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Identification and characterization of inter-species aging-related transcriptomic regulators

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Summary

Aging can be defined as time-dependent decline in organ functions concomitant with an increase in probability of death, and it begins after the organism has achieved its maximum reproductive competence. Aging is associated with multiple metabolic, cardiovascular, and neurodegenerative diseases in human population, and a growing body of evidence suggests that combating aging itself may be a better strategy to address these disorders than tackling them individually. At the same time, studies in model organisms indicate that many of the underlying molecular mechanisms of aging are evolutionarily conserved, and can be regulated resulting in considerable improvements in fitness and often longevity.

The genetic makeup of the organism determines the species-specific rate of aging and its potential for the maximum lifespan. In converting the genotype into phenotype transcription factors play crucial roles by controlling the rates of transcription of specific genes through direct binding to gene promoters. I here utilized data obtained by JenAge consortium in the trans-species approach using three model organisms (*Caenorhabditis elegans*, *Danio rerio*, and *Mus musculus*) to analyze aging-associated changes in the expression patterns of orthologous genes across these species. To identify the transcriptional regulators responsible for the aging related patterns of gene expression clustered during the JenAge study I created a bioinformatics pipeline that runs RSAT (Regulatory Sequence Analysis Tools) *in silico* promoter analyses. The identified transcription factors were screened for a strong effect on lifespan of *C. elegans*. This nematode is a popular model organism in aging studies due to its relatively short lifespan, multiple tools of genetic manipulation, and the fact that many lifespan-modulating pathways are conserved between both *C. elegans* and humans.

Here, a number of development-implicated transcription factors are identified in *C. elegans* as new transcriptional regulators modulating the rates of aging. I select the top candidates from the screen, *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *ceh-22*/F29F11.5, and specifically focus on *hlh-2*/M05B5.5 in detail. Its downregulation induced by RNA interference (RNAi) applied on day 1 of adulthood extends the mean lifespan by ~35%

and improves several fitness parameters. I provide evidence that this lifespan-extending effect is mediated among others by a creatine kinase ortholog in *C. elegans* *argk-1*/F44G3.2, an energy sensor *aak-2*/AMPK, and depends on the activities of *hsf-1*/HSF1, *skn-1*/NRF2, and *let-363*/TOR. I show that this mechanism involves reactive oxygen species (ROS) signaling and activation of autophagy.

These findings were supported in vitro by an inducible knockout model of TCF3 (E2A), the mammalian ortholog of *hlh-2*/M05B5.5, which shows alleviation of senescence in cell culture, raising the possibility that the mechanism through which *HLH-2* and TCF3 affect aging is conserved. Moreover, similarly to their effect in *C. elegans*, I observe that antioxidants interfere with the delayed senescence induced by TCF3 knockout, providing evidence for the conservation of the ROS signaling in this longevity phenotype.

Taken together, the results of this work advance our understanding of mechanisms of genetic regulation of aging and suggest that transcription factors regulating development can modulate the rates of aging once the organism has reached its maturity. These notions open new directions for future research in mammalian systems to determine how fully these pathways are conserved, to identify novel anti-aging targets, and in the end to contribute to human healthspan and well-being.

Riassunto

L'invecchiamento può essere definito come un declino tempo-dipendente delle funzioni organiche concomitante con un aumento della probabilità di decesso ed inizia dopo che l'organismo ha raggiunto la sua massima competenza riproduttiva. L'invecchiamento è associato a più malattie nella popolazione umana, quali metaboliche, cardiovascolari e neurodegenerative; un numero crescente di prove suggerisce che combattere l'invecchiamento stesso può essere una strategia migliore per curare questi disturbi piuttosto che affrontarli individualmente. Allo stesso tempo, diversi studi in organismi modello indicano che molti dei meccanismi molecolari sottostanti l'invecchiamento sono conservati in modo evolutivo, e che, una volta regolati possono migliorare considerabilmente parametri di fitness e spesso longevità.

La composizione genetica dell'organismo determina il tasso specifico d'invecchiamento della specie e il suo potenziale massimo di durata della vita. I fattori di trascrizione giocano ruoli cruciali nel convertire il genotipo in fenotipo, questo grazie alla loro abilità nel controllare i tassi di trascrizione di specifici geni attraverso il legame diretto con i promotori dei geni target. Ho qui utilizzato i dati ottenuti dal consorzio JenAge nell'approccio trans-specie, utilizzando tre organismi modello (*Caenorhabditis elegans*, *Danio rerio* e *Mus musculus*), per analizzare i cambiamenti associati all'invecchiamento nei pattern di espressione di geni ortologhi attraverso queste specie. Per identificare i regolatori trascrizionali responsabili dei pattern di espressione genica associati all'invecchiamento, raggruppati durante lo studio di JenAge, ho creato una pipeline di bioinformatica per l'analisi *in silico* del promotore che esegue RSAT (strumenti di analisi della sequenza normativa). I fattori di trascrizione identificati sono stati sottoposti a screening per un forte effetto sulla durata della vita di *C. elegans*. Questo nematode è un organismo modello molto popolare negli studi sull'invecchiamento grazie al suo ciclo vitale relativamente breve; è inoltre oggetto di molteplici strumenti di manipolazione genetica e molti dei meccanismi sottostanti alla durata della vita sono conservati tra *C. elegans* e uomo.

Un numero di fattori di trascrizione implicati nello sviluppo sono stati qui identificati in *C. elegans* come nuovi regolatori trascrizionali che modulano i tassi d'invecchiamento. Ho selezionato i migliori candidati dallo screening, *hlh-2*/M05B5.5, *lin-32*/T14F9.5 e *ceh-22*/F29F11.5, e mi sono focalizzato in particolare sullo studio dettagliato di *hlh-2*/M05B5.5. La sua down-regolazione indotta dall'interferenza del RNA (RNAi) applicata al primo giorno di età adulta prolunga la vita media del circa 35% e migliora inoltre diversi parametri di fitness. Ulteriormente, ho dimostrato come l'effetto della durata di vita prolungata è mediato da un ortologo della creatin-chinasi in *C. elegans argk-1*/F44G3.2, un sensore di energia *aak-2*/AMPK, e dipende dalle attività di *hsf-1*/HSF1, *skn-1*/NRF2 e *let-363*/TOR. Ho inoltre dimostrato che tale meccanismo coinvolge la segnalazione delle specie reattive dell'ossigeno (ROS) e l'attivazione dell'autofagia.

La down-regolazione dell'ortologo umano di *hlh-2*/M05B5.5, TCF3 (E2A) è in grado di limitare il processo di senescenza nella coltura cellulare, indicando la possibilità che il meccanismo attraverso cui *HLH-2* e TCF3 influenzano la durata della vita è conservato. Inoltre, analogamente al loro effetto in *C. elegans*, ho osservato che gli antiossidanti interferiscono con la senescenza ritardata indotta dal knockout di TCF3, fornendo prove per la conservazione della segnalazione dei ROS in questo fenotipo di longevità.

In conclusione, i risultati di questo lavoro non solo migliorano la nostra comprensione dei meccanismi di regolazione genica dell'invecchiamento, ma suggeriscono inoltre che i fattori di trascrizione, capaci di regolare lo sviluppo, possono anche modulare i tassi d'invecchiamento di un organismo una volta che ha raggiunto la sua maturità. Queste nozioni aprono nuove direzioni per la ricerca futura applicata a modelli di mammiferi per determinare in che misura questi percorsi siano conservati, per identificare nuovi obiettivi anti-invecchiamento, e infine per contribuire al benessere e alla salute umana.

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1 Introduction

1.1 Aging world population presents serious challenges to healthcare systems

The world's population aged 60 years and older is the fastest growing and is projected to rise from 841 million in 2013, to more than 2 billion by 2050, due to both increasing life expectancy at birth and the declining fertility rates (Chatterji et al. 2014). Accordingly, as age is the main risk factor for the aging-related diseases such as type 2 diabetes (Gunasekaran and Gannon 2011), cardiovascular disease (North and Sinclair 2012), cancer (De Magalhães 2013), osteoporosis (Epstein and Raisz 1988), osteoarthritis (Epstein and Raisz 1988), idiopathic pulmonary fibrosis (Nalysnyk et al. 2012), glaucoma (Y. H. Kwon et al. 2009), and neurodegenerative disorders (Kaeberlein 2013; Querfurth and LaFerla 2010), these disorders will increasingly define healthcare emphases, in both developed and developing countries. At the same time, the fact that aging itself influences so many diverse conditions is somewhat puzzling: what is this feature of aging that allows for such seemingly disparate pathological states to emerge within our cells and tissues? Multiple recent studies point at common molecular mechanisms underlying these diseases, to the point that in the field of studies of the processes of aging and the aging-associated diseases it is not entirely clear which is the cause and which the consequence. Addressing the common unifying biological root of aging-associated diseases rather than focusing on each individual disease may prove to be the winning strategy, at the same time leading to delayed aging, and such a strategy change has already been estimated to potentially bring a socio economic benefit of $7.1 trillion USD within the next 40 years (Goldman et al. 2013). Therefore, there appears to be imminent necessity to accelerate research in the field of aging in order to identify and characterize molecular pathways mechanistically linked to the aging process, and thus shorten the period of chronic disease and disability (the strategy also known as “compression of morbidity”)(Pitt and Kaeberlein 2015).

1.2 Current understanding of the molecular mechanisms/pathways related to aging

Aging can be defined as time-dependent decline in organ functions and increase in probability of death, notably after the organism has achieved its maximum reproductive competence (Roy et al. 2002). This definition is mostly descriptive, however, as it does not provide any insight into the very essence or reasons of the age-associated failure of homeostasis. This is because we still do not fully understand the nature of aging, or even have a reliable biological marker of aging. Yet, there have been proposed a number of theories of aging, not all mutually exclusive, and research in the past few decades has identified several molecular mechanisms or pathways which appear to be evolutionarily conserved and have the ability to modulate physiological aging in model organisms. Model systems are indispensable in aging research, as they provide a quick, convenient, and more or less ethical way to discover and test various interventions modulating longevity. The most common such organisms include yeast (*Saccharomyces cerevisiae*), worm (*Caenorhabditis elegans*), and fly (*Drosophila melanogaster*) due to the convenience of genetic manipulations and their very short lifespans compared to humans. Vertebrate model organisms such as zebrafish (*Danio rerio*), mouse *(Mus musculus*), and nonhuman primates allow us to imitate conditions closer to those that represent our own, human aging. It is thanks to these organisms that several genetic modulators of aging rate, as well as a few physiological and environmental interventions having effect on the progression of aging have been identified, and, importantly, have been found to follow the same evolutionarily conserved patterns/mechanisms throughout large evolutionary distances. Still, a lot of unsolved questions remain, with the biggest one being “what is aging: how do all these pathways come together to define such diverse lifespans seen in nature?” We will briefly summarize these conserved albeit only partially understood pathways and interventions in this section, and evaluate their relevance to understanding the underlying processes of human aging, as well as to combating aging-related loss of physiological function.

1.2.1 Calorie and dietary restriction

Calorie restriction (CR) is defined as a reduction in calorie intake without malnutrition, and it has been shown in various model systems to slow aging-related deterioration and to extend maximal and average life span (Masoro 2005; John R. Speakman and Mitchell 2011). The first mention of CR as a means to enhance life span dates back to early XX century (Osborne, Mendel, and Ferry 1917), and the first scientific paper to report that restricting food intake extended median and maximum life span and decreased the onset and severity of chronic diseases in rats soon followed (McCay, Crowell, and Maynard 1935). Although robust data in humans are still lacking, people practice CR. There are over 7000 members in The Calorie Restriction Society International which advises people that consuming 25% fewer calories than officially recommended will bring a longer and healthier life (Roth and Polotsky 2012).

Studies in various model organisms have recapitulated the beneficial effects of CR on health- and lifespan. DR (dietary restriction) by reducing glucose concentration in media extends both replicative and chronological lifespan in yeast (Kaeberlein et al. 2005; Wei et al. 2008). Various methods of DR in *C. elegans* including bacterial dilution in solid or liquid culture, use of chemically defined media, or reducing rates of pharyngeal pumping through genetic manipulation, increase mean lifespan by up to 85% (Lakowski and Hekimi 1998; Houthoofd et al. 2002; Szewczyk et al. 2006; Bishop and Guarente 2007). DR in fruit flies also leads to extended mean and maximum lifespan in a large number of different protocols (Partridge, Piper, and Mair 2005). Similarly, a multitude of studies presented evidence of CR increasing lifespan in rodent models, to different extents, depending on the level of calorie restriction, sex of the animals, genetic background, etc. (John R. Speakman and Mitchell 2011; J. R. Speakman, Mitchell, and Mazidi 2016). This CR-induced extended lifespan in mice and rats is accompanied by improved neurogenesis, better learning and memory, as well as a decrease in cancer occurrences (Richard Weindruch and Walford 1982; R. Weindruch et al. 1986; Donald K Ingram et al. 1987; J. Lee et al. 2000). These health benefits are however offset by impaired wound healing and susceptibility to infections in dietary restricted rodents (Reed et al. 1996; Kristan 2008). Among other mammals CR was also shown to have beneficial

effects on both median and maximal lifespan in cows (*Bos taurus*) and dogs (*Canis domesticus*) (Lawler et al. 2008, 2005; Kealy et al. 2002).

High expectations of the translatability of potential effects of CR and its relevance to human healthspan led to the establishment of two randomized controlled trials in non-human primates (Rhesus monkeys) in the 1990s at the NIA (National Institute on Aging) in Baltimore (LANE et al. 1992) and at the University of Wisconsin at Madison (D. K. Ingram et al. 1990). The trial at NIA applied 30% caloric restriction and failed to find any significant differences in the lifespan of monkeys, albeit demonstrated marked improvement in metabolic parameters and a delay in the onset of aging-related diseases (J. a Mattison et al. 2012). In contrast, the UW study did show lifespan extension of the calorie-restricted monkey, as well as delay in the onset of aging-related disorders such as cancer, cardiovascular diseases, and diabetes (Colman et al. 2009, 2014). The differences in the lifespan outcomes between the two trials are thought to have arisen from the differences in housing conditions and in the source of animals, yet both studies confirmed the overall health benefits of dietary restriction (J. A. Mattison et al. 2017). Practicing long term DR in humans is impractical due to such undesired side effects as constant hunger, feeling of being cold, and reduced libido, and in recent years considerable efforts have been poured into search for drugs that mimic the CR response. It became known that calories do not fully explain metabolic changes resulting from DR (Mair, Piper, and Partridge 2005), and that reducing intake of certain amino acids such as methionine may produce an effect similar to the full DR (Orgeron et al. 2014).

There are four downstream pathways that have been implicated in mediating the DR effect. They are as follows: 1) the IIS (insulin / insulin like growth factor (IGF-1) signaling) pathway; 2) the TOR (target of rapamycin) pathway; 3) the sirtuins pathway; and 4) the AMPK (adenosine monophosphate (AMP) activated protein kinase) pathway. All four of these pathways play important roles in mediating different aspects of the response, and they all likely interact with each other. The exact mechanisms of how they lead to health benefits are not completely understood, however it has been established that CR/DR results in enhanced autophagy and alleviated oxidative stress, both of which may be essential components of the beneficial health effects.

1.2.2 Insulin/insulin-like growth factor-1 (IIS) signaling

The first and probably the most studied signaling pathway implicated in influencing aging process in animals is the IIS pathway (Friedman and Johnson 1987; Holzenberger et al. 2003; C. Kenyon et al. 1993; C. Kenyon 2011). It was first discovered in *C. elegans* when a mutant of the *C. elegans* insulin receptor/ insulin-like growth factor-1 (IGF-1) homolog, *daf-2* was shown to extend lifespan more than two-fold (T. E. Johnson, Conley, and Keller 1988; C. Kenyon et al. 1993; Kimura et al. 1997). By that time its downstream effector, phosphoinositide kinase *age-1*, had already been identified through a forward genetics screen for longevity mutants (Klass 1983; Friedman and Johnson 1987). Further research of this pathway showed it to be a kinase cascade with such intermediate factors as PDK1 (phosphoinositide dependent kinase-1) and Akt (Morris, Tissenbaum, and Ruvkun 1996; Paradis and Ruvkun 1998; Paradis et al. 1999). It is thought that the main downstream effector of the IIS pathway is the transcription factor *daf-16* (ortholog of FOXO in mammals) whose activation leads to a change in expression levels of a very large number of other genes including those involved in longevity, heat shock survival and oxidative stress response (Gottlieb and Ruvkun 1994; K. Lin et al. 1997; Ogg et al. 1997; Paradis and Ruvkun 1998; K. Lin et al. 2001).

IIS studies continued to test if the mechanism is evolutionarily conserved, and found all of the pathway nodes identified in *C. elegans* in *Drosophila*. Importantly, these also showed a similar effect on longevity, albeit to a lesser extent: for instance, mutation of InR (the insulin-like receptor), the fly homolog of daf-2 and insulin/IGF-1 receptor, extended lifespan of female flies up to 85% (Tatar et al. 2001). Flies deficient in CHICO, the ortholog of insulin receptor substrate, are longer-lived, while overexpression of dFOXO, the daf-16 ortholog in *Drosophila,* having no statistically significant effect on male flies, increased the lifespan of female flies by 50% (Clancy et al. 2001; Tu, Epstein, and Tatar 2002; Giannakou et al. 2004).

The IIS pathway is well conserved, as mammals possess all the same nodes (INSR, PI3K, PDK1, AKT, and FOXO) as invertebrates, and yet IIS research in mammals showed more complexity in this signaling system and produced more conflicting results, albeit generally confirming the idea that insulin signaling affects longevity throughout

evolution. Firstly, mammals evolved IGF-1 signaling system alongside the insulin signaling to control metabolism and growth, and in the mouse there are two insulin and two insulin-like growth factors, whereas flies are known to have seven insulin types, and worms - 40. Mice possess two IGF receptors in addition to the insulin receptor, while flies and worms have only one insulin receptor each. Finally, in mice we find four genes encoding FOXO (Foxo1, Foxo2, Foxo4, and Foxo6) in contrast to a single FOXO gene in worms and flies (Papatheodorou, Petrovs, and Thornton 2014).

Initially insulin-signaling pathway was discovered to affect aging process during studies involving dwarf mice that have defects in pituitary function, and as a result lack growth hormone, prolactin, and thyroid-stimulating hormone. These mice have reduced circulating levels of insulin and IGF-1, which leads to increased insulin sensitivity, improved glucose tolerance, and other symptoms of delayed aging, including a generally longer lifespan compared to their control siblings (S. Li et al. 1990; Brown-Borg et al. 1996; Bartke 2006). Dwarf mice have reduced body size and exhibit many phenotypical features of calorie restricted mice, but their mechanism of longevity is different and thus additive when put on DR regimen (Bartke et al. 2001). Mice homozygously deficient for igf-1 and igf1r (IGF-1 receptor gene) exhibit growth deficiency and often die right after birth (J. P. Liu et al. 1993). In case of heterozygous knockout of IGF-1 receptor dwarfism is not present, and mice live on average 26% longer than their wild type littermates (Holzenberger et al. 2003). Homozygous mutants of insulin receptor develop severe hyperglycemia and die within 72 hours due to diabetic ketoacidosis (Accili et al. 1996).

Mammalian system is more complex than that of invertebrate models, with more tissue specific functions of genes that often affect the whole organism. Accordingly, tissue specific disruptions of insulin receptor produce different metabolic phenotypes and effects on lifespan. For instance, mice with muscle specific knockout of insulin receptor exhibit increased fat mass, serum triglycerides and free fatty acids, but normal blood glucose and glucose tolerance (Brüning et al. 1998), whereas mice with neuron specific knockout of insulin receptor (NIRKO mice) develop diet-sensitive obesity with increased body fat and decreased fertility (Brüning, Gautam, and Burks 2000). Liver specific insulin receptor knockout (LIRKO) mice show insulin resistance, glucose intolerance and an inability of insulin to regulate hepatic glucose production (Michael et al. 2000). Finally, mice with a

fat specific insulin receptor knockout (FIRKO mice) have reduced fat mass despite normal food intake, and have an increase in mean life-span of 18% (Blüher, Kahn, and Kahn 2003).

Studies in humans have shown that reduced IGF-1 signaling has predictive value for life expectancy, and is more often seen in longer-lived individuals (Bonafè et al. 2003; van Heemst et al. 2005; Milman et al. 2014). Research in Ashkenazi Jewish centenarians showed that an IGF1R gene polymorphism is associated with high serum IGFI levels, reduced activity of the IGFIR, and longer life (Suh et al. 2008). Finally, variations in the downstream transcription factor FOXO3A have been identified as associated with longevity in various populations by independent studies (Willcox et al. 2008; Flachsbart et al. 2009; Pawlikowska et al. 2009). Taken together, all these studies present evidence suggesting that IIS signaling is evolutionarily conserved, and that developing pharmacological interventions to improve insulin sensitivity or reduce levels of IGF-1 signaling has great potential for extending human healthspan.

1.2.3 TOR signaling

The story of mTOR (mechanistic target of rapamycin or mammalian target of rapamycin) began in the 1970s when soil samples from the Polynesian island of Rapa Nui were identified as having antifungal activity. The isolated compound was thus named rapamycin. It was widely studied as an immunosuppressant before its mechanism of action was discovered, and since then rapamycin has been approved for a variety of uses, including prevention of restenosis following angioplasty, and as a treatment for certain forms of cancer (S. C. Johnson, Rabinovitch, and Kaeberlein 2013). On the molecular level, rapamycin binds to a small protein FKBP12, which inhibits the kinase activity of the mTOR1 complex (Loewith et al. 2002). At the same time, long-term exposure to rapamycin also inhibits the assembly of the mTOR2 complex, which is believed to reduce the downstream Akt/PKB signaling (Sarbassov et al. 2006).

TOR is an evolutionarily conserved serine/threonine kinase that regulates cell growth, proliferation, and survival, in addition to playing a role in protein synthesis,

autophagy, and transcription (Wullschleger, Loewith, and Hall 2006). In mammals, mTOR is composed of two distinct complexes: mTOR1 and mTOR2, each having different subunit composition. The mTOR1 and mTOR2 complexes share the catalytic unit mTOR, its negative regulator DEPTOR (DEP-domain containing mTOR interacting protein), as well as mLST8 (mammalian lethal with Sec-13 protein 8) and TTI1/TEL2 complex (Jacinto et al. 2004; Kaizuka et al. 2010; Peterson et al. 2009). By themselves these complexes are composed of the following: mTOR1 contains RAPTOR (regulatory associated protein of target of rapamycin) and PRAS40 (proline-rich Akt substrate), whereas mTOR2 contains mSin1 (mammalian stress-activated map kinase interacting protein), RICTOR (rapamycin-insensitive companion of mTOR), and PROTOR (protein observed with rictor 1 and 2) (Hara et al. 2002; Jacinto et al. 2006; Sancak et al. 2007; Pearce et al. 2007).

mTOR is activated in nutrient- and energy-abundant conditions, integrating intra- and extracellular cues (such as amino acid levels, glucose, oxygen, and other growth factors including insulin/IGF-1) to stimulate growth and inhibit autophagy and other salvage pathways (S. C. Johnson, Rabinovitch, and Kaeberlein 2013; Wullschleger, Loewith, and Hall 2006). The major pathways downstream of mTOR1 include regulation of protein synthesis through activation of S6K (ribosomal S6 Kinase) and inhibition of 4E-BP1 (eukaryotic translation inhibition factor 4E binding protein); inhibition of autophagy through phosphorylation of ULK1; promotion of glycolysis through HIF1 (hypoxia-inducible factor), and upregulation of lipid biosynthesis through SREBP (sterol regulatory element binding protein) (Laplante and Sabatini 2012). mTOR2 regulates the cytoskeleton and also inhibits FOXO3A through AKT (Laplante and Sabatini 2012). Similarly to IIS, research in model organisms demonstrated that inhibition of TOR orthologs increases lifespan – replicative and chronological lifespan in yeast (Kaeberlein et al. 2005; Powers et al. 2006), as well as mean lifespan in worms (Vellai et al. 2003) and flies (Kapahi et al. 2004). As mentioned previously, one of the mTOR1 phosphorylation substrates is S6K, which in turn regulates a number of other substrates that modulate translation initiation and protein biosynthesis. Knockdown of rsks-1 (the *C. elegans* ortholog of S6K) extends mean lifespan by up to ~50%, and deletion mutants have reduced translation rate and longer lifespan (Hansen et al. 2007; Pan et al. 2007). In flies, overexpression of dominant-negative forms of dTOR or dS6K all lead to

considerable lifespan extension (Kapahi et al. 2004). Studies of S6K1 knockout in mice show that these animals have 9% longer mean lifespan as compared to their littermates (Selman et al. 2009). There are also multiple reports of mTOR signaling dysregulation implicated in a number of aging-associated diseases including diabetes, cancer, and neurodegeneration (Laplante and Sabatini 2012).

Rapamycin itself has been convincingly demonstrated to extend lifespan in multiple species ranging from yeast to mammals. Studies in yeast show increases in chronological and replicative lifespan already at low concentration of the compound (Powers et al. 2006; Medvedik et al. 2007). Relatively low concentrations of rapamycin extend lifespan in *C. elegans* by ~20% (Robida-Stubbs et al. 2012). Flies' lifespan extension has also been shown, with effect depending on the concentration of rapamycin and the diet used (Bjedov et al. 2010). There is multiple and solid evidence confirming that rapamycin extends lifespan in mice, including the studies performed through National Institute of Aging Interventions Testing Program (ITP). Genetically heterogeneous male and female mice fed with rapamycin at 20 months of age exhibited 9% and 13% increase in mean lifespan, respectively (Harrison et al. 2009). Feeding with rapamycin at earlier age increased median survival by 10% and 18% in males and females, respectively (Miller et al. 2011). Further research confirmed that the effect of rapamycin on lifespan is dose-dependent, with even higher concentration extending median lifespan by 23% and 26% in male and female mice, respectively (Miller et al. 2014). Finally, even transient rapamycin treatment of only 3 months at the age of 20 months increased lifespan of C57BL/6J mice by up to 60% and showed improvements in their muscle strength and motor coordination (Bitto et al. 2016). Rapamycin is an FDA approved drug used to prevent organ rejection in transplant recipients and known to delay the onset of many age-related conditions in mice. However, its administration to human patients, especially in high doses, results in undesirable side effects including poor wound healing, elevated blood cholesterol levels, reduced male fertility, and dermatological conditions such as edema and mouth ulcers (Mahé et al. 2005; Zuber et al. 2008; Johnston et al. 2008; McCormack et al. 2011). The metabolic side effects of chronic rapamycin administration, such as substantial impairment of glucose tolerance and insulin action, have been shown

to derive from disruption of mTOR2 and to be uncoupled from mTOR1 downregulation-induced longevity (Lamming et al. 2012).

There are multiple mechanisms by which rapamycin in particular and the inhibition of TOR in general extend lifespan, but the major effectors are hypothesized to be downstream of mTOR1. One potential important downstream mechanism of the mTOR1 axis is the control of autophagy whereby portions of cytoplasm are sequestered and digested in lysosomes. When nutrients are plentiful, mTOR1 is thought to inhibit autophagy, in mammals mainly by phosphorylation of Atg13 and ULK1/2 (Alers et al. 2012). It has generally been shown in different model organisms that inhibition of TOR by either starvation or rapamycin leads to upregulation of autophagy (Noda and Ohsumi 1998; Hansen et al. 2008; Tóth et al. 2008; Bjedov et al. 2010). Moreover, a number of other pathways implicated in lifespan extension converge on the activation of autophagy, and this is consistent with observations in different model systems that autophagy rates decline with age (Madeo, Tavernarakis, and Kroemer 2010).

1.2.4 Proteostasis

Now that metabolic pathways able to modulate the rate of aging are starting to be better understood, aging process can no longer be regarded simply as a stochastic, progressive decline. CR, IIS, TOR, and mitochondrial activity – all of these pathways have been implicated in lifespan extension in multiple species, and though genetic epistasis experiments show additive increases in lifespan when these different pathways are combined, it is tempting to hypothesize that their underlying downstream mechanisms might be similar: they may all converge on the way the cellular proteome is maintained.

A complex set of mechanisms such as regulators of translation, protein folding, trafficking and secretion, as well as protein degradation, prevents and eliminates proteome dysfunction in the cell (Balch and Morimoto 2008). However, cellular proteins still undergo misfolding and aggregation, and become dysfunctional through multiple ways including translational errors and stresses such as oxidation (carbonylation, oxidized methionine, and glycation) with age (Šoškić, Groebe, and Schrattenholz 2008).

This in turn may create increased demands on the cell's proteostasis machinery itself, creating a feedback loop and the phenotypes we ultimately observe as aging (Chiti et al. 2003; Stefani and Dobson 2003). In humans, for instance, protein misfolding and aggregation appears to be the defining feature of several neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease (Rubinsztein 2006). But even in non-diseased settings, protein aggregation still accumulates with age (David et al. 2010; Simonsen et al. 2008; Schöll et al. 2016). However, there is growing number of reports showing that aging pathways can directly modulate proteostasis to extend healthy lifespan using several mechanisms discussed in this chapter.

Firstly, reducing the rate of protein synthesis, a tightly regulated and highly energy consuming process, has been reported to extend lifespan during stress (Zid et al. 2009). Secondly, chaperone-mediated folding helps to mitigate protein unfolding in the cytosol, endoplasmic reticulum (ER), and mitochondria. Heat shock factor (HSF) is an important transcriptional hub that upregulates heat shock proteins (HSPs), proteases, co-chaperones, etc. in response to proteotoxic stresses (Vihervaara and Sistonen 2014). HSF1 and heat shock proteins' (HSPs) activity decreases during normal aging (Kayani, Morton, and McArdle 2008). Accordingly, overexpression of *C. elegans* ortholog of HSF1, *hsf-1* extends lifespan by 40%, while its mutations shorten lifespan and lead to phenotypes of accelerated aging (Hsu, Murphy, and Kenyon 2003; Garigan et al. 2002). Cellular compartments dealing with high flux, such as ER and mitochondria, developed their own stress response system termed UPR (unfolded protein response).

The two protein degradation pathways that remove and recycle permanently damaged proteins are ubiquitin-proteasome system (UPS) and the lysosome-mediated autophagy system. The UPS targets proteins tagged with a poly-ubiquitin chain for degradation in a large proteolytic complex called the proteasome. Multiple studies demonstrated positive association between proteasome activity and healthspan. Disruption of proteasome in *S. cerevisiae* reduces chronological lifespan; conversely, its overexpression extends lifespan (Q. Chen et al. 2004, 2006). Impairment of proteasome subunits severely shortens lifespan of C. elegans, while overexpression of pbs-5 (responsible for chymotrypsin like activity) extends lifespan by 25% (Niki Chondrogianni et al. 2015). Overexpression of another component of the proteasome, Rpn11, extends

lifespan of flies by 35% (Tonoki et al. 2009). In mammals, naked mole rats increase their proteasome activity rates 1.5 fold during aging, which may partly explain their lifespan of 25~28 years (Pe et al. 2009). Centenarians also show proteasome activity more similar to the younger than the older control donors, further confirming its importance for healthy aging (N. Chondrogianni et al. 2000).

The second mechanism of clearing damaged proteins is called autophagy: it is a process of cellular “eating” when cellular components are surrounded with a membrane and targeted to lysosomes for proteolytic breakdown (Ciechanover 2005). Inhibition of autophagy has been shown to shorten lifespan in several model organisms, and to lead to aging-associated phenotypes including elevated inflammation, neurodegeneration, and sarcopenia in mice (Rubinsztein, Mariño, and Kroemer 2011). Conversely, autophagy activation increases lifespan in flies and mice (Simonsen et al. 2008; Zheng et al. 2010; Pyo et al. 2013). Finally, as already mentioned in the previous sub-sections of this chapter, the most well-studies aging pathways such as CR, IIS, and TOR depend on activated autophagy for lifespan extension.

1.2.5 AMP-activated protein kinase / metformin

AMP-activated protein kinase (AMPK) is a well-conserved cellular energy sensor (Hardie and Hawley 2001). In low energy conditions AMPK restores energy homeostasis by activating catabolic processes and inhibiting energy-demanding processes, which results in increase in energy production and a coordinated decrease in ATP usage (Hardie, Ross, and Hawley 2012). It has been established that the most well-studied longevity pathways depend on the activity of AMPK (Burkewitz, Zhang, and Mair 2014). For instance, CRTC (cAMP response element binding protein-regulating transcriptional coactivator) requires AMPK activation for lifespan extension (Mair et al. 2011); AAK-2 (*C. elegans* ortholog of AMPK α subunit) is required for the longevity effects mediated by reduced IIS, and, conversely, DAF-16 is necessary for the longevity mediated by activated AMPK, suggesting a feedback loop (Apfeld et al. 2004). This connection is consistent with evolutionary perspective: given the conservation of both AMPK and the

CR response in times of nutrient scarcity, AMPK, with its ability to integrate multiple signaling and transcriptional pathways known to promote longevity, is a perfect candidate for the conserved mechanistic link between energetics and longevity.

Activating or inhibiting AMPK is sufficient to extend or shorten lifespan in model organisms. For instance, the loss of *aak-2* protein decreases lifespan of worms by 12%, and the elevated expression of *aak-2* increases lifespan (Apfeld et al. 2004). It is possible to directly or indirectly activate AMPK by a surprisingly large array of small molecules, including metformin, resveratrol, rapamycin, aspirin, etc. Metformin, one of the most widely prescribed treatments for type II diabetes, is an indirect AMPK agonist, and it also extends lifespan in C. elegans and mice (Onken and Driscoll 2010; Cabreiro et al. 2013; Anisimov et al. 2008). Long-term use of metformin has been associated with fewer incidences of cancer and cardiovascular diseases, and with improved insulin sensitivity (Barzilai et al. 2016).

Another well studied mechanism of longevity regulation also dependent on the activity of *aak-2* is the increased lifespan of nematodes overexpressing *sir-2.1* (Curtis, O'Connor, and DiStefano 2006); as both AMPK and sirtuins are cellular energy sensors, it is tempting to speculate that these factors could interact with each other during their pro-longevity actions.

1.2.6 Sirtuins

Sirtuins are a conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent silent information regulator 2 (Sir2) deacylases that initially attracted attention of scientists because their activity was implicated in lifespan-extending effects of dietary restriction (Wątroba and Szukiewicz 2016). The enzymatic activity of sirtuins requires cleavage of NAD+ at the glycosidic bond between nicotinamide and ADP-ribose, and serves as a molecular link between the energetic state of the cell and its energy metabolism pathways' downstream effectors (Shinichiro Imai and Guarente 2014). Sirtuins were shown to be required for the lifespan extension through moderate CR (0.5% glucose in yeast), but not necessary for the longevity induced by the severe CR (0.05%

glucose) (S.-J. Lin, Defossez, and Guarente 2000; Kaeberlein et al. 2005). Overexpression of sirtuins extends replicative lifespan in *S. cerevisiae* (Kennedy et al. 1995; Wierman and Smith 2014). SIR-2 homolog overexpression leads to increase in lifespan in *C. elegans* and *D. melanogaster* (Tissenbaum and Guarente 2001; Rogina and Helfand 2004). An important study from 2011 brought a lot of controversy as it questioned the role of sirtuins in aging by attributing the original results in the previous reports to the use of inappropriate controls and confounding genetic background (Burnett et al. 2011). It was later shown, however, that extension of lifespan upon overexpression of *sir-2.1* in worms does occur albeit to a much lesser extent (Viswanathan and Guarente 2011). Subsequent research in other species and laboratories further validated relevance of sirtuins in longevity and healthy aging (H. C. Chang and Guarente 2014).

In mammals there are seven sirtuins with different cellular localizations and functions. The most studied one is the closest homolog of the originally discovered sir2, SIRT1, whose main functions are mediating increased mitochondrial biogenesis, catabolism of triglycerides, regulation of gluconeogenesis etc. through deacetylating histones and such important cellular proteins as p53, PGC-1α, and FOXO (Wątroba and Szukiewicz 2016). Although whole organism overexpression of SIRT1 does not significantly extend lifespan in mice, it leads to better glucose homeostasis, reduced DNA damage, lower incidences of cancer, and delayed bone loss (Bordone et al. 2007; Banks et al. 2008; Herranz et al. 2010). At the same time, brain specific SIRT1 overexpression delays age-associated morbidities and extends median lifespan by 16% in females, and 9% in males, respectively, implicating sirtuins in the communication between the hypothalamus (as the signaling control center of aging) and the periphery (Satoh et al. 2013; Shin-ichiro Imai and Guarente 2016).

Similar to the search for compounds boosting AMPK, several screens to identify small molecules activating sirtuins have been performed, and one of the most widely known is a polyphenol called resveratrol. This compound administration leads to lifespan extension in a number of model organisms including *C. elegans*, *D. melanogaster*, and *N. furzeri* (Bhullar and Hubbard 2015). Although it has been shown to not extend lifespan in mice fed with standard chow, it rescues lifespan reduction mediated by high-fat diet (Baur et al. 2006; Pearson et al. 2008; Miller et al. 2011; Strong et al. 2013). Finally,

administration of resveratrol in humans was shown to improve cardiac function and glucose metabolism, and to delay cognitive decline (Bonkowski and Sinclair 2016).

NAD+ levels decrease with age in multiple organisms; at the same time, it is known that administration of a precursor of NAD+, nicotinic acid, improves cholesterol, blood lipid profiles, and is used for cardiovascular risk prevention (Lavigne and Karas 2013). As the activities of sirtuins are tightly linked to NAD+, a potentially promising approach to improve healthspan is to increase cellular NAD+ by boosting its precursors such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) (Cantó et al. 2012; Mouchiroud et al. 2013).

Much remains to be investigated concerning the mechanisms of sirtuin action in longevity, but the connection is not surprising: downstream targets of sirtuins include transcription factors and cofactors that control a number of important physiological pathways in mammals. In the context of energy metabolism, one particular connection deserves to be addressed: the link to mitochondria. Decreased SIRT1 activity reduces the functions of PGC-1α and FOXO1, inhibiting mitochondrial biogenesis, oxidative metabolism, and anti-oxidant defense pathways (Brunet et al. 2004; Rodgers et al. 2005). Moreover, it has been shown that sirtuin activities are involved in the mitochondrial unfolded protein response (UPRmt) pathway and mitophagy (Mouchiroud et al. 2013; Papa and Germain 2014). SIRT3 was implicated in UPRmt, and it was demonstrated that mitochondrial proteotoxic stress increases SIRT3 protein levels, activating mitophagy and anti-oxidant response (Papa and Germain 2014). It can tempting to speculate that disturbing the tight connection between NAD+ and sirtuins contributes to serious mitochondrial dysfunction, and consequently to the phenotypes of aging.

1.2.7 Mitochondria / ROS

Mitochondria have been shown to participate in all main aspects of aging: metabolic dysregulation, decline of stem cell functions, the so-called “inflammaging”(chronic, sterile, low-grade inflammation developing during aging), cellular senescence, etc., and mitochondrial function itself declines with age (Franceschi et al. 2018; Bratic and

Larsson 2013; Rando 2013; Theurey and Pizzo 2018; Srivastava 2017). At the same time, moderate inhibition of mitochondrial function in laboratory settings leads to lifespan extension in different model organisms including yeast, worms, flies, and mice (Kirchman et al. 1999; Feng, Bussière, and Hekimi 2001; S. S. Lee et al. 2003; Copeland et al. 2009; W. Yang and Hekimi 2010b). This inverse correlation of mitochondrial activity and lifespan is thought to function by triggering compensatory mechanisms related to ROS signaling and the mitochondrial unfolded protein response (UPR^{mt}) (Durieux, Wolff, and Dillin 2011; Yee, Yang, and Hekimi 2014). We can no longer view mitochondria as simple bioenergetics powerhouses of the cell, given all the evidence of their role in intracellular signaling, innate immunity, as well as regulating stem cell activity (N. Sun, Youle, and Finkel 2016). Moreover, the mitochondrial free radical theory of aging (MFTRA), stating that accumulation of cellular damage with increasing age results from the mitochondrial ROS, needs to be re-evaluated due to the overwhelming evidence of oxidative stress not being the primary or initial cause, but rather a consequence of aging (Srivastava 2017).

Many studies examined perturbations of superoxide dismutase (SOD) enzymes, the cells' internal defense against ROS. Inactivation of cytoplasmic and mitochondrial SOD shortens lifespan in yeast, fruit flies, and mice, but not worms (Longo, Gralla, and Valentine 1996; Phillips et al. 1989; Kirby et al. 2002; Duttaroy et al. 2003; Lebovitz et al. 1996; Elchuri et al. 2005; Wawryn et al. 1999; Unlu and Koc 2007; Doonan et al. 2008; W. Yang, Li, and Hekimi 2007; Van Raamsdonk and Hekimi 2009), whereas SOD overexpression extends lifespan only in flies (W C Orr and Sohal 1994; J. Sun and Tower 1999; J. Sun et al. 2002; William C. Orr et al. 2003). In mice there is no difference in lifespan with overexpression of any of the antioxidant enzymes applied (T.-T. Huang et al. 2000; Pérez, Van Remmen, et al. 2009; Jang et al. 2009; Pérez, Bokov, et al. 2009). In humans, antioxidants treatment had either no effect or even detrimental effect on health and disease susceptibility (Bjelakovic et al. 2007; Davies et al. 1982; Gomez-Cabrera et al. 2008). Conversely, ROS produced by reverse electron transport extends lifespan in flies, and mice heterozygous for cofactor ubiquinone Mclk+/- have increased levels of ROS and live longer than control animals (X. Liu et al. 2005; Lapointe and Hekimi 2008; Lapointe et al. 2009; Scialò et al. 2016). Mitochondria are the main source of ROS in the

cell, and oxidative damage does increase with age, but ROS have important signaling functions, and the underlying causes of aging appear to be upstream of oxidative stress.

There are, however, several other mechanisms of mitochondrial contribution to age-related physiological decline. One of such mechanisms is mitochondria-derived damage-associated molecular patterns (DAMPs), such as free mtDNA, which leave mitochondria and can induce inflammatory response leading to chronic inflammatory state termed “inflammaging” (Shimada et al. 2012; Oka et al. 2012; White et al. 2014). Another main candidate for a driver of aging is the link between mitochondrial decline and somatic stem cells (SSCs) dysfunction described in many tissues as part of the aging process (N. Sun, Youle, and Finkel 2016; Rossi et al. 2007; Conboy and Rando 2012; Carlson et al. 2009). Connecting the two is the fact that the SSCs decline is relying on point mutation accumulation in their mtDNA (Ahlqvist et al. 2012). This is consistent with the observation that mitochondria contribute to the metabolic control of stem cells pluripotency through specific mitochondrial metabolites, such as α-ketoglurate and NAD+ (Carey et al. 2015). Stem cells have been shown to possess a unique capacity to specifically segregate and recycle old mitochondria through increased mitophagy (N. Sun et al. 2015; Katajisto et al. 2015).

Generally, mitochondrial signaling pathways mediating longevity are highly interconnected and cross-regulated, making a complex network of regulators with multiple common targets often difficult to separate (Domise and Vingtdeux 2016; N. Sun, Youle, and Finkel 2016). For example, as already described above, DR has been shown to extend organismal lifespan and alleviate oxidative stress by modulating IIS, TOR, AMPK, and sirtuins signaling pathways (C. J. Kenyon 2010). Besides, for its lifespan extension effects DR requires autophagy that is also regulated by FOXO, TOR, AMPK, and SIRT1 signaling pathways (Martina et al. 2012; Lapierre et al. 2015; Zhang and Lin 2016).

In addition, mitochondria can affect other aging-related cellular phenomena, such as senescence. Just as SSC differentiation, senescence is characterized by profound metabolic changes, and induction or repression of senescence requires metabolic regulation often modulated through mitochondria (Quijano et al. 2012).

1.2.8 Cellular senescence

Cellular senescence plays physiological roles during normal organismal development, and it is necessary for tissue homeostasis; however, it also constitutes a stress response to the insults associated with aging (e.g. genomic instability and telomere attrition), and as such contributes to physiological dysfunction in old age (McHugh and Gil 2018). First identified by Hayflick and Moorhead in 1961 (L. Hayflick and Moorhead 1961), senescence is described as a multi-stage cellular program that induces a stable growth arrest accompanied by metabolic reprogramming, chromatin remodeling, increased autophagy, and secretion of pro-inflammatory factors termed as Senescence-Associated Secretory Phenotype (SASP) (McHugh and Gil 2018). The defining characteristic feature of senescence is the irreversible growth arrest, which is triggered by various stresses such as DNA damage, telomere attrition, mitochondrial dysfunction, and activation of oncogenes, and plays a role of a powerful mechanism of tumor suppression (Collado, Blasco, and Serrano 2007; Hanahan and Weinberg 2011; Campisi 2013). Senescence is implemented by the activation of $p16^{INK4a}$/Rb and p53/$p21^{CIP1}$ tumor suppressor networks, but has also physiological roles in wound healing and during normal development, cooperating with apoptosis to facilitate embryonic morphogenesis (Demaria et al. 2014; Muñoz-Espín et al. 2013; Storer et al. 2013).

Despite these physiological roles and its requirement for tissue homeostasis, senescence represents a stress response triggered by insults associated with aging (e.g. genomic instability or telomere attrition). Moreover, senescent cells themselves have decreased mitophagy, resulting in an “old,” defective mitochondrial network that also contributes to metabolic dysfunction in aging tissues (N. Sun, Youle, and Finkel 2016). It is known that senescent cells accumulate during normal aging in most tissues, and even postmitotic cells exhibit features of senescence such as SASP (Sapieha and Mallette 2018). Multiple reports link cellular senescence to aging and suggest that removal of these cells may postpone and alleviate aging-associated diseases (Soto-Gamez and Demaria 2017). Accordingly, targeted elimination of $p16^{INK4a}$-positive senescent cells through INK-ATTAC apoptosis system attenuated aging-associated phenotypes in skeletal muscle and adipose tissue in BubR1 hypomorphic progeroid model, albeit without

altering lifespan (Baker et al. 2008, Baker et al. 2011). The same method of senescent cells clearance in wild type mice showed lifespan extension between 17% and 35% in both sexes (Baker et al. 2016). Other reports with different methods of senescent cells clearance presented more evidence that reducing number of senescent cells leads to improvement in fitness and kidney function, and eventually creates a pro-regenerative environment alleviating such aging-associated diseases as osteoarthritis, pulmonary fibrosis, and atherosclerosis in mice (Childs et al. 2016; Baar et al. 2017; Jeon et al. 2017; Schafer et al. 2017).

Removal of senescent cells represents an attractive therapeutic avenue, and there is a new class of small drugs called senolytics being developed and tested to alleviate aging-related disease by selectively killing senescent cells (Zhu et al. 2015; Yosef et al. 2016; J. Chang et al. 2016). However, there still remain many potential problems to solve: for instance, senescent cell clearance in the mouse may not be a reliable model for recapitulating the physiological effects of clearance of senescent cells in humans. Removal of high number of senescent cells could have dangerous outcomes to human health by triggering atrophy and tissue dysfunction. On the other hand, targeting only $p16^{Ink4a}$-positive cells for elimination may not be sufficient as these do not represent all senescent cells population in the body (van Deursen 2014). Besides senolytics, a potentially promising bio-therapeutic approach would be to activate or reinforce the existing immune response normally clearing senescent cells but declining with age.

1.2.9 Biomarkers of aging

A major goal of aging research since the beginning of molecular studies in the field has been to identify biomarkers of aging which could be used to evaluate either genetic features influencing the rate of aging, or environmental factors accelerating it, such as exposure to toxins or lifestyle choices. It is useful to differentiate between types of biomarkers of aging: there are those that report on chronological age, and those that report on biological age, when “normal” for a defined species rate of aging is sped up, or slowed down. In contrast to biomarkers of chronological age which give quantitative

measure of age in units of time, biomarkers of biological age use units of the physiological progression from young to old to estimate age of an individual. As we are yet to define exactly what it entails to be physiologically young or old, however, the quest to identify biomarkers of biological age has so far been largely unsuccessful (Pitt and Kaeberlein 2015). A reliable biomarker of aging would greatly facilitate the research in development and translation of therapies to slow aging.

One frequently mentioned candidate for a biomarker of biological age is telomere length. Telomeres become shorter with age in human cells, and some models of aging suggest that telomere shortening is actually the driver of age-associated cellular senescence and physiological decline. Shortening of telomeres in leukocytes has been associated with chronological age, environmental influence such as stress and poor diet, with disease, and with all-cause mortality; however, limitations of telomere length measurement methods, and conflicting data from epidemiological studies revealed insufficient utility of telomere length as a reliable biomarker of biological age (Sanders and Newman 2013).

Another potential biomarker of aging emerged more recently and is so far proving to be remarkably robust, DNA methylation. Patterns of DNA methylation predict chronological age in any tissue across the entire life course. Some reports suggest that DNA methylation in blood can even be used to predict all-cause mortality after controlling for other risk factors (Marioni et al. 2015). The ‘epigenetic clocks’ of DNA methylation provide a link between developmental and maintenance processes, and biological aging, potentially establishing a unified theory of life course (Horvath and Raj 2018). As these measurements are sufficiently sensitive, they provide a useful approach to quantify not only chronological, but also biological age, and to test the effects of interventions meant to delay aging (Khan, Singer, and Vaughan 2017). It is important to remember, however, that some species have extremely little (e.g. *D. melanogaster* and *C. elegans*) or no (some yeast species) DNA methylation. Therefore, this epigenetic modification should strictly be considered a biomarker, as so far no causal relationship with the process of aging has been established.

1.3 Evolutionary theories of aging

Classical theories of aging have been mainly developed based on the data from short-lived species with increasing mortality and decreasing fertility after maturity; however, it is important to recognize great variation of aging patterns between species (Jones et al. 2014). Indeed, while many species undergo aging, some appear to show negligible or even negative senescence (i.e., no signs of physiological decline or even some physiological improvement with age) (Finch 2009; Vaupel et al. 2004). Many plants (over 90% of angiosperms), for instance, show no signs of aging (Baudisch et al. 2013). Aging, therefore, is not universal.

There is a large number of often vastly differing and mutually conflicting theories of aging which all attempt to provide a general theoretical framework that could accommodate available data on aging phenotypes and their underlying genetic backgrounds. Some of the most prominent examples include Wear and tear theory (first proposed by a German biologist Dr. August Weismann in 1882), Mutation accumulation theory (first proposed by Medawar in 1952), Free radicals theory (Harman 1956), Antagonistic pleiotropy theory (Williams 1957), Disposable soma theory (T. Kirkwood 1977), Rate of living theory (Brys, Vanfleteren, and Braeckman 2007), and Endocrine theory (Van Heemst 2010).

While nearly all theories of aging agree with the fact that the way aging is manifested is the accumulation of molecular damage inflicted mainly by ROS, as well as the negative impact of amyloid protein and glycation end-products, the most important point of disagreement comes from the explanation of evolutionary context of aging, namely whether aging is an evolved mechanism (so-called Programmed theories of aging, or just PA (Programmed aging)) or whether it is simply the result of entropy which inevitably destroys all non-living things with time (Non-programmed theories of aging, or NPA) (Jin 2010).

The proponents of Programmed aging have often attempted to frame the processes of aging within evolutionary constraints imposed by selective pressures. Evolutionary theorists speculated that aging may be genetically programmed, arguing that elimination of individuals after their peak of reproduction would be beneficial for the

species, as it would theoretically save resources for the more reproductively fit individuals and the young. The most compelling line of evidence is probably the notion that differences of aging rates within individuals of a species are incomparable with the enormous differences across species (Hayflick 1998). While a mayfly grows, reproduces and dies within a single day, some clones of grasses have been estimated to be 15,000 years old (Flatt and Partridge 2018). In addition, there are great differences between lifespans of similarly sized animals (e.g. mouse *M. musculus* vs. Brandt's bat or naked mole rat *H. glaber,* with difference of over 10 fold), which are unlikely in the otherwise similar background of stochastic assaults; and abrupt senescence of certain species (such as salmon and bamboo) that is impossible to explain by usual effects of entropy (Sergiev, Dontsova, and Berezkin 2015). On top of that there is overwhelming evidence of strong genetic influences on aging rate reflected by the ever increasing number of single-gene mutations found to affect the lifespan of eukaryotes ranging from yeast to mice (Friedman and Johnson 1987; C. Kenyon et al. 1993; Tissenbaum and Guarente 2001; Clancy et al. 2001; Tatar et al. 2001; Flurkey et al. 2001; Ikeno et al. 2003).

Critics of Programmed theories of aging note that aging occurs mainly after the peak of reproduction, and the progeny on which selection can act gets more and more scarce with the advancing age of individuals (Hayflick 2007). Even if group selection is taken into account, it is hard to imagine how the pro-aging genes would be selected for, given that individual-level selection is normally much stronger than group selection (Smith 1976). One of the most vocal critics of Programmed aging, and the founder of Disposable soma theory, Thomas B. L. Kirkwood, together with Axel Kowald ran computer simulations of some evolutionary models of Programmed aging and did not arrive at the same conclusions as the models' authors (Kowald and Kirkwood 2016).

At the same time, as more data enter the research field, the rift between the two camps appears to be closing with some new theories that attempt to provide a comprehensive view which would accommodate the seeming contradictions. At least two theories have emerged that appear to integrate the existence of genetic programs directing the process of aging with the notion that pressures of natural selection were likely not the main reason such programs have arisen (T. B. L. Kirkwood 2005). The first theory is the Medawar's Mutation accumulation theory which states that mutations leading

to age-related physiological decline could theoretically accumulate over successive generations provided that their effects would manifest a long time after the peak of reproduction. As such ages would seldom be actually reached, these mutations could escape negative selective pressure. The second is called Antagonistic pleiotropy theory (Williams 1957). It proposes that there may be genes whose expression is detrimental in old age but which still accumulate in populations because they are selected for their beneficial effects during growth and reproduction.

A more generalizable theory considering the influences of genetics on the process of aging is the Disposable soma theory (T. Kirkwood 1977) which postulates that the distinction between the immortal germ line and the mortal somatic cells is key, and that species have evolved to optimize the use of resources to survive and reproduce. As normal aging proceeds through entropy, genetic programs of any particular species protect from this damage just long enough for individuals to reach reproductive maturity. Consequently, in case of higher environmental stress those limited resources would be diverted from reproduction into somatic maintenance. This principle is generally consistent with the well-studied negative correlation between lifespan and fecundity (Partridge, Gems, and Withers 2005). The most prominent case of 'uncoupling' of the fecundity–longevity trade-off is reported in eusocial animals. For example, in naked mole rats queens and workers have almost the same lifespans even though queens can give birth to over 900 pups while workers do not reproduce (Flatt and Partridge 2018).

Finally, the Developmental theory of aging states that, though not a program per se, aging is simply an environmentally influenced continuation of the real, developmental program, and can thus be called a "quasi-program or, figuratively, a shadow of actual programs" (Blagosklonny 2013). This new view explicitly agrees with Non-programmed aging proponents that evolutionary pressure is unlikely to select for pro-aging genes. It departs, however, from the strictly stochastic explanation of the aging mechanisms, and acknowledges that the way a species will age strongly depends on its developmental program encoded by the genome of this species, and thus contradicts the main NPA tenet that science should focus on the ways to study and alleviate the symptoms of the inevitable decline instead of researching the underlying mechanisms of aging-related phenotypes (de Grey 2015).

1.4 Research limitations and possible approaches

Model organisms like yeast, fruit flies and nematodes present a convenient system to study aging. However, this approach has its limitations. First of all, there is considerable risk that pathways identified in simpler organisms are not conserved in mammals. Secondly, even when such pathways are identified, interventions in most cases are limited to boosting up or inhibiting actions of a single gene, which will rarely produce profound changes in a complex mammalian system with thousands of genes interconnected in an intricate network. At the same time, certain gene products (e.g., *daf-16*/FOXO) do have a broad range of activity and influence thousands of downstream effectors (Murphy et al. 2003). Such cases help understand general principles of aging-related mechanisms. We now understand, for instance, that there is inverse correlation between developmental and physiological mechanisms affecting growth and aging. Pathways connecting growth hormone, somatic growth, adult body size, and lifespan involve target of rapamycin (and in particular mTORC1), energy metabolism, insulin signaling, as well as changes in mitochondrial function, thermogenesis, and inflammation (Bartke 2017). NPA theories maintain that although such mechanisms affecting longevity are tightly regulated, they do not amount to aging programs, but merely supply adjustment mechanisms that add flexibility in times of stress (Kirkwood and Melov 2011). Within this trade-off framework lies inverse correlation of body size and longevity within a species (Calder 1984; Schmidt-Nielsen 1984; Promislow 1993; Austad 2005). However, positive correlation of body size and longevity among different species suggests that different mechanisms regulate a species' lifespan. In addition, independently of body mass, age at sexual maturity strongly correlates with average and maximum adult lifespan in many taxa, and among them, in mammals (Charnov 1990; Prothero 1993; Magalhaes, Costa, and Church 2007), which further hints at the notion that developmental patterns strongly influence aging rates. In light of these considerations, regulation of development in its relation to metabolism and aging phenotypes at maturity appears to present a promising venue of scientific enquiry.

1.5 The approach and aim of this study

In recent years, more and more powerful techniques in comparative genomics have become available due to the declining costs of DNA sequencing (de Magalhães, Finch, and Janssens 2010). This shift in experimental approaches towards systems biology led to multiple sequencing-based studies, including Genome-wide association studies (GWAS). While success in finding pro-longevity genes using such systems approach has been limited (e.g. in a GWAS of centenarians vs. younger controls, only one gene, APOE, has been found to achieve genome-wide significance (Brooks-Wilson 2013)), such slow speed of progress is understandable given that aging-associated phenotypes of physiological decline are among the most complex processes we have ever studied. At the same time, in a study which assumed a different approach allowing for more complexity, where the aging-related phenotypes were taken as associated with a complex multi-unit regulatory network, 62 correlated SNPs have been found likely associated with biological aging (Kulminski and Culminskaya 2013). It is thus tempting to speculate that while systems biology approach in general may become the most successful avenue of future aging research, simplistic one-gene view of possible treatments of aging-associated physiological decline must be overcome, and computer-assisted analyses and simulations may prove to yield better results.

In light of all the above mentioned considerations, I propose as the aim of this study to combine big data comparative genomics approach with the idea of evolutionarily conserved transcriptional regulators, which may, through a “quasi-programmed” pattern of “leftover” regulation, continue to function well after the age of reproductive prime, and contribute to the aging phenotypes, and ultimately death. We use a bioinformatics pipeline to analyze RNA deep sequencing data acquired through collaboration with the JenAge Consortium and derived from 3 species (nematode *C. elegans*, zebrafish *D. rerio*, and mouse *M. musculus*) at three time-points in their lifetime (Mansfeld et al. 2015), and to select transcription factors that likely regulate or contribute to the progression of age-associated decline, with the hope that better understanding of evolutionarily conserved transcriptomic modifiers of aging may help develop future treatments to postpone aging-related morbidity, and to increase human healthspan.

2 Results

2.1 Identification and validation of transcription factors that regulate genes differentially expressed with aging

2.1.1 JenAge study data was used as input for our bioinformatics pipeline

The initial dataset used in this research comes from a collaborative study with the JenAge Consortium (Hühne, Thalheim, and Sühnel 2014),(http://www.jenage.de/), and involves comparing gene expression patterns in three different well-established model organisms (the invertebrate nematode *C. elegans*, the vertebrate zebrafish *D. rerio*, and the mammalian mouse *M. musculus*, strain C57BL/6J), at three different time points roughly corresponding to young reproductive, mature, and old ages of these animals (Mansfeld et al. 2015). The aim of the study was to identify genes similarly regulated with age on a transcriptional level across evolutionarily distinct species, and try to identify the conserved mechanisms underlying physiological aging. A study with an almost identical approach (Comparing genomic expression patterns across species identifies shared transcriptional profile in aging) but with the use of a different technology has been done previously, and in that research a need for further examination using more data was emphasized. In short, the following methods were used. For RNA extraction *C. elegans* Bristol N2 pellets of approximately 2000 animals, as well as skin samples from individual zebrafish and mice were obtained. These RNA samples were quality checked and subjected to Illumina next-generation sequencing (RNA-seq), with 13–82 million reads per individual sample. As the final goal of the study was to get insight into the possible evolutionarily conserved mechanisms of aging regulation, only genes for which orthologs could be identified in all three species (*C. elegans*: 4,850; *D. rerio*: 6,064; *M. musculus*: 5,904) were considered as potential candidates and thus used in the data analysis. Differential gene expression analysis tools used to identify differentially expressed genes (DEGs) were edgeR and DESeq, and only the overlap DEGs with transcript levels showing statistically significant differences given by both programs were regarded as

such (DEG; *C. elegans*: 3608; *D. rerio*: 1721; *M. musculus*: 339). On a side note, DESeq has since been replaced by DESeq2 which gives fewer false positive results (Love, Huber, and Anders 2014), but it hadn't been available at the time, and later the results haven't been recalculated. The expression profiles of the entire set of DEGs were clustered into the optimal 6 patterns of genes that showed global upregulation (early or late), downregulation (early or late), or mixed (up- then down-regulation, and vice versa) with aging (I later also analyze these 6 patterns of changes in expression separately as 6 clusters). When overlapped, overall 13 upregulated and 16 downregulated genes were identified as consistently regulated during aging in all three species.

2.1.2 RSAT analysis gave a list of candidate aging-related genes to be further validated using lifespan assay

To isolate the transcriptional regulators responsible for the aging related patterns of gene expression identified in the JenAge study described above we have performed *in silico* RSAT (Regulatory Sequence Analysis Tools) promoter analyses (Turatsinze et al. 2008), separately for each species. In short, promoter regions of the DEGs in each of the 6 clusters (6 patterns of gene expression change with age described above) have been scanned using PWMs (Position Weight Matrices: motif sequences with calculated probabilities of each base for every position in a transcription factor binding site) from Transfac and JASPAR databases (Matys et al. 2006; Sandelin 2004; Mathelier et al. 2014) for possible transcription factor binding sites, and those transcription factors with the maximum number of predicted binding sites were scored the highest. The PWMs exist for nematodes and vertebrate species separately due to evolutionary distance, so the set of the 6 clusters of *C. elegans* DEGs (3608) was scanned with the nematodal set of PWMs, while the *D. rerio* DEGs (1721) and *M. musculus* DEGs (339) were scanned with the vertebrate set of PWMs, and the resulting TFs with orthologs were separately assessed for the upregulated and downregulated clusters based on the highest score from all three species combined (see the complete description of the procedure in Materials and Methods, and the tables with calculations in Appendix)(Table 1).

Downregulated DEGs		Upregulated DEGs	
Nematode PWM	Corresponding vertebrate PWM	Nematode PWM	Corresponding vertebrate PWM
EFL-1	E2F4	*EFL-1*	E2F4
mdl-1:*mxl-1*	MAX	*hlh-15*:*hlh-2*	NHLH1/:TCF3
mxl-3	MAX	*hlh-11*	TFAP2A
hlh-15:*hlh-2*	NHLH1/:TCF3	*BLMP-1*	PRDM1
hlh-11	TFAP2A	*Pha-4*	FOXA
BLMP-1	PRDM1	*mdl-1*:*mxl-1*	MAX
grh-1	GRHL1	*mxl-3*	MAX
Pha-4	FOXA	*grh-1*	GRHL1
lin-32:*hlh-2*	ATOH1	*SKN-1*	NFE2L2
SKN-1	NFE2L2	*DAF-16*	FOXO3
DAF-16	FOXO3	*lin-32*:*hlh-2*	ATOH1
DAF-12	VDR	*ceh-22*	NKX2-5.v2
ceh-22	NKX2-5.v2	*ces-2*	HLF
ces-2	HLF	*DAF-12*	VDR

Table 1: Top results of the RSAT analyses. Results from the clusters of differentially up- and downregulated genes during aging have been pooled together to produce this list. Visualized in the list are names of nematodal position weight matrices (PWMs), together with the corresponding vertebrate PWMs (in vertebrates no heterodimers are present).

PWMs of binding site motifs are not always represented by a single gene: many transcription factors perform their function only when bound to another protein or a group of proteins, or both alone/as a homodimer and as a heterodimer. We have thus selected more transcription factors for the screen than there were PWMs, 16 instead of 14. To keep the cross-species character of the study, the TFs without the corresponding orthologs in *D. rerio* or *M. Musculus* were not considered in the analysis. The remaining TFs were chosen for a lifespan screen using *C. elegans* nematodes.

It is important to note that after the lifespan screen had been completed the new vastly expanded set of nematodal PWMs was published, bringing the total number of PWMs we could use in our analysis from 39 to 324, or almost half of the estimated 763 sequence-specific transcription factors in *C. elegans* (Narasimhan et al. 2015). Although the present study had already been underway and we use the results of the original screen in the rest of this research, we did perform the RSAT analysis on the available DEGs' promoters using the updated matrices as well (Table 2), and the full tables with the results can be found in the Appendix. In this analysis PWMs were analyzed for each one of the 6 clusters of up- and downregulated differentially regulated genes separately. It is potentially a promising direction of further research to consider the top candidates of the updated tables for up- and downregulated DEGs. Although not the top TFs, the genes researched in this study are still among the top 10% in the updated tables, and therefore obtained results can be considered relevant and potentially valuable.

Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
lsy-2	lsy-2	lsy-2	EOR-1	klf-1	lsy-2
klf-1	lsl-1	mxl-3	klf-1	EOR-1	ceh-28
lsl-1	syd-9	lsl-1	lsy-2	sptf-3	lsl-1
EOR-1	ceh-28	mdl-1(ce):mxl-1(ce)	sptf-3	ZK337.2	EOR-1
sptf-3	EOR-1	EFL-1	lsl-1	lsy-2	syd-9
klf-2	mxl-3	DAF-12	klf-2	ZC328.2	klf-1
klf-3	klf-2	ref-1(ce)	klf-3	klf-2	klf-2
ceh-28	C27D6.4	syd-9	ZK337.2	zip-3	C27D6.4
ref-1(ce)	EFL-1	hlh-15(ce):hlh-2(ce)	egrh-3	Y53H1A.2	mxl-3
syd-9	efl-2	ceh-28	hlh-15(ce):hlh-2(ce)	gei-11	EFL-1

Table 2: Top 10 results of the RSAT analyses. All 6 clusters of differentially up- and downregulated genes during aging have been used separately for the RSAT screen. The new set of this round of RSAT analyses included 324 nematodal PWMs.

2.1.3 *C. elegans* as the optimal model for a longevity screen

The first studies utilizing *C. elegans* for research in longevity were done by Michael R. Klass, and he has identified the first five mutants affecting lifespan (Klass 1983). The advantages of using worms in this field of study include the ability to relatively easily and cheaply culture large quantities of worms in the lab, and their short and mostly invariant lifespan (average lifespan is approximately 17 days at 20 °C), which allows for identification of interventions and mutants that statistically significantly alter lifespan by only 10~15% or less (Tissenbaum 2015). Besides, *C. elegans* has well annotated genome, as well as an RNAi library covering approximately 80% of all genes which allows RNAi to be performed by simply feeding worms bacteria constructed in the way that they produce the desired dsRNA (Reverse Genetics in WormBook, Julie Ahringer, Wormbook 2006). The combination of these techniques made it possible to comprehensively screen the *C. elegans* genome for lifespan extending or shortening genes. As a result, more than

200 genes and interventions that affect lifespan in *C. elegans* have been identified, and evolutionarily conserved pathways (like insulin/IGF-1 signaling (IIS) pathway) that modulate lifespan not only in *C. elegans*, but also in flies and mice (Barbieri et al. 2003; Yen, Narasimhan, and Tissenbaum 2011) have been revealed (Tissenbaum 2015). Therefore, despite having a simple body plan, lacking defined blood, brain, or specialized fat cells, and being evolutionarily quite distant from *H. sapiens*, *C. elegans* offer the most optimal model organism system for aging research in general, and the most suitable one for the purposes of this particular study.

2.1.4 RNAi lifespan screen

We have tested the relevance of the 16 *C. elegans* transcription factors derived from the cross-species bioinformatics RSAT screen in regulating aging using lifespan assay experiments. In short, we synchronized an N2 worms population and had them grow to L4 stage of development when reproduction starts; this approach allowed us to test also the genes whose mutation had been reported as embryonic lethal because the downregulation starts after the development has completed. At that point we transferred the same age worms onto the fresh plates seeded with either control empty vector or experiment RNAi-harboring bacteria.

As a result, out of the 16 candidates 8 had lifespan extending effect, while 6 had lifespan-shortening effect. Two genes' RNAi, *pha-4*/F38A6.1 and *skn-1*/T19E7.2, had no effect on lifespan. This result supports the validity of our bioinformatics screen, as even by the most generous estimates only around 2.5% of all *C. elegans* genes are predicted to be modulating lifespan (Yanos, Bennett, and Kaeberlein 2012). Moreover, lifespans on RNAi of 9 of the genes (*mdl-1*/R03E9.1, *mxl-1*/T19B10.11, *mxl-3*/F46G10.6, *blmp-1*/F25D7.3, *pha-4*/F38A6.1, *daf-16*/R13H8.1, *daf-12*/F11A1.3, *skn-1*/T19E7.2 and *hlh-15*/C43H6.8) have already been reported in literature in relation to lifespan regulation with similar results, which further supports our approach (D. W. Johnson et al. 2014; Murphy et al. 2003; O'Rourke and Ruvkun 2013; Greer et al. 2010; Greer and Brunet

2009; Masse et al. 2005; P. L. Larsen, Albert, and Riddle 1995; Tullet et al., n.d.; Mansfeld et al. 2015).

2.1.5 Lifespan-extending genes

The following genes' RNAi treatment confers lifespan-extending effect with statistical significance of at least $p<0.05$ (Log-rank test): *hlh-2*/M05B5.5, *hlh-15*/C43H6.8, *mxl-1*/T19B10.11, *mdl-1*/R03E9.1, *mxl-3*/F46G10.6, *lin-32*/T14F9.5, *ceh-22*/F29F11.5, *ces-2*/ZK909.4 and *hlh-11*/F58A4.7 (Fig. 1); the table with statistics and percentages can be found in the Appendix.

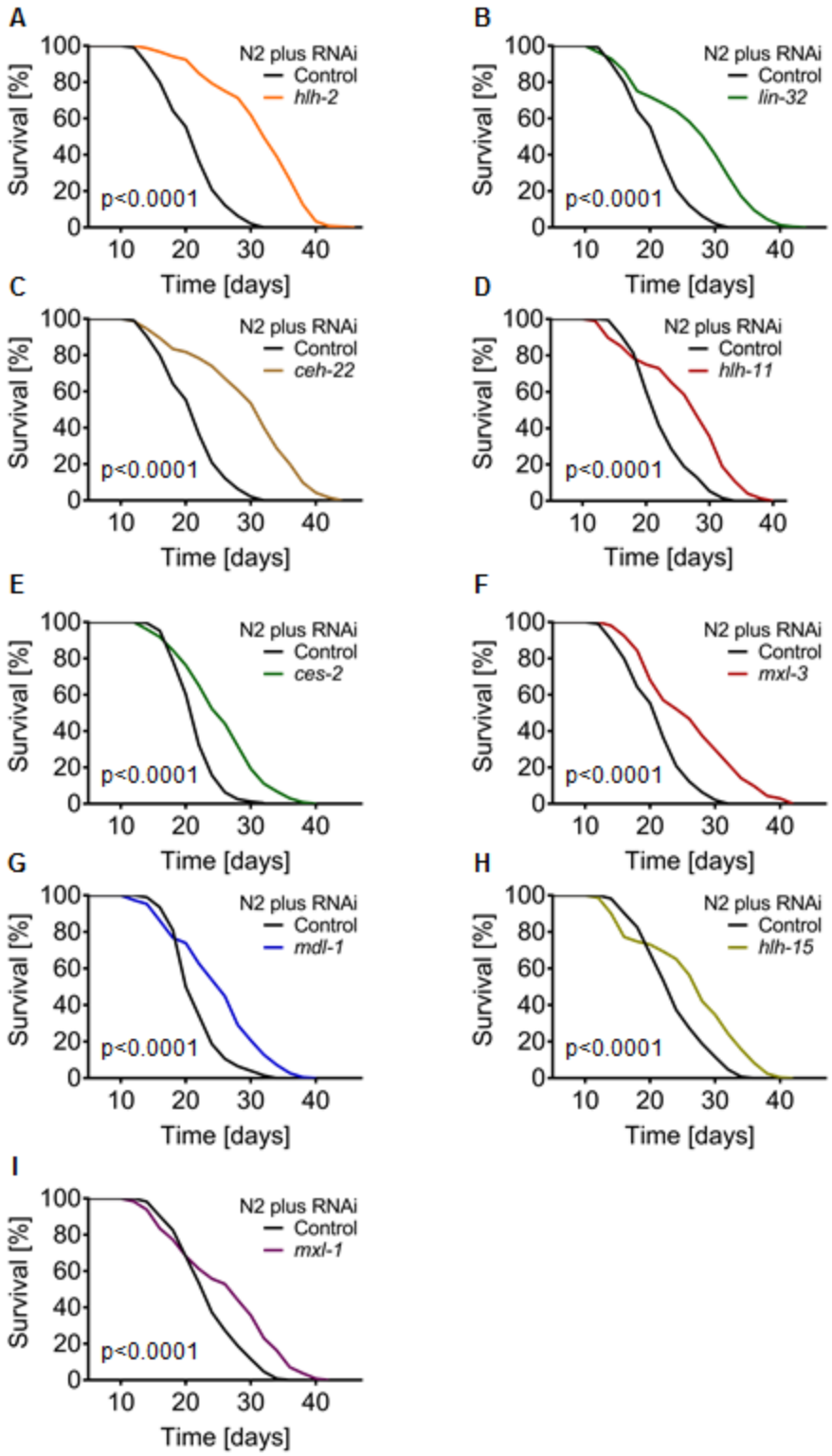

Figure 1: Lifespan extension upon RNAi gene knockdown of **(A)** *hlh-2*/M05B5.5, **(B)** *lin-32*/T14F9.5, **(C)** *ceh-22*/F29F11.5, **(D)** *hlh-11*/F58A4.7, **(E)** *ces-2*/ZK909.4, **(F)** *mxl-3*/F46G10.6,

(G) *mdl-1*/R03E9.1, **(H)** *hlh-15*/C43H6.8, and **(I)** *mxl-1*/T19B10.11 in the decreasing order of the effect.

Out of these, *hlh-15*/C43H6.8 was reported as controlling and epistatically synergizing with BCAT-1 to modulate physiological aging (Mansfeld et al. 2015); *MDL-1*:*MXL-1* complex was shown to function in both calorie restriction (CR) and the insulin signaling pathways (D. W. Johnson et al. 2014); and *mxl-3*/F46G10.6 has been described as a transcriptional switch coupling lysosomal lipolysis and autophagy to nutrient availability and controlling fat storage and aging in *Caenorhabditis elegans* (O'Rourke and Ruvkun 2013). We have thus focused on *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *ceh-22*/F29F11.5 as the transcription factors having the biggest lifespan-extending effect when knocked-down by RNAi. Interestingly, most of the reports about these 3 transcription factors describe them as important regulators of development (Krause, Greenwald, and Fire 1997; Portman and Emmons 2000; Thellmann, Hatzold, and Conradt 2003; C. Kenyon and Wrischnik 1997; Kuchenthal, Chen, and Okkema 2001; Chesney et al. 2009). The last 2 genes from the list, *ces-2*/ZK909.4 and *hlh-11*/F58A4.7, remain to be investigated and are potentially interesting candidates of lifespan-regulating transcription factors.

2.1.6 Lifespan-shortening genes

The following genes' RNAi confers lifespan-shortening effect: *efl-1*/Y102A5C.18, *blmp-1*/F25D7.3, *grh-1*/Y48G8AR.1, *daf-16*/R13H8.1, and *daf-12*/F11A1.3 (Fig. 2); the table with statistics and percentages can be found in the Appendix.

Out of these, *blmp-1*/F25D7.3 was reported as a pro-longevity transcription factor (Greer et al. 2010) where lifespan of N2 wild-type worms was found to be 20% reduced when *blmp-1*/F25D7.3 RNAi was applied; *daf-16*/R13H8.1 is a well-described transcription factor that acts in the insulin/IGF-1-mediated signaling (IIS) pathway that regulates dauer formation, longevity, fat metabolism, and stress response (Henderson and Johnson 2001; R. Y. N. Lee, Hench, and Ruvkun 2001; K. Lin et al. 2001); and *daf-12*/F11A1.3 affects dauer formation downstream of the TGF- and insulin signaling

pathways, and affects gonad-dependent adult longevity together with *daf-16*/R13H8.1 (P. L. Larsen, Albert, and Riddle 1995).

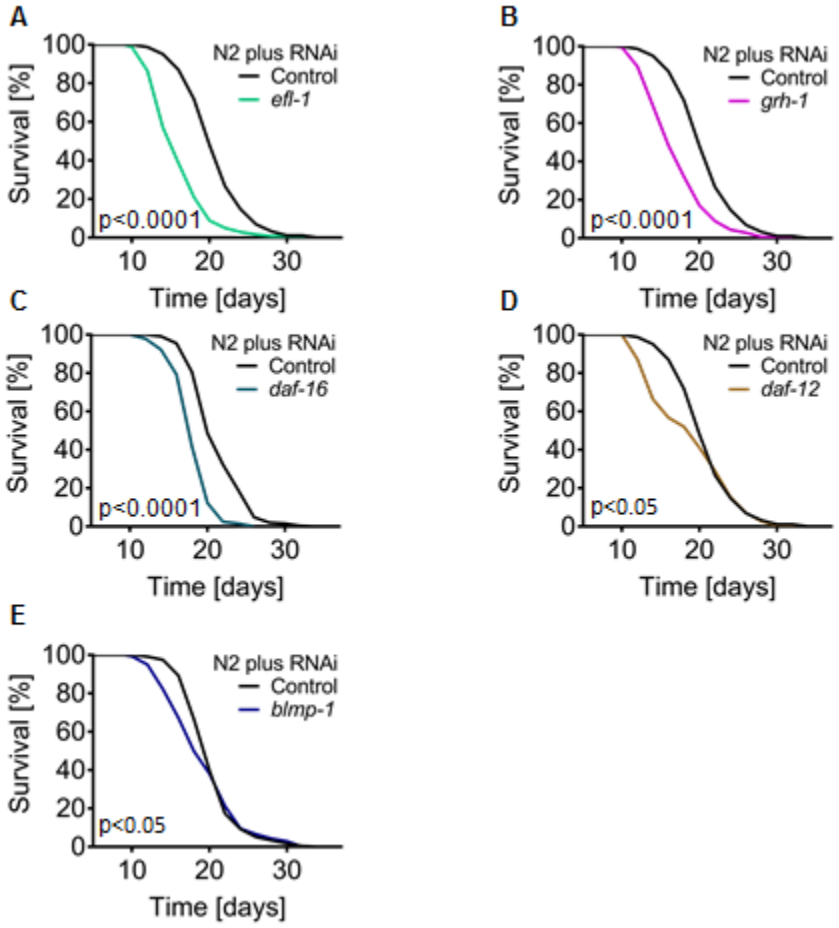

Figure 2: Lifespan analysis: lifespan shortening upon RNAi gene knockdown of **(A)** *efl-1*/Y102A5C.18, **(B)** *grh-1*/Y48G8AR.1, **(C)** *daf-16*/R13H8.1, **(D)** *daf-12*/F11A1.3, and **(E)** *blmp-1*/F25D7.3 in the decreasing order of the effect.

We have thus focused on *efl-1*/Y102A5C.18 as the gene whose RNAi knockdown confers the biggest life-shortening effect. During vulval development, *efl-1*/Y102A5C.18, a class B synMuv gene, acts with other synMuv genes of this class such as *dpl-1* and *lin-35*/Rb to antagonize receptor tyrosine kinase and Ras-mediated signaling (Ceol and Horvitz 2001a). While *grh-1*/Y48G8AR.1 is another interesting candidate, and it is currently being investigated in our laboratory, it is outside the scope of this study. Notably,

however, it, too, is mainly known as a development-regulating transcription factor required for normal cuticular synthesis and embryonic development (Paré et al. 2012).

2.1.7 Genes with no effect on lifespan

Finally, when RNAi of *pha-4*/F38A6.1 and *skn-1*/T19E7.2 was applied, we didn't see a statistically significant effect on lifespan (Fig. 3).

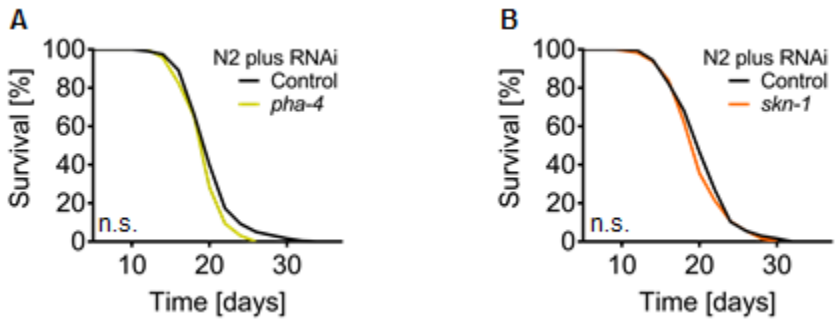

Figure 3: Lifespan analysis: no statistically significant effect on lifespan upon RNAi gene knockdown of **(A)** *pha-4*/F38A6.1 and **(B)** *skn-1*/T19E7.2.

This is in conflict with the published data on *pha-4*/F38A6.1 which was reported as an organ identity gene whose activity is necessary and sufficient for development of the pharynx/foregut; in addition, *pha-4*/F38A6.1 plays a key role in regulation of diet-restriction-induced longevity in adult nematodes (Greer and Brunet 2009). It is known that *skn-1*/T19E7.2 knock-out leads to lifespan shortening, and overexpression extends lifespan of worms (Tullet et al. 2017); however RNAi at L4 stage has not been reported to modulate lifespan in *C. elegans* to date.

It is possible that the difference in *pha-4*/F38A6.1 result stems from differences in culturing conditions of worms, which was reported to affect outcomes of experiments in different laboratories (Yanos, Bennett, and Kaeberlein 2012). To confirm the conflicting result the *pha-4*/F38A6.1 RNAi assay needs to be replicated, as the initial screen was done only once with each gene from the RSAT bioinformatics pipeline.

2.2 *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation show similar longevity-related phenotypes but likely act through different mechanisms

2.2.1 Basic helix-loop-helix (bHLH) transcription factors

As observed previously, the Developmental theory of aging finds itself in-between the conflicting points of view of NPA and PA theories. Though it claims that aging cannot be called a real genetic program because the aging process occurs mostly after the reproductive period and thus would experience little evolutionary pressure, it is nevertheless an environmentally influenced but highly dependent on the program of development process of biological inertia, a continuation of the real, developmental program, and can thus be called a “quasi-program or, figuratively, a shadow of actual programs” (Blagosklonny 2013). When viewed in this light, transcriptional regulation of development deserves more interest from the aging research. The basic helix-loop-helix (bHLH) transcription factor *hlh-2*/M05B5.5 we identified in our bioinformatics screen of the cross-species gene expression data is required for cell fate specifications occurring during embryonic and larval development which affect such important developmental processes as gonadogenesis, male tail formation, and programmed cell death (Krause, Greenwald, and Fire 1997; Portman and Emmons 2000; Thellmann, Hatzold, and Conradt 2003); *hlh-2*/M05B5.5 has been shown to dimerize with *lin-32*/T14F9.5, a neural-specific transcription factor that is required for development of several types of neurons (C. Kenyon and Wrischnik 1997), - and which is the product of another gene identified in our screen. In fact, many candidates from the screen (namely, *hlh-2*/M05B5.5, *lin-32*/T14F9.5, *mxl-1*/T19B10.11, *mdl-1*/R03E9.1, *mxl-3*/F46G10.6, *hlh-11*/F58A4.7, and *hlh-15*/C43H6.8), belong to the bHLH family, one of the largest families of dimerizing transcription factors, and interestingly all of them happen to lead to lifespan extension when knocked down (Fig. 1).

It is known that the majority of the 34 identified in *C. elegans* bHLH proteins exhibit highly specific dimerization as they interact with only a single other bHLH protein, and

hlh-2/M05B5.5 is of particular interest because it is one of only 2 proteins out of the 34 which interact with multiple other bHLH proteins (Grove et al. 2009). It thus serves as a central hub expressed in all cells, to which other, tissue-specific bHLH transcription factors bind. Moreover, many orthologs of these tissue-specific transcription factors are known to interact with the *hlh-2*/M05B5.5 ortholog (such as TCF3, Zgc, etc.) in other organisms as well (Grove et al. 2009).

2.2.2 Impaired *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 expression in *C. elegans* promotes healthspan

Given the unique character of *hlh-2*/M05B5.5 among bHLH transcription factors, and the fact that *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi, being binding partners in the context of several developmental processes, have shown the largest lifespan extending effect in the lifespan screen (Fig. 1), we set out to describe the phenotypes of these two genes' downregulation. We confirmed the effectiveness of RNAi at the expression levels of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 using real-time quantitative PCR with primers uniquely binding to these 2 gene products and not binding to the RNAi clones used during bacterial feeding (Fig. 4c,d). RNA for the qPCR samples was isolated 48 hrs after start of RNAi treatment. Gene knockdown had different effect on fertility of worms: while there is no significant effect on nematodes treated with *lin-32*/T14F9.5 RNAi, *hlh-2*/M05B5.5 RNAi, as may be expected given the importance of the transcription factor for early development, leads to almost complete sterility approximately 24 hrs after transferring the worms onto the plates seeded with RNAi bacteria (Fig. 4e,f). The number of unhatched eggs on *hlh-2*/M05B5.5 RNAi equals roughly ⅓ of the total number of progeny, and the rest of the eggs are not formed at all.

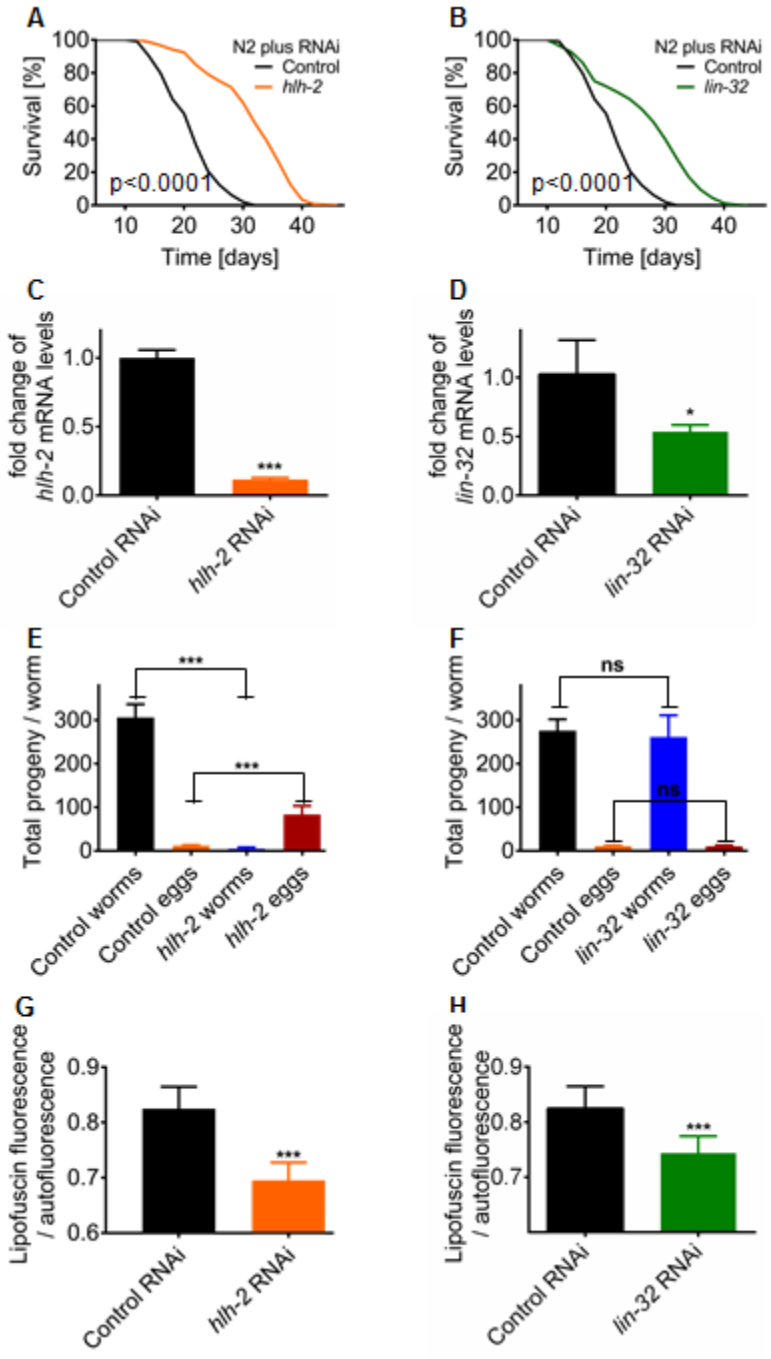

Figure 4: Lifespan analysis of N2 Bristol nematodes treated with **(A)** *hlh-2*/M05B5.5 (p<0.0001, log-rank test) and **(B)** *lin-32*/T14F9.5 (p<0.0001, log-rank test) RNAi; qPCR confirmation of gene knockdown using **(C)** *hlh-2*/M05B5.5 (p<0.0001, Student's t-test, n=3 biological replicates x 3 wells each) and **(D)** *lin-32*/T14F9.5 RNAi (p<0.05, Student's t-test, n=3 biological replicates x 3 wells each); fertility rates of **(E)** *hlh-2*/M05B5.5 (RNAi leads to almost complete sterility) (p<0.0001, Student's t-test, n=10 worms) and **(F)** *lin-32*/T14F9.5 RNAi (p<0.43, Student's t-test,

n=10 worms); reduction in aging pigment (lipofuscin) fluorescence induced by **(G)** *hlh-2*/M05B5.5 ($p<0.0001$, Student's t-test, n=3 biological replicates x 8 wells each) and **(H)** *lin-32*/T14F9.5 ($p<0.0001$, Student's t-test, n=3 biological replicates x 8 wells each) RNAi gene knockdown.

There have been reports questioning usefulness of lifespan assays alone, as even a very long but frail life would hardly be desirable: in the end we aim to extend healthspan, the functional and disease-free period of life (Hansen and Kennedy 2016). We have therefore tested if *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation would increase fitness of nematodes. For this purpose we first measured body bends per minute, a useful assay to assess youthfulness of organisms expressed in standard left and right movements on bacterial lawn (C. Huang, Xiong, and Kornfeld 2004). Both RNAi treatments led to a significant increase in body bends rate 5 days after transfer onto the RNAi plates (Fig. 5a,b). For the *hlh-2*/M05B5.5 RNAi we have additionally measured average and maximum locomotion speed at the same time, 5 days after starting RNAi treatment, and found significant increases in both parameters, which would indicate that more vigor is associated with *hlh-2*/M05B5.5 downregulation (Fig. 5c,d).

Additionally, we tested whether the increase in fitness observed in wild-type N2 worms would still hold true in the *C. elegans* neurodegenerative diseases model in which polyQ proteins are expressed throughout the nervous system (strain AM44 rmIs190 [F25B3.3p::Q67::CFP]). Many human neurodegenerative diseases are caused by the expansion of glutamine (Q, encoded by CAG) repeats (Brignull et al. 2006), and it has been shown that the nematode models' paralysis effects correlate with age and are affected by aging-modulating interventions such as downregulation of insulin signaling (Alexander et al. 2014). Percentage of paralyzed worms 8 days after transfer onto the RNAi plates was significantly lower in the *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi-treated worms (Fig 5e,f). Moreover, thrashing rates (a well-established assay where nematodes are placed in a drop of buffer, and the frequency of lateral swimming ("thrashing") movements is estimated) of *hlh-2*/M05B5.5 RNAi-treated AM44 mutant worms are higher than control, which suggests that formation of aggregates is alleviated with *hlh-2*/M05B5.5 downregulation (Fig. 5g). We haven't tested thrashing rates after treating worms with *lin-32*/T14F9.5 RNAi.

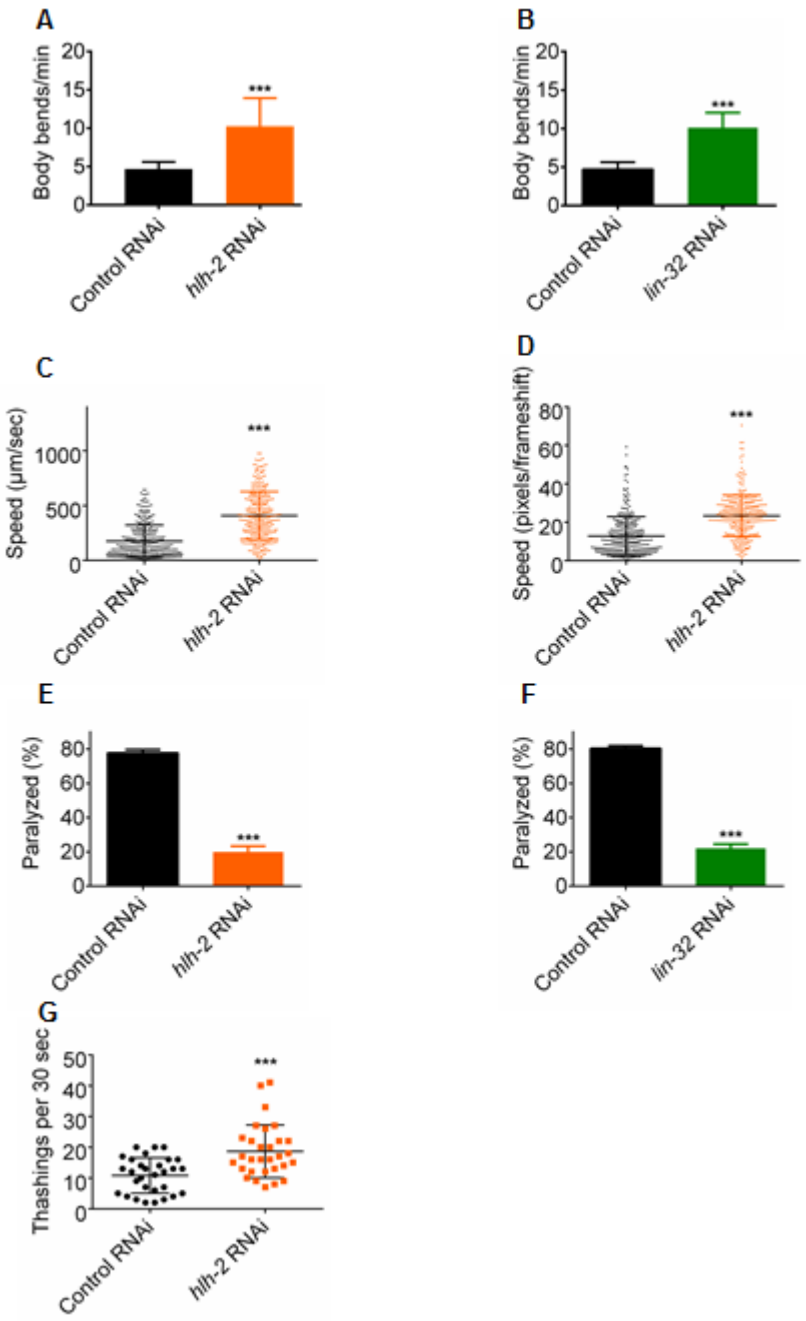

Figure 5: Body bends rate of N2 Bristol nematodes treated with **(A)** *hlh-2*/M05B5.5 ($p<0.0001$, Student's t-test, n=30 worms) and **(B)** *lin-32*/T14F9.5 RNAi ($p<0.0001$, Student's t-test, n=30 worms) **(C)** average ($p<0.0001$, Student's t-test, n~400 worms) and **(D)** maximum ($p<0.0001$, Student's t-test, n~400 worms) speed after *hlh-2*/M05B5.5 RNAi gene knockdown; percentage of paralyzed AM44 rmIs190 [F25B3.3p::Q67::CFP] mutant worms treated with **(E)** *hlh-2*/M05B5.5 ($p<0.0001$, Student's t-test, n=30 worms) and **(F)** *lin-32*/T14F9.5 RNAi ($p<0.0001$, Student's t-test, n=30 worms) **(G)** thrashing rates per 30 sec in *hlh-2*/M05B5.5 RNAi-treated worms ($p<0.0001$, Student's t-test, n=30 worms).

Another such *C. elegans* Alzheimer's disease model system comprises mutants expressing human β-amyloid peptide (Aβ). These strains CL802, CL4176 and CL2006, are useful due to their short life span, ability to develop muscle-associated deposits, and the concomitant progressive paralysis phenotype (Wu and Luo 2005). In the rapid paralysis system *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi treatment has either no or only a marginal effect (Fig. 6b,e). Rapid paralysis worms (CL4176) normally completely stop all movement 60 hrs after temperature shift from 15°C to 20°C, and this short time may not be sufficient for the RNAi system to remove enough transcripts from the cell to clear the functioning proteins. It is also possible that the effect of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation cannot fully stop such precipitous decline in function. The slow paralysis worms however show robust beneficial effect of both *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi (Fig. 6c,f).

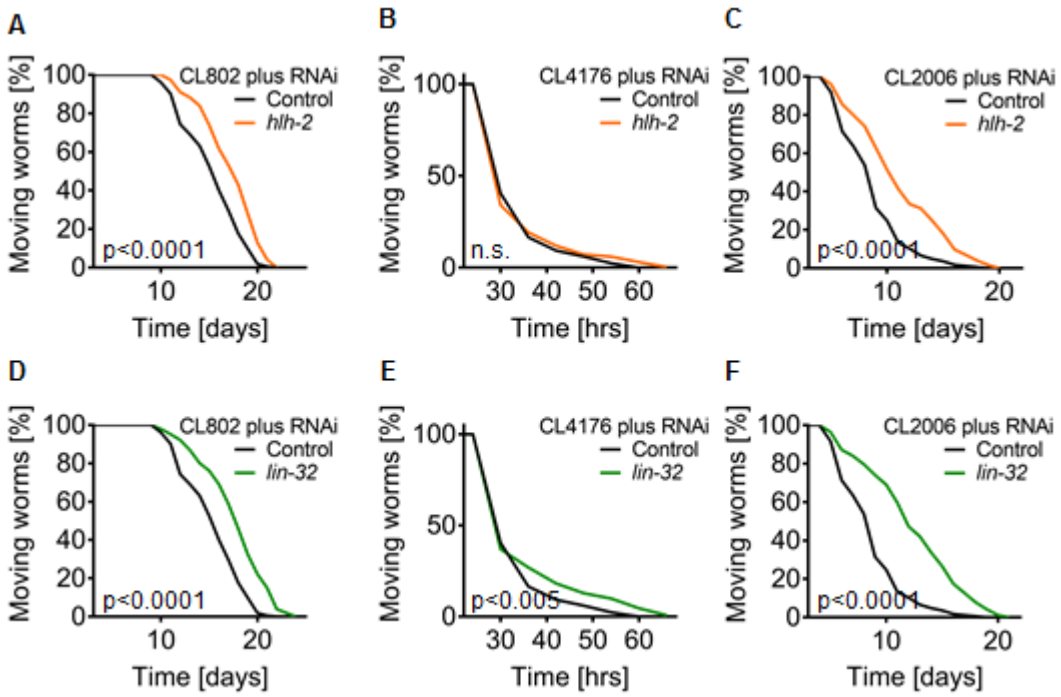

Figure 6: Lifespan analysis of *hlh-2*/M05B5.5 RNAi in **(A)** control (p<0.0001, log-rank test), **(B)** rapid paralysis (p=0.14, log-rank test), and **(C)** slow paralysis (p<0.0001, log-rank test) mutant nematodes; Lifespan analysis of *lin-32*/T14F9.5 RNAi in **(D)** control (p<0.0001, log-rank test), **(E)** rapid paralysis (p<0.005, log-rank test), and **(F)** slow paralysis (p<0.0001, log-rank test) mutant nematodes.

2.2.3 Downregulation of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 improves proteostasis, while upregulation shortens lifespan in *C. elegans*

Alleviation of symptoms induced by polyQ-aggregation or β-amyloid deposits suggests that systems responsible for proteostasis maintenance in the cells have been activated, such as proteasome-mediated targeted protein degradation or autophagy. We have first focused on the less specific system of protein quality control, namely autophagy, and used confocal microscopy imaging to detect lgg-1/C32D5.9-tagged autophagosome foci in a popular *C. elegans* autophagy reporter strain (Palmisano and Meléndez 2016) 5 days after transfer onto either control or *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi. The imaging was performed by our laboratory member Giovanna Grigolon.

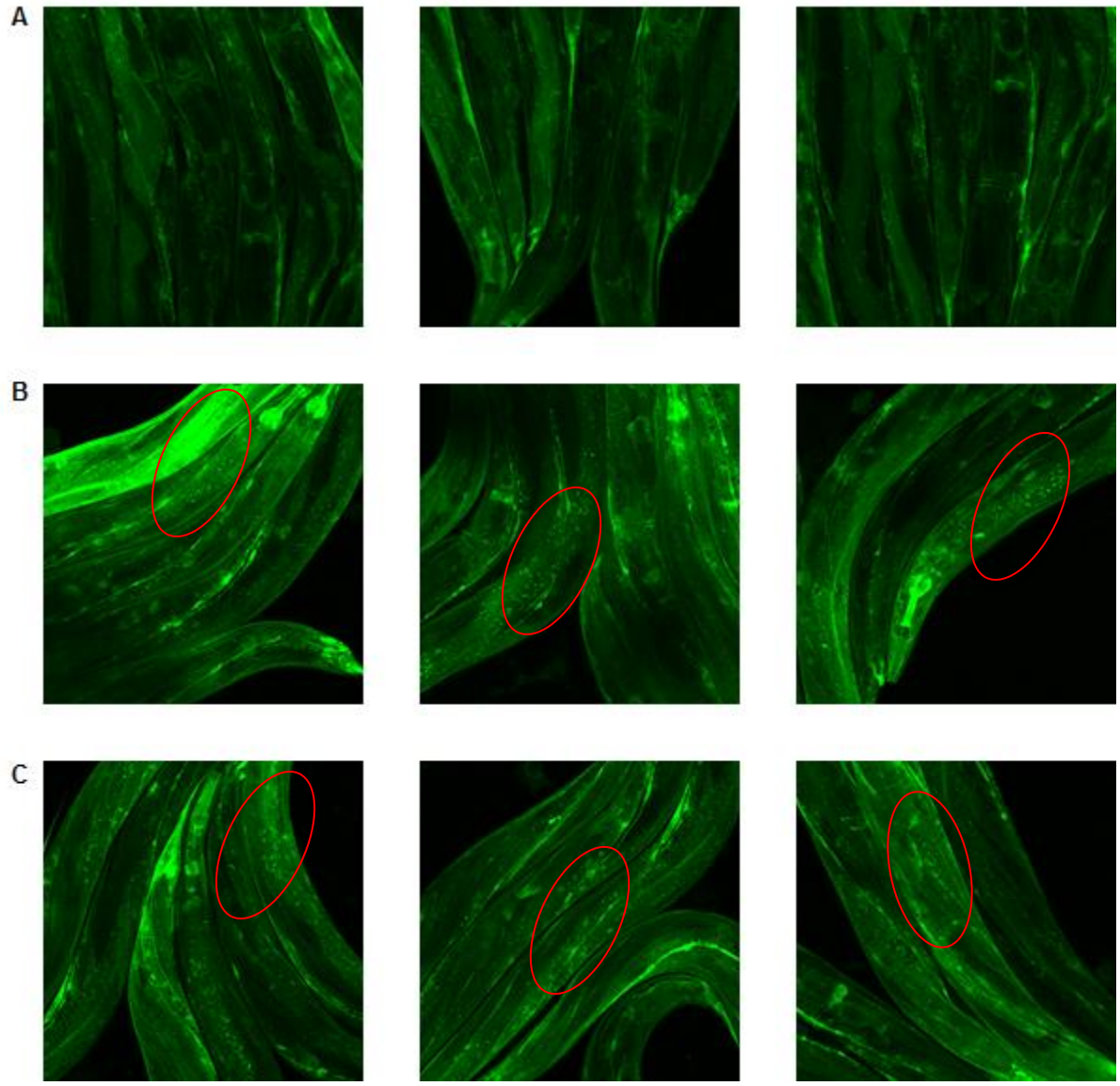

Figure 7: Fluorescent puncta visualize GFP-fused *lgg-1*/C32D5.9 incorporated into autophagosomes, and indicate autophagy activation in nematodes fed with **(A)** control RNAi; **(B)** *hlh-2*/M05B5.5 and **(C)** *lin-32*/T14F9.5 RNAi.

As can be observed in the confocal microscopy images (indicated with red ovals), there is an increase in GFP signal in the form of puncta in the worms treated with *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi (Fig. 7), suggesting elevated rates of autophagy in these animals. In the absence of uniform background precise quantification of the puncta is impossible, so autophagy activation is not definitively confirmed; in addition, *lgg-1*/C32D5.9 marker is only suggestive: an increase in the number of autophagosomes does not necessarily reflect an induction of autophagy (D. J. Klionsky et al. 2016); to show

autophagy activation with more certainty the use of multiple assays to infer the turnover of autophagosomes in the presence and absence of lysosomal degradation is recommended, which would help verify an increase in functional autophagy (Palmisano and Meléndez 2016).

Downregulation of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 at post-developmental stage leads to lifespan extension, which suggests that while necessary during development, these genes' activity may be detrimental for adult nematodes. In such case overexpression of these transcription factors at adult stage would lead to lifespan reduction. To test this hypothesis we applied heat shock-inducible overexpressor mutants of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5. One hour temperature shift to 33°C activates heat shock response and a heat stress-sensitive promoter which leads to upregulation of the downstream gene (Morton and Lamitina 2013). Heat shock itself had no statistically significant effect on either one of the strains used (Fig. 8). The *hlh-2*/M05B5.5 inducible overexpressor strain GS2968 worms have the same lifespan as the control N2 worms, while the *lin-32*/T14F9.5 inducible overexpressor strain UR189 worms live significantly shorter (Fig. 8). This is likely due to a leaky expression from the hsp promoter, and can be tested using qPCR, but we did not check *lin-32*/T14F9.5 expression at 20°C as compared to the N2 worms at the same temperature. When heat shock is applied, as expected, both *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 inducible overexpressor strains had statistically significant reduction of lifespan compared to the control worms incubated constantly at 20°C (Fig. 8).

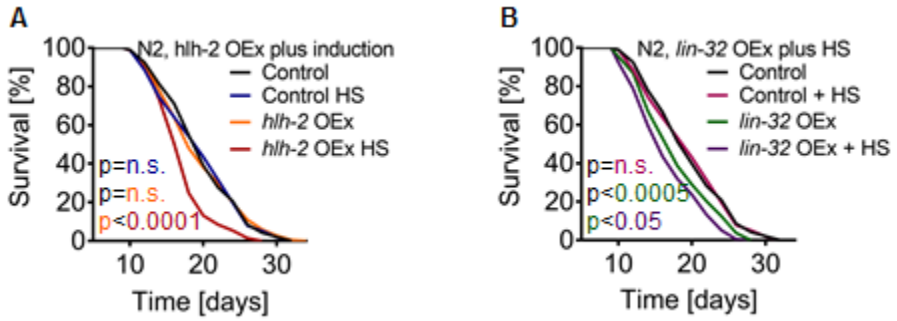

Figure 8: Lifespan analysis of heat shock (HS) conditional overexpressor of **(A)** *hlh-2*/M05B5.5 ($p<0.0001$, log-rank test) and **(B)** *lin-32*/T14F9.5 ($p<0.05$, log-rank test) versus control WT nematodes.

2.2.4 *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation-mediated lifespan extension depends on known longevity pathways

Having determined the phenotypic effects of changed expression of the transcription factors *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 we next sought to test whether these effects may be part of an already known pathway reported to modulate longevity in *C. elegans*. The most well-studied intervention comes from the research on insulin/insulin-like growth factor signaling (IIS) pathway involving a receptor *DAF-2*/InR and TF *daf-16*/FOXO. We have applied *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi at L4 stage to either *daf-16*/R13H8.1 or *daf-2*/Y55D5A.5 mutants and measured their lifespan.

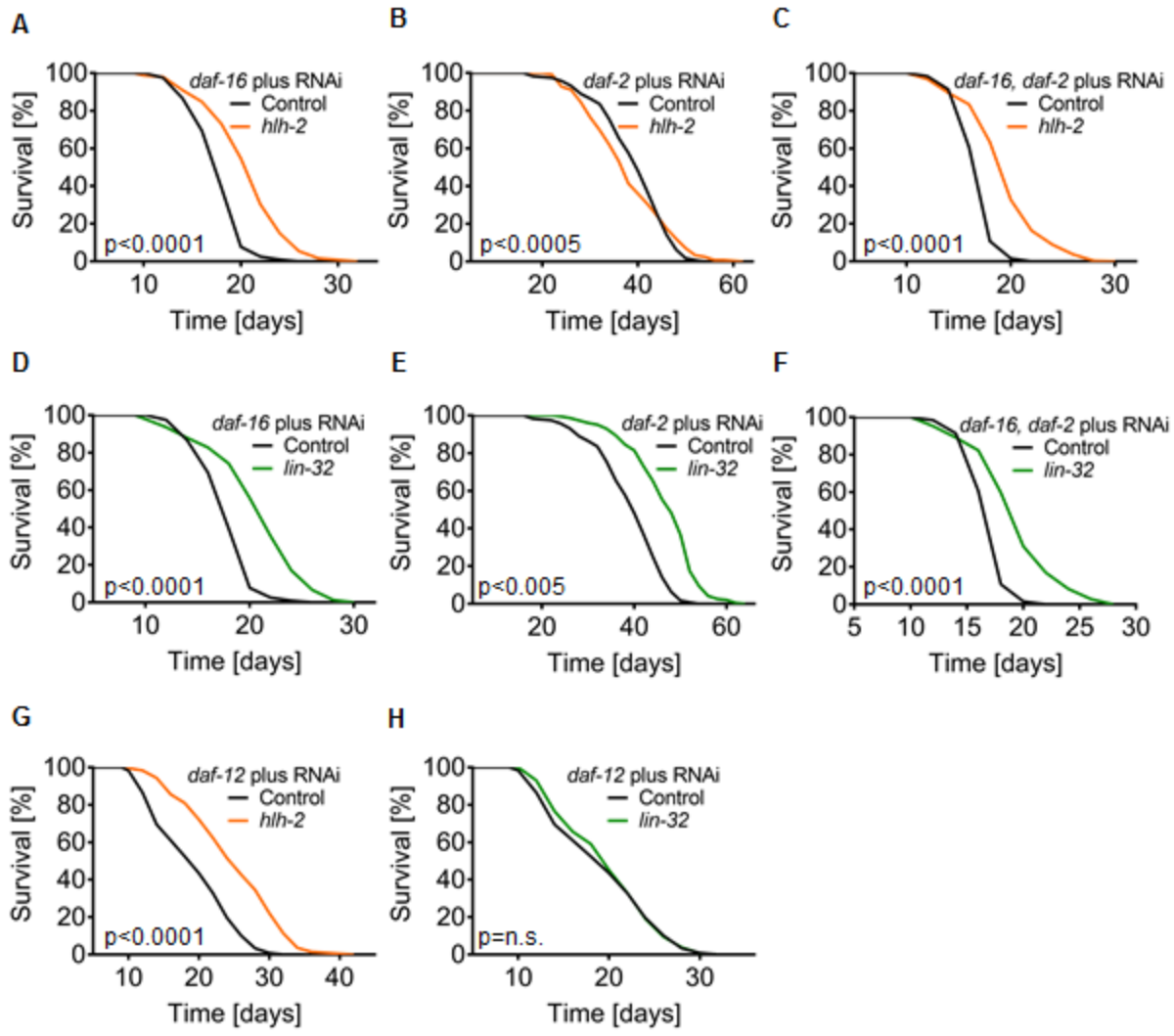

Figure 9: Lifespan analysis of *hlh-2*/M05B5.5 RNAi versus control RNAi in **(A)** *daf-16*(mu86) (p<0.0001, log-rank test), **(B)** *daf-2*(e1370) (p<0.0005, log-rank test) mutant, and **(C)** *daf-16*(mu86);*daf-2*(e1370) (p<0.0001, log-rank test) double mutant nematodes. Lifespan analysis of *lin-32*/T14F9.5 RNAi versus control RNAi in **(D)** *daf-16*(mu86) (p<0.0001, log-rank test), **(E)** *daf-2*(e1370) (p<0.0001, log-rank test) mutant, and **(F)** *daf-16*(mu86);*daf-2*(e1370) (p<0.0001, log-rank test) double mutant nematodes. Lifespan analysis of **(G)** *hlh-2*/M05B5.5 (p<0.0001, log-rank test) and **(H)** *lin-32*/T14F9.5 (p=0.43, log-rank test) RNAi versus control RNAi in *daf-12*(rh61rh411) mutant nematodes.

While the N2 wild-type worms live 30% and 26% longer when treated with *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi (Fig. 1), respectively, *DAF-16*/FOXO-deficient animals show almost identical lifespan extension of 30% and 27% on *hlh-2*/M05B5.5 and

lin-32/T14F9.5 RNAi, which would suggest that, as this lifespan extending effect is still present in the absence of functional *DAF-16*/FOXO, it is not dependent on IIS pathway (Fig. 9a,d). However, when we used the same approach of applying *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi to *DAF-2*-deficient worms, the results were not in line with this conclusion: even though *lin-32*/T14F9.5 RNAi produced lifespan extension, it was only a fraction of that in the wild-type worms (only 9%), and the *hlh-2*/M05B5.5 RNAi treatment led to lifespan shortening (Fig. 9b,e). In order to untangle these conflicting results we applied the same RNAi to the worms with mutation in both *daf-16*/R13H8.1 and *daf-2*/Y55D5A.5. In these experiments the resulting lifespan extension was 15% for *hlh-2*/M05B5.5 RNAi treatment and 14% for *lin-32*/T14F9.5 RNAi (Fig. 9c,f).

When *hlh-2*/M05B5.5 is knocked down at L4/YA stage of development worms become sterile approximately 24 hours after the start of the treatment. It has been reported that germline ablation leads to lifespan extension, which is consistent with the Disposable Soma theory of aging, and *daf-12* was reported as a key regulator of germline elimination-induced longevity, which also regulates *DAF-16* nuclear localization and activity (Hsin and Kenyon 1999). It could be that *hlh-2*/M05B5.5 RNAi-induced sterility is mainly responsible for the extended lifespan of worms with knocked down *hlh-2*/M05B5.5. To test this possibility we used worms mutant for *daf-12,* and applied *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi. Downregulation of *hlh-2*/M05B5.5 led to 30% lifespan extension in these mutants, excluding the possibility that this effect is achieved through *daf-12* activity (Fig. 9g). Unexpectedly, *lin-32*/T14F9.5 RNAi showed no statistically significant lifespan extension, implicating *daf-12* and possibly germline signaling in this longevity phenotype, even though *lin-32*/T14F9.5 downregulation has no apparent effect on fecundity (Fig. 9h, Fig. 4f).

Altogether, these lifespan assays suggest that the observed lifespan extending effect is *DAF-16*/FOXO-, and more generally, IIS-independent, although it may interfere with the upstream IIS signaling or act in parallel to *DAF-16*/FOXO-induced stimulation of protective mechanisms leading to longevity.

Data in mutants with polyQ-aggregation and autophagy marker suggest that proteostasis balance is more robust with post-developmental downregulation of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5. Certain designated transcription factors, and most notably *hsf-1*, regulate the multitude of genes involved in the protein quality control system (Labbadia and Morimoto 2014), and *HSF-1* transcription factor is central to the heat shock response and cytosolic proteostasis (Åkerfelt, Morimoto, and Sistonen 2010; J. Li, Labbadia, and Morimoto 2017). In the absence of *hsf-1* we can expect that the lifespan-extending effect of treatments that rely on the heat shock response would be abolished. Indeed, after *lin-32*/T14F9.5 RNAi treatment lifespan extension is only 2%, while *hlh-2*/M05B5.5 downregulation leads to a modest lifespan extension of 9% (Fig. 10a,b). The ubiquitin proteasome system (UPS) activity and autophagy are also important contributors to proteostasis involved in lifespan regulation. To test involvement of UPS we combined RNAi of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 with RNAi against pbs-3/Y38A8.2 or pbs-4/T20F5.2, both genes coding for proteasome subunits.

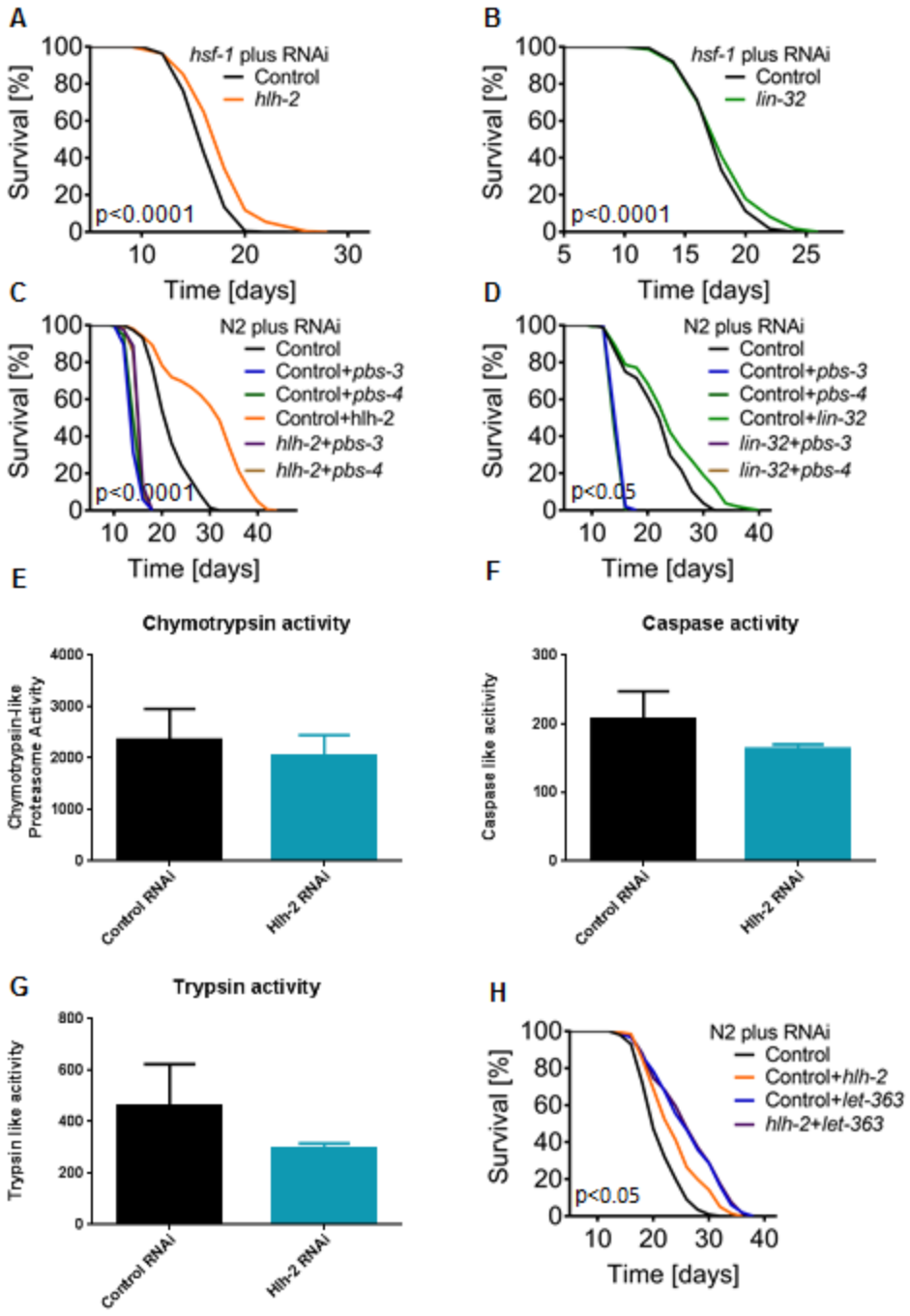

Figure 10: Lifespan analysis of *hsf-1*(sy441) mutant nematodes treated with **(A)** *hlh-2*/M05B5.5 (p<0.0001, log-rank test) and **(B)** *lin-32*/T14F9.5 (p<0.05, log-rank test) RNAi; Lifespan analysis of N2 Bristol nematodes treated with pbs-3/Y38A8.2 and pbs-4/T20F5.2 RNAi in the presence (purple, brown) or absence (blue, green) of **(C)** *hlh-2*/M05B5.5 (p<0.0001 and p<0.0001, log-rank test) and **(D)** *lin-32*/T14F9.5 (p=0.65 and p=0.84, log-rank test) RNAi; Activity of **(E)** chymotrypsin, **(F)** caspase, and **(G)** trypsin in proteasome activity assay of nematodes treated with *hlh-*

2/M05B5.5 versus control RNAi (all 3 tests p=n.s.); **(H)** Lifespan analysis of N2 Bristol nematodes treated with *let-363* RNAi in the presence (purple) or absence (blue) of *hlh-2*/M05B5.5 RNAi (p=0.74, log-rank test).

In both cases of proteasome subunits, addition of either *hlh-2*/M05B5.5 or *lin-32*/T14F9.5 RNAi led to no statistically significant lifespan extension, suggesting proteasome involvement. However, when proteasome activity was measured in *hlh-2*/M05B5.5 RNAi-treated worms (assay kindly performed by Meenakshi Ravichandran), no statistically significant chymotrypsin, caspase, or trypsin activity was detected (Fig. 10e,f,g). It is possible that interfering with proteasome subunits sickens nematodes to the extent impossible to rescue with this lifespan-extending intervention.

Finally, TOR signaling, also reported to account for the increased lifespan through proteostasis modulation (namely, it is responsible for decreased translation) is another pathway we have tested using administration of mixed RNAi bacteria. When *hlh-2*/M05B5.5 RNAi-harboring bacteria were added to *let-363*/B0261.2 RNAi, ortholog of human MTOR (mechanistic target of rapamycin), the lifespan extending effect was no stronger than with addition of *let-363*/B0261.2 RNAi-harboring bacteria alone, indicating that TOR signaling pathway is indeed involved in this intervention. Combination of *let-363*/B0261.2 RNAi with that of *lin-32*/T14F9.5 has not been tested yet but would be of interest. Taken together, these data suggest that both *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation-induced lifespan extension is *hsf-1*-dependent, and generally proteostasis is involved in mediating this effect.

AMP-activated protein kinase (AMPK) is a conserved cellular energy sensor necessary for IIS-mediated lifespan extension that restores energy homeostasis by, among other things, stimulating catabolic processes (Hardie, Ross, and Hawley 2012), and consistent with this it has been reported to inhibit TOR signaling (Motoshima et al. 2006). We tested *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi in *aak-2*/T01C8.1 (*C. elegans* ortholog of AMPK) mutant worms to check involvement of this energy sensing switch in the lifespan extension effect. Lifespan assays showed that *lin-32*/T14F9.5 RNAi-mediated

lifespan extension completely depends on *aak-2*/AMPK activity, while *hlh-2*/M05B5.5 RNAi extended lifespan 5% over the control, which indicates that *aak-2*/AMPK activity is highly important for both interventions' lifespan extending effect (Fig. 11a,b).

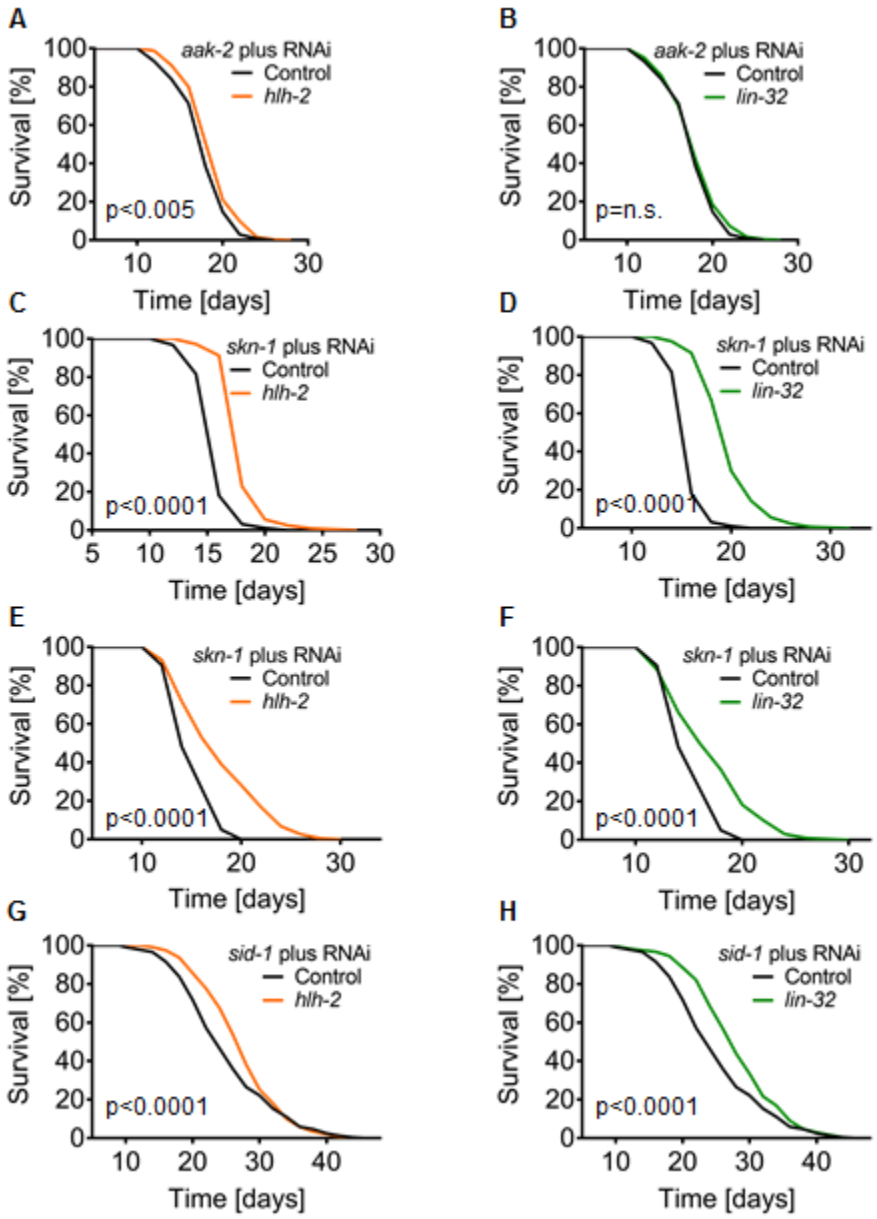

Figure 11: Lifespan analysis of *hlh-2*/M05B5.5 RNAi versus control RNAi in **(A)** *aak-2*(ok524) (p<0.005, log-rank test) **(C)** *skn-1*(zu67) (p<0.0001, log-rank test) **(E)** *skn-1*(zu135) (p<0.0001, log-rank test), and **(G)** sid-1(uIs60) (p<0.0001, log-rank test) mutant nematodes; Lifespan analysis of *lin-32*/T14F9.5 RNAi versus control RNAi in **(B)** *aak-2*(ok524) (p=0.17, log-rank test)

(D) *skn-1*(zu67) ($p<0.0001$, log-rank test) **(F)** *skn-1*(zu135) ($p<0.0001$, log-rank test), and **(H)** sid-1(uIs60) ($p<0.0001$, log-rank test) mutant nematodes.

2.2.5 *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation-mediated lifespan extension requires ROS signaling

Given such dependency on *aak-2* and its broad activity in the cell it is important to narrow down which of the *aak-2*-involved pathways is responsible for the *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation effect. On the one hand, *aak-2*/AMPK is required for the IIS-mediated longevity (Apfeld et al. 2004), while *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 activity is *daf-16*/FOXO independent (Fig. 9a,d). On the other hand, as noted earlier, *aak-2*/AMPK inhibits TOR signaling and activates autophagy in *C. elegans* (David et al. 2010). Furthermore, it is also required for the longevity effect of mitohormesis together with *skn-1*/T19E7.2 (ortholog of the mammalian Nrf2), a well-studied regulator of oxidative stress response (Schmeisser et al. 2013). *skn-1*/T19E7.2 encodes three isoforms, all of which share the DNA binding domain (www.wormbase.org). Two of the isoforms, *skn-1*a and *skn-1*c, are expressed and act in the intestine to mediate stress response (An and Blackwell 2003; Tullet et al., n.d.), while the third isoform, *skn-1*b, is required for dietary restriction-induced lifespan extension and functions in two sensory neurons (Bishop and Guarente 2007). When we applied *hlh-2*/M05B5.5 RNAi to *skn-1*(zu67) mutants, which specifically lack the *skn-1*a and *skn-1*c isoforms expressed in the intestine, we recorded lifespan extension of only 12%, whereas *lin-32*/T14F9.5 RNAi had the full effect of 26% (Fig. 11c,d). Interestingly, when the same RNAi was applied to *skn-1*(zu135) mutants which lack all 3 isoforms, the results were almost reversed: *hlh-2*/M05B5.5 RNAi led to 18% longevity induction, while *lin-32*/T14F9.5 RNAi had the effect of only 13% lifespan extension (Fig. 11e,f), which would suggest that *skn-1* is indeed involved in these interventions' lifespan-extending effect, and that the mechanisms are likely different for *hlh-2*/M05B5.5 and *lin-32*/T14F9.5. Finally, to test whether neuronal downregulation of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 can change their effect on longevity we used a mutant designed to have increased response of neurons to dsRNA delivered by feeding (Calixto

et al. 2010). Surprisingly, in this background we observed only 8% and 12% of lifespan extension exerted by *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi, respectively (Fig. 11g,h).

Given involvement of both *aak-2* and *skn-1* in *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation-mediated longevity, we aimed to further test whether mitohormesis and ROS signaling may be required for this phenotype (W. Yang and Hekimi 2010a). We added N-acetylcysteine (NAC) or butylated hydroxyanisole (BHA), an antioxidant precursor and an antioxidant, respectively, to the control, and *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi-seeded plates and measured lifespan to test whether addition of antioxidants which would interfere with ROS signaling can abolish the lifespan extension effect.

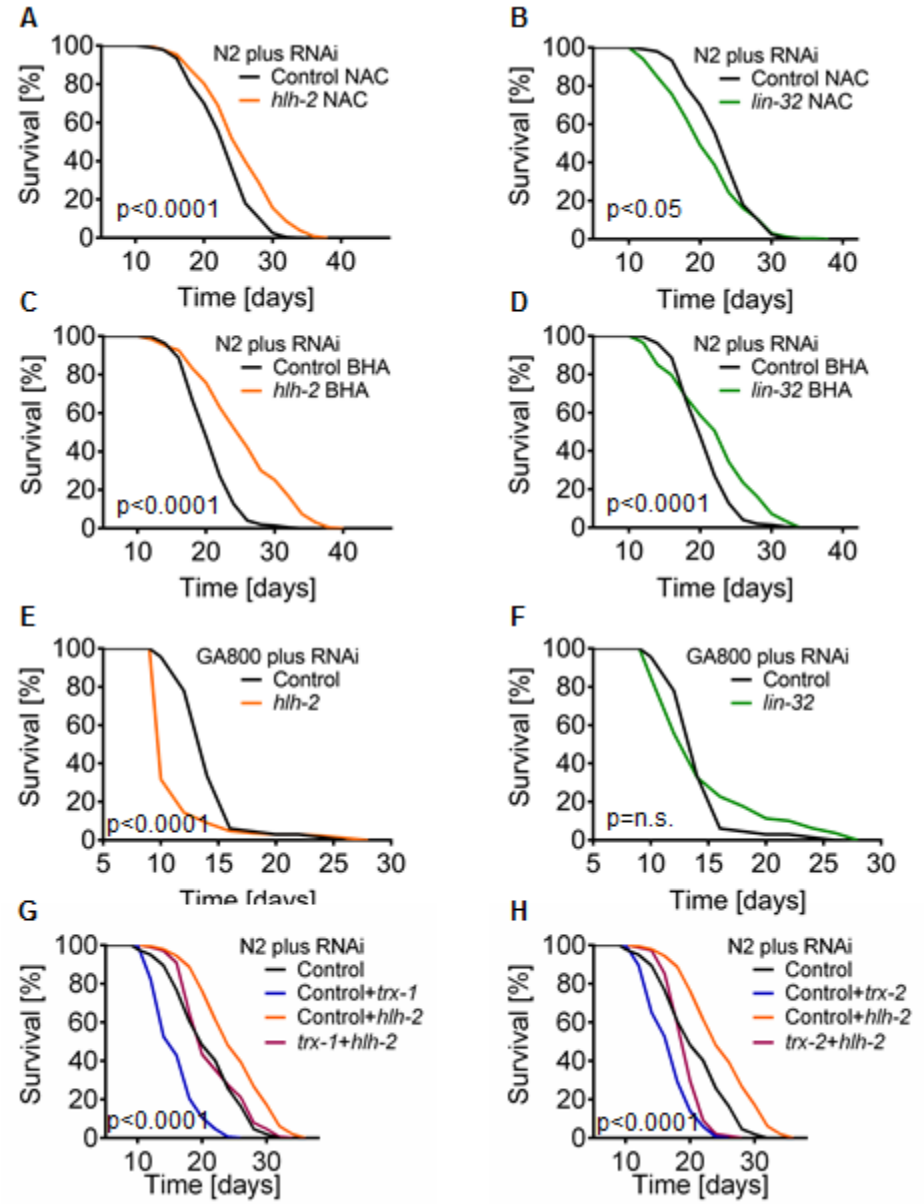

Figure 12: Lifespan analysis of *hlh-2*/M05B5.5 RNAi versus control RNAi in the presence of **(A)** NAC (p<0.0001, log-rank test) and **(C)** BHA (p<0.0001, log-rank test); Lifespan analysis of *lin-32*/T14F9.5 RNAi versus control RNAi in the presence of **(B)** NAC (p<0.05, log-rank test) and **(D)** BHA (p<0.0001, log-rank test); lifespan analysis of catalase overexpressing mutant treated with **(E)** *hlh-2*/M05B5.5 (p<0.0001, log-rank test) **(F)** *lin-32*/T14F9.5 (p=0.95, log-rank test) RNAi; **(G)** lifespan analysis of *trx-1*/B0228.5 RNAi in the presence (dark red) or absence (blue) of *hlh-2*/M05B5.5 RNAi (p<0.0001, log-rank test); **(H)** lifespan analysis of *trx-2*/B0024.9 RNAi in the presence (dark red) or absence (blue) of *hlh-2*/M05B5.5 RNAi (p<0.0001, log-rank test).

Confirming our expectations, with addition of NAC longevity modulation on *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi has decreased down to 10% and 8%, respectively (Fig. 12a,b). Addition of BHA reduced lifespan extension exerted by *lin-32*/T14F9.5 RNAi to 6%; however, it had only a fraction of the effect for *hlh-2*/M05B5.5 downregulation and still extended lifespan by 22% (Fig. 12c,d). We have used an additional system to confirm our results, namely a mutant overexpressing catalases 1, 2, and 3 - antioxidant enzymes that protect cells from reactive oxygen species (ROS) and are expected to interfere with ROS signaling, as well. Both RNAi treatments failed to induce any lifespan extension, with *hlh-2*/M05B5.5 RNAi exerting lifespan-shortening effect (Fig. 12e,f). Further testing the redox system involvement, we combined *hlh-2*/M05B5.5 RNAi with either control RNAi or *trx-1*/B0228.5, and in a separate experiment with *trx-2*/B0024.9 RNAi (*trx-1*/B0228.5 and *trx-2*/B0024.9 are redox proteins that function as protein-disulfide reductases; experiments testing loss of *trx-1* activity indicate that *trx-1* is required for normal adult *C. elegans* lifespan: www.wormbase.org). However, both combinations still had the usual lifespan-extending effect of approximately 30% compared with *trx-1*/B0228.5 or *trx-2*/B0024.9 RNAi alone, which would suggest that thioredoxins are likely not involved in the *hlh-2*/M05B5.5 downregulation-mediated longevity induction (Fig. 12g,h).

Metformin extends lifespan in *C. elegans* and was shown to be an indirect AMPK agonist (Onken and Driscoll 2010). It was also reported to extend lifespan through mitohormesis, and this effect depends on the activity of *prdx-2*/F09E5.15, one of the two *C. elegans* 2-Cys peroxiredoxins, peroxidase enzymes that contribute to the oxidative-stress response by reducing hydrogen peroxide (De Haes et al. 2014). We tested *prdx-2*/F09E5.15 involvement by applying *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi at L4 stage of *C. elegans* development. Both RNAi treatments led to 28% longevity induction, thus ruling out the possibility that this phenotype depends on *prdx-2*/F09E5.15 activity (Fig. 13a,b).

2.2.6 Mitochondrial involvement is likely in *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi-induced longevity

Some mutations in *C. elegans* have lifespan-extending effect by apparently extending the time it takes to undergo every stage of development, including aging itself, which fits with the Developmental theory of aging; one of such genes we tested was *clk-1*/ZC395.2, a highly conserved demethoxyubiquinone hydroxylase that is necessary for the biosynthesis of ubiquinone and is required for normal physiological rates of growth, development, behavior, and aging, as well as for normal brood sizes (Wong, Boutis, and Hekimi 1995). *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi was applied to *clk-1*/ZC395.2 mutant worms post developmentally, which led to 16% and 19% lifespan extension, respectively, suggesting that longevity phenotype of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation is mainly not mediated through *clk-1*/ZC395.2 (Fig. 13c,d).

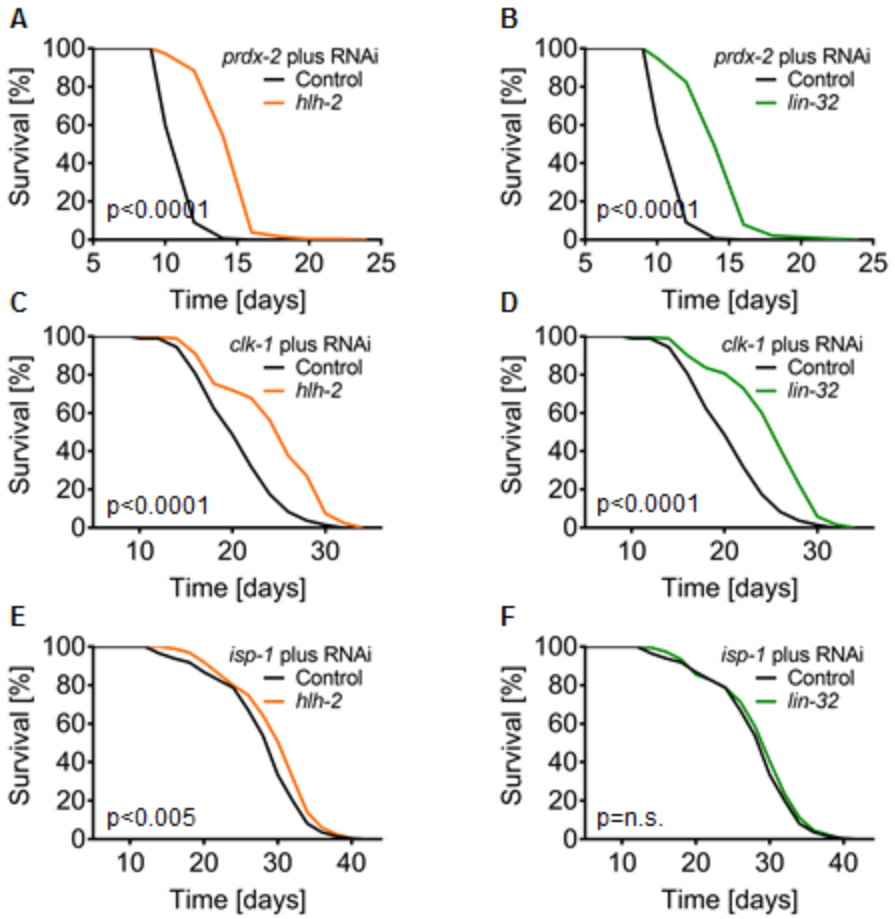

Figure 13: Lifespan analysis of *hlh-2*/M05B5.5 RNAi versus control RNAi in **(A)** *prdx-2*(gk169) (p<0.0001, log-rank test) **(C)** *clk-1*(qm30) (p<0.0001, log-rank test) and **(E)** *isp-1*(qm150) (p<0.005, log-rank test) mutant nematodes; Lifespan analysis of *lin-32*/T14F9.5 RNAi versus control RNAi in **(B)** *prdx-2*(gk169) (p<0.0001, log-rank test) **(D)** *clk-1*(qm30) (p<0.0001, log-rank test), and **(F)** *isp-1*(qm150) (p=0.25, log-rank test) mutant nematodes.

Another aging-modulating gene we tested in connection with the fact that it affects the rates of physiological processes like reproduction and development and through this slowing down of the normal wild type early life “rate of living” extends lifespan, is *isp-1*/F42G8.12 which codes for a subunit of the mitochondrial complex III in the mitochondrial membrane (Feng, Bussière, and Hekimi 2001). Mutants of *isp-1*/F42G8.12 show low oxygen consumption, as well as a decreased sensitivity to ROS, and increased lifespan (Feng, Bussière, and Hekimi 2001). When subjected to *hlh-2*/M05B5.5 RNAi post developmentally, these mutants showed only 6% lifespan extension, and *lin-32*/T14F9.5

RNAi failed to produce statistically significant longevity induction, further implicating mitochondrial involvement in this longevity phenotype (Fig. 13e,f).

2.2.7 Timing of *hlh-2*/M05B5.5 downregulation is crucial for the lifespan extension effect

After 24 hours on *hlh-2*/M05B5.5 RNAi worms become almost completely sterile indicating the high importance of presence of the functional protein during embryonic development. The RNAi is applied at L4 stage of development, which may interfere with the last developmental changes of the almost adult worms as well. To test the impact of *hlh-2*/M05B5.5 downregulation during different stages of *C. elegans* life we first applied the RNAi to synchronized populations of worms at either the usual 4th larval (L4) stage or several hours later when they have become young adults (YA).

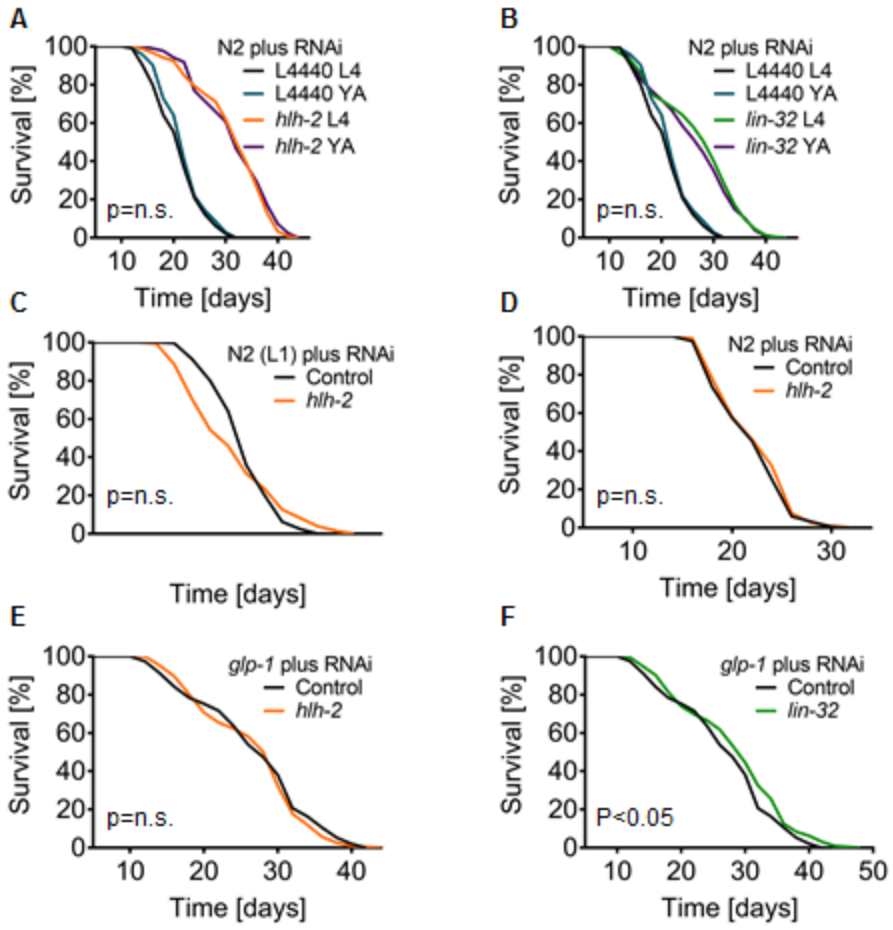

Figure 14: Lifespan analysis of N2 Bristol nematodes treated with **(A)** *hlh-2*/M05B5.5 (p=0.55, log-rank test) and **(B)** *lin-32*/T14F9.5 RNAi (p=0.39, log-rank test) at L4 versus YA stage of development; Lifespan analysis of *hlh-2*/M05B5.5 RNAi versus control RNAi started at **(C)** L1 stage of development (p=0.46, log-rank test); or **(D)** post-reproductive stage (p=0.27, log-rank test); Lifespan analysis of *glp-1*(e2141) mutant worms treated with **(E)** *hlh-2*/M05B5.5 (p=0.34, log-rank test) and **(F)** *lin-32*/T14F9.5 RNAi (p<0.05, log-rank test).

This timing of RNAi application did not lead to any statistically significant changes compared to the previously reported results, either for *hlh-2*/M05B5.5 or *lin-32*/T14F9.5 RNAi (Fig. 14a,b). To find out if earlier RNAi treatment (but after embryonic development has completed) would produce different results we also put worms on *hlh-2*/M05B5.5 RNAi at the 1st stage of larval development (L1), and just as expected this *hlh-2*/M05B5.5 downregulation failed to extend lifespan (Fig. 14c). This treatment apparently interfered with post-embryonic development, as the animals exhibited sick phenotypes, such as small size, explosions, protruding vulva, slow movement, etc. Interestingly, when

hlh-2/M05B5.5 RNAi was applied after the reproductive period had ceased, we could not see statistically significant lifespan extension, either (Fig. 14d). With the aim to further elucidate how germline elimination affects the studied longevity phenotype we used a mutant deficient for *glp-1*/F02A9.6, a transmembrane protein and member of the LIN-12/Notch family of receptors, which is required for cell fate specification in germline and somatic tissues in *C. elegans*. It is a temperature sensitive mutant normally cultured at 15C but completely sterile at 25C (Pepper, Killian, and Hubbard 2003). Both *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation treatments failed to produce statistically significant lifespan extension in the *glp-1*/F02A9.6 mutant background (Fig. 14e,f), which is a strong indication of the germline endocrine-mediated lifespan regulation involved in the longevity phenotype of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation; however, it is in conflict with the data obtained using *daf-12* and *daf-16*/R13H8.1 mutant worms, as both genes were reported to mediate germline endocrine-mediated lifespan regulation (Arantes-Oliveira et al. 2002), but *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation-mediated lifespan extension is both *daf-12*/F11A1.3- and *daf-16*/R13H8.1-independent.

2.2.8 *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 mediate lifespan extension independently of each other

Having obtained some data related to redox state involved in *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation-mediated lifespan extension (antioxidant treatment strongly reduces lifespan extension, and catalase overexpression background abolishes it completely; both RNAi interventions partly depend on *skn-1* activity) we sought to measure ROS levels 48 hours after transferring worms onto the RNAi-harboring bacteria-seeded plates. As expected, *hlh-2*/M05B5.5 RNAi-treated worms produced significantly more hydrogen peroxide which was detected in the supernatant after incubation; *lin-32*/T14F9.5 RNAi-treated worms' higher ROS was not significant, but elevated by trend (Fig. 15a). It is likely that this relatively minor spike of ROS signaling would be better detected by a more sensitive assay or by using a higher number of animals per group.

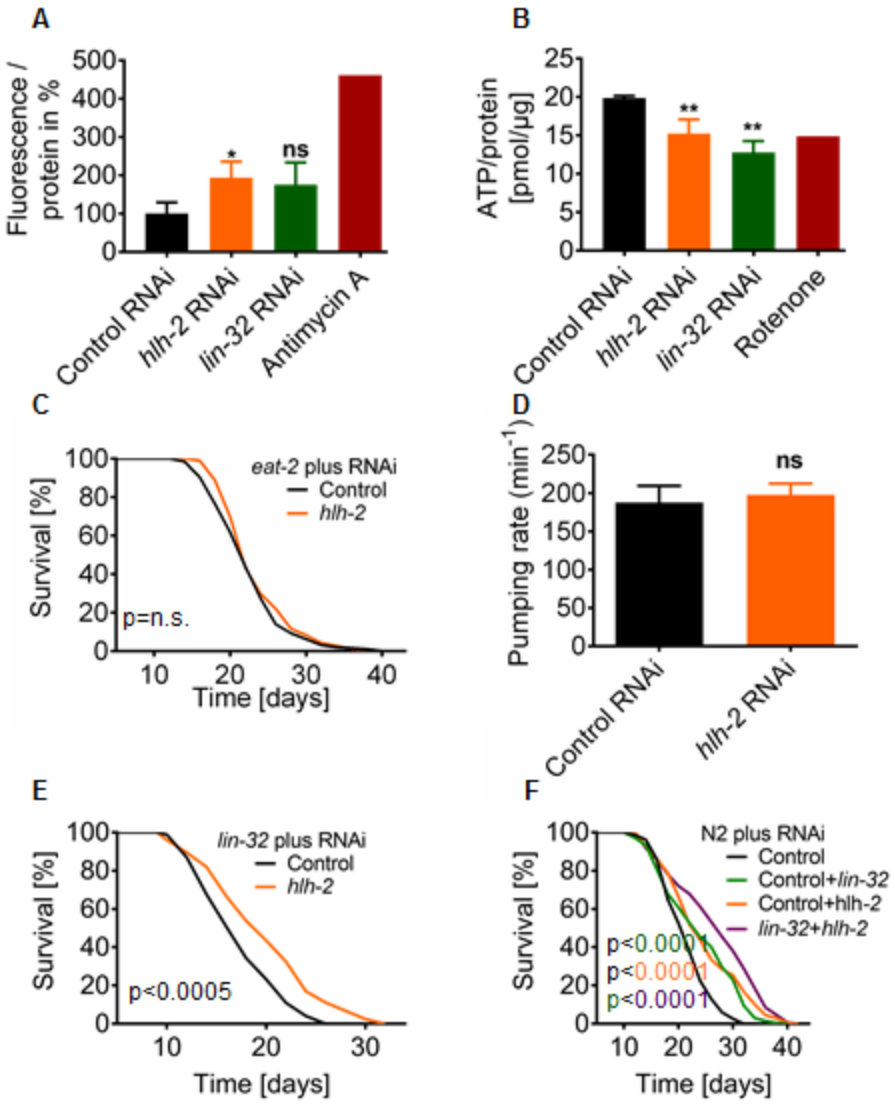

Figure 15: (A) Quantification of hydrogen peroxide in WT worms treated with control, *hlh-2*/M05B5.5 ($p<0.05$, Student's t-test, n=3 biological replicates x 3 measurements) or *lin-32*/T14F9.5 RNAi ($p=0.11$, Student's t-test, n=3 biological replicates x 3 measurements) with Antimycin A as positive control; **(B)** ATP determination in WT worms treated with control, *hlh-2*/M05B5.5 ($p<0.001$, Student's t-test, n=3 biological replicates x 3 measurements) or *lin-32*/T14F9.5 RNAi ($p<0.001$, Student's t-test, n=3 biological replicates x 3 measurements) with Rotenone as positive control; lifespan analysis of *hlh-2*/M05B5.5 RNAi in **(C)** *eat-2*(ad1116) ($p=0.39$, log-rank test) and **(E)** *lin-32*(u282) ($p<0.0005$, log-rank test) mutant nematodes; **(D)** pharyngeal pumping rates in *hlh-2*/M05B5.5 RNAi-treated WT nematodes ($p=0.60$, Student's t-test, n=8 worms x 3 measurements); **(F)** lifespan analysis of *lin-32*/T14F9.5 RNAi in the presence (purple) or absence (green) of *hlh-2*/M05B5.5 RNAi ($p<0.0001$, log-rank test).

Following the framework of mitohormesis system which depends on both *skn-1* and *aak-2* activity (Schmeisser et al. 2013) we next measured ATP levels in *C. elegans* treated with *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi 48 hours after transferring worms onto the RNAi-harboring bacteria-seeded plates. Both interventions led to statistically significant reduction in ATP compared to control, which supports the lifespan data suggesting that *aak-2* activation is required for the longevity phenotype (Fig. 15b).

Worms put on plates with *hlh-2*/M05B5.5 RNAi-harboring bacteria exhibit higher rates of locomotion speed and exploratory behavior, and often crawl off the bacterial lawn. To exclude the possibility that these animals have longer lifespan due to reduced food intake and the subsequent calorie restriction signaling we used a well-studied mutant deficient for *eat-2*/Y48B6A.4 gene, which codes for a protein functioning in pharyngeal muscle to regulate the rate of pharyngeal pumping (McKay et al. 2004). However this was indeed the case, as *hlh-2*/M05B5.5 RNAi could no longer induce statistically significant lifespan extension in this mutant background (Fig. 15c). To validate this result we measured pumping rate 5 days after start of the RNAi treatment to find out if these animals are indeed consuming less food than the control, but contrary to our expectations did not detect significant differences (Fig. 15d). More stringent, additional experiments (such as measuring the amount of bacteria consumed) may be necessary to rule out the possibility of calorie restriction mediating lifespan extension of *hlh-2*/M05B5.5 RNAi-treated *C. elegans*.

Overall, *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi-mediated phenotypes, though quite similar and producing mostly converging results, differed in a number of assays, namely fertility assay, and lifespan assays in *daf-2*/Y55D5A.5 and *daf-12*/F11A1.3 backgrounds. These 2 transcription factors have been reported as binding each other and functioning as a heterodimer (Portman and Emmons 2000), but it is not clear whether they also mediate lifespan extension while acting together, as part of the same mechanism. To address this question we first used mutant worms deficient for *lin-32*/T14F9.5 and tested whether downregulation of *hlh-2*/M05B5.5 would have additive effect on lifespan, thus showing that *hlh-2*/M05B5.5 works either alone as a homodimer or in combination with another heterodimer partner to affect lifespan in *C. elegans*. Worms on *hlh-2*/M05B5.5 RNAi-harboring bacteria lived 14% longer than control, which

suggested that this was indeed the case (Fig. 15e). Finally, when the two RNAi treatments were combined, the worms fed with the combination of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi-harboring bacteria showed statistically significant lifespan extension when compared to either control or each of the treatments alone, suggesting that lifespan extensions mediated by *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation may have different underlying mechanisms and is not fully explained by clearance of the *hlh-2*/M05B5.5:*lin-32*/T14F9.5 heterodimer (Fig. 15f).

2.2.9 Additional candidates from the RSAT screen, *efl-1* and *ceh-22*, regulate aging rates through known pathways

Three out of the 5 genes identified through the RSAT screen whose RNAi conferred lifespan-shortening effect (*efl-1*/Y102A5C.18, *blmp-1*/F25D7.3, *grh-1*/Y48G8AR.1, *daf-16*/R13H8.1, and *daf-12*/F11A1.3 (Fig. 2)) have already been reported as such and described in literature in detail (Greer et al. 2010; Masse et al. 2005; Pamela L Larsen, Albert, and Riddle 1995). The genes we have therefore focused on were *efl-1*/Y102A5C.18 and *grh-1*/Y48G8AR.1. The latter gene, *grh-1*/Y48G8AR.1, serves as the main research theme for another project and is studied by another member of our group. The former, *efl-1*/Y102A5C.18, has been described to act with other synMuv genes of this class such as *dpl-1* and *lin-35*/Rb to antagonize receptor tyrosine kinase and Ras-mediated signaling (Ceol and Horvitz 2001b). As the RNAi clone had not been available from a library at the time this research was started, we cloned a genomic region corresponding to the largest exon of the gene into the control vector L4440 (for details see Materials and Methods).

Its downregulation significantly reduced lifespan of *C. elegans* (Fig. 16a), while it had no effect on fertility rates – either on the number of eggs or the number of larvae (Fig. 16b). Similarly to the results obtained for *hlh-2*/M05B5.5, *efl-1*/Y102A5C.18 RNAi produced conflicting results concerning its involvement in IIS signaling pathway: while it had no effect on *daf-2*/Y55D5A.5 mutants' lifespan, it significantly shortened the lifespan of mutants deficient for *daf-16*/R13H8.1 (Fig. 16c,d).

Beta-lapachone has been shown to selectively induce apoptosis in cancer cells – without killing healthy cells – by activating S-phase checkpoint and by selective induction of an ortholog of *efl-1*/Y102A5C.18 in mammals, E2F1, a regulator of checkpoint-mediated apoptosis (Y. Li et al. 2003). If *efl-1*/Y102A5C.18 downregulation leads to lifespan shortening, its activation may lead to longevity induction. We tested this possibility by adding 2.5 μM or 25 μM β-Lapachone to the agar of the plates with bacteria. However, contrary to our expectations, the higher dose of the drug led to lifespan reduction, while the lower dose had no statistically significant effect (Fig. 16f). Finally, when applied at an earlier developmental stage (L1) *efl-1*/Y102A5C.18 RNAi still shortened *C. elegans* lifespan, albeit to a lesser degree (Fig. 16e).

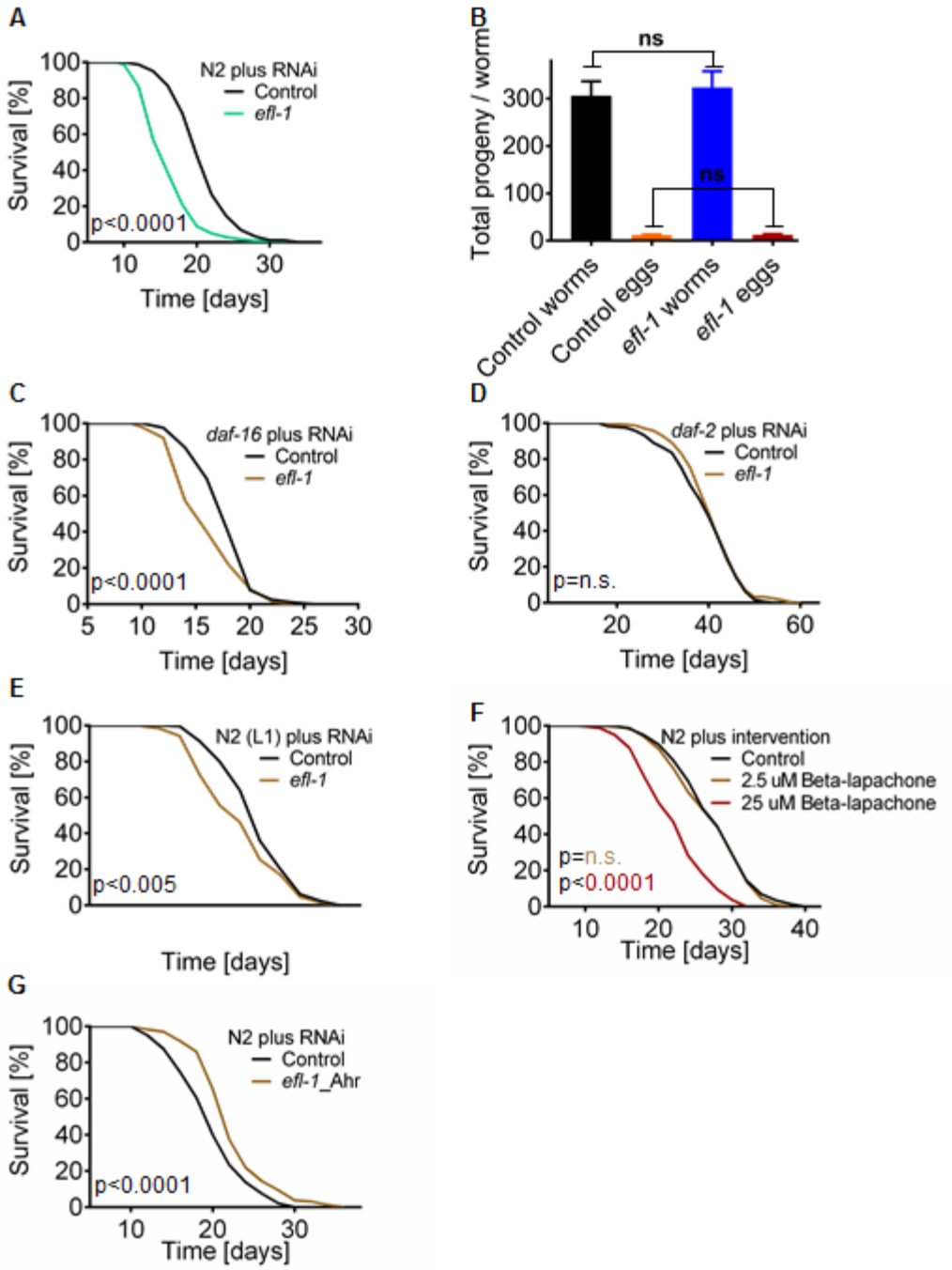

Figure 16: (A) Lifespan analysis of N2 Bristol nematodes treated with *efl-1*/Y102A5C.18 RNAi produced in our lab (p<0.0001, log-rank test) **(B)** fertility rates of nematodes treated with *efl-1*/Y102A5C.18 RNAi (p<0.25, Student's t-test, n=10 worms); Lifespan analysis of *efl-1*/Y102A5C RNAi versus control RNAi in **(C)** *daf-16*(mu86) (p<0.0001, log-rank test), **(D)** *daf-2*(e1370) (p=0.26, log-rank test) mutant nematodes; **(E)** Lifespan analysis of *efl-1*/Y102A5C RNAi versus control RNAi started at L1 stage of development (p<0.005, log-rank test); **(F)** Lifespan analysis of N2 Bristol nematodes treated with 2.5 µM (p=0.29, log-rank test) and 25 µM (p<0.0001, log-rank

test) β-Lapachone; **(G)** Lifespan analysis of N2 Bristol nematodes treated with commercially available *efl-1*/Y102A5C.18 RNAi ($p<0.0001$, log-rank test).

In 2014 Ahringer library of RNAi clones was updated, and *efl-1*/Y102A5C.18 clone became available for purchase. However, when we tested it in a lifespan assay it produced the result opposite to what we had observed previously: it significantly extended *C. elegans* lifespan (Fig. 16g). Moreover, the same result was later reported by another group in connection with *daf-16* regulation, both in *C. elegans* and in a mammalian system (Xie et al. 2014). It is not clear what is responsible for the differences in the effect these 2 RNAi clones have on lifespan; it is conceivable that our lab-produced clone had an off-target effect, or that different isoforms of *efl-1*/Y102A5C.18 are differently targeted by these RNAi clones. The results from the lifespan using β-Lapachone support the notion that *efl-1*/Y102A5C.18 downregulation extends lifespan in *C. elegans*.

We have already given an overview and provided some data on transcription factors with the main function during development that have a large effect on lifespan when downregulated or upregulated post developmentally. One more such transcription factor, *ceh-22*/F29F11.5, has been studied in connection with *hlh-2*/M05B5.5 and fits the same paradigm of the developmental theory of aging. This NK-2 family homeodomain factor with Drosophila homolog TINMAN and vertebrate homolog Nkx2 activates pharyngeal muscle gene expression and is required for normal pharyngeal development (Okkema et al. 1997). Interestingly, *CEH-22* has been reported to act in a common process with *HLH-2* to specify hermaphrodite distal tip cell (hDTC) (Chesney et al. 2009). We have thus aimed to investigate whether *ceh-22*/F29F11.5 RNAi-mediated lifespan extension works through a known lifespan regulating pathway, and whether it works in the same pathway with *hlh-2*/M05B5.5.

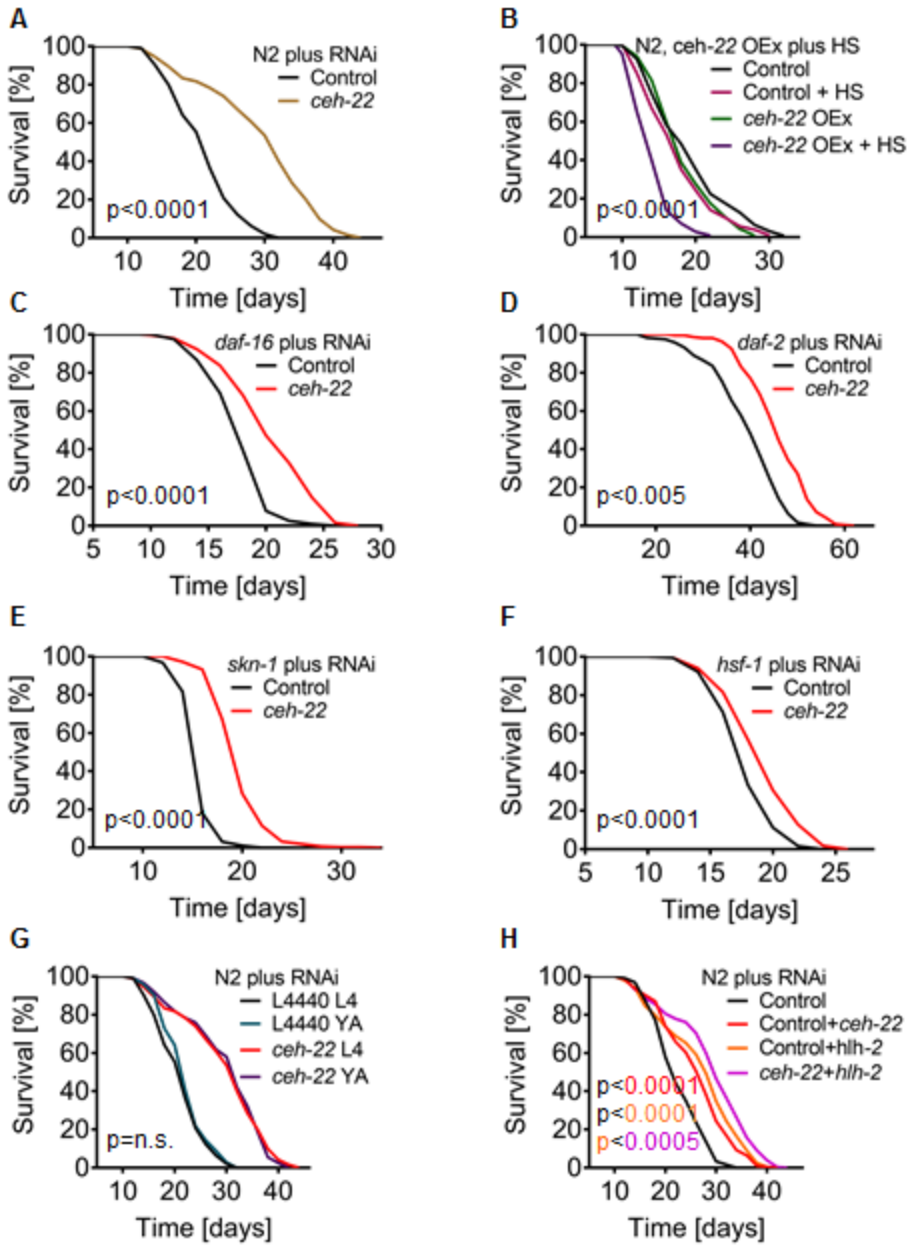

Figure 17: (A) Lifespan analysis of N2 Bristol nematodes treated with *ceh-22*/F29F11.5 RNAi (p<0.0001, log-rank test) **(B)** Lifespan analysis of heat shock (HS) conditional overexpressor of *ceh-22*/F29F11.5 versus control WT nematodes (p<0.0001, log-rank test); Lifespan analysis of *ceh-22*/F29F11.5 RNAi versus control RNAi in **(C)** *daf-16*(mu86) (p<0.0001, log-rank test), **(D)** *daf-2*(e1370) (p<0.005, log-rank test); **(E)** *skn-1*(zu67) (p<0.0001, log-rank test), and **(F)** *hsf-1*(sy441) (p<0.0001, log-rank test) mutant nematodes; **(G)** Lifespan analysis of N2 Bristol nematodes treated with *ceh-22*/F29F11.5 RNAi at L4 versus YA stage of development (p=0.65, log-rank test); **(H)** lifespan analysis of *ceh-22*/F29F11.5 RNAi in the presence (purple) or absence (red) of *hlh-2*/M05B5.5 RNAi (p<0.0001, log-rank test).

In N2 worms *ceh-22*/F29F11.5 downregulation induces at least 20% lifespan extension (Fig. 17a), which suggests that while necessary during pharyngeal development, this gene's activity may be detrimental for adult nematodes. In such case overexpression of this transcription factor at post developmental stage would lead to lifespan shortening. To test this hypothesis we applied short term temperature shift to either wild type N2 worms or heat shock-inducible overexpressor mutants of *ceh-22*/F29F11.5 (strain JK4074, kind gift from the Kimble Lab, (University of Wisconsin–Madison)). In a similar fashion to the experiment with overexpression of *hlh-2*/M05B5.5, heat shock itself had no statistically significant effect on either one of the strains used (Fig. 17b), and the *ceh-22*/F29F11.5 inducible overexpressor strain JK4074 worms have the same lifespan as the control N2 animals. When heat shock was applied, as expected, *ceh-22*/F29F11.5 inducible overexpressor strain worms had statistically significant reduction of lifespan compared to the control animals incubated constantly at 20°C (Fig. 17b).

Having determined the effects of changed expression of the transcription factor *ceh-22*/F29F11.5 we next sought to test whether these effects may be part of an already known pathway reported to modulate longevity in *C. elegans*. The most widely studied intervention comes from the research on insulin/insulin-like growth factor signaling (IIS) pathway involving a receptor *daf-2*/Y55D5A.5, downstream effector age-1/B0334.8 and the TF *daf-16*/R13H8.1. We have applied *ceh-22*/F29F11.5 RNAi at L4 stage to either *daf-16* or *daf-2* mutants and measured their lifespan. The results suggest that *ceh-22*/F29F11.5 RNAi-mediated lifespan extension is completely independent of the IIS pathway, as both *daf-16* or *daf-2* mutant worms had significantly longer lifespan with downregulation of *ceh-22*/F29F11.5 (Fig. 17c,d). The lifespan extension of 26% in *skn-1* mutant background (Fig. 17e) indicates that unlike *hlh-2*/M05B5.5, *ceh-22*/F29F11.5 is not part of ROS signaling pathway. Certain designated transcription factors, and most notably *hsf-1*/Y53C10A.12, regulate the multitude of genes involved in the protein quality control system (John Labbadia and Morimoto 2014). In the absence of *hsf-1*/Y53C10A.12 we can expect that the lifespan-extending effect of treatments that rely on the heat shock response would be abolished. Indeed, after *ceh-22*/F29F11.5 RNAi treatment lifespan

extension in *hsf-1*/Y53C10A.12 mutant background was observed at 8%, compared to over 20% in N2 background, suggesting that this longevity effect is at least partly mediated by *hsf-1*/Y53C10A.12 (Fig. 17f).

CEH-22/Nkx2.5 homeodomain TF is a key regulator of distal tip cell specification indicating the high importance of presence of the functional *ceh-22*/F29F11.5 protein during embryonic development (Lam, Chesney, and Kimble 2006). The RNAi is applied at L4 stage of development, which may interfere with the last developmental changes of the almost adult worms as well. To test the impact of *ceh-22*/F29F11.5 downregulation during different stages of *C. elegans* life we applied the RNAi to synchronized populations of worms at either the usual 4th larval (L4) stage or several hours later when they have become young adults (YA). Similarly to the results obtained while applying *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi, the lifespan extending effect did not significantly differ depending on whether *ceh-22*/F29F11.5 RNAi was used at L4 or YA stage of *C. elegans* development (Fig. 17g).

Transcription factors *CEH-22* and *HLH-2* have been reported to function within the same pathway to specify hermaphrodite distal tip cell (hDTC) in *C. elegans* (Chesney et al. 2009), but it is not clear whether they also mediate lifespan extension while acting together, as part of the same mechanism. To address this question we used combined RNAi treatment and measured lifespan to test for additive effect. The combination of *hlh-2*/M05B5.5 and *ceh-22*/F29F11.5 RNAi induced statistically significant increase in lifespan extension compared to either of the RNAi treatments alone (Fig. 17h), indicating that *hlh-2*/M05B5.5 and *ceh-22*/F29F11.5 downregulation-mediated longevity induction mechanisms likely act independently of each other.

To obtain more information on the downstream targets of *ceh-22*/F29F11.5 transcriptional regulation in an unbiased manner we have performed RNA deep sequencing in worms treated with *ceh-22*/F29F11.5 RNAi for 48 hours starting from the stage of reproductive competence. Resulting DEGs for *ceh-22*/F29F11.5 RNAi samples show a lot of similarity with those of *hlh-2*/M05B5.5 RNAi samples, yet the dominating biological theme from the DEGs of *ceh-22*/F29F11.5 downregulation is distinctly different: innate immunity and defense response. Indeed, 5 out of the 10 top DEGs (namely, *cpr-1*/C52E4.1, *lys-4*/F58B3.1, *lys-7*/C02A12.4, *dod-24*/C32H11.12, and F55G11.8

(www.wormbase.org)) are genes that have protective function against bacteria, and several of them have already been reported to affect lifespan in *C. elegans* (Murphy et al. 2003). It is thus tempting to speculate that strengthening innate immunity and antimicrobial response is what mainly responsible for the lifespan extending effect of *ceh-22*/F29F11.5 RNAi. Further study of this phenotype is needed to pinpoint the precise mechanism and elucidate the downstream effectors of *ceh-22*/F29F11.5 downregulation-induced longevity.

2.3 *argk-1*/F44G3.2 emerges as a downstream effector in the *hlh-2*/M05B5.5-related longevity mechanism

2.3.1 *C. elegans* ortholog of creatine kinase is identified as the top DEG in *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation treatments

Downregulation of *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *ceh-22*/F29F11.5 post developmentally leads to significant lifespan extension of approximately 30% in *C. elegans*. These three transcription factors likely act independently, as shown using combinations of respective RNAi-harboring bacteria (Fig. 15f, Fig. 17h). In order to elucidate the downstream effectors of these genes we performed RNA deep sequencing of whole worms cultured on either control L4440 or the respective RNAi treatments for 48 hours after transferring them onto the fresh plates with bacteria at L4 stage of development. The table below presents top 10 differentially expressed genes (DEGs) from each experiment (Table 3; the full DEGs tables are available in the Appendix).

hlh-2/M05B5.5	*lin-32*/T14F9.5	*ceh-22*/F29F11.5
***argk-1*/F44G3.2**	***argk-1*/F44G3.2**	asp-1/Y39B6A.20
cpr-4/F44C4.3	clec-209/Y19D10A.9	cpr-1/C52E4.1
egl-46/K11G9.4	F56A4.2	cpr-4/F44C4.3
arrd-1/T04B8.3	T24B8.5	lys-4/F58B3.1
cpr-1/C52E4.1	asp-1/Y39B6A.20	lys-7/C02A12.4
lys-4/F58B3.1	cysl-2/K10H10.2	dod-24/C32H11.12
cth-1/F22B8.6	spp-17/C54G6.5	cth-1/F22B8.6
ugt-63/C04F5.7	cth-1/F22B8.6	F22B8.7
fmi-1/F15B9.7	dhs-25/F09E10.3	***argk-1*/F44G3.2**
F21C10.9	icl-1/C05E4.9	F55G11.8

Table 3: Top 10 DEGs in *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *ceh-22*/F29F11.5 downregulated *C. elegans* samples. edgeR and DESeq2 results were overlapped to produce the lists for more stringent selection. The complete table is available in the Appendix.

We have focused on the top DEG of both *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation interventions, namely *argk-1*/F44G3.2. Notably, this gene is also among the top 10 DEGs in the list of *ceh-22*/F29F11.5 deep sequencing results (Table 3). Human orthologs of *argk-1*/F44G3.2 include CKMT2 (creatine kinase, mitochondrial 2), CKM (creatine kinase, M-type), CKB (creatine kinase B) and CKMT1B (creatine kinase, mitochondrial 1B). The product of *argk-1*/F44G3.2 is predicted to have kinase activity, and to be mainly expressed in the intestine of *C. elegans* (Spencer et al. 2011). A recent report also showed that *argk-1*/F44G3.2 is possibly a selective effector of TOR downregulation-mediated longevity, that it shows a limited expression pattern in a small number of cells located mainly in the head and tail but predominantly close to the anterior pharyngeal bulb, and that it functions together with *AAK-2*/AMPK (McQuary et al. 2016). Mammalian orthologs of *argk-1*/F44G3.2 function in the phosphocreatine - creatine kinase (PCr–CK) circuit system that provides energy buffer at sites of high energy utilization when the ATP demand outstrips the rate of production by mitochondrial respiration; that shuttles ATP from mitochondria to the locations of high energetic demands in the cell, and that serves as a sensor of low threshold ADP in order to maintain [ATP]/[ADP] ratio (Greenhaff 2001). In comparison to half a century of accumulating data on CK, relatively little is known about the function of *argk-1*/F44G3.2 in *C. elegans* cells. There are 5 putative arginine kinases identified in *C. elegans* to date (Fraga et al. 2015), with *argk-2*/W10C8.5 and isoform a of *argk-4*/F46H5.3 predicted to have mitochondrial localization by the MitoProt II mitochondrial localization prediction tool (Claros and Vincens 1996).

Deep sequencing and qPCR data showed that “middle aged” and older C elegans have higher expression of *hlh-2*/M05B5.5, and this could potentially be one of the drivers of the age-related decline (Fig. 18a,b). The dynamic of *argk-1*/F44G3.2 expression throughout the lifetime of *C. elegans* is such that starting from post reproductive stage it is expressed less and less (Fig. 18e,f), and based on what we know about the functions

of *argk-1*/F44G3.2 orthologs in mammals we can predict that low levels of *argk-1*/F44G3.2 may also lead to loss of vigor and fitness, - the well reported phenotypes of aging in multiple species.

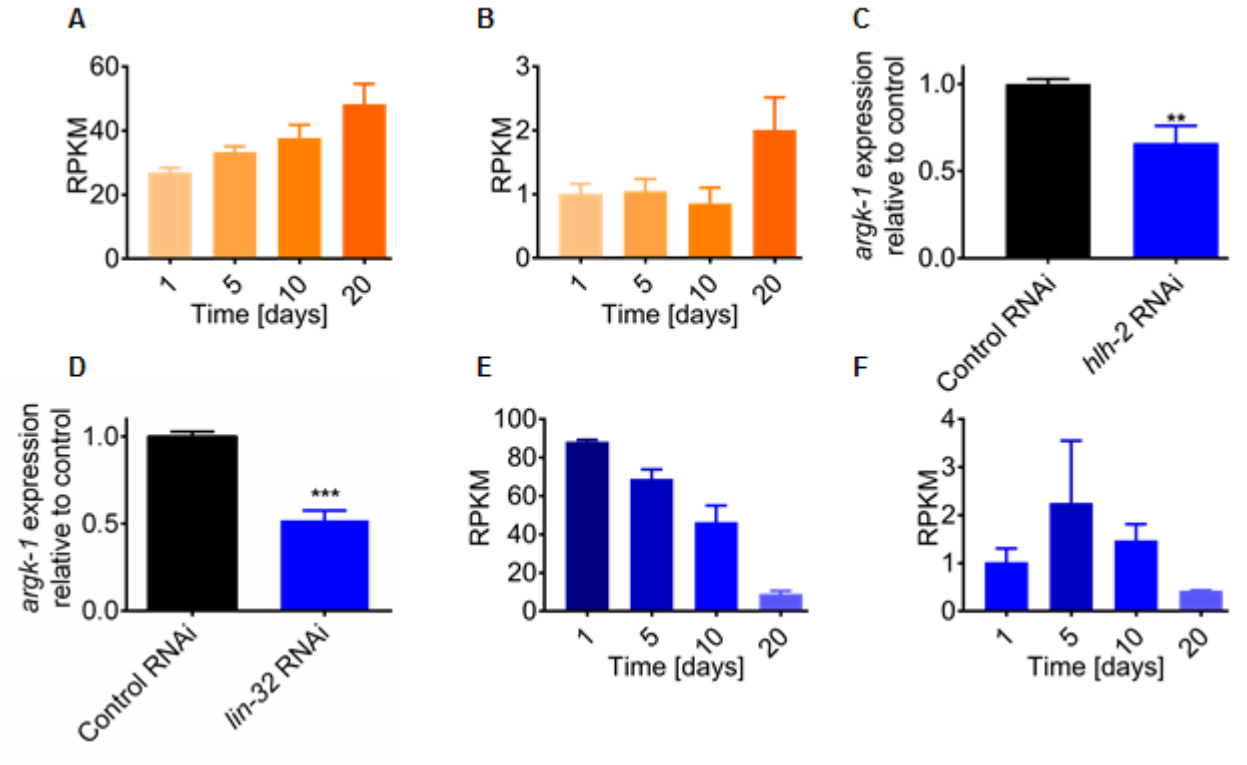

Figure 18: Expression levels of **(A)** *hlh-2*/M05B5.5 and **(E)** *argk-1*/F44G3.2 at 4 time points of *C. elegans* lifetime as output of RNA deep sequencing of WT nematodes; Expression levels of **(B)** *hlh-2*/M05B5.5 and **(F)** *argk-1*/F44G3.2 at 4 time points of *C. elegans* lifetime as measured by RT-qPCR in WT nematodes; Expression levels of *argk-1*/F44G3.2 in nematodes treated with **(C)** *hlh-2*/M05B5.5 ($p<0.001$, Student's t-test, n=3 biological replicates x 3 wells) and **(F)** *lin-32*/T14F9.5 RNAi ($p<0.0001$, Student's t-test, n=3 biological replicates x 3 wells) versus control RNAi.

The *argk-1*/F44G3.2 DEG was identified in both *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation interventions' deep sequencing experiment as the most significantly downregulated mRNA compared to control. In qPCR confirmation experiment we show that *argk-1*/F44G3.2 expression is indeed significantly reduced in both experiments: when either *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi is applied (Fig. 18c,d). How would the direct downregulation during adulthood affect lifespan?

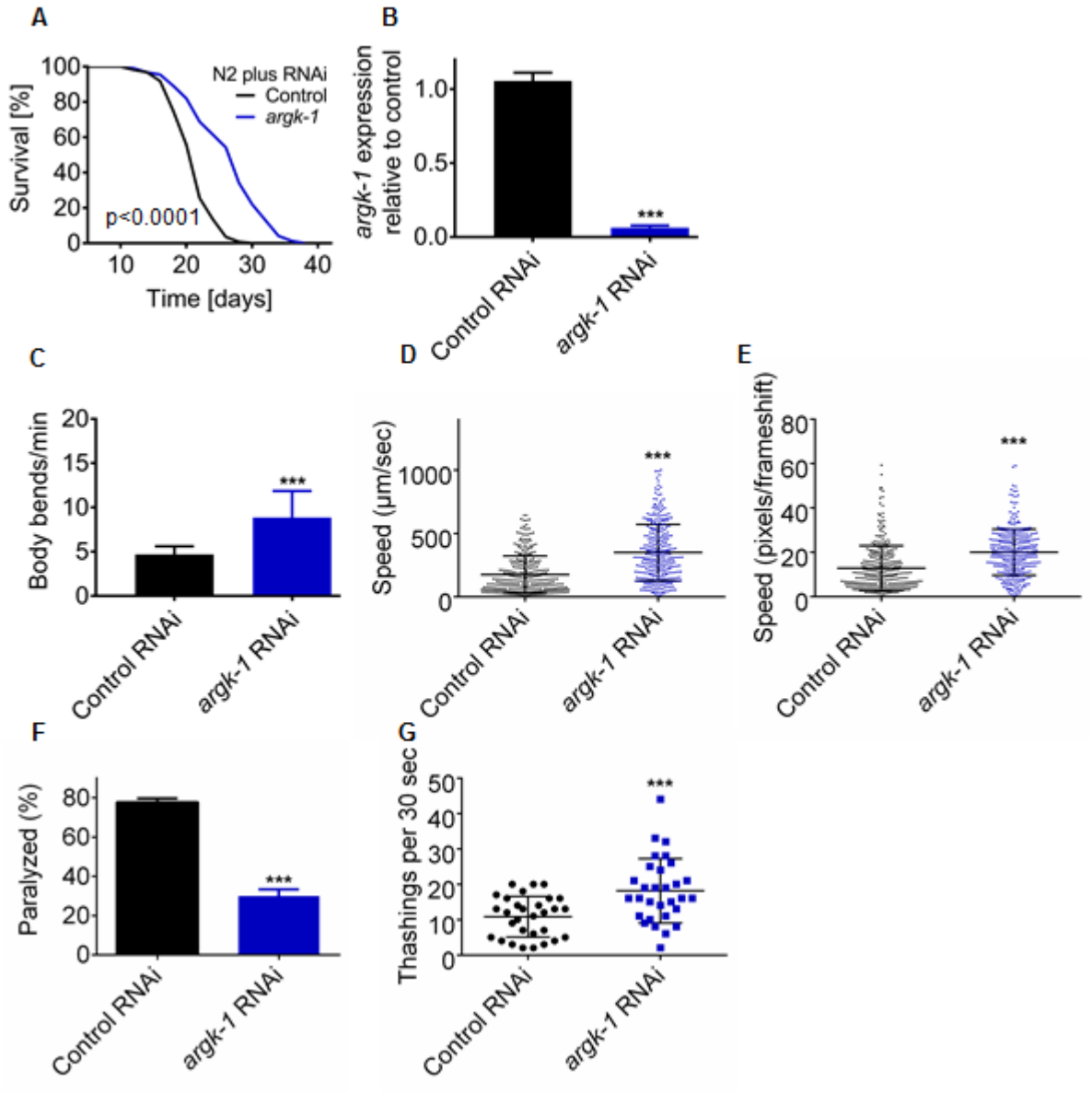

Figure 19: (A) Lifespan analysis of N2 Bristol nematodes treated with *argk-1*/F44G3.2 RNAi (p<0.0001, log-rank test) **(B)** qPCR confirmation of gene knockdown using *argk-1*/F44G3.2 RNAi (p<0.0001, Student's t-test, n=3 biological replicates x 3 wells each) **(C)** Body bends rate of N2 Bristol nematodes treated with *argk-1*/F44G3.2 RNAi (p<0.0001, Student's t-test, n=30 worms) **(D)** average (p<0.0001, Student's t-test, n~400 worms) and **(F)** maximum (p<0.0001, Student's t-test, n~400 worms) speed after *argk-1*/F44G3.2 RNAi gene knockdown **(F)** percentage of paralyzed AM44 rmIs190 [F25B3.3p::Q67::CFP] mutant worms treated with *argk-1*/F44G3.2 RNAi (p<0.0001, Student's t-test, n=30 worms) **(G)** thrashing rates per 30 sec in *argk-1*/F44G3.2 RNAi-treated worms (p<0.0001, Student's t-test, n=30 worms).

2.3.2 *argk-1*/F44G3.2 downregulation extends lifespan and improves health parameters in *C. elegans*

The lifespan assay confirmed that RNAi-mediated knockdown of *argk-1*/F44G3.2 leads to significant lifespan extension (Fig. 19a), and the downregulation has been confirmed by qPCR (Fig. 19b). To go further and provide evidence of increases in healthspan - as some long-lived mutants' usefulness has been questioned on the grounds that the lifespan increase may just be due to the extended period of aging-associated disability (Hansen and Kennedy 2016) - we have tested if *argk-1*/F44G3.2 downregulation would increase fitness of *C. elegans*. For this purpose we first measured worm body bends per minute, a useful assay to assess youthfulness of nematodes expressed in movement of the head of a worm in a half sigmoidal wave that produces forward movement and/or results in a bend in the body that moved halfway down the length of the animal (C. Huang, Xiong, and Kornfeld 2004). RNAi-mediated knockdown of *argk-1*/F44G3.2 led to a significant increase in body bends rate 5 days after transfer onto the RNAi plates (Fig. 19c). We have additionally measured average and maximum locomotion speed at the same time, 5 days after starting RNAi treatment, and found significant increases for both parameters, which would suggest better health and higher vigor associated with *argk-1*/F44G3.2 downregulation (Fig. 19d,e).

In addition, we tested whether the increase in fitness observed in wild-type N2 worms would also be maintained in the strain AM44 rmIs190 [F25B3.3p::Q67::CFP] - *C. elegans* neurodegenerative diseases model in which polyQ proteins are expressed throughout the nervous system (Alexander et al. 2014). Many human neurodegenerative diseases are caused by the expansion of glutamine (Q, encoded by CAG) repeats (Brignull et al. 2006), and it has been shown that the nematode models' paralysis effects correlate with age and are affected by aging-modulating interventions such as downregulation of insulin signaling (Alexander et al. 2014). There were more than twice more paralyzed animals on control RNAi compared to the *argk-1*/F44G3.2 RNAi-treated worms 8 days after transfer onto the RNAi plates (Fig 19f). Moreover, thrashing rates (a

well-established assay where nematodes are placed in a drop of buffer, and the frequency of lateral swimming ("thrashing") movements is estimated) of *argk-1*/F44G3.2 RNAi-treated AM44 mutant worms were significantly higher than those of the control, suggesting that formation of aggregates and the associated decrease in health and fitness are alleviated with *argk-1*/F44G3.2 downregulation (Fig. 19g).

2.3.3 Downregulation of *argk-1*/F44G3.2 improves proteostasis and associated resistance to paralysis

To test how quickly can *argk-1*/F44G3.2 knockdown affect protein aggregation-associated impairment we utilized another *C. elegans* Alzheimer's disease model system comprised of mutants expressing human β-amyloid peptide (Aβ). These strains (CL802, CL4176 and CL2006) are useful due to their short life span, ability to develop muscle-associated deposits, and the concomitant progressive paralysis phenotype (Wu and Luo 2005). In the rapid paralysis system *argk-1*/F44G3.2 RNAi treatment had a significant but modest effect and increased average number of moving worms (Fig. 20b). Rapid paralysis animals (CL4176) normally completely stop all movement 60 hrs after temperature shift from 15°C to 20°C, and this short period of time may not be sufficient for the RNAi system to have its full potential effect. It is also possible that the effect of *argk-1*/F44G3.2 RNAi cannot fully stop such precipitous decline in function. The slow paralysis strain (CL2006), on the contrary, showed considerable beneficial effect of *argk-1*/F44G3.2 downregulation (Fig. 20c).

Alleviation of functional impairment induced by polyQ-aggregation or β-amyloid deposits suggests that proteasome-mediated targeted protein degradation, or autophagy - systems responsible for proteostasis maintenance in the cell - has been activated. To test this possibility we have analyzed the less specific system of protein quality control, namely autophagy, and used confocal microscopy imaging to detect lgg-1/C32D5.9-tagged autophagosome foci in a popular *C. elegans* autophagy reporter (Palmisano and Meléndez 2016), 5 days after transfer onto either control L4440 or *argk-1*/F44G3.2 RNAi. The imaging was done by our laboratory member Giovanna Grigolon.

As shown in the confocal microscopy images (Fig. 20d,e), there is a slight increase in GFP signal in the form of puncta in the worms treated with *argk-1*/F44G3.2 RNAi, which suggests elevated rates of autophagy in these animals. We have already noted this point when describing visualization of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi-treated nematodes previously: autophagy activation cannot be definitively confirmed in these samples because precise quantification of the puncta would require uniform background difficult to obtain using this method.

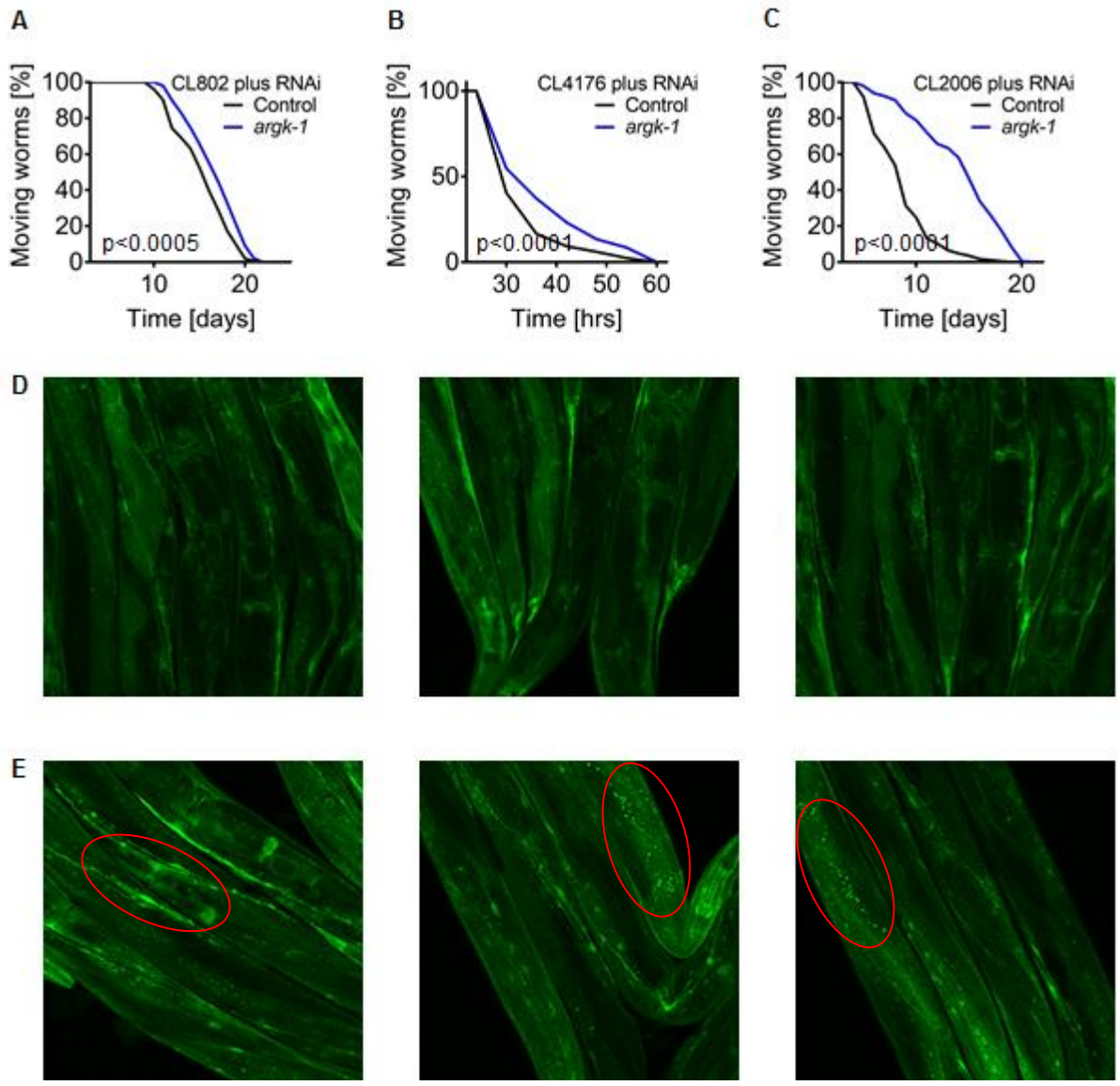

Figure 20: Lifespan analysis of *argk-1*/F44G3.2 RNAi in **(A)** control (p<0.0005, log-rank test), **(B)** rapid paralysis (p<0.0001, log-rank test), and **(C)** slow paralysis (p<0.0001, log-rank test) mutant nematodes; Fluorescent puncta visualize GFP-fused lgg-1/C32D5.9 incorporated into autophagosomes, and indicate autophagy activation in nematodes fed with **(D)** control RNAi and **(E)** *argk-1*/F44G3.2 RNAi.

Moreover lgg-1/C32D5.9 marker we used for the visualization of autophagy is only suggestive: an increase in the number of autophagosomes does not necessarily reflect an induction of autophagy (D. Klionsky and Deretic 2011); to show autophagy activation with more certainty assays to infer the turnover of autophagosomes in the presence and

absence of lysosomal degradation are recommended, which can help verify an increase in functional autophagy (Palmisano and Meléndez 2016).

2.3.4 *argk-1*/F44G3.2 RNAi-mediated lifespan extension is likely induced by all arginine kinase paralogs

We next compared the effects of constitutive change of expression on lifespan, as opposed to the RNAi applied at adulthood, and studied paralogs of *argk-1*/F44G3.2 because of high level of sequence similarity and possibly similar/overlapping functions among all of them (Table 4).

	F11G11.13	F44G3.2	F32B5.1	W10C8.5	F46H5.3a	ZC434.8
F11G11.13	100	53.22	80	50.92	49.36	67.8
F44G3.2	53.22	100	80	77.84	61.15	61.16
F32B5.1	57.37	80	100	97.62	65.54	63.27
W10C8.5	50.92	77.84	97.62	100	64.09	62.69
F46H5.3a	49.36	61.15	65.54	64.09	100	66.36
ZC434.8	67.8	61.16	63.27	62.69	66.36	100

Table 4: This Percent Identity Matrix visualizes sequence similarity among 6 putative paralogs of arginine kinase in *C. elegans*. Sequence data were downloaded from the database www.wormbase.org on 22.03.2018.

We used available mutants of *argk-1*/F44G3.2, *argk-2*/W10C8.5, *argk-3*/F32B5.1, and *argk-4*/F46H5.3, and backcrossed them to the wild type N2 *C. elegans* to avoid potential off-target effects of mutagenesis. Lifespan assays conducted using all these knockout strains of *argk-1*/F44G3.2, *argk-2*/W10C8.5, *argk-3*/F32B5.1, and *argk-4*/F46H5.3 (lifespans kindly performed by a former member of our lab Agnieszka Kaszlikowska) showed no change in longevity except a minor lifespan extension effect in

argk-2/W10C8.5 mutant worms (Fig. 21a,b,c,d), confirming previously published data on arginine kinase mutants' longevity (McQuary et al. 2016).

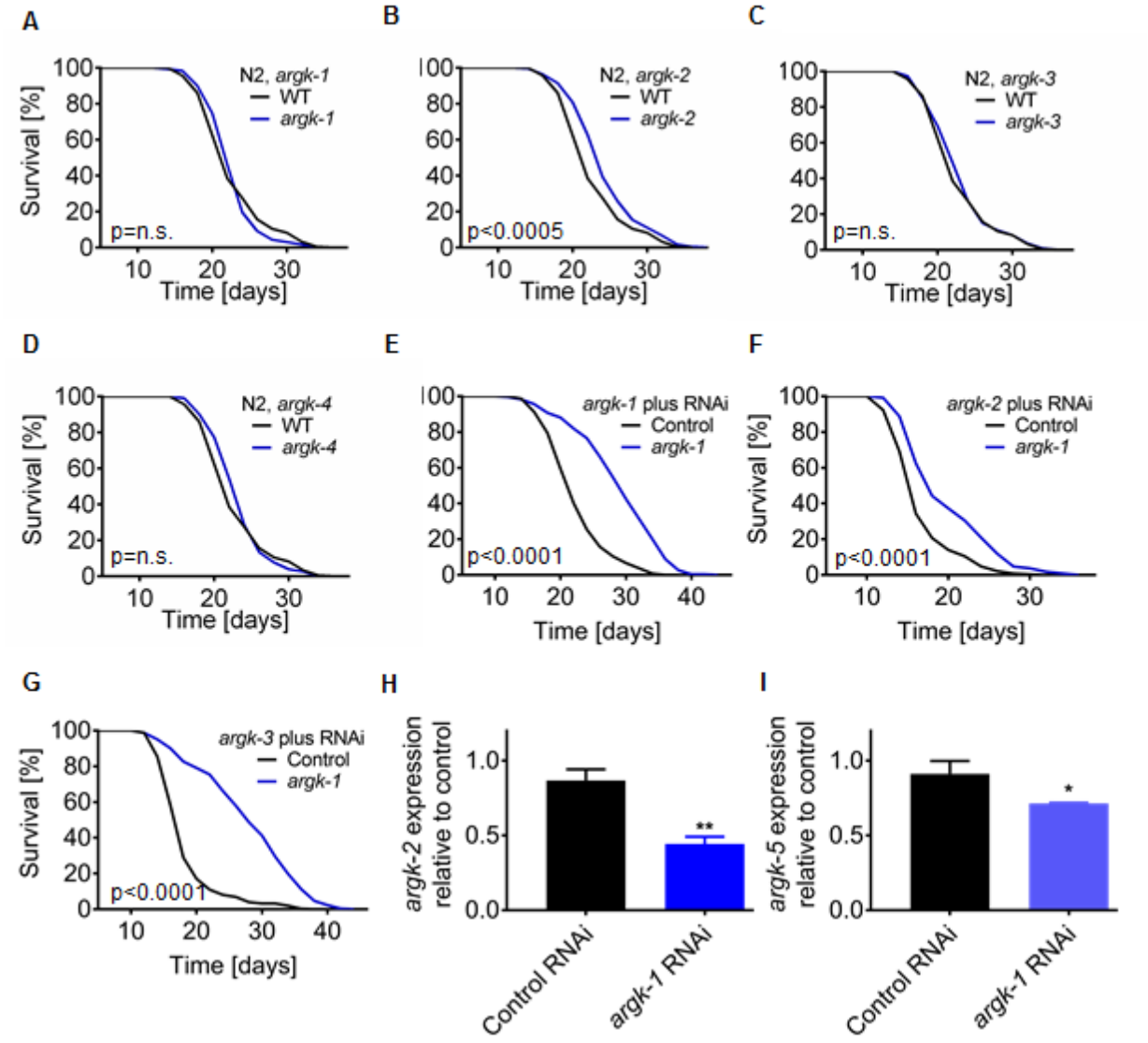

Figure 21: Lifespan analysis of nematodes deficient for **(A)** *argk-1*(ok2993) (p=0.58, log-rank test) **(B)** *argk-2*(ok2723) (p<0.0005, log-rank test) **(C)** *argk-3*(tm3937) (p=0.73, log-rank test) and **(D)** *argk-4*(ok3620) (p=0.41, log-rank test) versus WT worms; Lifespan analysis of *argk-1*/F44G3.2 RNAi versus control RNAi in **(E)** *argk-1*(ok2993) (p<0.0001, log-rank test), **(F)** *argk-2*(ok2723) (p<0.0001, log-rank test), and **(G)** *argk-3*(tm3937) (p<0.0001, log-rank test) mutant nematodes; Expression levels of **(H)** *argk-2*/W10C8.5 (p<0.001, Student's t-test, n=3 biological replicates x 3 wells) and **(F)** *argk-5*/ZC434.8 (p<0.05, Student's t-test, n=3 biological replicates x 3 wells) in WT nematodes treated with *argk-1*/F44G3.2 RNAi versus control RNAi.

As mentioned previously, there is considerable sequence similarity among arginine kinase paralogs in *C. elegans* (Table 4), and there is a possibility that the longevity effect of *argk-1*/F44G3.2 RNAi is actually mediated by a different gene other than *argk-1*/F44G3.2. We applied *argk-1*/F44G3.2 RNAi to the mutants of *argk-1*/F44G3.2, *argk-2*/W10C8.5, and *argk-3*/F32B5.1, and found that all three assays showed significant lifespan extension, with *argk-1*/F44G3.2 downregulation having the most pronounced effect in *argk-3*/F32B5.1 mutant animals (Fig. 21g), and notably also considerably increasing lifespan of *argk-1*/F44G3.2 mutants (Fig. 21e). When testing how specifically *argk-1*/F44G3.2 RNAi downregulates arginine kinase paralogs we also observed reduced expression of *argk-2*/W10C8.5 and *argk-5*/ZC434.8 (Fig. 21h,i), and while we haven't tested the expression levels of *argk-3*/F32B5.1 and *argk-4*/F46H5.3, these results suggest that *argk-1*/F44G3.2 RNAi likely impairs all 5 paralogs.

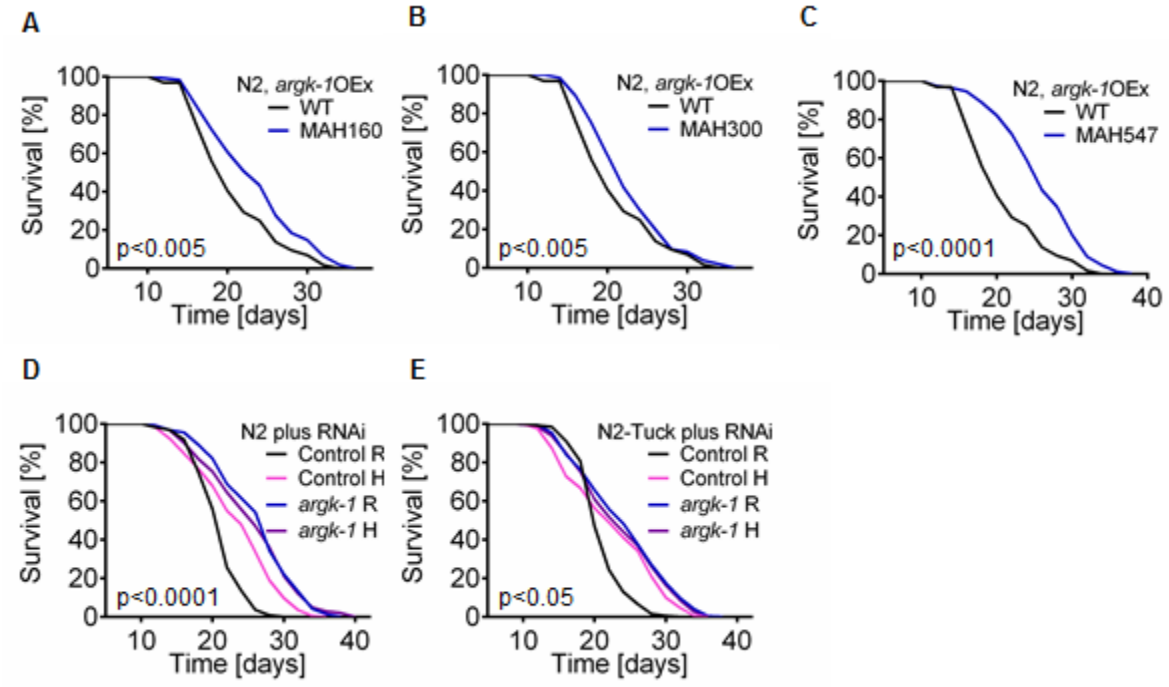

Figure 22: Lifespan analysis of **(A)** MAH160 (p<0.005, log-rank test), **(B)** MAH300 (p<0.005, log-rank test), and **(C)** MAH547 (p<0.0001, log-rank test) strains of *argk-1*/F44G3.2 overexpressor mutants versus WT nematodes; lifespan analysis of *argk-1*/F44G3.2 RNAi (clone from Malene Hansen lab) versus control RNAi (clone from Malene Hansen lab) in **(D)** N2 WT nematodes (p<0.0001, log-rank test), and **(E)** N2-Tuck WT nematodes (p<0.05, log-rank test).

2.3.5 Published reports of *argk-1*/F44G3.2 in relation to lifespan in *C. elegans* provide conflicting evidence

Downregulating *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 during adulthood leads to lifespan extension in *C. elegans*, and their conditional overexpression results in shortened lifespan. RNAi-mediated *argk-1*/F44G3.2 knockdown also extends lifespan, and we asked if overexpressing it may reduce longevity of nematodes. In the absence of a conditional heat-shock overexpressing strain we used constitutive overexpressor mutants under either a native promoter or a ubiquitously expressed sur-5 promoter. Contrary to our expectations, and confirming previously published results for these strains (McQuary et al. 2016), they had significantly longer lifespan than the control WT worms (Fig. 22a,b,c). Taken together, these results suggest that the enhanced longevity conferred by *argk-1*/F44G3.2 knockdown stems from a stress response-like reaction and not from the main function of the protein which appears to be rather contributing to *C. elegans* healthspan. On a side note, pharyngeal pumping data from the above-mentioned publication (McQuary et al. 2016) also indicate that at least one *argk-1*/F44G3.2 overexpressing strain may experience dietary restriction effect, which in turn possibly mediates lifespan extension.

Interestingly, the same publication (McQuary et al. 2016) had one phenotype that we couldn't replicate, namely *argk-1*/F44G3.2 RNAi having no statistically significant effect on *C. elegans* lifespan. Throughout repeated experiments performed by different members of the lab we consistently saw longer lifespan of *argk-1*/F44G3.2 RNAi- versus control RNAi-treated worms. To mutually understand what is responsible for this discrepancy we shared our wild type strain of *C. elegans* and the RNAi clones we used, and received in turn N2-Tuck (the WT strain used in the experiments for that publication), and the control L4440 and the *argk-1*/F44G3.2 RNAi clones used in the Hansen laboratory. Firstly, we observed considerable difference in lifespan between our control L4440 empty vectors-treated worms and the Hansen (H) control animals, which had significantly longer lifespan (Fig. 22d). While there was no difference between the lifespans of worms on our and Hansen *argk-1*/F44G3.2 RNAi, the difference between the controls alone reduced the lifespan-extending effect of *argk-1*/F44G3.2 downregulation

from 25% to only 8% (Fig. 22d). When N2-Tuck, the alternative WT *C. elegans* strain used in the Hansen lab was taken for the lifespan assay, lifespan-extending effect of *argk-1*/F44G3.2 RNAi compared to our control empty RNAi vector decreased to only 15%, while with the Hansen control RNAi there was no statistically significant lifespan extension (Fig. 22e), which essentially recapitulated the data from the publication (McQuary et al. 2016). We therefore concluded that the large difference we see between the published *argk-1*/F44G3.2 RNAi lifespan assay and those we conducted is completely explained by the combination of differences between, first, our control animals, and second, our control empty vector-harboring bacteria. Given the widespread use of the standard N2 strain available from *Caenorhabditis* Genetics Center (CGC), together with the more normally distributed Type II survivorship curve of the lifespan assay we commonly observe using our control RNAi – which should be the case for WT *C. elegans* under normal conditions – we would maintain the validity of our results.

2.3.6 *argk-1*/F44G3.2 downregulation-mediated lifespan extension depends on known longevity pathways

Having determined the effects of changed expression of *argk-1*/F44G3.2 on *C. elegans*' phenotypes we next sought to test whether these effects may be part of an already known pathway reported to modulate longevity in nematodes. We have omitted the most well-studied insulin/insulin-like growth factor signaling (IIS) pathway involving *daf-2*/Y55D5A.5 and *daf-16*/R13H8.1 as we didn't observe dependence on this pathway while investigating the proposed upstream effectors *hlh-2*/M05B5.5 and *lin-32*/T14F9.5. Lifespan extension mediated by *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 depended on transcription factor *hsf-1*/HSF1, therefore we tested lifespan of worms with *argk-1*/F44G3.2 knockdown during adulthood in *hsf-1*/Y53C10A.12-deficient animals and saw that the lifespan-extending effect of *argk-1*/F44G3.2 RNAi is abolished completely (Fig. 23a), suggesting that it is fully dependent on *hsf-1*/Y53C10A.12 signaling.

In addition to *hsf-1*/HSF1, lifespan-extending effect of *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation was also dependent on *aak-2*/AMPK, the *C. elegans*

ortholog of the conserved cellular energy sensor AMP-activated protein kinase (AMPK) necessary for IIS-mediated lifespan extension, that restores energy homeostasis by, among other things, stimulating catabolic processes (Hardie, Ross, and Hawley 2012). When *argk-1*/F44G3.2 RNAi was applied in *aak-2*/T01C8.1 mutant worms to check involvement of this energy sensing switch in the lifespan extension effect, we observed that it was completely abolished (Fig. 23b), indicating that *argk-1*/F44G3.2 RNAi-mediated lifespan extension fully depends on *aak-2*/T01C8.1 activity.

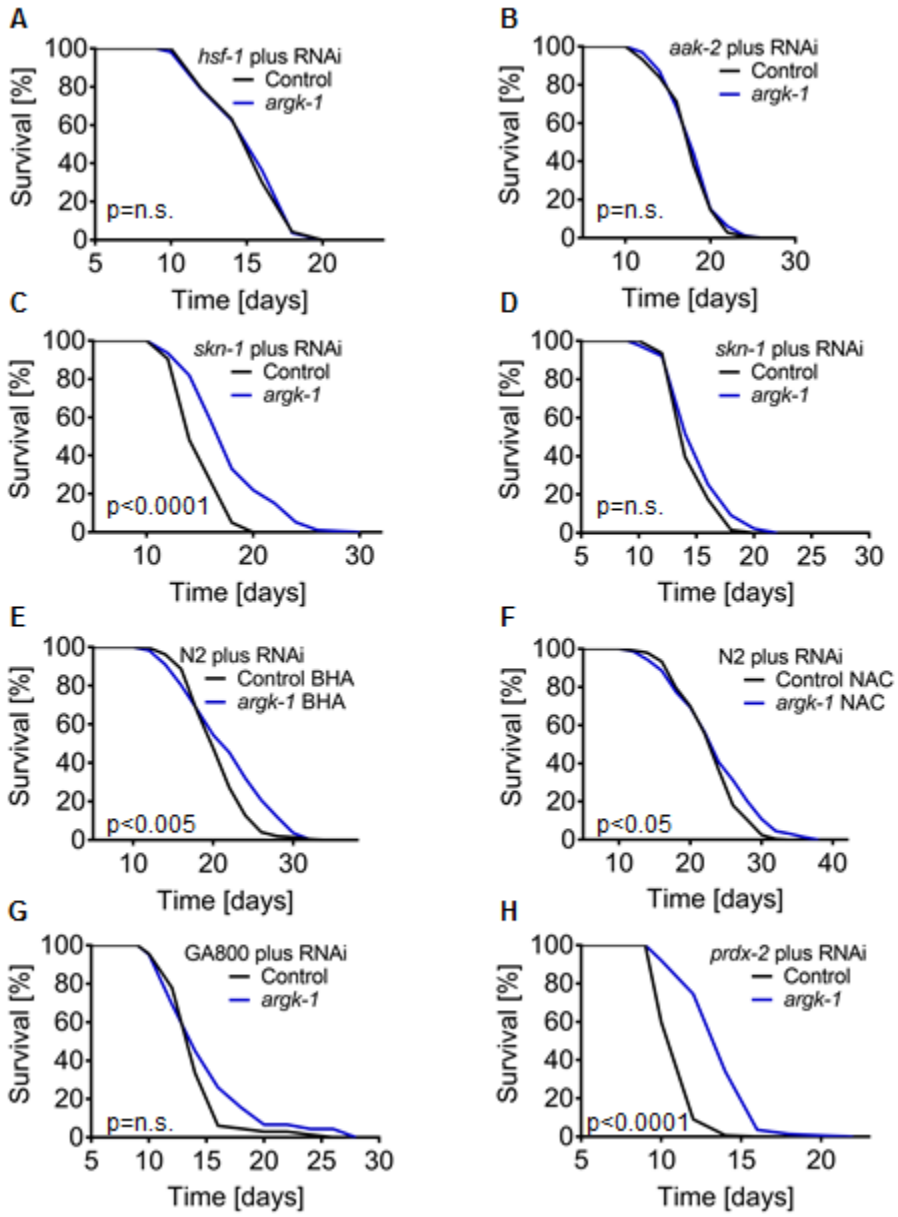

Figure 23: Lifespan analysis of *argk-1*/F44G3.2 RNAi versus control RNAi in **(A)** *hsf-1*(sy441) (p=0.66, log-rank test), **(B)** *aak-2*(ok524) (p=0.31, log-rank test), **(C)** *skn-1*(zu135) (p<0.0001, log-rank test), and **(D)** *skn-1*(zu67) (p=0.72, log-rank test); Lifespan analysis of *argk-1*/F44G3.2 RNAi versus control RNAi in the presence of **(E)** BHA (p<0.005, log-rank test) and **(F)** NAC (p<0.05, log-rank test); Lifespan analysis of *argk-1*/F44G3.2 RNAi versus control RNAi in **(G)** catalase overexpressing mutant (p=0.09, log-rank test) and **(H)** *prdx-2*(gk169) (p<0.0001, log-rank test) mutant nematodes.

Given such dependence on *aak-2*/AMPK, which is in line with the data obtained during the *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 downregulation experiments, as well as the results we saw previously in regards to *skn-1*/NRF2 signaling and the related ROS-dependent stress response phenotypes, we next tested if *argk-1*/F44G3.2 RNAi would have an effect on lifespan in the two *skn-1*/NRF2 mutant strains we used before, namely EU1[zu67] and EU31[zu135]. As noted in the description of our previous set of experiments using these two strains, two of the *skn-1*/T19E7.2 isoforms, *skn-1*a and *skn-1*c, are expressed and act in the intestine to mediate stress response (An and Blackwell 2003; Tullet et al. 2008), and are knocked out in the EU1[zu67] mutants, while the third isoform, *skn-1*b, is required for dietary restriction-induced lifespan extension and functions in two sensory neurons (Bishop and Guarente 2007), and it is also knocked out in addition to the other 2 isoforms in the EU31[zu135] mutant strain of *skn-1*/T19E7.2. When applied to *skn-1*(zu135) mutants, which lack all 3 isoforms of *skn-1*/T19E7.2, *argk-1*/F44G3.2 RNAi showed lifespan extension of 18%, precisely recapitulating the result on *hlh-2*/M05B5.5 RNAi (but not *lin-32*/T14F9.5 RNAi)(Fig. 23c). In the EU1[zu67] mutants lacking the *skn-1*a and *skn-1*c isoforms expressed in the intestine, we recorded no statistically significant lifespan extension, supporting the data on *hlh-2*/M05B5.5 RNAi and suggesting that *argk-1*/F44G3.2 is in fact a downstream effector of *hlh-2*/M05B5.5 downregulation-mediated longevity, and that the effect depends on *skn-1*/T19E7.2-mediated stress response in the intestine (Fig. 23d).

2.3.7 *argk-1*/F44G3.2 downregulation-mediated lifespan extension requires ROS signaling and likely involves mitochondrial dysfunction

Having confirmed the involvement of both *aak-2*/AMPK and *skn-1*/NRF2 in both *hlh-2*/M05B5.5 and *argk-1*/F44G3.2 downregulation-mediated longevity, we aimed to further test whether mitohormesis and ROS signaling may be required for this phenotype. We added a precursor to the antioxidant glutathione, N-acetylcysteine (NAC), or an antioxidant butylated hydroxyanisole (BHA), to the plates seeded with either control or *argk-1*/F44G3.2 RNAi-harboring bacteria, and measured lifespan to test whether addition of antioxidants would interfere with ROS signaling and the lifespan extension effect. With addition of BHA we observed 5% lifespan extension in *argk-1*/F44G3.2 RNAi samples compared to control, and NAC added to plates reduced the longevity phenotype of *argk-1*/F44G3.2 downregulation to 3% with $p<0.05$ (Fig. 23e,f), supporting our hypothesis. Moreover, we saw no statistically significant lifespan extension with *argk-1*/F44G3.2 RNAi in the catalase-overexpressing strain GA800, which is in line with the reasoning that catalases would disrupt ROS signaling required for longevity (Fig. 23g). However, similarly to the case of *hlh-2*/M05B5.5 downregulation, we still observed 22% lifespan extension in *prdx-2*-mutated worms when *argk-1*/F44G3.2 RNAi was applied (Fig. 23h), excluding the possibility that *prdx-2* is required for this longevity phenotype.

Slowing down the development has been shown to extend healthy lifespan in nematodes (Wong, Boutis, and Hekimi 1995; Feng, Bussière, and Hekimi 2001), albeit not all such mutations that slowed development, behaviors, and reproduction have been explained in relation to how exactly they extend lifespan. Even though the “rates of living” / metabolic rates have been reported to not be responsible for a number of such long-lived mutants (Brys, Vanfleteren, and Braeckman 2007), the general principle matches the framework of Developmental theory of aging. We have shown that slowed physiological rates may be implicated in *hlh-2*/M05B5.5 RNAi-mediated longevity before (Fig. 13e), and thus sought to test if *argk-1*/F44G3.2 might also function through the same pathways.

Lifespan assay in *clk-1*/ZC395.2-deficient *C. elegans* showed 22% lifespan extension on *argk-1*/F44G3.2 RNAi (Fig. 24a), in line with the results obtained on

hlh-2/M05B5.5 and *lin-32*/T14F9.5 RNAi (Fig. 24c,d). Applying *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi to the mutants of *isp-1*/F42G8.12 (another aging-modulating gene that affects behavior and the rates of physiological processes like reproduction and development, and codes for a subunit of the mitochondrial complex III in the mitochondrial membrane) (Feng, Bussière, and Hekimi 2001) led to 6% and no statistically significant longevity induction, respectively (Fig. 13e,f). Downregulation of *argk-1*/F44G3.2 led to only 4% lifespan extension compared to control (Fig. 24b), further implicating mitochondrial involvement in this longevity phenotype and closely matching the data obtained for *hlh-2*/M05B5.5 downregulation.

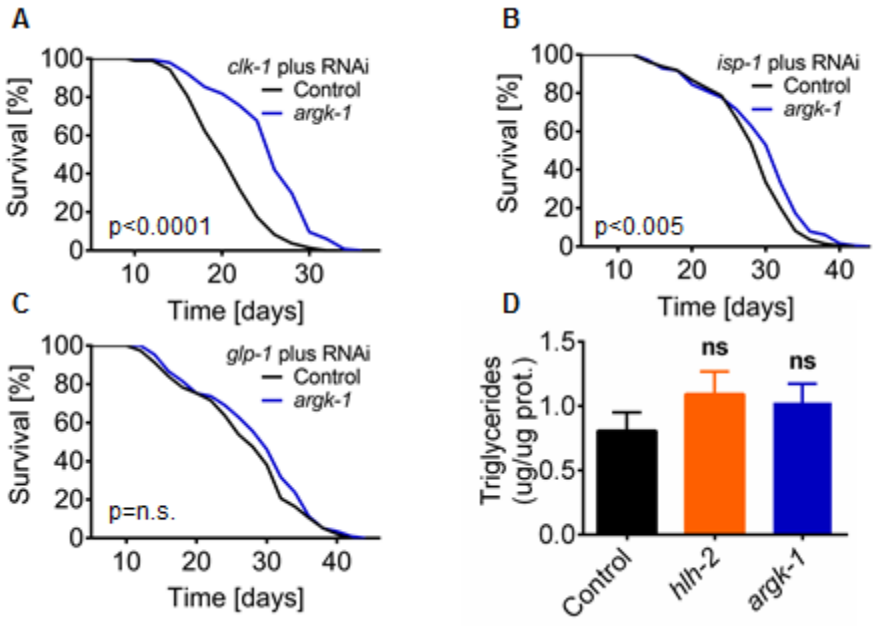

Figure 24: Lifespan analysis of *argk-1*/F44G3.2 RNAi versus control RNAi in **(A)** *clk-1*(qm30) (p<0.0001, log-rank test), **(B)** *isp-1*(qm150) (p<0.005, log-rank test), and **(C)** *glp-1*(e2141) (p=0.08, log-rank test); **(D)** effect of *hlh-2*/M05B5.5 RNAi (p=0.10, Student's t-test, n=3 worm pellets) and *argk-1*/F44G3.2 RNAi (p=0.14, Student's t-test, n=3 worm pellets) regarding fat content.

Lifespan extension by *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi was also abolished in *glp-1*/F02A9.6 mutant animals (Fig. 14e,f), implicating the germline endocrine-mediated lifespan regulation, with *argk-1*/F44G3.2 knockdown having the same effect of no statistically significant lifespan extension (Fig. 24c).

It has been shown previously that long-lived mutants lacking a germline have activated autophagy and mediate longevity signals through the lipase LIPL-4 activity (Lapierre et al. 2011). We measured triglyceride content in worms treated with *hlh-2*/M05B5.5 and *argk-1*/F44G3.2 RNAi but found no statistically significant differences compared to control, and rather a trend for an increase than a decrease (Fig. 24d). At the same time it is known that worms deficient for IIS signaling pathway genes, such as *daf-2* mutants, have higher fat content than wild type animals (Gerisch et al. 2001). It is likely that cells with fat stores produce signals that affect longevity, but as it is technically challenging to isolate such cells from *C. elegans*, the results obtained from general triglyceride assays produce information that is often difficult to interpret (Lemieux and Ashrafi 2016).

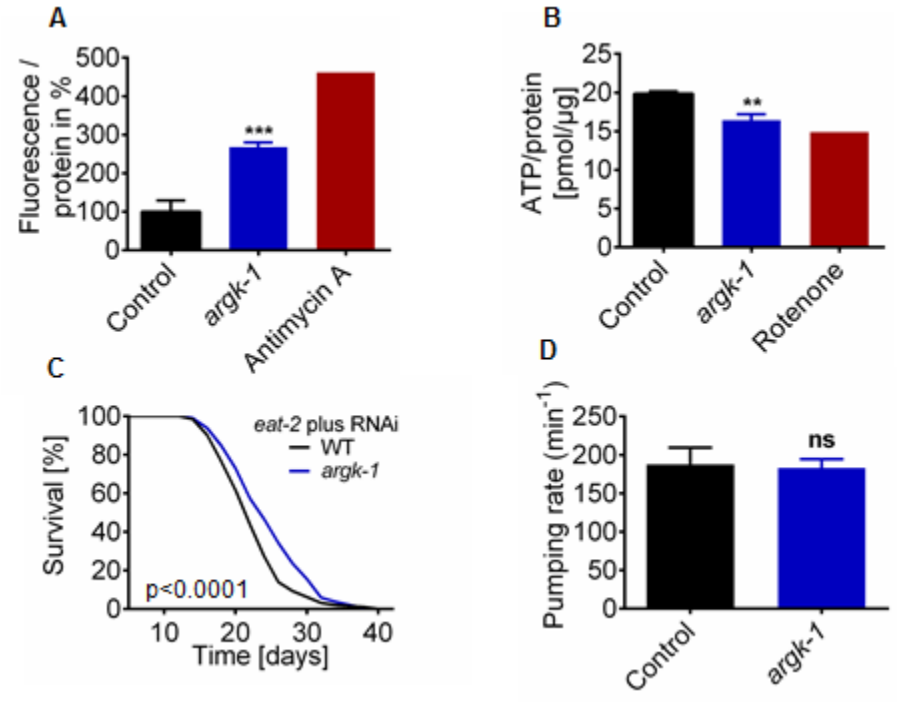

Figure 25: (A) Quantification of hydrogen peroxide in WT worms treated with control or *argk-1*/F44G3.2 RNAi (p<0.0001, Student's t-test, n=3 biological replicates x 3 measurements) with Antimycin A as positive control; **(B)** ATP determination in WT worms treated with control or *argk-1*/F44G3.2 RNAi (p<0.001, Student's t-test, n=3 biological replicates x 3 measurements) with Rotenone as positive control **(C)** lifespan analysis of *argk-1*/F44G3.2 RNAi in *eat-2*(ad1116)

(p<0.001, log-rank test) mutant nematodes; **(D)** pharyngeal pumping rates in *argk-1*/F44G3.2 RNAi-treated WT nematodes (p=0.30, Student's t-test, n=8 worms x 3 measurements).

We next sought to test the rest of the phenotypes recorded for *hlh-2*/M05B5.5 downregulation in *argk-1*/F44G3.2 RNAi-treated worms. Having obtained data suggesting ROS signaling involved in *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *argk-1*/F44G3.2 downregulation-mediated lifespan extension (namely, antioxidant treatment strongly reduces lifespan extension, and catalase overexpression background abolishes it completely; all three RNAi interventions partly depend on *skn-1* activity) we next measured ROS levels 48 hours after transferring worms onto the *argk-1*/F44G3.2 RNAi-harboring bacteria-seeded plates. As expected, *argk-1*/F44G3.2 RNAi-treated worms produced significantly more hydrogen peroxide detected in the supernatant after incubation (Fig. 25a). In line with the same paradigm of mitohormesis system which depends on both *skn-1* and *aak-2* activity (Schmeisser et al. 2013) we next measured ATP levels in *C. elegans* treated with *argk-1*/F44G3.2 RNAi 48 hours after transferring worms onto the RNAi-harboring bacteria-seeded plates. Downregulation of *argk-1*/F44G3.2 led to statistically significant reduction in ATP compared to control, which further supported the lifespan data indicating that *aak-2* activation is required for the longevity phenotype (Fig. 25b).

As noted before, nematodes put on plates with *hlh-2*/M05B5.5 RNAi-harboring bacteria exhibit higher rates of locomotion speed and exploratory behavior, often crawling off the bacterial lawn, and the same is true for *argk-1*/F44G3.2 RNAi-treated animals (data not shown). To exclude the possibility that these animals have longer lifespan due to reduced food intake and the subsequent calorie restriction signaling we used a well-studied mutant deficient for *eat-2*/Y48B6A.4 gene, which codes for a protein functioning in pharyngeal muscle to regulate the rate of pharyngeal pumping (McKay et al. 2004). Mutants deficient for *eat-2*/Y48B6A.4 had only 9% lifespan extension compared to control, suggesting that this may, at least in part, be the case (Fig. 25c). In order to validate this result we also measured pumping rate at 5 days of adulthood to find out if these animals were indeed consuming less food than the control, but contrary to our expectations we did not detect significant differences (Fig. 25d). It would be prudent to

say, however, that more stringent, additional experiments (such as measuring the amount of bacteria consumed) may be necessary to completely rule out the possibility of calorie restriction mediating lifespan extension of *hlh-2*/M05B5.5 and *argk-1*/F44G3.2 RNAi-treated *C. elegans*.

2.3.8 *hlh-2*/M05B5.5 and *argk-1*/F44G3.2 downregulation result in multiple common phenotypes and may function in the same pathway

To summarize the role of *argk-1*/F44G3.2 in relation to the *hlh-2*/M05B5.5 downregulation-mediated lifespan extension, the following data were obtained. First of all, *argk-1*/F44G3.2 has been identified as the most differentially expressed gene in the deep sequencing experiment of the worms treated with control or *hlh-2*/M05B5.5 RNAi for 48 hours during adulthood. Downregulation of *argk-1*/F44G3.2 produced many phenotypes that closely match those of *hlh-2*/M05B5.5 downregulation (Table 5).

At the same time we found levels of other paralogs of *argk-1*/F44G3.2 to be downregulated when *argk-1*/F44G3.2 RNAi is applied due to high levels of sequence similarity among them (Table 5), (Fig. 21h,i), such that when *argk-1*/F44G3.2 RNAi is applied to *argk-1*/F44G3.2-deficient *C. elegans* there is still statistically significant and considerable lifespan extension (Fig. 21e).

To further establish the connection between *hlh-2*/M05B5.5 and *argk-1*/F44G3.2 as its downstream effector we performed additional assays. We applied *argk-1*/F44G3.2 RNAi to the *hlh-2*/M05B5.5 overexpressing strain of *C. elegans* to help untangle the relationship between the two. In case *argk-1*/F44G3.2 is downstream of *hlh-2*/M05B5.5 we could expect that downregulation of *argk-1*/F44G3.2 would negate the effect of *hlh-2*/M05B5.5 overexpression and still considerably extend lifespan beyond the control without heat shock. If, however, the two are independent and act in parallel we might expect the usual lifespan-extending effect of *argk-1*/F44G3.2 which would just return the longevity to its control levels. In fact, this is what we observed, with *hlh-2*/M05B5.5 overexpression reducing lifespan by 25%, and *argk-1*/F44G3.2 RNAi having 26% lifespan increase, bringing the lifespan curve to the control values (Fig. 26a).

Phenotype	RNAi	
	***hlh-2*/M05B5.5**	***argk-1*/F44G3.2**
Lifespan extension	+	+
Increased body bends rate	+	+
Increased average speed	+	+
Increased maximum speed	+	+
Increased thrashing rate	+	+
Reduced paralysis rate	+	+
Decreased ATP content	+	+
Depends on *aak-2* (AMPK)	86%	100%
Increased ROS levels	+	+
Lifespan extension abolished with antioxidants	71%	90%
Depends on *skn-1* (Nrf2)	66%	100%
Depends on *hsf-1* (Nrf2)	74%	100%
No change in pharyngeal pumping rate	+	+
No significant effect on triglyceride content	+	+
Increased autophagy reporter signal	+	+
Lifespan extension abolished in catalase overexpressor mutants	100%	100%
Depends on *isp-1*	83%	84%
Depends on *glp-1*	100%	100%

Table 5: Common phenotypes between nematodes treated with *hlh-2*/M05B5.5 RNAi and *argk-1*/F44G3.2 RNAi.

In addition, we performed epistasis lifespan assays by combining *argk-1*/F44G3.2 RNAi with either *hlh-2*/M05B5.5 or *lin-32*/T14F9.5 RNAi-harboring bacteria at equal dilution to check for an additive effect of the combination, but did not see further extension of lifespan in either one of the combined treatments (Fig. 26b,c).

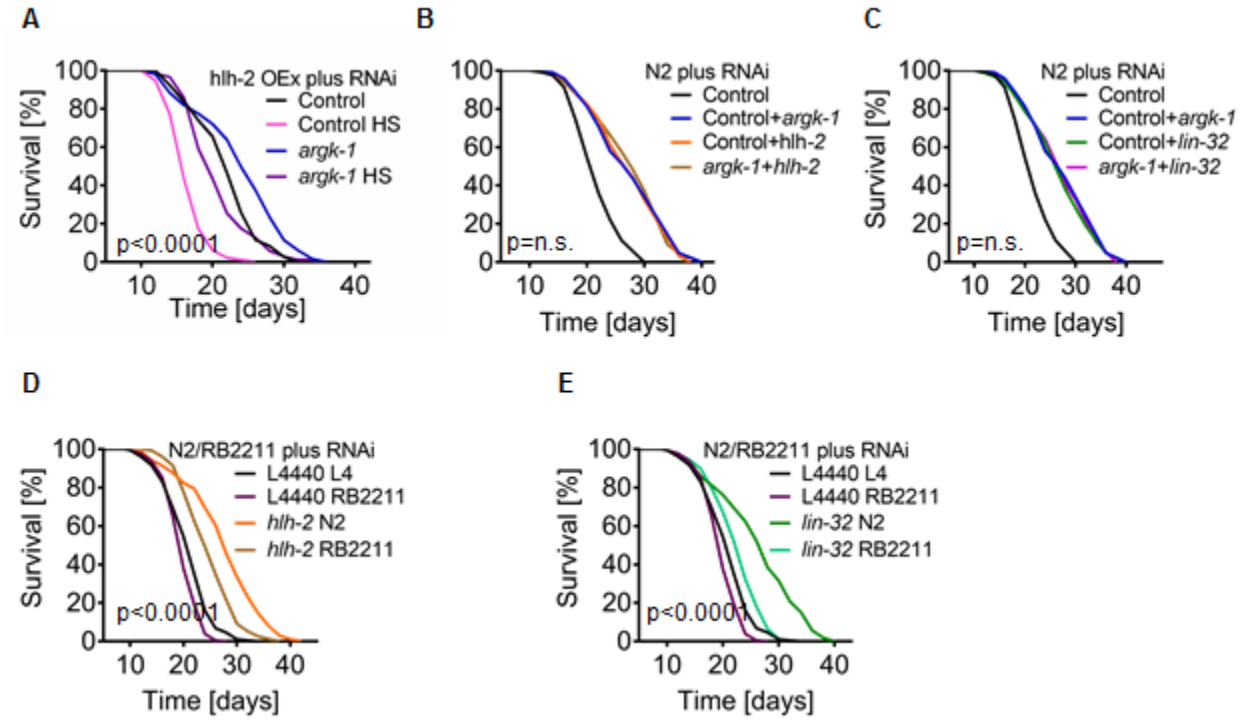

Figure 26: (A) Lifespan analysis of heat shock (HS) conditional *hlh-2*/M05B5.5 overexpressor nematodes treated with *argk-1*/F44G3.2 RNAi versus control RNAi (p<0.0001, log-rank test); **(B)** lifespan analysis of *argk-1*/F44G3.2 RNAi in the presence (brown) or absence (blue) of *hlh-2*/M05B5.5 RNAi (p=0.43, log-rank test); **(C)** lifespan analysis of *argk-1*/F44G3.2 RNAi in the presence (dark red) or absence (blue) of *lin-32*/T14F9.5 RNAi (p=0.15, log-rank test); **(D)** lifespan analysis of *hlh-2*/M05B5.5 RNAi versus control RNAi in WT N2 (p<0.0001, log-rank test) and *argk-1*/F44G3.2 mutant nematodes (p<0.0001, log-rank test); **(E)** lifespan analysis of *lin-32*/T14F9.5 RNAi versus control RNAi in WT N2 (p<0.0001, log-rank test) and *argk-1*/F44G3.2 mutant nematodes (p<0.0001, log-rank test).

It is important to note, however, that RNAi dilution effect has been reported to often take place and change effectiveness of gene knockdown (Rea, Ventura, and Johnson 2007; Kamath et al. 2000), and in fact has repeatedly been observed in our lab by several lab members (data not shown), such that in the absence of additive effect it is still advisable to perform additional experiments to verify the epistasis, for instance in mutant

animals. We tested both *lin-32*/T14F9.5 and *hlh-2*/M05B5.5 RNAi in the *C. elegans* strain deficient for *argk-1*/F44G3.2. Lifespan extension effect of *lin-32*/T14F9.5 downregulation was reduced from 26% to 14%, suggesting that *argk-1*/F44G3.2 is responsible for about half of the *lin-32*/T14F9.5 RNAi's effect on longevity (Fig. 26e). In contrast, *hlh-2*/M05B5.5 downregulation had the almost full effect of 25% lifespan extension compared to the 32% in wild type animals (Fig. 26d), which suggests that no more than a quarter of the *hlh-2*/M05B5.5 RNAi's lifespan extending effect can be attributed to *argk-1*/F44G3.2 downregulation, and other targets remain to be identified.

Finally, we propose the following model for the part of the lifespan-extending effect that can be attributed to *argk-1*/F44G3.2 as the downstream effector of *hlh-2*/M05B5.5 downregulation (Fig. 27).

hlh-2
↓
argk-1
↓
ATP
⊥
aak-2 (*AMPK*)
↓
ROS
↓
hsf-1 (HSF1), *skn-1* (NRF2)
↓
Healthspan

Figure 27: Summary of the hypothesized mechanism for *hlh-2*/M05B5.5- and *argk-1*/F44G3.2-mediated longevity. Downregulation of *hlh-2*/M05B5.5 leads to downregulation of *argk-1*/F44G3.2, which in turn results in a transient decrease in ATP levels. Energy sensor *aak-2*/AMPK activates transcription factors *hsf-1*/HSF1 and *skn-1*/NRF2 through ROS signaling, which upregulate protective mechanisms of the organism and ultimately lead to extension of lifespan and to the associated improvements in health parameters in nematodes.

Other pathways likely responsible for the *hlh-2*/M05B5.5 RNAi-mediated lifespan extension are TOR (with *glp-1* inhibiting TOR signaling and promoting autophagy and longevity (Sheaffer, Updike, and Mango 2008; Lapierre et al. 2011) and mitochondrial ROS production that triggers upregulation of sod-3 and sod-5 superoxide dismutases and leads to increases in physiologic rates and lifespan (Dues et al. 2017).

2.4 TCF3/E2A shows senescence-modulating activity with potential implications for organismal aging

2.4.1 Mammalian ortholog of *hlh-2*/M05B5.5 is an important transcriptional regulator of development and lymphopoiesis

We have established health-promoting effects of *hlh-2*/M05B5.5 downregulation at post-developmental stage in *C. elegans* nematodes, along with the most likely downstream pathways responsible for the increases in healthspan and lifespan of worms. To test the possibility that this mechanism may be evolutionarily conserved we decided to study the mammalian ortholog of *hlh-2*/M05B5.5, Tcf3 (E2A Immunoglobulin Enhancer Binding Factors E12/E47).

This gene encodes a member of the class I E family of basic helix-loop-helix (bHLH) transcription factors, and *hlh-2*/M05B5.5 was determined to be the only *C. elegans* E (class I) protein based on sequence homology and dimerization with Class II bHLH proteins (Krause, Greenwald, and Fire 1997). E proteins are transcription activators: they bind to regulatory E-box sequences on target genes as either heterodimers or homodimers, and *hlh-2*/M05B5.5 was reported as being able to form both (Sallee and Greenwald 2015). In humans, TCF3/E2A encodes two bHLH proteins, E12 and E47, which form through alternative splicing and contain different bHLH domains (Murre, Mccaw, and Baltimore 1989). The E proteins are ubiquitously expressed and can form heterodimers with various bHLH proteins, such as tissue-specific bHLH proteins like MyoD (Lassar et al. 1991) and broadly expressed inhibitory proteins like Id1 (Benezra et al. 1990). Several reports have shown that TCF3/E2A proteins take part in tissue-specific regulation by forming heterodimers with tissue-specific bHLH proteins (Murre et al. 1989; Lassar et al. 1991). TCF3/E2A regulates many developmental patterning processes such as central nervous system (CNS) and muscle development, and lymphopoiesis (Quong, Romanow, and Murre 2002; Massari and Murre 2000). Gene knockout studies showed that deletion of the TCF3/E2A gene resulted in a high frequency of postnatal death, sterility of the surviving TCF3/E2A KO females, and deficiency in B-lineage formation

(Bain et al. 1994; Zhuang et al. 1998). However, it was also shown that most tissue types and organs can still develop when TCF3/E2A gene is mutated (Massari and Murre 2000).

TCF3/E2A activity is regulated on transcriptional, as well as post-translational levels: when Notch is downregulated TCF3/E2A promotes the B cell lineage (Massari and Murre 2000), and E47 protein is degraded downstream of activated Notch in B cells (Nie et al. 2003; King et al. 2007). It is also known that hyper activity of TCF3/E2A, mediated either through dominant mutation or through loss of its negative regulator, is frequently associated with Burkitt's lymphoma (Schmitz et al. 2012). Moreover, TCF3/E2A transcription factor activates expression of p21, a target gene of p53 and an inhibitor of cell cycle progression, while depletion of TCF3/E2A impairs the cell cycle arrest response (Andrysik et al. 2013).

2.4.2 Strategy to use mammalian cell culture system to investigate effects of TCF3 impairment on senescence parameters

It has recently been shown that not only is TCF3 a close ortholog of *hlh-2*/M05B5.5, E47 protein encoded by TCF3/E2A is a functional ortholog of HLH2 protein in *C. elegans* (Sallee and Greenwald 2015). We have thus sought to investigate whether conditional knockout of TCF3/E2A may promote health effects similar to the *hlh-2*/M05B5.5 downregulation in nematodes. There are several Tcf3 knockout mouse models available, with most showing such phenotypes as postnatal lethality, premature death, and infertility (Bain et al. 1994; Zhuang, Soriano, and Weintraub 1994; Zhuang et al. 1998; K. Kwon et al. 2008), recapitulating the phenotypes we observed in *C. elegans* when *hlh-2*/M05B5.5 RNAi was applied during development or at the egg stage. We therefore used the tamoxifen-induced conditional knockout model mice (K. Kwon et al. 2008), and extracted embryonic fibroblasts with the aim to use these cells for metabolism and aging-related assays.

The strategy of conditional mutagenesis was as follows: the authors of the original publication created the construct with the floxed TCF3 allele that had loxP sites inserted upstream of exon 17 and downstream of exon 19, thus producing a Tcf3 null allele by

facilitating Cre-mediated excision of the two alternatively spliced exons 18 which code for the E12 and E47 DNA-binding domains (Zhuang, Soriano, and Weintraub 1994; K. Kwon et al. 2008)(Fig. 28).

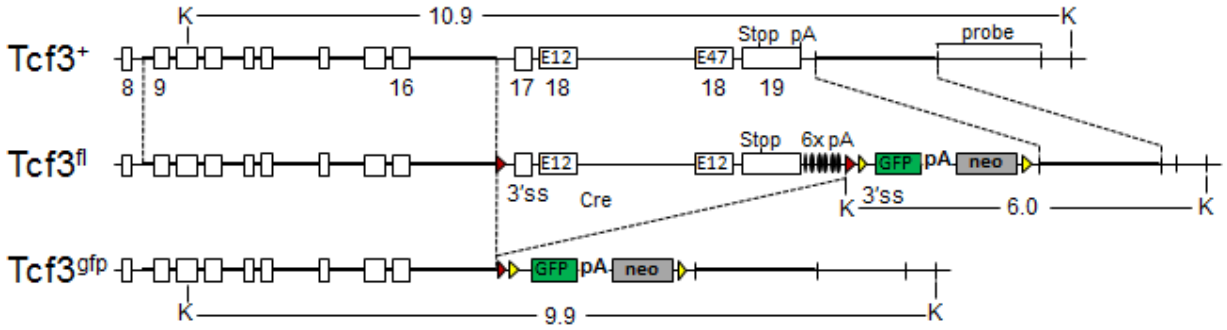

Figure 28: Structure of the targeted Tcf3 locus. The numbered exons are shown as open boxes. The two alternative exons 18 code for the DNA-binding domain of the E12 and E47 isoforms. Six copies of the SV40 polyadenylation (pA) region were inserted together with a Gfp gene downstream of the last Tcf3 exon 19. LoxP and frt sites are indicated by red and yellow arrowheads, respectively. The KpnI (K) fragment, which is indicative of each Tcf3 allele, is shown together with its length (in kb). (Adapted from Kwon *et al*., Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. Immunity, 2008).

A Gfp gene was inserted at the 3′ end of the Tcf3 in the way that Cre-mediated inactivation would lead to the expression of a TCF3-GFP fusion protein, with the six copies of the SV40 polyadenylation region downstream of the last Tcf3 exon preventing RNA splicing to the Gfp exon before the Cre-mediated deletion takes place. We crossed heterozygous $Tcf3^{fl/fl}$ mice with transgenic $CreER^{T2}$ lines to obtain $Tcf3^{fl/fl}CreER^{T2}$ embryos for fibroblast extraction, and added 4-hydroxytamoxifen (4-OHT) to a subset of cells for 7 days, with or without simultaneous treatment with an antioxidant precursor NAC to test if the effects of the knockout may be mediated through ROS, as was the case in the experiments with the homologous *hlh-2*/M05B5.5 in *C. elegans* (Fig. 12a,b).

Treatment	Genotype	
	WT CreER^{T2}	Tcf3$^{fl/fl}$ CreER^{T2}
--/(vehicle)		
NAC/(vehicle)		
--/4-OHT		
NAC/4-OHT		

Table 6: Experimental setup of Tcf3 KO in MEF. Cells homozygous for Tcf3 with loxP sites around exons 17 through 19 and positive for CreER^{T2} were selected for the experiment, with Tcf3 wild type and CreER^{T2} positive cells serving as control. Vehicle for 4-OHT was ethanol, and NAC was diluted in medium.

After 7 days of 4-OHT treatment a subset of cells was harvested and total protein was extracted for immunoblotting experiments. To confirm Tcf3 KO we used polyclonal antibody against TCF3, but initially saw the result opposite to the expected: TCF3 bands were observed only in the samples where Tcf3 KO was induced by addition of 4-OHT, either with or without treatment with NAC (Fig. 29a). As can be seen in the last blot (Fig. 29a) weaker bands are also present at slightly lower position of the expected 68 kDa molecular weight of TCF3. Authors of the original publication where this construct was first used speculated that when fused to GFP, TCF3 may be stabilized through disruption of homodimerization, tertiary structure, or post-translational modifications, and thus produce stronger signal; they also observed slight upward band shift of the GFP-fused protein in Tcf3 KO samples (K. Kwon et al. 2008). Indeed, when loading was adjusted, we could see the band shift quite clearly (Fig. 29b). To confirm that the KO takes place we conducted several additional immunoblotting experiments before moving on to test other proteins (Fig. 29c).

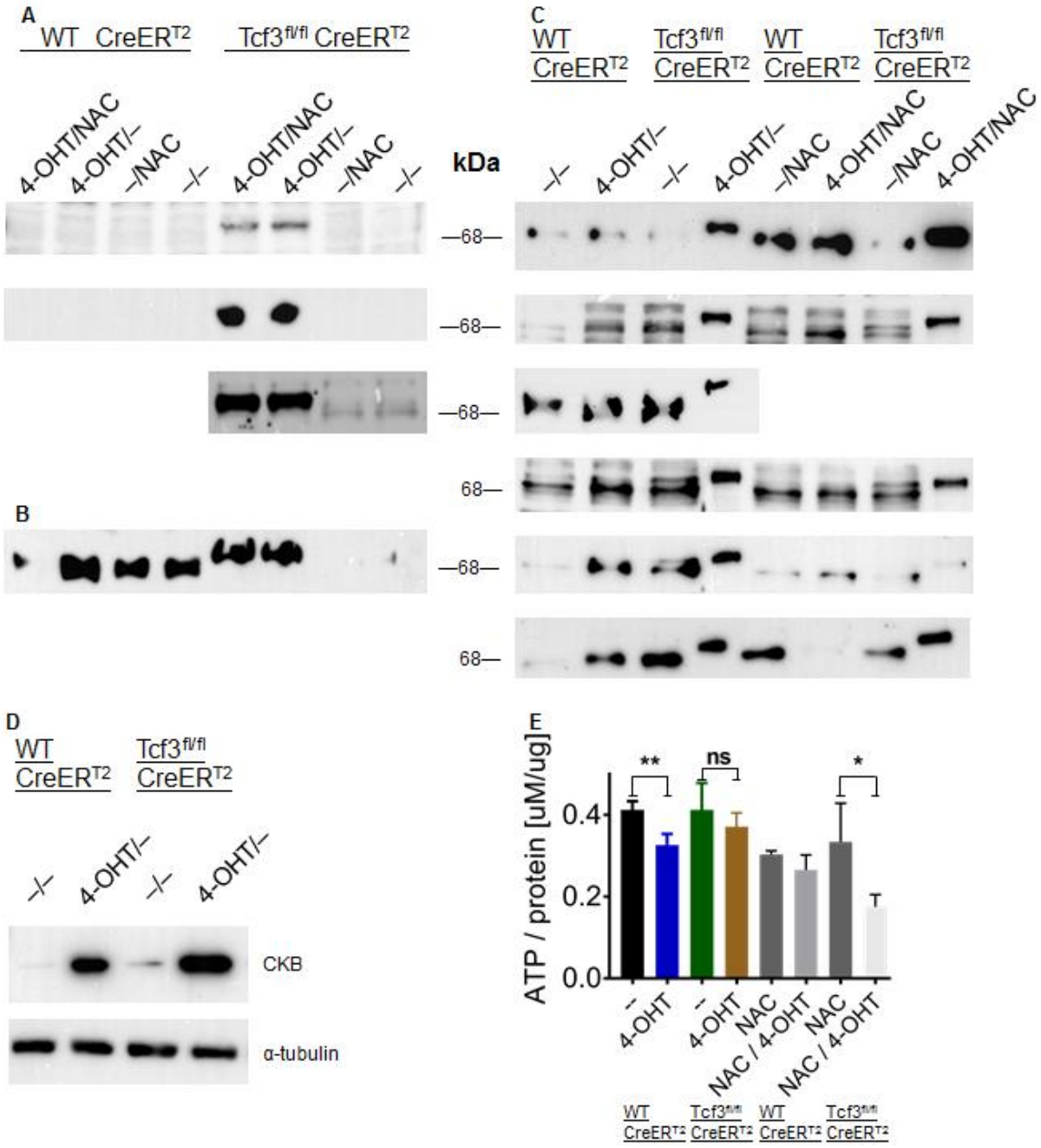

Figure 29: Immunoblot analysis. A polyclonal TCF3 antibody detects **(A)** only the TCF3-GFP fusion protein only in cells cultured from CreER^{T2} Tcf3$^{fl/fl}$ mice and treated with 4-OHT; **(B)** TCF3 and TCF3-GFP with a visible band shift; **(C)** TCF3 and TCF3-GFP with a visible band shift, while loading is still not equalized. **(D)** Immunoblot analysis: polyclonal CKB antibody produces stronger signal in 4-OHT-treated cells, and especially in the Tcf3 knockout cells; **(E)** ATP determination in 4-OHT-treated versus untreated cells (p=0.31, one way ANOVA, n=4 cell plates).

2.4.3 Tcf3 KO does not recapitulate many phenotypes observed after *hlh-2*/M05B5.5 RNAi

We hypothesized that one of the main downstream effectors of *hlh-2*/M05B5.5 is *argk-1*/F44G3.2, and to check if this mechanism is conserved in mammals we performed immunoblotting against CKB, one of the orthologs of *argk-1*/F44G3.2 in mice known to be expressed in embryonic cells. Contrary to our expectations and the data we obtained in *C. elegans*, there was no decrease in CKB signal in Tcf3 KO samples. We observed increased signal in all samples treated with 4-OHT, including the control samples, which is likely a reaction to 4-OHT itself, and a slight increase in the KO samples compared to control (Fig. 29d). Therefore it is impossible to say at the moment if CKB may be a target of TCF3. Furthermore, luciferase assay to determine changes in ATP did not detect statistically significant differences in Tcf3 KO samples compared to control (Fig. 29e).

To gain more information on the energy metabolism of Tcf3 KO cells we blotted against phosphorylated AMPK, and initially saw a strong increase in samples both with and without addition of NAC (Fig. 30a).

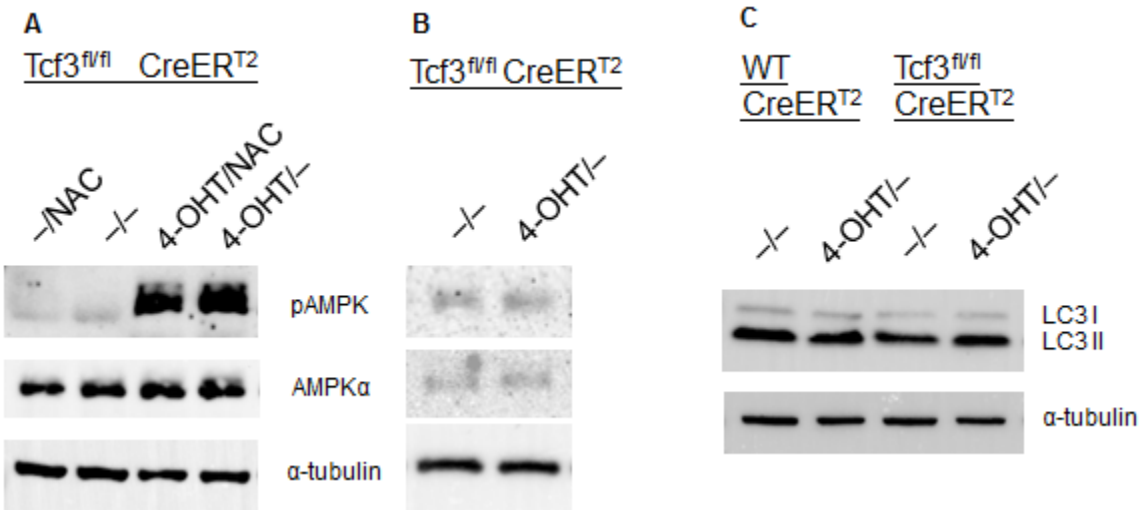

Figure 30: Immunoblot analysis. **(A)** A polyclonal phospho-AMPK antibody detects pAMPK only in cells treated with 4-OHT, both with and without addition of NAC; **(B)** No visible difference in pAMPK signal between cells treated with 4-OHT and the control cells; **(C)** No difference in LC3I to LC3II signal ration is observed between cells treated with 4-OHT and the control cells.

It has been recently reported, however, that addition of widely used for conditional gene knockout tamoxifen itself has the effect of AMPK activation in an estrogen receptor-independent manner (Snyder, Blair, and Koumenis 2017). To avoid this effect we have modified our protocol of Tcf3 KO by culturing the cells for additional 24 hours after 4-OHT removal to make sure tamoxifen signaling is not affecting the phenotype. When the new batch of the KO cells was tested for phosphorylated AMPK, we could no longer observe the change in signal intensity (Fig. 30b). This led us to the conclusion that Tcf3 KO has either no impact on AMPK-mediated signal transduction or this is a transient impact necessary to test in a precisely timed manner.

We next tested if elevated levels of autophagy, observed after *hlh-2*/M05B5.5 downregulation, can be detected in Tcf3 KO cells. One of the most common assays used to detect autophagy in mammals is comparing expression levels of LC3 I to those of LC3 II: a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to membranes of autophagosomes. These fuse with lysosomes, so that the autophagosomal components are lysed by hydrolases, with LC3-II degraded as well. In this way, lysosomal turnover of the autophagosomal marker LC3-II corresponds to autophagic activity, and detecting LC3 by immunoblotting can be a reliable method for detecting autophagy (Tanida, Ueno, and Kominami 2008). We have not observed any differences in LC3 I or LC3 II signals, however, which leads us to believe that autophagy is not induced after Tcf3 knockout in MEFs (Fig. 30c).

2.4.4 Tcf3 KO leads to decrease in senescence markers in MEFs

The primary phenotype resulting from knockdown of *hlh-2*/M05B5.5 in *C. elegans* during early adulthood is lifespan extension. Cell culture system, however, is considerably different in respect of assessment of aging and measuring lifespan. Generally, even when evaluating organismal aging in mammals, it is considered to be multifactorial in nature, with several associated causes, only one of which is thought to be cellular senescence, and the relationship between organismal aging and cellular senescence is far from clearly

defined (Kidd 2000; Sheikh et al. 2013; Bhatia-Dey et al. 2016). At the same time, there is strong evidence that senescent cells accumulate in organisms with age, at different rates in different organs, and cellular senescence can be considered a convenient readout serving as the status of cells' "longevity" or "youthfulness" (Jeyapalan and Sedivy 2008). Cellular senescence is defined as an irreversible cell cycle arrest triggered by various stressors, such as telomere erosion, DNA damage, oxidative stress, oncogene activation, or infection, and involving p16-pRB and p53-p21 tumor suppressor pathways (Mowla, Lam, and Jat 2014). It has been shown that naturally occurring senescent cells shorten healthy lifespan in mice, and clearance of p16-positive cells delays tumorigenesis and attenuates age-related deterioration of kidney, heart, fat, and other organs without apparent side effects (Baker, Childs, Durik, Wijers, Sieben, Zhong, A. Saltness, Jeganathan, Verzosa, Pezeshki, Khazaie, Miller, and van Deursen 2016).

We have sought to investigate the effect of Tcf3 KO in MEFs on cellular senescence markers, such as phosphorylated p53 and p16 levels, as well as speed of growth and senescence-associated beta-galactosidase staining (SA-β-gal). Indeed, TCF3-deficient cells probed for phosphorylated p53 showed weaker signal compared to control (Fig. 31a).

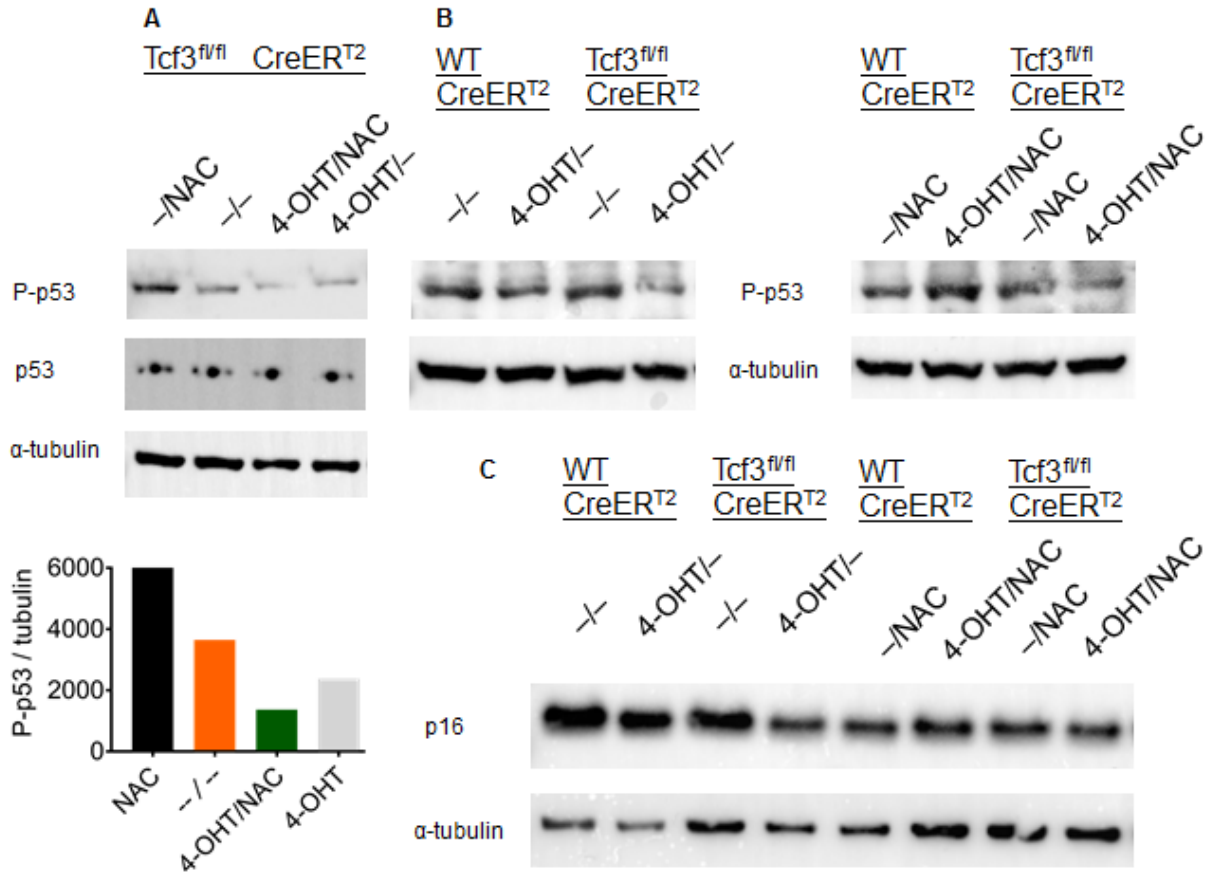

Figure 31: Immunoblot analysis. A decrease in phospho-p53 signal in cells treated with 4-OHT is **(A)** further strengthened, or **(B)** not changed by the addition of NAC; **(C)** antibody against p16 shows weaker signal in cells treated with 4-OHT, as well as in all cells where NAC was added to the medium.

Interestingly, addition of NAC, rather than abolishing the effect, decreased P-p53 signal even further (Fig. 31a). In contrast, in *C. elegans* addition of both NAC and BHA greatly reduced lifespan-extending effect of *hlh-2*/M05B5.5 RNAi, suggesting ROS as mediators of the pro-longevity signaling of *hlh-2*/M05B5.5 downregulation. The repeated experiments to confirm the results do not definitively show the stronger effect with addition of NAC, but still rule out that antioxidants abolish the effect of Tcf3 KO (Fig. 31b). NAC added to both 4-OHT-treated and the control cells led to reduced p16 signal, however, which is in line with published reports (Haendeler et al. 2004; Voghel et al. 2008). We can also see that TCF3-deficient cells have lower levels of p16/INK4A compared to control,

and are similar to those observed in samples treated with NAC (Fig. 31c). This observation is in agreement with the published data on TCF3/E2A transcription factor activating expression of p21, a target gene of p53 and an inhibitor of cell cycle progression (Andrysik et al. 2013). Taken together, these results suggest that Tcf3 KO leads to a decrease in senescence markers, possibly without being affected by the redox state of the cell.

2.4.5 Delayed senescence and improved growth induced by TCF3 impairment require ROS signaling

Normally cells of a particular type in a given species divide a certain number of times until they reach the state of senescence, and this is determined by their telomere shortening rate or stress conditions (L. Hayflick and Moorhead 1961; Parrinello et al. 2003). While senescence markers provide insight into the “aging/youthfulness” state of cells at a particular point in time, the closest equivalent in cell culture to the lifespan assay in *C. elegans* would be a growth curve of cell replicative lifespan showing speed of growth until the point of entering the senescent state plateau, and the number of passages at the plateau stage. We have conducted such an experiment with the same 8 conditions described in the Experimental setup (Table 6) and discovered that cells deficient for Tcf3 show the highest rate of growth (Fig. 32a). Moreover, consistent with the lifespan results in *C. elegans*, this phenotype is completely abolished in the presence of NAC (Fig. 32b). Addition of 4-OHT itself appears to inhibit growth of all cells, irrespective of the antioxidant precursor treatment, yet knockout of Tcf3 induced by 4-OHT leads to a considerable increase in proliferation rate (Fig. 32b). After passage 15 all samples spontaneously overcame replicative senescence and became immortalized, which is a normal behavior for MEFs and is consistent with data published in literature on MEF growth curves (H. Yang et al. 2017; Parrinello et al. 2003).

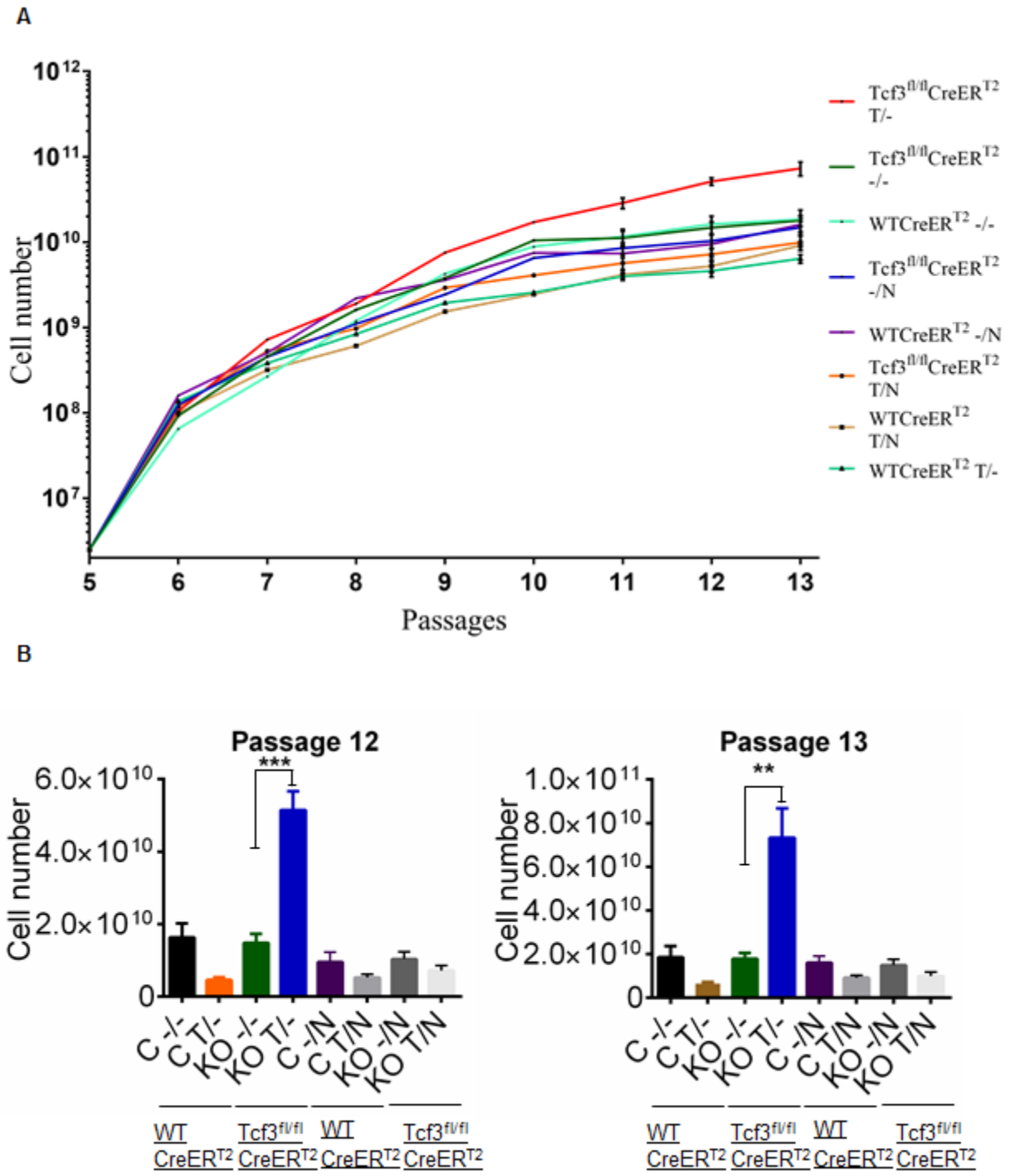

Figure 32: (A) Growth curve of MEFs from passage 5 until 13 shows faster growth of Tcf3 KO cells; bar graphs show higher growth speed of Tcf3 KO cells versus control at **(B)** passage 12 ($p<0.0001$, one way ANOVA, n=3 cell plates) and **(C)** passage 13 ($p<0.001$, one way ANOVA, n=3 cell plates).

Probably the most widely used and a very practical biomarker of cellular senescence is elevated activity of senescence associated β-galactosidase (SA-β-gal) (Bassaneze, Miyakawa, and Krieger 2008; Dimri et al. 1995; Matjusaitis et al. 2016), which was reported to reflect higher lysosomal mass of senescent cells (Kurz et al. 2000). We used this assay to stain Tcf3-KO and control cells at passage 12 to assess the relative number of senescent cells in culture.

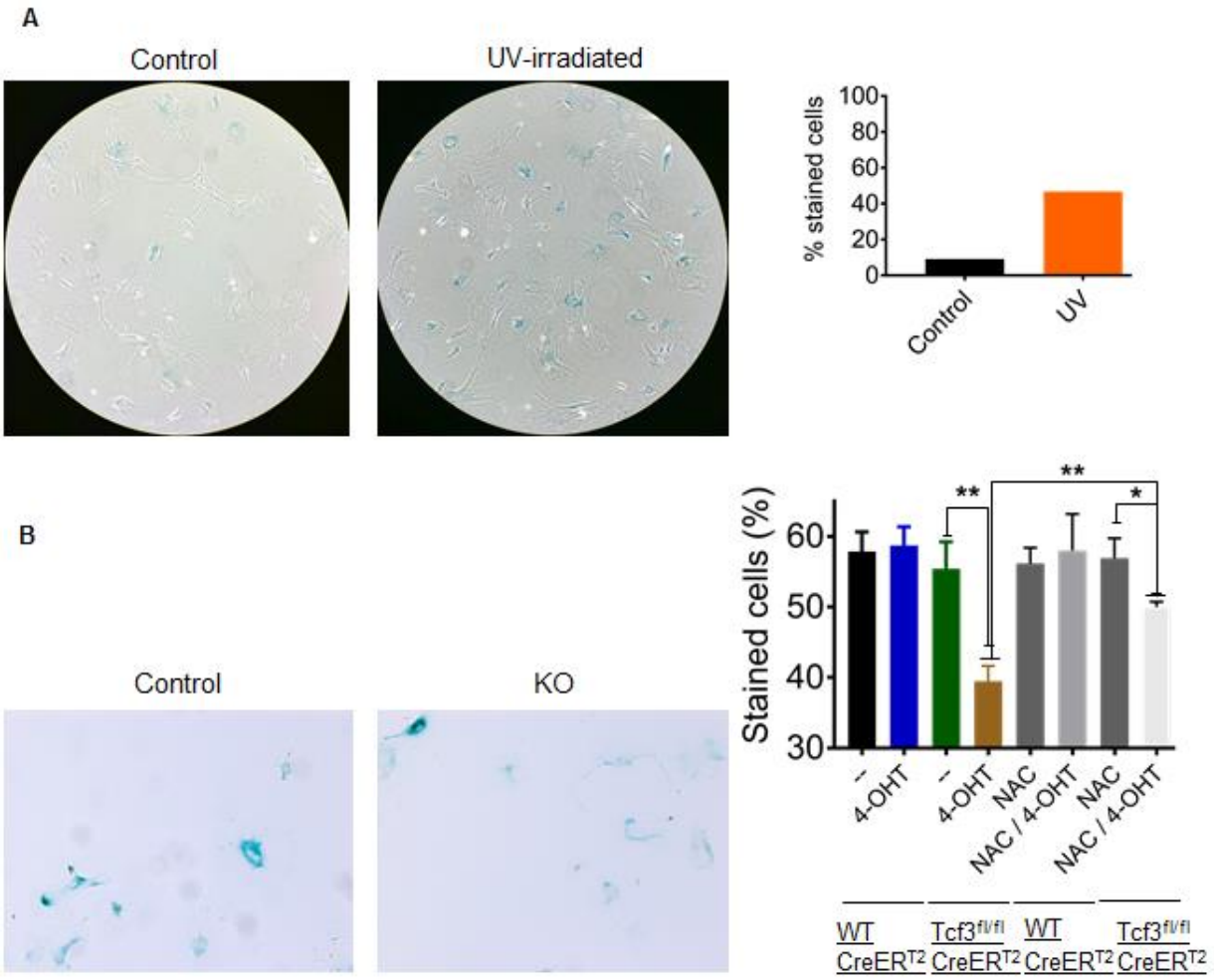

Figure 33: Senescence associated β-galactosidase (SA-β-gal) activity assay shows **(A)** higher signal in UV-irradiated primary MEFs, serving as positive control; **(B)** lower signal in Tcf3 KO cells compared to control ($p<0.001$, one way ANOVA, n=3 assays x 75 images each); lower signal in Tcf3 KO cells compared to Tcf3 KO cells with addition of NAC ($p<0.001$, one way ANOVA, n=3 assays x 75 images each); and lower signal in Tcf3 KO cells with addition of NAC compared to control with addition of NAC ($p<0.05$, one way ANOVA, n=3 assays x 75 images each).

To validate the assay, we first created positive control sample by irradiating primary mouse embryonic fibroblasts with UV (Fig. 33a).

At the senescent plateau stage of cell growth (passage 12) Tcf3 KO cells, as expected, showed statistically significant decrease in SA-β-gal staining compared to control (Fig. 33b). Addition of NAC significantly reduced this effect, but did not abolish it completely, which is consistent with the lifespan data in *C. elegans* where addition of an antioxidant considerably but not fully reduced the lifespan-extending effect of *hlh-2*/M05B5.5 downregulation (Fig. 33b). Altogether, these data support the notion that the results we obtained in nematodes can generally be translated into the mammalian system, albeit the downstream effectors and the overall mechanism is likely not conserved. A possible mechanism with multiple missing links could be visualized in the following scheme (Fig. 34):

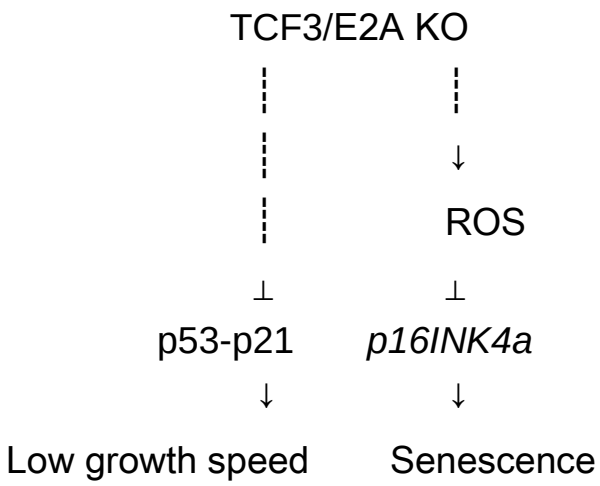

Figure 34: Summary of the hypothesized mechanism for TCF3/E2A-mediated decrease in senescence. Tcf3 KO through unknown mechanisms leads to transient induction of ROS, which results in inhibition of p16 and delay of senescence. In parallel, Tcf3 knockdown decreases p21 mRNA accumulation while enhancing PUMA mRNA induction, which inhibits cell cycle arrest mechanisms and ultimately results in faster cell growth.

3 Discussion

3.1 Theoretical framework of developmental regulation of aging

Different species have often vastly different lifespans, and emerging body of evidence suggests that it is the way the process of development of a given species unfolds and is influenced by environmental cues that is what determines its longevity (Johnathan Labbadia et al. 2017; Dillin et al. 2002; Rea, Ventura, and Johnson 2007; Ben-Zvi, Miller, and Morimoto 2009; Shemesh, Shai, and Ben-Zvi 2013). During aging (i.e., after the peak of reproduction and until death), only a small proportion of transcript levels change with age, and this trend is very similar in the vast majority of tested tissues and organisms (Stegeman and Weake 2017). At the same time, many aging-related changes that have profound consequences in later stages of life of an organism occur either during young adult / reproduction period or even earlier, during development. For instance, protein aggregation widely considered a hallmark of senescence and usually associated with old age is an early and non-random (i.e. programmed) event in *C. elegans*, and it has been reported that the transition into early adulthood in worms may be the critical threshold for proteostasis collapse (David et al. 2010), which itself represents an early molecular event of aging (Ben-Zvi, Miller, and Morimoto 2009). Moreover, these reports are relevant not only to the mostly post-mitotic adult *C. elegans* nematodes, but to other species as well, as stress responses to heat and oxidative stress in general were reported to be significantly correlated with early-adulthood transcriptional programs in both *C. elegans* and *D. melanogaster*, suggesting that changes in proteostasis-related gene expression are not only implemented in response to cumulative protein aggregation and damage common in old age but rather constitute inbuilt programs. These conserved events of aging are the result of developmentally timed transcriptional regulation in young adults (McCarroll et al. 2004). Remarkably, recent reports have shown that heat shock response (HSR) and unfolded protein response (UPR) genes' downregulation in somatic tissues of worms reaches ~80% already by day 2 or 3 of adulthood, and this precipitous decline happens within a 24-hour window and

correlates with a pronounced decrease of stress resistance, suggesting functional consequences of the HSR and UPR failure, that may even precede proteostasis collapse (Taylor and Dillin 2013; Shemesh, Shai, and Ben-Zvi 2013). HSR alone declines even faster in *C. elegans*: the precipitous fall of HSR activity occurs over 4 hours during the onset of reproductive maturity (Johnathan Labbadia and Morimoto 2016), and this programmed inhibition of HSR during transition to reproductive maturity later leads to the aging phenotypes we normally associate with passive physiological decline (Johnathan Labbadia et al. 2017). This process is not limited to nematodes: a similarly timed collapse of the HSR occurs in aged flies subjected to hyperthermia, and in adrenal cortex of aging rats, adding support to the early transcriptional dysregulation of stress responses as a conserved event in metazoans (Blake et al. 1991; Pappas et al. 2007). Such programmed change in proteostasis may serve a biological role, as has been shown in *S. Cerevisiae* (Saarikangas and Barral 2015).

Another line of evidence supporting developmental regulation of aging comes from studies of *C. elegans* mutants of mitochondrial genes. It has been found that mitochondrial activity during development determines stress resistance later in life of an organism (Dillin et al. 2002): for instance, a specific signal initiated during the L3/L4 larval stage of development, is sufficient to confer mild mitochondrial stress–dependent life extension in *C. elegans*. During this developmental stage the last somatic cell divisions normally occur in *C. elegans*, which is accompanied by massive mitochondrial DNA expansion. In light of recent reports directly linking cell cycle progression with mitochondrial activity in *C. elegans*, it is very likely that cell cycle checkpoint control is a key determinant of longevity in *C. elegans* mitochondrial mutants (Rea, Ventura, and Johnson 2007).

The best evolutionary explanation for these “programmed aging” mechanisms is ironically provided by the NPA theories, such as antagonistic pleiotropy model and Disposable soma theory: as organisms become ready for reproduction, it makes perfect evolutionary sense to divest as much energy and resources as possible from maintenance of the soma to the progeny, provided that these are species that do not nurture their young and have a clear separation between soma and germline (T. Kirkwood 1977; Gems and Partridge 2013; John Labbadia and Morimoto 2014). During

development, if the conditions are favorable and the food sources are plenty, the canonical kinase-mediated IIS signaling pathway leads from the DAF-2 receptor via PI3K, PDK-1, SGK-1 and AKT-1/2 to inhibit beneficial protective transcriptional effects of DAF-16/FOXO. Thus in a benign environment, normal development is maintained; however, during starvation or stress, inhibition of IIS signaling triggers the dauer program of developmental arrest and postponed reproduction (Ayyadevara et al. 2008). During normal reproduction most resources are redirected towards progeny, which is achieved by “switching off” a number of genes responsible for stress response and maintenance of homeostasis, and this in turn initiates the spiral down into entropy inevitably leading to death (Van Raamsdonk 2017).

3.2 Summary of the results

Within this framework of developmental regulation of aging it becomes apparent that testing known transcription factors required for development for their relevance to longevity may be a promising approach. Using data from the JenAge project and a bioinformatics pipeline utilizing tools such as RSAT (Regulatory Sequence Analysis Tools) (www.rsat.eu/), we have performed an unbiased screen of the available at the time position weight matrices corresponding to transcription factor binding sites reported or calculated to be within promoter regions of genes regulated by these mostly development-regulating transcription factors. We have identified several transcription factors already reported as having an important role in aging modulation, as well as several candidate genes mostly described as implicated in development. We then tested these identified genes' relevance using RNA interference that can be easily and effectively performed in *C. elegans* by feeding the worms with bacteria harboring RNAi vector. The most promising candidates' downregulation extended lifespan of *C. elegans* worms by up to 40%, giving support to the hypothesis of a developmental program setting the rates of physiological decline during and after reproduction. We have also run the same RSAT pipeline using the expanded set of PWMs, and identified potentially more relevant candidates which remain to be tested. Among these, the top hits include such genes as ***lsy-2*** (ubiquitously expressed novel C2H2 zinc-finger transcription factor required for the left-right asymmetry during development of the ASE neurons), ***klf-1*** (expressed in the intestine Kruppel-like (based on homology to mammalian transcription factors involved in regulation of cellular development and differentiation) C2H2 zinc finger transcription factor essential for fat regulation and possibly involved in apoptosis and the phagocytosis of apoptotic cells in the uterus and germline), ***lsl-1*** (based on protein domain information predicted to have nucleic acid binding activity), ***ceh-28*** (expressed in a single pharyngeal neuron, M4, homeodomain transcription factor of the NK-2 family which functions to ensure proper morphology of the pharyngeal g1 gland cells), ***syd-9*** (expressed in the muscle cell and the nervous system and localized to the nuclear speck protein predicted based on protein domain information to have nucleic acid binding activity), ***sptf-3*** (expressed ubiquitously in embryos and early larvae, and localized to the nucleus C2H2

zinc finger transcription factor required for cell fate specification, embryonic and larval development, and morphogenesis), ***klu-1*** (expressed in the anal depressor muscle, reproductive system, and the body wall musculature C2H2 zinc finger transcription factor), ***klf-2*** (Kruppel-like (based on homology to mammalian transcription factors involved in regulation of cellular development and differentiation) C2H2 zinc finger transcription factor homologous to human WT1, which when mutated leads to Wilms tumor), ***crh-2*** (expressed in the pharynx, spermatheca, hypodermis, nervous system, tail, intestine, and the body wall musculature transcription factor, ortholog of human CREB3L2 (cAMP responsive element binding protein 3 like 2)), and ***ref-1*** (bHLH transcription factor required for early larval development and head morphogenesis; in males, ref-1 is a target of MAB-3 transcriptional repression which in turn, promotes expression of *LIN-32*, a proneural bHLH transcription factor), among others (www.wormbase.org). It is possible that these genes present valuable candidates for further studies of developmental regulation of aging.

The three transcription factors identified through the screen that extend lifespan in *C. elegans* when downregulated by RNAi - *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *ceh-22*/F29F11.5 - function independently of each other, as confirmed by the combination of RNAi lifespan assays, even though they have been reported to function together during development (*hlh-2*/M05B5.5 with *lin-32*/T14F9.5 (Portman and Emmons 2000), and *hlh-2*/M05B5.5 with *ceh-22*/F29F11.5 (Chesney et al. 2009)). *lin-32*/T14F9.5 and *ceh-22*/F29F11.5 have also been shown to have independent functions, however, for instance *LIN-32* was also reported to act independently of *HLH-2* in the regulation of URX development (Romanos et al. 2017), and *CEH-22* is known to cooperate with Wnt signaling to specify a stem cell niche in worms (Lam, Chesney, and Kimble 2006). We have thus mainly focused on *hlh-2*/M05B5.5, which shows the largest lifespan extension and is the unique E protein in *C. elegans*. Downregulation of *hlh-2*/M05B5.5 leads to lower “aging pigment” fluorescence signal and improved fitness as measured in body bends rates and average and maximum speed at 5 days of adulthood in WT worms, and to increased resistance to paralysis in protein aggregation *C. elegans* models. It further shows higher occurrence of autophagic puncta at adulthood, suggesting that one of the 3 forms of autophagy (macroautophagy, microautophagy, and Chaperone mediated

autophagy) may be responsible for maintaining proteostasis after reproduction. Overexpressing *hlh-2*/M05B5.5 at the onset of reproduction leads to significant lifespan shortening, which may indicate that this transcription factor, having fulfilled its purpose during development, is still functioning during adulthood, and that, consistent with antagonistic pleiotropy model, this activity is detrimental to the worms' healthspan.

At present there have been at least 570 lifespan-extending genes identified in *C. elegans* (Tacutu et al. 2013), but most of them act through major signaling pathways that link the rate of aging to environmental factors: IIS signaling, TOR signaling, sirtuins, AMPK signaling, mitochondrial stress signaling, epigenetic mechanisms, or proteostasis (Uno and Nishida 2016). We tested most of these possibilities using epistasis experiments, and came to the conclusion that *hlh-2*/M05B5.5 downregulation-mediated lifespan extension is achieved mostly independently of IIS; however several other pathways may be involved. Lifespan in *daf-16*/R13H8.1 mutant animals showed exactly the same rate of lifespan extension as in WT worms, excluding the possibility that lifespan extending effect of *hlh-2*/M05B5.5 downregulation is in any way achieved through the boost of stress response activated by *DAF-16*/FOXO. At the same time, *hlh-2*/M05B5.5 RNAi failed to further extend lifespan in *DAF-2*-deficient worms, and even slightly reduced lifespan. Finally, in the *daf-16*/R13H8.1;*daf-2*/Y55D5A.5 double mutants it led to about 50% of the lifespan extending effect, suggesting that albeit independent of *daf-16*/R13H8.1, it still requires some downstream of *daf-2* effectors of IIS signaling acting in parallel with *DAF-16*, and they may also be part of another pathway. The other pathways implicated in the longevity effect are TOR, AMPK (notably, reported to be mutually required with *daf-16* in IIS pathway (Apfeld et al. 2004; Greer et al. 2007) and possibly explaining the results of the experiments with *daf-2* RNAi); mitochondrial dysfunction (*isp-1*), and proteostasis. The latter is likely not involved through its UPS system, as our epistasis experiments suggest, but rather through autophagy.

Loss of lifespan extension upon *hlh-2*/M05B5.5 downregulation applied in *glp-1* mutants and at post reproductive stage suggests that this signaling occurs during reproduction albeit may exerts its detrimental effects later throughout post reproductive life of nematodes. *glp-1* mutants show increased levels of autophagy and enhanced resistance to oxidative stress, implicating germline stem cell signaling in a range of

protective pathways (Lapierre et al. 2011; Arantes-Oliveira et al. 2002). Germline removal by laser microsurgery or genetic manipulation (for instance, in the *glp-1* mutants used in this study) increases lifespan; however, when the whole gonad is removed (that is, both the germline cells and the somatic gonad), the lifespan extending effect is abolished (Shemesh, Shai, and Ben-Zvi 2013; Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002). As both removal of the gonad and ablation of the germline lead to sterility, it is unlikely that the longevity induced by germline elimination is the result exclusively of resource reallocation towards progeny; rather, there is a body of research evidence suggesting that endocrine-mediated lifespan regulation takes place (Uno and Nishida 2016; Hsin and Kenyon 1999; K. Lin et al. 2001; Arantes-Oliveira et al. 2002). However, by the time *hlh-2*/M05B5.5 RNAi is applied (day 1 of adulthood) and the transcription factor cleared from cells (likely day 2 of adulthood, as sterility is induced at this time point (data not shown)), germline signaling should have already taken place and should not therefore be influenced by *hlh-2*/M05B5.5 downregulation. It has been shown that TOR pathway, *daf-12*, and *daf-12* specifically acting through inhibition of *daf-16* regulate longevity in animals without germlines (Antebi 2013b, 2013a; Shemesh, Shai, and Ben-Zvi 2013). Of these, we have shown that *hlh-2*/M05B5.5 RNAi extends lifespan independently of *daf-16* and *daf-12*, but likely through autophagy which acts downstream of inhibited TOR signaling (Jung et al. 2010).

Moreover, we have shown that the lifespan extension depends on *aak-2*/AMPK activity, which was reported to inhibit TOR pathway (Motoshima et al. 2006; McQuary et al. 2016) and to at least partially regulate autophagy in *C. elegans* and in mammals (Egan et al. 2011). Although there are reports showing that inhibiting TOR signaling increases the lifespan in *C. elegans* in a *DAF-16*-dependent manner (Jia 2004; Vellai et al. 2003), combining the inhibition of *daf-2* and *rsks-1* (a *C. elegans* S6 kinase and a target of TOR) has been shown to lead to additive lifespan extension in *C. elegans* (D. Chen et al. 2013), suggesting that the IIS and the TOR pathways act together to mediate lifespan extension each in its own distinct manner, and corroborating our results.

In addition to *aak-2*/AMPK, we showed that *hlh-2*/M05B5.5 RNAi-mediated lifespan extension depends on the activity of *hsf-1*/HSF1. Apart from the HSR known to be regulated by HSF-1, this transcription factor has also been reported to be involved in

activation of autophagy (Watanabe et al. 2017; Desai et al. 2013; Kumsta et al. 2017). Collectively, these results suggest that lifespan extension and health improvements seen after *hlh-2*/M05B5.5 downregulation are likely achieved through inhibition of TOR signaling and activation of autophagy with involvement of the transcription factor *hsf-1*/HSF1 and the energy sensor *aak-2*/AMPK.

Mutations targeting *skn-1*/NRF2 lead to a major decrease in *hlh-2*/M05B5.5 RNAi-mediated lifespan extension, and this effect is still present in a neuron-only RNA interference model worms, suggesting that neuronal signaling may be sufficient to induce the longevity phenotype. *SKN-1* and *AAK-2* mediate both lifespan extension induced by calorie restriction (CR) (Greer et al. 2007; Bishop and Guarente 2007) and mitohormesis (Hwang et al. 2014; Schmeisser et al. 2013), implicating a link between the two and the possibility that SKN-1 and AAK-2 might function together in this stress response mechanism. Data from assays with *hlh-2*/M05B5.5 RNAi-treated *isp-1* mutant worms which have elevated ROS and are long-lived due to SKN-1-mediated stress response support the possibility of mitohormesis mediating *hlh-2*/M05B5.5-linked longevity. This is further corroborated by the loss of longevity phenotype in worms treated with *hlh-2*/M05B5.5 RNAi in presence of antioxidants. Furthermore, we have shown ROS levels to be elevated 48 hours after *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi treatment start. The elevation is only weakly significant in case of *hlh-2*/M05B5.5 and by trend in *lin-32*/T14F9.5 RNAi treated worms, but we would argue that activation of the HSR, enhanced stress resistance and maintenance of proteostasis do not require substantial increases in ROS levels, and are not due to large scale oxidative damage to proteins (Johnathan Labbadia et al. 2017). Besides, we do not see the longevity phenotype completely abolished by introduction of antioxidants, and thus propose that mitochondrial dysfunction and ROS-mediated stress response involving SKN-1 and AAK-2 are likely responsible for only a part of this healthspan-promoting intervention. One of the possible downstream effectors in this pathway may be *cth-1*/F22B8.6, one of the top 10 DEGs in the *hlh-2*/M05B5.5 downregulation RNA deep sequencing experiment. ROS are potentially implicated because *cth-1*/F22B8.6 ortholog in humans - CTH - is an enzyme which breaks down cystathionine into cysteine which is the rate-limiting substrate in the

synthetic pathway for glutathione (Sastre et al. 2005). Interestingly, CTH has also been found to decrease with aging in rat lenses (Ferrer et al. 1990).

The results of RNA deep sequencing experiment performed with *hlh-2*/M05B5.5 downregulation samples showed very different DEGs, and we could not find any enriched function-related gene groups using such functional annotation tools as DAVID (https://david.ncifcrf.gov) or KEGG pathways (http://www.genome.jp/kegg/pathway.html). Therefore it is likely that the longevity phenotype of *hlh-2*/M05B5.5 RNAi is the result of an interplay of several distinct mechanisms, which is also evident from our experimental data using *hlh-2*/M05B5.5 RNAi. Results for *ceh-22*/F29F11.5 RNAi samples show more similarity with those of *hlh-2*/M05B5.5 than the *lin-32*/T14F9.5 RNAi samples DEGs, but the dominating biological theme from DEGs of *ceh-22*/F29F11.5 downregulation is innate immunity and defense response: 5 out of 10 top DEGs are genes that have protective function against bacteria, and several of them have been reported to affect lifespan in *C. elegans* (Murphy et al. 2003). It is tempting to speculate that antimicrobial response is indeed what is responsible for lifespan extending effect of *ceh-22*/F29F11.5 RNAi. As for *lin-32*/T14F9.5 DEGs, *cysl-2*/K10H10.2 depletion was shown to modify lifespan of nematodes 17-23% (Qabazard et al. 2013), and this may explain lifespan extension upon *lin-32*/T14F9.5 downregulation. In addition, *icl-1*/C05E4.9 - another DEG in the top 10 - was also reported to extend lifespan in *C. elegans*, implicating enhanced energy metabolism in this longevity phenotype (Yuan et al. 2012).

The top DEG from the *hlh-2*/M05B5.5 RNAi samples list, however, is *argk-1*/F44G3.2, and we have extensively studied its involvement and lifespan modulating mechanisms. Consistent with its #1 position in both DEGs list from the *hlh-2*/M05B5.5 and *lin-32*/T14F9.5 RNAi samples respective combinations of RNAi does not produce additive effect on lifespan, however due to RNAi dilution effect and mixed results in previous experiments RNAi combination experiments results must be taken into consideration with some caution. We found that *argk-1*/F44G3.2 RNAi likely affects all 5 paralogs of this gene in *C. elegans* due to their high sequence similarity, and that for lifespan extension arginine kinases must be impaired transiently, as constitutive mutants show no difference in longevity. Based on the predicted structure of the protein, ARGK-1 is hypothesized to be a cytosolic (as opposed to mitochondrial) arginine kinase probably

functioning in phosphocreatine shuttling system in tissues of high energy demand, such as neurons (Fraga et al. 2015; McQuary et al. 2016). There is no consensus on the importance of the respective ortholog in mammals, as experiments show, for example, that there is no major loss/gain of muscle function in creatine kinase KO animals and animals fed creatine analogues (Greenhaff 2001). At the same time, physiological importance of the phosphocreatine circuit fully operating in adult brain has been corroborated by experimental data indicating an important function for mitochondrial creatine kinase (Mi-CK) in the oxidative energy metabolism of the brain (Hemmer and Wallimann 1994).

Comparable to the *hlh-2*/M05B5.5 RNAi-treated worms, *argk-1*/F44G3.2 downregulation improves average and maximum lifespan, average and maximum locomotion speed, resistance to paralysis, and shows signs of activated autophagy in *C. elegans*. Experiments in mutants of most of *argk-1*/F44G3.2 paralogs suggest that it is the transient downregulation of arginine kinases that is responsible for improved health- and lifespan of nematodes, as constitutive knockouts behave similarly to WT animals, and overexpressing strains rather contribute to longevity of *C. elegans*. Pathways mediating the lifespan extending effect shadow the results we see in *hlh-2*/M05B5.5 RNAi-treated worms and likely include TOR signaling and proteostasis maintenance with lifespan extension by *argk-1*/F44G3.2 RNAi fully dependent on *aak-2*/AMPK, *hsf-1*/HSF1, and *let-363*/TOR. Moreover, just like in experiments using *hlh-2*/M05B5.5 RNAi, *argk-1*/F44G3.2 downregulation-mediated longevity depends on *skn-1*/NRF2 activity, and is substantially abrogated when antioxidants are present. Interestingly, we also saw no statistically significant lifespan extension with *argk-1*/F44G3.2 RNAi in the catalase-overexpressing strain, further implicating ROS signaling mediating the longevity phenotype. This is consistent with the literature describing thermodynamic efficiency of high-energy phosphate synthesis and channeling: active ATP/ADP exchange maintained by coupled Mi-CK contributes to proper functioning of the respiratory chain, which would otherwise generate higher levels of superoxide and ROS (Schlattner, Tokarska-Schlattner, and Wallimann 2013). It is also known that creatine exerts a strong indirect antioxidant effect by considerably reducing the mitochondrial ROS production, as well as maintaining high mitochondrial membrane potential (Meyer et al. 2006; Wallimann,

Tokarska-Schlattner, and Schlattner 2011). Further implicating mitochondrial involvement in *argk-1*/F44G3.2 RNAi-mediated longevity phenotype and closely matching the data obtained for *hlh-2*/M05B5.5 downregulation, we observed only marginal lifespan extension in mutants of *isp-1*/F42G8.12 (an aging-modulating gene that codes for a subunit of the mitochondrial complex III in the mitochondrial membrane). Finally, direct measurements confirmed elevated levels of ROS and decrease in ATP in *argk-1*/F44G3.2 RNAi-treated animals, adding to the multiple phenotypes that closely match those observed during *hlh-2*/M05B5.5 downregulation (Table 5).

3.3 Proposed novel mechanism of aging regulation

We hypothesize that the mechanism of the lifespan extension observed upon impairment of *hlh-2*/M05B5.5 could be represented with the following scheme (Fig. 35):

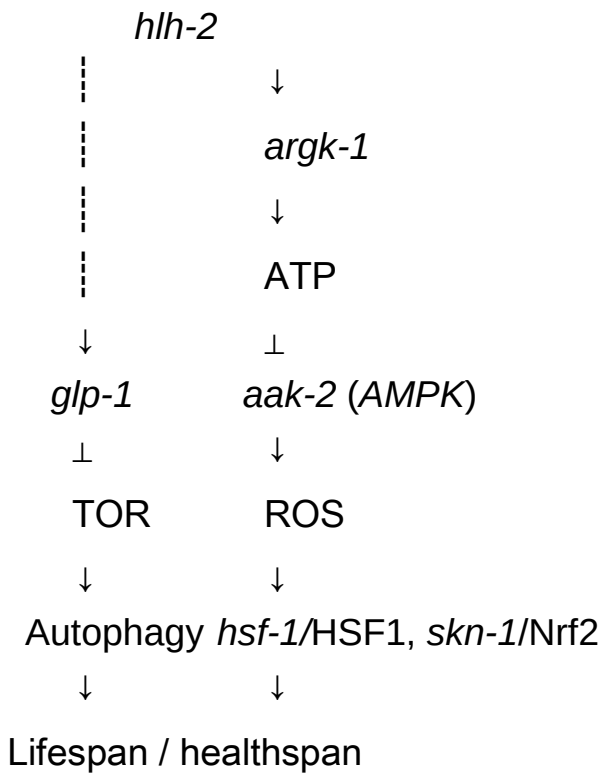

Figure 35: Summary of the hypothesized mechanism for *hlh-2*/M05B5.5-mediated longevity. Downregulation of *hlh-2*/M05B5.5 leads to sterility and inhibition of *glp-1*, which in turn downregulates TOR signaling and activates autophagy; in parallel, downregulation of *hlh-2*/M05B5.5 is followed by downregulation of *argk-1*/F44G3.2, which results in a transient decrease in ATP levels. Energy sensor *aak-2*/AMPK activates transcription factors *hsf-1*/HSF1 and *skn-1*/NRF2 through ROS signaling, which upregulate protective mechanisms of the organism and together with activated autophagy ultimately lead to extension of lifespan and to the associated improvements in health parameters in nematodes.

The mechanisms mediating longevity phenotype of *hlh-2*/M05B5.5 downregulation are likely not fully conserved in mammals, as is evidenced by a somewhat different function of the *hlh-2*/M05B5.5 mammalian ortholog, Tcf3 (E2A Immunoglobulin Enhancer

Binding Factors E12/E47)(Bain et al. 1994), and our experiments in mouse embryonic fibroblasts. Accordingly, we did not detect changes in CKB (one of the *argk-1*/F44G3.2 orthologs in mice) levels after Tcf3 knockout, ATP decrease, or autophagy or AMPK activation. However, intriguingly, the main phenotype of longevity, and some of the downstream effectors appear to match the results obtained in *C. elegans*: we have shown speed of growth of Tcf3 knockout cells to exceed that of control, and significantly lower levels of senescence, with both phenotypes showing dependence on ROS signaling. In case of CKB immunoblotting, it is possible that CKB downregulation is transient with further increase in the amount of the protein following stress response. In this scenario, more time points would be necessary to be checked to detect the dynamics of creatine kinase levels change in response to Tcf3 conditional knockout. It is also conceivable that other paralogs of creatine kinase are affected by Tcf3 impairment which may still result in the upregulation of CKB we see in our experiments. Another discrepancy needed to be addressed is the levels of P-p53 not affected by the presence of antioxidants. While it is known that ROS promote cellular senescence in human epidermal keratinocytes through epigenetic regulation of p16(INK4a) (M. Sasaki et al. 2014), and these data are matched by the results of our p16(INK4a) immunoblotting experiments where presence of antioxidants leads to a decrease in the levels of this senescence marker, p53 presents the other signaling pathway in senescence triggered by DNA damage response (DDR), and it has been shown to induce senescence and cell cycle arrest or apoptosis through controlling levels of ROS as it is upstream (T. M. Johnson et al. 1996). The activity of p53 is regulated through many mechanisms, but the one particularly relevant regarding the control of senescence and aging is the histone deacetylase Sirt1, which is strongly downregulated in senescent cells (T. Sasaki et al. 2006). It remains to be investigated whether sirtuins play a role in delayed senescence induced by Tcf3 knockout, but given the data we obtained through lifespan assays and SA-β-gal staining this may be a worthwhile direction of study (Vigneron and Vousden 2010).

While cellular senescence has been associated with organismal aging (Ressler et al. 2006; Jeyapalan et al. 2007), and the role of senescence in aging-associated dysfunction is often explained by its tumor suppressive function and the evolutionary concept of antagonistic pleiotropy (T. B. L. Kirkwood and Austad 2000; Campisi 2005),

the relationship between cellular senescence and organismal aging is far from clear and is an avenue of active research (Jeyapalan and Sedivy 2008). Whereas clearance of senescent cells was linked to delay in aging-related disorders and longevity (Baker et al. 2011), it was also shown that naked mole rats, the longest-lived rodents, retain all of the major types of cellular senescence response despite their longevity (Zhao et al. 2018); in addition, cellular senescence inhibition may not be a desirable effect in an organism in view of the tumor suppression function of senescence. At the same time, later in life of an organism, SASP rather contributes to the age-related dysfunction by turning neighboring cells into senescent when immune system can no longer handle their removal (Campisi 2013). It is thus difficult to draw general organismal aging level conclusions from our studies using cell culture, and more knowledge is necessary to understand if and how TCF3/E2A activity affects the rates of tissue physiological decline.

TCF3/E2A is widely expressed, it is required for normal functioning of immune system in adult organisms, and it plays crucial roles in regulation of cell commitment and differentiation in a variety of cell types including muscle cells, lymphocytes, and neurons (Slattery, Ryan, and McMorrow 2008). Consistent with our results, Tcf3 gene products were shown to play a role in regulating cell growth: E12/E47 proteins activate transcription of p21, a target of p53 and a cyclin-dependent kinase inhibitor (Sloan et al. 1996), suggesting a direct mechanism of TCF3/E2A cell growth regulation. TCF3/E2A has also been reported to indirectly regulate cell growth through apoptosis (Inukai et al. 1998), while overexpression of wild-type E47 suppressed the growth of NIH3T3 fibroblasts (Peverali et al. 1994). On the other hand, strong evidence indicates that TCF3 proteins play a role as important mediators of epithelial–mesenchymal transition (EMT) which is implicated in multiple maladies including kidney disease and tumor metastasis (Slattery, Ryan, and McMorrow 2008). Moreover, both total cellular and nuclear TCF3/E2A levels are decreased in aged mice due to accelerated E47/E12 protein turnover through UPS (which normally has decreased activity with age), as post-translational controls impair E47 protein expression in aged B-cell precursors (Riley, Blomberg, and Frasca 2005), suggesting a possible mechanism of combating detrimental effects of TCF3/E2A activity by the aging organism. Both expression levels and post-translational controls of TCF3 are very different in different cell types at different times of development and aging, and

are tightly regulated. Much remains to be discovered regarding these issues and other factors important to the transcriptional control during development and in senescence, and to the connection between the two. If properly understood at the molecular level, targeting this regulation would be a promising approach in tackling aging-related dysfunction in the rapidly aging human population.

3.4 Closing remarks

This thesis investigates functions and signaling pathways of transcription factors potentially regulating genes differentially expressed with aging in different species. This work shows a number of lifespan-extending *C. elegans* genes and gives insight into how they fit into the current understanding of aging-modulating pathways in this model organism. We specifically focused on HLH-2 transcription factor, showed some of its downstream effectors and investigated the exciting possibility that its downregulation leading to longevity and increased healthspan may be a mechanism that is evolutionarily conserved in mammals.

Functional HLH-2 and its orthologous protein TCF3/E2A are required for proper development of multiple tissues. We discovered that impairment of HLH-2 post-developmentally but only in early adulthood extends lifespan; we were also able to determine how this longevity is achieved and what pathways are likely involved. At the same time we are left with important questions concerning details and organismal context of HLH-2-related regulation. What tissues are mainly responsible for the longevity-inducing signaling? Are HLH-2 homodimers or heterodimers responsible for lifespan shortening in WT worms, and if it is the latter, which binding partners produce these heterodimers with HLH-2? Is it possible to dissociate the sterility effect of *hlh-2* knockdown and its longevity phenotype? How much of the HLH-2 downregulation-mediated longevity mechanism is conserved in mammals, and specifically, in humans? The work performed in this thesis advances our understanding of aging-associated transcriptional regulation, attempts to put it into the framework of general principles of development as the determinant of aging rates in adulthood, and allows us to begin to answer these questions. As research in this field is actively progressing, it will be exciting to see how advancement in the area of aging studies brings new understanding of the ways organismal aging is controlled and presents us with new interventions for healthy aging.

4 Materials and Methods

4.1 Chemicals

All chemicals were obtained from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

4.2 Nematode strains and maintenance

We used the following *C. elegans* strains obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA): Wild type, N2 (Bristol); EU31 *skn-1* (zu135), EU1 *skn-1* (zu67), PS3551 *hsf-1* (sy441), CF1038 *daf-16* (mu86), CB1370 *daf-2* (e1370), HT1890 *daf-16* (mgDf50) & *daf-2* (e1370), AA86 *daf-12* (rh61rh411), RB754 *aak-2* (ok524), MQ887 *isp-1* (qm150), MQ130 *clk-1* (qm30), VC289 *prdx-2* (gk169), CB4037 *glp-1* (e2141), DA1116 *eat-2* (ad1116), TU282 *lin-32* (u282), TU3311 sid-1 uIs60 [unc-119p::YFP + unc-119p::sid-1], GA800 wuIs151 [ctl-1 + ctl-2 + ctl-3 + myo-2::GFP], GF66 Q82 dgEx66 [(pAMS58) vha-6p::Q82::YFP + rol-6(su1006) + pBluescript II], CL802 Aβ (smg-1(cc546) I; rol-6(su1006)), CL4176 Aβ (smg-1(cc546); dvIs27 [myo-3p::A-Beta (1-42)::let-851 3'UTR) + rol-6(su1006)]), CL2006 Aβ dvIs2 [pCL12(unc-54/human Abeta peptide 1-42 minigene) + pRF4], IIDA2123 lgg-1 adIs2122 [lgg-1p::GFP::lgg-1 + rol-6(su1006)], RB2211 *argk-1* (ok2993), RB2060 *argk-2* (ok2723), and RB2598 *argk-4* (ok3620). The *argk-3* (F32B5.1) knockout strain 3937 *argk-3* (tm3937) was received from the International *C. elegans* Gene Knockout Consortium, Tokyo Women's Medical University School of Medicine. The following strains have been backcrossed for use in the lifespan assays: RB2211 (MIR127), RB2060 (MIR128), 3359 (MIR129), and RB2598 (MIR130). Heat-shock conditional *hlh-2*/M05B5.5 and *ceh-22*/F29F11.5 overexpressing strains (GS2968 *hlh-2* arIs63 [hsp16.2::*hlh-2*, ttx-3::gfp, dpy-20(+)] and JK4074 *ceh-22* qIs131 [hs::*ceh-22*b, ttx-3::dsRed]) are a kind gift of the Kimble lab (University of

Wisconsin–Madison). Heat-shock conditional *lin-32*/T14F9.5 overexpressing strain UR189 *lin-32* (him-5(e1490) bxIs14 V; fsEx148[hs*LIN-32*::GFP+unc-122::GFP]) is a kind gift of the Portman lab (University of Rochester Medical Center).

Nematodes were grown and maintained on Nematode Growth Media (NGM) agar plates at 20°C using E. coli OP50 bacteria as food source except for CB4037 which was maintained at 15°C. After plates were poured, they were dried, sealed and stored at 4°C. Freshly prepared E. coli were seeded on plates on the previous evening and allowed to dry and settle overnight.

4.3 RNA-mediated gene knockout (RNAi)

We applied alive E. coli HT115 to the worms during the RNAi-mediated gene knock-down experiments using the feeding method (Ingestion of bacterially expressed dsRNAs can produce speci®c and potent genetic interference in Caenorhabditis elegans, Timmons, Gene 2001). The clones for RNAi against *hlh-2*/M05B5.5, *hlh-15*/C43H6.8, *mxl-1*/T19B10.11, *mdl-1*/R03E9.1, *mxl-3*/F46G10.6, *lin-32*/T14F9.5, *ceh-22*/F29F11.5, *ces-2*/ZK909.4, *hlh-11*/F58A4.7, *pha-4*/F38A6.1, *daf-16*/R13H8.1, *daf-12*/F11A1.3, *blmp-1*/F25D7.3, *skn-1*/T19E7.2, *argk-1*/F44G3.2, *let-363*/B0261.2 (Ralph Baumeister), *trx-1*/B0228.5 and *trx-2*/B0024.9 were obtained from the ORF library v1.1 (Thermo Fisher Scientific, Waltham, MA, USA), while the clones for pbs-3/Y38A8.2 and pbs-4/T20F5.2 were obtained from the Ahringer library (Source Bioscience, Nottingham, UK). Clones for RNAi against *efl-1*/Y102A5C.18 and *grh-1*/Y48G8AR.1 have been constructed as follows: RNAi against *efl-1*/Y102A5C.18 was made by inserting a DNA region containing 248 bp matching the *efl-1*/Y102A5C.18 mRNA starting from position 161 into the L4440 empty vector; for RNAi against *grh-1*/Y48G8AR.1 we inserted a DNA region matching the *C. elegans* DNA on Chromosome I at positions 1,267,785~1,268,085 covering most of the largest exon of *grh-1*/Y48G8AR.1 gene. All clones were verified by sequencing (Microsynth AG, Balgach, Switzerland) before use. Bacterial clones were cultured in LB broth with 100 µg/ml ampicillin overnight, then seeded onto NGM plates containing 1 mM isopropyl-b-D-thiogalactoside (IPTG) and 100 mg/ml ampicillin (all from Applichem,

Darmstadt, Germany). After plates were poured and dried, they were sealed and stored at 4°C. Freshly prepared bacteria were spotted on plates on the previous evening and allowed to dry and settle overnight.

Incubations with RNAi clones started 64 h after synchronization of the population, by washing the synchronized, young adult worms and then transferring them suspended in S-Buffer to the respective treatment plates. To maintain synchronized populations during long-term experiments, worms were washed off the plates into 15 ml tubes every day of the reproductive period, allowed to settle and then washed again repeatedly until the supernatant was free of progeny. The clean worms were then transferred to the freshly prepared treatment plates.

4.4 Lifespan assays and compound treatment

All lifespans were performed at 20°C. In short, a *C. elegans* population was synchronized as described above at day 0 of the lifespan. Sixty-four hours after egg preparation, around 250 young adults were manually transferred to fresh incubation plates (50 worms per 55mm dish). For the first 8-10 days, worms were transferred every day and afterwards every other day. Nematodes that showed no reaction to gentle stimulation (prodding) with a pick were scored as dead. Worms that crawled off the agar surface or that displayed internal hatching or protruding vulva were censored.

NAC and BHA were dissolved in water (NAC, 500-fold stock solution, 500 mM) and DMSO (BHA, 1,000-fold stock solution, 25 mM), respectively. Nematodes (wild-type Bristol N2 and respective mutants) were propagated on agar plates containing antioxidants or respective solvent for 2 generations before start of the experiments.

Heat shock-induced overexpression for the lifespan assays using *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *ceh-22*/F29F11.5 overexpressing strains was performed as follows: 64 hours post egg synchronization, after the transfer of the L4 larvae onto the fresh plates with RNAi bacteria, the control and the heat shock treatment plates were put into the 20°C or in the 33°C incubator, respectively, for 1 hour, after which all plates were moved back

into the 20°C incubator. Heat shock treatment was repeated every 4 days until the end of the experiment.

4.5 Age pigment analysis

Age pigments, or lipofuscin fluorescence, in *C. elegans* have been reported to correspond with biological age (In vivo spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in Caenorhabditis elegans, Beate Gerstbrein, Aging Cell 2005). Worms were synchronized and treated for 10 days with control vector or *hlh-15*/C43H6.8, *lin-32*/T14F9.5 RNAi starting at L4 larvae stage. On day 10 worms were washed off the plates and distributed into 8 wells of a 96-well plate (Bioswisstec 96-well CG black with glass bottom, #5241). Age pigment auto-fluorescence was measured using a fluorescence plate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany; excitation: 340-10 nm, emission: 440-80 nm). We normalized the age pigment fluorescence to the stable auto-fluorescence signal of the worms (filters: excitation: 290-10 nm, emission: 330-10 nm; gain: 1800) as described (In vivo spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in Caenorhabditis elegans, Beate Gerstbrein, Aging Cell 2005).

4.6 Fertility assay

To determine fertility, nematodes were synchronized as previously described. The single L4 larvae were transferred onto single plates carrying control vector L4440 or *hlh-2*/M05B5.5, *lin-32*/T14F9.5, *efl-1*/Y102A5C.18 RNAi bacteria (10 plates per condition) and subsequently onto fresh plates every day of the reproductive period. Progeny were allowed to hatch and were counted.

4.7 Locomotion velocity assay

Worms were synchronized and after 64 hours treated for 10 days with either control vector L4440 or *hlh-2*/M05B5.5, *argk-1*/F44G3.2 RNAi bacteria. After 10 days, 10-15 worms were transferred to fresh plates and 30-second video clips were recorded with a Leica system (Leica M165FC microscope with Leica camera DFC 3000G). Subsequently, 20 independent videos per condition were analyzed with the wrMTrck plugin for Image J software as described (*C. elegans* maximum velocity correlates with healthspan and is maintained in worms with an insulin receptor mutation, Hahm et al., 2015). The pixel-to-distance ratio was calibrated, and average and maximum locomotion velocity values were then calculated.

4.8 Pharyngeal pumping rates assay

To determine the pumping rate, worms were synchronized and after 64 hours treated for 5 days with either control vector L4440 or *hlh-2*/M05B5.5, argk-*1*/F44G3.2 RNAi bacteria. The time it took a worm to perform 10 pumpings, as determined by the grinder movement in the terminal bulb, was recorder, and the number of pharyngeal pumpings per minute was calculated (Methods for measuring pharyngeal behaviors, Raizen et al., 2012). The worms were maintained on bacteria during the measurements, and each worm's pharyngeal pumping rate was quantified in triplicate.

4.9 MEF cell preparation and culture conditions

Mice homozygous for the Tcf3loxP mutation (Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development, Kwon, 2008) were bred with mice heterozygous for the tamoxifen-inducible Cre recombinase

(CreERT2, Taconic Farms Inc., Hudson, NY, USA) [Rapid generation of inducible mouse mutants, Seibler et al., 2003), and the progeny heterozygous for both Tcf3loxP mutation and the tamoxifen-inducible Cre recombinase were bred with the mice homozygous for the Tcf3loxP mutation to obtain mice homozygous for the Tcf3loxP and heterozygous for the tamoxifen-inducible Cre recombinase. MEFs were obtained from a single fetus that was homozygous for the Tcf3loxP and heterozygous for the CreERT2.

$Tcf3^{-/-}$ $CreERT2^{+/-}$ MEF cells and control $CreERT2^{+/-}$ fibroblasts were isolated from 13.5 postcoitum (p.c.) mouse embryos using a commercially available kit (Primary Mouse Embryonic Fibroblast Isolation Kit, #88279, Thermo Scientific) according to the manufacturer's instructions and were grown in Phenol Red-free Dulbecco's' modified Eagle's medium (DMEM) media containing 20% heat-inactivated fetal bovine serum at 37 °C in 5% CO_2/20% O_2.

For preparation of immortalized cells they were continuously passaged until they underwent crisis, all following previously described protocols (Preparation, culture, and immortalization of mouse embryonic fibroblasts, Xu, 2005). Cells were then aliquoted and frozen. One aliquot of these fibroblasts was exposed to tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 μM for 7 days to obtain a homozygous mutation of Tcf3.

4.10 Senescence-associated beta-galactosidase (SA-βgal) activity staining

Cellular senescence process is associated with organismal aging (Cellular senescence and organismal aging, Jeyapalan, 2008). Under the conditions of pH 6 beta-galactosidase activity is easily detectable in senescent, but undetectable in quiescent, immortal, or tumor cells (A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Dimri, 1995). MEF cells have been passaged 12 times when slowed growth suggested that most cells were undergoing senescence, and histochemical beta-galactosidase activity staining was performed using a commercially available kit

(Senescence Cells Histochemical Staining Kit, Catalog number CS0030, Sigma-Aldrich) according to the manufacturer's instructions.

4.11 Proteasome activity assay

The proteasome is responsible for the recycling of specific targeted, typically poly-ubiquitinated proteins, and for maintenance of the balance of protein synthesis and degradation (The proteasome: Overview of structure and functions, Tanaka, 2009). Control and TCF3-KO MEFs have been prepared, and chymotrypsin-like protease activity associated with the proteasome complex has been measured using a commercially available kit (Proteasome 20S Activity Assay Kit, Catalog number MAK172, Sigma-Aldrich) according to the manufacturer's instructions.

4.12 Amplex Red–based quantification of hydrogen peroxide

Synchronized worms were washed with S-buffer, pH 6 from 5 plates, and incubated in an Eppendorf tube in 100 µl of 50 mM sodium-phosphate buffer, pH 7.4, with 100 µM Amplex Red (Invitrogen, Carlsbad, USA) and 0.2 U/ml of horseradish peroxidase. Following incubation in the dark for 3 hours with constant gentle shaking, fluorescence intensity was measured in the supernatant (FLUOstar Omega, BMG Labtech, Offenburg, Germany; excitation: 544-10 nm, emission: 590-10 nm). The worms were washed with S-buffer, sonicated on ice, and centrifuged at 12000 g for 15 minutes at 4°C. The protein continent was determined for normalization using either Bradford or BCA assay (Pierce BCA protein assay, Thermo Fisher Scientific) according to standard protocols using appropriate standards.

4.13 ATP determination

Worms were harvested and immediately shock frozen in liquid nitrogen. The frozen pellet was ground in a liquid nitrogen-chilled mortar to the state of fine powder. Guanidinium-hydrochloric acid (Guanidium HCl) (4 M) was prepared, heated to 100 °C and then mixed with the frozen powder to abolish ATPase activity and to further lyse the samples. The mixture was boiled for 15 min at 100°C and then immediately centrifuged for 30 min at 13200 g at 4°C. The supernatant was diluted with ddH_2O at 1:200 ratio and analysed using a commercially available kit (CellTiter Glo; Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. For normalization of the luminescence signal, protein was determined as described below.

4.14 Immunoblotting

Frozen worm pellets were ground in a liquid nitrogen-chilled mortar along with phosphate buffer containing protease and phosphatase inhibitors (Roche, Penzberg, Germany) with addition of 2 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM PMSF and 2 mM EDTA. The extracts were centrifuged at 12000 g for 7 minutes at 4°C. The supernatant was used to determine the protein concentration, boiled in Laemmli buffer and used for SDS-PAGE.

Antibodies against TCF3 (kind gift from the Meinrad Busslinger lab (Institute of Molecular Pathology, Vienna), used in Ref.; at 1:5000 dilution), CKB (Abcam, ab108388; at 1:1000 dilution), phospho-AMPKα (Cell Signaling, #2535; at 1:1000 dilution), basal AMPKα (Cell Signaling, #2532; at 1:1000 dilution), LC3B (Cell Signaling, #2775; at 1:1000 dilution), phospho-p53 (Cell Signaling, #9286; at 1:500 dilution), basal p53 (Abcam, ab131442; at 1:1000 dilution), CDKN2A/p16INK4a (Abcam, ab211542; at 1:1000 dilution), and α-tubulin (Sigma, clone DM1A; at 1:5000 dilution) were used. Secondary antibodies were diluted 1:2000 before use on the membranes. Full-length images of immunoblots are available upon request.

4.15 Protein quantification

Protein content in nematodes and cells was determined by the Bradford method (A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, BRADFORD, 1976) or the BCA method (Measurement of Protein Using Bicinchoninic Acid, SMITH, 1985). Assays were performed in 96-well plates using commercial available kits (Bio-Rad Laboratories AG, Cressier, Switzerland, and Thermo Scientific).

4.16 Proteasome activity assay

The protocol was adapted from a previous publication (RPN-6 determines *C. elegans* longevity under proteotoxic stress conditions, Vilchez et al., 2012). In short, the worm pellets were ground in a liquid nitrogen-chilled mortar with proteasome activity assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl 2 , 0.5 mM EDTA, 2 mM ATP and 1 mM dithiothreitol). The lysate was centrifuged at 10,000 g for 15 min at 4°C. For each experiment, 50 µg of total protein lysate was transferred to a 96-well microtiter plate (BD Falcon), then respective fluorogenic substrates were added. The substrates Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Glu-AMC, and Ac-Arg-Leu-Arg-AMC (all from Enzo, Switzerland) were used at final concentrations of 100 µM to measure the chymotrypsin-like activity, caspase-like activity, and trypsin-like activity of the proteasome, respectively. Fluorescence (380 nm excitation, 460 nm emission) was monitored with a microplate fluorometer (FLUOstar Omega, BMG Labtech, Offenburg, Germany) every minute for 1 h at 25°C.

4.17 Optical microscopy

SA-βgal staining images were obtained using Zeiss AxioPhot upright Fluorescent Microscope (10x, 25 images per well, 3 wells per condition).

4.18 Confocal microscopy

Worms were mounted on freshly prepared agarose pads (3% agarose in S-buffer) and paralyzed using 20 mM tetramisole.

Confocal microscopy was performed using a Leica TCS SP8 microscope with inverted stand. The software used for image acquisition was Leica LAS X SP8 Version 1.0. Z-stacks of groups of worms were acquired by taking images every 500 nm using an HC PL FLUOTAR 10x/0.30 DRY objective and an argon laser with photomultiplier, collecting light from 500 to 541 nm. For image processing, maximum intensity projections were performed using Fiji ImageJ software.

4.19 RNA Extraction

Total RNA was isolated using QIAzol (Qiagen, Hilden, Germany) based on the phenol‑chloroform extraction method. Afterwards, the RNA was quantified photometrically with a NanoDrop 1000 (PeqLab, Erlangen, Germany) and stored at − 80°C until further use.

4.20 cDNA synthesis and qPCR

cDNA was prepared using High capacity cDNA reverse transcription kit with the use of Oligo(dT) primers following the manufacturer protocol (Applied Biosystems).

mRNA levels were quantified from 3 biological replicates and 3 technical replicates using SYBR Green select master mix (Applied Biosystems) fluorescence on a 384-well format plate in CFX96 real time system (Biorad). After an initial denaturation step

(95°C for 2 min), amplification was performed using 40 cycles of denaturation (95°C for 15 s) and annealing (60°C for 1 min). Samples were analyzed by ddCT method, with normalization to the reference gene Y45F10D.4 (Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in *C. elegans*, Hoogewijs et al., 2008); p values were calculated by two-tailed Student's t-test.

The following primers were used for this study:

hlh-2/M05B5.5 primers
FWD TGCCTGATTATTGGAGTGGA;
REV CTACGGAAGCAGGGGTTTC;
lin-32/T14F9.5 primers
FWD TCAGTCAGACCAACCATGAGC;
REV GGCTTTGCAGTGGAGTTGTC;
argk-1/F44G3.2 primers
FWD CGGCTCCAAACAGCATGAAG;
REV TTTTGCACCATCAGGACCGT;
argk-2/W10C8.5 primers
FWD CACTCTCACTCCACAGCAACTT;
REV TCCGAGCTTTGTACGCTTAGT;
argk-3/F32B5.1 primers
FWD GTCTCGTCGAAATGGCATCG;
REV GGCTTGAAGTTGCTGTGGAG;
argk-4a/F46H5.3 primers
FWD ATGTTACATCGATGCTCGAGAGT;
REV GCCTACTCGACCTCACTTCAC;
argk-5/ZC434.8 primers
FWD GAACGAACCGCCTGTTACAC;
REV TCCTTGGAGTTTCGTGTAGACTT;

4.21 Next-generation sequencing (RNAseq)

Total RNA was inspected for degradation using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). For library preparation an amount of 2 µg of total RNA per sample was processed using Illumina's TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's instruction. Sequencing was done on a HiSeq2500 in SR/50 bp/ high output mode. Libraries were multiplexed in five per lane; sequencing ends up with ~35 Mio reads per sample.

Sequence data were extracted in FastQ format and used for mapping approach. The FastQ files were mapped using Tophat versus the reference genome obtained from Ensembl (TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions, Kim et al., 2013). Uniquely mapped reads were counted for all genes using featureCounts (featureCounts: an efficient general purpose program for assigning sequence reads to genomic features, Liao et al., 2014). RPKM values were computed using exon lengths provided by featureCounts and the sum of all mapped reads per sample. DEG were identified using DESeq (Differential expression analysis for sequence count data, Anders and Huber, 2010), edgeR (edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Robinson et al., 2010), and DESeq2 (Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, Love, 2014). All three packages provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg approach for controlling for the false discovery rate (FDR). If both FDR values (by DESeq and edgeR, or by DESeq2 and edgeR in later analyses) were below P = 0.05, those genes were counted as differentially expressed.

Intersection of differentially expressed genes (DEGs) of *hlh-2*/M05B5.5 RNAi (padj<0.05 in edgeR and Deseq2) was performed using Venn analysis of the Biovenn tool (http://www.biovenn.nl/index.php).

4.22 Promoter analysis

The FASTA file containing proximal 1 kb upstream region of the differentially expressed genes was obtained using WormMart. The sequence files were scanned for one or more matches to the position weight matrices using the matrix-scan function of the pattern-matching program RSAT (regulatory sequence analysis tools) (Using RSAT to scan genome sequences for transcription factor binding sites and cis-regulatory modules, Turatsinze et al., 2008). The position-specific scoring matrices contain a nucleotide frequency at each position within binding sites and were obtained from the databases Transfac and Jaspar (JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles, Mathelier et al., 2014; TRANSFAC: transcriptional regulation, from patterns to profiles, Matys et al., 2003; JASPAR: an open-access database for eukaryotic transcription factor binding profiles, Sandelin et al., 2004). The threshold p-value, which indicates the risk of false positive predictions, was set to 0.0001.

4.23 Statistical analysis

Statistical analyses for all of the data except for lifespan assays was carried out using Student's t-test (unpaired, two-tailed) or ANOVA after testing for equal distribution of the data and equal variances within the data set. If the samples had unequal variance, the data was log-transformed before carrying out Student's t-test.

Experiments were performed in triplicate except where stated otherwise. For comparing significant distributions between different groups in the lifespan assays the statistical calculations were performed using JMP software version 9.0 (SAS Institute Inc., Cary, North Carolina, USA), applying the log-rank test. All of the other calculations were performed using Excel 2010 (Microsoft, Albuquerque, NM, USA). A p-value <0.05 was considered statistically significant. Data are expressed as mean ± standard deviations unless otherwise indicated.

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6 List of abbreviations

4-OHT	4-hydroxytamoxifen
Aβ	β-amyloid
AMPK	AMP-activated protein kinase
BHA	Butylated hydroxyanisole
bHLH	basic helix-loop-helix
CGC	Caenorhabditis Genetics Center
CKB	Creatine kinase (brain) (gene name)
CNS	Central Nervous System
CR	Calorie restriction
DDR	DNA damage response
DR	Dietary restriction
EMT	Epithelial–mesenchymal transition
GFP	Green fluorescent protein
HS	Heat shock
HSR	Heat shock response
IIS	Insulin/insulin-like growth factor signaling
KO	Knock-out
L4	Larval 4th (stage of development)
MEF	Mouse embryonic fibroblasts
Mi-CK	Mitochondrial creatine kinase
mTOR	Mechanistic or Mammalian target of rapamycin
NAC	N-acetylcysteine
NPA	Non-Programmed theories of aging, or Non-Programmed Aging
pA	polyadenylation
PA	Programmed aging theories, or Programmed Aging
PCr–CK	Phosphocreatine - creatine kinase system
PN	Proteostasis network
PWM	Position weight matrix
ROS	Reactive oxygen species
RSAT	Regulatory Sequence Analysis Tools
SA-β-gal	Senescence-associated beta-galactosidase

SASP	Senescence-associated secretory phenotype
TF	Transcription factor
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
WB	Western blot
WT	Wild type
YA	Young adult (stage of development)

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8 Appendix

The following materials are submitted in electronic version as supplementary information:

1. RSAT promoter analysis of aging-affected genes (JenAge study data) using the full set of 324 nematodal PWMs
2. Full lists of DEGs for the RNA deep sequencing of *hlh-2*/M05B5.5, *lin-32*/T14F9.5, and *ceh-22*/F29F11.5 RNAi-treated worms samples
3. Table with Results and Statistical Analyses of *C. elegans* Lifespan Assay data