Small Nucleoli and Reduced Ribosomal Biogenesis are Hallmarks of Longevity

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ABSTRACT

In the last decades a great deal of work in model organisms has revealed that lifespan of an organism is regulated by conserved signaling pathways including the insulin/IGF-1 and the target of rapamycin (TOR) signaling pathways that mediate longevity across species. A number of conserved transcription factors have been identified that work downstream of these pathways to modulate lifespan extension. However, it still remains elusive if there are common downstream targets and shared mechanisms that are affected by all these pathways to bring about a change in organismal lifespan. It is therefore of great interest to understand these convergent mechanisms because fine-tuning of the factors involved in these mechanisms may be sufficient to induce longevity and lead to resistance against age-associated diseases. Therapeutic interventions could be designed to modulate the function of these convergent mechanisms, which may prove to be sufficient to avail of the benefits of improved health and longevity.

In this study we found *ncl-1* as a convergent regulator of *C. elegans* lifespan working downstream of different longevity pathways. We identified *ncl-1* in RNAi based genetic screens as a suppressor of dietary restriction (DR) mediated longevity, using the *eat-2* mutant, which is the genetic model of DR in *C. elegans. ncl-1* encodes a cytoplasmic B-box zinc finger protein that inhibits rRNA and 5S RNA transcription and protein synthesis. Consistent with a role in ribosome biogenesis, NCL-1 regulates nucleolar size and *ncl-1* mutants have larger nucleoli especially in neuronal, muscle and hypodermal cells. Lifespan analyses revealed that *ncl-1* is required for longevity conferred by DR, reduced insulin/IGF-1 and reduced TOR signaling pathways. *ncl-1* was also required for mitochondrial and gonadal longevity models in *C. elegans* indicating that NCL-1 works as a common downstream regulator of longevity in *C. elegans.* Consistent with these findings, *ncl-1* over-expression was sufficient to promote longevity.

Since loss of *ncl-1* enlarges nucleoli of worms in different tissues, we investigated nucleolar size in different long-lived worms representing different longevity pathways in *C. elegans.* Strikingly we observed that all the long-lived

animals tested, exhibited smaller nucleoli in different tissues compared to wildtype. Among wildtype animals there is a high variation in nucleolar size, however we found that it is highly predictive for lifespan in an isogenic population. Consistent with smaller nucleoli, all these different long-lived animals possessed reduced ribosome biogenesis and reduced levels of the nucleolar marker FIB-1/Fibrillarin and these effects were reversed with the loss of *ncl-1*. In accord with reduced levels of FIB-1/Fibrillarin in long-lived animals, a modest reduction of FIB-1/Fibrillarin in wildtype animals increased their lifespan.

Consistent with our findings in *C. elegans,* we observed smaller nucleoli, reduced ribosome biogenesis and reduced FIB-1 levels in long-lived *Drosophila.* We also detected smaller nucleoli in different tissues of long-lived DR and IRS-1 knockout mice and also in humans who undergo modest dietary restriction coupled with exercise. Thus it is highly suggestive that smaller nucleoli and reduced ribosome biogenesis are hallmarks of longevity across species.

ZUSAMMENFASSUNG

Die wissenschaftliche Arbeit der letzten Jahrzehnte hat gezeigt, dass die Lebensdauer eines Organismus durch konservierte Signalwege reguliert wird. Zu diesen gehören beispielsweise der Insulin/IGF-1- und der TOR-(*engl*. target of rapamycin)-Signalweg, welche wichtige Rollen für die Langlebigkeit verschiedenster Spezies spielen. Es wurden viele konservierte Transkriptionsfaktoren identifiziert, welche diesen Signalwegen nachgeschaltet sind und die Langlebigkeit vermitteln. Jedoch ist es bislang ungeklärt ob all diese Signalwege gemeinsame Targets oder Mechanismen beeinflussen, um eine Änderung der Lebensdauer hervorzurufen. Es besteht großes Interesse daran, diese zusammenlaufenden Mechanismen zu verstehen, da auch nur kleinste Manipulationen an diesen Mechanismen ausreichend sein könnten um Langlebigkeit und Resistenz gegenüber altersbedingten Erkrankungen zu vermitteln. Therapeutische Eingriffe könnten darauf basieren, diese konvergenten Mechanismen zu modulieren und deren Vorteile für Gesundheit und Langlebigkeit nutzbar zu machen.

In der vorliegenden Arbeit haben wir mit *ncl-1* einen konvergenten Regulator der Lebensdauer des Modellorganismus *C. elegans* gefunden. Wir haben *ncl-1* in einem RNAi-basierten genetischen Screen als Suppressor der Langlebigkeit von *eat-2* Mutanten identifiziert. Die Langlebigkeit dieser Mutanten wird durch Beschränkung der Nahrungsaufnahme (*engl*. dietary restriction, DR) hervorgerufen. *ncl-1* kodiert ein zytoplasmatisches B-Box Zinkfingerprotein, welches die Synthese ribosomaler RNA und Proteinbiosynthese inhibiert. In Übereinstimmung mit einer Rolle in der Ribosom-Biogenese reguliert NCL-1 die Größe der Nukleoli und *ncl-1*-Mutanten weisen größere Nukleoli auf, vor allem im neuronalen, Muskel- und hypodermalen Gewebe. Unsere Analyse der Lebensdauer von verschiedenen langlebigen Wurmstämmen hat ergeben, dass *ncl-1* für die Langlebigkeit benötigt wird, welche durch DR, reduzierte Insulin/IGF-1- und TOR-Signalübertragung induziert wird. Des Weiteren war *ncl-1* auch für die mitochondriale und gonadale Langlebigkeit erforderlich, welches darauf hinweist, dass NCL-1 als allgemeiner

Regulator der Langlebigkeit in *C. elegans* agiert. Dementsprechend genügte die Überexpression von NCL-1, um Langlebigkeit in Würmern zu induzieren.

Da der Verlust von *ncl-1* zur Vergrößerung der Nukleoli in verschiedenen Geweben des Wurmes führt, untersuchten wir die Größe der Nukleoli in verschiedenen langlebigen *C. elegans* Mutanten, welche verschiedene Langlebigkeits-Signalwege repräsentieren. Dabei machten wir die erstaunliche Beobachtung, dass alle untersuchten langlebigen Mutanten kleinere Nukleoli in verschiedenen Geweben aufwiesen als Wildtyp-Würmer. Innerhalb der Wildtyp-Population herrschte eine recht große Variabilität in der Größe der Nukleoli. Jedoch erlaubte die Größe des Nukleolus eine Vorhersage der Lebenserwartung innerhalb einer isogenen Population. Konsistent mit der reduzierten nukleolären Größe wiesen alle langlebigen Tiere eine verringerte Biogenese von Ribosomen und niedrigere Level des nukleolären Markers Fibrillarin (FIB-1) auf, welches durch Verlust von *ncl-1* aufgehoben werden konnte. In Übereinstimmung mit dem verringerten FIB-1 Level in langlebigen Tieren konnte eine leichte Erniedrigung der FIB-1 Level die Lebensdauer von Wildtyp-Tieren erhöhen.

Im Einklang mit unseren Ergebnissen in *C. elegans* konnten wir kleinere Nukleoli, reduzierte Ribosomen-Biogenese und niedrigere FIB-1 Level in langlebigen Fruchtfliegen beobachten. Zusätzlich stellten wir eine signifikant reduzierte nukleoläre Größe in verschiedenen Geweben von langlebigen DR- und IRS-1-KO Mäusen sowie in Menschen, welche einer milden DR kombiniert mit physischer Anstrengung ausgesetzt waren, fest. Unsere Resultate weisen also stark darauf hin, dass kleinere Nukleoli und reduzierte Ribosomen-Biogenese Kennzeichen der Langlebigkeit in verschiedenen Spezies sind.

ABBREVIATIONS

AMPK	adenosine monophosphate kinase
Arf	alternative reading frame
BDR	bacterial dilution regimen
DA	dafachronic acid
DNA	deoxyribonucleic acid
DR	dietary restriction
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
EMS	ethylmethansulfonat
ER	endoplasmic reticulum
GFP	green fluorescent protein
IGF-1	insulin-like growth factor-1
IIS	insulin/IGF-1 like signaling
ILP	insulin like peptide
IRS-1	insulin receptor substrate-1
NGM	nematode growth medium
NHR	nuclear hormone receptor
OP50	*E. coli* strain OP50
PCR	polymerase chain reaction
qPCR	quantitative PCR
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
ROS	reactive oxygen species
TGF-β	transforming growth factor -β
TOR	target of rapamycin
UPR	unfolded protein response
UPS	ubiquitin proteasome system

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CHAPTER 1

INTRODUCTION

1.1 Genetics of Aging

Aging was formerly believed to be a completely random process occurring due to a decline in bodily functions with time. However our present understanding is that regulated longevity processes that promote and maintain cellular functionality and counteract aging, gradually become dysregulated and thereby lead to a progressive decline in organismal health. Recent research has provided considerable evidence that organismal lifespan is not random but a multifactorial process regulated by genetic and environmental factors. A large number of genes have been discovered to play vital roles in regulating organismal longevity. Among the first organisms used to study lifespan regulation using genetic approaches was the soil nematode *C. elegans* (Klass, 1977). The prime example of a genetic basis of lifespan regulation is single gene mutations that modestly reduce the insulin/IGF signaling pathway and cause a dramatic extension in *C. elegans* lifespan (Kenyon et al., 1993; Kimura et al., 1997). These seminal studies led to the prominence of genetics in the field of aging research to establish cause and effect relationships between gene function and length of life. Subsequently many other genes governing different pathways have been discovered in diverse model organisms that contribute to aging, many of which have also been found to be conserved in mammals (Lopez-Otin et al., 2013). Genetic factors have also been implicated in regulation of longevity in humans. Polymorphisms in the insulin/IGF pathway have been associated with exceptional longevity in centenarians (van Heemst et al., 2005). Single gene alterations can lead to drastic differences in rates of aging, as in the case of human progeroid syndrome (Navarro et al., 2006). Additionally, mutations in nuclear and mitochondrial DNA have been associated with deterioration of somatic stem cell function contributing to aging (Sharpless and DePinho, 2007). This growing body of evidence has established beyond doubt that lifespan of an organism has a genetic component. The field of molecular genetics of aging is moving ahead in the direction of unraveling the mechanisms of action and interaction of different genetic pathways impinging on aging with the long term goal of increasing health span and reducing the occurrence of age-associated diseases.

1.2 *C. elegans* as a Model Organism for Aging Research

C. elegans is a soil nematode which has been extensively studied in the field of aging research. It was established as a model organism for biological research by Sydney Brenner (Brenner, 1974). *C. elegans* is an exemplary model system for studying aging because of its short lifespan and powerful genetics. Average lifespan of this nematode worm is around three weeks, which makes it very convenient to do lifespan analyses in a relatively shorter period of time. Its small size (1 mm), growth in large numbers, and easy handling also make it a convenient model system to work with. Furthermore *C. elegans* is a self-fertilizing hermaphrodite with extensive genetic tools available, making genetic manipulation and crosses relatively easy. This is particularly important for performing genetic epistasis and synergy experiments to establish genetic pathways. Another useful advantage is that gene function can be specifically and potently knocked down using RNA inhibition (RNAi), whereby double stranded RNA against a given gene reduces its expression, an approach first discovered in C. *elegans* resulting in the Nobel prize (Andrew Fire and Craig C. Mello) (Fire et al., 1998). RNAi is fairly easy and straightforward; feeding specific short double-stranded RNA expressing bacteria effectively knocks down the target gene (Timmons et al., 2001). This advantage can be ideally used in designing high-throughput RNAi based genetic screens (Boutros and Ahringer, 2008). Another benefit that *C. elegans* offers is that it consists of only around 1000 somatic cells at maturity, which has allowed its cellular development to be traced in entirety (Sulston and Horvitz, 1977; Sulston et al., 1983). Its transparency also allows live imaging of worms without tedious procedures of sample preparation for imaging. Moreover, *C. elegans* was the first multicellular organism to have its whole genome sequenced (Consortium, 1998). This allowed for the identification of a large number of protein coding genes that have homologs in mammals and thus can be studied in the context of aging and disease.

1.3 Pathways that Regulate Longevity

1.3a Insulin/IGF-1 Signaling (IIS) Pathway and Longevity

By far the most extensively investigated signaling pathway in the context of aging is insulin/IGF-1 signaling (IIS) pathway. The first evidence that IIS pathway regulates longevity came with the isolation of single gene mutations that extend lifespan (Klass, 1983). Some of these loci were subsequently followed up, including *age-1*, encoding worm ortholog of PI3K (Morris et al., 1996), whose mutation induced an 80% increase in longevity (Friedman and Johnson, 1988). Later, the *daf-2* locus, encoding the worm ortholog of the insulin/IGF receptor (Kimura et al., 1997) was also shown to extend lifespan by 2-3 fold (Kenyon et al., 1993).

Several lines of molecular genetic evidence supported the view that *age-1* and *daf-2* worked in the same pathway. For example there was no additive influence on lifespan when both *age-1* and *daf-2* were mutated together (Dorman et al., 1995; Larsen et al., 1995). Additionally, the longevity conferred by both *age-1* and *daf-2* mutants required the same factors namely *daf-16* and *daf-18*, which encode worm orthologs of FOXO and Phosphatase and Tensin Homolog (PTEN) (Lin et al., 1997; Ogg et al., 1997; Ogg and Ruvkun, 1998). Both *daf-16/*FOXO and *daf-18/*PTEN work downstream of *daf-2* in the IIS pathway. The IIS pathway relays its effects by regulating the phosphorylation status of DAF-16/FOXO. When active, signaling through the IIS pathway leads to the activation of phosphoinositide 3-kinase AGE-1/PI3K, which phosphorylates phosphatidylinositol 4,5,-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-triphosphate (PIP3) (Morris et al., 1996). In the presence of PIP3, 3–phosphoinositide-dependent kinase PDK-1 (Paradis et al., 1999) phosphorylate AKT/PKB (Paradis and Ruvkun, 1998) which together with the serine/threonine kinase SGK-1 phosphorylates DAF-16/FOXO (Hertweck et al., 2004). Phosphorylated DAF-16/FOXO is thus exported out of the nucleus and retained in the cytoplasm (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). In contrast, down-regulation of this kinase cascade in turn leads to nuclear localization of DAF-16/FOXO which then induces the expression of its target genes thereby leading to longevity and stress resistance (Murphy et al., 2003). Other more

recently identified transcriptional mediators of IIS signaling include SKN-1/NRF2, HSF-1, and PQM-1 (Hsu et al., 2003; Tepper et al., 2013; Tullet et al., 2008). Insulin like peptides (ILPs), which are the ligands of DAF-2, form the components of the endocrine system. 40 of these ILPs have been described in *C. elegans*, which are variously expressed in neurons, gut, and gonad, and several of which affect lifespan e.g. *daf-28* (Li et al., 2003) and *ins-7* (Murphy et al., 2007). Some ILPs such as INS-1 and INS-18 act antagonistically to DAF-2 and thereby over-expression of these INS-1 and INS-18 phenocopies the effects of *daf-2* reduction of function (Pierce et al., 2001). Remarkably, the lifespan regulation by the IIS signaling pathway is evolutionarily conserved. In *Drosophila* and also in mice, a reduction in IIS signaling leads to an extension in lifespan (Bartke, 2008; Clancy et al., 2001; Kappeler et al., 2008; Selman et al., 2008; Tatar et al., 2001). Moreover, in humans, polymorphisms in the insulin/IGF pathway have been linked with longevity in centenarians (van Heemst et al., 2005).

In addition to regulating longevity, the IIS pathway also regulates larval arrest called dauer in worms. Components of *C. elegans* IIS first emerged from forward genetic screens designed to identify mutants with abnormal regulation of dauer arrest (Riddle et al., 1981). *daf-2*, *age-1* and *akt-1; akt-2* double null mutants all arrest non-conditionally as dauers at 25°C and 27°C, unlike wildtype worms which grow normally at 25°C and show low levels of dauer arrest at 27 °C (Alam et al., 2010; Gems et al., 1998; Kimura et al., 1997; Morris et al., 1996; Oh et al., 2005) and these dauer-constitutive phenotypes of these mutants are suppressed by *daf-16*/FOXO null mutations (Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Vowels and Thomas, 1992). IIS signaling also regulates stress resistance in *C. elegans; daf-2* mutants are more resistant to different stress conditions including heat (Lithgow et al., 1994; Lithgow et al., 1995), oxidative stress (Honda and Honda, 1999, 2002) and hypoxia (Scott et al., 2002). Finally, another physiological aspect regulated by the IIS pathway is fat metabolism. *daf-2* mutants possess elevated fat content compared to wildtype worms (Ashrafi et al., 2003; Kimura et al., 1997; O'Rourke et al., 2009; Ogg et al., 1997; Perez and Van Gilst, 2008) attributed to increased *de novo* lipogenesis (Perez and Van Gilst, 2008) and dependent on *daf-16/*FOXO (Ashrafi et al., 2003;

Ogg et al., 1997; Perez and Van Gilst, 2008). Therefore the IIS pathway regulates a myriad of processes some of which contribute to its longevity phenotype.

1.3b Mitochondrial Function and Longevity

Another intriguing longevity pathway is that of mitochondrial longevity wherein a partial reduction of mitochondrial activity leads to lifespan extension. These findings suggest that reduced metabolic rate can result in organismal lifespan extension. A number of genes have been identified which when mutated lead to a partial reduction in mitochondrial function and respiration and thereby cause an extension in lifespan in yeast, worms, flies and mice (Copeland et al., 2009; Dell'agnello et al., 2007; Pospisilik et al., 2007). One of the first examples establishing a causal relation between metabolic rate and aging in *C. elegans* was the long-lived *clk-1* mutant. *clk-1* encodes a homolog of COQ7 enzyme involved co-enzyme Q biosynthesis (Ewbank et al., 1997; Lakowski and Hekimi, 1996). *clk-1* mutants have slower rates of respiration and growth, and live longer than wild type. Similarly *C. elegans isp-1* encodes an iron sulfur protein in mitochondrial complex III whose mutation leads to impaired electron transport function and an extension of lifespan (Feng et al., 2001) (Yang and Hekimi, 2010). Large-scale RNAi screens also identified knockdown of mitochondrial functions to extend lifespan (Dillin et al., 2002; Lee et al., 2003). However, there are also mutations in ETC known that can cause lifespan shortening e.g. *mev-1* mutants, which have a mutation in the succinate dehydrogenase cytochrome *b560* subunit of mitochondrial respiratory chain complex II (Ishii et al., 1998), live shorter than wildtype. Therefore it is not well understood how a reduction in mitochondrial function can regulate aging in a positive or a negative way.

Initially it was thought that the longevity effect upon reduced mitochondrial function results from a reduction in the production of Reactive Oxygen Species (ROS). ROS are produced primarily through mitochondrial respiration and can cause damage to cellular macromolecules including DNA and proteins. This explanation is consistent with the free radical theory of aging by Denham Harman, which proposes

that an accumulation of cellular damage induced by oxidative damage and ROS causes aging (Harman, 1956). However, just the opposite was observed; long-lived mitochondrial mutants had increased levels of superoxide, and mild levels of pro-oxidant paraquat extended lifespan (Yang and Hekimi, 2010). Moreover, other interventions that provoke mild ROS increase lifespan, leading to the mitohormesis hypothesis (Schulz et al., 2007) - that low levels of ROS stimulate general defense response. Therefore, it still remains unresolved if mitochondrial longevity works through elevated or reduced levels of ROS and if increased or decreased levels of ROS are beneficial for longevity and aging (Gems and Partridge, 2013).

Another aspect that connects mitochondria and lifespan regulation is the mitochondrial unfolded protein response or UPR^{MT}. UPR^{MT} is a mitochondrial–to-nuclear signal transduction pathway, initiated by the accumulation of unfolded proteins in the mitochondria and resulting in the induction of specific stress response proteins that work to restore mitochondrial nuclear balance (Benedetti et al., 2006; Martinus et al., 1996; Merkwirth et al., 2016). UPR^{MT} was first associated with aging when it was reported that UPR^{MT} is essential for the lifespan extension of long-lived mitochondrial mutants, *isp-1* and *clk-1* (Durieux et al., 2011). A number of studies have provided more evidence about the involvement of UPR^{MT} in aging. For example a recent study reported that UPR^{MT} components and mitochondrial biogenesis are up-regulated in long-lived Complex IV deficiency *Surf1*$^{-/-}$ mice (Pulliam et al., 2014). Another study reported that a knockdown of mitochondrial ribosomal protein 5 (mrps-5) strongly induced UPR^{MT} and extended lifespan in worms in a UPR^{MT} dependent manner (Houtkooper et al., 2013). Despite all this evidence, it is not clearly understood how this stress response mechanism can promote longevity.

1.3c Reproduction and Longevity

In addition to IIS and mitochondrial longevity models, it is also well established that germline stem cell removal leads to extended lifespan. In *C. elegans* it was discovered that laser microsurgical ablation of the germ cells could extend the lifespan of the animal by 60%. However, somatic gonad cells were required for this effect; removal of the entire gonad including the somatic gonad cells abolished this longevity benefit (Hsin and Kenyon, 1999). Genetic manipulation rendering worms germlineless using *glp-1*/Notch receptor knock-out also extends lifespan while mutations that lead to over-proliferation of germline such as *gld-1* shorten lifespan (Arantes-Oliveira et al., 2002). Gonadal longevity, as it is called, is dependent on many factors namely DAF-16/ FOXO (Hsin and Kenyon, 1999), steroid receptor DAF-12/FXR and its ligands (Gerisch et al., 2007; Gerisch et al., 2001; Hsin and Kenyon, 1999), DAF-18/PTEN (Berman and Kenyon, 2006), HSF-1 (Hansen et al., 2005), SMK-1/SMEK-1 (Wolff et al., 2006), PHA-4/FOXA (Lapierre et al., 2011) and SKN-1/Nrf (Steinbaugh et al., 2015). DAF-16/FOXO, the major transcriptional regulator of the IIS pathway, is nuclear localized in the intestine and neurons upon reduction in the activity of the IIS pathway. However, upon germline loss DAF-16/FOXO is shuttled into the nucleus only in the gut indicating that the gut is the major tissue in mediating the systemic effect of lifespan increase in the gonadal longevity model (Yamawaki et al., 2010). In addition to the above-mentioned factors, which work mostly through the IIS pathway, there are other factors that have been discovered to be playing key roles in the gonadal longevity model e.g KRI-1, which is an ankyrin repeat and FERM domain-containing protein orthologous to human KRIT1 (Berman and Kenyon, 2006) and TCER-1, which is a transcription elongation/splicing regulator orthologous to human TCERG1 (Ghazi et al., 2009). Additionally, the TOR pathway which is a nutrient sensing pathway and regulates lifespan across multiple species, has been associated with the maintenance of germline stem cell proliferation through ribosomal protein S6 Kinase (S6K) in worms (Korta et al., 2012). Germline loss triggers down-regulation of TOR and consistent with its role in gonadal longevity, TOR RNAi extends lifespan of wildtype

worms but does not further extend the lifespan of *glp-1* worms (Lapierre et al., 2011). Moreover, gonadal longevity is implicated in regulating fat metabolism, requiring the activity of *lipl-4*, which encodes a triglyceride lipase and is required for extended lifespan of *glp-1* animals (Wang et al., 2008). Additional evidence pointing towards the involvement of fat metabolism in regulating gonadal longevity came when FAT-6, which is an acyl-CoA desaturase, was found to be required for longevity. Germline loss induces FAT-6 to higher levels, which works by desaturating stearic acid to oleic acid. The loss of longevity in germlineless animals upon *fat-6* knockdown can be restored by nutritional supplementation of oleic acid (Goudeau et al., 2011) giving a direct evidence of fat metabolism being regulated upon germline removal and its involvement in mediating longevity upon germline loss. Fatty acids likely signal through nuclear receptors NHR-80 and NHR-49, which work in a complex to regulate fat metabolism, β-oxidation and longevity (Goudeau et al 2011, Folick et al 2015). Regulation of lifespan by the gonad is present in other species as well e.g. in *Drosophila* loss of germ cells in adults is known to modulate the IIS pathway and increase lifespan (Flatt et al., 2008). In mice it was shown that a transplantation of ovaries from young to old mice increased the lifespan of the old mice by 31-44% providing more evidence of a link between the reproductive system and lifespan even in mammals (Cargill et al., 2003; Kagawa et al., 2010). These studies indicate an evolutionarily conserved link between reproduction and aging.

1.3d Endocrine Signaling and Longevity

Endocrine mechanisms coordinate development and physiology in response to environmental cues and internal signals and this regulation is crucial for sustaining organismal physiology, reproduction and lifespan. The most majorly studied endocrine pathway regulating physiology and lifespan has been the IIS pathway (discussed above). However, recent work in model organisms has brought to light some other endocrine mechanisms that affect aging and other life history traits. An important example is nuclear hormone receptors (NHRs) and their lipophilic ligands that have been shown to affect aging in different model organisms (Fielenbach and Antebi, 2008; Flatt et al., 2005; Rottiers and Antebi, 2006; Tatar et al., 2003). NHRs normally comprise a ligand binding domain and a DNA binding domain. There are various NHRs present across species; humans possess 48 NHRs while lower organisms like *Drosophila* and *C. elegans* have 18 and 284 respectively, a bulk of which remain poorly characterized. In *C. elegans* the most widely studied NHR is DAF-12 which is a homolog of human vitamin D receptor, farnesoid-X and liver X receptor (VDR, FXR, LXR). It has been described to regulate diapause formation, developmental timing and longevity and has ben identified as the major target of the dafachronic acid (DA) pathway in *C. elegans* (Antebi, 2013; Antebi et al., 2000).

Worms respond to nutritional conditions through the sensory head neurons via cGMP and serotonergic signaling (Birnby et al., 2000; Thomas et al., 1993). Under extreme conditions like over-crowding, elevated temperature or food shortage worms enter a diapause state called dauer. Several pathways have been described to regulate dauer formation and multiple genes have been identified in dauer screens (Albert and Riddle, 1988; Morris et al., 1996; Pierce et al., 2001; Riddle et al., 1981; Wolkow et al., 2002). In these studies major pathways that regulate lifespan like transforming growth factor β (TGFβ), IIS and TOR have also been identified eventually impinging on the DA pathway for dauer decision (Hu, 2007). Sensory perception regulates synthesis and secretion of TGF-β (Ren et al., 1996) and ILPs (Li et al., 2003) activating downstream signaling cascades and

thereby regulating steroidal hormone signaling (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). IIS and TGFβ are active under favorable conditions (Murakami et al., 2001), which eventually lead to DA production via the DA pathway (Wollam et al., 2011). The final decision of DA synthesis is under tight regulation via multiple upstream inputs including serotonin (Sze et al., 2000), acetylcholine (Lee et al., 2014) and TOR (Jia et al., 2004). DA thus produced binds to the receptor DAF-12 and promotes normal reproductive growth (Antebi et al., 2000; Motola et al., 2006). In the absence of DA, DAF-12 binds to DIN-1 and leads to dauer formation and extended lifespan (Ludewig et al., 2004). In accord with this, DAF-12 mutants cannot enter the dauer diapause and are short lived (Antebi et al., 2000). Therefore, DA and DAF-12 act in conjunction to govern dauer diapause decisions and regulate lifespan in worms. This endocrine signaling network is not only limited to regulating *C. elegans'* physiology but also plays a similar role in higher organisms like insects and mammals. Both IIS and TGFβ pathways regulate ecdysteroidogenesis in insect metamorphosis and steroidogenesis in mammalian puberty (Tennessen and Thummel, 2011).

1.4 Dietary Restriction and Aging

Environmental interventions like DR have long been known to regulate lifespan in different organisms. DR is a reduction of food intake without malnutrition, which often confers health benefits and extends lifespan. The first seminal study that demonstrated that DR could bring about an extension in health and lifespan was done in rodents (McCay et al., 1935). In this study rats were fed *ad libitum* or a low calorie diet and the major objective of the study was to determine the effect of retarding growth on lifespan and body size. It came as a surprise to see DR rats showed average lifespan extension of more than 60% compared to *ad libitum* fed rats. This groundbreaking discovery paved the way for the field of DR to emerge and people tried different methods of employing DR on different organisms and found out that the effects were conserved. These DR experiments have been successfully reproduced in higher organisms like primates as well. Limiting the caloric intake by 10-30% compared to the baseline-unrestricted intake reduces the incidence of diabetes, cancer, cardiovascular disease, and brain atrophy and also slows ageing in rhesus monkeys (Colman et al., 2009). Although another study saw no effect on mortality, they observed less incidence of age-related disease (Mattison et al., 2012). The fundamental assumption, that DR can extend the mean and maximum lifespan and delay the onset of age-associated disorders, has been demonstrated in many organisms including yeast, worms, flies and mammals (Anderson and Weindruch, 2010; Kennedy et al., 2007; Piper and Bartke, 2008) (Figure 1). Even in humans undergoing voluntary DR, the risks for developing cardiovascular diseases and cancer are reduced (Fontana et al., 2010). DR in humans results in similar molecular changes as those seen in laboratory animals including reduced myocardial stiffness, reduced body temperature, down-regulation of *pi3k*/*akt*/*foxo* etc (Cava and Fontana, 2013; Mercken et al., 2013). The fact that different organisms show improvement in most aspects of health during aging has instigated great interest in this field. Indeed, now research is also proceeding ahead in the direction of understanding the basic mechanisms underlying DR so that DR mimetics can be designed, which might mimic the same benefits as DR without actually undergoing

drastic alteration in the diet.

There are various ways of carrying out DR in different organisms. For example in yeast, the most predominant way of performing DR is glucose deprivation. A drop in glucose concentration from 2% to 0.01% increases the mean and maximum lifespan in yeast (Lin et al., 2000). In *C. elegans* there are eight different procedures of manipulating the diet that all lead to an extension in lifespan to varying degrees, allowing comparison among the different DR regimens. The major DR regimens include dilution of the bacterial food, deprivation of peptones from the diet etc (Greer and Brunet, 2009; Hosono et al., 1989). Additionally, a genetic model for DR in *C. elegans* is the *eat-2* mutant, in which the pharyngeal pumping rate and hence food intake are reduced, leading to approximately 20% increase in lifespan (Lakowski and Hekimi, 1998). Strikingly, different DR regimens act through different downstream factors, which eventually leads to an increase in lifespan. Thus, diverse DR regimens evoke somewhat independent genetic pathways, depending on how DR is induced. The notion that reduced intake of calories can lead to lifespan extension upon DR gained ground by whole organism transcriptomic studies in *Drosophila*. It was reported that reduction in caloric intake reverted the transcriptional changes that occur during aging (Pletcher et al., 2002). However this theory was challenged when reduced intake of different nutrients rather than overall caloric intake, was shown to exhibit the effects of DR (Mair et al., 2005). Based on the current evidence, ratio of protein to carbohydrate in the diet of the fruit fly regulates lifespan. Lower ratios like 1/16 increase lifespan while higher ratios like 1/2 shorten it (Fanson and Taylor, 2012; Grandison et al., 2009). Other reports also show that methionine deprivation extends the lifespan of flies (Troen et al., 2007) and mice (Miller et al., 2005; Sun et al., 2009). In rodents, a reduction in food by 30% to 40% leads to lifespan extension (Wanagat et al., 1999). Reduced food intake has been associated with lower incidence of age-related diseases including cardiovascular disease, sarcopenia, neoplasia and type-2 diabetes and prolonged youthful metabolic state (Anderson and Weindruch, 2012; Colman et al., 2009; Kemnitz, 2011; Masoro, 2005).

1.5 Regulators of Dietary Restriction Mediated Longevity

Though the molecular mechanisms by which DR confers health benefits and extends lifespan are largely unknown, some regulators that mediate DR response have been identified. One of the major factors that is known to mediate DR longevity in *C. elegans* is *pha-4* which encodes a fork head box A (FOXA) transcription factor (Panowski et al., 2007). Knockdown of *pha-4* abrogates longevity conferred by bacterial dilution regimen and of *eat-2* mutants without affecting the increased lifespan by reduced IIS. This suggests that *pha-4* specifically plays a regulatory role in mediating DR longevity. Another important factor that is required for sDR (solid DR), one of the DR regimens in *C. elegans,* is *aak-2* (Greer et al., 2007). *aak-2* codes for the α-catalytic subunit of the important energy homeostasis regulator AMP kinase or AMPK. When energy levels drop leading to an increase in AMP to ATP ratio, AMP binds to the γ regulatory subunit of AMPK. Binding of AMP results in phosphorylation of a threonine residue on the α subunit thereby activating AMPK, which increases the ATP production and reduces the consumption of ATP by suppressing energy-consuming processes and thereby maintains energy homeostasis (Hardie, 2011). In addition to DR, *aak-2* is also required for the longevity of *rsks-1* mutants providing evidence that DR and the TOR pathway confer longevity through the same mechanism (Selman et al., 2009). Another transcription factor shown to be important for DR in *C. elegans* is hypoxia inducible factor 1 or HIF-1 (Chen et al., 2009). During normoxic conditions HIF-1 undergoes proteasomal degradation. *egl-9* encodes for the proline hydroxylase that mediates this degradation of HIF-1. Therefore, a loss of function of *egl-9* stabilizes and increases the levels of HIF-1 (Epstein et al., 2001). Interestingly, loss of *egl-9* abolishes DR mediated longevity and the longevity of *rsks-1* mutants. Consistent with this finding, *hif-1* mutants were shown to be long-lived and there was no further extension of lifespan when *hif-1* worms were subjected to DR (Chen et al., 2009). Besides these factors, the nuclear factor-erythroid-related-factor or Nrf ortholog SKN-1 in worms is induced in ASI neurons which signals cell non-autonomously to other tissues thereby mediating longevity under DR (Bishop and Guarente, 2007). Lifespan

extension by inhibiting the TOR pathway through Rapamycin treatment also requires the activity of SKN-1 (Robida-Stubbs et al., 2012).

Genetic screens in *C. elegans* using the DR model *eat-2* have been extensively carried out by a number of laboratories to identify new genes that are involved in the DR pathway. Such a screen performed by the Kenyon lab identified four genes namely *sams-1*/S-adenosyl methionine synthetase, *rab-10*/RAB-like GTPase, *drr-1*,*-2*/dietary restriction response. These genes extend the lifespan of insulin signaling FOXO transcription factor (*daf-16*) mutant but are incapable of extending the lifespan of *eat-2* mutants suggesting overlapping mechanisms with DR (Hansen et al., 2005). In another set of genetic screens performed in our lab, we identified for the first time a nuclear hormone receptor, NHR-62 to be required for DR longevity in *eat-2* worms and in bacterial dilution regimen (Heestand et al., 2013). Loss of NHR-62 altered the metabolic phenotypes of *eat-2* including fat levels and autophagy.

1.6 Dietary Restriction, TOR Pathway and Protein Synthesis in Aging

Of all the nutrient-sensing pathways, the Target of Rapamycin or the TOR pathway has been prominently associated with DR. The TOR pathway is a central pathway that senses nutrients (amino acids, sugars etc.), promotes cell growth and suppresses degradation and recycling processes like autophagy when the food is plentiful (Kapahi et al., 2010). TOR inhibition extends lifespan across different species all the way from yeast to mice (Harrison et al., 2009; Kaeberlein et al., 2005; Kapahi et al., 2004; Sheaffer et al., 2008; Vellai et al., 2003) (Figure 1). There have been many studies done that link the TOR pathway with DR. For example inhibition of TOR and DR show similar physiological effects and there is no additive longevity observed when the TOR pathway is suppressed under DR conditions in different organisms (Bjedov et al., 2010; Hansen et al., 2007; Kapahi et al., 2004). There is also some evidence in mammals that supports the notion of TOR being involved in DR. A mutation in murine S6 kinase, which is the downstream target of TOR, extends lifespan and shows a similar transcriptional profile as that of DR, which implies that TOR regulates the effects of DR (Selman et al., 2009). TOR regulates numerous processes that might be involved in DR mediated longevity, a prime example being protein synthesis. TOR stimulates protein synthesis by modulating key components of the translation machinery. In response to nutrients, TOR phosphorylates and activates ribosomal subunit S6 kinase which in turn phosphorylates and activates translation elongation factor 2 (eEF2) kinase thereby promoting global translational levels (Wang et al., 2001). Consistent with this, reduced food intake causes suppression of the TOR pathway and thus reduction in translation and increase in lifespan (Kapahi et al., 2010). In line with the reduction of the TOR pathway leading to longevity, reduced levels of S6 kinase extends lifespan in yeast, worms, flies and mice (Hansen et al., 2007; Kaeberlein et al., 2005; Kapahi et al., 2004; Selman et al., 2009). In *C. elegans* the longevity conferred by the loss of *rsks-1/*S6K depends on several factors including *pha-4/*FOXA (Sheaffer et al., 2008), *hif-1* (Chen et al., 2009) and *hsf-1* (Seo et al., 2013). A plausible explanation for the longevity benefits

observed with the down-regulation of TOR is that TOR knockdown leads to reduction in protein synthesis rates, which makes animals live longer. An overall reduction in protein synthesis is known to extend lifespan (discussed below). Concordantly, rates of protein synthesis are reduced in *rsks-1/*S6K worms (Ching et al., 2010; Hansen et al., 2007; Pan et al., 2007), which might be the reason behind their longevity.

In addition, TOR also phosphorylates and inhibits 4E-binding protein (4E-BP), which represses translation by binding to translation initiation factor eIF4E. *C. elegans* does not possess a structural 4E-BP homologue (Syntichaki et al 2007). In *Drosophila*, 4E-BP is not required for increased lifespan in response to rapamycin and one form of DR (Partridge et al., 2011) although its effects on translation have been implicated in a second type of DR through modulation of mitochondrial activity (Zid et al., 2009). TOR may relay its effects on aging indirectly through other processes such as stress resistance, endoplasmic reticulum stress signaling and metabolic alterations (Kapahi et al., 2010) but it is not completely understood how these processes may slow aging.

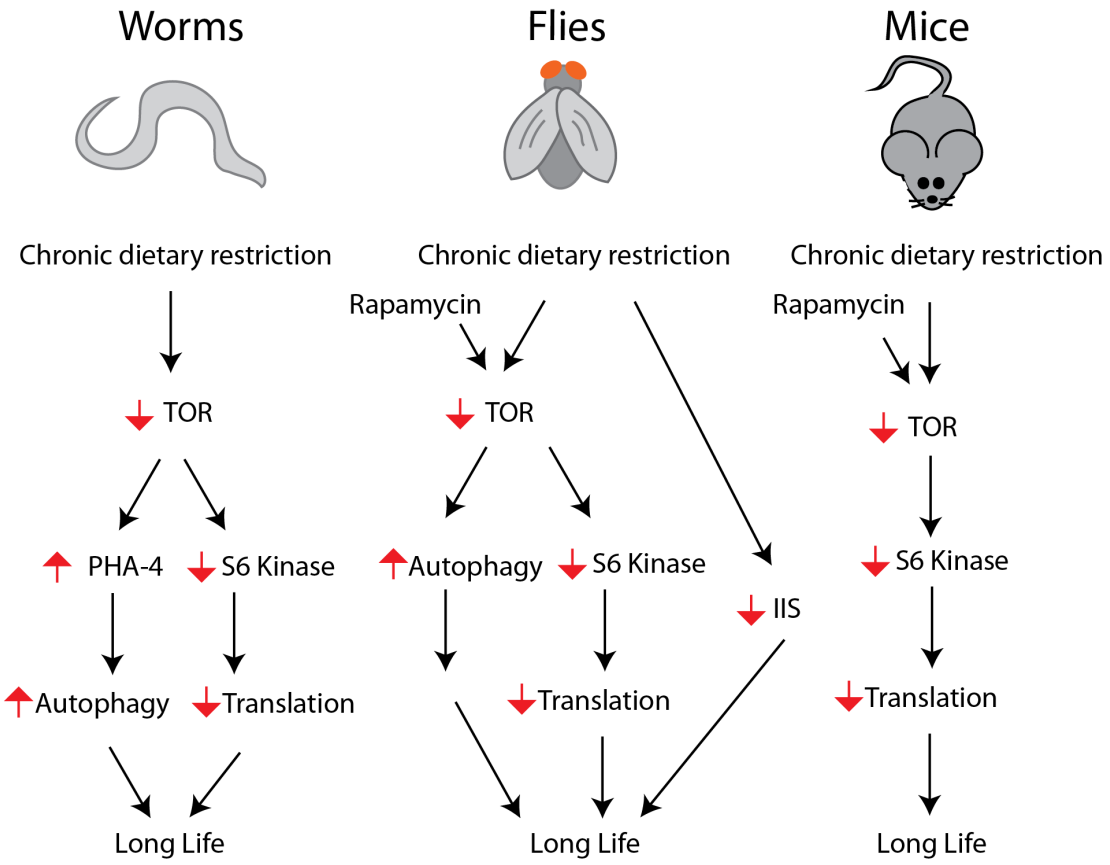

Figure 1. DR extends lifespan across species. The TOR pathway modulates the effects of DR in multiple species in part by regulating autophagy and also S6 kinase activity and thereby translation. (Adapted from (Kenyon, 2010)

Another cellular process that regulates lifespan is protein synthesis. Protein synthesis links nutritional state with overall organismal growth and is regulated by the TOR pathway as discussed above. In addition, protein synthesis is also regulated by the IIS pathway (Proud, 2006; Shamji et al., 2003; Sonenberg et al., 2000). Reduced nutrients lower the level of insulin which in turn decreases the activity of Ser/Thr kinase Akt preventing Akt from phosphorylating and activating S6 kinase. Therefore IIS and TOR pathways cross talk in regulating protein synthesis levels via the common mediator S6 kinase. Furthermore inputs from the MAPK signaling pathway also regulate protein synthesis. Signaling through the MAPK pathway promotes the assembly of the initiation factor complexes and also activates the elongation machinery for translation of mRNA (Hsieh and Papaconstantinou, 2004;

Proud, 2007). Besides IIS and TOR pathways which when suppressed extend lifespan, knockdown of a few other factors leads to reduced translational levels and thereby promotes longevity. For example knockdown of several ribosomal proteins including RPS15, RPL6 and RPL19 reduces translation and increases lifespan (Hansen et al., 2007). Reduction in the levels of translation initiation factors eIF2β, eIF4G and eIF4E also leads to an extension in lifespan (Hansen et al., 2007; Syntichaki et al., 2007). However the reasons why organismal lifespan is increased with a modest reduction in protein synthesis are still unclear. Conceivably, a reduction in protein synthesis conserves cellular energy reserves. It is well known that translation is a high-energy consuming process (Proud, 2002). A single amino acid addition into a growing polypeptide requires the energy derived from the hydrolysis of four ATP molecules. Therefore a reduction in translation can conserve cellular energy reserves that can be channeled towards the mechanisms of repair and maintenance, leading to prolonged cell survival and resistance against stress conditions. Nevertheless, the exact molecular mechanism of how different pathways coordinate under nutritional stress to down-regulate translation and extend lifespan is not clearly understood.

1.7 Cellular Processes that Mediate Longevity

Besides the signaling pathways that were discussed afore, certain cellular processes can also play a role in regulating lifespan of an organism. Prime example of this regulation is the mechanism of improving protein homeostasis or proteostasis. Cells have inherent mechanisms of keeping their proteome in balance. The two main processes by which this is carried out biologically are autophagy and proteasomal degradation. Macroautophagy (hereafter referred to as autophagy) is the process of regulated degradation and recycling of unnecessary or dysfunctional cellular components occurring by isolation of targeted cellular components within a double-membrane vesicle called an autophagosome. The autophagosome fuses with a lysosome and the contents are degraded and recycled. Proteasomal degradation of the unneeded or damaged proteins happens through the Ubiquitin Proteasome System (UPS) which involves the tagging of proteins with ubiquitin and further degradation of these tagged proteins via the 26S proteasome. It is known that proteostasis mechanisms decline with age and in accord with this improving protein quality control leads to longevity benefits. It is also known that protein homeostasis is enhanced in long-lived animals (Morimoto and Cuervo, 2014). Therefore it is easy to imagine that a lot of diseases involving protein aggregation are age-associated and generally appear in old age e.g. neurodegenerative diseases like Alzheimer's and Parkinson's. Thus a lot of contemporary research focuses on improving protein quality control in order to find therapeutic interventions to combat such diseases. The following paragraphs discuss the current understanding of proteostasis particularly focusing on autophagy and proteasomal degradation.

Autophagy is an important cellular mechanism acting as a bulk degradation process in metabolic stress conditions like starvation, DNA damage, oxidative and endoplasmic reticulum (ER) stress (Egan et al., 2011; Rubinsztein et al., 2011). Autophagy induction using pharmacological agents or genetic methods increases lifespan in worms, flies and mice (Harrison et al., 2009; Kenyon, 2010). Autophagy is not only required in almost all the longevity models studied (Gelino and Hansen, 2012) but is also sufficient for lifespan extension. For example, the longevity of *daf-2*

worms is reversed with the loss of BEC-1, which plays a role in localizing autophagy proteins to pre-autophagosomal membranes (Melendez et al., 2003). Autophagy is also required for longevity conferred by calcineurin deficiency (Dwivedi et al., 2009) and depletion of p53 homolog CEP-1 (Tavernarakis et al., 2008) in *C. elegans.* Similarly, lifespan extension in worms achieved by genetic or pharmacologic activation of the SIRT1 ortholog SIR-2.1 (Morselli et al., 2010), or treatment with the acetyltransferase inhibitor spermidine (Eisenberg et al., 2009) is abrogated if autophagy is inactivated. Important regulatory genes of autophagy including *Atg1* and *Atg8* when overexpressed in *Drosophila* promote longevity (Rubinsztein et al., 2011). *Atg5* over-expression in mice induces autophagy and increases lifespan (Pyo et al., 2013). Autophagy is also required for DR mediated longevity in *C. elegans* (Jia and Levine, 2007). In conjunction with its requirement for longevity in worms, another study reported that autophagy is induced upon dietary restriction in mice (Morselli et al., 2010). In addition to extending lifespan, multiple studies have reported that autophagy modulation leads to beneficial effects in diseases occurring due to protein aggregation. For example, in flies and mice autophagy induction using rapamycin enhances the clearance of aggregates of tau and Aβ (Berger et al., 2006; Caccamo et al., 2010). Similarly, rapamycin induced autophagy also clears α-synuclein aggregates in cultured cells (Webb et al., 2003). Another study showed that overexpression of *beclin-1*, which plays a crucial role in autophagy, ameliorates aggregation of α-synuclein (Spencer et al., 2009). And lastly it has also been shown that pharmacological interventions that induce autophagy lead to a drastic improvement of Huntington's disease phenotype (Sarkar et al., 2007). All this evidence points towards the fact that an inherent improvement in proteostasis via autophagy can lead to an improvement in healthspan and also an increase in lifespan of organisms.

Another key component for maintaining proper proteostasis is the UPS. Protein quality control mechanisms carried out by the UPS decline with age. There are various levels reported at which the UPS activity declines with age including decreased expression of the components of the UPS (Lee et al., 1999), alteration of the proteasomal subunits (Ferrington et al., 2005), disassembly of the proteasomal

machinery (Vernace et al., 2007) or inactivation of the UPS through protein aggregates (Andersson et al., 2013). There have been several studies conducted which provide a clear link between protein clearance mechanisms and aging. And this holds true for different species. For example proteasome inhibitor treatment of fibroblasts reduces their replicative lifespan and induces senescence like features (Torres et al., 2006). Likewise RNAi knockdown of 19S and 20S subunits of the proteasome in *C. elegans* shortens lifespan (Ghazi et al., 2007; Vilchez et al., 2012). And conversely if the activity of the UPS is increased, this leads to increased lifespan in various organisms. In *S. cerevisae,* over-expression of UMP1/POMP, which is a proteasomal chaperone, stabilizes the activity of the UPS and leads to increased viability during stationary phase (Chen et al., 2006). *rpn11* is a multi-protein complex involved in the ATP-dependent degradation of ubiquitinated proteins and its overexpression in *Drosophila* inhibits the age-dependent decline of 26S/30S proteasome activity and thereby increases lifespan. Similarly overexpression of *rpn6,* a subunit of the 19S proteasome, increases lifespan of worms (Tonoki et al., 2009; Vilchez et al., 2012). Well-established *C. elegans* models of longevity have been associated with elevated UPS activity. For example a recent study reported that decreased IIS in worms leads to an induction of proteasomal activity (Matilainen et al., 2013). Another study reported that the CUL-1 E3 ubiquitin ligase complex (Skp-1-Cul-1-F-Box) is required for longevity conferred by reduction in the IIS pathway (Ghazi et al., 2007). Similarly HECT E3 ubiquitin ligase in *C. elegans* called WWP-1 acts as a positive regulator of lifespan in response to DR (Carrano et al., 2009). DR also induces the expression of the 19S proteasomal subunit Psmc3/Rpt5 and the proteasome activator PA28 (Lee et al., 1999). Intriguingly results from the studies on higher animals are consistent with the observations in the lower organisms. Interesting studies reported elevated UPS activity in naturally occurring exceptionally long-lived animals like the naked mole rat (Perez et al., 2009) and the giant clam (Ungvari et al., 2013).

In addition to regulating lifespan, several reports have evidenced clear links between age-related neurodegenerative diseases and proteasomal activity (Zabel et al., 2010). For example, the inclusion bodies observed in Alzheimer's, Parkinson's,

Huntington's Disease and Amyotrophic Lateral Sclerosis contain very high amounts of ubiquitin suggesting an association between proteasomal dysfunction and neurodegeneration (Huang and Figueiredo-Pereira, 2010; Matsuda and Tanaka, 2010). Proteasome activity is decreased in the brains of patients suffering from Alzheimer's disease (Keck et al., 2003). Moreover elevating the UPS activity has been proven beneficial for Huntington's disease model. Higher levels of *rpn6* in worms and *rpn11* in flies decrease the toxic protein aggregates in the Huntington's disease model (Tonoki et al., 2009; Vilchez et al., 2012). Hence there is substantial evidence that increased protein quality control leads to an increase in health-span by ameliorating a number of age-associated diseases and in addition also increases lifespan across different species.

Besides cellular processes leading to protein quality control, our lab recently demonstrated that metabolic pathways can also play a role in regulating proteostasis. Activation of the hexosamine pathway either genetically or by the supplementation of N-glycan precursor, N-acetylglucosamine in the medium increases lifespan of *C. elegans*. Moreover activation of this pathway leads to an improvement in the pathology of distinct neurotoxic disease models. This improved health state and increased lifespan depend on elevated ER-associated protein degradation, proteasomal activity and autophagy (Denzel et al., 2014). Hence activation of the hexosamine pathway leads to improved protein quality control and in the future may be a promising pathway to target for developing therapeutic interventions against age-associated proteotoxic diseases.

1.8 Convergent Mechanisms Regulating Longevity

As described earlier there are a number of genes and pathways that regulate longevity across different species and some of them act independently of each other. However there is recent evidence that highlights the crosstalk across different models of longevity. The remarkable molecular conservation of the components linked with different longevity pathways allows us to understand longevity from a multi-species perspective. The degree to which distinct longevity pathways converge on common downstream factors is not entirely understood yet and there have been a handful of these convergent factors recently identified that get functional inputs from different pathways governing lifespan. HLH-30 was described recently as one such factor which acts downstream of multiple longevity pathways and is required for lifespan extension in these distinct models of longevity in *C. elegans* (Lapierre et al., 2013). HLH-30 is the worm homolog of the mammalian transcription factor EB (TFEB). TFEB was recently reported to act as a pivotal factor regulating lysosomal biogenesis, autophagy and fat metabolism in mammals (Sardiello et al., 2009; Settembre et al., 2013; Settembre et al., 2011). *C. elegans* HLH-30 also regulates the expression of multiple autophagy and lysosomal genes and thereby modulates the autophagy process (Lapierre et al., 2013). In various longevity models, HLH-30 is shuttled to the nucleus where it drives the expression of multiple autophagy genes. Over-expression of *hlh-30* is sufficient to induce longevity in wildtype worms (Lapierre et al., 2013). In the same study it was also reported that the levels of TFEB were higher in the liver extracts of mice subjected to DR (Lapierre et al., 2013), suggesting that TFEB could impart some of the benefits of DR.

Recently our lab identified another protein complex belonging to the Myc superfamily that works downstream of the major longevity pathways and acts as a convergent node in longevity regulation in *C. elegans*. Myc family members MML-1 (Myc/mondo-like) and its partner MXL-2 (Max, Max-like) were identified in RNAi based suppressor screens for suppression of germline-mediated longevity in worms (Johnson et al., 2014; Nakamura et al., 2016). MML-1 and MXL-2 are required for

longevity conferred by multiple longevity pathways including the IIS pathway and DR (Nakamura et al., 2016). The study further reported that MML-1 and MXL-2 work in transcriptional cascades down-regulating TOR activity, which in turn activates autophagy and HLH-30 activity in the nucleus. This study reveals a fundamental regulatory network largely affecting worm lifespan.

Another convergent mechanism, which is required for lifespan extension in almost all longevity pathways in *C. elegans* is autophagy. Autophagy, as discussed above in detail, is a major cellular process for degradation and subsequent recycling of proteins and organelles in eukaryotic cells. Autophagy is induced in major longevity models including IIS, TOR and DR and is required for extended lifespan in these pathways (Hansen et al., 2008; Melendez et al., 2003). Autophagy is also required for rapamycin to extend lifespan in *Drosophila* (Bjedov et al., 2010) making it an important convergent mechanism for lifespan extension in different species.

ROS levels have also been associated with lifespan in different longevity models. The free radical theory of aging has been challenged by numerous studies that have reported elevated levels of ROS to be required for lifespan extension. For example ROS promote lifespan extension in germline-deficient worms (Wei and Kenyon, 2016). In yeast and worms it has been shown that DR induces mitochondrial respiration and oxidative stress (Mesquita et al., 2010; Schulz et al., 2007; Sharma et al., 2011). In worms it was also reported that treatment with juglone, which generates ROS extends lifespan in a manner dependent on DAF-16/FOXO (Heidler et al., 2010). There are contradicting studies about the role of superoxide dismutase (SOD), which is a ROS scavenger, on lifespan (Back et al., 2012; Liochev, 2013). However there is evidence that longevity due to overexpression of SOD is not caused by decreased oxidative damage (Cabreiro et al., 2011), favoring the notion that ROS accumulation does not affect lifespan negatively. Since there is ample evidence in favor of an association between elevated ROS levels and lifespan, it can be proposed as a convergent mechanism that regulates aging across multiple species and in different pathways.

1.9 Nucleolar and Ribosomal Proteins in Aging

The nucleolus is the most obvious sub-nuclear structure where ribosomal RNA (rRNA) and ribosomal subunits are synthesized. Within the nucleolus, ribosomal DNA (rDNA) is transcribed by RNA polymerase I (RNA Pol I). Transcription over rDNA region generates precursor rRNA or pre-rRNA molecules that are further processed to generate mature 28S, 18S, and 5.8S rRNAs. These rRNAs together with ribosomal proteins are exported out of the nucleus to the cytoplasm for the purpose of translation (Figure 2) (Boulon et al., 2010). The size of the nucleolus is a measure of the rate of ribosome biogenesis. Thus actively proliferating cells that require higher levels of ribosomes and elevated global translational levels possess large nucleoli whereas nucleolar size decreases in metabolically inactive cells (Derenzini et al., 1998). Since nucleolus is the site of ribosome biogenesis, it plays an active role in determining the metabolic state of a cell. There is growing evidence that the processes regulated by the nucleolus play important roles in regulating aging.

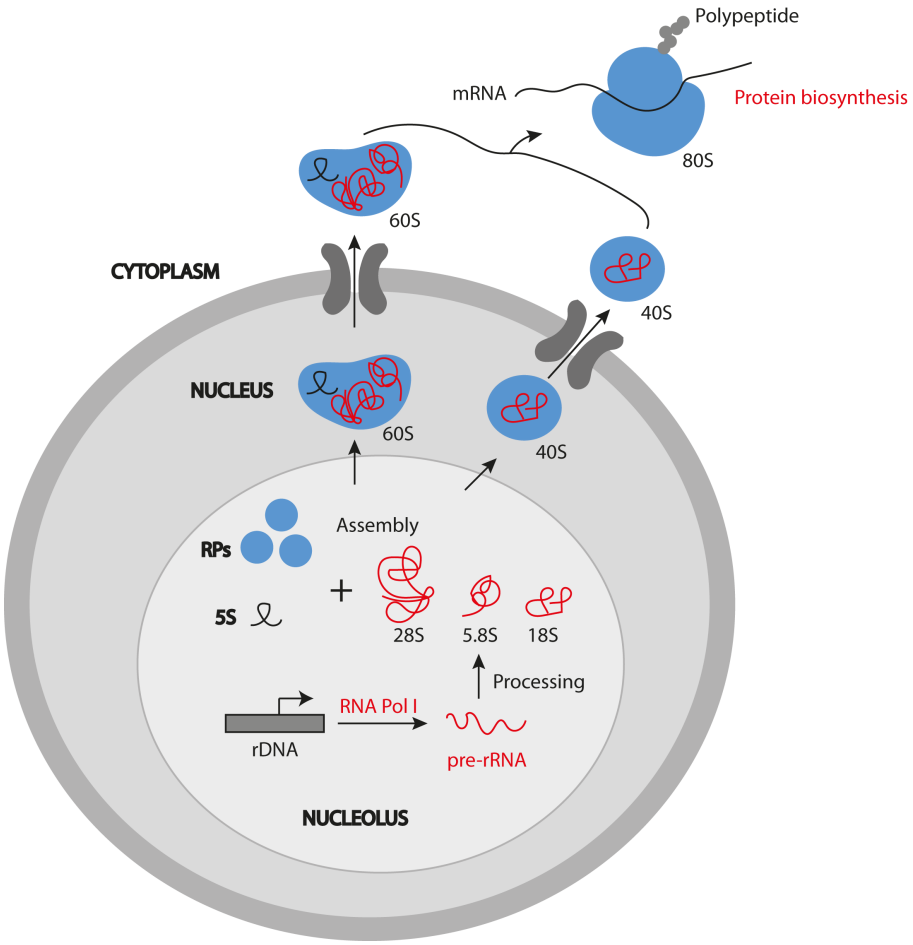

Figure 2. Ribosome biogenesis at a glance. Pre-rRNAs generated by RNA polymerase I transcription in the nucleolus are processed into 28S, 5.8S and 18S rRNAs and assembled together with ribosomal proteins (RPs) and 5S rRNAs to form pre-60S and 40S ribosomal subunits. These subunits are translocated to the cytoplasm where they mature and carry out translation.

For example the TOR pathway in addition to regulating lifespan also regulates ribosome biogenesis, which implies that changes in nucleolar function can affect lifespan of an organism (Martin et al., 2006). Rapamycin treatment, which inhibits the TOR pathway, has been shown to inhibit synthesis and processing of rRNA and also promotes its decay. Furthermore, it was reported that mTORC1 and its components raptor and mTOR are present in nucleoli and that rapamycin treatment induces loss of these components from nucleoli (Iadevaia et al., 2012). There is also evidence in *C. elegans* that reduction in TOR signaling reduces nucleolar size (Sheaffer et al., 2008). Further interesting data that supports the evidence for the

link between nucleolus and aging comes from the yeast gene *sgs1,* which encodes a helicase like protein that localizes to the nucleolus. *sgs1* is closely related to the human Werner gene (*WRN*) (Watt et al., 1996). Mutations in the *WRN* gene in humans lead to a pre-mature aging disease called the Werner Syndrome. And intriguingly, WRN protein is also localized in the nucleolus of human cells (Gray et al., 1998). *sgs1* mutations cause pre-mature aging in yeast and together with pre-mature aging there is also a significant enlargement and fragmentation of nucleoli (Sinclair et al., 1997). In line with this, there have been studies that have reported that an alteration in ribosome biogenesis can also affect aging. Replicative lifespan of yeast increases by altering ribosome biogenesis through the knock down of genes encoding 60S ribosomal subunit proteins. Furthermore, inhibiting 60S subunit biogenesis using small molecule inhibitors also increases the replicative lifespan of yeast (Steffen et al., 2008). And it is known that knock down of different ribosomal proteins and a modest down-regulation of protein synthesis leads to lifespan extension in *C. elegans* (Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). In addition, it was shown that RNAi mediated knock down of nucleolar GTPase NOG-1, which is required for 60S subunit biogenesis, leads to longevity in worms. Consistent with this, overexpression of NOG-1 reduced wildtype lifespan (Kim et al., 2014). All this evidence points towards a role of nucleolus and ribosome biogenesis in regulating lifespan of an organism. Another recent study also reported a role of nucleolus in aging in *Drosophila.* In this study, a basic helix-loop-helix transcription factor called Mnt was reported to regulate the expression of various genes that are involved in ribosome biogenesis. Over-expression of Mnt in skeletal muscle improved the climbing ability of flies in old age indicative of improved health. Moreover Mnt over-expression in skeletal muscle also increased lifespan. Expression profiles revealed a down-regulation of the genes encoding nucleolar proteins and reduced levels of rRNA upon Mnt over-expression. RNAi against genes involved in rRNA processing and ribosome biogenesis increased lifespan indicating nucleolar function in determining lifespan (Demontis et al., 2014).

Although there is enough evidence to back the connection of nucleolar processes in regulating aging, the exact mechanism of how these effects are brought

about is not clearly understood. There are various hypotheses that still need to be validated. For example it is known that an increase in Arf and p53 proteins promotes cancer resistance and also increases lifespan in mice (Matheu et al., 2007). Arf is alternative reading frame protein, which is induced upon mitogenic stimuli such as aberrant growth. p53 is an extensively studied tumor suppressor gene which maintains the stability of the genome by preventing mutations that can lead to tumor. p53 is induced by the inhibition of ribosome biogenesis (Le Bouteiller et al., 2013) and it can be argued that the cancer resistance and longevity with decreased ribosome biogenesis may be caused by the elevated levels of p53. Likewise, reduced metabolic rates and a drop in free radical production have been linked to aging and lifespan implying a link between ribosome biogenesis and aging (Hulbert et al., 2007).

1.10 Aims of the Study

As discussed above, a number of pathways and cellular processes contribute to longevity across multiple species. Single gene mutations can lead to significant longevity benefits and improved health quality. But how these pathways interact with each other and whether the inputs from these pathways converge is largely unknown, despite the discovery of some important players such as HLH-30 and MML-1/MXL-2. The overarching aim of my thesis work is to identify novel players that act in mediating longevity across different longevity pathways. Furthermore I seek to understand what are the processes that the different longevity pathways converge on to bring about lifespan extension. This study can reveal the common nodes that work downstream of various pathways. If one is able to understand these convergent mechanisms in detail, it could prove useful to develop diagnostic or therapeutic strategies to target factors involved in these pathways, to combat age-related diseases and improve health-span into old age.

For this study, genetic screens carried out by a former colleague (Bree Heestand) identified a number of mediators DR longevity in *C .elegans.* I started to characterize one of these genes called *ncl-1,* which encodes a homolog of mammalian TRIM2 and Drosophila BRAT/tumor suppressor. NCL-1 affects nucleolar size and rRNA production in *C. elegans* (Hedgecock and Herman, 1995).

The following were the major aims of my thesis:

Aim 1. Determine the role of *ncl-1* in various longevity pathways, and thereby see if it regulates a convergent axis of lifespan regulation.

Aim 2. Explore the physiologic effects and genetic interactions of *ncl-1* and longevity pathways on nucleolar physiology and ribosome biogenesis.

Aim 3. Investigate if the effects observed in *C. elegans* are also conserved in higher organisms.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth, Maintenance and Culturing of *C. elegans* Strains

All the worm strains were grown using standard procedures on nematode growth medium (NGM) in petri dishes seeded with *E. coli* strain OP50 (Brenner, 1974). NGM is composed of NaCl, peptone, agar, KPO4, cholesterol, CaCl, MgSO4, and water. OP50 bacteria used as the food source for *C. elegans* are uracil-auxotrophs which allows for the slow growth of the bacterial lawn. All *C elegans* strains were grown and maintained at 20°C unless otherwise noted. Strains carrying *glp-1(e2141)* mutation were maintained at 15°C and shifted to 25°C for inducing germlineless phenotype. The strains used for the experiments were: N2 (wildtype), *eat-2(ad465), ncl-1(e1865), ncl-1(e1942), eat-2(ad465);ncl-1(e1865), eat-2(ad465);ncl-1(e1942), isp-1(qm150), isp-1(qm150);ncl-1(e1942), glp-1(e2141), glp-1(e2141);ncl-1(e1942), daf-2(e1370), daf-2(e1370);daf-16(mgDf50), hlh-30(tm1978), cguIs001* (FIB-1::GFP) (Lee et al., 2010), *eat-2(ad465)+cguIs001, ncl-1(e1942)+ cguIs001* and *eat-2(ad465);ncl-1(e1942)+ cguIs001. dhEx1007 ncl-1* extra-chromosomal transgenic strain was generated by injecting fosmid DNA WRM0611AC10 (*ncl-1*::TY1 EGFP) (30 ng/µl) and a co-injectable marker (*myo-2::mcherry* at 10 ng/µl) in N2 strain and further crossed into *eat-2(ad465)*, *eat-2(ad465);ncl-1(e1865)* and *eat-2(ad465);ncl-1(e1942)* backgrounds. The transgenic worms were maintained by selecting the worms showing the expression of the co-injected marker. Worm maintenance also entailed decontaminating strains from time to time to get rid of contaminants, which was done by bleaching. In this method, contaminated plates were washed off with M9 physiological buffer and the worms were collected in a falcon tube. The falcon tubes were briefly centrifuged so that the worms were collected at the bottom. M9 buffer was replaced by the bleach solution containing sodium hypochlorite, potassium hydroxide and water. Worms were incubated with the bleach solution for around 10 minutes at room temperature and afterwards the samples were washed twice with M9 buffer. At the end of the treatment only the eggs of the animals survive which were then seeded on plates containing *E. coli* OP50 bacteria.

2.2 Lifespan Analyses

Lifespan analyses were performed at 20°C as previously reported (Gerisch et al., 2001). Worms were age-synchronized by 6-8 hours of egg-lays. Lifespan experiments were started by using a total of 120 worms. The experimental worms were moved to 6 cm petri dishes at the density of 15 worms per plate. Worms were scored for movement every other day. Until the end of the reproductive span, worms were moved to fresh plates every other day. Animals that crawled off the plates, burst due to a ruptured vulva or had internal hatching of the eggs were censored from the experiment. RNAi lifespan analysis experiments were carried out following previously described protocol (Kamath et al., 2001). All RNAi treatments were performed throughout development and adulthood except *let-363/*TOR and *fib-1,* which were initiated on the first day of adulthood. Finally the data was plotted to calculate mean, median, and maximum lifespans and log-rank (Mantel-Cox) analysis was used to determine significance between the lifespan curves (Supplementary Table 2).

2.3 Bacterial Dilution Regimen (BDR) for Lifespan Analyses

For BDR lifespan analyses, the method followed was the same as described previously (Panowski et al., 2007). 90 worms were used for each bacterial concentration to be tested and the worms were scored every 3-4 days. The worms were transferred to freshly prepared bacterial conditions on each day of scoring. The liquid media also called the BDR media was made up of NaCl, K_2HPO_4, KH_2PO_4, cholesterol and water. Additionally, antibiotics carbenicillin (50ug/ml), kanamycin (10ug/ml), and tetracycline (1ug/ml) were added to inhibit bacterial replication and thus maintain the concentration of bacteria. The experiment was carried out in 12 well cell culture dishes. For the first two weeks of the experiment FUdR (100 mg/mL) was added to the BDR media to prevent progeny production. Mantel-Cox Log Rank method was used for statistical analysis (Supplementary Table 2). Bacterial concentration was estimated by serial dilutions, subsequent plating and counting colony forming units (CFUs).

2.4 Western Blotting

Day 1 adult worms (50) were collected in M9 and Laemmli lysis buffer was added and the samples were snap-frozen in liquid nitrogen. The samples were then boiled at 95°C for 5 minutes, ultrasonicated for 10 cycles and loaded on 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels. After separation, proteins were blotted on a nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System (BioRad). The membranes were then blocked for an hour at room temperature in 5% milk in Tris-buffered Saline and Tween20 (TBST) and probed with the following antibodies against: RPS-6 (abcam® ab70227, 1:1000), RPS-15 (antibodies-online.com ABIN503870, 1:1000), Fibrillarin (Novus Biologicals NB300-269, 1:1000) and β-Actin (abcam® ab8224, 1:5000). The antibodies were diluted in TBST with 1% Bovine Serum Albumin (BSA). The membranes were incubated in the primary antibody overnight at 4°C. Specific secondary antibodies (mouse or rabbit) were used at a concentration of 1:4000 in TBST with 1% BSA at room temperature for one hour. The membranes were developed with Western Lightening® Plus-Enchanced Chemiluminescence Substrate (PerkinElmer). The membranes were imaged then with ChemiDoc Imager (BioRad)

[For all Western Blots: n=50 worms/replicate, 3 independent replicates]

For Drosophila western blots, 5 females were homogenised in 100 µl of RIPA lysis buffer carrying 1X Complete mini protease inhibitor (EDTA free) (Roche). The homogenized lysates were cleared by centrifugation. Protein content was determined by following the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). 30 µg of total protein was loaded on precast gels (Bio-Rad Any KD, Mini-PROTEAN® TGX™). The proteins were transferred to nitrocellulose membranes using Trans-Blot® Turbo™ Transfer System (BioRad). The membranes were then blocked for an hour at room temperature in 5% milk in Tris-buffered Saline and Tween20 (TBST) and probed with the same antibodies as above following the same dilutions.

[For all Western Blots: n=5 flies/replicate, 3 independent replicates]

2.5 qRT-PCR

One hundred age-matched day 1 adult worms were collected in TRIzol (Invitrogen) and snap-frozen in liquid nitrogen. RNA extraction was performed by using RNeasy Mini Kit (QIAGEN). Extracted RNA samples were analyzed by NanoDrop 2000c (peqLab) to validate purity and determine quantity of RNA. cDNA was subsequently synthesized from the extracted RNA using iScript cDNA Synthesis Kit (Bio-Rad). The samples were loaded along with the primers onto a 384 well plate using JANUS automated workstation (PerkinElmer). Power SYBR Green master mix (Applied Biosystems) was used for qRT-PCR on a ViiA7 384 Real-Time PCR System machine (Applied Biosystems). *ama-1* served as the endogenous control. qPCR primer sequnces are given in Supplementary Table 1

2.6 rRNA Analyses

Levels of rRNA were monitored by running total RNA, extracted from 100 worms as described above (2.5 qRT-PCR section), on agarose gels. NorthernMax® Kit protocol was followed for running RNA gels. The extracted RNA was mixed with three volumes of formaldehyde load dye from the kit. The samples were incubated at 65°C for 15 min and were loaded into the wells of an agarose gel prepared for RNA grade work. 1 µl of commercial Ethidium Bromide was added to the samples right before loading them on the gel. The gel was run at ~5 V/cm for two hours. Alpha Innotech MultiImage II was used for imaging the gel.

[For RNA extraction: n=100 worms/replicate, 3 independent replicates]

2.7 Immunofluorescence

Immunofluorescence was performed on 10 µm thick cryo-sections of mouse tissues derived from kidney, liver and brain by fixing the samples with 4% Paraformaldehyde for 15 minutes at room temperature (RT) followed by three washes with PBS at RT. The samples were then blocked with 5% Normal Donkey Serum in PBS with 0.1% Triton-X for 30 minutes at RT followed by an over-night incubation at 4°C with the primary antibody against Fibrillarin (abcam® ab166630,

1:200). After three subsequent washes with PBS, the samples were then probed with the secondary anti-rabbit antibody at RT for one hour followed by three more washes with PBS. The samples were mounted with ProLong® Gold Mounting Medium containing DAPI (ThermoFisher Scientific) and imaged with Axio Imager Z1 (Zeiss) and a laser-scanning confocal microscope (TCS SP5-X; Leica). Immunofluorescence quantification represents three independent biological replicates with each replicate representing 3 mice (DR) and 2 mice (IRS1 KO). Imaging and quantification of the experiments were performed in a blinded manner.

For *Drosophila,* guts and fat bodies were dissected out in PBS followed by immediate fixation with 4% PFA in PBS and permeabilization for 10 minutes at RT with 0.3% Triton X-100 in PBS (PBST). Blocking, primary and secondary antibody incubation were done in 5% BSA in PBST using Fibrillarin (Novus Biologicals NB300-269, 1:250) as the primary antibody and goat anti-mouse conjugated to Alexa Fluor 488 (Invitrogen Inc., 1:1000) as the secondary antibody. Hoechst 33342 was applied at 1:1000 for staining nuclei. Tissues were extensively washed with PBST after antibody treatments and finally mounted on glass slides with 80% glycerol in PBS. The quantification represents three independent biological replicates with each replicate representing 5 dissected flies. Imaging and quantification of the experiments were performed in a blinded manner.

For staining human muscle biopsies, samples were thawed at RT. Then the samples were blocked with 5% milk in PBS with 0.05% Tween (PBST) for 30 minutes at RT, followed by three washes with PBST. The primary antibody, Rabbit-anti-Fibrillarin (abcam® ab166630, 1:600 in PBST), was incubated overnight at 4°C. After three washes with PBST, samples were incubated with the secondary goat-anti-rabbit-conjugated-Alexa647 antibody (Molecular Probes, 1:1000 in PBST) for 1 hour at RT, followed by three washes in PBST and one wash in PBS containing DAPI (0.5µg/mL, Sigma-Aldrich, Saint Louis, Missouri, USA). Slides were mounted with Aqua Poly-Mount® (Polysciences Inc, Niles, Illinois, USA). All samples were stained on the same day with the same antibody mixes.

For cells, after appropriate starvation the medium was washed off and the cells were washed twice briefly with PBS. Then the cells were fixed with 4% PFA at

4°C for 10 minutes followed by a wash with PBS. Then 1 ml of ice-cold methanol (100%) was added and the cells were incubated for 10 minutes at -20 °C. The cells were washed in PBS twice and further blocked in 5% BSA in PBS for one hour at room temperature. Anti-Fibrillarin (abcam® ab166630, 1:200) antibody served as the primary antibody and was added for one hour at room temperature followed by three washes each lasting five minutes with PBS. Then the secondary antibody anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen Inc., 1:500) was applied to the samples and the samples were incubated in the dark for one hour at room temperature. The samples were then washed three times with PBS and mounted on the slides using ProLong® Gold Mounting Medium containing DAPI (ThermoFisher Scientific). Imaging of the cells was performed at 63X magnification with Axio Imager Z1 (Zeiss).

2.8 Imaging and Quantification

DIC microscopy was used to perform all the nucleolar imaging. Hypodermal, germ cell and pharyngeal muscle nucleoli of age-matched day 1 adults were imaged using 100X magnification with Axio Imager Z1 (Zeiss) (Supplementary Table 3). Worms carrying FIB-1::GFP and NCL-1::GFP transgenes were imaged using 63X magnification with Axio Imager Z1 (Zeiss). Immunofluorescent images were acquired using a laser-scanning confocal microscope (TCS SP5-X; Leica), equipped with a white light laser, a 405- diode UV laser, and a 100X objective lens (HCX Plan-Apochromat CS 100X oil, 1.46 NA). For human muscle biopsies, a total 15 representative fields with a 63X objective from each muscle sample were obtained, using the DM5500 fluorescent microscope (Leica) and the LAS AF software (version 2.3.6, Leica). Anti-Fibrillarin was detected with the Y5 cube, and nuclei were detected with the A4 cube. The area of the nucleolar and nuclear regions was quantified manually with the freehand tool, and subsequently the ratio of nucleolar/nuclear area was calculated. For the human samples, the average ratio of nucleolar/nuclear area (from an average of 100.4 (± 28.9) nuclei) per sample was used for the analyses.

2.9 *Drosophila melanogaster* Experiments: DR, Rapamycin Treatment and *dilp2-3,5*

DR in *Drosophila melanogaster* was performed by feeding a total of 50 hatched flies with 0.5x SYA food compared to ad libitum food supply of 2x SYA for 10 days (Bass *et. al.,* 2007). Rapamycin treatment was performed by dissolving Rapamycin in absolute ethanol and mixing it with SYA food at a final concentration of 200 μM and fed to a total of 50 age-matched flies. For control food, ethanol alone was added. Both DR and Rapamycin treatment were performed for 10 days before harvesting the flies for experiments. The treatments were performed separately in 3 different vials serving as 3 independent biological replicates. Long-lived dilp2-3,5 (Grönke *et. al.,* 2010) and control wDah flies were harvested on day 1 of adulthood. The flies were dissected and immunofluorescence was performed on the dissected tissues as described above.

2.10 DR and IRS1 KO Mice

The mice used for the experiments were handled according to the guidelines of LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany). C57BL/6 male mice were maintained under 12 hour light:12 hour dark schedule and were fed standard chow diet (SC) - 4.5g SC/animal/24 hours (ssniff® Spezialdiäten GmbH) until 10 weeks of age and then subjected to DR at 75% food intake (3g SC per animal/24 hours) compared to ad libitum fed control mice. The DR regimen was continued for 1 month and the mice were sacrificed at the age of 14 weeks along with the ad libitum fed controls to perform cryosectioning for the analysis of nucleoli. The tissues sampled with sectioning were kidney and liver. C57BL/6 IRS1 KO male (Selman et. al., 2008) and WT control male mice were maintained similarly on SC diet. The animals were sacrificed at the age of 12 months to perform cryo-sectioning for the analysis of nucleoli. The tissues sampled with sectioning were kidney and brain. For both the experiments cryo-sectioning was performed horizontally across the entire tissue. This nature of processing aided in observing the effect of the treatments across different cell types in each tissue.

2.11 Dietary Restriction and Exercise Intervention in Human Volunteers

Samples for nucleolar staining were obtained from the biomaterial collected in the Growing Old Together Study, a 13-weeks lifestyle intervention in older adults, consisting of 12.5% caloric restriction and 12.5% increase in physical activity, resulting in an average weight loss of 3.3kg. The study design, inclusion and exclusion criteria, and changes in metabolic parameters have been described previously (van de Rest et al., 2016). For the current study we used samples from 5 men and 5 women selected based on the greatest weight loss due to the intervention and the availability of muscle tissue from before and after the lifestyle intervention. This subgroup had an average age of 62.4 years (± 4.1) and lost an average of 6.8kg (± 1.3) due to the intervention. Characteristics of this subgroup are detailed in Supplementary Table 4.

All participants signed a written informed consent for participating in this study. All experiments were performed in accordance with the relevant regulations and guidelines. The medical ethical committee of the Leiden University Medical Center approved this study. This trial (NTR3499) was registered at the Dutch Trial Register (www.trialregister.nl).

2.12 Muscle Biopsies and Sectioning

Muscle biopsies were collected from the *vastus lateralis* muscle before and after the lifestyle intervention. Biopsies were collected 40-45 minutes following a standardized liquid meal (Nutridrink™, Nutricia Advanced Medical Nutricion, Zoetermeer, The Netherlands) in the morning after at least 10 hours of fasting. Under local anesthesia, an incision was made 10cm cranial of the patella on the lateral side of the upper leg. A biopsy needle (3mm thick) was inserted to obtain the muscle biopsy. The muscle biopsy was immediately frozen in liquid nitrogen and stored at -80°C prior to cryo-sectioning.

Cryosections of 16µm were made with the CM3050-S cryostat (Leica, Wetzlar, Germany), pasted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and stored at -20°C prior to staining.

2.13 Cell Culture

Primary mouse embryonic fibroblasts (MEFs) and primary human keratinocytes were used for the cell culture experiments. The cells were thawed and grown in Dulbecco's Modified Eagle Medium (DMEM)-GlutaMAX™ (Life Technologies) containing 20% fetal calf serum (FCS). The cells were used at passage 3 for starvation experiments followed by immunofluorescence. Starvation protocol was followed which included washing off the old medium from the cells and further washing the cells with PBS. Then Earle's Balanced Salt Solution (EBSS) (ThermoFisher Scientifc) starvation medium was added on experimental cells while the normal DMEM growth medium was added on the control cells. After 3 hours of starvation, MEFs were harvested for fixation and immunofluorescence further. Primary human keratinocytes were starved for 8 hours and the medium used was DMEM and Ham's F12 (Sigma-Aldrich D6421). The cells were similarly processed as pMEFs.

2.14 Worm Size Measurements

Day one adult worms were used for body size measurements. Worms were anesthetized using 0.01% sodium azide, mounted on slides and imaged with 10X magnification. This magnification ensured that an entire worm would fit in the focal plane. The length of the worms was quantified using the measure tool of Axio Imager Z1 (Zeiss) microscope software.

2.15 Blinding of Experiments

All the lifespan analysis experiments were performed in a blinded manner. For blinding, the strain names were concealed during scoring, analyzing and plotting the

data. Nucleolar imaging and quantification were also performed with concealed strain names.

Drosophila nucleolar size analysis was performed in a blinded manner. Two different people were involved in performing the experiment. One individual carried out fly feeding and mutant strain maintenance and the samples were passed on blinded for imaging and quantification to the second experimenter.

Mouse nucleolar size analysis was also carried out blinded. Three different people were involved in performing the experiments. One experimenter maintained the mice while carrying out the feeding/treatments and sacrificed the mice for sectioning. The experiment was blinded henceforth. The sectioning was carried out blinded by the second experimenter. The blinded sections were stained, imaged and quantified by the third experimenter.

Two different experimenters performed human muscle biopsy staining. The whole experiment including staining, imaging and image quantification were performed completely blinded.

CHAPTER 3

RESULTS

3.1 *ncl-1* is Required for DR-Induced Longevity

In order to identify regulators of DR mediated longevity in *C. elegans*, RNAi based suppressor screens were performed by our lab on the DR genetic model *eat-2(ad465)*. NHR-62 was identified in these screens as being the first nuclear hormone receptor involved in DR longevity (Heestand et. al., 2013). In addition to NHR-62, RING finger protein, NCL-1 was identified in these genetic screens to be required for DR longevity revealing another potential regulator. *ncl-1* encodes a cytoplasmic zinc finger protein that inhibits rRNA and 5S RNA transcription and protein synthesis (Frank and Roth, 1998). NCL-1 is an ortholog of the TRIM2/BRAT tumor suppressor, containing B-box, ring finger, and NHL repeats. Even though the expression of *ncl-1* is largely cytoplasmic, it regulates nucleolar size in worms by as yet an unknown mechanism. Loss of *ncl-1* leads to enlarged nucleolar size especially in neuronal, muscle and hypodermal cells (Hedgecock and Herman, 1995). Consistent with its role in nucleolar size regulation, *ncl-1* loss leads to increased rRNA and protein synthesis indicating that *ncl-1* plays a role in ribosome biogenesis and thereby regulates translation. In addition, NCL-1 also regulates body size of worms; *ncl-1* animals are 9% larger than wild-type worms, possess 22% more protein and twice as much rRNA as wild-type animals (Frank and Roth, 1998).

Since *ncl-1* was identified in an RNAi based screen for DR longevity, we went on further to validate its role with *ncl-1* null mutants. Two different *ncl-1* mutant alleles were used namely *ncl-1(e1865)* and *ncl-1(e1942)*. Both the alleles carry point mutations in the coding region of the gene, resulting in a non-functional truncated protein. We generated *eat-2;ncl-1* double mutants and performed lifespan analyses to confirm if the longevity conferred by DR is abrogated with the loss of *ncl-1.* With the loss of *ncl-1*, the longevity of *eat-2* was completely abolished suggesting that *ncl-1* is required for *eat-2* longevity (Figure 3A,B). Since there are various methods of carrying out DR in worms, we resorted to Bacterial Dilution Regimen (BDR) to induce a DR response in order to investigate if the longevity conferred by BDR is also abrogated with the loss of *ncl-1*. We performed BDR experiment by feeding seven different concentrations of OP50 bacteria to wildtype and *ncl-1* worms and

monitored their lifespan. The highest concentration corresponding to ad-libitum feeding was 5.85×10^{9} CFU/ml. The results show that the loss of *ncl-1* also suppresses the longevity induced by BDR (Figure 3C). The optimal concentration where the graph shows a peak and maximum lifespan extension in wildtype worms was found out to be 6.5×10^{8} CFU/ml. At this concentration, wildtype worms lived significantly longer than *ncl-1* mutants implying that *ncl-1* is also required for BDR longevity. Taken together, these results clearly show that *ncl-1* plays a pivotal role in mediating DR longevity in *C. elegans*.

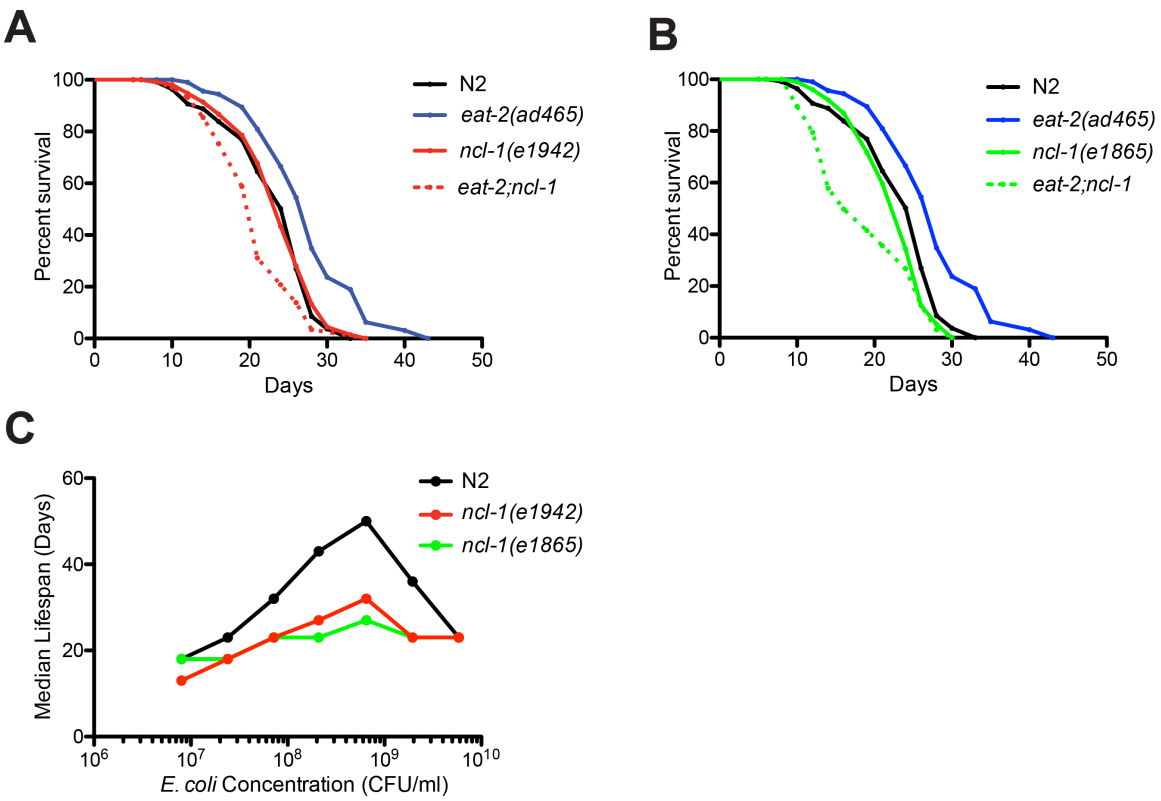

Figure 3. *ncl-1* is required for DR longevity. (A,B) *eat-2(ad465)* is significantly longer lived than wildtype (N2) ($p<0.0001$). Loss of *ncl-1* suppresses the longevity of *eat-2 (ad465)* worms ($p<0.0001$). (C) Wildtype (N2) worms are significantly longer lived compared to *ncl-1* upon bacterial dilution cross 7 different concentrations ($p<0.0001$). p-values calculated by log-rank test.

To further study the role of *ncl-1* in the context of regulating lifespan under DR conditions, we obtained a *gfp* fused *ncl-1* translational reporter construct from the TransgeneOme project (Sarov et al., 2012). The construct was injected into wildtype worms and thus two independent transgenic strains were generated which carried

the construct as an extra-chromosomal array. The expression of *ncl-1* was seen to be largely cytoplasmic across different tissues including neurons, pharynx, body wall muscle, seam cells and vulva (Figure 4A). These transgenic lines served as over-expression strains for *ncl-1* in N2 background. We next wanted to validate the function of *ncl-1* transgene. Therefore, *ncl-1* transgenic worms were crossed in *ncl-1* mutant background and nucleolar size was examined. As discussed above, *ncl-1* mutants possess significantly larger nucleoli compared to wildtype worms (Figure 4B,C). As expected, *ncl-1* transgene rescued the large nucleolar phenotype in *ncl-1* mutants back to wildtype levels (Figure 4B,C).

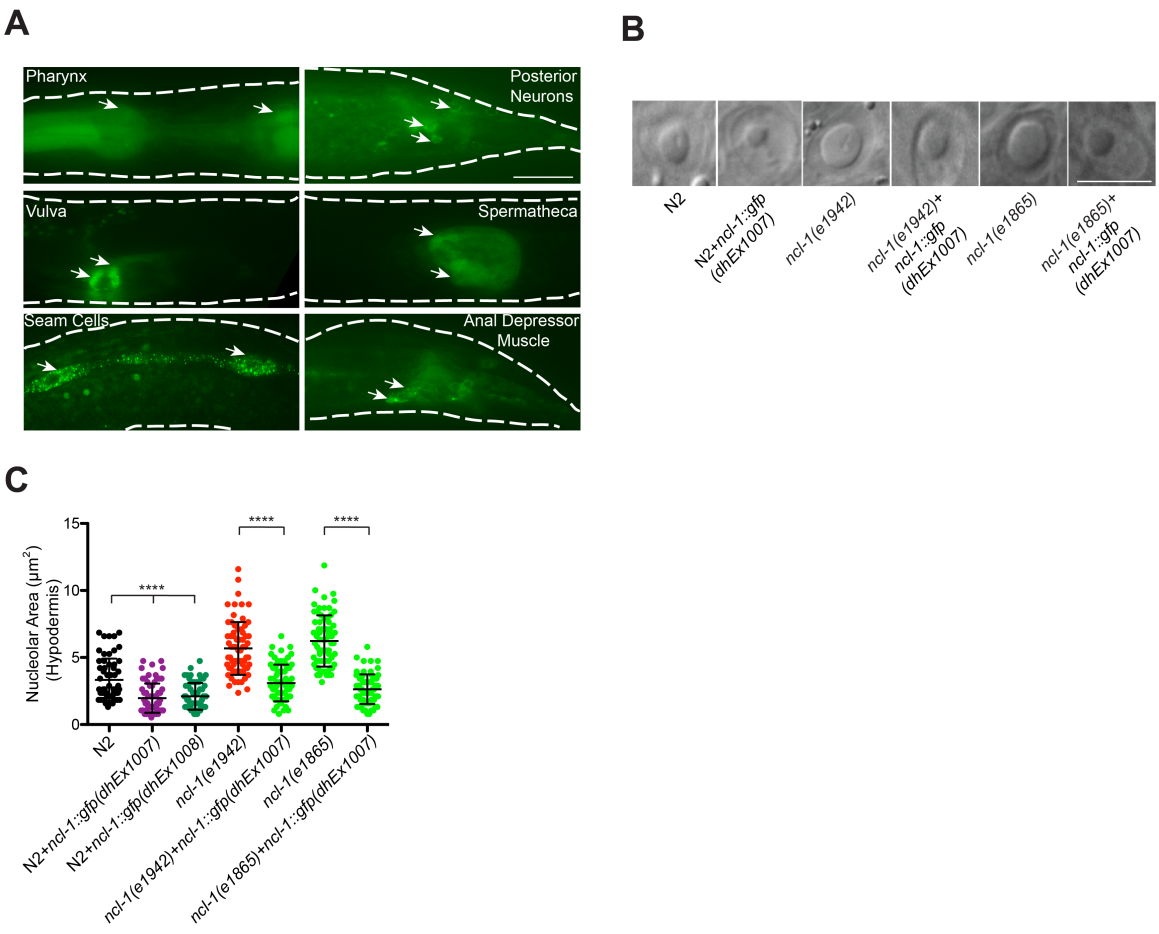

Figure 4. Transgenic expression of *ncl-1* rescues large nucleolar size of *ncl-1* mutants. (A) The arrows indicate the expression of *ncl-1* across multiple tissues. Scale bar represents 20 µm. (B,C) Over-expression of *ncl-1* decreases nucleolar size in N2. Restoring *ncl-1* transgenically rescues the big nucleolar phenotype of *ncl-1* mutants. Scale bar represents 5 µm. **** p<0.0001, unpaired t-test.

We next crossed the *ncl-1* transgenic line in *eat-2;ncl-1* background and monitored the lifespan. Upon restoring functional *ncl-1* transgenically, longevity of *eat-2;ncl-1* was restored back to *eat-2* levels (Figure 5A,B). Furthermore, over-expression of *ncl-1* in wildtype worms increased lifespan however there was no additive longevity observed in *eat-2* suggesting an overlapping mechanism (Figure 5C,D). Lifespan analysis with over-expression of *ncl-1* in N2 was performed using two independent transgenic strains both of which showed similar lifespan extension (Figure 5C). These results clearly suggest that *ncl-1* plays an important role in DR mediated lifespan extension and that an over-expression of this gene is sufficient to drive longevity. These results also validate the functionality of transgenic *ncl-1.*

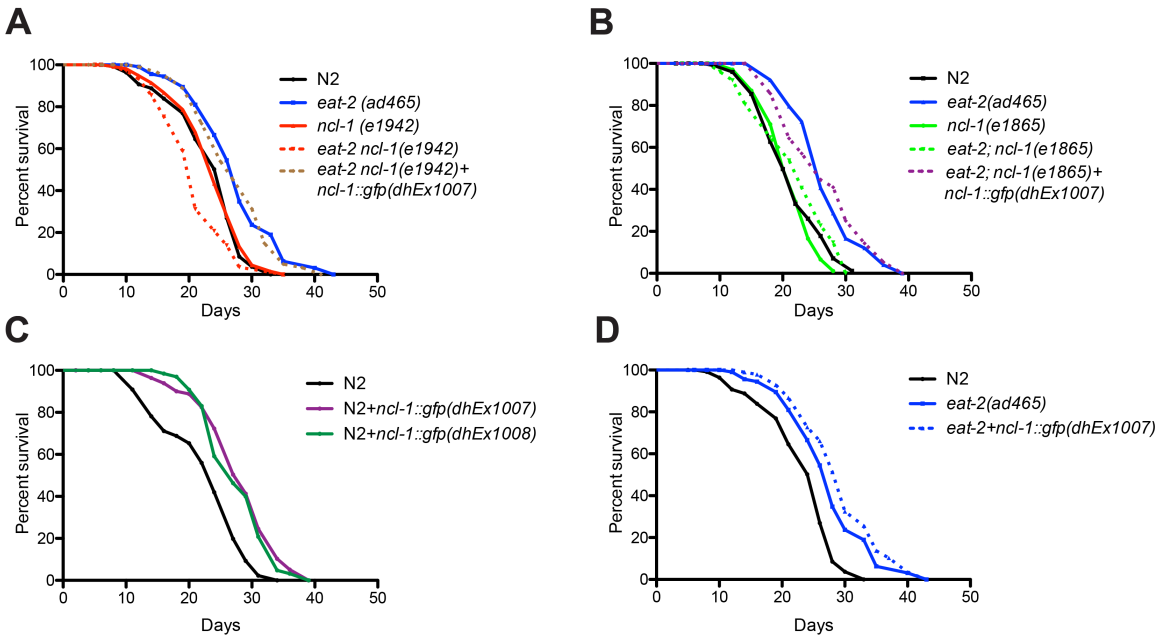

Figure 5. Over-expression of *ncl-1* extends lifespan. (A,B) Survivorship curves of Wildtype (N2), *eat-2, ncl-1, eat-2;ncl-1* and *eat-2;ncl-1* carrying wildtype *ncl-1* transgene. *ncl-1* transgene in *eat-2;ncl-1* rescues the longevity of *eat-2;ncl-1* mutants ($p<0.0001$). (C,D) *ncl-1* overexpression in wildtype (N2) background extends lifespan ($p<0.0001$) but there is no significant lifespan extension in *eat-2* with *ncl-1* overexpression. p-values calculated by log-rank test.

3.2 *ncl-1* Regulates Body Size of *C. elegans*

In addition to extending lifespan, DR reduces body size of different organisms. Body size of *eat-2(ad465)* mutants is significantly smaller compared to wildtype worms. However, *ncl-1* loss rescues the smaller body size of *eat-2* worms back to almost wildtype levels. *eat-2;ncl-1* double mutants are significantly larger in size compared to *eat-2(ad465)* single mutants (Figure 6). These results suggest *ncl-1* plays a role in regulating body growth and that the loss of *ncl-1* restores the small body size of *eat-2* back to wildtype levels. *eat-2(ad465)* worms have a slower pharyngeal pumping rate which leads to reduced food intake and *ncl-1* mutation in *eat-2(ad465)* worms does not affect this trait. These data uncouple the body size and food intake; both *eat-2(ad465)* and *eat-2;ncl-1* worms eat less, yet *eat-2;ncl-1* mutants grow bigger in size than *eat-2(ad465)* single mutant.

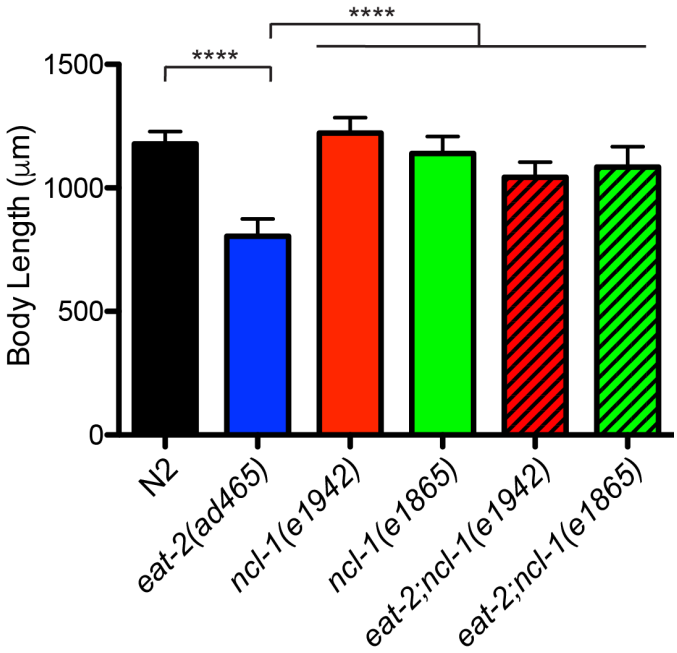

Figure 6. *ncl-1* regulates body size. Loss of *ncl-1* restores the body size of *eat-2* worms back to wildtype levels. N2 vs *eat-2*, p<0.0001; *eat-2* vs *eat-2,ncl-1* (both alleles), p<0.0001. p-values were calculated by unpaired t-test.

3.3 *ncl-1* Plays a Role in Other Longevity Models

Since there are already some well characterized longevity pathways known, we next asked if *ncl-1* also modulates longevity in these known longevity models. We started by studying the role of *ncl-1* in the TOR pathway. The TOR pathway has been closely linked with DR and is already known to mediate lifespan extension under DR conditions (Hansen et al., 2007). Therefore we tested if the loss of *ncl-1* would also abrogate lifespan extension under TOR inhibition conditions. We performed RNAi against *let-363*/TOR kinase on wildtype and *ncl-1* mutants and monitored the lifespan. TOR RNAi was started on the first day of adulthood since TOR knockdown causes developmental arrest when initiated egg-on. Interestingly, *ncl-1* mutants had a significantly shorter lifespan than the wildtype counterparts upon *let-363*/TOR RNAi knockdown (Figure 7A,B). These results imply that *ncl-1* works downstream of the TOR pathway and is required for mediating lifespan extension upon knockdown of TOR.

Reduced insulin signaling (IIS pathway) and reduced mitochondrial activity are known to extend lifespan (Dillin et al., 2002; Kenyon et al., 1993). Next, we asked if *ncl-1* would be required for lifespan extension in these already known longevity pathways. To address this question we performed RNAi mediated knock down of the *C. elegans* insulin/IGF receptor *daf-2*, to investigate the involvement of *ncl-1* in the IIS pathway. To examine the role of *ncl-1* in the mitochondrial longevity pathway we crossed *ncl-1* in *isp-1* mutant background. *isp-1* encodes an iron sulfur protein in mitochondrial complex III and a mutation of this gene leads to impaired electron transport function and an extension in lifespan (Yang and Hekimi, 2010). As reported earlier, RNAi against *daf-2* increased the lifespan of wildtype worms significantly. However *ncl-1* mutants were not as long lived as the wildtype worms upon *daf-2* knock down indicating that *ncl-1* is partially involved in regulating the lifespan upon reduced insulin signaling (Figure 7C,D). A similar trend was observed in the lifespan of *isp-1* compared to *isp-1;ncl-1* also*. isp-1* worms were long-lived and *isp-1;ncl-1* mutants had an intermediate lifespan, still longer lived than wildtype however significantly shorter lived than *isp-1* single mutants (Figure 7F). These data

indicate that *ncl-1* plays a partial but significant role in modulating lifespan upon reduced insulin signaling and reduced mitochondrial activity.

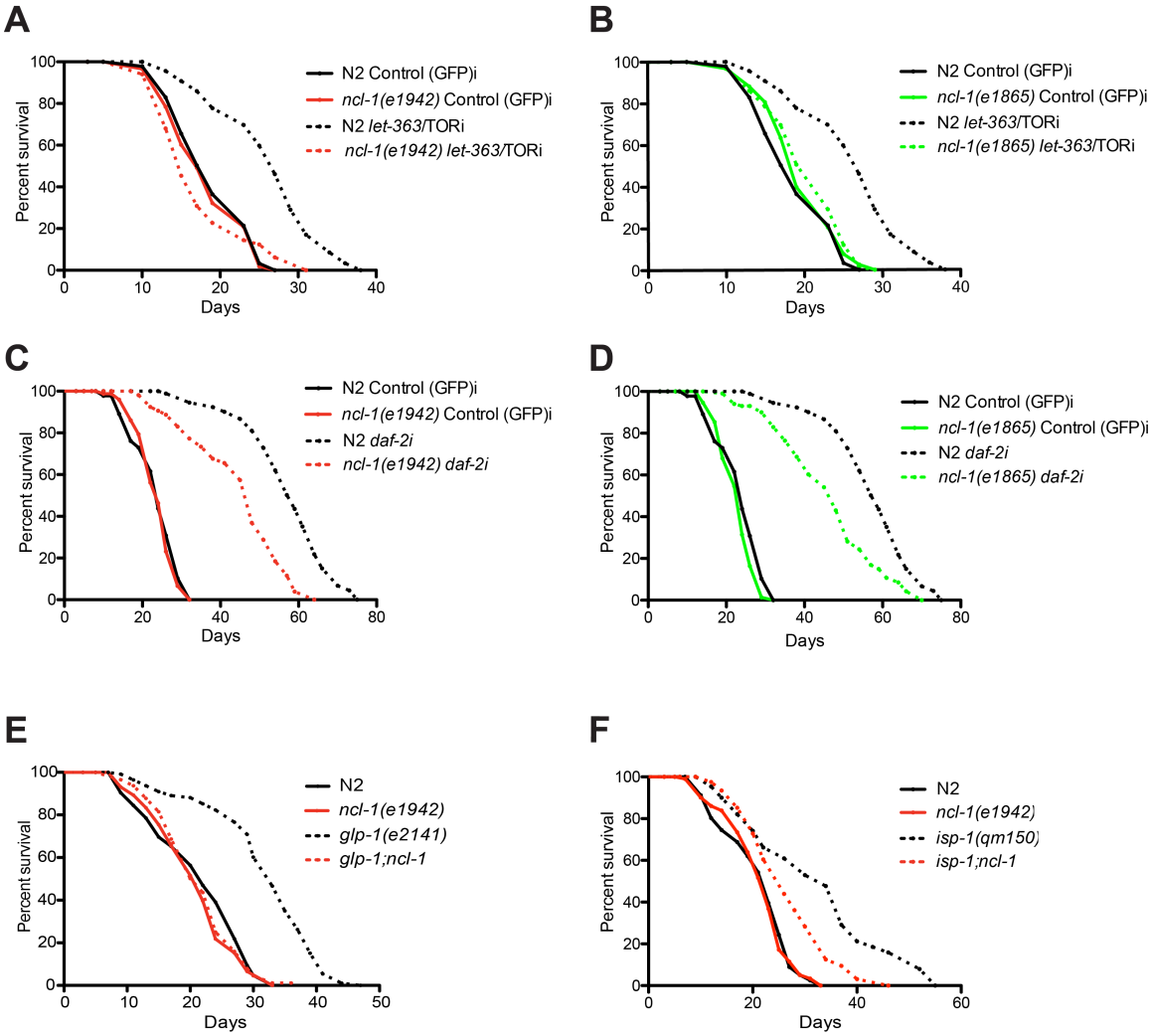

Figure 7. *ncl-1* is required for multiple longevity pathways. (A,B) *let-363*/TOR RNAi extends lifespan of wildtype (N2) worms but not of *ncl-1* mutants ($p<0.0001$). (C,D) *ncl-1* mutants are not as long lived as wildtype (N2) upon *daf-2* RNAi ($p<0.0001$). (E,F) *glp-1(e2141)* and *isp-1(qm150)* are significantly longer lived than *glp-1;ncl-1* ($p<0.0001$) and *isp-1;ncl-1* ($p=0.0016$). p-values calculated by log-rank test.

Another well-characterized model of longevity is the gonadal longevity model. As discussed above germline ablation extends lifespan in diverse organisms suggesting its evolutionary conservation. There are two ways of removing germline in worms discussed in detail above. There is a laser microsurgical method by which germline precursor cells can be ablated in larval stage 1 or L1 worms and this removes the germline in the animals entirely. A genetic way of inducing germline removal is by

growing the worms harboring *glp-1* mutation at 25°C. *glp-1* codes for a notch transmembrane receptor in *C. elegans* and its disruption and subsequent growth at 25°C renders the worms germlineless. Both these treatments of germline removal induce significant longevity. We asked if *ncl-1* is responsible for mediating this longevity. We generated *glp-1;ncl-1* double mutants to address this question. Since both these genes lie close together on Chromosome III, the genetic cross was tedious. Nevertheless we were able to generate a double mutant with one of the *ncl-1* alleles. We next monitored the lifespan and interestingly *ncl-1* loss totally abolished the longevity of germlineless *glp-1* animals (Figure 7E). These results show that *ncl-1* is required for mediating longevity conferred by germline removal.

Taken together, our data indicate that *ncl-1* plays a role in modulating lifespan upon DR, reduced TOR pathway, reduced IIS pathway, reduced mitochondrial activity and also upon germline removal. Longevity conferred by DR, reduced TOR signaling and germline removal are completely abolished in the absence of *ncl-1* indicating a crucial role played by *ncl-1*, whereas it plays a partial role in mediating longevity upon reduced IIS pathway and reduced mitochondrial activity suggesting that there are other mechanisms independent of *ncl-1* which orchestrate this lifespan extension. Nevertheless, *ncl-1* plays into all the well-known mechanisms that increase lifespan in *C. elegans.* Therefore it acts as a universal regulator of longevity across different longevity models pointing towards a convergent mechanism that leads to lifespan extension and is regulated by inputs from *ncl-1.*

3.4 Smaller Nucleoli Associate with Longevity in *C. elegans*

The nucleolus is the site of rRNA transcription and ribosome biogenesis. It was previously described that NCL-1 regulates nucleolar size in *C. elegans* and *ncl-1* mutants have larger nucleoli especially in neuronal, muscle and hypodermal cells (Hedgecock & Herman, 1995). In both *ncl-1(e1865)* and *ncl-1(e1942)* nucleoli are larger compared to wildtype worms (Figure 8A). Since we found that *ncl-1* is required for mediating lifespan extension across multiple longevity pathways and that protein synthesis rates per se regulate lifespan as noted earlier, it was intriguing for us to investigate the nucleolar size in different longevity pathways and if there existed any connection between the size of the nucleolus and lifespan in general. We started off by examining the nucleoli of *eat-2(ad465)* mutants. We measured the nucleolar size in the superficial hypodermal cells around the posterior bulb of pharynx in age-synchronized N2, *eat-2,* and *eat-2;ncl-1* worms. Strikingly we observed that *eat-2(ad465)* possessed smaller nucleoli compared to wildtype and the small nucleolar size was rescued in *eat-2;ncl-1* suggesting that *ncl-1* is epistatic to *eat-2* for both nucleolar size and longevity (Figure 8B). Next we examined the nucleoli of the worms subjected to BDR. And interestingly we observed a similar effect; a reduction in bacterial concentration led to a reduction in nucleolar size with a corresponding increase in lifespan (Figure 8C). We could only use three concentrations namely 5.85×10^9 CFU/ml (ad-libitum), 1.95×10^9 CFU/ml and 6.5×10^8 CFU/ml for the nucleolar size analysis because at concentrations below 6.5×10^8 CFU/ml the worms were getting developmentally arrested because of reduced food. For BDR lifespan analysis this problem did not arise because day one adult worms were subjected to the different food concentrations. This result points towards a negative correlation between lifespan and nucleolar size wherein food reduction mediated lifespan increase is accompanied with a corresponding drop in nucleolar size. We further wondered if this effect on nucleolar size was confined to DR or if it was a general trait of long-lived animals. To investigate this possibility, we examined nucleoli of other long-lived worms namely *daf-2(e1370)*, *isp-1(qm150)*, *glp-1(e2141)*, and N2 upon *let-363*/TOR RNAi. Strikingly, nucleoli in all these long-

lived worms were reduced in size compared wildtype worms (Figure 8D). Along with these long-lived worms we also checked the nucleoli of the short lived *daf-2;daf-16* mutants in order to get more evidence about the effect on nucleoli. Longer lived *daf-2* worms had smaller nucleoli compared to *daf-2;daf-16* which is significantly shorter lived than *daf-2*, further supporting the notion that long-lived animals possess smaller nucleoli and vice versa (Figure 8D). We also tested the nucleolar size of the long-lived worms over-expressing *ncl-1*. We observed that over-expression of *ncl-1* reduced the size of nucleoli compared to wildtype worms (Figure 4B,C). We also investigated nucleoli in other tissues to get more convincing evidence of this effect. We examined the nucleolar size in pharyngeal muscle and germ cells in all these long-lived mutants. We observed a similar; nucleoli of pharyngeal muscle and germ cells were also reduced in size in long-lived mutants compared to wildtype (Figure 8E,F). Here again we observed that the pharyngeal muscle and germ cell nucleoli of the long-lived *daf-2* were significantly smaller than the short lived *daf-2;daf-16* further adding to the evidence of nucleolar size being reduced in long-lived animals (Figure 8E,F). These results also indicate that nucleolar size is affected across different tissues in long-lived mutants and leads to the assumption that may be the concerted effect of reduced nucleoli in different tissues causes the animals to attain longevity, which needs to be further explored.

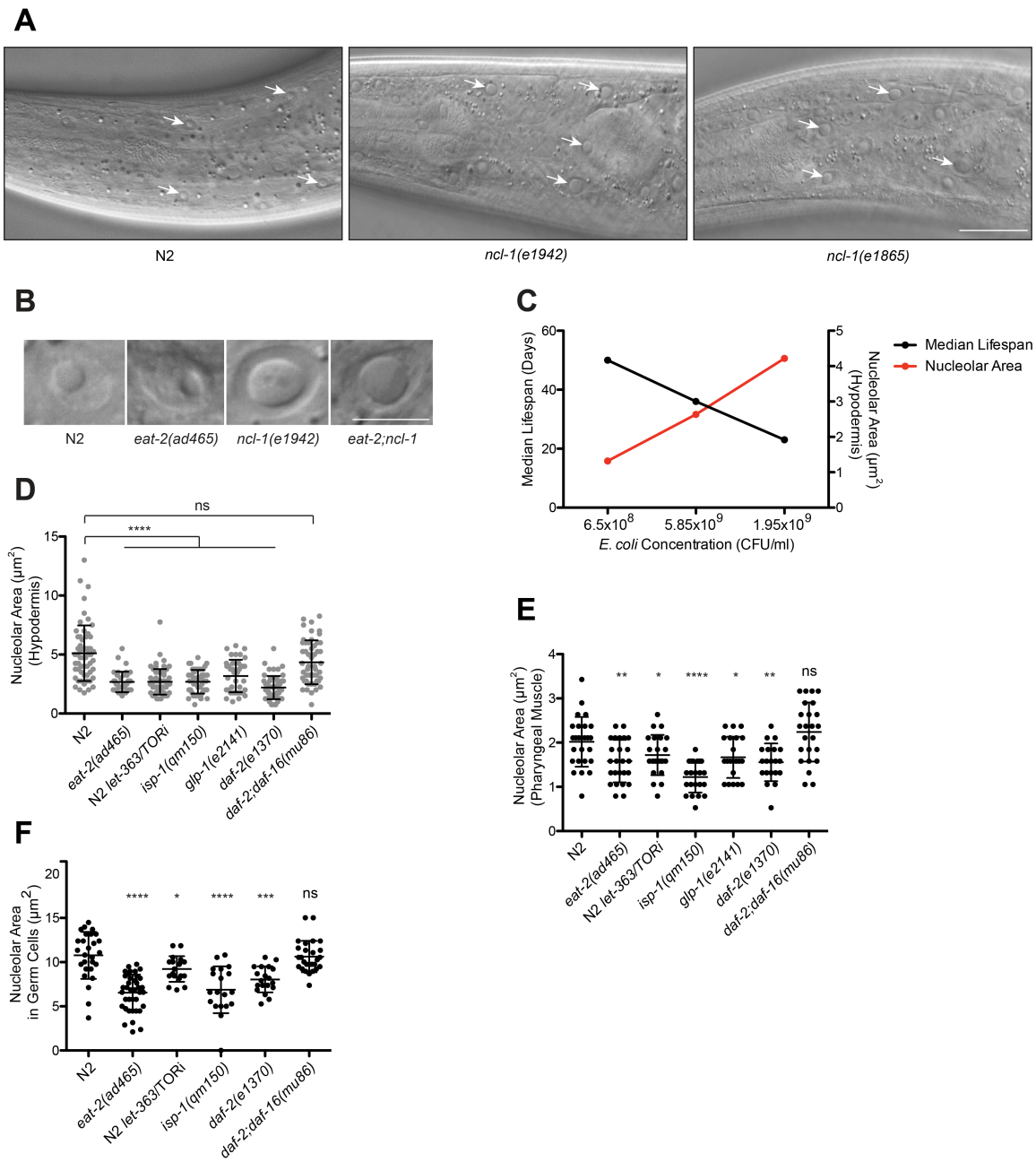

Figure 8. Nucleolar size inversely correlates with longevity. (A) *ncl-1* mutants possess larger nucleoli compared to N2. Hypodermal nucleoli are depicted here. Scale bar represents 20 μm. (B) *eat-2(ad465)* animals have smaller nucleoli while *ncl-1(e1942)* and *eat-2;ncl-1* animals possess larger nucleoli compared to N2. Scale bar represents 5 μm. (C) Nucleolar size is reduced upon bacterial food reduction with a corresponding increase in lifespan. ($p<0.0001$, log-rank test). (D,E,F) *eat-2(ad465)*, TOR RNAi, *isp-1(qm150)*, *glp-1(e2141)* and *daf-2(e1370)* animals possess smaller nucleoli while *daf-2;daf-16* have nucleoli similar to N2 in the hypodermis pharyngeal muscle and germ cells. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, ns: non-significant, unpaired t-test.

Isogenic wildtype worms show considerable variance in life expectancy, with some animals dying as early as day 10 and others as late as day 30, despite culture in a uniform environment. The basis of this variance however has remained elusive. We also found that wildtype animals showed variance in nucleolar size, and therefore asked if these differences associate with lifespan in wildtype populations. To address this question, we imaged the nucleoli of age-matched worms on the first day of adulthood, recovered them on single plates and monitored their lifespan individually (Figure 9A). A mild anesthetic was used for imaging live worms and then they were recovered on OP50 bacteria. From the images of individual worms nucleolar size was measured and strikingly, the animals with larger nucleoli lived shorter compared to the animals with smaller nucleoli. This piece of data further substantiates the idea of an association between longer lifespan and smaller nucleoli (Figure 9B). Taken together, our data draws an inverse correlation between nucleolar size and lifespan. This phenomenon can thus be used as a proxy for lifespan and the lifespan of an animal can be approximated on day one of adulthood based on the size of its nucleoli.

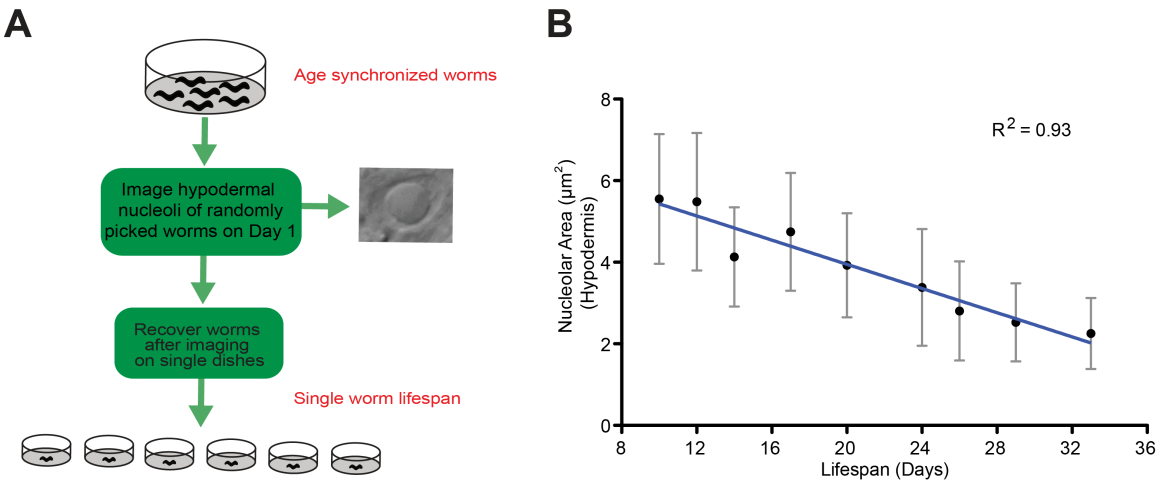

Figure 9. Nucleolar size can approximate *C. elegans'* life expectancy (A,B) Schematic diagram illustrating the experiment, which shows that longer-lived worms exhibit small nucleoli and vice versa (Pearson correlation coefficient R^2 = 0.93).

3.5 Reduced Nucleolar Function Associates with Longevity

It was very striking to discover the negative correlation between lifespan and nucleolar size. Therefore to have mechanistic insights as to how smaller nucleoli might be linked to aging, we examined how *ncl-1* and various long-lived mutants affected nucleolar function. It was previously reported that the expression of nucleolar marker *fib-1* increases drastically upon *ncl-1* loss (Lee et al., 2012). *fib-1* encodes the *C. elegans* ortholog of Fibrillarin which is an rRNA 2'-O-methyltransferase enzyme. It is an essential component of the U3 small nucleolar ribonucleoproteins (snoRNPs) complex that functions in rRNA processing. We used transgenic worms expressing FIB-1 fused to GFP for our analysis (Lee et. al., 2010). The GFP signal in these worms is punctate and each punctum represents a nucleolus (Figure 10A). We crossed these worms into *ncl-1* mutant background. As reported earlier, we could see an increase in FIB-1::GFP levels upon *ncl-1* loss (Figure 10A,B). And conversely, FIB-1 goes down in *ncl-1* over-expressing worms (Figure 10C). This indicates that NCL-1 acts as a suppressor of FIB-1. Next we asked if the levels of FIB-1 are altered in long-lived mutants. This would give us an indication of the activity of NCL-1 in these worms. We started with *eat-2*, specifically asking if DR regulates the levels of FIB-1. To address this question, we crossed the transgenic worms carrying FIB-1::GFP into *eat-2* background and surprisingly we found that the levels of FIB-1 were highly reduced in *eat-2* (Figure 10A,B). To study epistasis, we further crossed FIB-1::GFP into *eat-2;ncl-1* double mutant background and looked at the expression of FIB-1. Strikingly in the double mutant *eat-2;ncl-1* the levels of FIB-1 were dramatically high again like in *ncl-1* single mutant suggesting that *ncl-1* is epistatic over *eat-2* in terms of FIB-1 regulation (Figure 10A,B). We could also confirm these results by western blotting using an antibody detecting the endogenous FIB-1 protein ruling out any effects that could occur due to dysregulation of the FIB::GFP transgene. We observed lower levels of FIB-1 in *eat-2* and higher amount FIB-1 in *ncl-1* compared to wildtype (Figure 10C).

Next we wanted to confirm the levels of FIB-1 in other long-lived animals as well. We used FIB-1::GFP transgenic strain to address this question. We crossed FIB-

1::GFP strain in *daf-2(e1370)*, *isp-1(qm150)* and *glp-1(e2141)* genetic backgrounds. And we observed a significant down-regulation of the GFP signal compared to wildtype indicating that the levels of FIB-1 are reduced in these long-lived worms (Figure 10D). Additionally TOR knockdown via RNAi in wildtype worms with FIB-1::GFP also reduced the GFP signal (Figure 10D). However the reduction of FIB-1::GFP was totally abolished with the loss of *ncl-1* in all these long-lived mutants (Figure 10D). We also confirmed these findings by western blotting to assess the levels of endogenous FIB-1 in *daf-2*, *isp-1* and *glp-1* mutants and also in animals subjected to TOR RNAi. Consistent with the findings observed using FIB-1::GFP, we saw that FIB-1 was down regulated in all these long-lived animals compared to wildtype (Figure 13A,B). However loss of *ncl-1* reversed these effects and increased the levels of FIB-1 in these long-lived worms (Figure 13A,B).

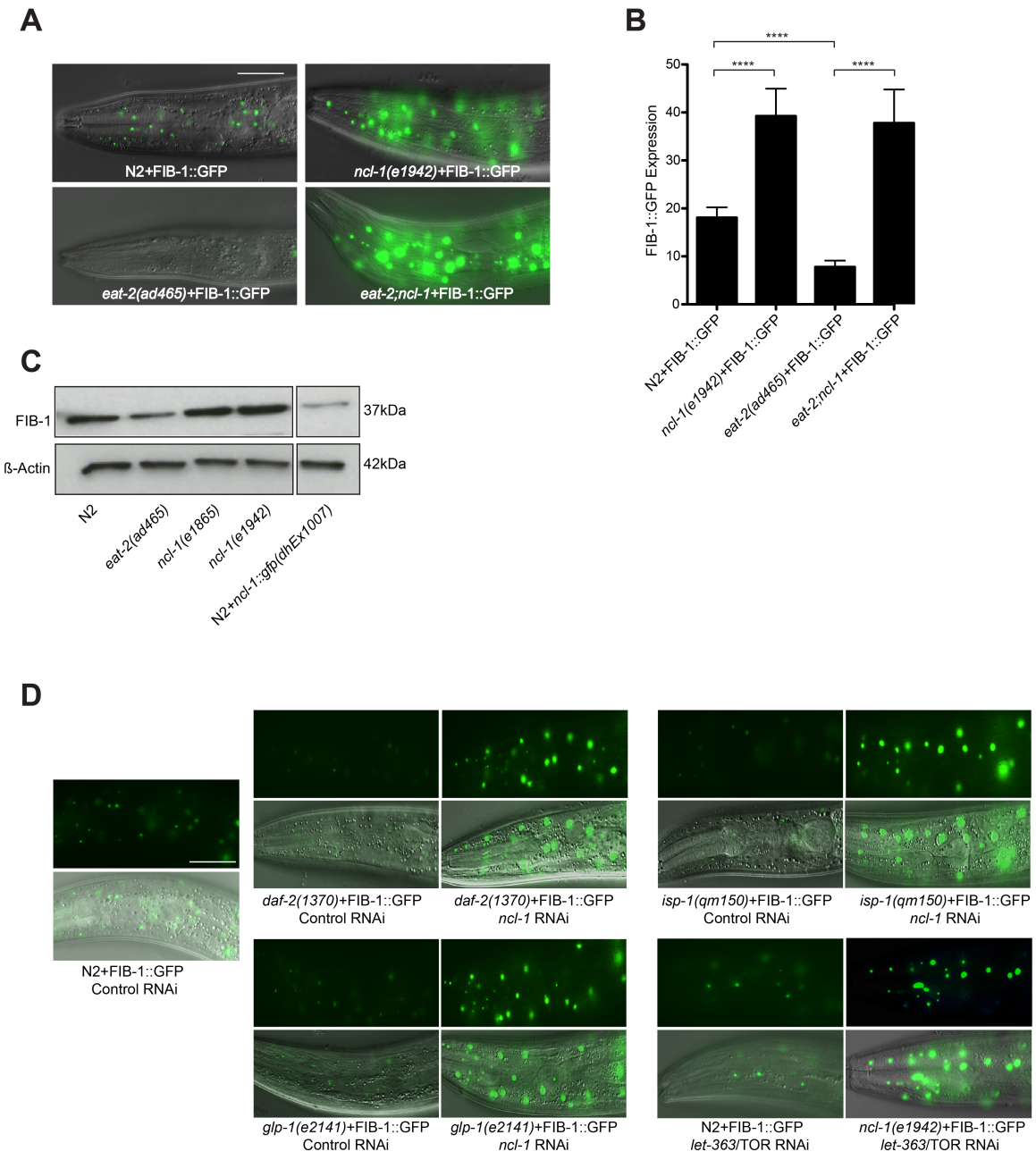

Figure 10. *ncl-1* and longevity mutants regulate fibrillarin. (A,B) FIB-1::GFP is strongly down-regulated in *eat-2(ad465)* animals but up-regulated in *ncl-1* and *eat-2;ncl-1* double mutants. (C) Western Blot showing reduced levels of FIB-1 in *eat-2(ad465)* and upon *ncl-1* over-expression and increased levels in *ncl-1* mutants. (D) FIB-1 levels are reduced in long-lived *daf-2(e1370)*, *isp-1(qm150)*, *glp-1(e2141)* and *let-363*/TOR RNAi animals and this effect is lost with the loss of *ncl-1*. Scale bar represents 20 µm. **** P<0.0001, unpaired t-test.

Since the levels of FIB-1 went down in all the longevity mutants that we tested here, this in turn led us to ask if reducing *fib-1* levels per se would lead to longevity. *fib-1* is an essential gene and *fib-1* mutants are not viable. Therefore we

performed RNAi to knockdown *fib-1.* However we observed that the RNAi treatment initiated egg-on was making the worms sick and some of the worms were also getting developmentally arrested. Thus we initiated the RNAi knockdown of *fib-1* in the adulthood and interestingly we found that *fib-1* knock down led to a modest but significant lifespan extension of wildtype worms (Figure 11A). We also performed *fib-1* RNAi on *eat-2* worms but there was no additive longevity in *eat-2* upon *fib-1* RNAi (Figure 11A) indicating an overlapping mechanism.

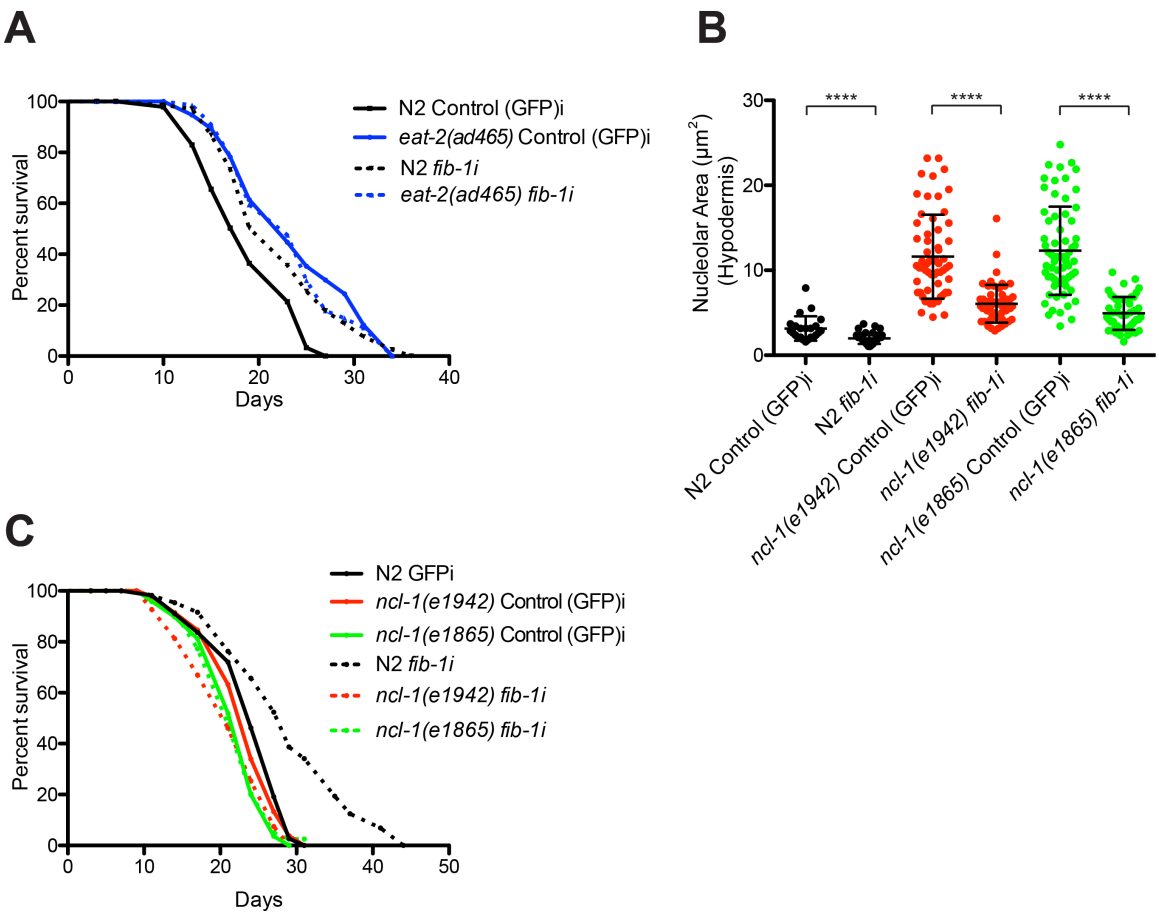

Figure 11. Reduction of *fib-1* levels induces longevity. (A) N2 worms are long-lived when subjected to *fib-1* RNAi but *eat-2(ad465)* is not further long-lived with *fib-1* RNAi (P = 0.0004, log-rank test). (B) *fib-1* RNAi reduces the nucleolar size of N2 and *ncl-1* mutants. (C) N2 worms but not *ncl-1* mutants are long-lived upon *fib-1* RNAi (P<0.0001, log-rank test). **** P<0.0001, unpaired t-test.

We next asked how *fib-1* genetically interacts with *ncl-1*. To confirm this we went on to study genetic epistasis between *ncl-1* and *fib-1.* We knocked down *fib-1*

by RNAi in *ncl-1* mutants and checked the nucleolar size of *ncl-1.* Interestingly we found that reduction of *fib-1* significantly reduced the big nucleolar size of *ncl-1,* however not to wildtype levels (Figure 11B). And the nucleolar size of wildtype worms was also reduced with *fib-1* RNAi compared to control RNAi (Figure 11B). These results indicate that *fib-1* is genetically downstream of *ncl-1.* However to our surprise we did not observe longevity in *ncl-1* mutants upon *fib-1* knock down (Figure 11C). We interpret these results with the caveat that we cannot carry out epistasis experiments with *fib-1* null mutants. Taken together, these data suggest that *fib-1* is negatively regulated across different longevity pathways and that reduction of *fib-1* is sufficient to extend lifespan. Since *fib-1* is negatively regulated by *ncl-1*, these data may suggest that *ncl-1* is activated across these longevity pathways and its activation inhibits *fib-1* in these long-lived worms. These findings are in-line with our data, which indicates *ncl-1* regulates a convergent mechanism focused on the nucleolus, and is required for longevity in all the long-lived mutants investigated here. Since FIB-1 is regulated in the longevity mutants our data further suggest that *fib-1* works in the same convergent pathway downstream of *ncl-1*.

A major function of nucleolus is ribosome biogenesis. We therefore asked if ribosome biogenesis is affected in *ncl-1* and long-lived mutants. We started by investigating the levels of ribosomal RNA (rRNA). We extracted RNA from the same number of worms with different genotypes (*ncl-1*, long-lived mutants and long-lived mutants in *ncl-1* background). Interestingly we observed that the mutation of *ncl-1* increased rRNA levels in worms. And rRNA levels were reduced in long-lived mutants *eat-2(ad465)*, *isp-1(qm150)*, *glp-1(e2141)*, *daf-2(e1370)* and upon TOR RNAi (Figure 12A,B). Furthermore, rRNA levels were also reduced in worms over-expressing *ncl-1* (Figure 12C). With the loss *ncl-1*, the reduction in rRNA levels in long-lived worms was lost and double mutants displayed rRNA levels almost similar to wildtype (Figure 12A,B). Interestingly, rRNA levels were similar to wildtype in *daf-2;daf-16* which lives like wildtype and not as long as *daf-2* single mutant (Figure 12A,B).

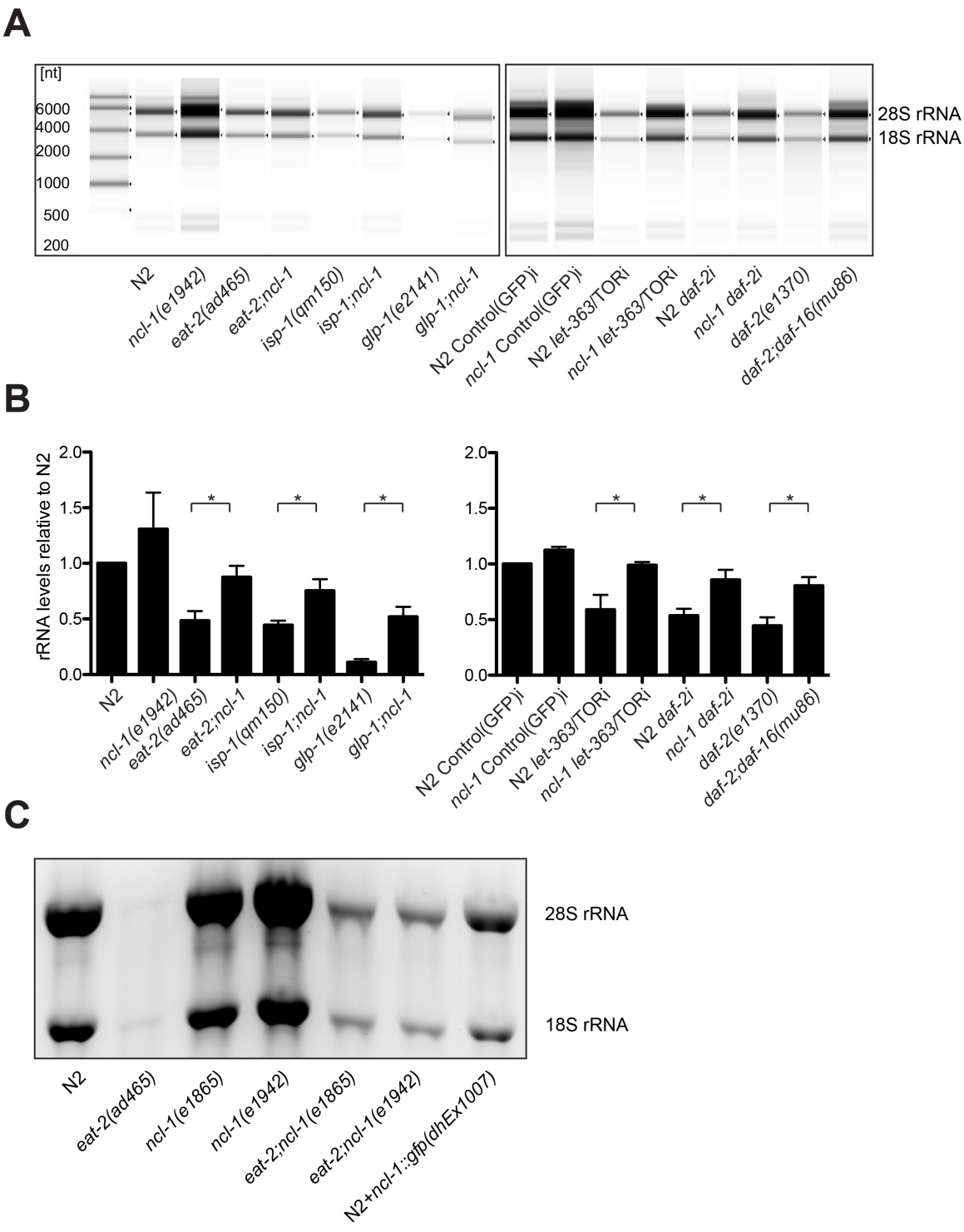

Figure 12. *ncl-1* and longevity mutants affect rRNA levels. (A,B) rRNA levels are increased in *ncl-1* mutants and reduced in long-lived *eat-2(ad465)*, *isp-1(qm150)*, *glp-1(e2141)*, *daf-2(e1370)* and TOR RNAi worms. Loss of *ncl-1* rescues this effect and increases rRNA levels. *daf-2(e1370)* has significantly lower rRNA levels compared to *daf-2;daf-16(mu86).* (C) rRNA levels are reduced in worms over-expressing *ncl-1.* * P<0.05, unpaired t-test

We further went on to investigate the levels of ribosomal proteins to get more insights on ribosomal biogenesis in *ncl-1* and long-lived mutants. We observed the same trend with ribosomal proteins RPS6 and RPS15. Both these proteins were significantly reduced in all the long-lived mutants examined and also in the worms over-expressing *ncl-1.* (Fig. 13A,C,D,E). *ncl-1* loss partially reversed this effect increasing RPS6 and RPS15 in the long-lived animals similar to the effects observed on rRNA levels (Fig. 13A,C,D). Ribosomal protein levels were similar to wildtype in *daf-2;daf-16* and significantly higher than *daf-2* single mutant (Fig. 13F). These data suggest that reduced nucleolar size also associates with reduced nucleolar activity in terms of ribosome biogenesis. Our findings further suggest reduced ribosome biogenesis associates with longevity and is exhibited by different long-lived mutants. Loss of *ncl-1* in these long-lived mutants increases their rRNA and RPS6 and RPS15 levels similar to the effects observed on FIB-1.

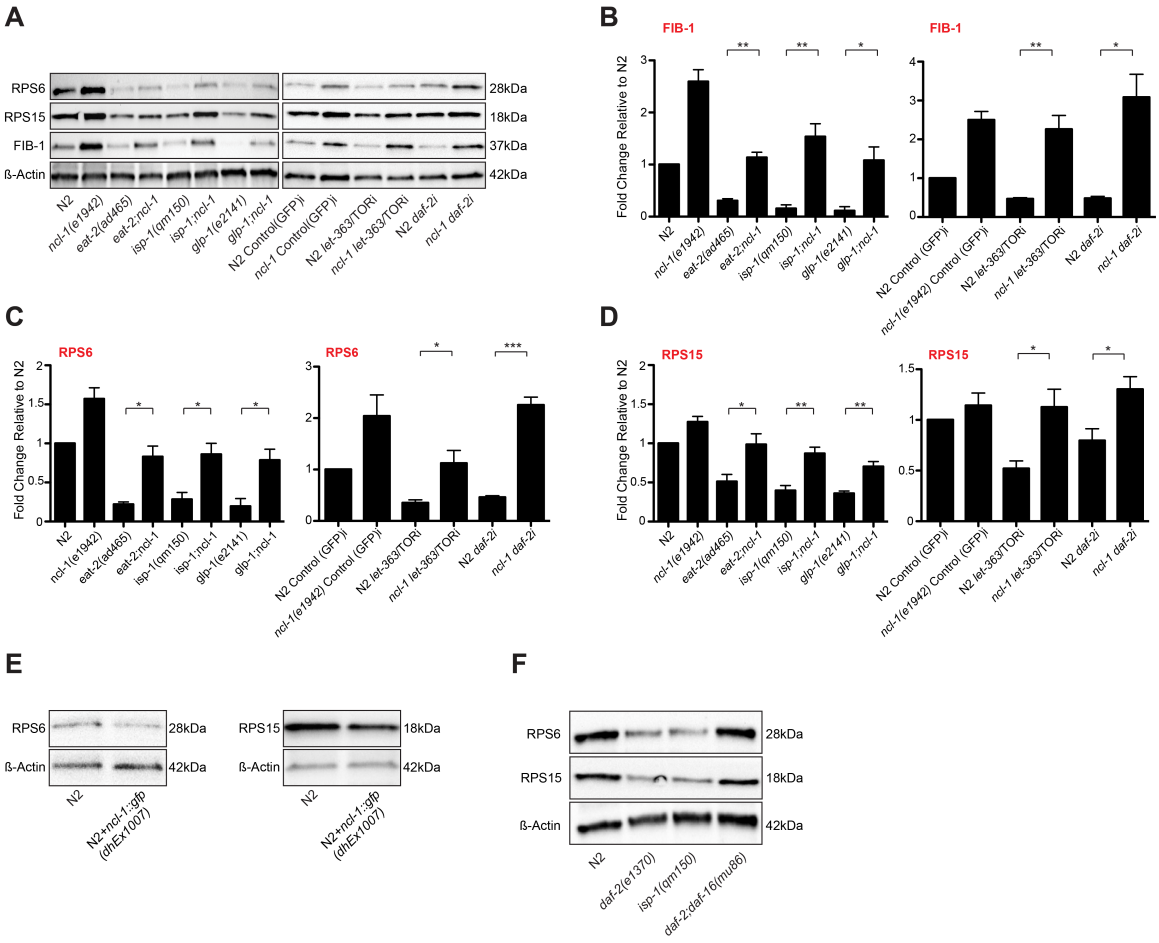

Figure 13. *ncl-1* and longevity mutants affect FIB-1 and ribosomal protein levels. (A,B,C,D) FIB-1 and ribosomal proteins RPS6 and RPS15 are down-regulated in long-lived *eat-2(ad465)*, *isp-1(qm150)*, *glp-1(e2141)*, *daf-2(e1370)* and TOR RNAi worms. *ncl-1* mutation in these long-lived worms significantly abolishes this effect. (E) *ncl-1* over-expression leads to a reduction in RPS6 and RPS 15. (F) RPS6 and RPS15 are lower in *daf-2(e1370)* and higher in *daf-2;daf-16(mu86).* * P<0.05, ** P<0.01, *** P<0.001, unpaired t-test.

Our results are also in line with a previous study that reported that TOR knockdown in *C. elegans* reduced nucleolar size (Sheaffer et al., 2008). Based on this we wanted to investigate if the inhibition of the TOR pathway would also reduce the size of the large nucleoli of *ncl-1* mutants. We subjected *ncl-1* mutants to RNAi against *let-363*/TOR. And interestingly, reduction in TOR levels did not reduce the size of the large nucleoli in *ncl-1* mutants (Figure 14B). In line with our previous data where we found *ncl-1* to be required for lifespan extension upon TOR inhibition (Figure 7A,B), our findings suggest that *ncl-1* is genetically downstream of TOR. We extended this nucleolar size epistasis to the rest of the long-lived mutants as well. We examined the nucleoli of *ncl-1* upon *daf-2* RNAi, *eat-2;ncl-1*, *isp-1;ncl-1* and *glp-1;ncl-1* and we observed that the reduced nucleolar size of the long-lived mutants was rescued by *ncl-1* mutation (Figure 14A).

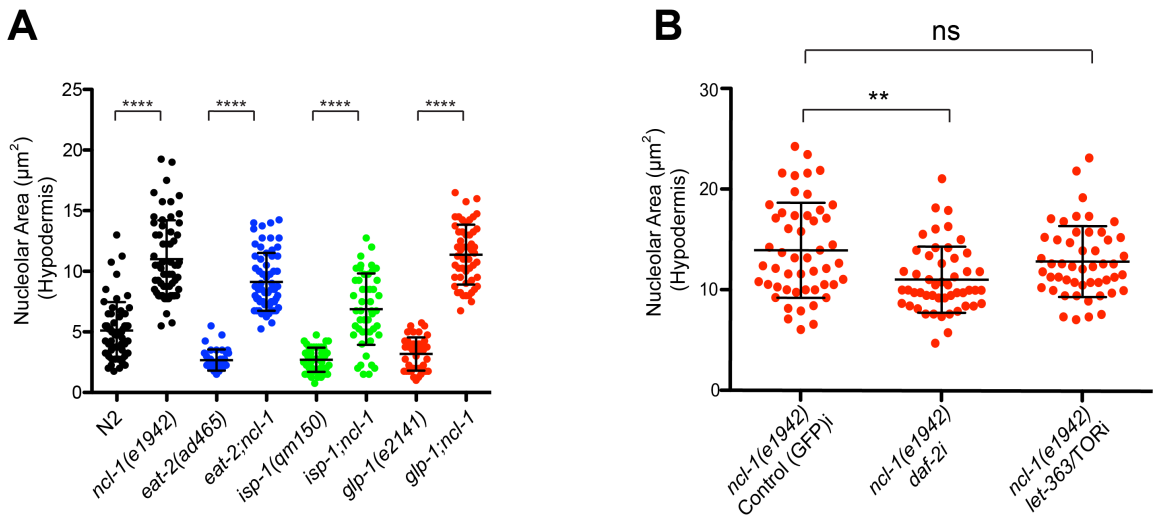

Figure 14. Nucleolar Size epistasis between *ncl-1* and long-lived mutants. (A) Small nucleoli of *eat-2(ad465), isp-1(qm150)* and *glp-1(e2141)* are enlarged with the loss of *ncl-1* but not to the level of *ncl-1* single mutants. TOR RNAi in *ncl-1* mutants does not rescue the large nucleolar phenotype of *ncl-1* mutants, however *daf-2* RNAi induces a significant reduction of nucleoli of *ncl-1* mutants. ** P<0.01, **** P<0.0001, ns: non-significant, unpaired t-test.

3.6 *ncl-1* is Not Regulated in Long-Lived Worms

Since our findings suggest that *ncl-1* plays an important role in mediating lifespan extension in different long-lived mutants, we wanted to test if the levels of NCL-1 are regulated in these mutants. We started by checking the relative mRNA levels of *ncl-1* in *eat-2* and N2 worms by qPCR but we did not see any significant change (Figure 15A). Next we monitored the levels of NCL-1 in *eat-2* background using the *ncl-1::gfp* transgenic construct in *eat-2* and compared the levels of NCL-1 using the same transgenic line in wildtype background. Surprisingly, the levels did not differ significantly (Figure 15B). We next subjected the *ncl-1* transgenic worms to RNAi against *let-363/*TOR but we did not observe significant differences in the levels of NCL-1 (Figure 15B). We also followed the same strategy using *daf-2* RNAi. We treated wildtype worms expressing transgenic *ncl-1* with *daf-2* RNAi and surprisingly we did not see any changes yet again (Figure 15B). Taken together, these results suggest that *ncl-1* is not directly regulated under DR, TOR and IIS pathway knockdown. We therefore speculate that the activity of *ncl-1* might be post-translationally regulated. Further studies will be needed to study its regulation in detail.

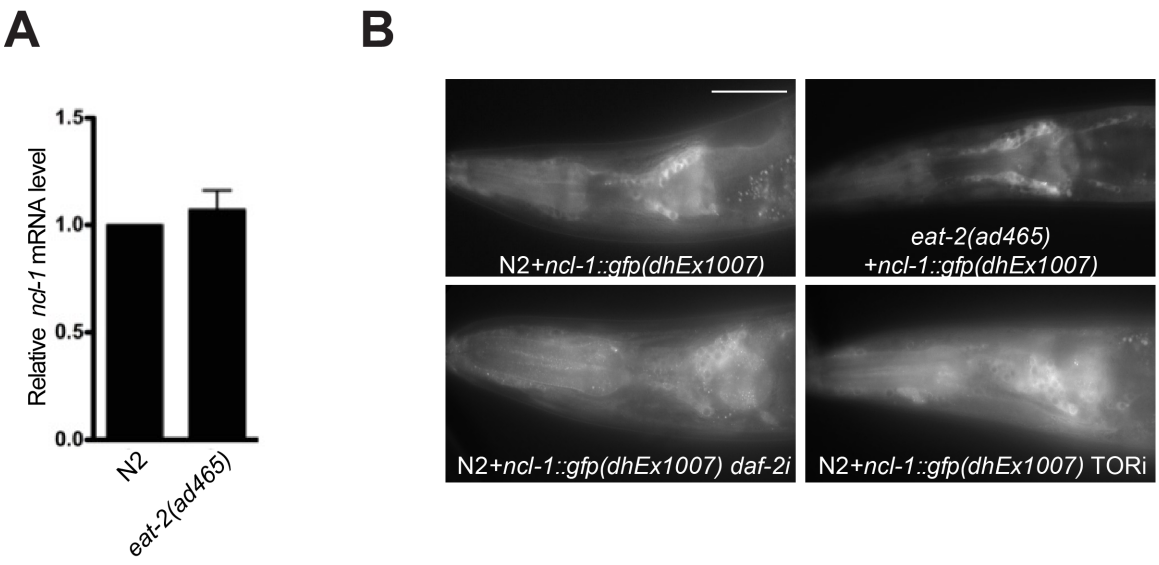

Figure 15. *ncl-1* is not regulated in long-lived mutants. (A) qPCR results depicting similar mRNA levels of *ncl-1* in N2 and *eat-2(ad465).* (B) *ncl-1* transgenic line shows similar expression levels in *eat-2(ad465)* and upon *daf-2* and TOR RNAi. Scale bar represents 20 μm.

3.7 Nucleolar Size in Other Long-Lived Organisms

Our findings about reduced nucleolar size in longevity mutants in *C. elegans* incited us to investigate if this phenomenon also existed in higher organisms. We started a collaboration with Prof. Linda Partridge's lab (MPI-AGE, Cologne), Chirag Jain performed the fly experiments. To start with, we examined the nucleoli of *Drosophila* subjected to DR. Wildtype flies were fed two different concentrations of food; 2X yeast concentration which served as the ad-libitum diet and 0.5X yeast concentration which served as the DR condition. The flies were reared on these food conditions for 12 days and afterwards the intestine and fat body of these flies were dissected out. Immunofluorescence using an antibody staining Fibrillarin revealed a significant reduction in nucleolar size in both intestine and fat body of DR flies compared to the ad-libitum fed flies (Figure 16A,B). We further extended our analysis to the long-lived *Drosophila* insulin-like peptides (DILPs) knockout flies. *Drosophila* possesses seven DILPs and their loss, which in turn inhibits the IIS pathway, has been associated with increased lifespan (Gronke et al., 2010). We stained the intestine and fat-body of age-matched long-lived *dilp2-3,5* and *wDah* control flies with anti-Fibrillarin antibody. The results were consistent with our findings in worms; *dilp2-3,5* flies had significantly smaller nucleoli compared to wildtype flies (Figure 16A,B). We also performed antibody staining on the intestine and fat-body of flies exposed to 200μM Rapamycin. It is known that Rapamycin treatment extends lifespan of *Drosophila* (Bjedov et al., 2010). Nucleolar size in intestinal and fat-body cells was significantly smaller in the flies exposed to Rapamycin compared to EtOH vehicle control flies (Figure 16A,B). These results are in accord with the results that we obtained in *C. elegans.* Long-lived flies also possess smaller nucleoli across multiple tissues.

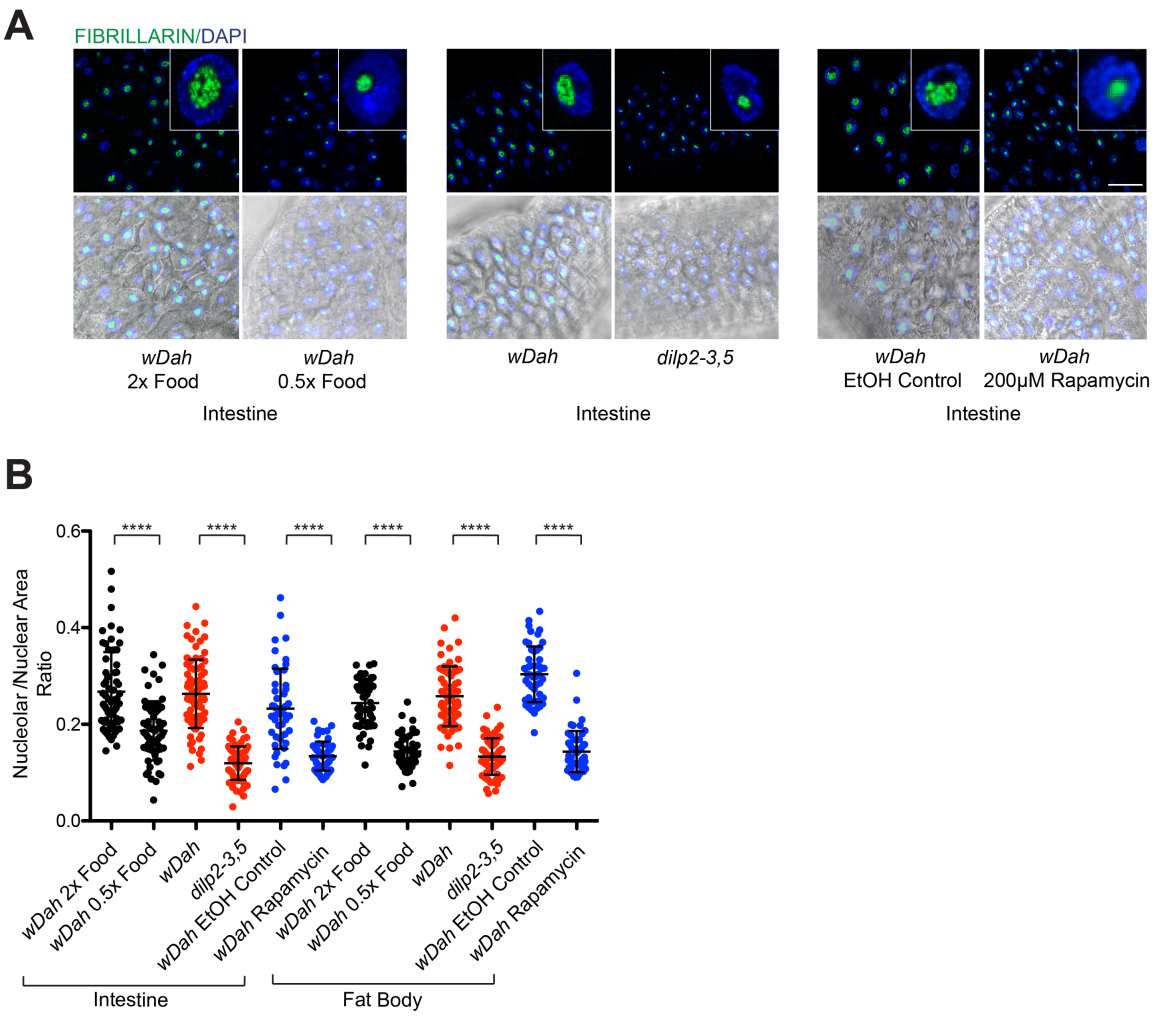

Figure 16. Long-lived *Drosophila* possess small nucleoli. (A) *Drosophila* intestinal nucleoli stained with anti-fibrillarin antibody. (B) Long-lived dietary restricted, Rapamycin treated and *dilp2-3,5* mutant flies exhibit smaller nucleoli compared to controls in intestine and fat body. Nucleoli are stained in green and nuclei are DAPI stained in blue. **** P<0.0001, unpaired t-test. Scale bar represents 10 μm.

After observing the striking conservation of the phenomenon of reduced nucleoli in long-lived flies we further wanted to investigate the levels of Fibrillarin and ribosomal proteins in these long-lived flies. Using western blotting we observed that Fibrillarin and RPS6 and RPS15 were down-regulated in long-lived flies similar to our results in *C. elegans* (Figure 17). Although we observed a significant reduction in RPS6 and RPS15 in flies that were long-lived due to DR and reduced IIS signaling, there were no significant alterations in the levels of RPS6 and RPS15 in flies subjected to Rapamycin treatment unlike our results obtained in *C. elegans* where

TOR down-regulation via RNAi decreased the levels of RPS6 and RPS15 (Figure 17A,C,D). However we observed that Fibrillarin levels were significantly reduced in all the long-lived flies that we examined here compared to controls (Figure 17A,B).

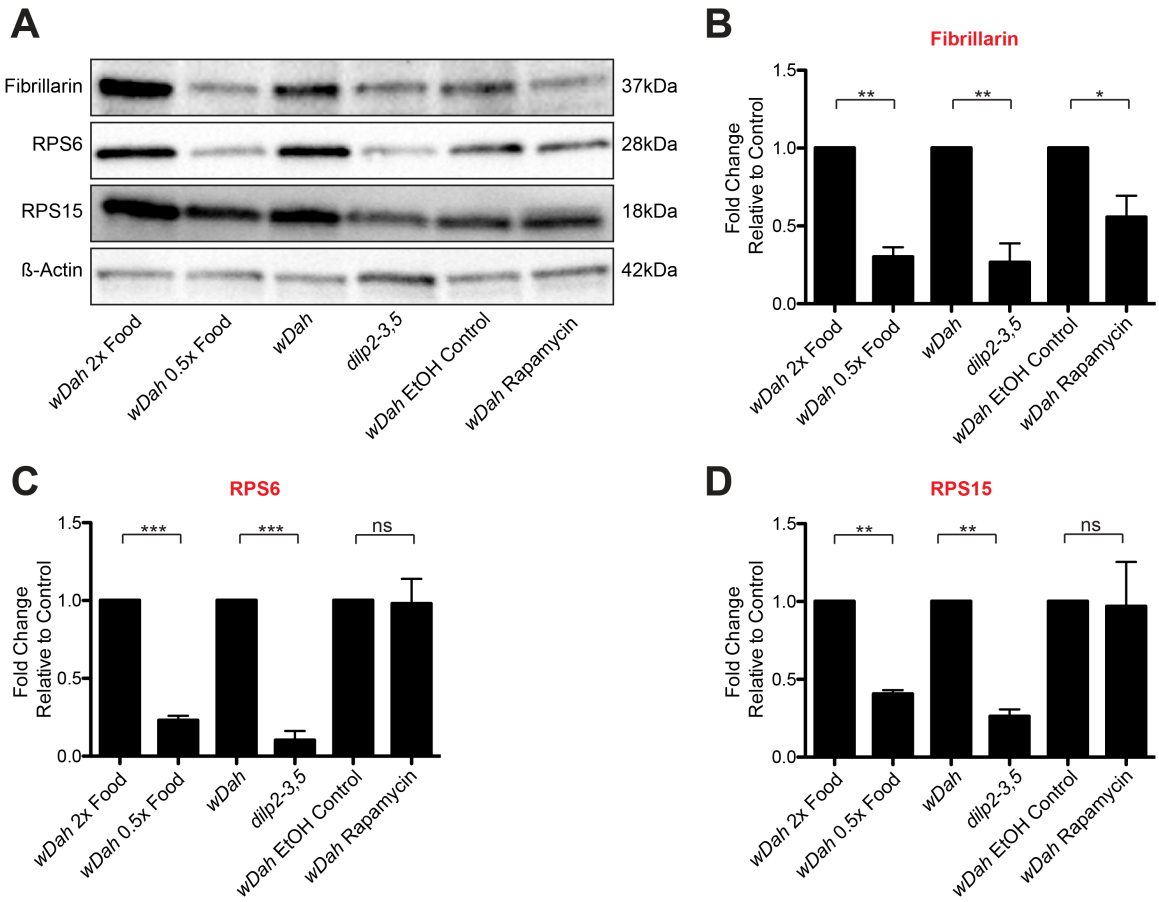

Figure 17. Fibrillarin and ribosomal proteins are reduced in long-lived flies. (A,B) In flies subjected to DR and Rapamycin treatment and in *dilp2-3,5* mutants Fibrillarin levels are significantly down compared to their respective controls. (A,C,D) RPS6 and RPS15 are significantly reduced in DR flies and *dilp2-3,5* mutants but not in Rapamycin treated flies. * P<0.05, ** P<0.01, *** P<0.001, ns: non-significant, unpaired t-test.

We further asked if the effects of reduction in nucleolar size are also true in the case of long-lived mice. We collaborated with Dr. Roman Mueller's lab (CECAD-Cologne) where Dr. Martin Spaeth performed DR treatment on wildtype mice for 28 days. They were exposed to 75% of normal chow diet as against wildtype that had 100% food availability. After 28 days of DR, the mice were sacrificed and stained on liver and kidney sections with anti-Fibrillarin antibody. We observed a significant reduction in nucleolar size of DR mice compared to ad-libitum fed mice in both liver

and kidney (Figure 18 and 19).

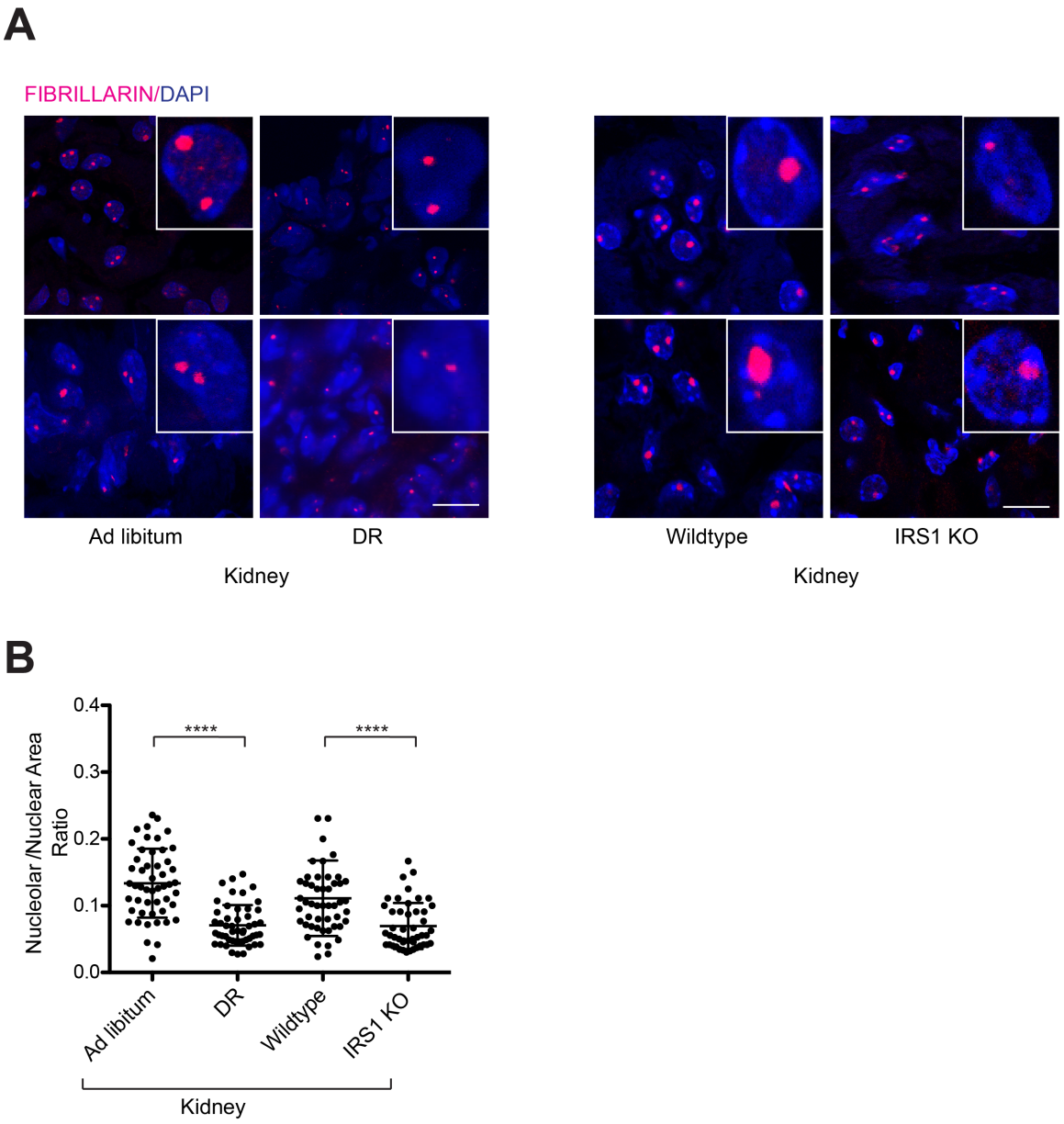

Figure 18. Nucleolar size is reduced in kidneys of long-lived mice. (A,B) Mice exposed to DR and IRS-1 KO mice possess smaller nucleoli in kidney tissue. Nucleoli are stained in magenta and nuclei are DAPI stained in blue. **** P<0.0001, unpaired t-test. Scale bars represent 10 μm.

We further resorted to another long-lived mouse model, the long-lived IRS-1 KO mouse (we obtained tissues of IRS-1 KO mice from Joana Goncalves, Linda Partridge lab). IRS-1 encodes the Insulin Receptor Substrate-1 protein, which plays a critical

role in transmitting signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors to the intracellular pathways and a mutation of IRS-1 reduces insulin signaling and enhances lifespan in mice (Selman et al., 2008). Antibody staining revealed that IRS-1 knockout mice exhibited smaller nucleolar size in brain and kidney compared to wildtype counterparts (Figure 18 and 19). These results suggest that even in mammals longevity is inversely associated with the size of nucleoli in different tissues. This points towards an evolutionarily conserved nucleolar function that is associated with longevity in different organisms. It still remains to be understood if a reduction in nucelolar size and function is a cause or consequence of aging.

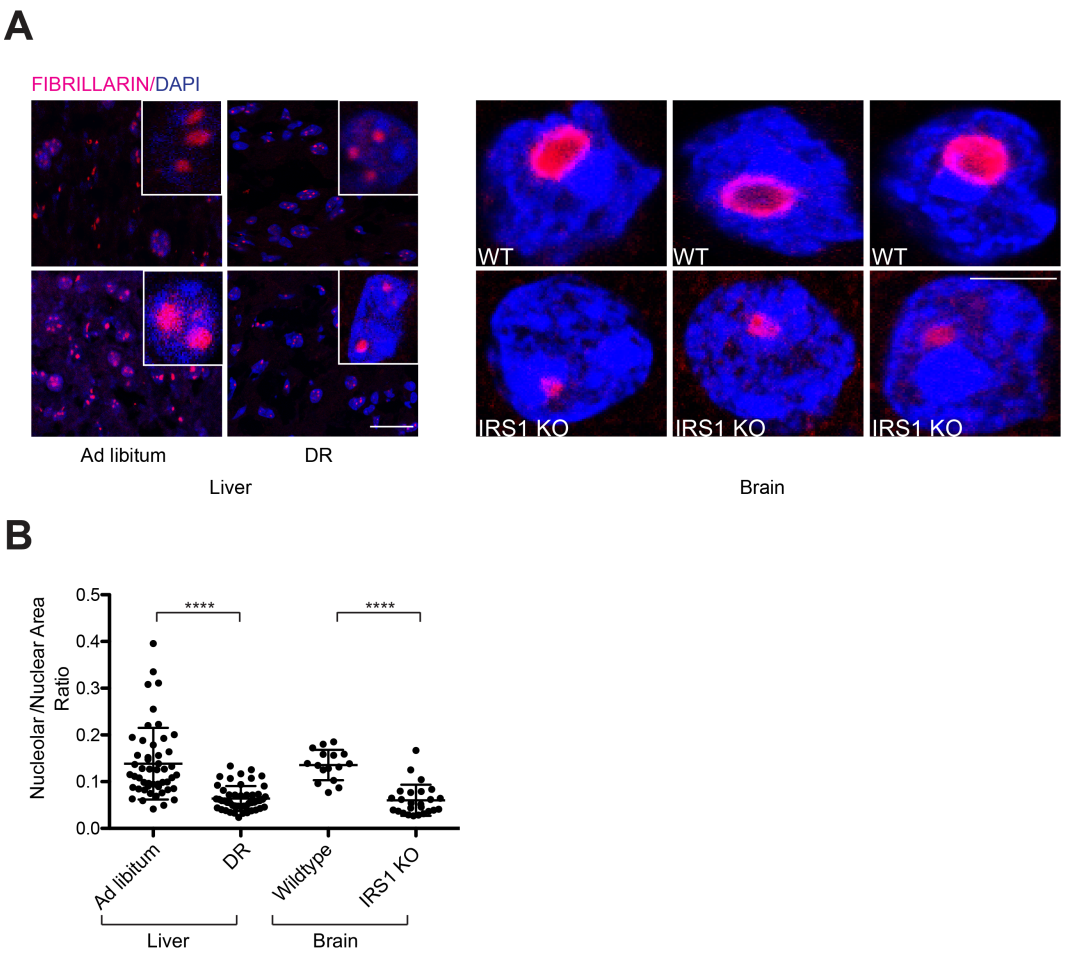

Figure 19. Nucleolar size is reduced in liver and brain tissues of long-lived mice. (A,B) Mice exposed to DR and IRS-1 KO mice possess smaller nucleoli in liver and brain tissue respectively. Nucleoli are stained in magenta and nuclei are DAPI stained in blue. **** P<0.0001, unpaired t-test. Scale bars represent 10 μm (liver sections) and 5 μm (brain sections).

3.8 Nucleolar Size in Humans Undergoing DR

Having observed a striking reduction in the size of the nucleolus of long-lived animals from lower model system to mammals, we were intrigued to ask if the effects might be conserved in humans as well. We started a successful collaboration with Prof. P. Eline Slagboom's lab at the Leiden Medical Center. Yotam Raz from the Slagboom lab stained muscle biopsies of humans who had taken up a healthy life style regime, referred to as the Life Style Intervention, which included a reduction of caloric intake by 12.5% and an increase in exercise by 12.5%. The intervention was carried out for a period of 13 weeks. (Details of the intervention are given in Chapter 2). After the intervention, the subjects experienced a weight reduction, lowered blood glucose levels and reduced body mass index (BMI). There was an average 7.7 kg weight loss in men and 6.0 kg in women. Additional clinical characteristics of this group of men and women are detailed in Supplementary Table 4. Muscle biopsies were taken from these individuals before and after the intervention from the *vastus lateralis* muscle. Fibrillarin staining of these muscle biopsies revealed that the nucleolar size of the individuals declined significantly after going through the intervention (Figure 20). Samples of age matched five men and five women were analyzed for this experiment.

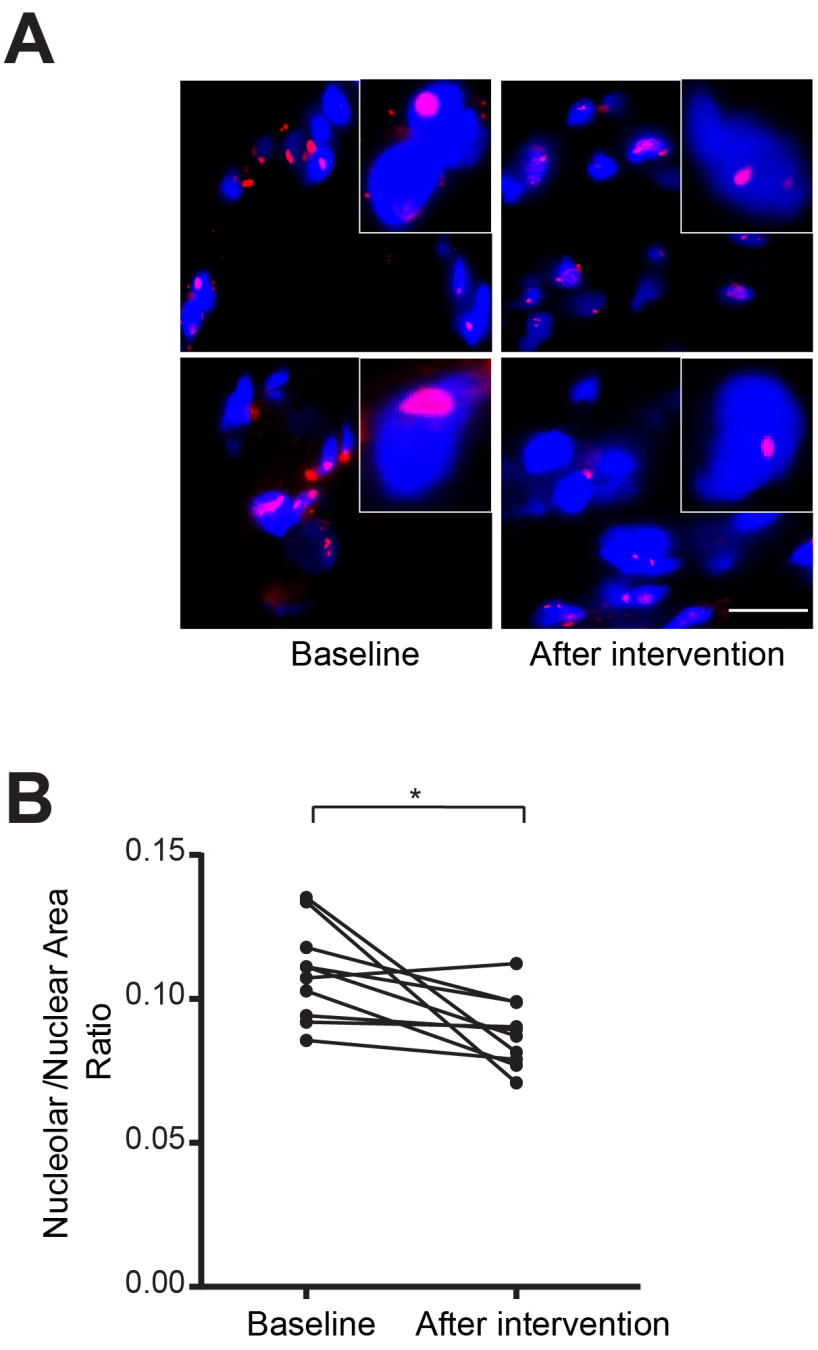

Figure 20. Nucleolar size is reduced in muscle biopsies of humans undergoing DR. (A,B) Humans exposed to 12.5% reduced caloric intake and 12.5% increased exercise exhibit reduced nucleoli after the intervention. Nucleoli are stained in magenta and nuclei are DAPI stained in blue. * P<0.05, unpaired t-test. Scale bars represent 20 μm.

3.9 Nucleolar Size in Mammalian Cells

We further wanted to investigate the effect of nutrient deprivation on nucleolar size in primary Mouse Embryonic Fibroblasts (MEFs) and primary human keratinocytes. We subjected these cells to starvation and then harvested them, fixed them and performed antibody staining on these cells using anti-Fibrillarin antibody. We observed that nucleoli of both primary MEFs and primary human keratinocytes became fragmented upon starvation when compared with non-starved control cells (Figure 21). This might indicate a stress response that the cells exhibit upon starvation. Since starvation is a very harsh treatment, it thus is not quite comparable to DR. Moreover, DR is generally performed on organisms for their entire lifetime or for substantially longer periods of time, for them to exhibit longevity and improved health span response. Nutrient deprivation for a few hours might again not be equivalent to DR in terms of time period. And finally DR mounts a combined systemic response, which collectively leads to improvement in health and extension in life. It is very difficult to mimic this response on an individual cell level in vitro. These reasons might explain why we did not observe a reduction in nucleoli in cells.

Primary cells, and not any of the widely used cancer cell-lines like HeLa cells, were used for this experiment because of the fact that cancer cells are known to possess a large nucleolus and consequently translational levels are highly up-regulated in those cells. We wanted to mimic a physiological response at the cellular level and wanted to investigate if we could observe effects of starvation leading to reduced nucleolar size in different kinds of primary cells.

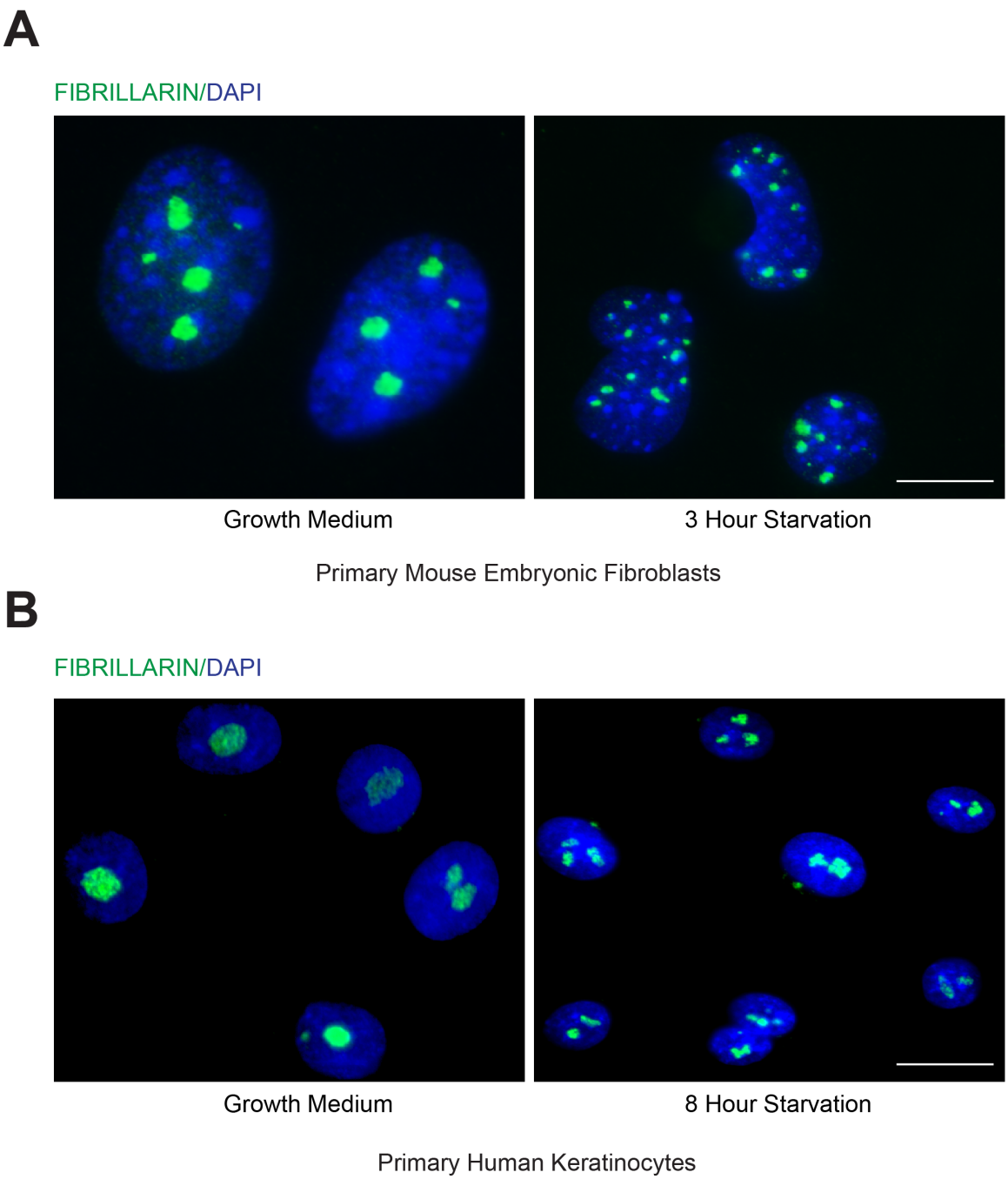

Figure 21. Mammalian cells exhibit nucleolar fragmentation upon starvation. Primary MEFs (A) and primary human keratinocytes (B) undergo nucleolar fragmentation upon 3 hour and 8 hour starvation respectively. Scale bars represent 10μm.

CHAPTER 4

DISCUSSION

4.1 *ncl-1* as a Regulator of Lifespan Across Multiple Pathways

Our RNAi based genetic screens identified *ncl-1* as a novel regulator of DR mediated longevity in *C. elegans*. We confirmed these findings further with *ncl-1* mutants where we found that *ncl-1* mutation in long-lived *eat-2(ad465)* worms reverses their longevity. We confirmed these findings with two independent alleles of *ncl-1* both of which are point mutations rendering the protein non-functional. We however observed that *eat-2,ncl-1* double mutants lived even shorter than wildtype worms. This effect can be due to a synthetic phenotype that affects the double mutants and not the single mutants alone. There are multiple ways of inducing a DR response in *C. elegans. eat-2(ad465)* is a genetic model of DR and additionally there are a number of dietary interventions that can be employed to induce the state of DR in worms. These methods mostly have to do with a direct reduction of food that is fed to worms. We performed a method called BDR in which we diluted bacteria and induced DR in liquid culture. *ncl-1* mutants did not respond to the longevity inducing effects of BDR while the wildtype worms were significantly longer lived when subjected to BDR. Between the two alleles of *ncl-1* mutants also we observed slight differences in terms of their response to BDR. *ncl-1(e1942)* exhibited slight longevity initially leading to a low peak for lifespan extension against bacterial dilution. However with further reduction in bacteria there was no longevity observed and the curves for wildtype and *ncl-1(e1942)* mutant separated significantly. *ncl-1(e1865)* on the other hand exhibited a very blunted response for all the bacterial dilutions. These results point towards the crucial role played by *ncl-1* in mediating longevity under DR conditions.

It is not only under DR where *ncl-1* plays a key role in inducing longevity but we also found that *ncl-1* is required for long life of other long-lived mutants. It is well known that a down-regulation of the TOR pathway has life extending benefits across multiple species (McCormick et al., 2011). Investigating the TOR pathway in the context of *ncl-1* function was intriguing for us because the TOR pathway is closely associated with DR. Our results clearly show that *ncl-1* is required for lifespan extension mediated by the inhibition of the TOR pathway. *ncl-1* mutants do

not exhibit longevity upon TOR inhibition unlike wildtype animals. This places *ncl-1* genetically downstream of the TOR pathway. In addition to DR and TOR inhibition modest inhibition of the IIS pathway, mitochondrial activity and germline loss lead to lifespan extension in different organisms. We investigated if *ncl-1* plays a role in modulating longevity in these established models of lifespan increase. Our results show that *ncl-1* is a critical factor required in these longevity pathways as well. *ncl-1* mutation in *daf-2(e1370)* and *isp-1(qm150)* partially but significantly reduces lifespan of these long-lived mutants indicating that a part of the mechanism that leads to lifespan extension of these worms relies on the function of *ncl-1.* Furthermore longevity of *glp-1(e2141)* was totally abolished in *glp-1;ncl-1* double mutants suggesting that *ncl-1* is required for lifespan extension in *glp-1(e2141)* animals. These results indicate that *ncl-1* is an important factor in modulating longevity across multiple longevity pathways. A large number of genes have been implicated in regulating longevity in different pathways but how all these pathways cross-talk with each other and if there are downstream mechanisms that are shared by different longevity pathways still remains elusive. In this study our discovery of *ncl-1* as being a universal regulator of lifespan extension across multiple longevity pathways points towards the fact that the cellular mechanisms regulated by *ncl-1* are significantly shared by different pathways that extend lifespan.

4.2 Nucleolar Size as a Potential Predictor of Life Expectancy in *C. elegans*

ncl-1 regulates nucleolar size in worms. Since we identified the role of *ncl-1* as a convergent factor for lifespan regulation across multiple pathways we were intrigued to confirm if the mechanism by which *ncl-1* works, acts as a downstream convergent mechanism for lifespan regulation in these longevity pathways. For understanding this we went ahead to establish if the downstream convergent mechanism by which *ncl-1* functions converges somehow on nucleolar function. The nucleolus is the cellular site of ribosome biogenesis. It is the most prominent subnuclear structure where ribosomal RNA (rRNA) and ribosomal subunits are synthesized. These cellular processes are energetically very demanding and thus are coordinated in a very strict manner. Within the nucleolus, ribosomal DNA (rDNA) is transcribed by RNA polymerase I (Pol I). The precursor rRNA (pre-rRNA) thus generated is further processed and modified to produce 28S, 18S, and 5.8S rRNAs. The mature rRNAs are then assembled with ribosomal proteins and shuttled to the cytoplasm for protein synthesis (Boulon et al., 2010; van Sluis and McStay, 2014). The size of the nucleolus varies between cells and in turn points towards the rate of ribosome biogenesis. Actively proliferating cells possess large nucleoli because of higher demands of ribosome biogenesis and protein synthesis whereas the size of the nucleolus decreases upon cell cycle arrest where the cells are in quiescence.

In order to investigate the nucleolar function in long-lived mutants we started by analyzing the size of nucleoli in these mutants. We made use DIC optics to capture high magnification images of different tissues where we could visualize and measure nucleoli directly. Strikingly we observed that all the examined long-lived mutants in our study namely *eat-2(ad465)*, *daf-2(e1370)*, *let-363/*TOR knockdown, *glp-1(e2141)* and *isp-1(qm150)* possessed smaller nucleoli in multiple tissues including hypodermis and pharyngeal muscle. This was indicative of reduced nucleolar function. And interestingly when crossed in *ncl-1* mutant background the small nucleolar size was enlarged again. Notably, these long-lived mutants also lose their longevity with the loss of *ncl-1* (discussed in section 4.1). These observations

were very exciting and pointed towards a negative correlation between nucleolar size and lifespan. In other words, worms representing distinct longevity pathways possessed smaller nucleoli and were significantly longer-lived but when their nucleolar size was perturbed genetically they lost their longevity. We also observed the same effects with worms subjected to DR using BDR regimen. Lifespan of animals increased upon bacterial food reduction but this increase in lifespan was accompanied with a corresponding drop in their nucleolar size. And we obtained another piece of evidence in favor of this notion of reduction in nucleolar size in long-lived worms. *daf-2(e1370)* worms are long-lived and when *daf-2* worms harbor a mutation of *daf-16* (FOXO homolog), they lose their longevity. When we analyzed the nucleoli of *daf-2(e1370)* and *daf-2;daf-16(mu86)* worms we found that the nucleoli of *daf-2(e1370)* worms were much smaller than *daf-2;daf-16(mu86)* which exhibited nucleoli similar to wildtype. This effect was seen to be true in multiple tissues. These results again suggest that nucleolar size and longevity are inversely linked to one another. These data also point towards the fact that this concept of inverse correlation between nucleolar size and lifespan also holds true in the case of environmental interventions that reduce nucleolar size like food reduction and is not only limited to genetic interventions.

These intriguing findings between nucleolar size and lifespan made us wonder if nucleoli could be predictive for life expectancy. Isogenic wildtype worms grown under similar conditions show considerable variation in lifespan with some worms dying as early as day 10 and some others living all the way upto day 30. And in isogenic wildtype worms interestingly there exists a significant variation in nucleolar size also. Therefore we reasoned if this disparity in lifespan could be attributed to differences in nucleolar size. We explored this by imaging the nucleoli of age-matched worms and following each worm through its lifespan. Strikingly we observed that the animals with smaller nucleoli lived considerably longer than the ones with large nucleoli. This strong correlation can be used in approximating the life expectancy of *C. elegans*.

4.3 Reduced Nucleolar Fibrillarin Expression and Longevity

Since we observed small nucleoli associate with longevity and that nucleolar size could essentially have the potential of predicting the lifespan of *C. elegans,* we wondered if nucleolar function was affected in long-lived worms. We looked at a nucleolar marker *fib-1*/Fibrillarin, which is a nucleolar protein with an important role in pre-rRNA processing during ribosomal biogenesis. It is known that *fib-1* is up-regulated in *ncl-1* mutants (Lee et. al., 2012). It was also shown by a recent study from the same lab that *ncl-1* directly regulates *fib-1.* They reported that *ncl-1* binds to the 3' UTR of *fib-1* and thereby down-regulates the expression of *fib-1.* Consequently, *ncl-1* loss abolishes this check on *fib-1* levels and thus *fib-1* is highly up-regulated in *ncl-1* mutants (Yi et al., 2015). Since *fib-1* is directly regulated by *ncl-1* and *ncl-1* is required for longevity in multiple lifespan extending pathways, we wondered if *fib-1* is regulated in long-lived mutants. Using the transgenic FIB-1::GFP strain, we observed a significant down-regulation of FIB-1 in *eat-2(ad465), daf-2(e1370), glp-1(e2141), isp-1(qm150)* and in animals with TOR knock-down. These results are in accord with our findings that *ncl-1* is required in these mutants for their long life. These results indirectly hint towards a higher activity of *ncl-1* in these mutants, which in turn down-regulates *fib-1.* We observed a similar down-regulation of FIB-1 in long-lived *ncl-1* over-expressing worms. Interestingly, this effect of reduction in FIB-1 levels was lost when we knocked down *ncl-1* in these long-lived mutants, adding more evidence that the activity of *ncl-1* is required in these mutants for down-regulation of *fib-1.* The effects on *fib-1* regulation are convincing because we saw similar effects using western blotting against the endogenous protein. Furthermore the effects on *fib-1* are similar to the effects seen on nucleolar size; in long-lived mutants nucleolar size is smaller and loss of *ncl-1* enlarges it again. These findings suggest a mechanistic role that *ncl-1* plays in regulating lifespan by reducing the expression of *fib-1* and maintaining the smaller size of nucleoli which is lost in *ncl-1* mutants and both *fib-1* expression and nucleolar size increase and that may be the reason behind loss of longevity. The detailed mechanistic role needs to be further explored as to how this *ncl-1 fib-1*

cascade regulates nucleolar size and in turn how nucleolar size plays into lifespan regulation.

Since FIB-1 was seen to be down regulated in all the long-lived worms examined, we wondered if *fib-1* could play a role in regulating lifespan. Indeed, we observed that *fib-1* knock-down via RNAi was sufficient to induce a modest but significant lifespan extension in wildtype worms. And *fib-1* RNAi also reduced the nucleolar size of wildtype worms, going in conjunction with our previous findings that long-lived worms possess smaller nucleoli. In order to study epistasis between *ncl-1* and *fib-1,* we examined the nucleoli of *ncl-1* mutants and observed that *fib-1* RNAi reduced the large sized nucleoli of *ncl-1* mutants however not back to wildtype levels. These results suggest that *fib-1* functions downstream of *ncl-1.* However, we did not see an increased lifespan of *ncl-1* mutants when subjected to *fib-1* RNAi which was surprising because the nucleolar size experiment suggested *fib-1* works downstream of *ncl-1.* Since *fib-1* is an essential gene we cannot draw convincing conclusions from these results because of our failure to use *fib-1* loss of function mutants, which are inviable. RNAi against *fib-1* through development also leads to larval arrest of worms. Therefore, the RNAi treatment was initiated from the day one of adulthood.

Furthermore, FIB-1 acted as a handy indirect tool to be used as a read-out to gauge the activity of *ncl-1.* Our qPCR expression analysis and transgenic expression of *ncl-1::gfp* did not show any regulation of *ncl-1* in long-lived worms yet we observed that it was required for the longevity of these mutants. So we speculate that there might be some post-translational modification occurring on NCL-1, which might be regulating its activity. We will try to understand this in detail using mass spectrometric approaches to identify post-translational modifications on NCL-1 and how they further modulate the expression of the protein.

4.4 Reduced Ribosome Biogenesis and Longevity

The next step was to investigate ribosome biogenesis in long-lived worms and *ncl-1* mutants because ribosome biogenesis is a key process happening in the nucleolus. We examined ribosome biogenesis indirectly by looking at the levels of rRNA and ribosomal proteins. We observed the same trend in rRNA and ribosomal protein levels as in FIB-1. Long-lived mutants *eat-2(ad465), daf-2(e1370), glp-1(e2141), isp-1(qm150)* and animals with TOR knock-down exhibited lower levels of rRNA and ribosomal proteins RPS6 and RPS15 indicative of reduced ribosome biogenesis in these worms. It is plausible to think that ribosome biogenesis is reduced in long-lived worms because it is a highly-energy consuming process and a reduction in ribosome biogenesis would spare energy reserves to operate other physiological processes. It is already known that a reduction in protein synthesis and knockdown of a handful of ribosomal proteins extends lifespan in *C. elegans* (Hansen et. al., 2007; Syntichaki et. al., 2007). We wondered if we could rescue this reduction of rRNA and ribosomal protein levels seen in long-lived worms by mutating *ncl-1* gene in these worms. And indeed that is exactly what we observed. Long-lived worms have a reduction in rRNA and ribosomal protein levels which is partially but significantly rescued with the loss of *ncl-1* again following the same trend that we observed in FIB-1 expression. Additionally, long-lived *ncl-1* over-expressing worms exhibit reduced levels of rRNA and ribosomal proteins, again similar to our observations of reduced FIB-1 levels in these worms. And finally we also observed that rRNA and ribosomal proteins were significantly higher in *daf-2;daf-16(mu86)* mutants compared to *daf-2(e1370)* alone, again hinting towards ribosome biogenesis being perturbed in *daf-2* mutants upon the introduction of *daf-16* mutation which might be the cause of their reduced lifespan.

Overall our results suggest that long-lived mutants have a reduction in nucleolar size, FIB-1, rRNA and ribosomal protein levels. This is indicative of a reduced ribosomal function being associated with longevity (Figure 22). And in turn if we down-regulate *fib-1* we can induce longevity, which indicates that reduced nucleolar function may be the causal factor for longevity. We need to further

investigate the mechanistic details to get a clear understanding of how reduction of ribosome biogenesis brings about an increase in lifespan.

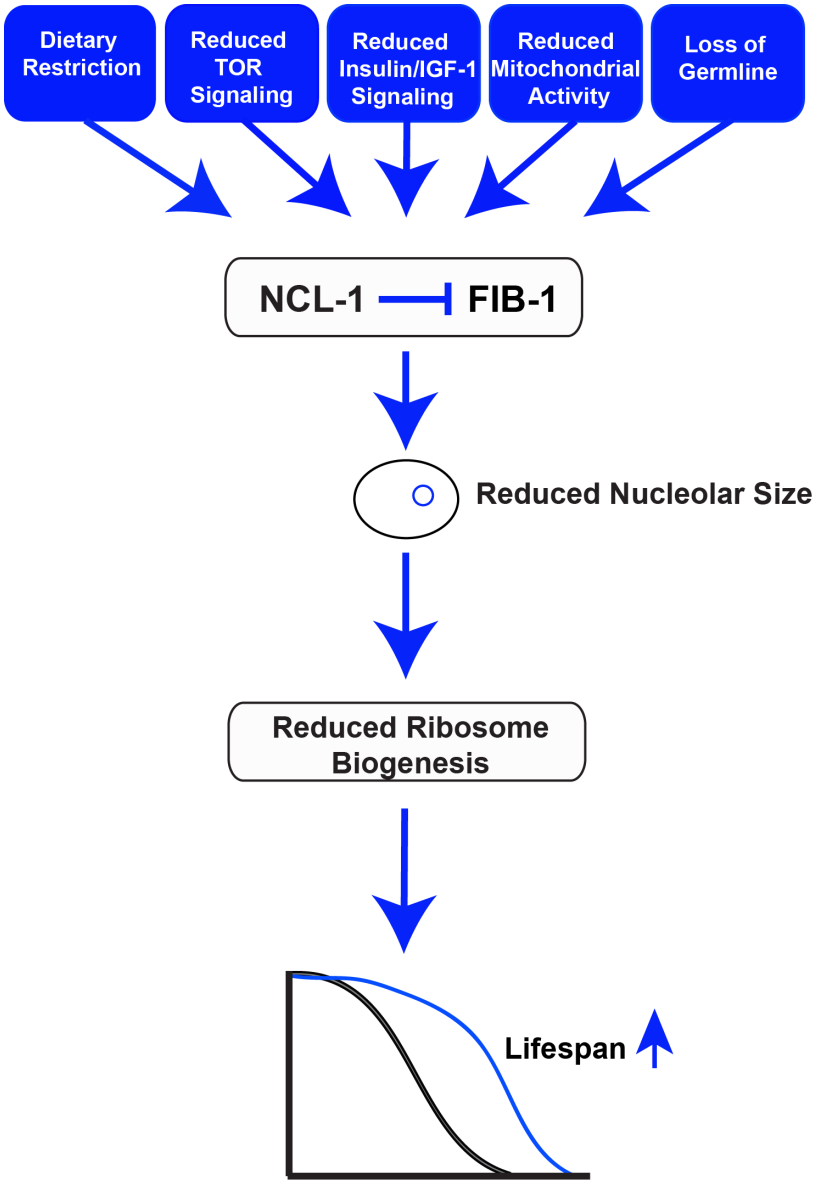

Figure 22. NCL-1 functions downstream of longevity pathways to regulate nucleolar size and longevity. NCL-1 down-regulates FIB-1 and mediates lifespan extension across different pathways. Long-lived mutants possess small nucleoli and reduced ribosome biogenesis and these effects are mediated by NCL-1.

4.5 Nucleolus and Longevity in Higher Organisms

Given our interesting results in *C. elegans,* we wanted to explore if longevity pathways relay their effects on the nucleolus in other organisms as well. We looked at the nucleoli of long-lived *Drosophila melanogaster.* We subjected *Drosophila* to DR and Rapamycin treatment both of which extend lifespan (Bass et al., 2007; Kapahi et al., 2004). We observed that these long-lived flies possessed smaller nucleoli in fat body and intestine compared to their respective controls. We further tested a genetic model of longevity in flies; *dilp2-3,5* mutant flies which harbor a deletion of insulin-like-peptide (Gronke et. al., 2010). These flies have a modest down-regulation of the IIS pathway, which in turn renders them long-lived. We detected smaller nucleoli in *dilp2-3,5* mutants compared to *wDah* control flies in fat body and intestine. These results are very exciting for the reason that we could observe similar nucleolar changes in long-lived *Drosophila* like we noticed in worms. Further going to the molecular level, we spotted reduced levels of Fibrillarin and ribosomal proteins RPS6 and RPS15 in long-lived DR and *dilp2-3,5* flies compared to control flies. A distinct exception was Rapamycin treatment where we did not observe significant changes in RPS6 and RPS15. However Rapapmycin treatment did reduce Fibrillarin levels like in DR and in *dilp2-3,5* mutants. These results are again in conjunction with our findings in worms where we observed longevity to be associated with reduced ribosome biogenesis.

Next we wondered if we could detect effects on nucleolar size in long-lived mammals. We resorted to investigating these effects in long-lived mice. We examined the nucleoli of long-lived DR mice. We observed that these mice had reduced nucleoli in the liver and the kidney. We also tested it in the long-lived genetic model; the IRS-1 knockout mice (Selman et. al., 2008), which lack insulin like receptor (IRS) 1. IRS1 is a major intracellular effector of the IIS receptors and knockout of IRS1 extends lifespan of mice. We noticed that IRS1 knockout mice also had smaller nucleoli in the kidney and the brain.

Furthermore, we investigated muscle biopsies from humans voluntarily undergoing reduced caloric intake and increased exercise for 13 weeks. This pro-

health intervention also reduced the size of the nucleoli in the muscles of these individuals significantly.

Therefore our results from *Drosophila,* mice and humans are in agreement with our data from *C. elegans.* Small nucleoli seem to be a cellular hallmark of longevity and reduced ribosome biogenesis and reduced Fibrillarin levels are the signatures that associate with longevity at least in *C. elegans* and *Drosophila.*

CHAPTER 5

FUTURE PERSPECTIVES

5.1 Investigating the Mechanism of Nucleolar Function in Aging

Our findings clearly show that nucleolar size and nucleolar function are affected in long-lived mutants. Ribosome biogenesis which is a major function of the nucleolus seems to be down-regulated in different long-lived worms and mutations that abrogate longevity of these worms, perturb this effect on ribosome biogenesis. On the contrary we observe that *ncl-1* over-expression reduces rRNA and protein synthesis rates. But it is unclear if and how these processes contribute to longevity. To address this issue, we will examine interactions of *ncl-1* mutation together with various mutations/RNAi knockdowns that decrease protein synthesis and rRNA production. Specifically, we will examine the cap binding subunit translation initiation factor 4f *ife-2*, the eIF-4G homolog *ifg-1* and various ribosomal subunits, whose modest knockdown promotes longevity. We will also examine *rrn3*, a subunit of RNA Polymerase I involved in rRNA synthesis. If these genes work downstream of NCL-1, then their knockdown should reverse the phenotypes of *ncl-1* null mutants. Alternately if they work upstream, *ncl-1* loss would reverse their phenotypes. We will monitor nucleolar size, fibrillarin production, protein synthesis rates, rRNA levels, and longevity in various single and double mutants to get detailed mechanistic insights.

5.2 Investigating Nucleolar Regulation of Proteostasis

We hypothesize that NCL-1 not only affects protein synthesis, but also protein quality control. Accordingly, our preliminary data suggest that autophagy is down-regulated in *ncl-1* mutants. To unravel potential roles in protein quality control, we would like to examine how *ncl-1* mutation and *fib-1* knockdown impact autophagy, by monitoring autophagic puncta containing LGG-1/ATG8 and SQST-1/p62. Further we will measure proteasome activity by biochemical methods as well as measuring the stability of *in vivo* model substrates using a transgenic strain containing a proteasomal substrate fused to GFP. We also want to test whether *ncl-1* and *fib-1* affect induction of stress markers for the endoplasmic reticulum unfolded protein response (UPR) (*hsp-4*), mitochondrial UPR (*hsp-6, hsp-60*), and the cytosolic heat shock response (*hsp-70*), or influence the stability of endoplasmic reticulum associated degradation (ERAD) substrates.

Other aspects of protein quality control tightly associated with ribosomal function include compartmental sorting and translational fidelity (Gamerdinger et al., 2015; Sherman and Qian, 2013). We will test whether *ncl-1* missorts mitochondrial proteins into the ER with existent split GFP reporters, or compromises the fidelity of protein synthesis by monitoring translational error read through rates on luciferase reporters. Moreover the mTOR signaling pathway is known to affect protein synthesis and protein quality control. We will test the idea that NCL-1 works within the TOR pathway by checking TOR outputs, including phospho-S6K levels, phospho-ULK1/UNC-51 and HLH-30/TFEB nuclear localization.

Disrupted proteostasis can also increase susceptibility to proteotoxic disease. We will also examine how *ncl-1* mutants respond to challenge by Aβ42, polyglutamine repeats, and α-synuclein, which model Alzheimer's, Huntington's, and Parkinson's disease, respectively, in *C. elegans*. In addition we will determine the influence of these proteotoxic species on nucleolar size. These experiments may allow us to link nucleolar function and age-related disease.

5.3 Investigating the Mechanism Underlying NCL-1 Action

The biochemical mechanisms underlying NCL-1 action are currently unclear. It is not known how this cytosolic protein regulates nucleolar functions and what its molecular targets are. Recent evidence points towards a role as an RNA binding protein, associated with the 3' UTR of germline expressed genes (Yi et. al., 2015). RING domains are also implicated in ubiquitin-mediated proteolysis. To clarify NCL-1 mechanism we will immunoprecipitate NCL-1, and identify associated proteins by mass spectrometry. Associated proteins will be validated by pairwise immunoprecipitation, interaction domains characterized, and complexes analyzed. Interestingly, yeast two hybrid data from *C. elegans* and *Drosophila* indicate a conserved interaction of NCL-1/BRAT with the argonaute homologs, NRDE-3/AGO1 (string-db.org), respectively, suggesting a potential link to the RNAi machinery. We will test NRDE-3 as a candidate protein interactor. In addition, we will perform pulldown experiments, and sequence associated RNAs to see if NCL-1 has RNA targets. Associated proteins or RNAs will be functionally analyzed by mutation or RNAi knockdown, for effects on protein synthesis, protein quality control, RNAi silencing, nucleolar function, and longevity.

5.4 Identifying Novel Genes Regulating Nucleolar Function and Longevity

Our findings clearly show that FIB-1 is down regulated and lifespan extension is promoted in different longevity models in a *ncl-1* dependent manner. However it is not understood how FIB-1 is suppressed in these longevity pathways. We aim to get a clear understanding of the mechanism by performing genetic screens using FIB-1::GFP translational reporter. The transgenic strain will be subjected to EMS mutagenesis, which introduces random mutations in the genome. And we will screen for the worms with reduced FIB-1::GFP levels. Whole genome sequencing will be performed on these worms to identify the causal gene mutations. These genes will be further characterized by assessing their role in aging, nucleolar size/function and ribosome biogenesis/protein synthesis. This method will be helpful in identifying novel lifespan regulating genes that have a role in nucleolar function. Additionally this strategy will also be employed to identify new factors that regulate *ncl-1*/*fib-1* axis.

5.5 Probing Other Nucleolar Functions in Aging

Besides regulating ribosome biogenesis nucleolus is also involved in other critical processes including assembly of signal recognition particle (SRP) and regulation and induction of nucleolar stress under cellular stress conditions (Boulon et al., 2010). We will investigate both these roles of the nucleolus in the context of aging and the involvement of *ncl-1* in these functions. It is well known that upon nucleolar stress, which can be caused by DNA damage, cytotoxic drugs or transcriptional inhibition etc, p53 is induced which serves as the marker of nucleolar stress (Boulon et al., 2010). There are several reports that have associated increased activity of p53 with aging (Donehower, 2005). Concordantly in *C. elegans* it has been reported that loss of p53 homolog *cep-1* promotes longevity (Arum and Johnson, 2007) dependent on autophagy (Tavernarakis et al., 2008). We will investigate if *ncl-1* affects the levels of *cep-1* and if *cep-1* is regulated in long-lived worms. We will also confirm if *ncl-1* is required for longevity induced by *cep-1* loss. These experiments will be useful in confirming if nucleolar stress is induced upon *ncl-1* loss. We will also check if the SRP function is altered in *ncl-1* mutants. We will use antibodies against different subunits of the SRP to check by western blotting if the levels differ in long-lived animals and *ncl-1* mutants. These experiments will reveal if *ncl-1* is required for the SRP pathway.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1: qPCR Primers

Gene	Primer	Sequence
ncl-1	*ncl-1* Forward	CCAGCAGCAAATGACTTTTTGA
	ncl-1 Reverse	TTGTGCTCGTCCGATTGGT

Supplementary Table 2: Lifespan Analyses

Strain/Treatment	Median Lifespan	Median Difference from Control (%)	Worms*	P value	Reference Control.
N2	26		91/120		
eat-2(ad465)	28	+7.69	68/120	<0.0001	vs N2
ncl-1(e1942)	24	-7.69	75/120	ns	vs N2
ncl-1(e1865)	24	-7.69	98/120	0.03	vs N2
eat-2;ncl-1(e1942)	21	-25	40/120	<0.0001	vs *eat-2*
eat-2;ncl-1(e1865)	16	-42.85	72/120	<0.0001	vs *eat-2*
N2	25		100/120		
eat-2(ad465)	30	+20	48/120	0.002	vs N2
ncl-1(e1942)	25	0	83/120	ns	vs N2
ncl-1(e1865)	25	0	91/120	ns	vs N2
eat-2;ncl-1(e1942)	25	-16.66	43/120	<0.0001	vs *eat-2*
eat-2;ncl-1(e1865)	17	-43.33	79/120	<0.0001	vs *eat-2*
N2	24		89/120		
eat-2(ad465)	27	+12.5	64/120	0.002	vs N2
ncl-1(e1942)	24	0	82/120	ns	vs N2
ncl-1(e1865)	24	0	79/120	ns	vs N2
eat-2;ncl-1(e1942)	24	-12.5	58/120	<0.0001	vs *eat-2*
eat-2;ncl-1(e1865)	17	-37.07	94/120	<0.0001	vs *eat-2*

BDR					
N2 (5.85×10^{9}CFU/ml)	23		76/90		
N2 (1.95×10^{9}CFU/ml) 5.85×10^{9}	36	+56.52	83/90	<0.0001	vs
N2 (6.5×10^{8}CFU/ml) 1.95×10^{9}	50	+38.88	82/90	<0.0001	vs
N2 (2.1×10^{8}CFU/ml) 6.5×10^{8}	43	-14	89/90	<0.0001	vs
N2 (7.2×10^{7}CFU/ml) 2.1×10^{8}	32	-25.50	86/90	<0.0001	vs
N2 (2.4×10^{7}CFU/ml) 7.2×10^{7}	23	-28.12	84/90	<0.0001	vs
N2 (8×10^{6}CFU/ml) 2.4×10^{7}	18	-21.73	81/90	0.0003	vs
ncl-1(e1942) 5.85×10^{9} (5.85×10^{9}CFU/ml)	23	0	58/90	ns	vs N2
ncl-1(e1942) 1.95×10^{9} (1.95×10^{9}CFU/ml)	23	-36.11	76/90	<0.0001	vs N2
ncl-1(e1942) 6.5×10^{8} (6.5×10^{8}CFU/ml)	32	-36	85/90	<0.0001	vs N2
ncl-1(e1942) 2.1×10^{8} (2.1×10^{8}CFU/ml)	27	-37.20	88/90	<0.0001	vs N2
ncl-1(e1942) 7.2×10^{7} (7.2×10^{7}CFU/ml)	23	-28.12	83/90	<0.0001	vs N2
ncl-1(e1942) 2.4×10^{7} (2.4×10^{7}CFU/ml)	18	-21.73	84/90	0.0092	vs N2
ncl-1(e1942) 8×10^{6} (8×10^{6}CFU/ml)	13	-27.77	86/90	ns	vs N2
ncl-1(e1865) 5.85×10^{9} (5.85×10^{9}CFU/ml)	23	0	77/90	ns	vs N2
ncl-1(e1865) 1.95×10^{9} (1.95×10^{9}CFU/ml)	23	-36.11	85/90	<0.0001	vs N2
ncl-1(e1865) 6.5×10^{8} (6.5×10^{8}CFU/ml)	27	-46	78/90	<0.0001	vs N2
ncl-1(e1865) 2.1×10^{8} (2.1×10^{8}CFU/ml)	23	-46.51	79/90	<0.0001	vs N2

ncl-1(e1865) 7.2×10^7 (7.2×10^7CFU/ml)	23	-28.12	86/90	<0.0001	vs N2
ncl-1(e1865) 2.4×10^7 (2.4×10^7CFU/ml)	18	-21.73	75/90	0.0253	vs N2
ncl-1(e1865) 8×10^6 (8×10^6CFU/ml)	18	0	87/90	ns	vs N2
N2 GFPi	21		94/120		
N2 *let-363*/TORi GFPi	28	+33.33	82/120	<0.0001	vs N2
ncl-1(e1942) GFPi	26		79/120		
ncl-1(e1942) *363*/TORi *let-363*/TORi	23	-17.85	82/120	0.0046	vs N2 *let-*
ncl-1(e1865) GFPi	23		92/120		
ncl-1(e1865) *363*/TORi *let-363*/TORi	26	-7.14	83/120	<0.0001	vs N2 *let-*
N2 GFPi	24		90/120		
N2 *let-363*/TORi GFPi	29	+20.83	65/120	<0.0001	vs N2
ncl-1(e1942) GFPi	22		45/120		
ncl-1(e1942) *363*/TORi *let-363*/TORi	27	-6.89	79/120	<0.0001	vs N2 *let-*
ncl-1(e1865) GFPi	22		65/120		
ncl-1(e1865) *363*/TORi *let-363*/TORi	24	-17.24	92/120	<0.0001	vs N2 *let-*
N2 GFPi	19		81/120		
N2 *let-363*/TORi GFPi	27	+42.10	60/120	<0.0001	vs N2
ncl-1(e1942) GFPi	17		54/120		
ncl-1(e1942) *363*/TORi *let-363*/TORi	15	-44.44	49/120	<0.0001	vs N2 *let-*
ncl-1(e1865) GFPi	19		90/120		
ncl-1(e1865) *363*/TORi	23	-14.81	78/120	<0.0001	vs N2 *let-*

let-363/TORi

N2 GFPi	25		99/120		
N2 *daf-2i* GFPi	55	+120	83/120	<0.0001	vs N2
ncl-1(e1942) GFPi	21		84/120		
ncl-1(e1942) daf-2i daf-2i	34	-38.18	77/120	<0.0001	vs N2
ncl-1(e1865) GFPi	21		104/120		
ncl-1(e1865) daf-2i daf-2i	45	-18.18	42/120	<0.0001	vs N2
N2 GFPi	20		87/120		
N2 *daf-2i* GFPi	58	+190	86/120	<0.0001	vs N2
ncl-1(e1942) GFPi	20		64/120		
ncl-1(e1942) daf-2i daf-2i	51	-12.06	58/120	<0.0001	vs N2
ncl-1(e1865) GFPi	20		94/120		
ncl-1(e1865) daf-2i daf-2i	47	-18.96	79/120	<0.0001	vs N2
N2 GFPi	24		62/120		
N2 *daf-2i* GFPi	59	+145	69/120	<0.0001	vs N2
ncl-1(e1942) GFPi	24		31/120		
ncl-1(e1942) daf-2i daf-2i	48	-18.64	47/120	<0.0001	vs N2
ncl-1(e1865) GFPi	24		74/120		
ncl-1(e1865) daf-2i daf-2i	48	-18.64	73/120	<0.0001	vs N2
N2	22		66/120		
ncl-1(e1942)	20		64/120		
glp-1(e2141)	33	+50	102/120	<0.0001	vs N2
glp-1;ncl-1	22	-33.33	92/120	<0.0001	vs *glp-1*
N2	24		83/120		
ncl-1(e1942)	24		57/120		
glp-1(e2141)	40	+66.66	102/120	<0.0001	vs N2

glp-1;ncl-1 1	24	-40	92/120	<0.0001	vs *glp-*
glp-1(e2141)	37		76/120	<0.0001	
glp-1;ncl-1 1	25	-32.43	103/120	<0.0001	vs *glp-*
N2	23		93/120		
ncl-1(e1942)	23		89/120		
isp-1(qm150)	29	+26.08	39/120	<0.0001	vs N2
isp-1;ncl-1 1	25	-13.79	103/120	0.002	vs *isp-*
N2	23		93/120		
ncl-1(e1942)	23		89/120		
isp-1(qm150)	34	+47.82	38/120	<0.0001	vs N2
isp-1;ncl-1 1	26	-23.52	69/120	0.0016	vs *isp-*
isp-1(qm150)	34		29/120	<0.0001	
isp-1;ncl-1 isp-1	22	-35.29	70/120	<0.0001	vs
N2	24		86/120		
N2+*ncl-1::gfp (dhEx1007)*	29	+20.83	78/120	<0.0001	vs N2
N2+*ncl-1::gfp (dhEx1008)*	27	+12.50	63/120	<0.0001	vs N2
N2	16		78/120		
N2+*ncl-1::gfp (dhEx1007)*	22	+37.50	57/120	<0.0001	vs N2
N2	17		63/120		
N2+*ncl-1::gfp (dhEx1007)*	23	+35.29	48/120	<0.0001	vs N2
N2	26		91/120		
eat-2(ad465)	28	+7.69	68/120	<0.0001	vs N2
ncl-1(e1942)	24		75/120		

eat-2;ncl-1 eat-2	21	-25	40/120	<0.0001	vs
eat-2;ncl-1+ eat-2 ncl-1::gfp(dhEx1007)	27	+3.57	78/120	ns	vs
eat-2+ncl-1::gfp 2 (dhEx1007)	30	+7.14	62/120	ns	vs *eat-*
N2	20		87/120		
eat-2(ad465)	26	+30	66/120	<0.0001	vs N2
ncl-1(e1865)	20		94/120		
eat-2;ncl-1 eat-2	23	-11.53	77/120	<0.0001	vs
eat-2;ncl-1+ eat-2 ncl-1::gfp(dhEx1007)	26	0	56/120	ns	vs
N2 GFPi	24		90/120		
N2 *fib-1i* GFPi	27	+12.50	56/120	<0.0001	vs N2
N2 GFPi	19		81/120		
N2 *fib-1i* GFPi	23	+21.10	47/120	0.0004	vs N2
N2 GFPi	19		81/120		
N2 *fib-1i* GFPi	29	+52.63	87/120	<0.0001	vs N2
N2 GFPi	24		89/120		
N2 *fib-1i* GFPi	29	+20.83	87/120	<0.0001	vs N2
N2 GFPi	24		90/120		
N2 *fib-1i* GFPi	26	+8.33	75/120	0.0004	vs N2

* no. of dead worms/total no. of worms analyzed
p-values for statistical analyses were calculated using Mantel-Cox Log Rank test.

Worms that escaped the dishes, had internal hatching or had bursting of vulva were censored from the experiment.

Supplementary Table 3: Nucleolar Size Analyses

Strain/Condition	Average nucleolar area (μm²)	Standard Deviation	% Change relative to N2 or N2 Control RNAi
Nucleoli in hypodermis			
N2	5.390	± 2.480	
eat-2(ad465)	2.821	± 0.919	-47.65
N2 *let-363*/TORi	2.835	± 1.141	-47.40
isp-1(qm150)	2.845	± 1.053	-47.22
glp-1(e2141)	3.354	± 1.447	-37.76
daf-2(e1370)	2.321	± 1.032	-56.92
daf-2;daf-16(mu86)	4.583	± 1.965	-14.98
N2 Control RNAi	4.492	± 1.150	
N2 *fib-1i*	2.131	± 0.779	-52.56
Nucleoli in pharyngeal muscle			
N2	2.017	± 0.562	
eat-2(ad465)	1.581	± 0.481	-21.60
N2 *let-363*/TORi	1.719	± 0.455	-14.79
isp-1(qm150)	1.222	± 0.350	-39.42
glp-1(e2141)	1.669	± 0.466	-17.25
daf-2(e1370)	1.555	± 0.426	-22.91
daf-2;daf-16(mu86)	2.240	± 0.668	+11.05

Nucleoli in germ cells

N2	10.769	± 2.657	
eat-2(ad465)	6.549	± 2.050	-39.18
N2 *let-363*/TORi	9.227	± 1.451	-14.32
isp-1(qm150)	7.264	± 2.119	-32.54
daf-2(e1370)	8.047	± 1.471	-25.27
daf-2;daf-16(mu86)	10.633	± 1.779	-1.269

BDR

Strain and Condition	Average nucleolar area (μm^2)	Standard Deviation	% Change relative to N2 ($5.85x10^9$)
N2 ($5.85x10^9$)	4.218	± 1.725	
N2 ($1.96\ x10^9$)	2.757	± 0.988	- 34.61
N2 ($6.50\ x10^8$)	1.531	± 0.365	- 63.70

Supplementary Table 4: Characteristics of human volunteers following lifestyle intervention

	Growing Old Together participants (N=10)
Age, years	62.4 (4.1)
Females, N (%)	5 (50)
Lipid lowering medication, N (%)	3 (30)
Antihypertensive medication, N (%)	3 (30)
Intervention parameters	
Baseline weight, kg	81.0 (12.8)
After intervention weight, kg	74.2 (12.1)
Baseline glucose, mmol/L	5.1 (0.4)
After intervention glucose, mmol/L	4.9 (0.5)
Baseline SBP, mmHg	146.5 (14.9)
After intervention SBP, mmHg	136.4 (9.4)
Baseline BMI, kg/m^2	28.4 (2.7)
After intervention BMI, kg/m^2	26.0 (2.4)

Means (standard deviations) are provided unless otherwise stated.

APPENDIX

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Work Contribution

I performed all the *C. elegans* work myself except for the genetic screens in which *ncl-1* was identified as a candidate for mediating DR longevity. These genetic screens were carried out by Dr. Bree Heestand and Dr. Wei Liu (former Antebi lab members at Huffington Center on Aging, Baylor College of Medicine, Houston TX, USA). *Drosophila* work was done in collaboration with Prof. Linda Partridge's lab (MPI-AGE, Cologne). Chirag Jain (Partridge lab, MPI-AGE) performed all the fly experiments. DR mouse experiments were performed in collaboration with Dr. Roman Mueller's lab (CECAD, Cologne) where Dr. Martin Spaeth (CECAD, Cologne) performed DR treatment on mice and sacrificed them after 28 days on DR. I carried out all the other experiments including antibody staining, imaging and quantification myself. Finally in collaboration with Prof. P. Eline Slagboom's lab at (Leiden University Medical Center (LUMC), Netherlands) experiments on human muscle tissue were done. Yotam Raz together with H. Eka Suchiman carried out antibody staining, imaging and quantification on the human tissues.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass sie abgesehen von unten angegebenen Teilpublikationen–noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt.

Die von mir vorgelegte Dissertation ist von Prof. Dr. Adam Antebi betreut worden.

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Köln, September 2016

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(c) Publikationen

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Shubha Vij, Jochen C. Rink, Hao Kee Ho, Deepak Babu, Michael Eitel, Vijayashankaranarayanan Narasimhan, **Varnesh Tiku**, Jody Westbrook,Bernd Schierwater and Sudipto Roy. Evolutionarily Ancient Association of the FoxJ1 Transcription Factor with the Motile Ciliogenic Program. PLoS Genet., 8, no. 11, (2012)

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