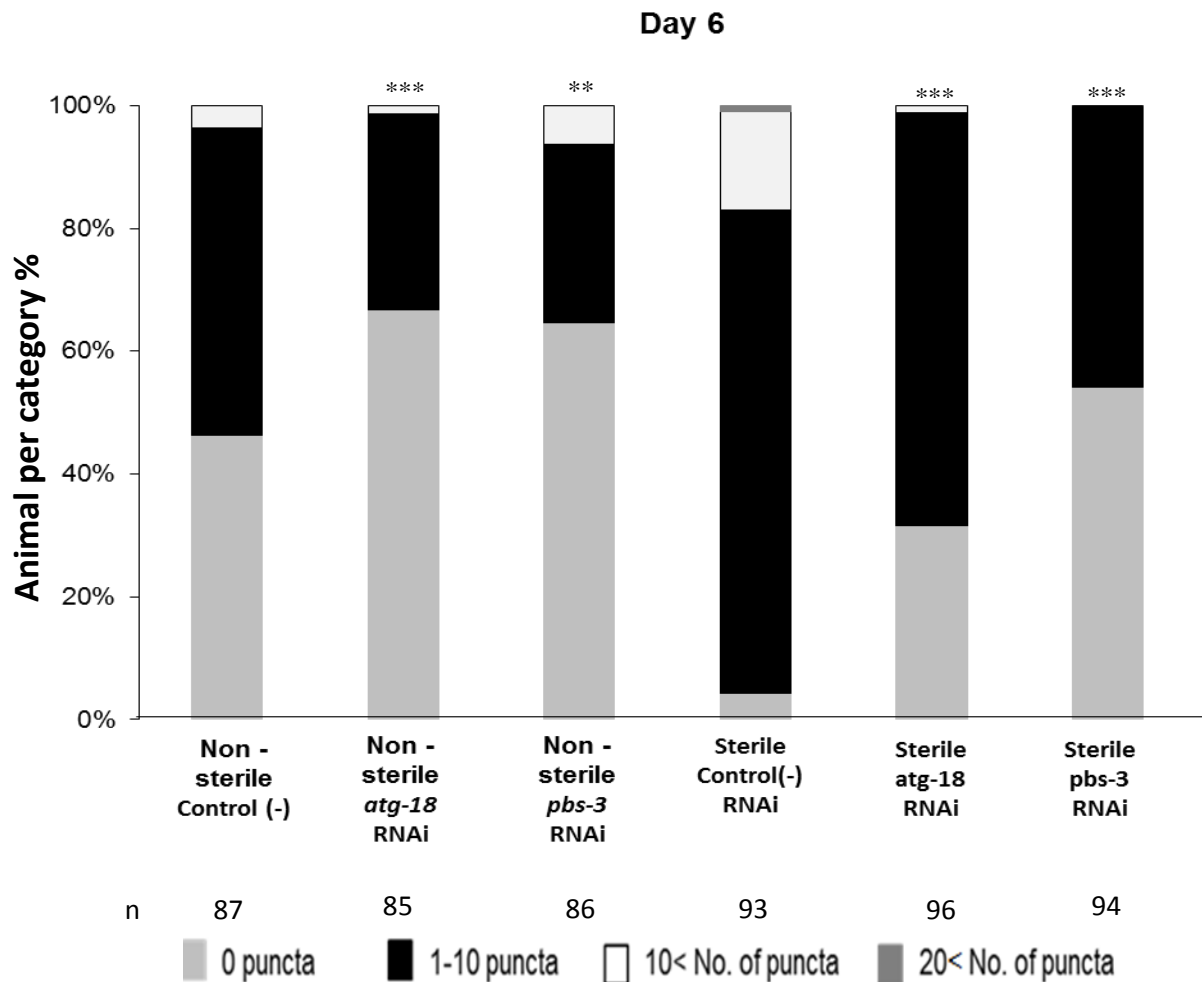
Figure 20: *atg-18* and *pbs-3* RNAi does not lead to more LBP-2 accumulation. Sterile worms that were fed *atg-18* and *pbs-3* RNAi did not had significantly higher rates of LBP-2 puncta accumulation than wild type worms that were fed the same RNAi. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 0 puncta category and 10 <No. of puncta was significant on day 6, Fisher's exact chi-square test P <0.01 (for Non-sterile pbs-3 RNAi), **p <0.01 (for Non-sterile atg-18 RNAi) and ***p <0.001 (for Sterile atg-18 and pbs-3 RNAi). Number of worms evaluated is shown at the bottom. Details in appendix table 14.

For the experiment in figure 21, I decided to pick out other candidates from the Fares and Greenwald, 2001a. In that publication these were other factors that were shown to regulate the coelomocyte function and the RNAi can affect the coelomocyte function to varying degrees. In this experiment I only tested sterile worms and I also used *gfp* RNAi and *her-1* as an additional negative control. *her-1* is required by the worms for sex determination (Hunter and wood, 1992; Streit *et al.*, 1999) and hence was used as an additional negative control. When the worms were observed on day 6 at 25°C, worms feeding on *dyn-1* and *rab-5* RNAi had the most number of worms with more than 10 puncta 3.9% and 7% respectively. These worm populations also had significant number of worms in 1-10 puncta category, 68.6% and 67.5% respectively. *rme-6* and *vps-54* also had a significant number of worms with puncta; with 59% of the worms on rme-6 and 57.6% of worms on *vps-54*.
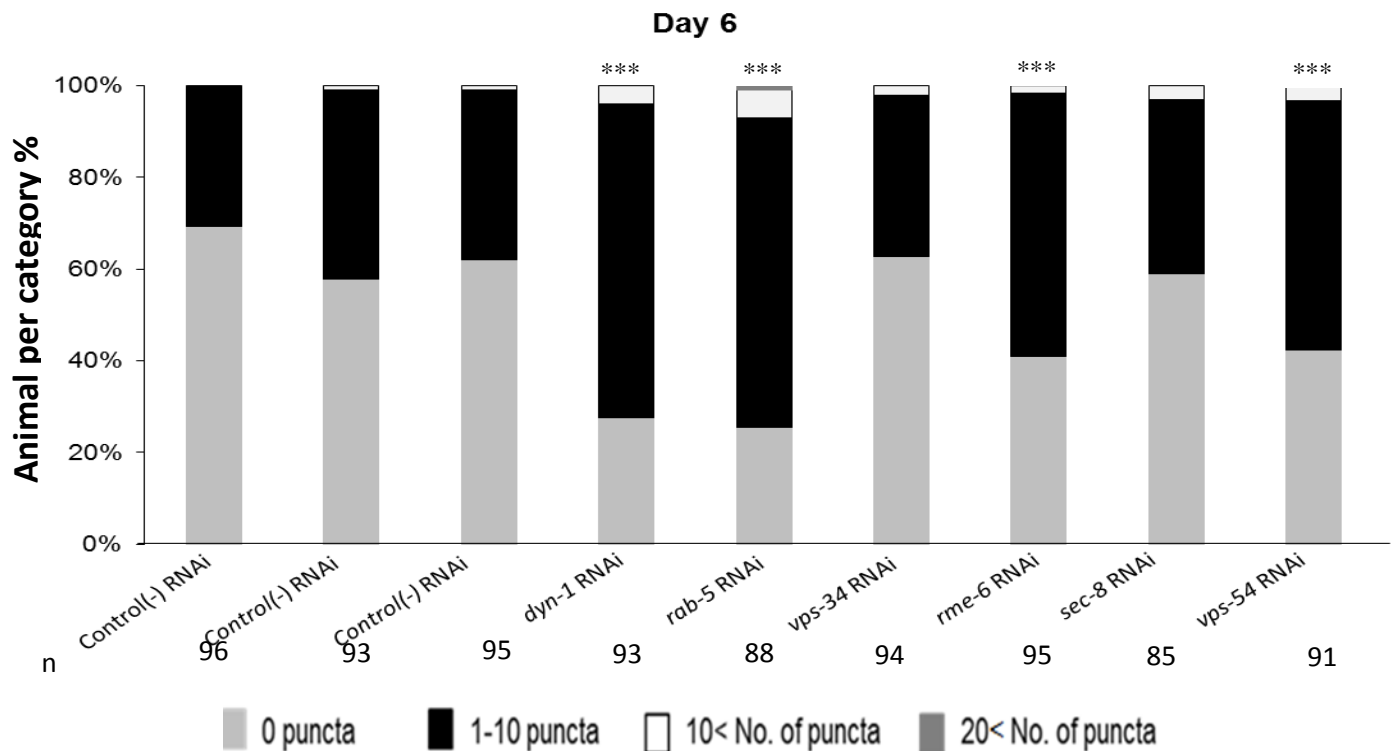
Figure 21: *dyn-1*, *rab-5*, *rme-6* and *vps-54* RNAi cause more LBP-2 accumulation. Sterile worms were fed *dyn*-1, *rab*-5, *rme*-6 and *vps*-54 RNAi had significantly higher rates of LBP-2 puncta accumulation than the control RNAi. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 0 puncta category and 10 <No. of puncta was significant on day 6, Fisher's exact chi-square test ***p <0.001 (for all four RNAi). Number of worms evaluated is shown at the bottom. Details in appendix table 15.

Before setting up the RNAi screen for secreted factors that modulate extracellular aggregation; I conducted a series of pilot screens to refine my scoring system and further evaluated my controls. In one such screen (Fig. 22), 4 random candidates were selected and these candidates were 1.1A7, 1.2D7, 1.1A11 and 1.1C3. When the worms were observed on day 6, both the positive controls (*rme-6* and *dyn-1*) for increased extracellular accumulation had a significant increase in the rate of LBP-2 accumulation.
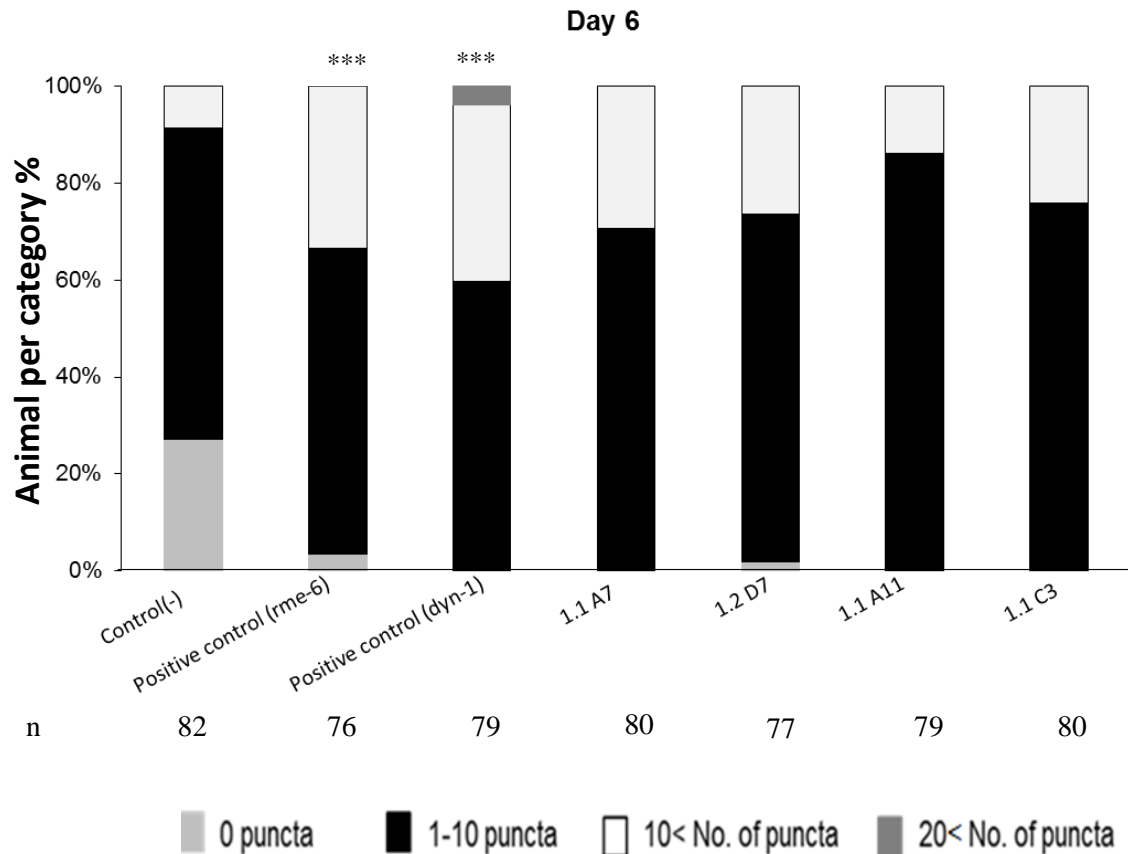
Figure 22: The pilot screen to test the scoring system and setup for the RNAi screen. The positive controls and the negative controls were tested. In addition few randomly picked clones were also picked to test. The name of the clone is as per their 96 well plate number and their well position on the plate. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 0 puncta category and 10 <No. of puncta was significant on day 6, Fisher's exact chi-square test ***p <0.0005 (*rme-6*) and ***p <0.0001 (*dyn-1*). Number of worms evaluated is shown at the bottom. Details in appendix table 16.

3.1.7 Sterile strain on l4440 does not have an extended lifespan

Before beginning the RNAi screen, I wanted to see if there is any difference in lifespan different between the sterile and the non-sterile strains. I was also interested to see if OP50 or l4440 had any effect on the lifespan. Both DCD23 and DCD130 were transferred to 25°C at L1 and then transferred to 20°C at L4. The worms were kept at a constant temperature of 20°C until the end of their lifespan. The DCD130 worm has a longer lifespan when grown on OP50 as 50% of its population was surviving until day 17. While with DCD130 on l4440 50% of the population was surviving until day 13. With the non-sterile strains DCD23 on OP50 and l4440 the survival of 50% of the population was only until day 14 and day 11 respectively. Therefore, there is an extended lifespan with the sterile strain when these worms are growing on OP50, although no change is observed when the same strain is growing on l4440.
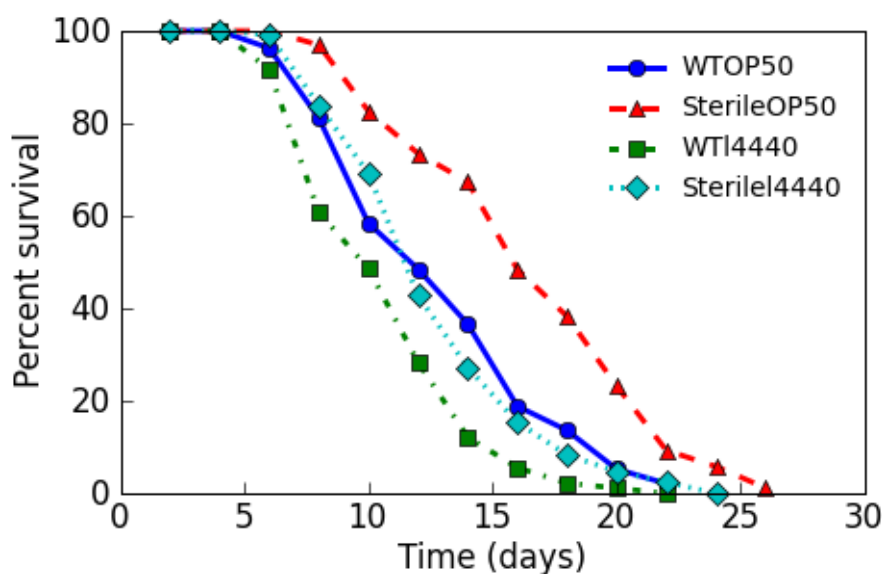
Figure 23: Lifespan evaluation of non-sterile and sterile LBP-2 transgenics on different food sources. Non-sterile LBP-2 worms and sterile LBP-2 worms feeding on l4440 control RNAi bacteria as a food source had similar lifespans. There was a little significant increase in lifespan when sterile worms were grown on OP50 versus non-sterile worms on OP50 (p value 0.001). Details in appendix table 17.

Section 2

3.2.1 RNAi screen for modulators of age dependent extracellular aggregation

RNAi screen is a commonly used method to investigate the functions of different genes in *C. elegans* (Poulin *et al*., 2004; Boutros and Ahringer, 2008). Although there are different ways to introduce the RNA into the organism, the most commonly used method is through feeding. I was interested in finding out the factors that might regulate the extracellular protein quality control. In this section, I conducted an RNAi screen to look for such a factor.

3.2.1.1 Pilot screen for modulators of extracellular aggregation

Before setting up the main RNAi screen for factors that modulate extracellular aggregation; I conducted a series of pilot screens with ~200 secreted factors to refine my scoring system and further evaluated my controls. In addition, during the pilot screen a robust scoring system was established and was further refined during the repeated pilot screens. For the screen the sterile LBP-2 transgenic worms were used. The worms were age synchronised and after the L4 larval stage, they were transferred to RNAi plates which were seeded with different RNAi clone. For the screen, there were 3 replicate plates per RNAi clone. There were ~35 worms on each replicate plate. After the worms were transferred to the replicate plate, they were kept at 25°C and observed for aggregation on day 6 or day 7 of adulthood. For scoring the plates for the screen, the plates with the RNAi clones were compared to the negative and the positive clones for increased aggregation. If the worms on each plate from the RNAi clone replicate had more or equal amount of LBP-2 puncta accumulation when compared to the positive control, that plate was marked as a positive plate. The RNAi clones with two or more positive plates out of the three replicates were scored as positive clones. The other clones were counted as negative clones or clones without an effect.

3.2.1.2 Screen for the modulators of extracellular aggregation

The screening for modulators of extracellular aggregation was done in many rounds. In total, RNAi clones of 1508 secreted factors were screened. To find these factors, a thorough search was conducted through the wormbase database to search for all the *C. elegans* genes with a transmembrane or signal domain. 3299 such genes were found. These genes were then analysed using the TMHMM v 2.0 prediction servers. TMHMM is a method for predicting transmembrane helices based on the hidden Markov model. The sequences from the wormbase database were scrapped using a python scrapping library and were stored. The sequence was extracted from the file and submitted to the server in text form. The script ran one sequence at a time and the result were returned to an excel file (appendix script 1). Using the result from the TMHMM servers, the numbers of factors were reduced to 1508. The RNAi for these 1508 clones were used for the RNAi screen. From these 1508 clones, there were 1346 clones that showed no effect on the extracellular LBP-2 accumulation. In these 1346 clones no significant increase in the extracellular LBP-2 puncta accumulation was observed. For 162 RNAi clones, I observed a very clear effect on the extracellular aggregation as there were more LBP-2 puncta in the worms growing on the 162 clones when compared to the negative control l4440. These 162 clones were to be further evaluated to see whether their effect was reproducible and significant.

Whole Screen
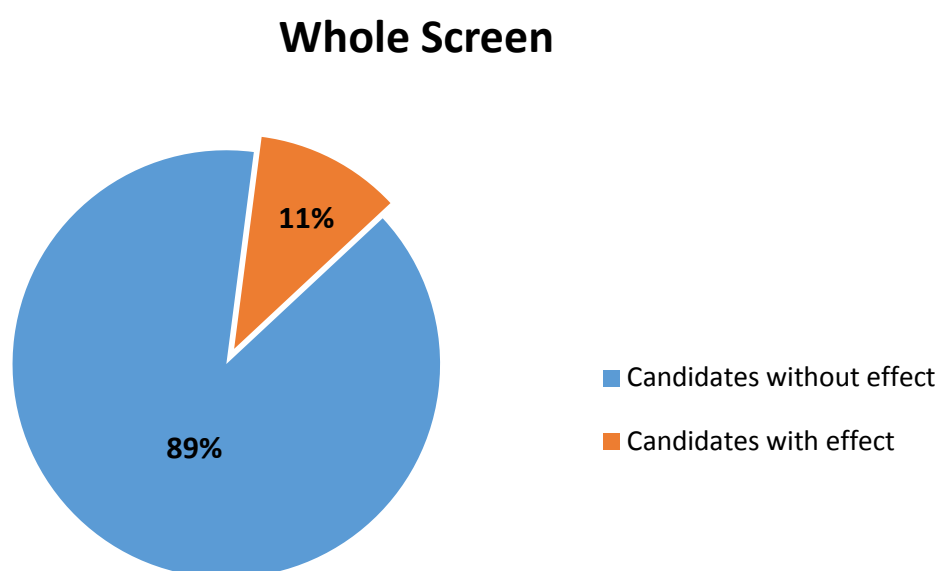
Figure 24: RNAi screen. RNAi screen to look for factors that regulate extracellular protein aggregation was conducted. From the 1508 clones, 1346 clones had no effect on the extracellular protein accumulation and 162 clones had an effect.

3.2.1.3 Repeat screen for further analysis of the candidates

From the RNAi screen for modulators of extracellular aggregation, 162 RNAi clones were discovered. These 162 clones were further screened in a secondary validation screen with new and more stringent scoring system so the number of the candidates can be further narrowed down and only the candidates with the strongest effect remain. There were 4 plates per clone with ~30 worms per plate. When the worms on a plate showed an effect, the plate was considered to be a positive plate. Therefore the scoring categories differed as per different number of positive plates. In the repeat screen, 103 clones out of the 162 showed no effect when re-evaluated with the new scoring methods. There were 33 clones with 3 positive replicate plates and 4 positive replicate plates in this repeat screen. Of these 33 clones, 15 clones were also scored positive in the three replicate plates in the initial screen. These 15 clones were picked for additional analysis.
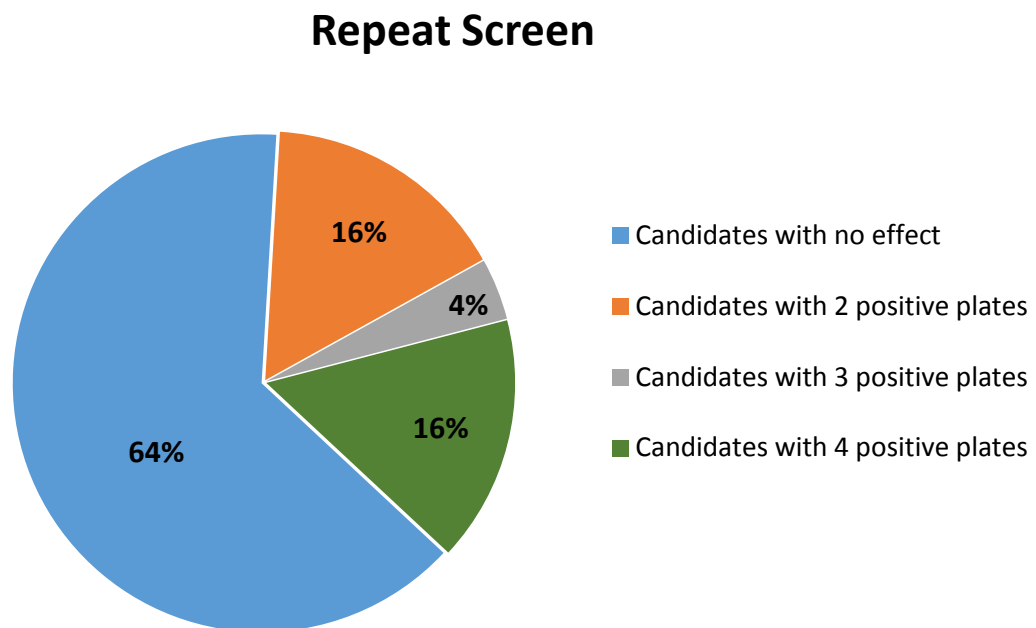
Repeat Screen

Figure 25: Repeat screen of the positive clones from the RNAi screen. The repeat screen with the 162 positive clones from the RNAi screen was conducted. From the repeat screen 103 clones did not have any effect, 26 clones had only 2 positive plates, 7 clones had 3 positive plates and 26 candidates had all 4

3.2.2 Fifteen potential candidates that modulate extracellular aggregation

The 15 clones from the initial screen and the repeat RNAi screen were more extensively evaluated for their effect on the extracellular LBP-2 accumulation by counting the number of worms in the population with different levels of puncta. When they were evaluated on day 6 of their adulthood, the worms growing on ECRC_7 RNAi had the highest number of worms i.e. 29.6% in the 11-20 worms category. The amount of LBP-2 puncta accumulation observed in ECRC_13, 3, 5, 1, 10, 4, 2, 7, 15, 9, and 1 was significantly higher when compared to the control l4440 ($p<0.001$). Although less significant, this was also true for the amount of LBP-2 accumulation in ECRC_8 and ECRC_14 (higher $p<0.01$ and $p<0.05$ respectively). However, the rate of accumulation in the worms that were fed ECRC_12 and ECRC_6 was not significant. Henceforth I had a list of potential candidates as modulators of extracellular accumulation.

Day 6
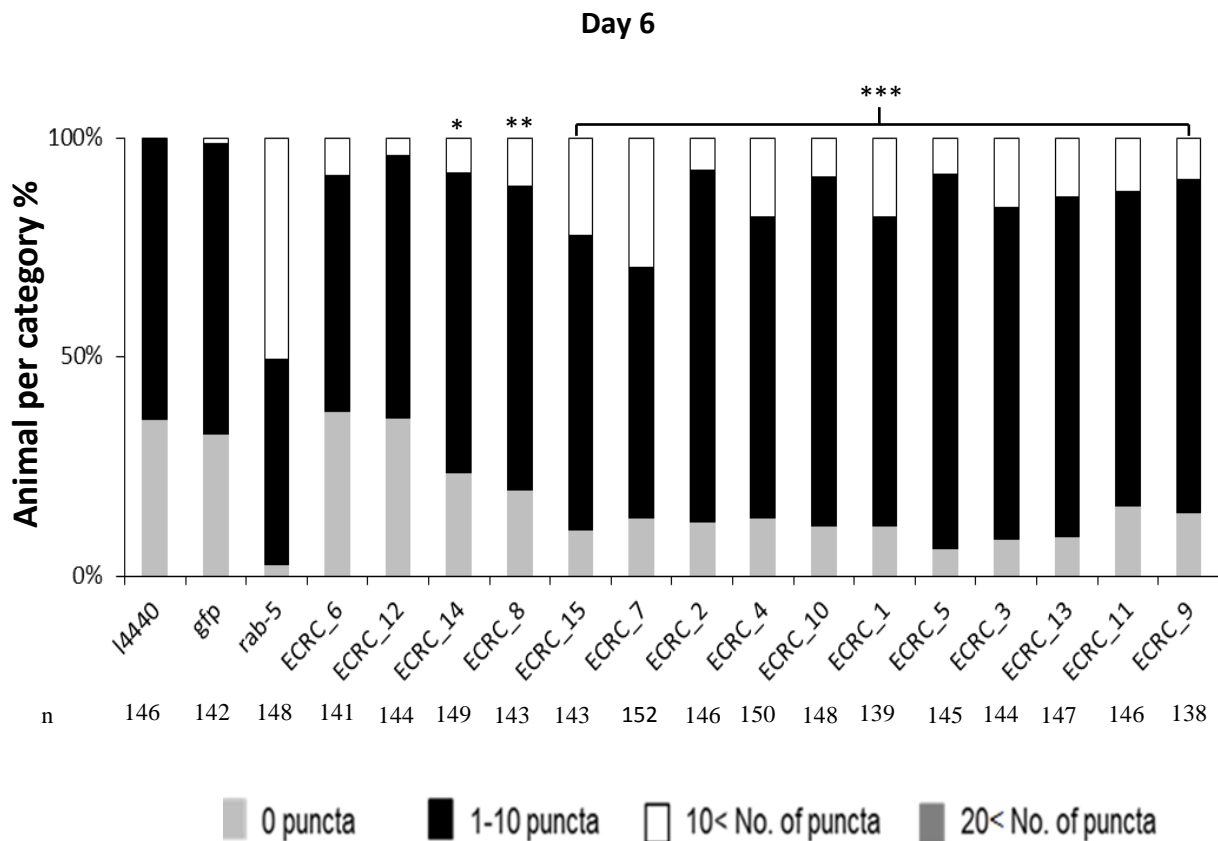
Figure 26: 13 candidates from the RNAi screen were significant in evaluation. Out of the 15 potential candidates as a regulator of extracellular protein accumulation, 13 candidates were positive. The RNAi of these positive candidates caused a significant increase in the rate of accumulation of extracellular LBP-2 puncta. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 0 puncta category and 10 <No. of puncta was significant on day 6, Fisher's exact chi-square test *p <0.05 (ECRC_14), **p <0.01 (ECRC_8) and ***p <0.001 (ECRC_1,2,3,4,5,7,9,10,11,13,14,15). Number of worms evaluated is shown at the bottom. Details in appendix table 18.

The two clones from the earlier screen which did not have a significant effect on the rate of LBP-2 accumulation were tested again. On day 6, there was a there was a significantly higher amount of LBP-2 puncta in the ECRC_3 (p<0.05) and ECRC_12 (p<0.05) when compared to

the negative control l4440. ECRC_6 RNAi did not lead to a significant increase in the rate of LBP-2 puncta accumulation.
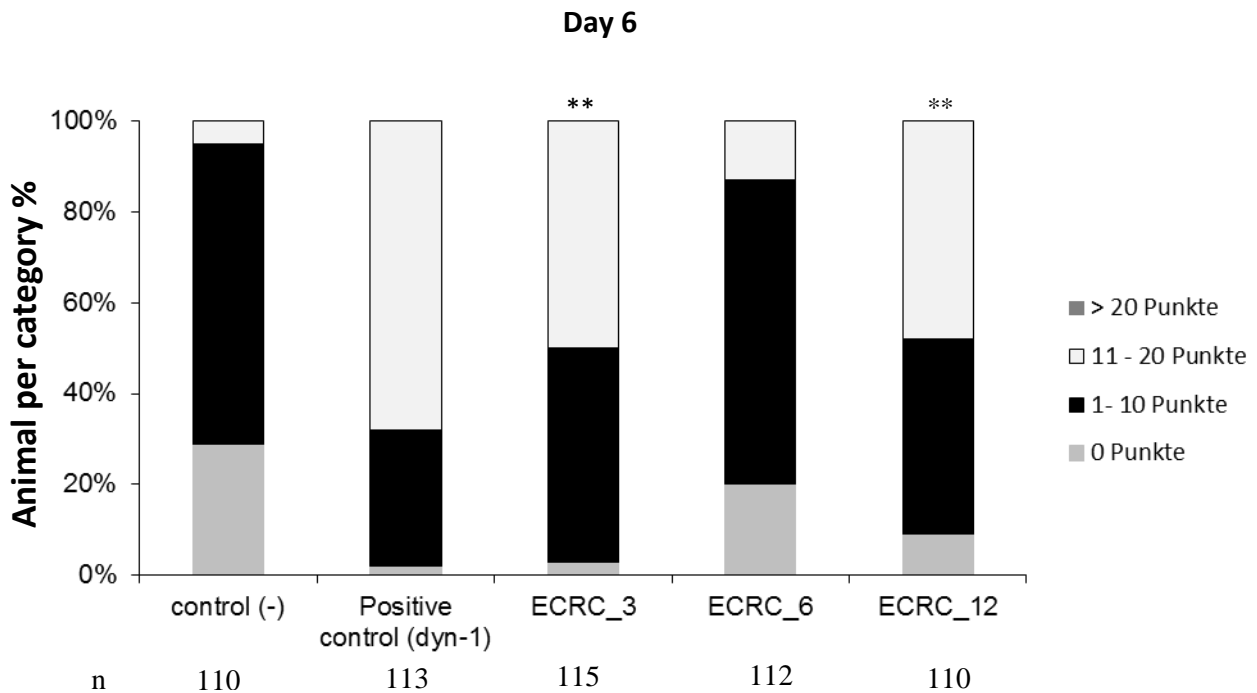
Figure 27: ECRC_12 RNAi leads to a significant increase in extracellular LBP-2 puncta. Evaluation of ECRC_6 and ECRC_12 showed that ECRC_12 RNAi causes a significant increase in the rate of LBP-2 puncta accumulation. The worms were classified into four groups depending on the number of LBP-2::tagRFP puncta present in the head. For statistical analysis, the categories with puncta were compared to the category without any puncta and the categories with less than 10 puncta was compared with the category with more than 10 puncta. The difference between 0 puncta category and 10 <No. of puncta was significant on day 6, Fisher's exact chi-square test P <0.005 (ECRC_3, ECRC_12). Number of worms evaluated is shown at the bottom. Details in appendix table 19.

Section 3

3.3.1 Functional analysis of the candidates for regulators of extracellular accumulation

From the genome wide RNAi screen, I now had 15 potential modulators of extracellular aggregation. I was interested in characterising them functionally and to dissect the mechanism through which these candidates affected the extracellular aggregation.

For this experiment I wanted to look into the effect of these candidates on the endocytotic uptake by the coelomocytes. Coelomocytes are 3 pair of scavenger cells which are located in the in the pseudocoelomic cavity. Coelomocytes are known to uptake substances by endocytosis from the body cavity of *C. elegans* (Fares and Greenwald, 2001a).

I decided to use the GS1912 *(myo-3p::ssGFP + dpy-20(+))* worm strain for this experiment, as this strain has already been used in various studies related to endocytotic uptake by coelomocytes (Fares and Greenwald, 2001a, 2001b). In this strain the green fluorescent protein containing a secretory signal is secreted into the body cavity by the muscle cells and taken up by the coelomocytes by endocytosis (Fares and Greenwald, 2001a). Therefore, in this experiment I planned to feed these worms RNAi for all of the 15 candidates and the controls to see whether those RNAi have any effect on the amount of uptake by the coelomocytes.

Initially the worms were tested to find out the best temperature, time point and the scoring system. For the experimental setup, the worms were kept at 25°C and were analysed on day 1 of adulthood. For evaluating the effect of the RNAi the best measure was to count the number of worms with no visible fluorescence in the coelomocytes. The RNAi screen was tested 5 times and the results were culminated from all the 5 repetitions. Neither of the potential candidates had any significant effect on the endocytotic function of the coelomocytes. The number of worms without coelomocytes was higher in ECRC_12, 1 and 3; although, this difference was not significant. Therefore, none of the 15 potential candidates showed any effect on extracellular accumulation by regulating the function of the coelomocyte.
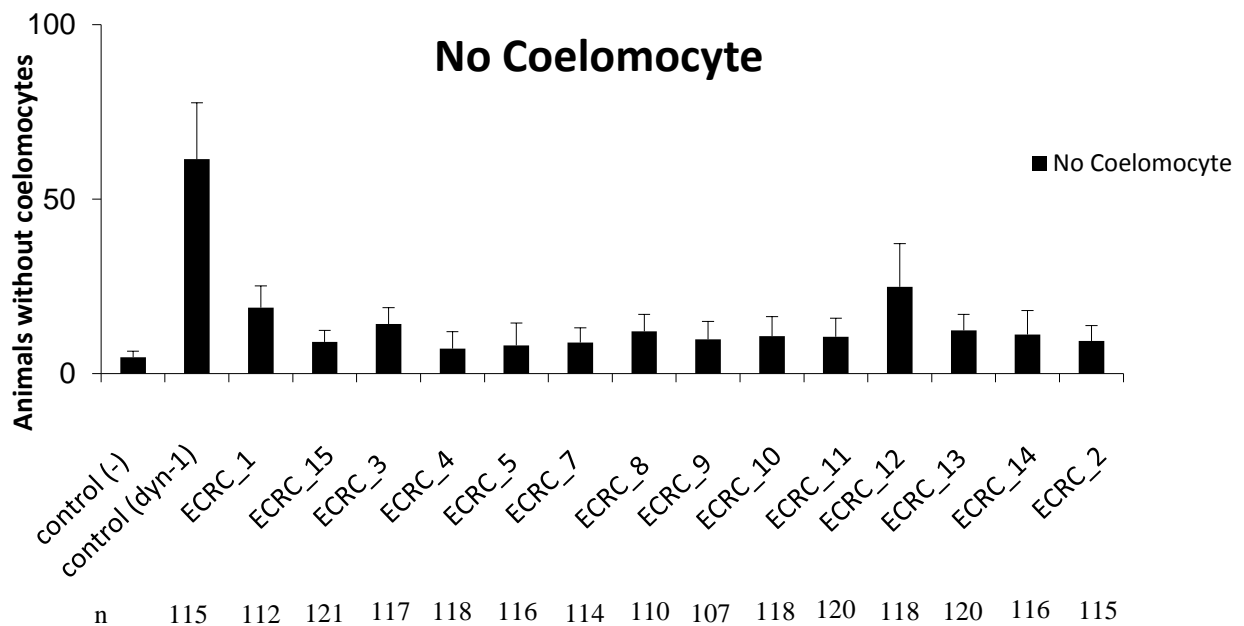
Figure 28: Neither of the candidates leads to an increase in LBP-2 puncta via disrupting coelomocyte function. Therefore the candidates do not play a role in endocytosis by the coelomocyte. Animals were grouped as per the lack of visibility of their coelomocyte; *myo-3p::ssGFP + dpy-20(+)* worms were used as they secrete green fluorescent protein in the body cavity which is then taken up by the coelomocytes. Details in appendix table 20. Bars: mean ± sem.

3.3.2 Disruption of extracellular proteostasis does not affect the lifespan of the organism

Lifespan analysis is a commonly used method to look into the effect of a certain gene/protein in the worms (Apfeld and Kenyon, 1999; Kenyon *et al.*, 1993). To investigate if downregulating the modulators of extracellular LBP-2 aggregation affects aging, I performed a lifespan assay using the sterile transgenic strain expressing LBP-2 exposed to RNAi against the different candidates.

For this the worms were moved from 25°C to 20°C at L4 onto the respective RNAi. For lifespan assessment the number of live worms were counted every second day. The positive control *rab-5* had an adverse effect on the lifespan of the worms (Fig. 29). On the other hand, no adverse effect on lifespan was observed with the RNAi from other potential candidates

being similar to the negative control (Fig. 29). Though the lifespan of worms with ECRC_5 was 2 days shorter, this effect was not significant (Fig. 29(B)).

A

B

C

D
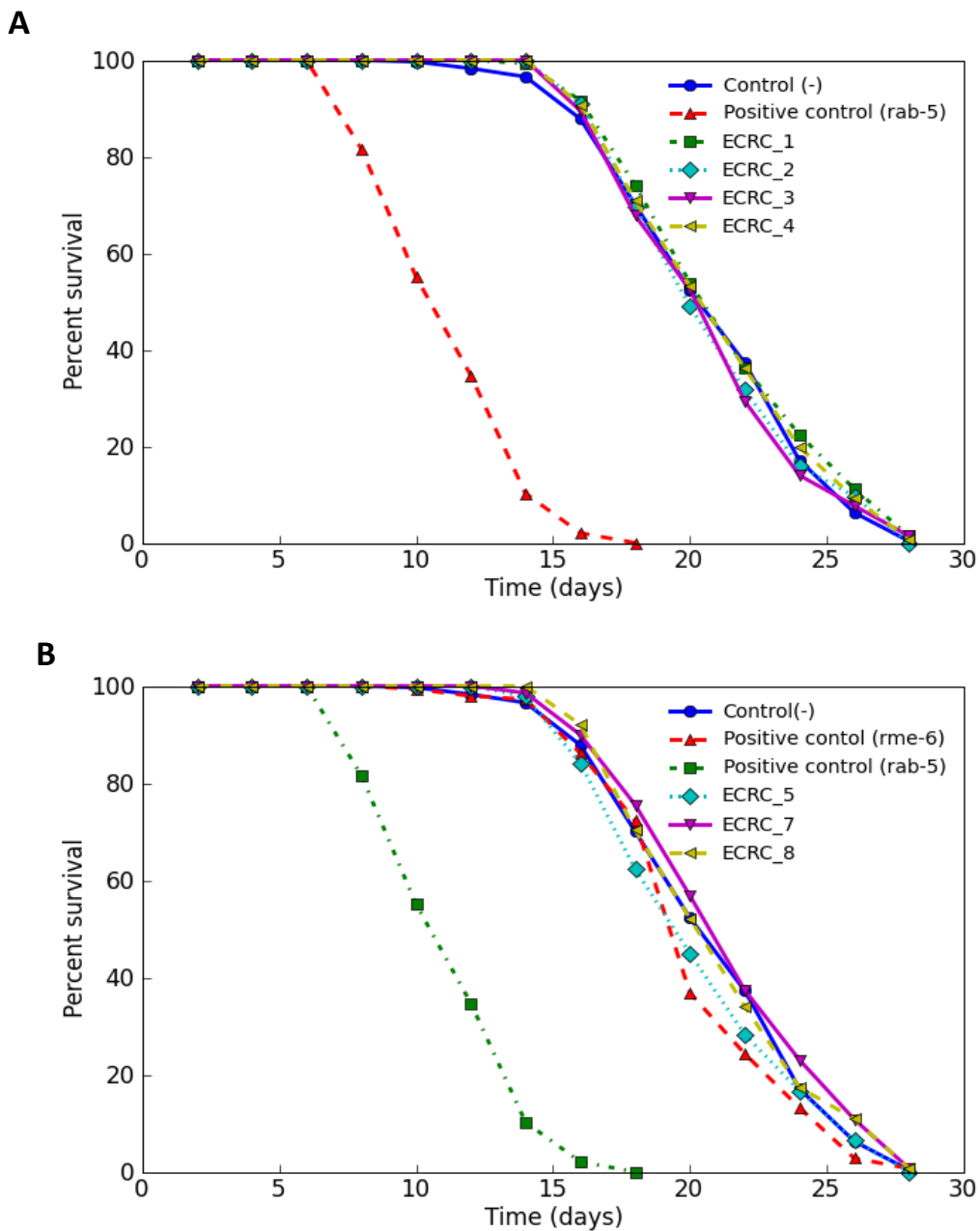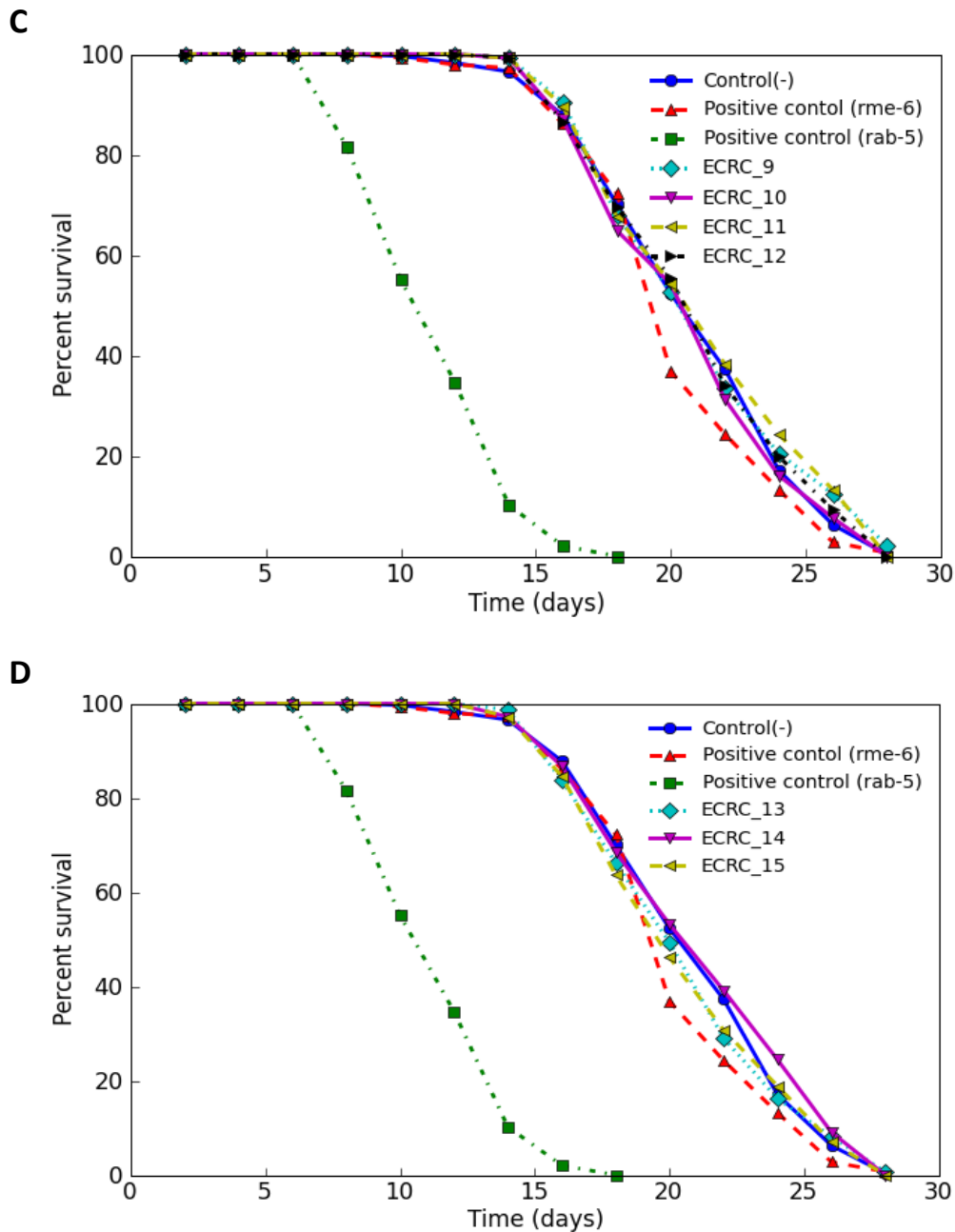
Figure 29: The candidates do not affect the lifespan of the worms. A) ECRC_1, 2, 3 and 4 were observed for their effect on the lifespan of the worms. *rab-5* and *rme -6* RNAi, a positive control caused shortening of the lifespan of the worms. **B)** The RNAi of candidate ECRC_5, 7, and 8 did not cause any change in the lifespan of the worms. **C)** The RNAi of ECRC_9, 10, 11, 12 did not cause a change in the lifespan of the worms. **D**) No change in the lifespan was observed when the worms were fed RNAi of ECRC_13, 14 and 15. Details in appendix table 21

3.3.3 Disrupting extracellular proteostasis affects heat stress sensitivity in *C. elegans*

Studies have shown that homologs of genes involved in stress response in *C. elegans* play a key role in the development of age-related diseases (Hsu *et al*., 2003; Anckar and Sistonen, 2007; Rodriguez *et al*., 2013). I was interested to validate the role of the potential candidate in the regulation of heat stress resistance in the worms. To set up such an experiment, I tried testing the temperature sensitive sterile worms first. The worms were kept at 25°C until day 6 and on day 6 they were moved to 34°C and then the number of living worms were evaluated at different time point (Fig. 30). However, after repeated attempts, I could not find a consistent middle point to make a count of the number of worms that survived.
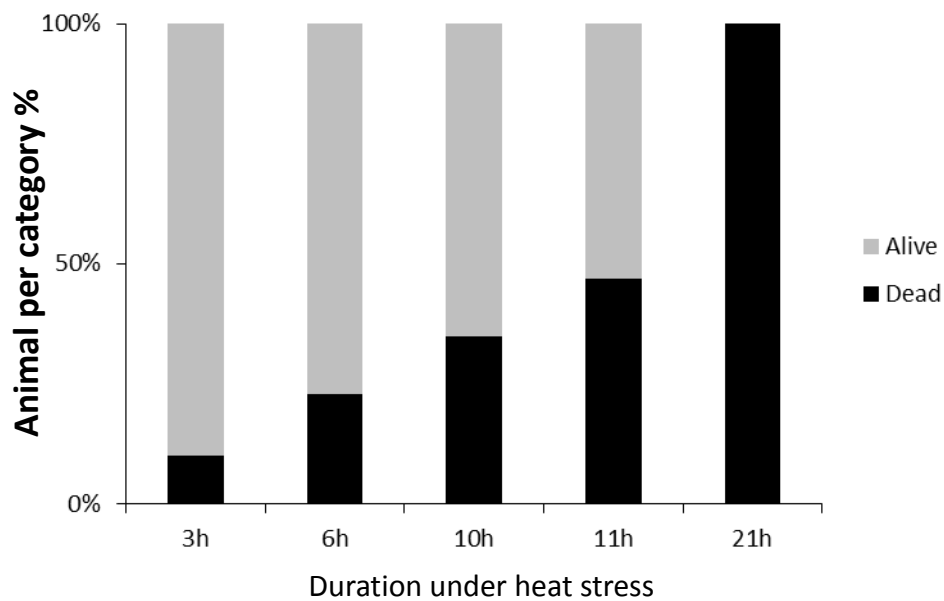
Figure 30: Sterile worm survival for heat stress resistance. The temperature sensitive sterile worms show a gradual decline in the living population under the heat stress at 34°C. After 3hours only 90% worms were alive. After 11hours 53% of the worm population was still alive and after 21hours under heat stress, 100% of the worm population was dead. Details in appendix table 22.

With non-sterile worms I tried the heat stress at day 1. The worms were grown at 25°C and then transferred to 34°C for the heat stress. I also grew the worms on l4440, *gfp* and *cup-4* RNAi. As I wanted to see if inhibiting the endocytotic uptake by the coelomocyte would influence the heat stress resistance in the worms. On day 1, after 13 hours 98% of the worms growing on *gfp* RNAi were alive, while 96% of the worms growing on l4440 were alive and 90% of the worms growing on *cup-4* RNAi were alive. After 23 hours, there was a significant decline in the population of worms growing on either RNAi. 40% of the worms growing on *gfp* RNAi were alive, while 38% and 20% of the worms growing on l4440 and *cup-4* RNAi respectively were alive. However, even after repeated trials this setup I could not get a consistent time point to analyse the worms.
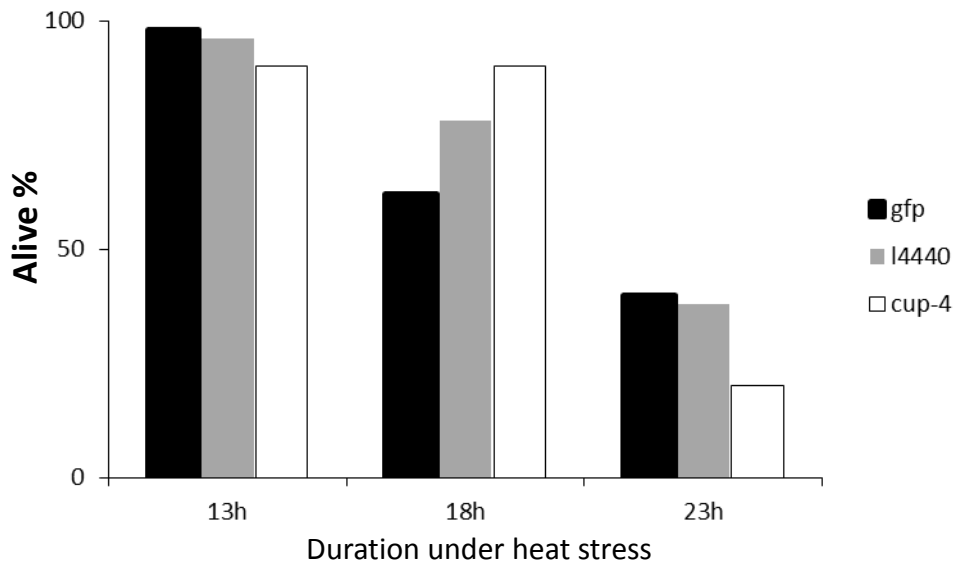
Figure 31: Wild-type worm survival for heat stress resistance. The wild-type worms show a gradual decline in the living population under the heat stress at 34°C. After 13 hours 98%, 96% and 90% of the worms growing on *gfp*, l4440 and *cup-4* RNAi respectively were alive. After 18 hours 62%, 78% and 90% of the worm population growing on was *gfp*, l4440 and *cup-4* RNAi respectively was alive and after 23 hours under heat stress, 40%, 38% and 20% of the worm population growing on was *gfp*, l4440 and *cup-4* RNAi respectively was alive. Details in appendix table 23.

Therefore, I decided to carry out a different setup for the experiment. For this the sterile worms were kept at 20°C from L4 stage till day 8 of adulthood when they were moved to 35°C on day 8 of adulthood. With this setup I managed to find a consistent time point of 50% survival of the worms. There was a significant decrease in the heat resistance in the worms feeding on ECRC_1, 3 and 4 RNAi; these worm populations had the maximum number of

worms that were dead. ECRC_14, 5, 9, 12 and 13 RNAi also led to a significant decrease in the heat stress survival resistance of the worms albeit to a lesser extent. ECRC_2, 7, 8, 10, 11 and 15 had no effect on heat stress resistance.

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n   117   120   111   115   107   113   102   109   110   111   105   114   119   112   98   108
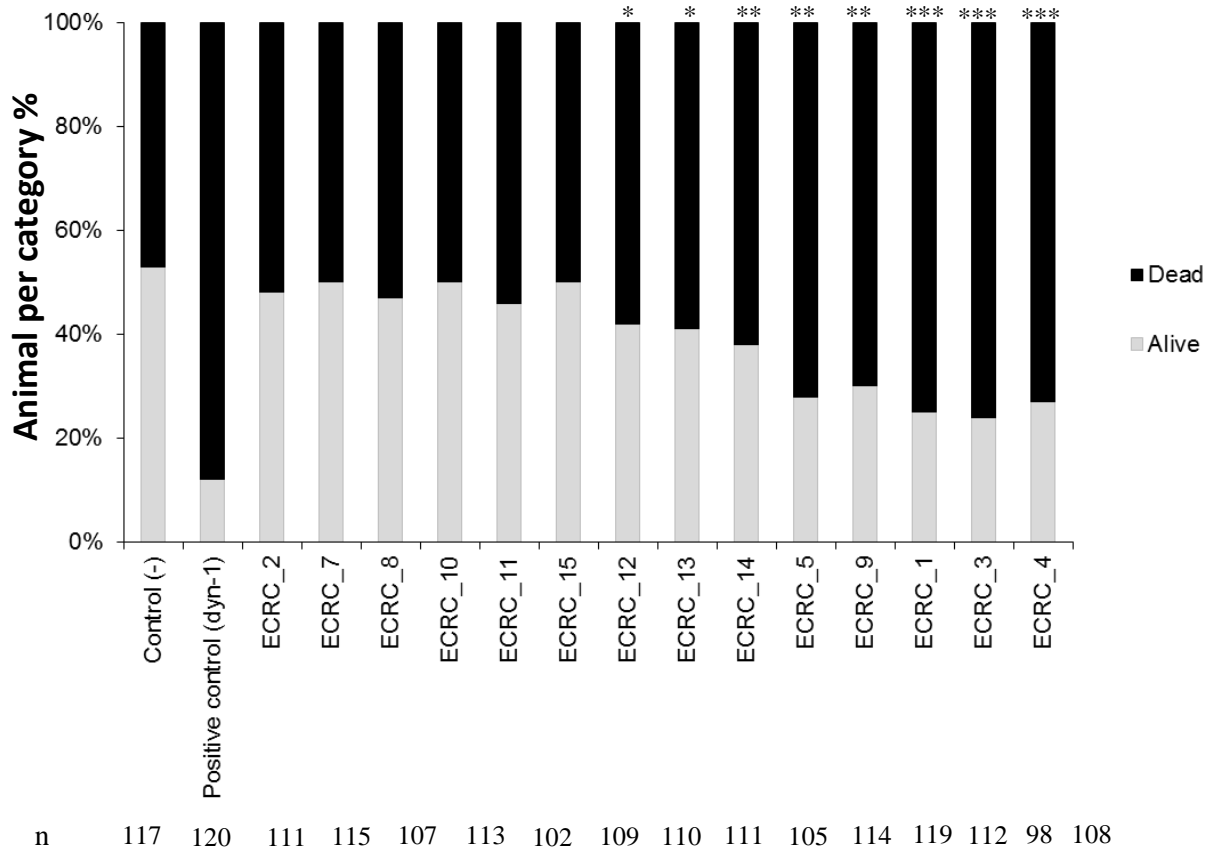
Figure 32: Eight candidates cause a change in the heat stress resistance. The worms that were fed ECRC_1, 3, 3, 5, 9, 12, 13, 14 RNAi had significantly lower levels of heat stress resistance. The heat stress resistance was measured by the survival of the 8 days old worms which were fed different RNAi and then exposed to 34°C. The worms were classified into two groups, the worms that were alive and the worms that were dead. For statistical analysis, the number of alive worms was compared to the number of dead worms. The difference between alive and dead category was significant, Fisher's exact chi-square test P <0.05 (ECRC_12 and 13), P <0.01 (ECRC_14, 5 and 9) and P <0.001 (ECRC_1, 3, and 4). Number of worms evaluated is shown at the bottom. Details in appendix table 24.

Discussion

In this study, I investigated the potential regulators of extracellular protein aggregation. I carried out an RNAi screen of secreted factors to search for modulators of extracellular protein aggregation. From the RNAi screen, 15 different regulators of extracellular protein aggregation were discovered. The effect of these potential regulators on ageing and functional pathways was also investigated.

4.1 Model organism for extracellular aggregation

In this study, I wanted to observe age mediated protein insolubility. Therefore, I used Lipid Binding Protein or LBP-2 as a protein of interest. I decided to use LBP-2 as it has been shown to become insoluble with age (David *et al*., 2010). LBP-2 encodes a homolog of fatty acid binding protein As-p18, which is found in *Ascaris suum* and is a member of intercellular fatty acid binding protein family. LBP-2 plays a critical role in sequestering potential toxic fatty acids and their peroxidation products. LBP-2 also contains putative secretory signals. When they conducted a study on the expression pattern of *lbp-2* by using a *plbp-2::gfp* transcriptional reporter, the expression was restricted to body wall muscle which is adjacent to the pseudocoelom. Thus, suggesting that the LBP-2 protein is produced in the body wall muscle before being secreted into the pericenteric fluid of the pseudocoelom (Plenefisch *et al*., 2000). In the experiment design, LBP-2 protein was tagged with tagRFP and hence any accumulation of the LBP-2 could be quantified in the worms. I also observed that the localization pattern of *Plbp-2::lbp-2::tagrfp* in the model worms was similar to another worms model where GFP was secreted from the body wall muscle into the pseudocoelom (Fares and Greenwald, 2001a). Initially, I investigated if there was any difference in the rate of extracellular LBP-2 accumulation between the two integrated strains and then if there was a difference between the integrated and non-integrated strain. This was done to validate that the rate of LBP-2 accumulation in the integrated strains and to see if there was any difference in the rate of LBP-2 accumulation when *Plbp-2::lbp-2::tagrfp* is integrated in the worms genome and when it is in the extrachromosomal array. There was a slight but insignificant difference between the integrated and the non-integrated strain. Suggesting, that whether the

lbp-2::tagrfp is in the extrachromosomal array or integrated in the genome, the rate of LBP-2 accumulation was the same. After testing my model organism with the tagged protein of interest, I was interested in the effect of food source on the levels of extracellular accumulation. Under standard laboratory conditions, the food source of the worms is an *E. coli* strain OP50. For RNAi screens, the *E. coli* strain HT115 is transfected with dsRNA (double stranded RNA) of interest and is fed to the worms. The HT115 bacterial strain also contains IPTG inducible T7 polymerase (Timmons *et al*., 2001). For any studies involving RNAi, l4440 is commonly used as a negative control as it contains just the empty vector. As my aim was to conduct a RNAi screen, I decided to check whether the bacterial strains had any effect on the extracellular LBP-2 accumulation. There was no significant difference in the rate of LBP-2 accumulation between the worms fed with either strain during the early or middle time-point of adulthood. However, during the late stages of adulthood there was a marked difference with an increase in the number of LBP-2 puncta in the worms that were fed OP50 compared to the worms that were fed l4440. One possible reason for such a difference in the number of puncta could be the difference in toxicity of the two *E. coli* strains. Earlier studies have shown that regular food source used in the laboratory is slightly toxic to the worms. *E. coli* cells can accumulate and block the pharynx and the intestine of the worms (Garigan *et al*., 2002). Another possibility could be that there is some unspecific RNA interference by the l4440 vector backbone which reduced the rate of extracellular LBP-2 accumulation at later stages. To check whether this effect is due to unspecific RNAi, I used a worm strain with mutation in the *sid-1* gene. SID-1 is a transmembrane protein that is critical to systemic RNAi in *C. elegans* (Feinberg and Hunter 2003; Winston *et al*., 2002). When the *sid-1* mutant worms were fed either of the two *E. coli* strains, during the early stages of lifespan, their rate of LBP-2 accumulation was similar to the worms without the mutation. However, there was more aggregation in the worm on OP50 during later stages of their lifespan. This higher rate of LBP-2 accumulation was similar to what was observed in the worms without the mutation. Therefore, it could not have been due to unspecific RNAi. Hence it is more likely that the composition of different the E. coli strains used to feed the worms has an effect on LBP-2 accumulation late in life.

4.2 Extracellular protein accumulation is slowed down by Insulin/IGF-1 signalling pathway

In early ageing associated studies with *C. elegans,* it was discovered that mutations in *daf-2* gene doubles the life span of worms (Kenyon *et al*., 1993; Dorman *et al*., 1995). In *C. elegans*, the *daf-2* gene encodes for the insulin-like growth factor 1 (IGF-1) receptor. The cloning of *daf-2* uncovered the importance of the insulin/insulin-like growth factor-1 signalling (IIS) pathway in the regulation of ageing (Kaletsky and Murphy, 2010). In addition to the regulation of ageing, IGF-1 signalling has also been associated with initiation of cell protective function (Lee *et al*., 2001). On the other hand mutation in *daf-2* results in making the worms highly resistant to oxidative stress (Honda and Honda, 1999) and heat stress (Lithgow *et al*., 1995). In my studies, I found out that in the *daf-2(e1370)* mutants the rate of accumulation of the extracellular LBP-2 puncta was significantly slower than the rate of accumulation in the wild type of the same age. This effect was sustained with age and even at a later time point when half of the wild type worms were dead. The rate of extracellular LBP-2 puncta accumulation remained significantly slower in the *daf-2(e1370)* mutants.

In addition, the suppression of insulin/IGF-1 signalling by RNAi produced similar albeit smaller effects on the rate of LBP-2 accumulation. One possibility for such an effect is that in the long lived *daf-2(e1370)* mutants, the mechanisms responsible for the maintenance of the protein homeostasis are working more efficiently and are stable over a long period of time than in the wild type, where after a certain point of age similar mechanisms becomes overloaded. Previous studies have already shown that autophagy increases in the *daf-2* mutants and that it protects against protein specific aggregation (Hansen *et al*., 2008; Melendez *et al*., 2003; Florez-McClure *et al*., 2007). Another possibility is that the suppression of insulin/IGF-1 signalling could results in the overall reduction in the rate of LBP-2 accumulation through reduction in the rate of protein synthesis. Inhibition of the insulin/IGF-1 signalling can lead to reduced rates of protein synthesis in some organisms (Sharp and Bartke, 2005; Tatar *et al*., 2003). However, previous study has shown that the global rate of protein synthesis is not reduced in the *daf-2(e1370)* mutants (Hansen *et al*., 2007). Therefore, it is unlikely that a change in the rate of LBP-2 accumulation is due to a change in the rate of protein synthesis. Another explanation of the slower rate of LBP-2

accumulation in the *daf-2(e1370)* mutants is through protective function of transcription factors such as heat shock factor. Heat shock factor (HSF-1) has been shown to promote longevity in worms when over expressed, whereas it reduces the lifespan when knocked down (Morley and Morimoto, 2004). HSF-1 regulates the expression of a group of heat-shock proteins in response to various stress or pathological conditions. *hsf-1* is critical for the insulin/IGF-like signalling-mediated lifespan extension. It also promotes thermo-tolerance and longevity in *C. elegans* in a *daf-16* dependent manner. Henceforth, the reduction in the rate of extracellular protein accumulation could be through the DAF-2 either directly or indirectly via its interaction with the hsf-1.

4.3 Higher extracellular LBP-2 aggregation in MOAG-4 mutant

MOAG-4 is a highly conserved modifier of aggregation and henceforth I tested the worms to investigate the effect of MOAG-4 on extracellular protein accumulation. A previous study with *C. elegans* had shown that MOAG-4 functions as a positive regulator of aggregate formation on worm model for polyglutamine diseases (van ham *et al*., 2010). To test its effect on extracellular protein accumulation, I crossed the wild-type worms expressing *Plbp-2::lbp-2::tagrfp* with *moag-4* genetic deletion mutants and compared the rate of LBP-2 accumulation between the strains with and without the mutation. Rate of LBP-2 accumulation was significantly higher in the *moag-4* mutants at different points of adulthood compared to the wild type strains. Even though it is known that MOAG-4 plays an important role in intracellular function (van Ham *et al*., 2010), little is known about its mechanism. Study by van Ham *et al*., (2010) has shown that protein aggregation via MOAG-4 is independent of the intracellular *hsf-1* and *daf-16* pathways. Further dissection of the mechanism by which MOAG-4 influences aggregation needs to be addressed to underline its function in the extracellular protein accumulation.

4.4 Increase in temperature leads to higher rate of aggregation

Various studies have shown an association of change in the temperature and regulation of the lifespan of the worms. A direct correlation is suggested between the temperature and lifespan i.e., increase in temperature and reduction in lifespan and vice versa (Klass, 1977; Lee and Kenyon, 2009). Therefore, I was interested to look into the effects of temperature on extracellular LBP-2 accumulation. In my study, I found that the rate of extracellular LBP-2 accumulation was significantly higher at higher temperature. This result supports earlier studies where they have shown that the formation of aggregates depends on experimental conditions like temperature, ionic strength and pH (Calamai *et al*., 2005). It is likely that the increase in the rate of aggregation is due to formation of more unstable aggregates which often increase with temperature (Nguyen and Hall, 2004).

To look for potential modulators of extracellular aggregation, an RNAi screen for regulators was carried out. For carrying out the screen, temperature sensitive sterile mutant worms were required as the screens would require examining a large number of worms on multiple plates at a certain age. This would not be possible with non-sterile worms for two reasons; progeny of the non-sterile worms on the same plate would get mixed with the original worm population and it would be hard to track the accurate age of the worm. Secondly, with a large number of worms and their progeny on the same plate there would be competition for food between the worms, resulting in exposure of the work to stress and starvation state. It would eventually askew the results of the worm screen prior to the intended time point. Mutation of *spe-9* gene has been known to affect gamete recognition, adhesion and signalling (Singson *et al*., 1998), whereas *fer-15* mutants have been shown to produce defective sperms (Friedman and Johnson, 1988; Klass, 1983). Therefore, I used *spe-9(hc88);fer-15(b26)* double mutant (Melov *et al*., 1995). However, when the double mutant were crossed with the wild-type worms containing the integrated *lbp-2::tagrfp,* the resulting worms were not completely sterile. Worm with mutation with the *fem-1* gene produce oocytes instead of sperms (Kenyon *et al*., 1993). Henceforth, I decided to use another temperature sensitive sterile mutant strain, a *fer-15(b26);fem-1(hc17)* double mutant (Garigan *et al*., 2002). When this sterile strain was crossed with the wild type strain containing lbp-2::tagrfp, the progeny was sterile at the higher temperature of 25°C. Further, I investigated whether there was a difference in lifespan

between the two strains i.e., sterile and wild-type. From the examination I found out, that even though there was an increase in the lifespan of the sterile strain on OP50, this increase was not significant. Thus, suggesting that there was no significant difference in the lifespan of either of the worm strains. Therefore, I had a model organism fit for carrying out the RNAi screen as this temperature sensitive sterile strain would be easy to maintain and track, which would facilitate carrying out the large RNAi screen.

To setup the RNAi screen targeting secreted factors, I required a positive control for increased extracellular accumulation; a factor that would significantly increase the rate of LBP-2 puncta accumulation. It would be used for comparison during the screen and would facilitate the selection of factors that would significantly increase the extracellular LBP-2 accumulation. Coelomocytes are scavenger cells that continuously and non-specifically endocytose fluid from the pseudocoelom (Fares and Greenwald 2001b; White, 1988). *C. elegans* have six coelomocytes in the pseudocoelom (body cavity) of adult hermaphrodites. Studies have shown that GFP secreted into the pseudocoelom from many tissues and body wall muscle cells is endocytosed and degraded by coelomocytes (Fares and Greenwald, 2001a; Fitzgerald and Greenwald 1995; Grant and Greenwald 1997). Also, ablation of coelomocytes results in a failure to endocytose pseudocoelomic GFP (Fares and Greenwald, 2001b). Therefore, I was interested to look into the endocytotic function of the coelomocytes and used the RNAi against the genes that are known to play a role in the endocytosis. In the wild type worms, *rme-1* RNAi (Grant *et al*., 2001) is shown to be efficient to cause an age dependent increase in the amount of LBP-2 punkte. I also tried other RNAi like *sec-6* (Terbush *et al*., 1996) and *cup-4* (Fares and Greenwald, 2001b) but they showed no significant effect on the accumulation of the LBP-2 puncta.

The temperature sensitive sterile worm with *Plbp-2::lbp-2::tagrfp,* which I prepared were tested with the *rme-1* RNAi, the positive control for increased aggregation which would be used in the RNAi screen. However, with the sterile worm, *rme-1* caused no significant increase in the rate of extracellular accumulation when compared to the negative control. In addition, I also used RNAi against *atg-18,* which is a part of the core machinery and is essential for autophagy (Barth *et al*., 2001; Guan *et al*., 2001; Rieter *et al*., 2013). Similarly, I tested RNAi against *pbs-3*, a proteasomal subunit part of the 20S core. However, neither one of them increased the rate of LBP-2 puncta accumulation. Following this I decided to test

other RNAi clones against genes that have been shown to play a role in endocytosis, such as *dyn-1* (Clark *et al*., 1997), *rab-5* (Grant and Hirsh, 1999), *vps-34* (Stack and Emr, 1994), *rme-6* (Grant and Hirsh, 1999), *sec-8* (Terbush *et al.,* 1996), *vps-54* (Conboy and Cyert, 2000). *her-1* is required for sex determination in the worms (Hunter and wood, 1992; Streit *et al*., 1999) and it was used as an additional negative control. *dyn-1* and *rab-5* showed maximum amount of LBP-2 puncta whereas a significant increase in the rate of extracellular puncta was shown with *rme-6* and *vps-54*. The reason for the difference in the effectiveness of the RNAi's could be due the fact that there is a difference in effectiveness when an organism is injected or when it is fed an RNAi (Fares and Greenwald, 2001b).

4.5 RNAi screen for factors regulating extracellular aggregation.

Before carrying out the RNAi screen, I carried out a pilot screen (Boutros and Ahringer, 2008; Sharma and Rao, 2009). With the pilot screen a robust scoring system was established and which was later used in the main RNAi screen. The RNAi screen (referred as main screen) was carried out with 1508 RNAi clones from the Ahringer RNAi feeding library (Fraser *et al*., 2000; Kamath and Ahringer, 2003; Kamath *et al*., 2003). The clones were picked for the Ahringer RNAi library as it has very high levels of reliability (Qu *et al*., 2011)**.** The 1508 RNAi clones were selected against proteins that were predicted to be secreted. In the main screen each RNAi had three replicates. Out of the total clones, 162 RNAi clones had a positive effect on the LBP-2 accumulation. A secondary validation screen was carried out using the 162 RNAi clones. The scoring system was changed for this secondary validation screen (referred as repeat screen) to make the criteria more stringent so as to narrow down the list of potential regulator of extracellular accumulation. In the repeat screen, each RNAi clone had 4 replicates. From this secondary screen, only 33 clones from all the replicates had a positive effect on the LBP-2 accumulation. From the result of the main screen and the repeat screen, I only chose those positive clones that showed a positive effect in all replicates from the main screen and the repeat screen. There were 15 RNAi clones that were positive in both these screens. Therefore, they were picked for further analysis. Furthermore, I evaluated the effect of these 15 clones on the extracellular LBP-2 accumulation in the wild type LBP-2 worms. Apart from the two clones (ECRC_6 and ECRC_12), with the RNAi of the other 13 clones (ECRC_1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 13, 14, 15) there was a very significant increase in the LBP-2 puncta accumulation. Furthermore, to validate the effect of the two RNAi clones

(ECRC_6 and ECRC_12), I re-evaluated them. While carrying out the re-evaluation, the worms which were fed with ECRC_12 had significantly higher rates of LBP-2 puncta accumulation, while the worms which were fed ECRC_6 did not have a significantly higher rate of LBP-2 accumulation.

4.6 Increase in extracellular puncta accumulation is not due to disruption of coelomocyte uptake

After the RNAi screen, the functional analysis of the potential candidates of extracellular protein quality control was carried out to identify the pathways through which they regulate the extracellular protein accumulation. Initially, I decided to examine whether any of these candidates might have a positive effect on the extracellular accumulation through regulation of the endocytotic function of the coelomocytes. Therefore, the candidates were tested for their effect on the endocytotic uptake of secreted proteins by the coelomocytes. My earlier results already showed that reducing the efficacy of coelomocyte function resulted in higher rates of aggregation of LBP-2. I decided to use *myo-3p::ssGFP + dpy-20(+)* worm strain as it has already been used for the genetic analysis of endocytotic uptake by the coelomocytes (Fares and Greenwald, 2001a; 2001b). Therefore, in this experiment I fed these worms RNAi for all of the 15 (ECRC_1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15) candidates to see whether those RNAi induced any effect on the uptake by the coelomocytes. I conducted multiple screens, but none of the candidates had any significant effect. Thus, suggesting that none of the candidates causes an increase in LBP-2 accumulation by disrupting the coeolomocyte uptake.

4.7 The candidates to not lead to an increase of lifespan

In addition, I checked for the effect of the candidates on the lifespan of the worms. As previously stated, lifespan analysis is a commonly used method to see the effect of a certain gene and protein on ageing (Apfeld and Kenyon, 1999; Kenyon *et al.*, 1993; Kenyon, 2010). I was interested to understand the role of these candidates in the extracellular accumulation and its corresponding effect on the lifespan of the organism. From the experiment, none of the candidates were observed to have any effect on the lifespan of the worms, although one of the candidates led to a shorter lifespan by two days (ECRC_5), but this difference was not significant. Thus, suggesting that the accumulation of extracellular proteins may not impact the rate of aging of the worms.

4.8 Seven candidates had a significant effect on heat stress resistance

Using *C. elegans* as a model for stress response research has highlighted the genetic and molecular mechanisms underlying complex human diseases (Rodriguez *et al.*, 2013). Stress responses involve genes and pathways that are involved in a number of complex human diseases such as cancer and neurodegenerative diseases, including Alzheimer's and Parkinson's (Doonan *et al.*, 2008; Fujii *et al.*, 2011; Kourtis *et al.*, 2012; Mendillo *et al.*, 2012; Tissenbaum, 2012; Uttara *et al.*, 2009; Yen *et al.*, 2011). To find out more about the pathways through which these candidates function, I decided to do a heat stress analysis on the worms. With the RNAi of the candidates, I wanted to investigate the role of the different candidates towards the heat stress resistance in worms. Seven candidates out of the 14 had a significant effect on the heat stress resistance in the worms, suggesting that these candidates potential play an important role in the heat stress resistance in the worms most likely by regulating extracellular protein aggregation.

Conclusion

Protein homeostasis is important for the overall development, maintenance and survival of an organism. Additionally, protein homeostasis has also been shown to be essential for an efficient cellular response to stress and in the fight against neurodegeneration. A lot of research has been done into understanding the processes that are involved in maintaining this quality control within the cell. However, only recently have we started looking into the ways through which protein homeostasis can be maintained in the extracellular space. So far research has suggested that a small but growing family of secreted chaperones that are involved in the maintenance and disposal of misfolded proteins outside the cell.

In my study, I found out that the Insulin/IGF-1 signalling pathway effects the extracellular protein aggregation. In *C. elegans* diminishing the insulin/IGF-1 signalling can increase the lifespan more than twofold and the worm also remains active and much longer than normal. This effect can be observed in the worms with the reduction-of-function mutations in *daf-2* receptor (Kenyon, 2010). In the *daf-2* mutants, the rate of extracellular LBP-2 accumulation was significantly lower than the wild type. This slower rate was maintained with age. This lower slower rate of extracellular accumulation was also observed when the Insulin/IGF-1 signalling pathway was supressed using RNAi. However, the effect with RNAi was not as potent as seen in the mutant. In my study I also found out that the rate of extracellular protein accumulation was significantly higher in the MOAG-4 mutant. Interestingly, I also found that higher temperature has a positive effect on extracellular protein accumulation, similar to the effects of higher temperature on intracellular protein accumulation. I also carried out a RNAi screen to look for regulators of extracellular protein aggregation. Using the RNAi screen I found 14 potential regulator candidates of extracellular accumulations. To do a functional analysis and see how they affect the extracellular accumulation, I checked for their effect on the endocytotic function of the coelomocyte and found that none of the 14 regulator candidates have any effect of the endocytotic function of the coelomocyte. Additionally, I also looked at the effect of these 14 candidates on the lifespan of the worms; I found that none of the potential regulators had any significant effect on the lifespan of the worms. Finally, conducted a heat stress analysis with the 14 candidates, I wanted to analyse what

kind of role to these factors play in the heat stress resistance of the organism. I found out that 7 out of the 14 candidates had a significant effect on the heat stress resistance in the worms, which suggests that these candidates potential play an important role in the heat stress resistance in the worms.

In my research I have tried to look into some factors that are required for extracellular protein quality control as such regulators will be important for maintain the normal functioning of the body, protect it from the disease pathologies that can arise from the misfolding and aggregation of proteins and defend it against diseases and to achieve healthy ageing. Therefore, it is important to find out about these factors and the mechanisms of their function as they will help in creating effective therapies to problems related to aggregating proteins and ageing.

Future Direction

Protein misfolding and aggregation are involved in a number of human diseases. There are a number of factors that can cause a protein to become misfolded. Misfolded proteins lose their normal biological activity which results in loss of critical function. Misfolded proteins also have a propensity to form aggregates which become toxic for the cell. A number of diseases such as cancer, Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis and Parkinson's disease are caused due to the accumulation of toxic protein aggregates which are detrimental to the cells and leads to cellular dysfunction and neuronal death. A number of environmental and genetic factors play a role in the conditions responsible for the maintenance of protein homeostasis (Douglas and Dillin, 2010). However, ageing remains the most important factor. As an age dependent decline of the proteostasis can be observed in a number of organisms.

To further study the effects of these modifiers on protein aggregation, one can look into the effect of overexpression of these candidates on the LBP-2 accumulation. *C. elegans* are used to study the effect of overexpression of genes in ageing related studies (Burnett *et al*., 2011; Cabreiro *et al*., 2011). With such a study one observe the effect of these candidates on the extracellular protein aggregation and to what extent does the over expression of the candidates reduce the extracellular aggregation. In addition, one can also find out if there is any difference in the efficiency of these candidates when it comes to prevention of extracellular protein aggregation.

In my study, I looked into the effects of extracellular protein aggregation on ageing. I found that neither of the candidates have any significant effect on the lifespan of the worms. However, one can study the motility and body movements of the organism as another parameter to see the age dependent effect of extracellular protein aggregation. As motility has been used in number of studies (Duhon and Johnson, 1995; Hosono *et al*., 1980), it is a well-established physiological parameter to evaluate the effect of ageing on the worm (Huang *et al*., 2004). Therefore, one can evaluate the effects of age dependent extracellular protein

aggregation on the motility of the worm. In addition, one can analyse the age dependent effects on body movement in the worms with the overexpression of the candidates compared to the wild type as it has been seen that the rates of mortality and movement decline were correlated (Samuelson *et al*., 2007). Therefore body movement is a reliable measure of healthy ageing of the organism.

Alzheimer's disease (AD) is a progressive neurological disorder that results in irreversible loss of neurons. The brain of an Alzheimer's disease affected patient is characterized by accumulation of intracellular neurofibrillary tangles (Kosik *et al*., 1986) and extracellular senile plaques primarily composed of β-amyloid peptide (Glenner and Wong, 1984). *C. elegans* have been extensively used in AD associated research. In the *C. elegans* β-amyloid model with expression of muscle-associated human Aβ1–42 deposits they found out that there was a delay of Aβ1–42-mediated paralysis by heat shock treatment (Link, 2006). In another model, neuronal Aβ expression induces which induced defect in chemotaxis (Wu *et al*., 2006). Studies have also been done using *C. elegans* expressing human Aβ$_{3-42}$ in muscle tissue (Link, 1995; Link *et al*., 2001) and Alzheimer's Drug Discovery screening for β-amyloid peptide-induced toxicity (Lubin and Link, 2013). However, there is no successful *C. elegans* model for extracellular β-amyloid accumulation. If such a model was available, one could use this to study the effects of the modifiers of extracellular protein accumulation on the β-amyloid. Such a study can give us further insight into AD and its therapy.

The findings from my studies and the following work can give us a lot of insight into the extracellular quality control and its effects on ageing and diseases. However, to test the viability of the factors found in this study, they need to be tested in mammalian models.

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Appendix

Section 1

Table 1: Aggregation in worms with integrated LBP-2. The age dependent LBP-2 puncta accumulation was quantified. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 6 of the result.

	DCD22		Percentage	DCD23		Percentage
Day 2	Total	129		Total	131	
	0 puncta	127	98.4	0 puncta	127	96.9
	1-10 puncta	2	1.5	1-10 puncta	4	3
	10< No. of puncta	0	0	10< No. of puncta	0	0
Day 8	Total	114		Total	100	
	0 puncta	20	17.5	0 puncta	12	12
	1-10 puncta	82	71.9	1-10 puncta	61	61
	10< No. of puncta	12	10.5	10< No. of puncta	27	27
Day 12	Total	97		Total	93	
	0 puncta	3	3	0 puncta	5	5.4
	1-10 puncta	20	20.6	1-10 puncta	15	16.1
	10< No. of puncta	74	76.2	10< No. of puncta	73	78.5

Table 2: Aggregation in worms with integrated and non-integrated LBP-2. The age dependent LBP-2 puncta accumulation was quantified. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 7 of the result.

	DCD23		Percentage	DCD1		Percentage
Day 4	Total	125		Total	120	
	0 puncta	111	88.8	0 puncta	108	90
	1-10 puncta	14	11.2	1-10 Puncta	12	10
	10< No. of puncta	0	0	10< No. of puncta	0	0
Day 8	Total	114		Total	102	
	0 puncta	14	12.2	0 puncta	19	18.6
	1-10 puncta	90	78.9	1-10 Puncta	77	75.4
	10< No. of puncta	10	8.8	10< No. of puncta	6	5.9
Day 12	Total	90		Total	67	
	0 puncta	1	1.1	0 puncta	0	0
	1-10 puncta	21	23.3	1-10 Puncta	22	32.8
	10< No. of puncta	68	75.5	10< No. of puncta	45	67.1

Table 3: Effect of food source on LBP-2 accumulation. The age dependent LBP-2 puncta accumulation was quantified to observe the effect of different food source. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 9 of the result.

		OP50	Percentage	L4440		Percentage
Day 2	Total	117		Total	117	
	0 puncta	117	100	0 puncta	117	100
	1-10 puncta	0	0	1-10 Puncta	0	0
	10< No. of puncta	0	0	10< No. of puncta	0	0
Day 8	Total	102		Total	87	
	0 puncta	41	40.1	0 puncta	37	44
	1-10 puncta	54	52.8	1-10 Puncta	47	55.9
	10< No. of puncta	7	6.9	10< No. of puncta	0	0
Day 20	Total	59		Total	47	
	0 puncta	0	0	0 puncta	0	0
	1-10 puncta	4	6.7	1-10 Puncta	23	48.9
	10< No. of puncta	55	93.2	10< No. of puncta	24	51

Table 4: Effect of food source in *sid-1* mutants. The age dependent LBP-2 puncta accumulation was quantified to see the effect of food source in worms with gene silencing. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 10 of the result.

		sid-1(-)(OP50)	Percentage	*sid-1(-)*(l4440)		Percentage
Day 6	Total	91		Total	94	
	0 puncta	9	9.9	0 puncta	12	12.8
	1-10 puncta	73	55.8	1-10 puncta	74	78.7
	10< No. of puncta	9	9.9	10< No. of puncta	8	9.9
	20> No. of puncta	0	0	20> No. of puncta	0	0
Day 14	Total	65		Total	61	
	0 puncta	0	0	0 puncta	2	3.3
	1-10 puncta	29	38.5	1-10 puncta	42	68.8
	10< No. of puncta	25	44.6	10< No. of puncta	14	22.9
	20> No. of puncta	9	13.8	20> No. of puncta	3	4.9

Table 5: Effect of *daf-2* mutation of lbp-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *daf-2* mutation. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 11 of the result.

		WT	Percentage	*daf-2(-)*		Percentage
Day 2	Total	129		Total	128	
	0 puncta	121	93.8	0 puncta	114	89
	1-10 puncta	8	6.2	1-10 Puncta	14	10.9
	10< No. of puncta	0	0	10< No. of puncta	0	0
Day 8	Total	90		Total	113	
	0 puncta	1	1.1	0 puncta	3	2.6
	1-10 puncta	59	65.5	1-10 Puncta	110	97.3
	10< No. of puncta	30	33.3	10< No. of puncta	0	0
Day 14	Total	56		Total	75	
	0 puncta	0	0	0 puncta	0	0
	1-10 puncta	3	5.3	1-10 Puncta	39	52
	10< No. of puncta	53	94.6	10< No. of puncta	36	48

Table 6: Effect of *daf-2* mutation of lbp-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *daf-2* mutation. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 12 of the result.

		WT	Percentage	*daf-2(-)*		Percentage
Day 2	Total	125		Total	125	
	0 puncta	108	94.4	0 puncta	115	92
	1-10 puncta	7	5.6	1-10 Puncta	10	8
	10< No. of puncta	0	0	10< No. of puncta	0	0
Day 8	Total	76		Total	102	
	0 puncta	2	2.6	0 puncta	17	16.7
	1-10 puncta	71	93.4	1-10 Puncta	81	79.4
	10< No. of puncta	3	3.9	10< No. of puncta	4	3.9
Day 14	Total	56		Total	95	
	0 puncta	0	0	0 puncta	0	0
	1-10 puncta	0	0	1-10 Puncta	67	70.5
	10< No. of puncta	56	100	10< No. of puncta	28	29.4

Table 7: Effect of *daf-2* RNAi of lbp-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *daf-2* RNAi. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 13 of the result.

		Control RNAi	Percentage	*daf-2* RNAi		Percentage
Day 8	Total	80		Total	100	
	0 puncta	20	25	0 puncta	27	27
	1-10 puncta	60	75	1-10 Puncta	73	73
	10< No. of puncta	0	0	10< No. of puncta	0	0
Day 14	Total	60		Total	84	
	0 puncta	0	0	0 puncta	0	0
	1-10 puncta	37	61.7	1-10 Puncta	65	77.4
	10< No. of puncta	23	38.3	10< No. of puncta	19	22.6
Day 18	Total	35		Total	68	
	0 puncta	0	0	0 puncta	0	0
	1-10 puncta	4	11.4	1-10 Puncta	28	41.1
	10< No. of puncta	31	88.6	10< No. of puncta	40	58.8

Table 8: LBP-2 aggregation in MOAG-4 mutant. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *moag-4* mutation. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 14 of the result.

		WT	Percentage	*moag-4(-)*		Percentage
Day 2	Total	128		Total	126	
	0 puncta	128	100	0 puncta	105	83.3
	1-10 puncta	0	0	1-10 Puncta	21	16.7
	10< No. of puncta	0	0	10< No. of puncta	0	0
Day 6	Total	98		Total	87	
	0 puncta	69	70.4	0 puncta	10	11.5
	1-10 puncta	29	29.6	1-10 Puncta	76	87.3
	10< No. of puncta	0	0	10< No. of puncta	1	1.1
Day 10	Total	63		Total	55	
	0 puncta	0	0	0 puncta	0	0
	1-10 puncta	48	76.1	1-10 Puncta	16	29
	10< No. of puncta	15	23.8	10< No. of puncta	39	70.9

Table 9: Effect of temperature on LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of difference of temperature. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 15 of the result.

		WT (20°C)		Percentage	WT (25°C)		Percentage
Day 2	Total	126			Total	127	
	0 puncta	126		100	0 puncta	127	100
	1-10 puncta	0		0	1-10 Puncta	0	0
	10< No. of puncta	0		0	10< No. of puncta	0	0
Day 6	Total	106			Total	100	
	0 puncta	95		89.6	0 puncta	19	19
	1-10 puncta	11		10.4	1-10 Puncta	79	79
	10< No. of puncta	0		0	10< No. of puncta	2	2
Day 10	Total	79			Total	76	
	0 puncta	0		0	0 puncta	0	0
	1-10 puncta	74		93.7	1-10 Puncta	38	50
	10< No. of puncta	5		6.3	10< No. of puncta	38	50

Table 10: *rme-1* RNAi and LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *rme-1* RNAi. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 16 of the result.

		Control RNAi		Percentage	*rme-1* RNAi		Percentage
Day 6	Total	125			Total	123	
	0 puncta	0		0	0 puncta	1	0.8
	1-10 puncta	113		90.4	1-10 puncta	75	61
	10< No. of puncta	12		9.6	10< No. of puncta	47	38.2
Day 8	Total	104			Total	94	
	0 puncta	0		0	0 puncta	0	0
	1-10 puncta	37		35.6	1-10 puncta	17	18
	10< No. of puncta	67		64.4	10< No. of puncta	77	81.9

Table 11: *sec-6* and *cup-4* and LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *rme-1* RNAi. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 17 of the result.

		Control RNAi		Percentage	sec-6 RNAi		Percentage	cup-4 RNAi		Percentage
Day 6	Total	102			Total	100		Total	100	
	0 puncta	0		0	0 puncta	0	0	0 puncta	6	6
	1-10 puncta	93		91.1	1-10 puncta	89	89	1-10 puncta	92	92
	10< No. of puncta	9		8.8	10< No. of puncta	11	11	10< No. of puncta	2	2
	20> No. of puncta	0		0	20> No. of puncta	0	0	20> No. of puncta	0	0
Day 8	Total	95			Total	95		Total	98	
	0 puncta	0		0	0 puncta	0	0	0 puncta	0	0
	1-10 puncta	36		37.9	1-10 puncta	36	37.9	1-10 puncta	40	40.8
	10< No. of puncta	55		57.9	10< No. of puncta	50	57.9	10< No. of puncta	51	52
	20> No. of puncta	4		4.2	20> No. of puncta	9	9.4	20> No. of puncta	7	7.1

Table 12: Effect of homologues of extracellular chaperone on LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *try-1* and *tep-1*. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 18 of the result.

		Control RNAi		Percentage	try-1 RNAi		Percentage	tep-1 RNAi		Percentage
Day 4	Total	106			Total	105		Total	103	
	0 puncta	85		80.1	0 puncta	75	71.4	0 puncta	83	80.6
	1-10 puncta	21		19.8	1-10 Puncta	30	28.5	1-10 Puncta	20	19.4
	10< No. of puncta	0		0	10< No. of puncta	0	0	10< No. of puncta	0	0
	20> No. of puncta	0		0	20> No. of puncta	0	0	20> No. of puncta	0	0
Day 8	Total	101			Total	103		Total	99	
	0 puncta	9		8.9	0 puncta	20	19.4	0 puncta	5	5
	1-10 puncta	91		90	1-10 Puncta	83	80.6	1-10 Puncta	90	90.9
	10< No. of puncta	1		0.9	10< No. of puncta	0	0	10< No. of puncta	4	4
	20> No. of puncta	0		0	20> No. of puncta	0	0	20> No. of puncta	0	0

Table 13: Effect of *rme-1* RNAi on LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *rme-1* RNAi in sterile and non-sterile worms. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 19 of the result.

	Day 6					Day 8				
	Total	0 puncta	1-10 puncta	10< No. of puncta	20> No. of puncta	Total	0 puncta	1-10 puncta	10< No. of puncta	20> No. of puncta
Non-sterile control (-) RNAi	90	7	61	22	0	90	0	38	47	5
Percentage		7.8	67.8	24.4	0		0	42.2	52.2	5.6
Non-sterile *rme-1* RNAi	92	1	47	44	0	88	0	23	55	10
Percentage		1	51	47.9	0		0	26.1	62.5	11.4
Sterile control (-) RNAi	90	2	66	22	0	90	0	38	39	13
Percentage		2.2	73.3	24.4	0		0	42.2	43.3	14.4
Sterile *rme-1* RNAi	87	1	63	23	0	87	0	34	48	5
Percentage		1.1	72.4	26.4	0		0	39	55.1	5.7

Table 14: Effect of *atg-18* and *pbs-3* RNAi on LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of *pbs-3* and *atg-18* RNAi in sterile and non-sterile worms. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 20 of the result.

		Day 6			
	Total	0 puncta	1-10 puncta	10< No. of puncta	20> No. of puncta
Non-sterile control (-) RNAi	87	40	44	3	0
Percentage		46	50.6	3.4	0
Non-sterile *atg-18* RNAi	85	57	27	1	0
Percentage		67	31.8	1.2	0
Non-sterile *pbs-3* RNAi	86	56	25	5	0
Percentage		65.1	29	5.8	0
Sterile Control RNAi	93	4	74	15	1
Percentage		4.3	79.6	16.1	1
Sterile *atg-18* RNAi	96	30	65	1	0
Percentage		31.2	67.7	1	0
Sterile *pbs-3* RNAi	94	51	43	0	0
Percentage		54.2	45.7	0	0

Table 15: Effect of different RNAi on LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of various RNAi in worms. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 21 of the result.

	Day 6				
	Total	0 puncta	1-10 puncta	10< No. of puncta	20> No. of puncta
Control (-) RNAi	96	66	30	0	0
Percentage		68.7	31.2	0	0
Control (-) RNAi	93	54	38	1	0
Percentage		58	40.8	1	0
Control (-) RNAi	95	59	35	1	0
Percentage		62.1	36.8	1	0
dyn-1 RNAi	93	25	64	4	0
Percentage		26.9	68.8	4.3	0
rab-5 RNAi	88	22	59	6	1
Percentage		25	67	6.8	1.1
vps-34 RNAi	94	59	33	2	0
Percentage		62.7	35.1	2.1	0
rme-6 RNAi	95	39	54	2	0
Percentage		41	56.8	2.1	0
sec-8 RNAi	85	50	32	3	0
Percentage		58.8	37.6	3.5	0
vps-54 RNAi	91	38	50	3	0
Percentage		41.8	54.9	3.3	0

Table 16: A pre pilot screen to test the scoring system for RNAi screen. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 22 of the result.

	Total	0 puncta	1-10 puncta	10< No. of Puncta	20< No. of Puncta
Day 6					
Control (-)	82	22	53	7	0
Percentage		26.8	64.6	8.5	0
Positive control (*rme-6*)	76	3	48	25	0
Percentage		3.9	63.2	32.9	0
Positive control (*dyn-1*)	79	0	47	29	3
Percentage		0	59.5	36.7	3.8
1.1 A7	80	0	56	24	0
Percentage		0	70.00	30.00	0
1.2 D7	77	1	56	20	0
Percentage		1.30	72.73	25.97	0
1.1 A11	79	0	68	11	0
Percentage		0	86.08	13.92	0
1.1 C3	80	0	60	20	0
Percentage		0	75.00	25.00	0

Table 17: Lifespan evaluation in sterile and non-sterile worms. The total number of alive, dead and censored worms at different time point of their lifespan is shown. Censored worms are the worms that died due to non-natural conditions like crawling up the side of the dish and drying. The data corresponds to figure 23 of the result.

Wild-type worms on OP50					Sterile worms on OP50			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	120	0	0		0	120	0	0
2	119	0	1		2	120	0	0
4	108	0	11		4	115	0	5
6	102	4	2		6	109	0	6
8	82	16	4		8	99	3	7
10	59	23	0		10	83	14	2
12	49	10	0		12	71	12	0
14	35	12	2		14	62	7	2
16	18	17	0		16	41	18	3
18	13	5	0		18	31	9	1
20	5	8	0		20	18	13	0
22	0	5	0		22	6	12	0
					24	3	3	0
					26	0	3	0
Wild-type worms on l4440					Sterile worms on l4440			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	120	0	0		0	120	0	0
2	120	0	0		2	119	0	1
4	108	0	12		4	119	0	0
6	92	9	7		6	117	1	1
8	60	31	1		8	97	18	2
10	48	12	0		10	76	17	4
12	28	20	0		12	44	29	3
14	11	16	1		14	23	16	5
16	5	6	0		16	13	10	0
18	2	3	0		18	7	6	0
20	1	1	0		20	4	3	0
22	0	1	0		22	2	2	0
					24	0	2	0

Table 18a: Effect of candidates on LBP-2 aggregation. The age dependent LBP-2 puncta accumulation wa quantified to see the effect of RNAi candidate in worms. The data corresponds to figure 26 of the result.

	Day 6			
	Number of worms in punkte category			
	0 Puncta	1-10 Puncta	11-20 Puncta	> 20 Puncta
Control (-)	52	94	0	0
Percentage	35.6	64.4	0	0
Negative Control (*gfp*)	46	94	2	0
Percentage	32.4	66.2	1.4	0
Positive Control (*rab-5*)	4	69	75	0
Percentage	2.7	46.6	50.7	0
ECRC_6	53	76	12	0
Percentage	19.6	67	13.4	0
ECRC_12	52	86	6	0
Percentage	36.1	59.7	4.2	0
ECRC_14	35	102	12	0
Percentage	23.5	68.4	8	0
ECRC_8	28	99	16	0
Percentage	19.6	69.2	11.2	0
ECRC_15	15	96	32	0
Percentage	10.5	67.1	23.4	0
ECRC_7	20	87	45	0
Percentage	13.2	57.2	29.6	0
ECRC_2	18	117	11	0
Percentage	12.4	80.1	7.5	0
ECRC_4	20	103	27	0
Percentage	13.3	68.7	18	0
ECRC_10	17	118	13	0
Percentage	11.5	79.7	8.8	0

	Day 6			
	Number of worms in punkte category			
	0 Puncta	1-10 Puncta	11-20 Puncta	> 20 Puncta
ECRC_1	16	98	25	0
Percentage	11.5	70.5	18	0
ECRC_5	9	124	12	0
Percentage	6.2	85.5	8.3	0
ECRC_3	12	109	23	0
Percentage	8.3	75.7	16	0
ECRC_13	13	114	20	0
Percentage	8.8	77.6	13.6	0
ECRC_11	23	104	18	1
Percentage	15.8	71.2	12.3	0.7
ECRC_9	20	105	13	0
Percentage	14.5	76.1	9.4	0

Table 19: Effect of three candidate RNAi on LBP-2 aggregation. The age dependent LBP-2 puncta accumulation was quantified to see the effect of three candidate RNAi in worms. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 27 of the result.

	Day 6			
	Number of worms in punkte category			
	0 Puncta	1-10 Puncta	11-20 Puncta	> 20 Puncta
Control (-)	32	73	5	0
Percentage	29	66.3	4.5	0
Positive Control (*dyn-1*)	3	34	76	0
Percentage	2.6	30	67.2	0
ECRC_3	4	54	57	0
Percentage	3.5	47	49.5	0
ECRC_6	22	75	15	0
Percentage	19.6	67	13.4	0
ECRC_12	10	48	52	0
Percentage	9	43.6	47.2	0

Table 20: Effect of candidate RNAi on coelomocyte uptake. The effect of candidate RNAi on the coelomocyte uptake was evaluated as per the no of worms without coelomocyte. Total number of worms, the number of worms per category and their percentage are displayed. The data corresponds to figure 28 of the result.

		Day 6		
	Total	**No Coelomocyte**	**Coelomocytes except 3rd**	**3rd Coelomocytes**
Control (-) RNAi	342	16	5	321
Percentage		4.7	1.5	93.9
Control (*dyn-1*)	345	212	73	60
Percentage		61.4	21.2	17.4
ECRC_1	360	68	38	254
Percentage		18.9	10.6	70.6
ECRC_15	363	33	25	305
Percentage		9.1	6.9	84.0
ECRC_3	358	51	38	269
Percentage		14.2	10.6	75.1
ECRC_4	364	26	69	269
Percentage		7.1	19.0	73.9
ECRC_5	360	29	61	270
Percentage		8.1	16.9	75.0
ECRC_6	349	47	58	244
Percentage		13.5	16.6	69.9
ECRC_7	361	32	48	281
Percentage		8.9	13.3	77.8
ECRC_8	363	44	60	259
Percentage		12.1	16.5	71.3
ECRC_9	345	34	64	247
Percentage		9.9	18.6	71.6
ECRC_10	353	38	71	244
Percentage		10.8	20.1	69.1
ECRC_11	342	36	69	237
Percentage		10.5	20.2	69.3
ECRC_12	346	86	61	199
Percentage		24.9	17.6	57.5
ECRC_13	340	42	56	242
Percentage		12.4	16.5	71.2
ECRC_14	358	40	49	269
Percentage		11.2	13.7	75.1
ECRC_2	342	32	59	251
Percentage		9.4	17.3	73.4

Table 21: Lifespan evaluation in non-sterile worms. The total number of alive, dead and censored worms a different time point of their lifespan is shown. The data corresponds to figure 29 of the result.

Worms on Control(-)					Worm on *rab-5*			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	150	0	0		0	156	0	0
2	150	0	0		2	156	0	0
4	150	0	0		4	151	0	5
6	150	0	0		6	147	0	4
8	150	0	0		8	120	27	0
10	147	1	2		10	81	39	0
12	144	2	1		12	51	30	0
14	142	1	1		14	15	36	0
16	127	15	0		16	3	12	0
18	98	29	0		18	0	3	0
20	71	27	0					
22	51	20	0					
24	20	31	0					
26	9	11	0					
28	0	9	0					

Worm on *rme-6*					Worms on ECRC_1			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	156	0	0		0	155	0	0
2	156	0	0		2	155	0	0
4	151	0	5		4	152	0	3
6	149	0	2		6	149	0	3
8	146	0	3		8	147	0	2
10	144	1	1		10	144	0	3
12	142	2	0		12	143	0	1
14	140	1	1		14	142	1	0
16	124	16	0		16	131	11	0
18	104	20	0		18	106	25	0
20	53	51	0		20	77	29	0
22	35	18	0		22	52	25	0
24	19	16	0		24	32	20	0
26	4	15	0		26	16	16	0
28	0	4	0		28	0	16	0

Worms on ECRC_2					Worms on ECRC_3			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	155	0	0		0	155	0	0
2	155	0	0		2	155	0	0
4	150	0	5		4	151	0	4
6	149	0	1		6	144	0	7
8	147	0	2		8	143	0	1
10	145	0	2		10	143	0	0
12	145	0	0		12	143	0	0
14	145	0	0		14	143	0	0
16	132	13	0		16	128	15	0
18	101	31	0		18	97	31	0
20	71	30	0		20	75	22	0
22	46	25	0		22	42	33	0
24	23	23	0		24	20	22	0
26	14	9	0		26	9	9	0
28	0	14	0		28	0	11	0

Worms on ECRC_4					Worms on ECRC_5			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	154	0	0		0	154	0	0
2	154	0	0		2	154	0	0
4	148	0	6		4	149	0	5
6	141	0	7		6	140	0	9
8	141	0	0		8	138	0	2
10	141	0	0		10	137	0	1
12	141	0	0		12	137	0	0
14	141	0	0		14	134	3	0
16	128	13	0		16	115	19	0
18	100	28	0		18	85	30	0
20	75	25	0		20	61	24	0
22	51	24	0		22	38	23	0
24	28	23	0		24	22	16	0
26	13	15	0		26	8	14	0
28	13	13	0		28	0	8	0

Worms on ECRC_7					Worms on ECRC_8			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	155	0	0		0	156	0	0
2	155	0	0		2	156	0	0
4	147	0	8		4	151	0	5
6	140	0	7		6	145	0	6
8	140	0	0		8	139	0	6
10	140	0	0		10	139	0	0
12	139	0	1		12	139	0	0
14	137	2	0		14	138	0	1
16	125	12	0		16	127	11	0
18	105	20	0		18	97	30	0
20	79	26	0		20	72	25	0
22	52	27	0		22	47	25	0
24	32	20	0		24	24	23	0
26	15	17	0		26	15	9	0
28	0	15	0		28	0	15	0
Worms on ECRC_9					Worms on ECRC_10			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	157	0	0		0	153	0	0
2	157	0	0		2	153	0	0
4	144	0	13		4	144	0	9
6	143	0	1		6	141	0	3
8	139	0	4		8	135	0	6
10	139	0	0		10	135	0	0
12	139	0	0		12	134	0	1
14	136	1	2		14	130	1	3
16	124	12	0		16	114	16	0
18	93	31	0		18	85	29	0
20	72	21	0		20	71	14	0
22	46	26	0		22	41	30	0
24	28	18	0		24	21	20	0
26	17	11	0		26	10	11	0
28	0	17	0		28	0	10	0

Worms on ECRC_11					Worms on ECRC_12			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	151	0	0		0	156	0	0
2	151	0	0		2	156	0	0
4	147	0	4		4	150	0	6
6	146	0	1		6	147	0	3
8	146	0	0		8	143	0	4
10	140	0	6		10	143	0	0
12	136	0	4		12	142	0	1
14	135	1	0		14	140	1	1
16	122	13	0		16	122	18	0
18	92	30	0		18	98	24	0
20	74	18	0		20	78	20	0
22	52	22	0		22	48	30	0
24	33	19	0		24	28	20	0
26	18	15	0		26	13	15	0
28	0	18	0		28	0	13	0
Worms on ECRC_13					Worms on ECRC_14			
No. of days	Alive	Dead	Censored		No. of days	Alive	Dead	Censored
0	158	0	0		0	155	0	0
2	158	0	0		2	155	0	0
4	153	0	5		4	147	0	8
6	151	0	2		6	145	0	2
8	150	0	1		8	145	0	0
10	150	0	0		10	145	0	0
12	148	0	2		12	143	0	2
14	146	2	0		14	139	4	0
16	124	22	0		16	124	15	0
18	98	26	0		18	98	26	0
20	73	25	0		20	76	22	0
22	43	30	0		22	56	20	0
24	24	19	0		24	35	21	0
26	12	12	0		26	13	22	0
28	0	12	0		28	0	13	0

Worms on ECRC_15			
No. of days	Alive	Dead	Censored
0	156	0	0
2	156	0	0
4	150	0	6
6	147	0	3
8	147	0	0
10	143	0	4
12	143	0	0
14	139	4	0
16	121	18	0
18	91	30	0
20	66	25	0
22	44	22	0
24	27	17	0
26	10	17	0
28	0	10	0

Table 22: Survival of worms under heat stress. A setup for heat stress resistance for sterile worms on day 6. The worms were stressed at 34°C. The number of worms alive and the number of worms dead with different length of time under heat stress were evaluated. The data corresponds to figure 30 of the result.

Day 6			
	Total	Alive	Dead
3 hours	100	93	7
Percentage		93	7
6 hours	100	80	20
Percentage		80	20
10 hours	100	62	38
Percentage		62	38
11 hours	100	55	45
Percentage		55	45
21 hours	100	0	100
Percentage		0	100

Table 23: Survival of worms under heat stress. A setup for heat stress resistance for non-sterile worms on day 1. The worms were stressed at 34°C. The number of worms alive and the number of worms dead with different length of time under heat stress were evaluated. The data corresponds to figure 31 of the result.

Day 1		
13 hours		
	Total	**Alive**
gfp	100	98
Percentage		98
l4440	100	96
Percentage		96
cup-4	100	90
Percentage		90
18 hours		
	Total	**Alive**
gfp	100	62
Percentage		62
l4440	100	78
Percentage		78
cup-4	100	90
Percentage		90
23 hours		
	Total	**Alive**
gfp	100	40
Percentage		40
l4440	100	38
Percentage		38
cup-4	100	20
Percentage		20

Table 24: Effect of candidate RNAi on heat stress resistance. The number of worms alive and dead with different length of time under heat stress was evaluated. The data corresponds to figure 32 of the result.

Day 8			
	Total	**Alive**	**Dead**
Control (-)	117	62	55
Percentage		53	47
Positive control (*dyn-1*)	120	15	105
Percentage		12.5	87.5
ECRC_1	112	29	83
Percentage		25.9	74.1
ECRC_2	111	54	57
Percentage		48.6	51.4
ECRC_3	98	24	74
Percentage		24.5	75.5
ECRC_4	108	30	78
Percentage		27.8	72.2
ECRC_5	114	32	82
Percentage		28.1	71.9
ECRC_7	115	57	58
Percentage		49.6	50.4
ECRC_8	107	50	57
Percentage		46.7	53.3
ECRC_9	119	36	83
Percentage		30.3	69.7
ECRC_10	113	57	56
Percentage		50.4	49.6
ECRC_11	102	47	55
Percentage		46.1	53.9
ECRC_12	110	46	64
Percentage		41.8	58.2
ECRC_13	111	46	65
Percentage		41.4	58.6
ECRC_14	105	41	64
Percentage		39	61
ECRC_15	109	55	55
Percentage		50.46	50.46

Script 1: The php script used for sorting the candidates. The php script used for generating the list of candidates after evaluating all the factors found on the wormbase database which were signalp positive through the TMHMM database. The script was used to run all the factors against the TMHMM to remove the factors with transmembrane domains

```php
//lets take input from user..


if(count($argv) < 1) {
  print "Usage: php ProteinSequenceScrapper.php {input file}\n";
  exit;
}


$inputfile = $argv[1];


if(!file_exists($inputfile)) {
  print "ERR - Input file ".$inputfile." doesn't exists\n";
  exit;
}


$data = file_get_contents($inputfile);


$data = str_replace("\r\n",'\n', $data);


$sequences = explode("\n\n",$data);


if(count($sequences) ==0) {
        print "ERR no sequences found in an input file\n";
        exit;
}


$curlcmd = "curl";
```

```php
if($windows) {

  $curlcmd = "echo off & curl";

}


$curlcmd .= " --form-string \"outform=-short\"";

$curlcmd .= " --form-string \"configfile=/usr/opt/www/pub/CBS/services/TMHMM-
2.0/TMHMM2.cf\"";


foreach($sequences as $sequence) {

  $curlreq = $curlcmd . " --form-string \"SEQ=".trim($sequence)."\"" . "
\"http://www.cbs.dtu.dk//cgi-bin/webface2.fcgi\" -L -s -o -";

  $res = array();

  exec($curlreq,$res,$rescode);

  foreach($res as $r) {

        $r = trim($r);

        $tpos = strpos($r, "<title>");

    if($tpos === 0) {

      $bits = explode("</span>",$r);

      if(count ($bits) < 2) {

        print  "--- Found problem with title in html.. probably it has changed now?---\n--- HTML
response is ---\n";

        print_r($res);

      exit;

      }

      $text = substr($bits[0],$tpos+7);

      //now all we need to do is to get the

      $jobid = trim(substr($text,strlen("Job status of")));


      print "---Found JobId as ".$jobid."---\n---Now polling the server and wait till server is ready ---\n";

      $tried = 0;

      while(true) {
```

```php
$polreq = "curl \"http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi?ajax=1&jobid=$jobid\"";

$pres = array();

print $polreq;

exec($polreq,$pres,$polco);

$res = $pres[0];

$resobj = (array)json_decode($res,true);


if($resobj["status"] == "queued") {

        print "--Server still processing will ask the server again in a bit--\n";

        if($tried == 25) {

          print "---Giving up as tried so many times---\n";

          break;

        }

        print "---Waiting 3 sec before asking again---\n";

        sleep(3);

        $tried++;

}else{

        //we got result lets just print it..

}



    }


    break;

  }else{

        print "--- Found problem with sevrer response.. probably it has changed the html now?---\n";

  }

 }

}
```

Section 2

Instruments and consumables

Table 25a: Instruments and consumables used.

Company	Model	Serial No
Eppendorf AG (Hamburg, Germany)	Centrifuge 5424R	5404AQ906917
Eppendorf AG (Hamburg, Germany)	Centrifuge 5424	5424AJ238107
Eppendorf AG (Hamburg, Germany)	Thermomixer Comfort 5355	5355AO054220
Eppendorf AG (Hamburg, Germany)	Thermomixer Comfort 5355	5355AO254214
Leica (Wetzlar, Germany)	MDG33/10450123	5763559
VWR (Darmstadt, Germany)	VWR ministar silverline	110050404
Integra Biosciences AG (Zizers, Switzerland)	Integra Pipetboy	155 017
VWR (Darmstadt, Germany)	VWR INCU-Line IL10	1100729
WLD-TEC GmbH (Arenshausen, Germany)	Flame 100	12210317
Eppendorf (Hamburg, Germany)	New Brunswick^{TM} Innova 40R	010851208
Sigma-Aldrich (St. Louis, Missouri, United States)	Consort EV202	100569
Campingaz (Cambrai, France)	CV470 plus	F14031

Company	Model	Serial No.
VWR (Darmstadt, Germany)	VWR STD1000 Shaker	120322001
VWR (Darmstadt, Germany)	VWR INCU-Line IL10	1100729
Liebherr (Bulle Switzerland)	GKV4310 Index 20B/001	81.598.628.5
Liebherr (Bulle, Switzerland)	GGV5010 Index 40/001	81.375.745.0
BINDER		11-20027
Systec (Winnenden, Germany)	HX210	2220
Miele (Gütersloh, Germany)	G7893	RS232
Keison (Chelmsford, United Kingdom)	Stuart CB161	R200000595
IKA (Staufen German)	RH basic-2	0003339000
VWR (Darmstadt, Germany)	Nutating mixer	P110383
Eppendorf (Hamburg, Germany)	New Brunswick Scientific C585-86	100573530111
UVP (Cambridge, United Kingdom)	CL-1000 Ultraviolet Crosslinker	C111711-002
Sutter Instrument Co. (California, USA)	P-97	P97-5183
Bio-Rad Laboratories (California, USA)	C1000 Touch Thermal Cycler	CT001310
Bio-Rad Laboratories (California, USA)	C1000 Touch Thermal Cycler	CT001310
Audion Elektro (Weesp, Netherlands)	300FJR-Z	4515484.2

Company	Model	Serial No.
Thermo Fisher Scientific (Massachusetts, United States)	Nanodrop 2000	8848
Bio-Rad Laboratories (California, USA)	Universal Hood II	721BRO5640
Thermo Fisher Scientific (Massachusetts, United States)	7312 B1A	117665-18/363106
Thermo Fisher Scientific (Massachusetts, United States)	7312 B1A	117665-18/363106
Peqlab Biotechnologie (Erlangen, Germany)	EV231	100642
Thermo Fisher Scientific (Massachusetts, United States)	HERAEUS MEGAFUGE 40R	41302234
Eppendorf (Hamburg, Germany)	New Brunswick Scientific U725-86G	1016-0837-0711
Keison (Chelmsford, United Kingdom)	Stuart CB1611	200000665
Keison (Chelmsford, United Kingdom)	Stuart CC162	4900000194
Leica Microsystems (Wetzlar, Germany)	M165FC	
Leica Microsystems (Wetzlar, Germany)	DFC310FX	
Leica Microsystems (Wetzlar, Germany)	DFC310FX	

Company	Model	Serial No.
Carl Zeiss AG (Oberkochen, Germany)	Axio Vert.A1	
Carl Zeiss AG (Oberkochen, Germany)	Axio HPX-120C	
Carl Zeiss (Oberkochen, Germany)	Axio Observer Z1	
Carl Zeiss (Oberkochen, Germany)	Axiocam MRm	
Carl Zeiss (Oberkochen, Germany)	RS232	410361-1000-000
Thermo Fisher Scientific (Massachusetts, United States)	EMD Millipore Milli-Q™ Integral Systems	Z00Q0V0T0

Table 25b: Chemicals used.

Company	Product
Invitrogen (Carlsbad, CA, USA)	Agarose UltraPure®
Becton Dickinson and Co. (Franklin Lakes, NJ, USA)	Bacto^{TM} Yeast Extract
Becton Dickinson and Co. (Franklin Lakes, NJ, USA)	Bacto^{TM} Tryptone
Becton Dickinson and Co. (Franklin Lakes, NJ, USA)	Bacto^{TM} Peptone
Merck (Darmstadt, Germany)	$CaCl_2$
AppliChem (Darmstadt, Germany)	Carbenicillin
Sigma-Aldrich (St Louis, Mo, USA)	Cholesterol
Sigma-Aldrich (St Louis, Mo, USA)	Citric Acid Monohydrate
Merck (Darmstadt, Germany)	$CuSO_4$
Becton Dickinson and Co. (Franklin Lakes, NJ, USA)	Difco Bacto Agar
New England Biolabs (Ipswich, MA, USA)	dNTP Mix
Carl Roth (Karlsruhe, Germany)	Dimethylsulfoxide
Merck (Darmstadt, Germany)	EDTA
Sigma-Aldrich (St Louis, Mo, USA)	Ethanol
Sigma-Aldrich (St Louis, Mo, USA)	Ethidium Bromide
Merck (Darmstadt, Germany)	$FeSO_4$

Company	Product
Carl Roth (Karlsruhe, Germany)	Glycerol
Merck (Darmstadt, Germany)	HCl
Merck (Darmstadt, Germany)	KH_2PO_4
Merck (Darmstadt, Germany)	Methanol
Merck (Darmstadt, Germany)	$MgCL_2$
Merck (Darmstadt, Germany)	$MgSO_4$
Merck (Darmstadt, Germany)	$MnCl_2$
Merck (Darmstadt, Germany)	NA_2HPO_4
Merck (Darmstadt, Germany)	NaCl
Roche (Basel, Switzerland)	Phosphatase inhibitor
AppliChem (Darmstadt, Germany)	PMSF
Sigma-Aldrich (St Louis, Mo, USA)	Sodium Hypochloride
Sigma-Aldrich (St Louis, Mo, USA)	Sucrose
AppliChem (Darmstadt, Germany)	TAE-Buffer 50x
AppliChem (Darmstadt, Germany)	Triton X-100
Sigma-Aldrich (St Louis, Mo, USA)	Tween20
Sigma-Aldrich (St Louis, Mo, USA)	$ZnSO_4$

Table 25c: Antibodies used.

Antibodies			
Anti-tagRFP	Rabbit	1:5000	Evrogen
Anti-rabbit-IgG	Goat	1:5000	New England Biolabs

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