Investigating the roles of SKN-1B and RNA polymerase III in ageing.

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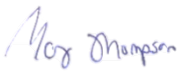
Submitted to the University of Kent for the degree of PhD in Cell Biology.

Submitted by Maximilian Alexander Thompson, PhD in the School of Biosciences, Lab of Jennifer Tullet, April 2019. All work shown here is my own, unless duly noted otherwise.

Signed 

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

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5’ RACE: 5’ Rapid amplification of cDNA ends

CGC: *C. elegans* Genetics Center, University of Minnesota

daf-c/daf-d: Dauer Formation Constitutive/Defective

DiI: 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate

DR: Dietary restriction

- bDR: bacterial DR
- lDR: liquid DR
- sDR: solid DR
- msDR: modified solid DR
- dd – Dietary Deprivation
- IF – Intermittent Fasting

ERAD: ER associated degradation

FBS: Fetal Bovine Serum

GFP: Green fluorescent protein

GPRC: G-protein coupled receptors

ILPs: Insulin Like Peptides

LB: Lysogeny/Luria Broth media

NGM: Nematode Growth Media

PCR: Polymerase Chain Reaction

RT-qPCR: Real Time Quantitative Polymerase Chain Reaction

rIIS: reduced Insulin/IGF1-like signalling

SL1/2: Spliced leader 1/2

TOR: Target of Rapamycin

UCE: Upstream Control Element

UPR^{ER}: Unfolder Protein Response – Endoplasmic Reticulum

Abstract

The diseases of ageing represent an increasingly severe threat to human health, and most societies are experiencing an unprecedented demographic shift towards an older population. In this thesis the nematode worm *C. elegans* is used to examine two aspects of the ageing process. Firstly, it investigates the function of *skn-1b. skn-1* is the *C. elegans* orthologue of mammalian NRF proteins and is required for normal longevity and stress resistance. The *skn-1b* isoform is expressed in two ASI neurons and is known to be required for the longevity resulting from the life-extending intervention dietary restriction, however little is known about the mechanisms by which this is possible. This study shows that *skn-1b* expression peaks at the L2 stage and that its levels and patterns are modulated by food levels in the external environment with *daf-11* being an important mediator of this. Signalling from ASI neurons via secreted ILPs and TGF-β ligand appears disrupted in animals lacking *skn-1b*, and animals lacking *skn-1b* display a cell non-autonomous failure to properly activate the UPR^{ER} when challenged with tunicamycin. It also reports that the SKN-1B protein is a functional transcription factor, but that *skn-1b* is not required for oxidative stress resistance, normal lifespan or brood size.

In the second part of this thesis we identify RNA polymerase III as limiting lifespan in *C. elegans*, showing that knockdown of Pol III function using *rpc-1* RNAi can extend lifespan either when active in the whole worm or just the intestine. Inhibition of Pol III protects the *C. elegans* intestine showing that at least one ageing pathology is delayed. Finally, this thesis shows that this lifespan extension is separable from that resulting from inhibition of

Ribosomal Protein S6 Kinase (*rsks-1).* Taken together, the findings presented here shed more light on the role of transcriptional regulators in the ageing process.

Introduction.

1.1. Ageing as a global issue

Ageing, and its pathological manifestations through the diseases of ageing presents an increasingly major challenge to societies across the globe. Many western countries expect a near doubling in the percentage of their population which is over 60 years old between 2000 and 2050 (Powell and Cook, 2009). This challenge is not limited to the west, either – China and India which between them account for nearly 25% of the global population each expect the percentage of over 60s in their societies to approximately triple in the same period (Ang, 2013). In the context of rising powers such as these, how a country responds to this demographic shift will have profound economic and geopolitical consequences as well as the obvious increased burden on public health. In many ways, the increased prevalence of age-related disease is simply a symptom of a series of successes in addressing the major causes of premature death. Human deaths by war, communicable diseases, famine and genocide have all radically decreased over the last few centuries in much of the world (Ang, 2013; Norheim *et al.*, 2015). Concurrently, average human lifespan has undergone a period of rapid extension which has been driven by the development of antibiotics, vaccination and improvements in the provision of healthcare services. For the last 160 years, female life expectancy has consistently risen by 3 months per year, with the longest-lived population in the world in 1840 being Swedish women who could expect to live an average of 45 years. That record is now held by Japanese women who can expect to live to 85 (Oeppen and Vaupel, 2002). The Gompertz-Makeham law of mortality, named after the 18th century actuaries William Makeham and Benjamin Gompertz, describes two main factors in the

determination of lifespan in humans. Firstly, there is the Makeham function – that is the non-age dependent causes of death (Izsák and Gavrilov, 1995). Recent reductions in deaths attributable to progress in fighting communicable diseases and prolonged periods of great power peace have therefore largely contributed to the extension of human lifespan by reducing the Makeham function. For example, this age-independent hazard approximately doubled for many European countries during world war 1 (Gavrilov, Gavrilova and Krut'ko, 2017). The Gompertzian aspect of mortality is the age-related increase in the likelihood of death, and more closely describes "ageing" as it is described here since this is necessarily a product of biological changes which occur in the body as we age (Gompertz, 1825). This function has not been static either, with most of the increases in average lifespan prior to the 1950s being attributable to a reduction in the Makeham function, but since then the phenomenon of "rectangularisation" of survival curves has been occurring where more deaths are clustered near the apparent maximum lifespan in a population, pushing the average lifespan up and compressing the period in which death tends to happen. It is important to note that this does not necessarily correspond to a compression of morbidity in old age, so much as better healthcare for those at this stage of life (Nusselder and Mackenbach, 1996).

The impact of ageing on human health is profound. Of the approximately 150,000 people who die every day, 100,000 of them die of age related disease (Lopez *et al.*, 2006). Age is a major risk factor in the development of cancer (Smetana *et al.,* 2016) and there are many age related pathologies including Alzheimer's disease, Parkinsons disease, diabetes and others. Therefore, the study of ageing process is of great importance to human health.

1.2. *C. elegans* as a model organism in ageing studies

One major challenge when studying mammalian ageing is the timescales involved in doing so. Two trials of dietary restriction (DR) protocols in the primate *Rhesus macaques* lasted in excess of 30 years, only to deliver conflicting and somewhat confusing findings; one study showed no effect of their DR protocol on these animals (Mattison *et al.*, 2012) while another concurrent study showed a lifespan extension and better health in these animals (Colman *et al.*, 2014) although the consensus seems to have settled on the notion that DR can achieve a small lifespan extension in these animals (Anderson *et al.*, 2017). For this reason, simpler less long-lived model organisms such are flies, worms and yeast offer a significant advantage for lifespan studies. In this study we use the nematode worm *C. elegans* which has proved a valuable tool in the study of ageing. First developed as a model organism for the study of development and the nervous system by Sydney Brenner (Brenner, 1974), *C. elegans* has become a key model organism in numerous research areas. Adult *C. elegans* are approximately 1mm long and are easily viewable with a dissecting microscope (Brenner, 1974). Homogenous populations of *C. elegans* can easily be maintained due to the fact that most animals are self-fertilising hermaphrodites, however male animals do appear at low frequency and male enriched populations can be maintained by mating with hermaphrodites (Brenner, 1974). The presence of males means that strains can be easily crossed, while resulting mutants can be easily isolated from single hermaphrodites. *C. elegans* is post mitotic, with 959 somatic cells (Sulston and Horvitz, 1977) including 302 neurons (White *et al.*, 1986) of known embryonic and postembryonic lineages (Sulston *et al.*, 1983), making very precise study of the development of these animals possible down to the level of individual cell fates . Additionally, the full connectome of the *C. elegans* nervous

system has been reported which means that the animal is a very useful for neuronal studies since known connections between known neurons allow the identification of neuronal circuits (Corsi, Wightman and Chalfie, 2015). The *C. elegans* genome was the first multicellular eukaryotic genome to be sequenced, further increasing its utility as a model organism (The C. elegans Sequencing Consortium, 1998). *C. elegans* has a short generation time going from an egg to an adult in 3.5 days and is amenable to many sorts of study including RNAi and exogenous expression of chosen genes (Hillier *et al.*, 2005). *C. elegans* has proved to be a valuable tool in ageing, with important insights including the implication of insulin signalling in ageing being generated in *C. elegans* (Friedman and Johnson, 1988; Kenyon *et al.*, 1993) and being found to be widely conserved, including in mice (Bluher, 2003; Taguchi, Wartschow and White, 2007; Selman, 2008) and potentially in humans (Suh *et al.*, 2008; Willcox *et al.*, 2008; Flachsbart *et al.*, 2009; Pawlikowska *et al.*, 2009; Soerensen *et al.*, 2015). This led to the identification of other lifespan regulating genes involved in different longevity processes being identified in *C. elegans,* including Target of Rapamycin (TOR) (Vellai *et al.*, 2003), mutants that decrease mitochondrial function (Van Raamsdonk *et al.*, 2010) and others. There is a reasonably high degree of conservation between nematodes and humans with 60-80% on human genes being represented by a *C. elegans* orthologue (Corsi, Wightman and Chalfie, 2015), which means that studies in the worm have real relevance to human disease.

1.3. Dietary restriction and ageing

In the 1930s, the American government became worried that the great depression and the chronic hunger which resulted would damage the health of the American population in the long term. A series of experiments were conducted on rats which were fed severely restricted diets (the diets themselves were varied in order to limit growth of rats subjected to them). This work demonstrated that animals in the dietary restricted group were in fact long lived and displayed slower appearance of pathologies (M. McCay and F. Crowell, 1934; McCay, Mary and Maynard, 1935). Longevity by dietary restriction (DR) has since been shown to be conserved to all model organisms tested, including yeast (Jiang *et al.*, 2000; Lin, Defossez and Guarente, 2000; Anderson *et al.*, 2003; Kaeberlein *et al.*, 2004; Sinclair, 2005), worms (Klass, 1977; Lakowski and Hekimi, 1998; Houthoofd *et al.*, 2002; Fontana, Partridge and Longo, 2010), flies (Partridge, Green and Fowler, 1987; Chippindale *et al.*, 1993; Society and Sciences, 1996), mice (Weindruch *et al.*, 1986; Masoro, 2000) and rhesus monkeys (Mattison *et al.*, 2012; Colman *et al.*, 2014; Anderson *et al.*, 2017). The downstream genetic pathways which mediate dietary restriction longevity are complex and there are differences between model organisms and DR protocols (Mair and Dillin, 2008). The main pathways of interest in DR longevity are pathways regulated by the nutrient sensor Target of Rapamycin (TOR), insulin signalling and the NAD+ dependent histone deacetylase sirtuin proteins (Kapahi, Kaeberlein and Hansen, 2017), with an important caveat being that in *C. elegans,* which we focus on here, the longevity achieved by reducing insulin signalling (rIIS) is separable from that resulting for the most commonly used (and oldest) DR protocols in the worm (Houthoofd *et al.*, 2003), but not others.

1.3.1 Dietary restriction protocols in *C. elegans*

C. elegans have been used to examine underlying mechanisms of dietary restriction, resulting in a significant body of work on DR in *C. elegans* (Houthoofd *et al.*, 2003; Bishop and Guarente, 2007a). One issue with conducting dietary restriction experiments in *C. elegans* is the fact that there are numerous methodologies which are used to achieve DR in this model. Indeed, there are numerous DR assays published which differ in the bacterial food source, the addition of various different antibiotics, the use of liquid or solid media to perform the assay and the day in adulthood at which DR is initiated (Kapahi, Kaeberlein and Hansen, 2017). Figure 1.1 shows some common DR protocols, but by no means an exhaustive list. The differences between different dietary restriction protocols in terms of the requirement of different pathways adds significant complexity to interpreting dietary restriction lifespan data. The bDR and iDR protocols use liquid NGM media and various antibiotics to prevent bacterial growth, whereas the sDR, msDR, dietary deprivation and intermittent fasting use standard solid NGM plates and focus on dilution (or removal) of the bacterial food source (Kapahi, Kaeberlein and Hansen, 2017). Perhaps surprisingly, the requirement for key longevity associated pathways seems to vary between them (Greer and Brunet, 2009; Mair *et al.*, 2009). This is particularly notable in the case of insulin signalling, which (in *C. elegans*) was initially considered to regulate lifespan via mechanisms distinct from those regulated by dietary restriction due to the lack of requirement of *daf-16* for *eat-2* longevity (Lakowski and Hekimi, 1998) and the lack of requirement for *daf-16* and additive effect of *daf-2* mutation with some DR protocols (Houthoofd *et al.*, 2003). Thus, earlier experiments supported a clear-cut model where DR longevity is neatly separable from insulin and IGF-1 like signalling (IIS).

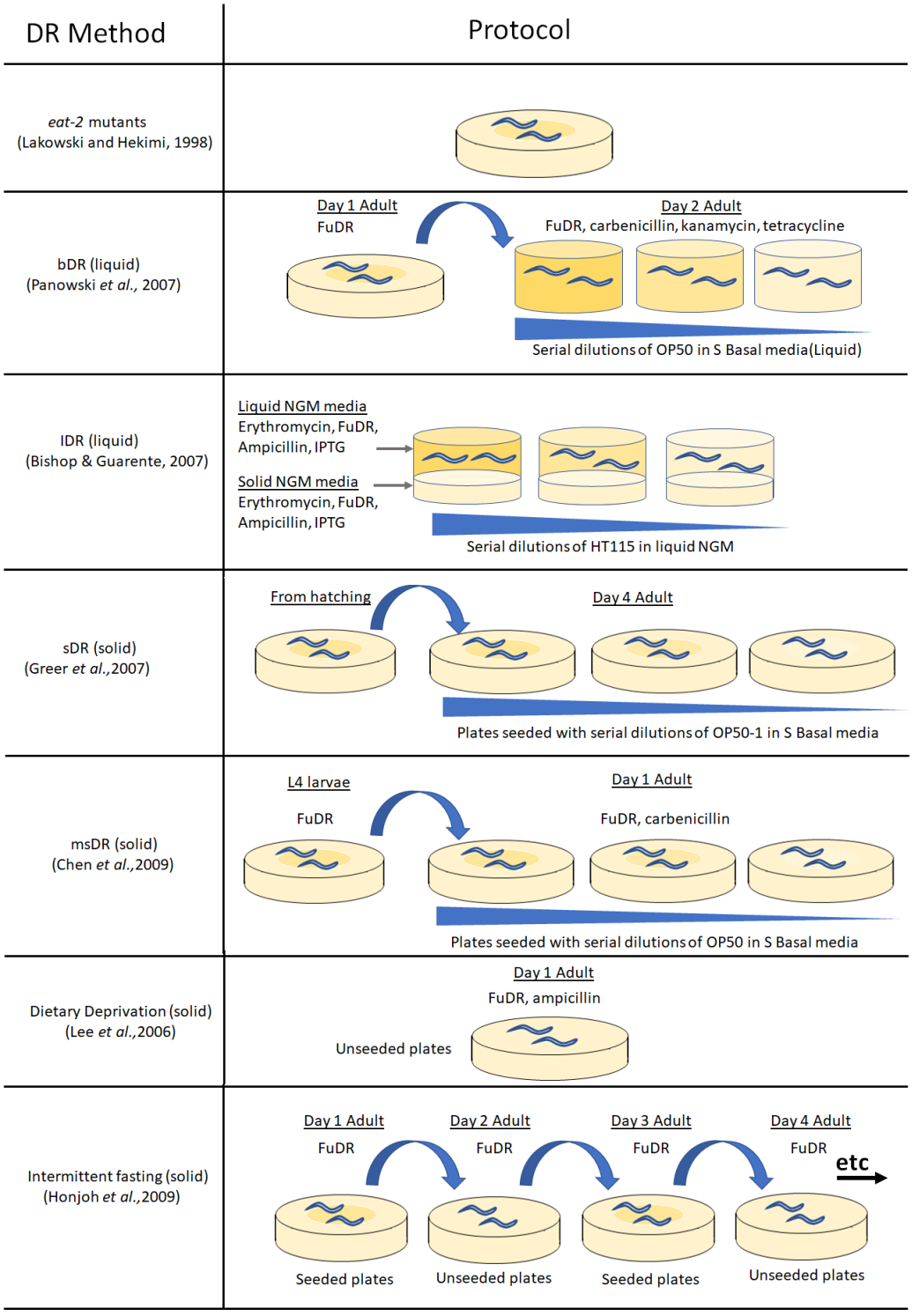

Figure 1.1: Various Dietary Restriction protocols from the literature: Various methods and protocols of achieving DR in C. elegans. Mutations in eat-2 reduce bacterial uptake (Lakowski and Hekimi, 1998), bDR (Panowski et al., 2007) and lDR (Bishop and Guarente, 2007b) are liquid DR protocols, while sDR (Greer et al., 2007), msDR (Chen, Thomas and Kapahi, 2009), dietary deprivation (Lee et al., 2006) and intermittent fasting (Honjoh et al., 2009) are conducted on solid NGM.

What has emerged in recent years has been a significantly messier picture of the separation between DR and IIS longevity, where *daf-16* is required for intermittent fasting protocols and the sDR method (Greer and Brunet, 2009; Honjoh *et al.*, 2009) in which diluted bacteria is seeded onto solid plates rather than in liquid as with other DR protocols.

1.4. Target of Rapamycin (TOR)

In this research we focus on ageing, and TOR signalling is a target of huge interest in ageing research which is an important effector of DR longevity (Hansen *et al.*, 2007) capable of extending lifespan in all model organisms tested (Kapahi *et al.*, 2010). TOR is a nutrient sensitive regulator of numerous processes related to growth and cellular maintenance (Kenyon, 2010).

Rapamycin was first identified as an antifungal compound produced by the bacteria *Streptomyces Hygroscopicus*, isolated from soil samples from Easter island (or, Rapa Nui in the local language) (Vézina, Kudelski and Sehgal, 1975). It was later observed to have potent activity as an immunosuppressor which led to the use of rapamycin and rapamycin derived drugs (rapalogues) in organ transplants (e.g Gurk-Turner, Manitpisitkul, & Cooper, 2012). TOR itself was identified first by working backwards from rapamycin to identify its binding to FKBP12 and the interaction of this complex with the protein which they termed target of rapamycin (TOR) in three concurrent studies and yeast and mammalian cells (Brown *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995), which has subsequently been identified as strongly conserved in all eukaryotes. In terms of molecular function - mechanistically, TOR has been most studied in yeast and in human cell lines with much of the specifics of its interactions in worms being inferred from sequence homology to these models.

TOR is a serine threonine protein kinase which acts as one of the central regulators of cellular metabolism. It exists in two complexes – TORC1 and TORC2, both of which share some conserved components but also associate with unique ones which impart specific activities and targets on TORC1 and TORC2 (Figure 1.2). TORC2 is significantly less sensitive to rapamycin (Kennedy and Lamming, 2016). The role of TOR can be summarised as controlling cell growth and metabolism in response to nutrient status, upstream signalling pathways, energy levels and stress (Kenyon, 2010).

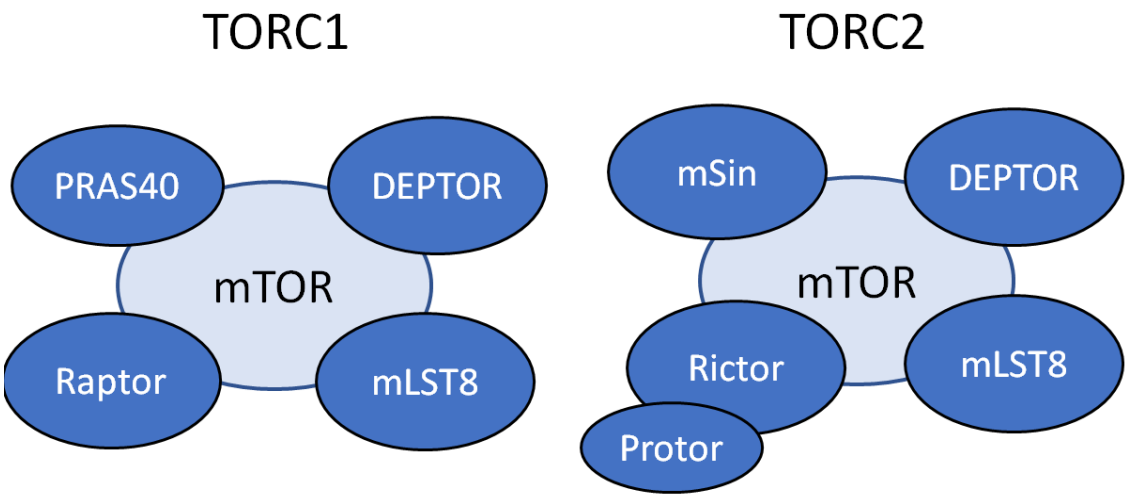

Figure 1.2: TORC1 and TORC2 complexes. TORC1 includes PRAS40, Raptor, DEPTOR and mLST8. TORC2 consists of RICTOR, PROTOR, mSin as well as DEPTOR and mLST8.

1.4.1. Overview of TORC1 activity

TORC1 is a central regulator of metabolism and acts to integrate numerous signals, which in effect means that TORC1 is itself regulated by numerous processes. Key outputs of increased TOR signalling are translation (Hay and Sonenberg, 2004) and reduced autophagy (Wullschleger, Loewith and Hall, 2006). One important aspect of the activity of TORC1 is that it enacts pro-growth processes in conditions where there exist the nutrients and necessary components to support this. TORC1 activity requires the presence of sufficient levels of amino acid levels, particularly levels of the BCAA branch chain amino-acids are important in regulating it (Hara *et al.*, 1998). The requirement of amino acid sufficiency overrides growth factor based stimulation of TORC1 activity, meaning that TORC1 will only proceed with its pro-growth activities when sufficient amino acid levels will support them (Kapahi *et al.*, 2010). This occurs on the lysosomal surface, which TORC1 is recruited to by the Rag GTPases (Kim *et al.*, 2008). Amino acids cause the Rag-GTPase heterodimer to enter its active conformation and promotes TOR localisation to the lysosome, thus enforcing the requirement for amino acids for TORC1 localisation to the lysosome and its activation (Jewell, Russell and Guan, 2013). In terms of nutrient availability, TORC1 also is regulated by glucose levels, with higher levels of glucose leading to TORC1 localisation to the lysosome (Efeyan *et al.*, 2012). TORC1 at the lysosomal surface additionally requires the activity of Rheb-GTP which is in turn inhibited by the TSC1/2 heterodimer. This interaction with TSC1/2 is important for TORC1 function since TSC itself is a target for numerous other TORC1 regulating pathways, including AMP-activated protein Kinase (AMPK) in response to reduced ATP levels (Mihaylova and Shaw, 2012), the MAPK pathway via ERK (Ma *et al.*, 2005), GSK3 in the wnt pathway (Majid, Saini and Dahiya, 2012), REDD as a result of hypoxia (Horak *et*

al., 2010), AKT downstream of insulin signalling (Menon *et al.*, 2014) and TORC2, also via phosphorylation of AKT. It is principally by the regulation of the TSC complex that TORC1 integrates the output of cellular energy sensors such as AMPK (which responds to the AMP/ADP:ATP ratio) (Gwinn *et al.*, 2008) and insulin (which responds to glucose levels) into the regulation of cell growth (Menon *et al.*, 2014) Thus, TORC1 is regulated by numerous pathways which share the characteristic of responding to changes in conditions which necessitate a change in the rate of cell growth or autophagy (Figure 1.3).

TORC1 is then able to regulate a large variety of biochemical processes in response to these stimuli. Translation in particular is heavily regulated by TORC1 activity, since increased translation is required for growth (Kapahi *et al.*, 2010). Direct inhibitory phosphorylation of eukaryotic initiation factor 4E-binding protein (4E-BP) by TORC1 causes its dissociation from eukaryotic initiation factor 4E (eIF4E), which allows eIF4E to associate with the translational initiation complex (Martelli *et al.*, 2011) thus promoting translation. Similarly, S6K is activated by phosphorylation by TORC1 which allows it to promote translation by phosphorylation of ribosomal S6 and eIF4B (Pullen and Thomas, 1997). Of particular note to this thesis, TORC1 also enters the nucleus and associates with RNA polymerase III promoters where it provides an inactivating phosphorylation of Maf-1, an inhibitor of RNA polymerase III, which ultimately results in active TORC1 promoting the transcription of Pol III targets (Kantidakis *et al.*, 2010). Pol III targets include rRNA and tRNAs which are required substrates for translation (Weinmann and Roeder, 1974).

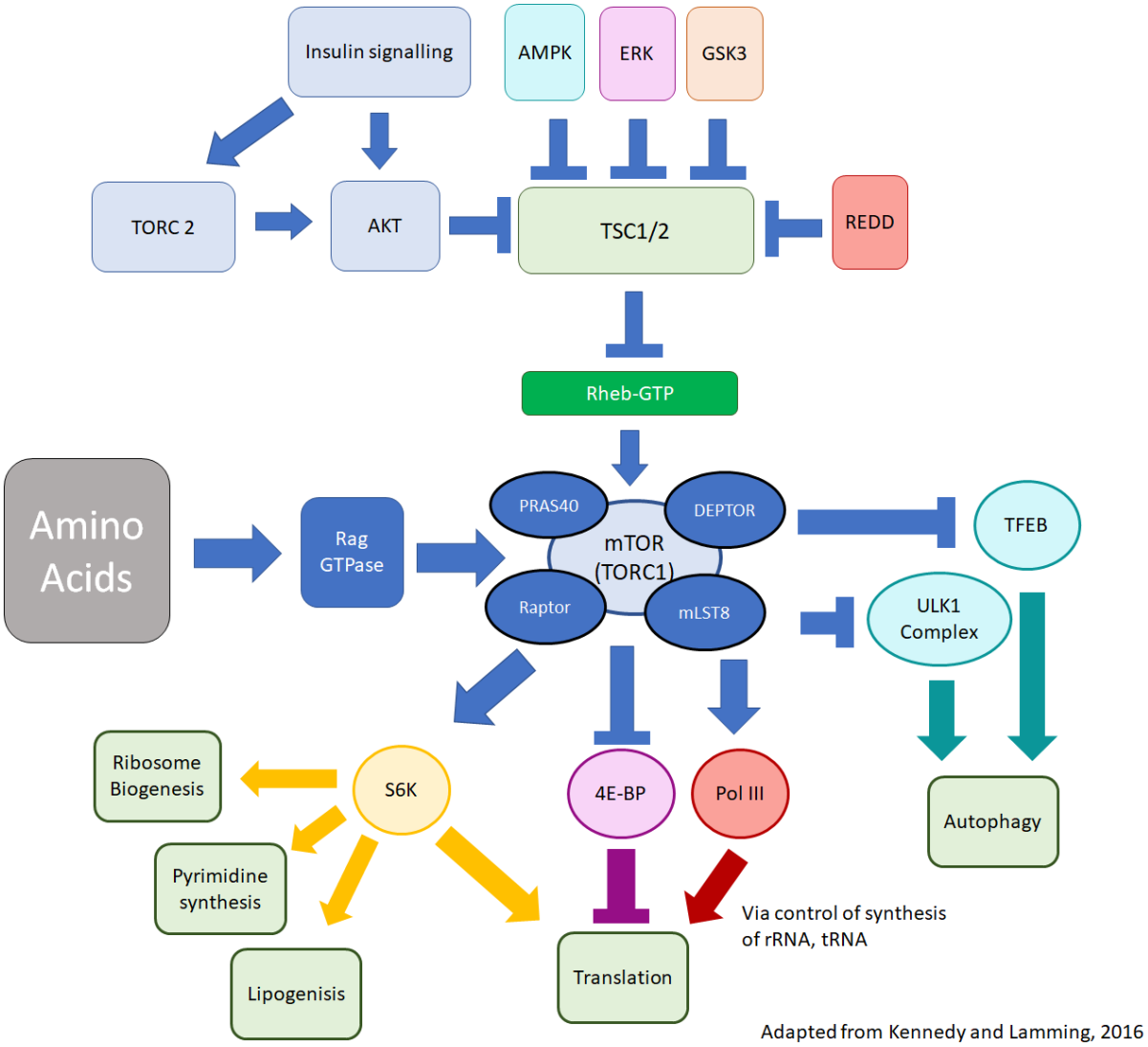

Figure 1.3: Simplified representation of the regulation of TORC1 and key processes it regulates. TORC1 is regulated by numerous nutrient sensing processes. Much of this is mediated by the regulation of the TSC complex. Downstream of reduced insulin signalling AKT, provides inhibitory phosphorylation of TSC thus releasing rheb from inhibition, as does AMP-activated protein kinase (AMPK), extracellular regulated kinase (ERK), Glycogen synthase kinase 3 and regulated in development and DNA damage responses 1 (REDD-1). Translation is regulated via activating phosphorylation of ribosomal protein S6 kinase and Pol III as well as inhibitory phosphorylation of eif-4E-binding protein. Autophagy is regulated by TORC1 via transcription factor EB (TFEB) and Unc-51 like autophagy activating kinase (ULK1).

As well as promoting translation, TORC1 inhibits autophagy, a process by which the cell recycles cellular components including damaged mitochondria and other organelles (Wullschleger, Loewith and Hall, 2006; Jia and Levine, 2007). TORC1 interacts with the ULK-1 kinase complex and provides inhibitory phosphorylation events which prevent this complex from localising to the plasma membrane where it is required to interact with the preautophagosome for autophagy to occur (Alers *et al.*, 2011). TORC1 also inhibits the action of the autophagy promoting transcription factor TFEB by inhibitory phosphorylation which causes it to be retained in the cytoplasm rather than enter the nucleus (Napolitano and Ballabio, 2016). Thus, TOR causes an increase in translation and a downregulation of autophagy (Figure 1.3). Crucially, the action of TOR on both translation and autophagy are ones which would be predicted to act against longevity since interventions which reduce translation (Pan *et al.*, 2007a) or increase autophagy (Nakamura and Yoshimori, 2018) are pro longevity.

1.4.2. Overview of TORC2 activity

The role of TORC2 is a little less clear, partly due to its relative insensitivity to rapamycin (caused by RICTOR masking the rapamycin sensitive domain in the TOR protein) and a lack of other potent TORC2 specific inhibitors (Loewith *et al.*, 2002). This means that the well-studied effects of rapamycin are principally functions of TORC1, and equivalent experiments are difficult to perform in on TORC2 resulting a slower progress in determining both the regulation and targets of TORC2 (Gaubitz *et al.*, 2016). Another reason for the focus on TORC1 is that while TORC1 is crucial in cancer development (Beauchamp and Platanias, 2012) and of huge interest in ageing research (McCormick, Tsai and Kennedy, 2011), TORC2 is less critical in these processes. TORC2 seems to respond to membrane tension and nutrients in order to regulate the formation of the plasma membrane and other diverse processes (Kabeche, Howard and Moseley, 2015).

What is known is that TORC2 is regulated downstream of insulin signalling by the activation of PI3K and synthesis of PIP3, and in line with this idea TORC2 localises to the plasma membrane (Berchtold and Walther, 2009). In yeast, TORC2 is regulated by membrane tension and this is suspected to be the case in mammalian cells as well, since mTOR dependent AKT phosphorylation is increased as a result of membrane tension (Kaufmann *et al.*, 2004). There is some controversy about localisation of TORC2 to non-plasma membrane tissues in mammalian cells including the ER and mitochondria (Gaubitz *et al.*, 2016) in terms of whether this is an experimental artefact or a genuine indication of functions in those tissues. Various post-translational modifications modulate TORC2 activity; there are numerous phosphorylation events reported in TORC2 subunits, including by AKT (Humphrey *et al.*, 2013) and GSK3 (Chen *et al.*, 2011) suggesting that TORC2 may be responding to some

of the same cues that TORC1 does. Acetylation events also regulate TORC2 activity, and there is a link between nutrient balance and TORC2 activity via the acetylation of RICTOR, since the general acetylation capacity of the cell is linked to its nutrient status (Choudhary *et al.*, 2014). One group of candidate effectors of TORC2 are the AGC group of kinases, which includes AKT and the protein kinase A, G and C families (Gaubitz *et al.*, 2016). mTORC2 is often considered to be an effector of insulin signalling via its multiple phosphorylation of AKT downstream of PI(3)K (e.g. Boutouja, Stiehm, & Platta, 2019), and indirectly regulates mTORC1 via this interaction (Kennedy and Lamming, 2016). However, this is an incomplete view of the complex relationship between TORC1 and TORC2 – at least in mammalian systems the phosphorylation of AGC kinases modulates their substrate specificity more than simply directly activating them (Gaubitz *et al.*, 2016). As well as AKT/SGK-1, TORC2 regulates numerous protein kinase C family enzymes which in turn regulate diverse cellular functions. Finally, in fitting with its known regulation by tension of the plasma membrane TORC2 also regulates the cytoskeleton by a series of different mechanisms, making it a player in the remodelling of the cytoskeleton in response to both mechanical stimuli and signalling pathways (Dos D. Sarbassov *et al.*, 2004; Jacinto *et al.*, 2004).

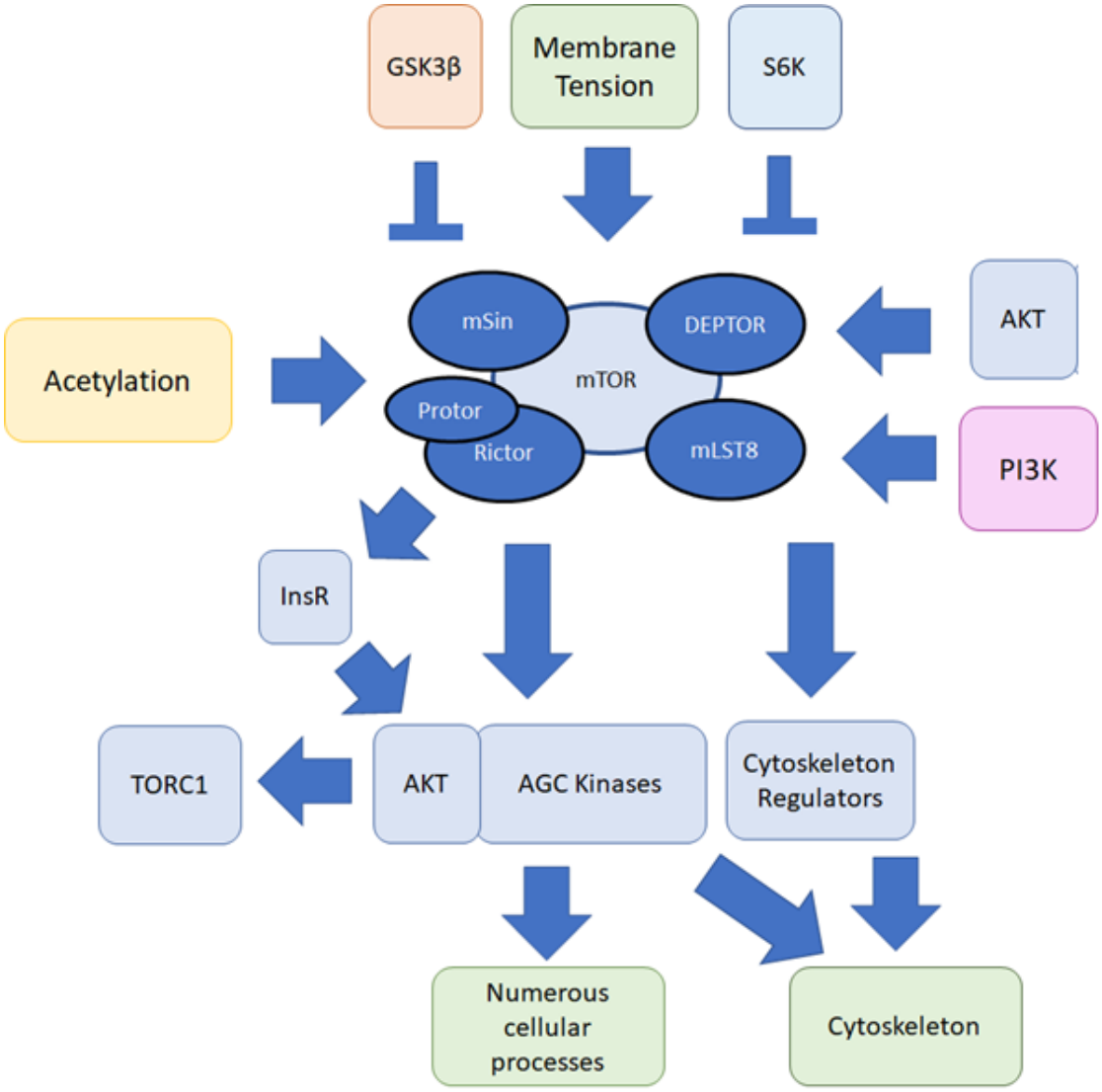

Figure 1.4: Simplified model of TORC2 Signalling. Inhibitory phosphorylation of TORC2 by Glycogen synthase kinase 3 and ribosomal protein S6 kinase acts against TORC2, while activating phosphorylation by AKT and formation of PIP3. Membrane tension also results in TORC2 activation, as does activating acetylation of RICTOR links TORC2. TORC2 positively regulates AGC kinases including AKT which places TORC2 upstream of TORC1. TORC2 also regulates numerous proteins which regulate the cytoskeleton.

1.4.3 TOR as a regulator of the ageing process.

The ability of reduced TORC1 activity to extend lifespan was first demonstrated in *C. elegans* where it was shown that *let-363* mutants (*let-363* encodes the *C. elegans* orthologue of TOR, CeTOR) can more than double lifespan of this model organism, and that RNAi against *let-363* can achieve the same (Vellai *et al.*, 2003). This longevity seems to be independent of insulin signalling since *daf-16*, which is required for rIIS longevity is not required for the longevity of *let-363* mutants (Vellai *et al.*, 2003). Furthermore, the longevity resulting from dietary restriction via *eat-2* mutation is not additive with *let-363* mutation suggesting that they are working in the same pathway. This suggests that TOR is a major effector in dietary restriction longevity in *C. elegans* (Hansen *et al.*, 2007), although it is worth noting the differences in pathway requirement between DR protocols in *C. elegans* mentioned in section 1.3.1. In the budding yeast *Saccharomyces cerevisiae,* deletion of TOR1 results in an increase of both the chronological (Kaeberlein *et al.*, 2005) and replicative (Kaeberlein *et al.*, 2005) lifespan. Pharmacological TOR inhibition by rapamycin is also capable of extending yeast lifespan, suggesting that TOR pathway components may make good drug targets for interventions against the diseases of ageing (Powers, 2006; Medvedik et al., 2007). As in *C. elegans*, this is not additive with dietary restriction longevity (Kaeberlein *et al.*, 2005) again suggesting that these two genes fall in the same pathway. In *D. melanogaster*, this situation is also largely the same with mutations in *dTOR*, *dTSC1*, *dTSC2* and *dS6K* extending lifespan, which is radically reduced if flies are con-currently dietary restricted (Kapahi *et al.*, 2004). This places TOR downstream of DR in *D. melanogaster*. In worms (Hansen *et al.*, 2007) and flies (Bjedov *et al.*, 2010) this lifespan extension is thought to be due in part to the downregulation of translation resulting from

TOR inhibition, although autophagy downstream of TOR is almost entirely required for the lifespan extending effect of this intervention in *C. elegans* (Jia and Levine, 2007; Hansen *et al.*, 2008). Excitingly, this is conserved in mammals with Rapamycin treatment (Harrison *et al.*, 2009; Miller *et al.*, 2011), decreased TOR activity (Lamming *et al.*, 2012; Wu *et al.*, 2013) and S6K mutation (Selman *et al.*, 2009) all extending lifespan in mice, strongly suggesting that reduced mTORC1 function in mice is capable of extending life in a vertebrate model organism. At least two separate studies (Selman *et al.*, 2009; Wu *et al.*, 2013) have shown S6K to be an important effector of longevity downstream from reduced TORC1 signalling, suggesting that reduced translation may also contribute to the lifespan extension resulting from TOR inhibition in mice. Upregulation of autophagy in mice promotes healthy lifespan, however whether autophagy contributes to longevity downstream of mTORC1 in mice remains unclear (Fernández *et al.*, 2018). Furthermore, analysis of S6K deficient mice showed that changes of gene expression significantly overlap with those seen in mice subjected to DR, suggesting that TORC1 inhibition is involved in DR longevity in these animals (Selman *et al.*, 2009). This lifespan extension is thought to be due in part to the downregulation of translation resulting from TOR inhibition (Hansen *et al.*, 2007), although autophagy downstream of TOR is almost entirely required for the lifespan extending effect of this intervention in *C. elegans* (Jia and Levine, 2007; Hansen *et al.*, 2008). As previously mentioned, the energy sensor AMPK regulates TORC1, and notably AMPK is required for DR longevity (Greer *et al.*, 2007) and lifespan extension by S6K inhibition (Selman *et al.*, 2009), implying that the interaction between TOR and AMPK is an important aspect of DR longevity.

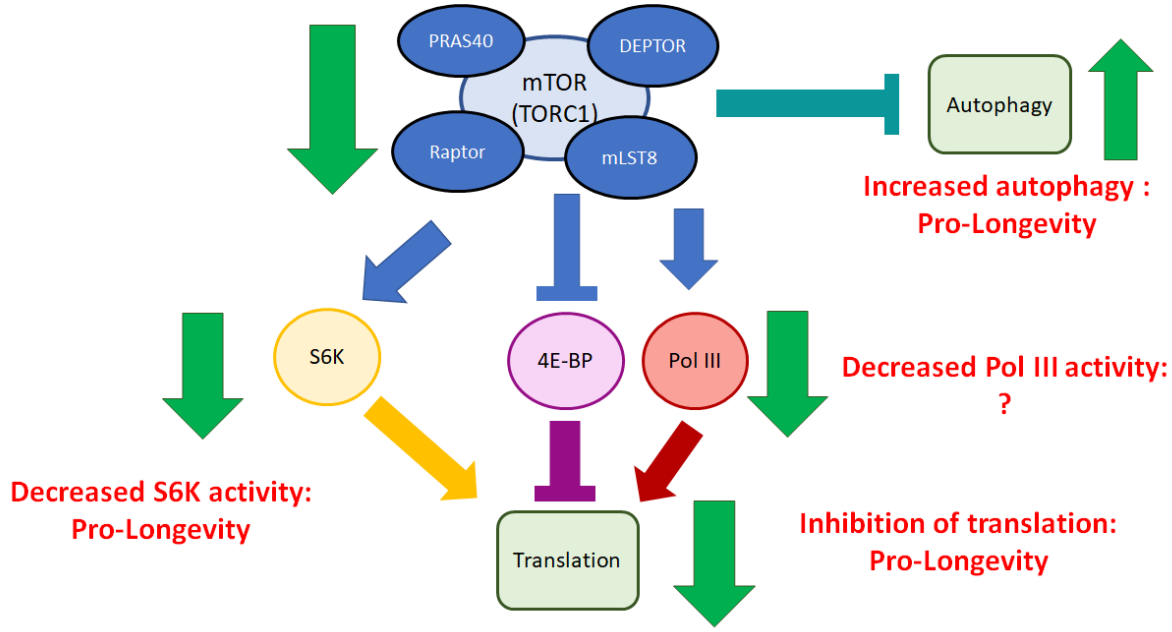

Figure 1.5: The lifespan extending effects of TORC1 inhibition in *C. elegans*. Decreased TORC1 activity by rapamycin or other interventions extends lifespan via; decreased S6K (Pan *et al.*, 2007b; Selman *et al.*, 2009), increased autophagy (Hansen *et al.*, 2008; Lapierre *et al.*, 2013), and reduced activity of eIF4E (inhibited by 4E-BP) is pro longevity, as is generally reduced translation levels (Hansen *et al.*, 2007).

1.5. Insulin and IGF1 like signalling in *C. elegans* ageing.

Insulin and IGF1 like signalling (IIS) is a highly conserved process which links nutrient status to growth and metabolism. There is a strong degree of evolutionary conservation in the general IIS pathway from humans to *C. elegans* and other model organisms, although there are also some important differences (Papatheodorou, Petrovs and Thornton, 2014). Insulin signalling is initiated by the interaction between insulin like peptides released as a cell-non autonomous signal from distant cells and their receptor, DAF-2 (Kimura *et al.*, 1997; Li, Kennedy and Ruvkun, 2003; Murphy, 2013). This results in the activation of the *C. elegans* orthologue of the mammalian class 1 phosphatidylinositol 3-kinase (PI3K) group of kinases, AGE-1. AGE-1 produces the membrane associated phospholipid signalling molecule PIP3 (phosphatidylinositol (3, 4,5)-bisphosphate) from PIP2 (phosphatidylinositol (4,5)-bisphosphate) in the plasma membrane (Morris, Tissenbaum and Ruvkun, 1996) triggering a signalling cascade via PDK-1 (Paradis *et al.*, 1999) which ultimately results in the activating phosphorylation of AKT1/2 (Paradis and Ruvkun, 1998) and SGK1 (Hertweck, Göbel and Baumeister, 2004). AKT-1 phosphorylates the transcription factors DAF-16 (Ogg *et al.*, 1997) and SKN-1 (Tullet *et al.*, 2008) which enact a transcriptional program associated with longevity and attenuated growth (Murphy, 2013). Genetic interventions which cause a downregulation of insulin signalling in *C. elegans* reliably increase lifespan, with the longest lived *age-1* mutants living up to tenfold of a wild type lifespan (Kenyon *et al.*, 1993; Arantes-Oliveira, 2003). The pro-longevity effect of reducing insulin signalling is conserved in *D. melanogaster* (Clancy, 2001; Tatar, 2001; Giannakou, 2004) and mice (Blüher, Kahn and Kahn, 2003; Kappeler *et al.*, 2008; Selman, 2008; Yuan *et al.*, 2009) and there is the suggestion that some alleles of FOXO3A (*daf-16* in *C. elegans*) are overrepresented in long

lived humans, with this data being confirmed by numerous studies around the world, including in long lived cohorts of humans from Californian, new Englander, Ashkenazi Jewish (Pawlikowska *et al.*, 2009), Japanese Hawaiian (Willcox *et al.*, 2008), Italian (Anselmi *et al.*, 2009) and Chinese (Li *et al.*, 2009) populations.

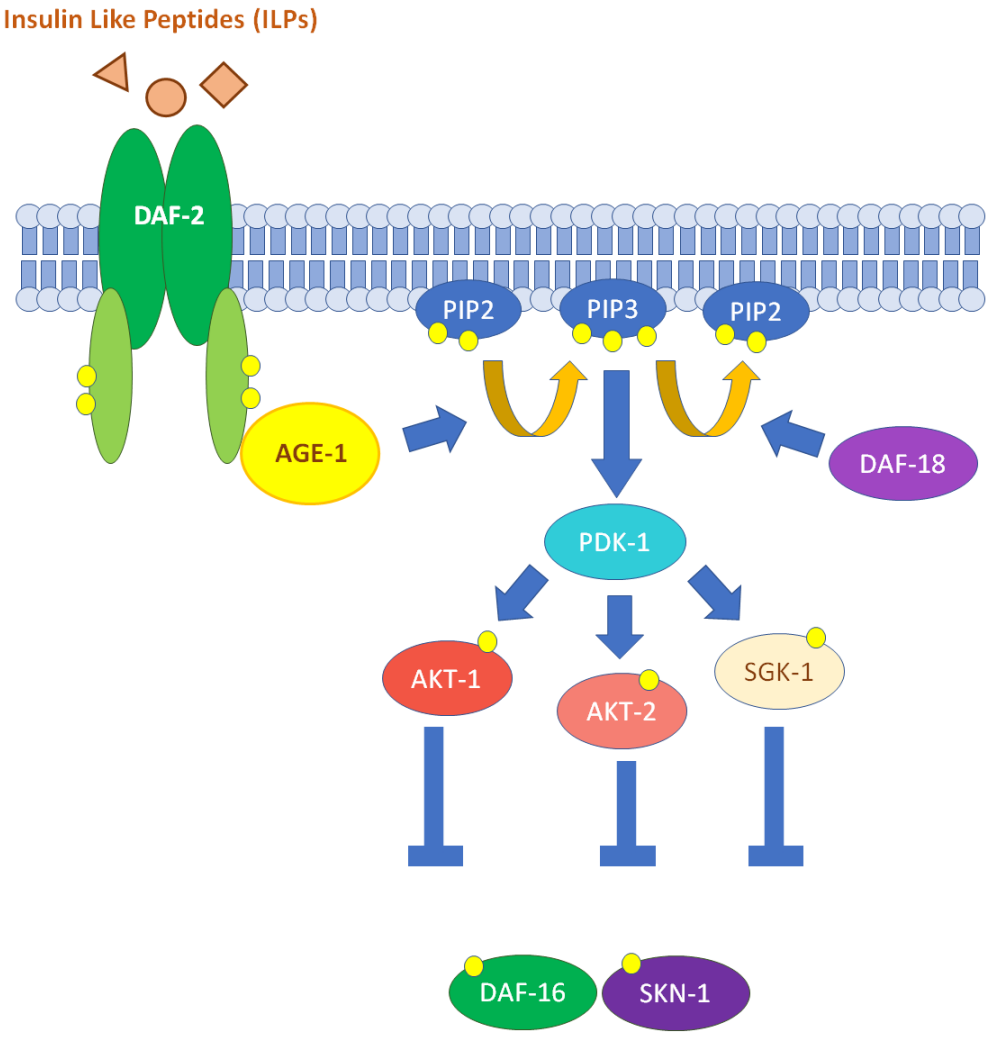

Figure 1.6: Insulin IGF-like signalling in *C. elegans*: DAF-2 is activated (or repressed) by its ligands, ILPs. This results in the activation of AGE-1 which drives the conversion of PIP2 to PIP3 and ultimately leads to the inhibitory phosphorylation of transcription factors SKN-1 and DAF-16. Yellow circles represent phosphorylation.

1.5.1. Insulin Like Peptides (ILPs) in *C. elegans.*

One major point of difference between the nematode and mammalian insulin signalling is that while mammalian systems features relatively few members of the insulin superfamily with the human genome containing 10 such genes (Lau and Chalasani, 2014), the significantly smaller *C. elegans* genome contains 40 such genes – *daf-28* and *ins-1 – ins-39* (Murphy, 2013). These insulin-like peptides are all thought to be ligands for the single *C. elegans* orthologue of the mammalian INSR and IGF1R (Lau and Chalasani, 2014). While it was originally suspected that these ILPs may simply contribute redundancy to the system, it is now clear that individual ILPs have divergent functions and that this complexity provides the insulin signalling system in *C. elegans* with the capacity to respond to different stimuli in a complex manner (Fernandes de Abreu *et al.*, 2014). While some ILPs are better studied, many ILPs functions remain largely unknown and their function as *daf-2* ligands is inferred from homology to insulins in other organisms (e.g. (Duret *et al.*, 1998)). ILPs are secreted from many cell types in the worm and seem to serve different purposes in different tissues. For example, in the intestine INS-7 seems to agonise DAF-2 while itself being transcribed downstream of insulin signalling resulting a positive feedback loop which propagates and amplifies a downregulation of IIS in this tissue (Murphy, Lee and Kenyon, 2007). However, the same ILP INS-7 actually seems to operate as a DAF-2 antagonist in some neuronal tissues where it is involved in the memory of and behavioural response to external pathogens. In this case INS-7 is expressed in URX neurons and antagonises DAF-2 in the RIA neuron via a synaptic connection, opposing learning. In normal conditions INS-6 secreted from the ASI neurons inhibits *ins-7* transcription thus enabling olfactory learning, but in dauer animals *ins-6* is downregulated, with the result being a downregulation of olfactory memory and the

likely result that in extreme conditions the animals may still move to food which is of low quality, even if they have been exposed to it before (Z. Chen *et al.*, 2013). This is a relevant example of ILPs being used as neurotransmitters rather than simply being pro- or anti- *daf-16* and *skn-1* activity, which illustrates the complexity of interpreting changes in ILP secretion from neurons. ILPs are widely expressed in neuronal tissues and different neurons produce different ILPs, allowing the regulation of behaviour by these peptide hormones and the construction of complex neuronal circuits utilising ILPs and other signalling molecules. Some ILPs are known to be regulated by signals from the external environment, such as DAF-28 which is regulated by perception of external peptides (Kaplan *et al.*, 2018). A recent study demonstrated that these ILPs are set up into a regulatory network which governs dauer entry/exit, lifespan, thermotolerance, pathogen resistance and reproductive longevity (Fernandes de Abreu *et al.*, 2014). This study shows that different combinations of ILPs have effects on one or several of these processes when mutated together which are much more pronounced that the sum of the individual mutations, suggesting that the signals transduced in this network are complex and rely on the simultaneous regulation of numerous ILPs. Some ILPs have also been shown to be regulated by the TGF-β/DAF-7 pathway regulating dauer formation (Shaw *et al.*, 2007), linking these two pathways.

1.5.2. DAF-2

The *daf-2* gene was first investigated by the Riddle lab during the characterisation of the pathway regulating dauer formation in *C. elegans*, which was later identified as the insulin signalling pathway (Kimura *et al.*, 1997). Animals deficient in *daf-2* are highly *daf-c* (Riddle, Swanson and Albert, 1981) . Interest in this pathway was increased when it was observed that mutants in insulin signalling were long-lived – while the earlier discovery that *age-1*

mutation extended lifespan (Friedman and Johnson, 1988) was attributed to reduced fecundity, the discovery by Cynthia Kenyon that the point mutation *daf-2(e1370)* could double wild-type lifespan provided a watershed moment in ageing science since this triggered the recognition that lifespan could be radically manipulated in model organisms (Kenyon *et al.*, 1993). DAF-2 is composed of two heterodimers each containing an α and β subunit which associate to form the holoreceptor (Kimura *et al.*, 1997). The *C. elegans daf-2* gene shares approximately 30% homology with the human insulin receptor and the basic structure (two heterodimers with an extracellular α domain) is conserved across species (Kimura *et al.*, 1997). Since animals with deletion mutations of *daf-2* are not viable, point mutations of *daf-2* which partially reduce function are often used. The severity of these mutant alleles varies, and such mutations are classified as class 1 (more severe) or class 2 (less severe) *daf-2* mutants (Gems *et al.*, 1998). This seems to be a result of the location of the mutation on the DAF-2 protein, with each class of mutant clustering around various key domains of the DAF-2 protein (Patel *et al.*, 2008).

1.5.3. DAF-16/FOXO

One major effect of insulin signalling in *C. elegans* is the inhibition of the pro-longevity *daf-16.* This gene encodes a transcription factor with orthologues conserved across species, including human FOXOs (Lee, Hench and Ruvkun, 2001). In conditions where insulin signalling is activated, AKT receives an activating phosphorylation by PDK-1 which cause it to in turn phosphorylate DAF-16 (Paradis *et al.*, 1999). This prevents the DAF-16 protein from entering the nucleus and results in its sequestration in the cytoplasm (Cahill *et al.*, 2001). The longevity effect of *daf-2* mutants is dependent on the activity of *daf-16* (Lin, 1997; Ogg *et al.*, 1997), and *daf-16* mutants are *daf-d* (Riddle, Swanson and Albert, 1981).

The pro-longevity effects of *daf-16* are widely conserved across all model organisms (Sun, Chen and Wang, 2017) and as previously mentioned, intriguing data from human population studies have shown that certain alleles of FOXO3A are highly over-represented in long lived humans (reviewed in (Kenyon, 2010)) making interventions in insulin signalling a highly interesting point of intervention in the ageing process. However, downstream of *daf-16* there is no clear single target which mediates the longevity effect that *daf-16* promotes – approximately 1700 genes are positively regulated by *daf-16* and a similar number of genes negatively regulated according to a recent meta-analysis (Tepper *et al.*, 2013) of several studies (Lee *et al.*, 2003; McElwee, Bubb and Thomas, 2003; Murphy *et al.*, 2003; Halaschek-Wiener *et al.*, 2005; Wook Oh *et al.*, 2006). It is not therefore clear exactly which targets of *daf-16* regulate longevity and which are involved in unrelated cellular processes (Tullet, 2014). Interestingly, while in mammals the roles of the FOXO orthologues of DAF-16 are divided between different genes, in *C. elegans* DAF-16 roles in longevity are divided between several isoforms, with both DAF-16A and DAF-16D/F promoting longevity separately and in complementary ways in different tissues (Kwon *et al.*, 2010).

1.6. SKN-1 isoforms, regulation and function.

The *skn-1* gene encodes a transcription factor which is the functional orthologue of mammalian Nrf bZip transcription factors (also referred to as NF-E2 related factors) (Walker *et al.*, 2000). While mammalian Nrf proteins must dimerise in order to bind the DNA via their ZIP motif, in *C. elegans* there is no ZIP domain and the need for dimerization is removed by a more efficient DNA binding motif (Kophengnavong, Carroll and Blackwell, 1999). Indeed, there is significant divergence in sequence and structure between mammalian NRFs and SKN-1. Crucially, there are 4 possible isoforms of *skn-1* (*a*-*d*) with three of these (*a*-*c*) having been detected *in vivo* (Figure 1.7). These isoforms are significantly divergent in structure, expression patterns, cellular localisation and function with some isoforms more closely resembling individual NRF proteins in this regard. Many studies use mutant alleles which knock out all *skn-1* isoforms which, while valid, means that some known *skn-1* functions are hard to attribute to any one isoform (Figure 1.7). In terms of its transcriptional regulation, SKN-1A is expressed from an operon promoter upstream, while SKN-1C is thought to be expressed from its own unique promoter (An and Blackwell, 2003; An *et al.*, 2005) and SKN-1B is also predicted to be expressed from a unique promoter (Bishop and Guarente, 2007b). The SKN-1 DNA binding domain is conserved between all four isoforms and consists of a Cap N Collar domain (CnC), a basic region and a flexible NH_2 terminal arm which interacts with the DNAs minor groove (Kophengnavong, Carroll and Blackwell, 1999). Since it does not dimerise, the consensus SKN-1 binding site differs from the mammalian antioxidant response element (ARE) and SKN-1 will bind to a single sequence of WWTRTCAT, with the NH_2 arm binding to the WWT via the minor groove (Blackwell *et al.*, 2015).

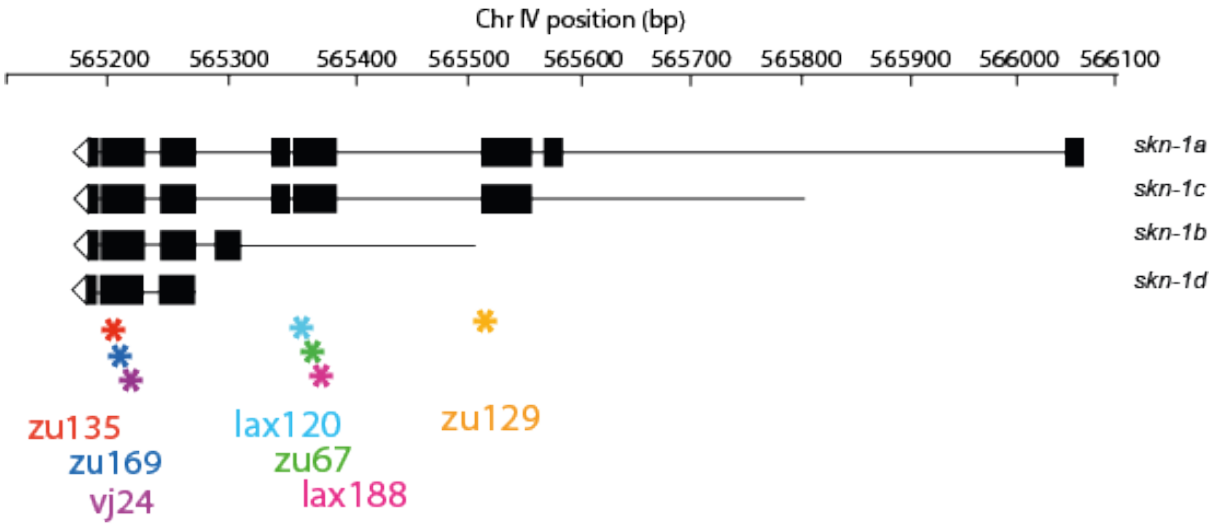

Figure 1.7: Classical alleles of *skn-1*: Some commonly used mutations in *skn-1* used for studying the function of this gene. The *zu135, zu169,* zu67 *and zu129* (Bowerman, Eaton and Priess, 1992) and *vj24* (Staab *et al.*, 2013) mutations introduce premature stop codons. *zu135* , *zu169* and *vj24* therefore abolish all *skn-1* expression, while *zu67* and *zu129* abolish *skn-1a/c* expression. The *lax120* and *lax188* are substitutions which cause constitutively active *skn-1a/c* to be expressed (Paek *et al.*, 2012).

SKN-1 is essential for early embryonic development – it is unevenly expressed at the 4 cell stage where it is involved in initiating the development of the endodermal and mesodermal tissues (Bowerman *et al.*, 1993), and strains carrying mutations that result either in the elimination of the *a* and *c* isoforms or all skn-1 isoforms simultaneously are embryonic lethal unless balancers are used (Bowerman, Eaton and Priess, 1992). While *skn*-1 was initially studied for its role in development, a series of post developmental roles for *skn-1* were later identified. The phase II detoxification response in *C. elegans* acts to detoxify reactive molecules, including products of the phase I detoxification response and reactive oxygen species (ROS) (*C. elegans* detoxification responses reviewed in (Lindblom and Dodd, 2006)). SKN-1 shares a degree of similarity (particularly via the conservation of the DIDLID motif and

aspects of the DNA binding domain) with the stress responsive mammalian NRFs, bZIP transcription factors which mediate the response to oxidative stress, regulating effector genes which share a common ARE (Antioxidant Response Element). Therefore, the post embryonic role of *skn-1* in oxidative stress was investigated by An & Blackwell in 2003. They showed that *skn-1* is required for the expression of various phase II detoxification genes including *gcs-1*, and further that animals lacking *skn-1a/c* are sensitised to oxidative stress. Follow up studies showed that numerous phase II detoxification genes are regulated by *skn-1*, including *gst-4* which is now often used as a readout of *skn-1* activity due to its strong upregulation (Oliveira *et al.*, 2009; Park, Tedesco and Johnson, 2009). These analyses further showed that numerous detoxification related genes are directly regulated by *skn-1*, including genes in phase I, II & III detoxification pathways.

Another important function of *skn-1* is the maintenance of proteostasis in the animal. SKN-1A responds to proteasome dysfunction (Lehrbach and Ruvkun, 2016) or unfolded protein (Lehrbach and Ruvkun, 2019) by its release from the ER as discussed in section 1.6.1 and acts to upregulate proteasome genes. Numerous genes associated with the UPR^{ER} in particular are regulated by SKN-1 with *xbp-1, ire-1, atf-5* and *hsp-4* all directly regulated by the UPR, and some UPR related transcription factors also regulate *skn-1* (notably *xbp-1* and *atf-6*) (Glover-Cutter, Lin and Blackwell, 2013). It isn't currently clear whether the regulation of the UPR^{ER} genes is mediated by any one isoform in particular, and this would be an interesting avenue to explore in the future since the basic assumption has been that SKN-1C is likely responsible for this, but recent findings place SKN-1A as a key effector of a new arm of the cytosolic UPR (Lehrbach and Ruvkun, 2019). Additionally it is now known that the UPR^{ER} can be activated cell non-autonomously by neuronal activation of *xbp-1s* (Taylor and Dillin, 2013), meaning that a role for the neuronal *skn-1b* is also possible. Studies of SKN-1

targets have also identified other proteostasis network components including chaperones and lysosomal proteins as SKN-1 targets (Oliveira *et al.*, 2009).

SKN-1 is also implicated in the regulation of fat storage, with *skn-1* mutant animals storing excessive fat (Pang *et al.*, 2014; Steinbaugh *et al.*, 2015). Consistent with this, in starvation conditions mitochondrially located SKN-1 seems to be important for utilising fat reserves (Paek *et al.*, 2012).

In terms of its impact on the ageing process, SKN-1 is a very interesting molecule because it is required for the life extending effect of at least three of the main pro-longevity interventions in *C. elegans*. Downstream of insulin signalling, *skn-1c* is required for the full longevity resulting from *daf-2* mutation (Tullet *et al.*, 2008; Ewald *et al.*, 2014), *skn-1* is required for the longevity that results from the inhibition of the TOR pathway (Robida-Stubbs *et al.*, 2012) and *skn-1b* in ASI neurons is required for the longevity resulting from at least one dietary restriction protocols (Bishop and Guarente, 2007b). More recent analysis of *skn-1* targets found that a major set of targets of *skn-1* are collagens, and that some of the regulated collagen genes are partially required for *skn-1* dependant longevity phenotypes, pointing to an important and understudied role for *skn-1* in collagen remodelling (Ewald *et al.*, 2014).

1.6.1. SKN-1A function

SKN-1A is the largest protein encoded by *skn-1* (Figure 1.8). SKN-1A seems to mostly serve to activate *skn-1* target processes in response to proteasome dysfunction. The response of *C. elegans* to proteasome dysfunction is to upregulate genes encoding proteasome subunits, detoxification processes, immune response and to change their behaviour such that they

avoid their bacterial food (Lehrbach and Ruvkun, 2016). SKN-1A seems to be the functional orthologue of the (better studied) mammalian NRF1 and indeed the mechanism by which NRF-1 is activated is conserved to *C. elegans*. In worms and humans, NRF1/SKN-1A is attached to membranes including ER and mitochondrial membranes by a membrane localisation domain, and constitutively targeted for degradation by the proteasome by the ERAD pathway (ER associated degradation) (Radhakrishnan, den Besten and Deshaies, 2014; Sha and Goldberg, 2014; Lehrbach and Ruvkun, 2016). In the case of proteasome dysfunction this pathway fails and accumulated SKN-1A on these membranes is cleaved by the protease DDI-1, releasing the majority of the protein into the cytoplasm where it can ultimately enter the nucleus and enact the *skn-1* associated program (Lehrbach and Ruvkun, 2016). In this way *skn-1* is able detect proteasome dysfunction and enables the cell to react to this. Generally, *skn-1a* is expressed in intestinal tissues although it has been detected in some neurons during larval development (Bishop and Guarente, 2007b; Tullet *et al.*, 2008).

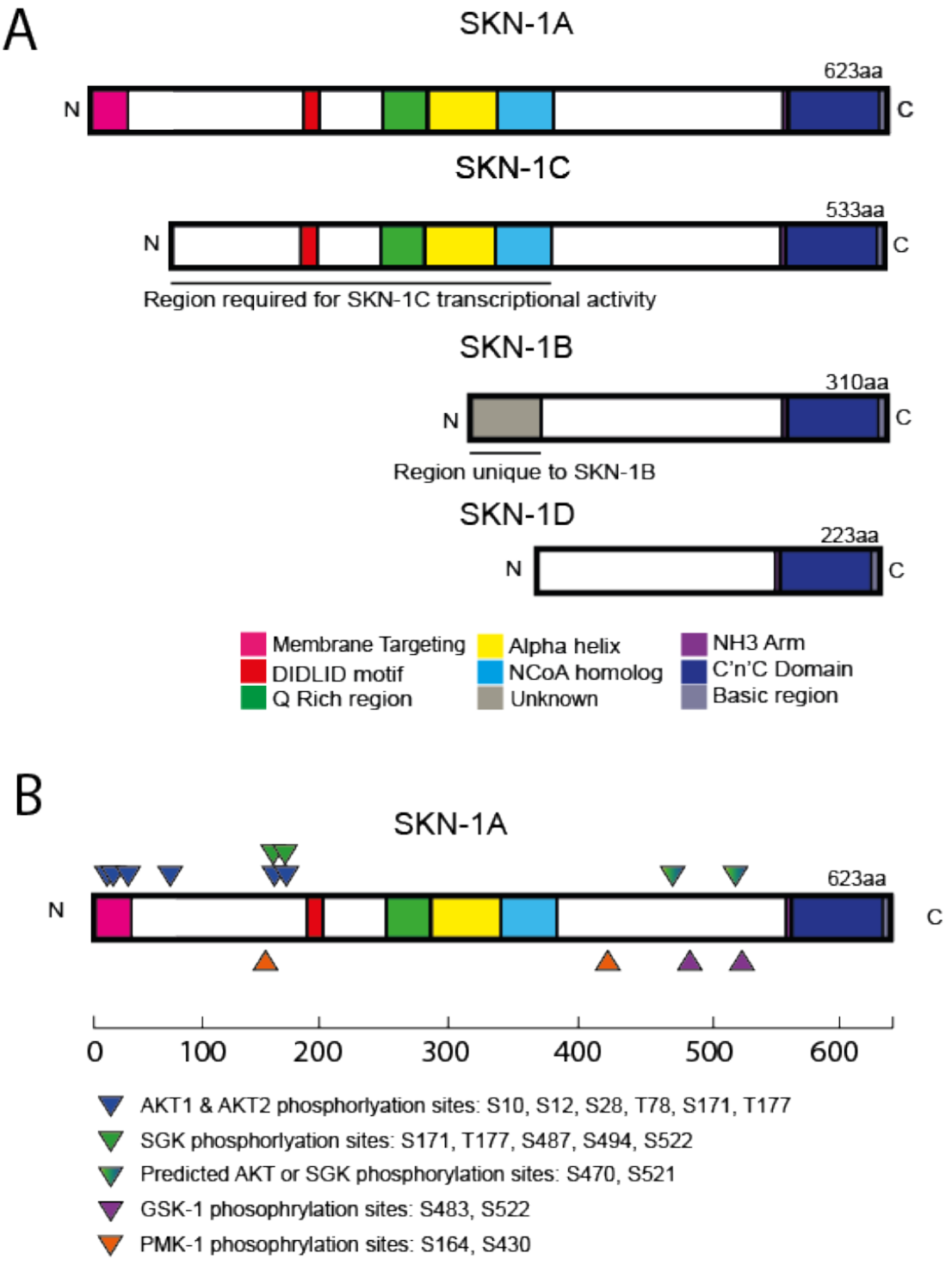

Figure 1.8: The four protein isoforms of SKN-1. A: Displayed in descending order of size. Point mutations in the DIDLID motif radically decrease SKN-1 function (Walker *et al.*, 2000) and this motif is conserved to mammalian NRF proteins. The alpha helix and Glu-rich regions form a second transactivation domain, and the region of NCoA homology is required for interaction with p300 and forms a third transactivation domain (Walker *et al.*, 2000). The DIDLID motif, glu-rich region, alpha helix and region of NCoA homology however are not present in SKN-1B or (should it exist *in vivo*) SKN-1D. **B:** SKN-1 phosphorylation sites mapped onto the SKN-1A protein (AA positions adjusted for SKN-1A).

1.6.2. SKN-1C

The SKN-1C isoform is by far the best studied isoform of SKN-1. The main reason for this is that SKN-1C seems to be the primary effector of most of the effects associated with the *skn-1* gene generally. It is expressed mostly in the intestine where it is sequestered in the cytoplasm in normal conditions, and RNAi against *skn-1c* prevents expression of a SKN-1B/C::GFP transgene in the intestine but not ASI neurons (Bishop and Guarente, 2007b). Many different post translational modifications can control the nuclear entry of SKN-1C, including its phosphorylation (Fig 1.8B) by AKT1/2, SGK, GSK3, PMK-1 (An *et al.*, 2005; Inoue *et al.*, 2005; Tullet *et al.*, 2008; Blackwell *et al.*, 2015), O-GlcNAcylation (Li *et al.*, 2017) and indirectly via WDR-23 which targets SKN-1 for proteasomal degradation (Choe, Przybysz and Strange, 2009) and can be up or down regulated as a means of controlling *skn-1* activity. The activity of SKN-1C is highly dependent on the DIDLID motif, with point mutations in this region radically reducing the activity of SKN-1C *in vitro* (Walker *et al.*, 2000), and this motif is conserved all the way to mammalian NRFs. The intestinal activity of *skn-1a/c* is required for the canonical *skn-1* functions of stress resistance and normal lifespan with *skn-1(zu67)* and *skn-1(zu135)* mutants being sensitive to a variety of stresses and exhibiting shortened lifespans (An and Blackwell, 2003). SKN-1C acts in the intestine as an important effector of insulin signalling, where it is directly phosphorylated by AKT1/2 and SGK (Fig 1.8) downstream of activated insulin signalling and thus prevented from entering intestinal nuclei. When insulin signalling is reduced, as in *daf-2* mutant animals, SKN-1C constitutively accumulates in intestinal nuclei (Tullet *et al.*, 2008; Ewald *et al.*, 2014). Intestinal activity of *skn-1a/c* is required for rIIS longevity resulting from *daf-2(e1368)* with *skn-1(zu67)* abolishing this longevity, and that resulting from *sgk-1* RNAi as well as abolishing oxidative

stress resistance resulting from rIIS (Tullet *et al.*, 2008). Overexpression of *skn-1b/c* in the intestine is also capable of extending lifespan – these data together suggest that *skn-1c* in the intestine contributes to lifespan downstream of rIIS (Tullet *et al.*, 2008). Intestinal SKN-1 (likely SKN-1C) responds to oxidative stress and is regulated by the MAPK signalling cascade which acts to internalise external signals related to some stresses, including oxidative stress. This cascade ultimately activates PMK-1 which then provides activating phosphorylation of SKN-1 (Fig 1.8) and enables the nuclear entry of SKN-1 and results in the upregulation of *gcs-1*, a phase II detoxification gene (Inoue *et al.*, 2005). Acting in the opposite direction, GSK-3 provides inhibitory phosphorylation of SKN-1, preventing its nuclear entry in normal conditions. If this inhibition is released by loss of GSK-3, SKN-1 will enter constitutively enter the nucleus and activate stress response pathways (An *et al.*, 2005).

1.6.3. SKN-1B

SKN-1 was originally observed to be expressed in the ASI neurons and the intestine, with SKN-1 in the ASI neurons being constitutively nuclear while SKN-1 in the intestine could become nuclear in response to activating processes (An, 2003). In their 2007 study of *skn-1b* (which remains one of only a few papers to examine this isoform) Bishop & Guarente identified the tissue specificity of the expression of *skn-1b* and *skn-1c*, showing that a SKN-1B/C::GFP (GFP – Green fluorescent protein) transgene carrying strain still shows neuronal but not intestinal GFP when *skn-1c* is silenced and intestinal but not neuronal GFP when *skn-1b* is silenced (Bishop and Guarente, 2007b). Thus, it is deduced that *skn-1b* is expressed principally in ASI neurons and *skn-1c* is expressed in the intestine. Structurally, *skn-1b* contains a unique exon which is not present in any other isoforms of *skn-1*, with currently unknown functions. This seems to hint at divergent posttranslational regulation of *skn-1b*

and we note that unlike other *skn-1* isoforms, *skn-1b* is constitutively nuclear - however pending proper characterisation of this exon we cannot know its function. Further to this, SKN-1B protein is lacking many of the known domains required for the activity of SKN-1A and SKN-1C including the crucial DIDLID motif, as well as parts of the protein required for the interaction with WDR-23 (Tang and Choe, 2015), p300 (Walker *et al.*, 2000) and numerous phosphorylation sites (Blackwell *et al.*, 2015). In the characterisation of the SKN-1C transcriptional activity in Walker *et al.* (2000) the DNA binding domain of SKN-1 is shown to be incapable of inactivating transcription alone, and SKN-1C missing the DIDLID motif and the alpha helix was shown to be largely inert (Walker *et al.*, 2000). With all these domains entirely missing in SKN-1B (Figure 1.8) there has been the suggestion in the literature that the capability of SKN-1B to drive transcription is in question (Tang and Choe, 2015). In terms of transcriptional regulation, it has been suggested by 5' RACE (rapid amplification of cDNA ends) that *skn-1b* and *skn-1c* are driven from their own unique promoters while *skn-1a* is expressed from an upstream operon promoter, meaning that the individual transcriptional regulation of these isoforms is possible and supporting the idea of divergent function (Bishop and Guarente, 2007b).

The main conclusion of the aforementioned Bishop and Guarente study was that *skn-1b* expression in ASI neurons is required for *C. elegans* to respond to the IDR protocol of dietary restriction which they developed in that study (Bishop and Guarente, 2007b). To demonstrate this, they removed all isoforms of *skn-1* and then showed that the transgenic rescue of *skn-1b* in ASI neurons was sufficient to restore responsiveness to DR (Bishop and Guarente, 2007b). This is clearly a very interesting result for ageing researchers, particularly since this isoform of SKN-1 is expressed only two neurons, suggesting that signals sent by these cells downstream of *skn-1b* to the rest of the animal are required for DR longevity. It is

not yet known how the activity of *skn-1b* in ASI neurons is able to exert an effect on other tissues, nor is it known whether *skn-1b* is involved in rIIS or TOR longevity. Another study which examined neuronal activity of *skn-1* seems to demonstrate that loss of *skn-1* results in defective AIY morphology and defective behaviour and salt chemotaxis (Wilson *et al.*, 2017). They observed that *skn-1b* "rescue" in AIY neurons does not rescue such phenotypes however pan-neuronal expression of *skn-1b* does. It is therefore also possible that *skn-1b* plays a role in the development of other neurons through cell non-autonomous signals which remain unidentified, or that *skn-1b* can partially rescue functions performed by other *skn-1* isoforms.

1.6.4. SKN-1D

This isoform has not been detected *in vivo* and is predicted based upon the presence of an SL1 site at the end of this possible transcript (Wormbase version WS259). If it is expressed *in vivo* it has the potential to confuse conclusions based upon the use of *skn-1* transgenes – transgenes previously thought to encode SKN-1B or SKN-1B/C etc would now carry the additional SKN-1D isoform, and the risk exists that some functions attributed to other SKN-1 isoforms could be those of SKN-1D (Arnaboldi & Kishore, 2018).

1.6.5. SKN-1 targets

Analysis of *skn-1* target genes reveals that numerous processes are regulated by *skn-1.* The best understood of these are genes which mediate the oxidative stress response to certain stressors including sodium arsenite and TBOOH, with *skn-1* mutant animals being sensitised to these stresses. Notable targets of *skn-1* of this sort include *gst-4* (Glutathione S-transferase 4) which is the most highly upregulated gene by *skn—1* (Riva P Oliveira *et al.*,

2009). This particular gene acts to facilitate the reduction of oxidative stressors by glutathione. Due to its being the most radically regulated gene target of *skn-1*, *gst-4* activity has sometimes been used as a proxy for *skn-1* activity (Riva P Oliveira *et al.*, 2009). Later analysis of *skn-1* targets by microarray revealed an unexpected set of targets – *skn-1* regulates a large number of collagen genes, to the extent that collagens and other extracellular matrix associated genes seem to be the major target of *skn-1,* with genes involved in the oxidative stress response being the second most highly enriched group in line with previous studies of *skn-1* (An and Blackwell, 2003; Ewald *et al.*, 2014). The *skn-1* mediated expression of many of these collagen genes is required for the full longevity resulting from rIIS and dietary restriction suggesting that collagen remodelling downstream of *skn-1* is an important pro-longevity output of *skn-1* (Ewald *et al.*, 2014).

The isoforms of *skn-1* therefore show significant differences in their regulation, localisation, structure and function. In ageing, *skn-1* is a target of great interest but there remains work to be done in determining the role played by each isoform of *skn-1* in the ageing processes which it is known to influence.

1.7. Amphid neurons in the worm.

C. elegans has 302 neurons, and as with all cells in the worm the specific cells and cell divisions which generate them are known (Hobert, 2005). On top of this, *C. elegans* is the only organism for which the full neural connectome has been described, where all of the synaptic connections and neurons which make up the worm nervous system have been mapped and are freely viewable online (http://wormwiring.org/). The relative simplicity of the *C. elegans* brain and our high degree of confidence about its structure means that the worm is the perfect system for investigating neuronal function. In order to properly respond to their environment, *C. elegans* need to be able to sense it and in the worm this is achieved by sensilla (White *et al.*, 1986).

C. elegans sensilla consist of the dendrites of numerous neurons which are surrounded and protected by a single sheath cell forming a bundle of dendrites as well as a socket cell for the interface with the external environment and to enable sensory dendrites to interact with the external environment via protruding cilia to sense whatever cues they are able to detect (Altun & Hall, 2010).

Highly relevant to this project are the amphid neurons, which are a group of neurons which populate the amphid sensilla, primitive sensory organs which are the primary means by which the worm detects olfactory, chemical and thermal signals (Bumbarger *et al.*, 2009). Each amphid is composed of 12 neurons (ADF, ADL, AFD, ASE, ASG, ASH, ASI, ASJ, ASK, AWA, AWB, AWC). Amphids are arranged in pairs with one on the left and one on the right and thus there are two of each amphid neuron. These are distinguished by suffixing L or R to each cell, for example the ASIL is the ASI neuron in the left amphid sensilla (Altun and Hall, 2010).

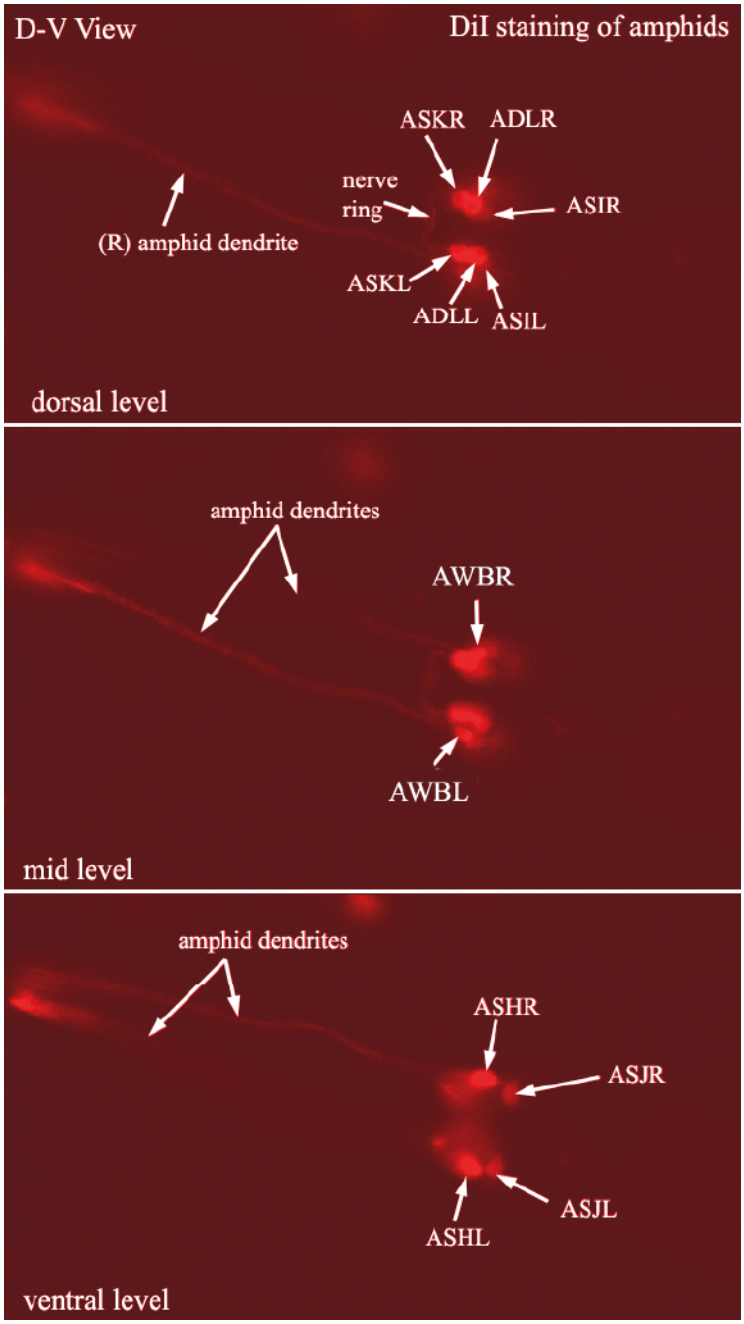

Figure 1.9: Cells of the amphid sensilla: DiI stained amphid neurons showing the structure of the amphid sensilla including the amphid dendrites and ASI, ADL, ASK AWB, ASH and ASJ neurons. Directly reproduced from wormatlas. (http://www.wormatlas.org/EMmethods/DiIDiO.htm)

1.7.1. Chemosensory neurons in lifespan and stress

Of the 302 neuron pairs in *C. elegans*, 60 are able to detect chemosensory signals from the external environment via direct interaction with the external environment through the sensory cilia (Inglis, 2006; Lin *et al.*, 2017). Intriguingly, these chemosensory neurons exert a high level of control over longevity and stress responses, suggesting that cues from the external environment are integrated into the control of pathways leading to longevity or stress resistance (and often, both). Laser ablation of the ASI and ASG neuron pairs is able to extend lifespan and the longevity resulting from ASI ablation is in turn dependent on the ASJ and ASK neurons (Alcedo and Kenyon, 2004). This seems to suggest the existence of an anti-longevity signal originating from the ASI neurons and in a circuit with the ASJ and ASKs (Lin *et al.*, 2017). Blocking the release of neuropeptides from the ASIs is sufficient to recapitulate this effect (Chisnell and Kenyon, 2018), suggesting that this may be the mechanism by which such a signal is initiated in the nervous system, although downstream signals are more obscure. One fascinating aspect of the control of longevity by external signals is the ability of food cues to suppress the longevity resulting from DR. Exposing *C. elegans* to the supernatant of OP50 cultures is sufficient to shorten the lifespan of dietary restricted animals (Artan *et al.*, 2016), suggesting that it is not just signals related to internal energy balance that regulate DR but also signals which are related to external food availability. This is also the case in *D. melanogaster*, where food derived odours are similarly capable of shortening the lifespan of DR animals (Libert *et al.*, 2007).

1.7.2. ASI neurons.

The ASI neuron pair is comparatively well studied, with defined roles in chemotaxis to lysine and various ions (Bargmann and Horvitz, 1991), regulating male exploratory behaviour (White and Jorgensen, 2012), thermo-sensation (Beverly, Anbil and Sengupta, 2011), innate immunity (Liu, Sellegounder and Sun, 2016) and locomotion (Sun *et al.*, 2011). ASI neurons are notable for the fact that of all the cells in the worm, the ASI neurons express more G-protein coupled receptor genes than any other (Vidal *et al.*, 2018), suggesting that these cells are capable of processing complex information about the outside environment. These neurons have been identified as key cells in the regulation of the longevity, and laser ablation of these cells produces a lifespan extension (Alcedo and Kenyon, 2004), as does blocking their export of signalling molecules (Chisnell and Kenyon, 2018). ASI neurons are also required for the longevity resulting from dietary restriction in *C. elegans* since the activity of *skn-1b* in these cells is required for this (Bishop and Guarente, 2007b). One major function of the ASI neurons is to regulate the TGF- β pathway which regulates dauer entry (Thomas, Birnby and Vowels, 1993), longevity (Shaw *et al.*, 2007) and behavioural responses to pathogenic bacteria (Meisel *et al.*, 2014). The TGF-β ligand in *C. elegans* is encoded by *daf-7*, and the ASI neurons are the sole source of the DAF-7 protein in the worm (Thomas, Birnby and Vowels, 1993). This is required for dietary restriction longevity, suggesting that one way in which ASI neurons communicate to other tissues to enact DR longevity is via DAF-7 (Fletcher and Kim, 2017) but setting up the slightly paradoxical situation in which ASI neurons and their export of signalling molecules are required for DR longevity, but blocking the export of signalling molecules from them also causes a form of longevity. Many neurons secrete insulin like peptides, and this is the case with ASI neurons which secrete DAF-28 and

8 other ILPs which can signal to distant tissues or other neurons (Murphy, Lee and Kenyon, 2007; Chen *et al.*, 2013; Fernandes de Abreu *et al.*, 2014, Wormatlas ASI neurons). ASI neurons are therefore an important hub in the regulation of longevity by the nervous system and given their known roles in chemosensation and likely numerous unknown roles based on their high concentration of GPCRs (G-protein coupled receptors), it is possible that information about the external environment is being integrated into these decisions.

1.8. Overview of the activity of RNA polymerases I, II and III.

Translation play a major role in the regulation of ageing, with not only pro-growth pathways with numerous targets such as IIS and TORC signalling being involved in ageing but also direct regulators of translation being capable of extending lifespan if inhibited. Together, the RNA polymerases function to provide translation with essential inputs via the various transcriptional products they form. In eukaryotic organisms, the control of transcription is divided between three RNA polymerases (John, Birnstiel, & Jones, 1969). RNA polymerase I controls the synthesis of the rRNA precursor which is then processed into mature rRNAs, RNA Polymerase II controls mRNA synthesis for protein coding genes and various microRNAs, and RNA polymerase III transcribes tRNAs, ribosomal 5S RNA and some small non-coding RNAs. RNA polymerases I, II and III contain a conserved common core which is involved in interaction with DNA as well as various unique subunits which confer the specificity seen in the type of promoters with which the different polymerases interact (Vannini & Cramer, 2012). Further, each RNA polymerase interacts with a set of unique general transcription factors in order to properly form their different pre-initiation complexes (Bywater *et al.*, 2013). Together, intricate regulation of these RNA polymerases is able exert multiple levels of regulation on levels of protein synthesis (Figure 1.10) occurring in response to the needs of the cell and the energetic and nutritional environment in which the organism finds itself (Moir & Willis, 2013). More recently, Pol I and Pol III a have been observed to be consistently dysregulated in cancers and interventions against the activity of these polymerases by targeting subunits or associated factors may hold potential in the treatment of cancers (Bywater *et al.*, 2013).

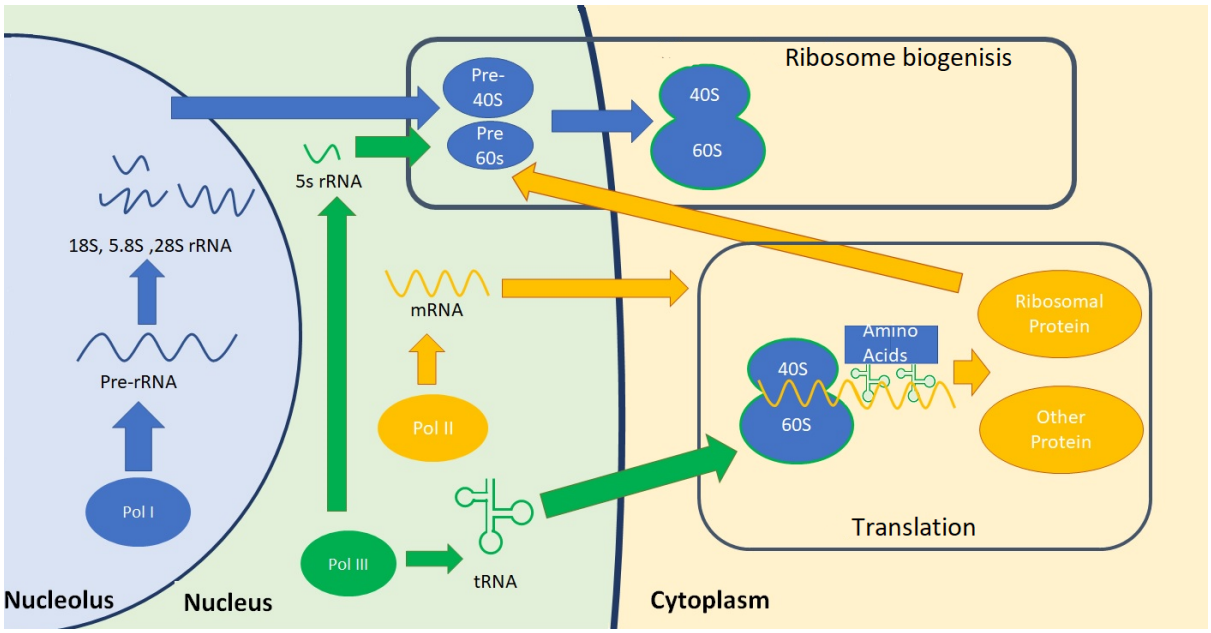

Figure 1.10: RNA Polymerases in translation and ribosome biogenesis. Pol I functions in the nucleolus to transcribe pre-rRNA from multiple copies of the same gene which is processed to form 18S, 5.8S and 28S rRNA. This is exported to the cytoplasm where along with the Pol III product 5S rRNA and numerous ribosomal proteins and assembly factors they form the scaffold of the pre-40S and pre-60s ribosome subunits. These are exported to the cytoplasm and further processed in a complex and tightly regulated process of maturation. The ribosomes can then produce protein from mRNA transcripts produced by Pol II and Pol III provides the tRNAs which recruit amino acids for this.

1.8.1 RNA Polymerase I

RNA polymerase I is a 14 subunit complex which transcribes 60% of all RNA in mammalian cells and synthesises multiple copies of a single polycistronic rRNA precursor which is then cleaved into the 18S, 5.8S ,28S mature rRNA (Engel *et al.*, 2013). This pre rRNA gene exists as numerous tandem repeats, the number of which varies greatly between species. The human

genome containing 400 such repeats (Zentner *et al.*, 2011), the budding yeast *S. cerevisiae* contains 150 (Kobayashi *et al.*, 1998), while plants usually contain more copies with the pea plant *P. sativum* containing ~4000 copies (Ingle, Timmis and Sinclair, 1975). The *C. elegans* genome, which is the model organism we examine here contains 55 copies (Ellis, R.E., Sulston, J.E., and Coulson, 1986). Pre rRNA transcription is the initiating event in ribosome biogenesis, which also requires 5S rRNA transcribed by pol III and numerous ribosomal proteins transcribed by Pol II (Fig 1.10) (Engel *et al.*, 2013). While Pol I is strongly conserved, there is significant divergence in the promoter sequences with which the Pol I interacts to initiate transcription of the pre-rRNA between species (Grummt, 2003). Unlike Pol II or III, Pol I transcription occurs in the nucleolus, and indeed accounts for the existence of the nucleolus, which lacks a membrane and is thus not technically a separate compartment from the nucleus. Instead, the dense concentration of macromolecules resulting from the first stages of ribosome biosynthesis, including the generation of Pol I transcripts accounts for the visibility of nucleolus which will disappear if Pol I in inhibited (White, 2005). The Pol I promoter consists of the core promoter and an upstream control element (UCE) which both interact with dimerised upstream binding factor (UBF) to initiate Pol I recruitment. Dimerised UBF interacting with the UCE and core promoter then recruits the SL1 complex which contains the TATA-box binding protein (TBP) and at least 5 TBP associated factors (TAFs). The resulting UBF-SL1 complex is stable and will then recruit initiation competent RNA Polymerase I which is Pol I bound by RRN3 which mediates the interaction with UBF-SL1 (Figure 1.11). Once Pol I is recruited initiation can take place and the pre-rRNA is generated (Russell and Zomerdijk, 2006; Bywater *et al.*, 2013).

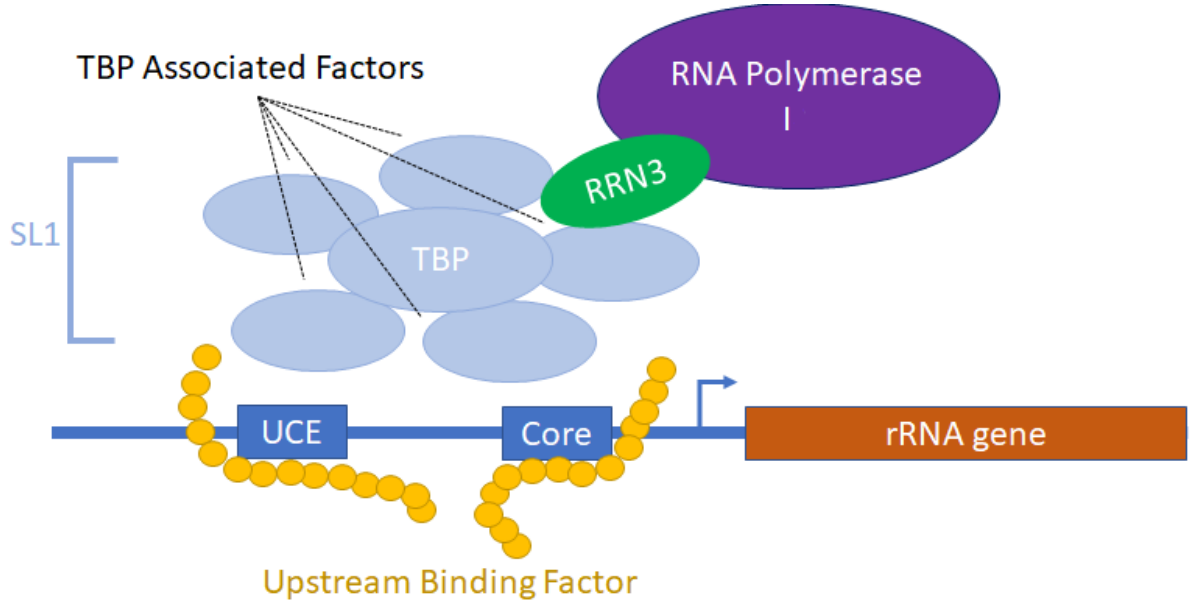

Figure 1.11: RNA Polymerase I promoter pre-initiation complex: Upstream Binding Factor UBF is recruited to the UCE and Core promoter, which then recruits the TATA-box binding protein containing SL1 to the promoter. The UBF-SL1 complex can then recruit initiation competent (i.e RRN3 bound) RNA Polymerase III to form the preinitiation complex.

1.8.2 RNA Polymerase II

RNA polymerase II is a 12 subunit complex required for the synthesis of the mRNAs corresponding to all protein coding genes as well as many microRNAs and short non-coding RNAs (Paabo, 1988; Cramer, 2001; Lee *et al.*, 2004). For this reason, it is arguably the most studied of all three polymerases. Additionally, Pol II is required for the efficient capping, splicing and polyadenylation of pre-mRNAs as they are processed by RNA-processing factors which interact with the C-terminal domain of Pol II. This activity is unique to RNA polymerase II and transcripts which contain these features (cap, polyA) can be identified as Pol II products (Meinhart and Cramer, 2004). In order to initiate transcription Pol II interacts with general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH to form the

preinitiation complex, often in interaction with other gene specific transcription factors (Orphanides, Lagrange and Reinberg, 1996). TFIID contains the TATA binding protein TBP which is also present in the Pol I and Pol III preinitiation complex and binds to the TATA box to initiate Pol II recruitment. TFIIA and TFIIB then associate with TFIID in order to stabilise its interaction with DNA and TFIIB can then recruit Pol II. TFIIF serves to position Pol II at the TSS and TFIIE and TFIIH are required from the melting and stabilisation of the DNA (Roeder, 2003). Once initiation takes place, the RNA polymerase II elongation complex will then travel along the DNA generating a strand of pre-mRNA which is then processed into mature RNA by a variety of factors in the aforementioned Pol II C-terminal dependent process (Sims, Belotserkovskaya and Reinberg, 2004). RNA Polymerase II is also thought to have an important role in regulating heterochromatin formation by its transcription of specific siRNAs which are involved in this process (Kato *et al.*, 2005).

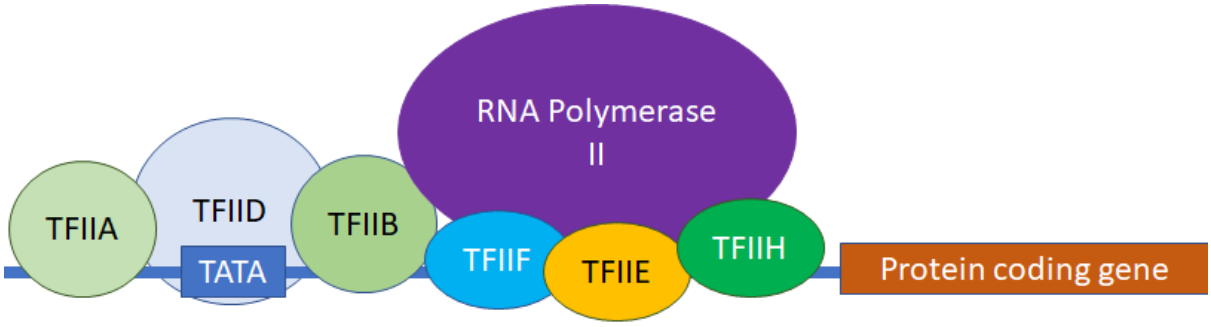

Figure 1.12: Pol II promoter and pre-initiation complex: TFIID binds to TATA box via TBP then recruits TFIIA and TFIIB, the latter of which recruits Pol II and TFIIF. TFIIH mediates DNA melting and TFIIE stabilises DNA in the melted configuration.

1.8.3 RNA Polymerase III

RNA polymerase III is a 17 subunit complex which is responsible for the transcription of tRNAs, 5s rRNA and other small non coding RNAs (Weinmann and Roeder, 1974). Of direct relevance to this study is the fact that RNA Polymerase III structure is centred around the two largest subunits, C160 and C128 in *S.cerevisae* where Pol III is best characterised. The C160 domain is involved in directly binding the DNA and is located near the centre of the Pol III holoenzyme (Flores *et al.*, 1999). RNA Polymerase III promoters are quite unusual in that most of these promoters require sequence elements downstream of the transcription start site, within the transcribed region. Briefly, Pol III promoters are divided into type I, II and III promoters. Type I promoters are restricted to rRNA genes encoding the 5S rRNA, while type II promoters are associated with tRNA genes. Both type I and II promoters require internal control regions for their transcription. Type I promoters contain two binding domains called the A and B blocks within the transcribed region, which interact with TFIIIC which then recruits TFIIIB which in turn recruits Pol III. Type II promoters contain the A block and another sequence, the C block. The C block first recruits TFIIIA which then recruits and stabilised TFIIIC which then recruits the other components of the PIC in the same was as in type I promoters. Type III promoters are only found in vertebrates and control various other Pol III products in these species, such as human and mouse U6 snRNA. Type III promoters are upstream of the transcribed gene and rely on interaction of TBP with a TATA box and of a proximal sequence element with the protein SNAPC, which interacts with a differently configured form of TFIIIB, which then recruits Pol III (Bywater *et al.*, 2013). Interestingly, since this is restricted to vertebrates it seems that this promoter structure has emerged

relatively recently in evolutionary time, and mimics more traditional promoter structures (Paule and White, 2000).

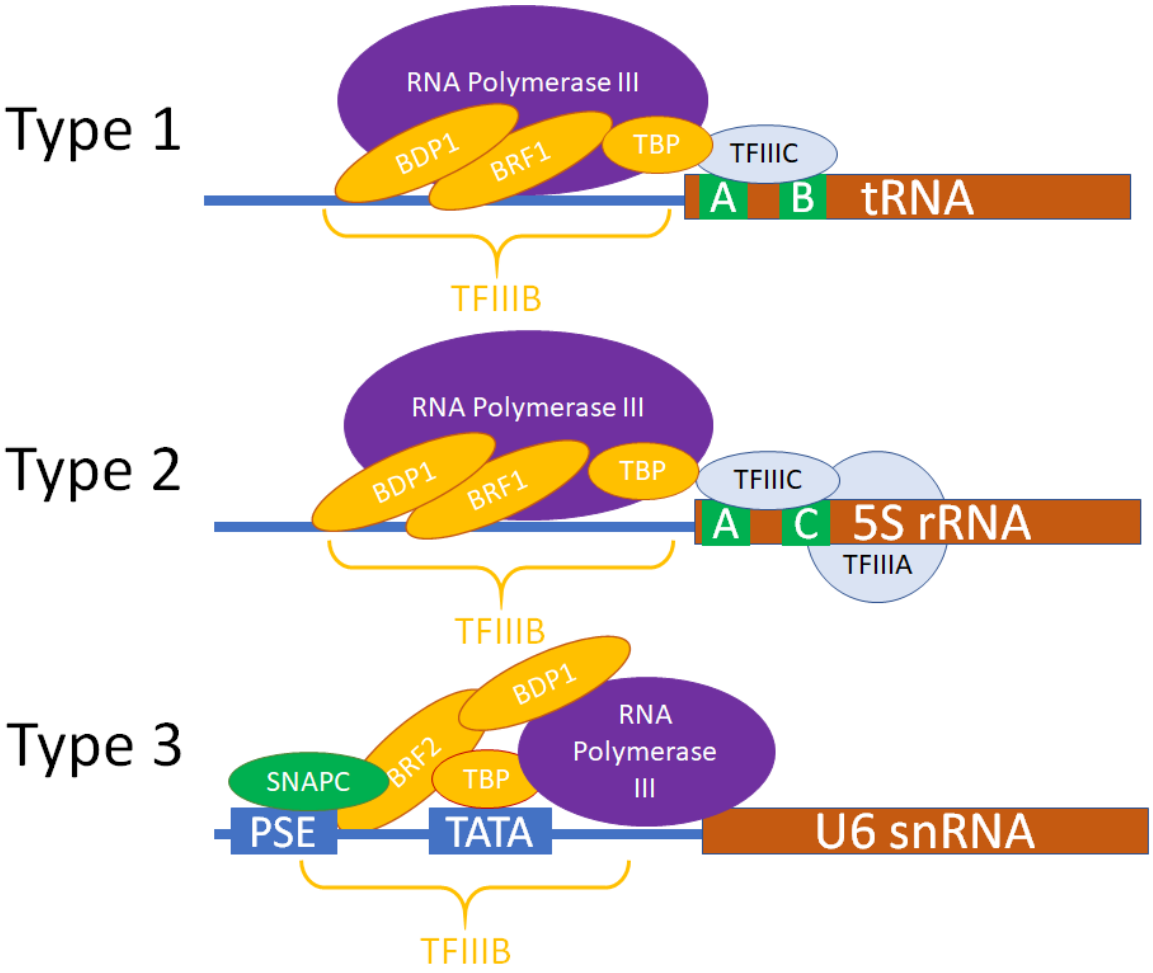

Figure 1.13: RNA Polymerase III promoter and pre-initiation complex structures. Type 1: A and B blocks recruit TFIIIC which recruits TFIIIB which then recruits Pol III. Type 2: C block recruits TFIIIA which stabilises TFIIIC in its interaction with A and C block, initiation then proceeds as with type I. Type 3: A different form of TFIIIB containing BRF2 rather than BRF1 is recruited via its interaction with a TATA box via TBP and its interaction with SNAPC via BRF2. Pol III is then recruited by TFIIIB.

1.8.4 Regulation of RNA Polymerase III by the TORC1

One way in which Pol III activity is modulated by nutrient status and stress is by TORC1. TORC1 is known to physically interact with the promoters of Pol III target genes via its association with TFIIIC, an interaction demonstrated by the reciprocal co-immunoprecipitation of TORC1 and TFIIIC (Kantidakis *et al.*, 2010; Tsang, Liu and Zheng, 2010). At Pol III promoters, TORC1 regulates Pol III activity via phosphorylation of Maf-1. Maf-1 is an inhibitor of Pol III activity in its dephosphorylated form. Once phosphorylated by TORC1, Maf-1 is no longer capable of inhibiting Pol III transcription and Pol III activity can proceed. Upon starvation or other stress, Maf-1 is dephosphorylated due to reduced TORC1 dependent phosphorylation and will prevent Pol III activity, thus linking tRNA and 5S rRNA production to nutrient status. TORC1 is the major target of the drug rapamycin, and this inhibitory phosphorylation of Maf-1 is abolished by rapamycin treatment (Michels *et al.*, 2010). As downregulation of TORC1 and treatment with rapamycin have been associated with longevity in *C. elegans* and other model organisms as discussed previously, this raises the possibility that RNA Polymerase III may be working downstream of TORC1 in the lifespan extension seen with TORC1 inhibition. Here, I present data from experiments which I performed in *C. elegans* which formed part the study which we published along with the Alic and Heinemann labs in Nature in 2017, "*RNA polymerase III limits longevity downstream of TORC1*".

Materials & Methods

2.1. Maintenance of *C. elegans*

2.1.1. NGM preparation

Nematode growth media (NGM) was prepared by adding 3g NaCl, 17g Agar and 2.5g peptone to an appropriately sized duran bottle and making up to 1L with distilled H_2O. The media was autoclaved at 120°C and cooled to 55°C before the addition of 1ml 1M $MgSO_4$, 1ml 1M $CaCl_2$, 1ml 5mg/ml cholesterol in ethanol and 25ml 1M KPO_4 and made up to 1L with autoclaved water to make up for any evaporation. At this point any relevant antibiotic or other additions to the media (e.g. ITPG and Ampicillin for RNAi plates) could be added, and for OP50-1 plates 1ml of 25mg/ml streptomycin stock was added. 6cm plates were poured with 15ml volume unless otherwise specified.

2.1.2. *C. elegans* husbandry

Animals were grown on standard NGM media using OP50 (A uracil auxotroph *E. coli* B strain) *E.coli* as a food source – this strain is described in the original Brenner (1974) study (Brenner, 1974) and has been used as the standard bacterial food source in most *C. elegans* labs since then. The full genotype of this strain is not described in Brenner (1974), common databases (wormbase, CGC), nor in any papers we have been able to find and is thus not shown here. More information on the base OP50 strain can be found in the worm breeders gazette (http://wbg.wormbook.org/2009/12/01/the-genome-sequence-of-e-coli-op50/) as can further information on OP50-1 (http://www.wormbook.org/wli/wbg8.2p56/). We used

the streptomycin resistant OP50-1 variant, obtained directly from the CGC. This strain was identified via the selection of streptomycin resistant OP50 and is not thoroughly defined (see WBG link above). This was grown on NGM containing 25ug/ml streptomycin. OP50-1 was cultured in Miller LB media (Fisher Bioreagents, BP1426-500 Tryptone 10g/L, Yeast extract 5g/L, NaCl 10g/L) overnight at 37°C and 200µl of the resulting culture was seeded onto NGM + strep 6cm plates two days before use to allow time for the bacteria to grow. Worms were manipulated mostly by "picking" whereby animals are individually picked up using a length of platinum wire melted into a glass pipette. OP50-1 bacteria collected on the flattened end of the platinum wire is used to collect worms and transfer them to the required plate or other destination. Platinum picks were sterilised in a Bunsen burner before re-use to prevent cross contamination or infection. Stocks were maintained without starvation by picking 5-8 L4 or young adult animals to new NGM plates before starvation. A Leica M80 dissecting scope was used next to Bunsen burners in order to enable visualisation of *C. elegans*, with the burners providing an upwards airflow to reduce contamination by floating particles. Stocks were mostly cultured at 20°C, with the exception of *daf-2(e1370), daf2-(e1368), daf-2(e1391), daf-11(m47)* and derivative strains which were cultured at 15°C due to dauer phenotypes at 20°C preventing their culture in these conditions. 15°C, 20°C and 25°C storage and incubations were always performed in VelpScientifica FOC 215 E Cooled Incubators at the appropriate temperature.

2.1.3. Freezing and thawing of *C. elegans* stocks

One major advantage of *C. elegans* over other model organisms is that they can be stored indefinitely at -80°C. In order to freeze a strain, plates were allowed to starve inducing large numbers of progeny to enter the L1 arrest state which is able to survive freezing at -80°C.

Starved L1 larvae from 5 6cm NGM plates were pooled by washing off animals in M9 (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre. Sterilized by autoclaving.) and a 1:1 dilution with 2x freezing media (30% glycerol, 5.5mg/ml NaCl, 6.8mg/ml autoclave then add 0.3% 0.1M $MgSO_4$) in the required number of cryo-tubes (thermofisher) and placed in a freezing container ("Mr Frosty", Fisher) in a -80°C freezer. Strains were thawed on ice and immediately pipetted onto seeded NGM plates, with 500µl of the thawed stock being placed onto each plate. Animals were recovered at an appropriate temperature for the strain and moved to a new NGM plate as soon as possible.

2.1.4. Bleach drop cleaning of stocks

In the event of bacterial or fungal contamination of the plate, the infection was removed mostly by the bleach drop technique. In this protocol, a 7:8 mixture of thin bleach and 4M NaOH is used to kill the infection while allowing embryos to survive. 5-10 µl of this bleach solution was spotted onto the edge of a seeded NGM plate and 8-10 gravid adult animals from the contaminated plate were picked into the bleach drop. Plates were left upright at the appropriate temperature for the strain until visible offspring develop and these animals were then moved onto a new NGM plate.

2.1.5. Bleach Prep

Bleach preps were used to synchronise populations of *C. elegans* and as an alternative technique to remove contaminations from badly infected strains. Populations containing gravid adults were washed off of plates in M9 media as required into 15ml falcon tubes and allowed to settle for 5min and M9 media removed. 1ml of 7:8 bleach: 4M NaOH was added and animals were vortexed for 5min. Bleach was immediately quenched by making up to

15ml with M9, and tubes were centrifuged at 3000rpm for 5min using a Thermo Heraeus Megafuge 16R Centrifuge. Three further identical wash steps were performed, at the resulting eggs were diluted in 1ml M9 and split between two NGM plates to hatch.

2.1.6. RNA interference

RNAi was first discovered in *C. elegans* (Fire *et al.*, 1998) and while this can be achieved in various ways, we used the RNAi feeding method (Timmons and Fire, 1998). In this strategy, the *E.coli* HT115 [F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lacUV5 promoter -T7 polymerase] strain is used which contains IPTG inducible T7 RNA polymerase. This is used to drive the expression of a chosen dsRNA in the *E.coli.* Upon ingestion, this dsRNA is processed by the endogenous *C. elegans* RNAi machinery which results in specific degradation of the targeted RNA and silencing of the corresponding gene. Tissue specific RNAi in the intestine was achieved by using the VP303 strain which is *rde-1* null with this rescued in the intestine. RDE-1 is an argonaut protein in *C. elegans* which is required for processing of exogenous RNA in the RNAi pathway. In VP303, *rde-1* is then rescued under the control of the promoter of the intestinal specific *nhx-2*, resulting in intestinal specific competence to RNAi silencing (Espelt *et al.*, 2005). NGM + amp (50mg/ml) plates seeded with HT1115(DE3) bacteria either expression empty PL4440 or PL4440 carrying the relevant RNAi insert were used to induce RNAi by feeding. The *rpc*-1 RNAi clone (gene code C42D4.8) was obtained from the Ahringer *C. elegans* RNAi library.

2.2. Strains

Most strains were acquired for the Caenorhabditis Genetic Centre (CGC) at the University of Minnesota, with some other strains being acquired from the National Bioresource Project for the nematode at Tokyo Women's Medical University School of Medicine. Many other strains were given to us by other labs, notably including the Gems lab at University College London. COP1835 and COP1836 were generated by Knudra Transgenics, and QL196 was generously gifted to us by Queelim Ch'ng of Kings College London.

Strain	Genotype	Source
CGCM	N2 (male)	CGC
GA1058	*skn-1b(tm4241)*	Gems lab
GA82	*daf-2(e1370)*	Gems lab
GA1059	*daf-2(e1370)*; *skn-1b(tm4241)*	Gems lab
JMT31	*daf-2(e1370)*	GA82 backcrossed to CGCM
JMT32	*daf-2(e1370)*; *skn-1b(tm4241)*	GA1059 backcrossed to CGCM
DR1572	*daf-2(e1368)*	CGC
GA1060	*daf-2(e1368); skn-1b(tm4241)*	Gems lab
DR1574	*daf-2(e1391)*	CGC
JMT5	*daf-2(e1391);skn-1b(tm4241)*	JMT5 x GA1058
VP303	*rde-1(ne219) V; kbIs7[Pnhx-2::rde-1 + rol6(su1006)]*	CGC
RB1205	*rsks-1(ok1255)*	CGC
GA1017	*WuEx217[Pskn-1b::skn-1b::GFP]*	Gems lab
DA1116	*eat-2(ad1116)*	CGC
GA1077	*eat-2(ad1116); WuEx252[Pskn-1b::skn-1b::GFP]*	Gems Lab
COP1835	*knu1835[skn-1b::wrmScarlet]*	Knudra Genetics
COP1836	*knu1836 [skn-1b::wrmScarlet]*	Knudra Genetics
GR1455	*mgIs40[Pdaf-28::GFP]*	CGC
JMT52	*skn-1b(tm4241); mgIs40[Pdaf-28::GFP]*	GR1455 x GA1058
HT1702	*wwEx66 [Pins-7::GFP + unc-119(+)]*	CGC
JMT51 *skn-1b(tm4241)*	*skn-1b(tm4241) wwEx66 [Pins-7::GFP + unc-119(+)]*,	HT1702 x GA1058
QL196	*drcSI61 [unc-119;Ptph-1::mCherry]; drcSI7[unc-119;Pdaf-7::Venus]*	Ch'ng lab
JMT50	*drcSI7[unc-119;Pdaf-7::Venus]*	QL196 x CGCM
JMT75	*skn-1b(tm4241); drcSi7[Pdaf-7::venus]*	JMT50 x GA1058
DR47	*daf-11(m47)*	CGC
JMT79	*daf-11(m47); WuEx252 [Pskn-1b::skn-1b::GFP]*	DR47 x GA1058
SJ4005	*zcIs4[Phsp-4::GFP]*	CGC
JMT80	*skn-1b(tm4241); zcIs4[Phsp-4::GFP]*	SJ4005 x GA1058

Table 2.1: List of *C. elegans* strains and their sources. Crossed strains all generated by the author.

2.3. *C. elegans* assays

2.3.1. Lifespan Assays

In preparation for lifespan assays animals were synchronized without the use of bleach by picking L4 animals from busy but not starved plates with animals being grown at the appropriate temperature and with adequate food for three generations prior to such experiments, since it is known that starvation induces epigenetic changes which extend lifespan over several generations (Rechavi *et al.*, 2014). L4 animals were picked directly onto lifespan plates and this was recorded as "day 0". Lifespan plates contained ~20 animals per plate, with each experimental condition having 100 animals total split between 5 plates. Animals were scored every second day as "alive", "dead" or "censored". The alive/dead status of a worm was scored according to their responsiveness to a light touch with a pick to the animals head – animals which were not otherwise moving and failed to respond to the light touch were scored as dead. Censored animals were either those which left the plate or died from causes other than ageing (e.g. dried out on plate edge, gut explosion). In most of our lifespans, 10µM FuDR was used to prevent progeny production however in lifespans with no FuDR animals were moved every second day for their reproductive period (5-10 days depending on strain, detected by the laying of dead eggs). FuDR use is noted on the appropriate figures. Statistical analysis was performed by log-rank test, using the open source website OASIS (Yang *et al.*, 2011) and its successor OASIS 2 (Han *et al.*, 2016).

2.3.2. Brood size assay

Animals were individually picked onto seeded NGM plates at either L4 stage or as eggs depending on the assay in question. One animal was picked per plate, with each

experimental condition having 10 animals were assay. Each day, animals were moved to new plates and the old plate (containing progeny from that day) was incubated at 20°C for one day before progeny was counted. This provided age-specific fecundity data as well as total brood size. The assay was discontinued when an animal began laying unfertilized oocytes which were identified visually based on their morphology.

2.3.3. RNAi development assay

5 animals were placed onto NGM + amp (50mg/ml) plates seeded with HT1115(DE3) bacteria either expression empty PL4440 or PL4440 carrying the relevant RNAi insert. Offspring were examined for phenotypes and imaged on a dissecting scope. At L4 stage, 5 animals were moved to a new plate the procedure was repeated. In this way three generations of development on RNAi were observed.

2.3.4. Oxidative stress survival assay

Animals were synchronized to day 1 adult by picking L4s the day prior to the experiment and placing the animals at 20°C. In a 96 well plate 100 µl of 5mM sodium arsenite in M9 was pipetted in the relevant number of wells (M9 only in control wells) and 100 µl of an OP50-1 overnight culture was added to produce a final concentration of 2.5mM arsenite. Animals were directed picked into the wells, with 5 wells of 10 animals being the standard for each experimental condition. Animals were scored as alive or dead based upon movement every 8-16 hours depending on the assay, and survival curves were generated from this data. Censored status was only applied to animals which could no longer be located, and this was very rare. Statistical analysis was performed by log-rank test using Oasis 2 (Han *et al.*, 2016).

2.3.5. Bacterial Deprivation

Animals were synchronized by picking L4s and were immediately moved to blank NGM plates. After 1 hour, the same animals were picked with no bacteria to new blank NGM plates to minimize the amount of bacteria carried over. After 16 hours, the BD protocol is complete and the animals were analysed by whichever techniques were to be used. In our DiI colocalization assays, animals were incubated in 2mg/ml DiI in M9 with no bacteria in order to maintain the BD conditions during DiI treatment.

2.3.6. Tunicamycin UPR induction

Animals were synchronized to D1 adult by picking L4s the day before the experiment. Animals were picked 100µl of 25ng/µl tunicamycin in M9 for 4 hours, then pipetted out onto seeded NGM plates, dried next to a Bunsen burner and the animals immediately imaged. The tunicamycin stock was prepared in DMSO, so control animals were picked into M9 + DMSO to the same concentration.

2.3.7. Pumping Assays

Pumping assays were performed to confirm the *eat-2(ad1116)* status of animals used in this study, while this data is not shown it was required to genotype *eat-2(ad1116)* in the generation of relevant strains. Animals of L4 stage were examined for their pumping rates by manually counting the movements of the pharyngeal grinder using a counter and a dissecting microscope. Number of pumps per minute was calculated, and reduced pumping rates confirmed the *eat-2(ad1116)* status of these animals. In later assays, *eat-2 (ad1116)* animals were selected based on non-quantified observation of pumping rates.

2.3.8. Smurf assay for intestinal integrity

The smurf assay was performed largely as it was by those who first developed it for use in *C. elegans* (Gelino *et al.*, 2016). Animals were grown to L4 stage and picked into 200µl of OP50 liquid culture from an overnight mixed with blue dye (2.5% w/v in water FD&C blue #1) for 3h. Animals were pipetted onto seeded NGM plates and immediately moved to new seeded NGM plates and allowed to move freely for 1h to allow dye to leave the intestine if it had not invaded the body cavity. Animals were imaged on a Leica DMR scope and images were scored using a semi quantitative scale where 0 = no dye crossing gut barrier, 5 = entire animal dyed.

2.4. Molecular Biology

2.4.1. Single worm genotyping

It was necessary to obtain lysates of single or multiple animals through the project, mostly in order to obtain material for PCR. For multiple worm lysis 8 -10 animals from a homogenous population were used, while many genotyping experiments require lysates from single worms for genotyping PCR. In either case the animals were lysed in 5µl worm lysis buffer (50mM KCl, 10mM Tris (pH 8.3), 2.5mM $MgCl_2$, 0.45% NP40, 0.45% Tween20, 0.01% Gelatin, 0.1mg/ml proteinase K) in a Bio-rad T100 thermal cycler using our lysis protocol (70°C: 60min → 95°C: 15min → 12°C: ∞).

Pre-made PCR mixes, mostly 2x PCRBIO Taq Mix Red (Ingredients listed in the manual as: PCRBIO Taq DNA Polymerase, 6mM $MgCl_2$, 2mM dNTPs, enhancers, stabilizers and a red dye) were used. PCR reactions were prepared with 10µl Taq Mix, 3µl of 10mM primer mix (10mM in MilliQ water) for the specific reaction in question and 2µl of milli-Q H_2O. This was added to either 5µl of worm lysate or a DNA solution of the same volume. The reaction mix was placed into a Bio-rad T100 thermal cycler and subjected to the chosen PCR protocol (94°C: 60s → 94°C: 30s → X°C (differs between primer sets):30s → 72°C: 60s → Repeat previous 3 steps 28-32x →72°C: 7min →12°C: ∞). PCR products were then analysed on 1-2% agarose gels using Promega 1Kb ladder. Visualised using a SynGene GBox.

2.4.2. RNA extraction

Animals were washed off of their plates in M9 buffer and washed 3X by adding 10 ml of M9, allowing animals to settle, removing as much M9 and possible then performing the next wash in the same way. Animals were lysed (and proteins denatured) in TRIzol reagent in a Disrupter Genie (Zymo) for 30 minutes. 75µl of 1-Bromo-3-chloropropane was added and the sample was incubated for 3min at room temperature. This mixture was then spun down (12,000 rpm, 4°C, 15min) and the RNA containing phase was transferred to a new RNAse free tube. An equal volume of isopropanol was used to precipitate the RNA which was washed with 400µl ethanol and left to air dry. Dried RNA was dissolved in 10ul milli-Q water, heated at 60°C for 10 minutes to dissolve and stored at -80°C. RNA concentration and purity was analysed by Nanodrop 2000 (thermofisher), with 260/280 ratios of 1.8 – 2.1 deemed acceptable and 260/230 ratios of greater than 2. Phenol contamination was removed by using the RNA cleanup application of the Qiagen RNAeasy kit.

2.4.3. cDNA synthesis

Prior to cDNA synthesis RNA must be cleaned by the removal of genomic DNA by DNAse I treatment. The digest was prepared with 1µg of RNA, 1µl of 10x DNAse I buffer, 1ul of DNAse I (1U/µl) and DPEC water up to 10µl. DNaseI digest was performed at room temperature for 15 minutes and inactivated with 1 µl EDTA and heating to 65°C for 10min. This digest product was then directly used as the starting material for cDNA synthesis. cDNA synthesis was performed by preparing an initial mix of 1µl oligo(dT) 12-18, 1µg total RNA, 1µl dNTPmix. The reaction mix was heated in a Bio-rad T100 thermal cycler to 65°C for 5min and chilled on ice. Next, 4µl 5x first strand buffer and 2µl 0.1M DTT were added and incubated for 2 min at 42°C. 1µl of superscript II RT was added and samples were incubated

at 42°C for 50 min then inactivated by heating to 70°C for 15min. Finally, 1µl of *E.coli* RNAse H was added and incubated at 37°C for 20min. This cDNA mix was diluted up to 100µl and stored at -20°C until use.

2.4.4. RT-qPCR

qPCR primers were designed with the aid of NCBI PrimerBlast (www.ncbi.nlm.nih.gov/tools/primer-blast/), with primers being selected which cross exon-exon boundaries if possible. Primer sets were optimized by gradient qPCR to identify optimal melting temperatures (T_m) and primer efficiency was confirmed to be between 95% and 105% as per BioRad's guidelines via the generation of a standard curve.

RT-qPCR reaction mixes were prepared with 2µl cDNA, 5µl SYBR green, 2.6µl H_2O and 0.4µl of relevant primers. Each sample was prepared in triplicate. mRNA levels were normalized to the geometric mean of 3 housekeeping genes – *pmp-3, cdc-42* & *Y45F10D.4 (Table 2.2)*.

A Bio-rad CFX96(well) Connect R-T system was used to run these assays. qPCR protocols were optimized and annealing temperatures varied. However the basic template protocol below was used.

95°C 2min → (95°C 20s → X°C 20s → 70°C 20s → plate read) X 44 cycles → melt curve 55-95°C

Where X is the anneal temperate determined by gradient PCR and/or predicted T_m.

For ILP screen (Table S3), RNA was extracted from 6x N2 and 6x *skn-1b(tm4241)* samples as described previously and sent to qStandard who performed the qPCR using their own internal procedures. Primers listed in Table 2.2 and Table 2.3.

2.5. Luciferase Assay

2.5.1. Plasmid construction

Construction of SKN-1 binding site luciferase reporter plasmid

Complimentary custom oligos (Sigma) containing 3 of the consensus SKN-1 binding site (TTATCAT) flanked by Nhe1 and Sma1 restriction sites were annealed to form the insert. Oligos were dissolved in annealing buffer (10mM Tris, 50µM NaCl, 1mM EDTA) to a final stock concentration of 100 µM and equal volumes of each were mixed and processed in a thermocycler for the following steps : (95°C: 2 min → 95°C to 25°C cool off at -1°C per minute: 70 min → 4°C until collection). The resulting oligo was cleaned up using a QIAquick PCR purification kit. Both the SKN-1 binding site oligo and the PGL-3 Basic plasmid were digested with Nhe-1 and Sma-1 (NEB, as per manufacturer's instructions) cleaned – PGL-3 by gel extraction (QIAquick Gel Extraction kit) and digested SKN-1 binding site oligo by PCR purification (QIAquick PCR purification). The insert was ligated into the plasmid using T4 ligase (Promega) with the final reaction containing: *Vector DNA: 100ng, Insert: 17ng, Ligase 10x buffer: 1µl, Nuclease free H_2O up to 10 µl*. Since Sma1 produces a blunt end this was incubated at 20°C for 16 hours. 25 µl of JM109 cells were transformed with 3 µl of ligated plasmid (4°C – 30min, 42°C – 1min, 4°C – 1min) and cells were outgrown for 60min in 500µl LB media at 37°C. Transformed JM109 then plated on LB + ampicillin (100µg/ml) agar overnight and grown at 37°C overnight. Colonies surviving on ampicillin plates carry the complete SKN-1 binding site PGL3 plasmid. Resulting plasmids were sequenced by GATC SupremeRun sequencing.

Construction of SKN-1B and SKN-1C expression vectors

Our lab had a pEX-A plasmid containing *skn-1b* cDNA (eurofins) flanked by Nhe-1 and Sma1 restriction sites .The *skn-1b* cDNA was removed from this plasmid by Nhe1 and Sma1 digest (as shown above) and isolated by gel extraction (QIAquick gel extraction kit). PCI-Neo mammalian expression vector was cut with the same restriction enzymes and *skn-1b* cDNA ligated into this plasmid using the same ligation protocol as shown above. cDNA of *skn-1c* was ordered from Eurofins Genomics, who directly inserted it into the PCI-neo plasmid.

2.5.2. Transient transfection

Hek-293T cells were grown to 70% confluence in a 24 well plate in standard tissue culture conditions using MEM media (Gibco) supplemented with 2mM Glutamine and 10% v/v FBS (Fetal Bovine Serum) . Cells were then washed with PBS and a mixture of OPTI-MEM media (Gibco) and an appropriate mixture of lipofectamine 2000 (thermofisher) and the required plasmids (equal volumes lipofectamine & plasmid) for 5 hours before the media was replaced with normal MEM (Gibco).

2.5.3. Luciferase assay

Prior to starting all reagents were equilibrated to room temperature. Hek-293T cells were scraped off into 200 µl PBS and 75 µl of this was transferred to 96 well clear bottomed plate (Corning) in triplicate. 1 volume (75 µl) of luciferase reagent (Promega) was added to each well and the plate was incubated in a light free environment for 10 minutes, before luciferase fluorescence was recorded using a CLARIOstar plate reader (580nm). Immediately after reading, 1 volume of Dual Glo Stop and Glo reagent was added and incubated in a light

free environment for 10 minutes before reading Renilla fluorescence (480nm). Firefly fluorescence was normalised to Renilla.

2.6. Microscopy

2.6.1. Leica DMR & Quantitative fluorescence

For most microscopy in this project including all quantitative fluorescence assays a Leica DMR microscope with a CoolLED lightsource and a Leica DFC9000 GT camera was used. For GFP and Venus fluorescence a Leica N2.1 filter cube was used (Ex:515-560 Em:538/46). In order to visually identify autofluorescence, the I3 filter cube (Ex:450-490 Em: LP515) was used to render auto-fluorescence yellow while GFP remained green. Images were not taken with i3 due to the camera being monochrome. Animals were synchronized at day 1 adult picking L4 animals the day before, and animals were imaged at day 1 adulthood unless otherwise specified. Animals were mounted on a 2% agarose pad on a microscope slide in 5µl tetramisole hydrochloride. ASI neurons were visualized at 40x magnification and whole body fluorescence was visualized at 20x magnification. Images were processed using the FIJI distro of ImageJ (https://fiji.sc/). For quantitative fluorescence of ASI neurons and the intestine, the tissues of interest were manually selected as an ROI (meaning that it was drawn around as a region of interest, using the ROI tool on imageJ) and mean grey value and integrated densities were recorded. Equivalently sized areas of background were recorded for each image and images were blank subtracted prior to analysis. ASIs were individually scored. For the DiI colocalization assays, animals were incubated in 2mg/ml DiI in M9 for 1.5 hours before being imaged.

2.6.2. Confocal microscopy

Animals were synchronized to day 1 adult as before and mounted onto a microscope slide in CyGel spiked with 0.6% tetramisole hydrochloride to prevent movement of live animals. Two confocal microscopes were used for imaging – most confocal imaging was performed using a Zeiss LSM880/Elyra/Axio Observer.Z1 confocal microscope with the airyscan acquisition mode with the 60x lens. Images were processed with ZenBlue software. We also used a spinning disk confocal in order to reduce acquisition time and bypass an issue with the animals moving slightly during the longer airyscan acquisition times. The spinning disk was an Olympus IX73 with a Plan Apo 60x 1.40 NA lens mounted on a PIFOC z-axis focus drive (Physik Instrumente, Karlsruhe, Germany) and illuminated using LDI light sources (89 North) with appropriate filters in line with a CrEST. Images from this scope were processed using MetaMorph software with the assistance of Tara Eastwood in the Mulvihill lab (UKC).

Target		Forwards Primer	Anneal Temp
skn-1b Genotyping	F	gctgattgaatggaaaacaatcata	58
	In	gcttatcctcctttatttgctctc	
	R	tctaggttaacaaccctctgc	
PCI-neo Sequencing	F	agttcaattacaagcttaagg	60
GFP genotyping	F	ggagagggtgaaggtgatgc	65
	R	agggcagattgtgtggacag	
pL4440	F	gttttcccagtcacgacgtt	58
	R	tggataaccgtattaccgcc	
qPCR control *cdc-42*	F	ctgctggacaggaagattacg	61
	R	ctcggacattctcgaatgaag	
qPCR control *pmp-3*	F	gttcccgtgttcatcactcat	61
	R	acaccgtcgagaagctgtaga	
qPCR control Y45F10D.4	F	gtcgcttcaaatcagttcagc	61
	R	gttcttgtcaagtgatccgaca	
venus genotyping	F	atgcttcgccagatacccag	63
	R	gggcagattggtaggacagg	
qPCR *skn-1b*	F	aacaggtggatcaacacggc	60
	R	ttttgcattccaatgtaggc	
qPCR *skn-1a*	F	agtgcttctcttcggtagcc	60
	R	gaggtgtggacgatggtgaa	
qPCR *skn-1a/c*	F	gagagaaggggcacacgacaa	60
	R	tcgagcattctcttcggcag	
daf-7 genotyping	F	ttatggagagaacccgtcgc	65
	R	actgtgagtgtggcctgaaat	
qPCR *daf-28*	F	agtccgtgttccaggtgt	63
	R	tgttgcgatgtcaattcctt	

Table 2.2: Primer sequences and T_m for the work presented here. Excluding qStandard qPCR primers (Table 2.2) and those used for assays not shown.

ILP	Primer	Sequence
daf-28	daf-28 F	agtccgtgttccaggtgt
	daf-28 R	tgttgcgatgtcaattcctt
ins-1	ins-1 F	gcactggattaaccgctttc
	ins-1 R	cacaacattctgtcgcaattc
ins-2	ins-2 F	cgtgcctcaagagtccaga
	ins-2 R	atgcagcaaatatgcgaaagg
ins-3	ins-3 F	tcactccctgatggccagat
	ins-3 R	ctattttccagccacagcact
ins-4	ins-4 F	aaaatcaactctcccgagca
	ins-4 R	gcaatgtccatgtcctcttgt
ins-5	ins-5 F	caaggcttcatcctgtcgtc
	ins-5 R	tcaaatgggcagcagatttct
ins-6	ins-6 F	catctctttagtcatggctgtctg
	ins-6 R	ggacaacaagcagatcttatgtagtc
ins-7	ins-7 F	gcatgcgaatcgaatactgaagtta
	ins-7 R	gttttcgaatgaagtcgtcgg
ins-8	ins-8 F	acacacatctttagaggagtcc
	ins-8 F	agacttgctttccgcacaat
ins-9	ins-9 F	ccgctgtggaagaaaactct
	ins-9 R	agagatcctgttccgtaccc
ins-10	ins-10 F	tcctcgtaacattggctccc
	ins-10 R	cagtctgtcaatgctccaatct
ins-11	ins-11 F	taatcatgaaggtgatgcaga
	ins-11 R	ggtgcagctcgctaacattt
ins-12	ins-12 F	gcgatgaagtcgttgtgtagt
	ins-12 R	cttggcaacacttagttgcag
ins-13	ins-13 F	ctgttattccaatcgtgctct
	ins-13 R	tcgaacaacaagtcacgagta
ins-14	ins-14 F	atgcgatgcaaagttcatttcg
	ins-14 R	tgagctttggagcagtgagat
ins-15	ins-15 F	attgtggtttttgctcttttggc
	ins-15R	ggctcaacgattccccaca
ins-16	ins-16 F	ctgcctcctcacactgtcag
	ins-16 R	atttctgcatcactttcagttcc
ins-17	ins-17 F	atcactcgggacactatccga
	ins-17 R	gcaaatcaagttgaaagcgtca
ins-18	ins-18 F	taacacaatggtccaccgac
	ins-18 R	cacattttcatgcgtccgtc
ins-19	ins-19 F	agagtatgcgtgaaggatatagatc
	ins-19 R	acttcctcactgcaaatatgcttc
ins-20	ins-20 F	gctcgaatgttgatgataacct
	ins-20 R	ttcattctggacagcaccgt

ins-21	ins-21 F	aagcggtcaaacacattcgg
	ins-21 R	tgagaatccgagtaccccatc
ins-22	ins-22 F	tacgtcagaactctgtgtgga
	ins-22 R	gcttggatactctccggtaga
ins-23	ins-23 F	cagagcttcacgttcgtagg
	ins-23 R	tgggcacaatcgcattatgt
ins-24	ins-24 F	gcactatgccatgtcttctcg
	ins-24 R	agtcccatctccttgaggct
ins-25	ins-25 F	ctccagctttctgaagccaaa
	ins-25 R	ttgtggcaatgtcaacgctt
ins-26	ins-26 F	cggaactatgatgcactcgt
	ins-26 R	aatagcttcatggttgggca
ins-27	ins-27 F	gccgcttaattccctatgtcta
	ins-27 R	gacagcaagccttttggactt
ins-28	ins-28 F	cttggctctgctcgcaatag
	ins-28 R	ggcaatcagttcgtggtgag
ins-29	ins-29 F	ttgaactagcttcgtgggca
	ins-29 R	gcaagatttgaaggacagcaca
ins-30	ins-30 F	cagttcacgctgatgacctc
	ins-30 R	aagacgaatggtgtctgggg
ins-31	ins-31 F	gtttgccagaccggagaaaa
	ins-31 R	ttgatgtcttcaccgcacac
ins-32	ins-32 F	taggcgactaagtaagacggt
	ins-32 R	gttgcgatgtcctcctcctt
ins-33	ins-33 F	caacagctccgatgaagtctc
	ins-33 R	cgtcgtcgaagaggctatgt
ins-34	ins-34 F	gacagtcttccattccgaaagc
	ins-34 R	ccagatggagctttggttgtagt
ins-35	ins-35 F	caataatcgtcactgccaacg
	ins-35 R	atcaccgttgagccacatcc
ins-36	ins-36 F	cacccagaagggaaattggtt
	ins-36 R	tttgggcagcataattcacga
ins-37	ins-37 F	gccttccttccctatcgttc
	ins-37 R	actgctgcgatctttcttccc
ins-38	ins-38 F	tttctcctcgtctgcatcgc
	ins-38 R	gagatttttcatcgggggtaaaacta
ins-39	ins-39 F	gtttccattccacaaggctaatacc
	ins-39 R	tcttctgcattgcctccatgtt

Table 2.3: qStandard ILP qPCR primers: qStandard previously ran this assay for Queelim Ch'ngs group and reused the same primers from that study (Fernandes de Abreu *et al.*, 2014).

Investigations into the expression, regulation and molecular function of *skn-1b*

Since *skn-1b* was shown to be required for (one protocol of) dietary restriction longevity (Bishop and Guarente, 2007b) – one of the best studied mechanisms of lifespan extension and a subject of great scientific interest – there has been surprisingly little new discovered about this isoform. The first part of our study of *skn-1b* consists of a more general characterisation of the function and regulation of this transcription factor, in order support further hypotheses about specific roles which *skn-1b* plays in the animal which are examined in chapter four. In particular, we focus on identifying how *skn-1b* is regulated and how loss of *skn-1b* influences normal adult physiology. Our aims in this section are as follows:

1. To record *skn-1b* expression patterns under the control of its own promoter in normal conditions, during development and in bacterial deprivation conditions.
2. To examine the biological effects of *skn-1b(tm4241)* mutation via phenotypic analysis.
3. To directly test the hypothesis that *skn-1b* as an active transcription factor.

3.1. *skn-1b* can be expressed from its own promoter and localises to ASI neurons.

As discussed in the introduction (section 1.6.3 & 1.7.2) *skn-1b* in ASI neurons was shown to be required for the longevity resulting from (one protocol of) dietary restriction by Bishop & Guarente (2007). This study demonstrated that a major site of *skn-1b* activity is the ASI neurons and demonstrated that *skn-1b* is predominantly expressed in ASI neurons. Furthermore, using 5' RACE they found evidence that *skn-1b* is expressed from its own promoter. Whether it is actually possible to express *skn-1b* from this promoter, and its expression patterns under the control of its own promoter rather than known ASI specific promoters remained to be investigated. First, the translational reporter *WuEx217 [Pskn-1b::skn-1b::GFP]* (created by Jennifer Tullet, in the Gems lab, UCL) which is expressed as an extra-chromosomal array was used to examine *skn-1b* expression patterns under the control of its own promoter. This transgene contains the 2kb containing the predicted promoter region upstream of *skn-1b,* followed by *skn-1b* genomic DNA and *gfp*. This results in the expression of a SKN-1B::GFP fusion protein under the control of a putative *skn-1b* promoter. We examined the expression patterns of the resulting fusion protein by confocal microscopy. Under normal conditions, we observed that *skn-1b* is restricted to the ASI neurons, and we were unable to identify GFP fluorescence in other tissues by visual inspection, although this was not quantified (Figure 3.1 A). This tightly regulated tissue specific expression of *skn-1b* in one pair of neurons points towards the likelihood of a role for *skn-1b* that is likely to be involved in separate processes – either by performing a similar stress resistance role in tissues with different functions, or by direct involvement in ASI specific processes. Next, since extrachromosomal transgenes are somewhat poorly

understood in terms of their regulation, we sought to visualise levels of endogenous *skn-1b*. To this end we used a strain generated by crispR on our behalf by Kundra transgenics, COP1835 [*skn-1b::wrmScarlet*] in which a scarlet reporter was inserted at the *skn-1b* translational start site leading to endogenous *skn-1b* being tagged with *wmrScarlet* (Figure 3.1 B). We examined expression patterns of these animals and observed the same expression pattern of ASI expression only. These data together confirm that in normal conditions *skn-1b* is only expressed at detectable levels in ASI neurons, with potentially important implications for *skn-1b* function. Additionally, since we go on to use our extrachromosomal transcriptional reporter as the basis of various assays, the fact that we see the same expression patterns in COP1835 helps to support the safety of these conclusions. COP1835 expression was significantly fainter than that of our transcriptional reporter, with *skn-1b::wrmscarlet* being only barely visible by our Leica DMR and was visualised by spinning disk confocal microscopy (Olympus IX73, detailed in methods)

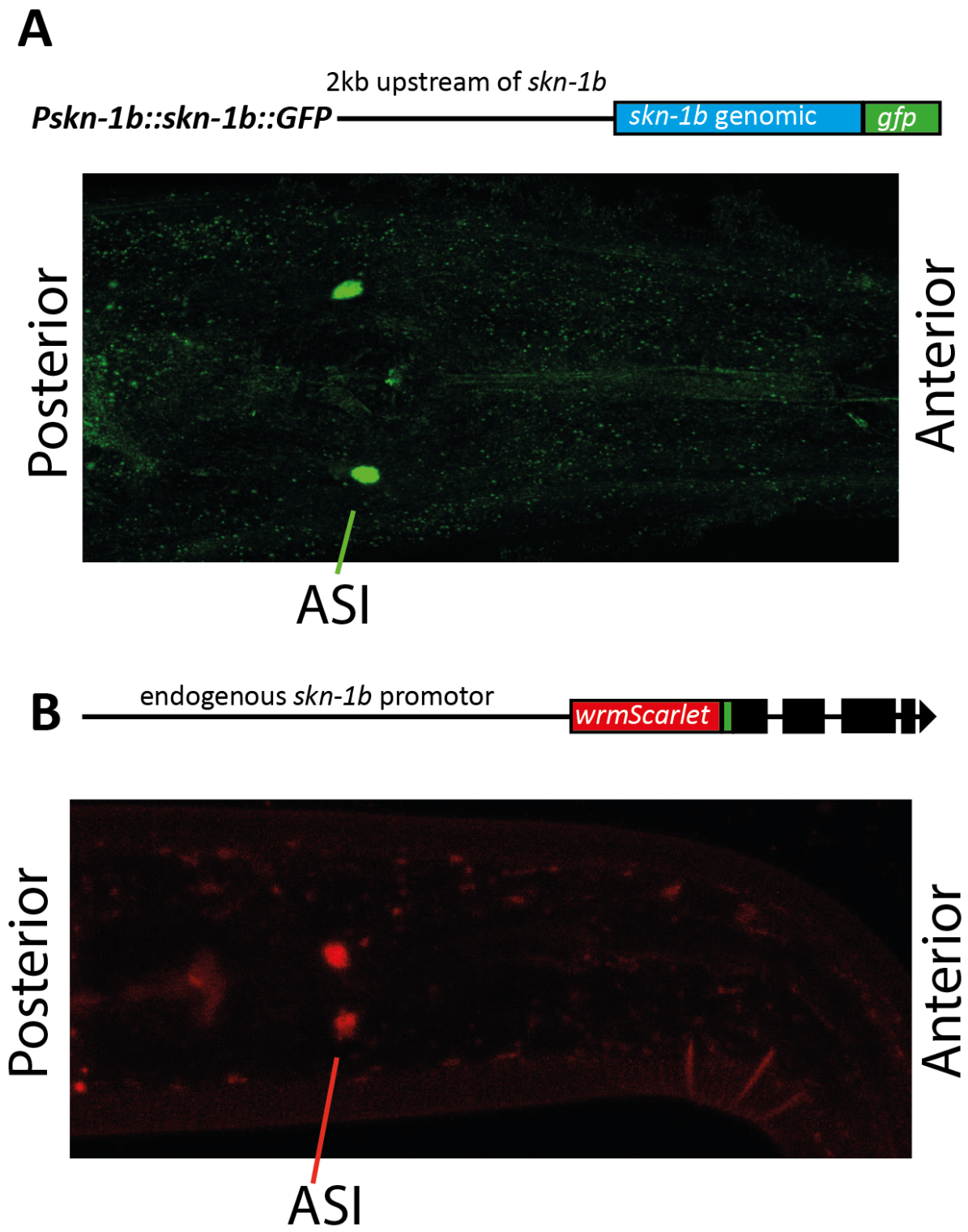

Figure 3.1: SKN-1B::GFP expression patterns in day 1 adult animals.

A: SKN-1B::GFP expression in N2*[Pskn-1b::skn-1b::GFP]* imaged by airyscan using Zeiss LSM880/Elyra/Axio Observer.Z1 confocal microscope, 63x mag. **B:** SKN-1B::WrmScarlet expression in COP1835 imaged by Olympus IX73 microscope 60x magnification.

3.2. SKN-1B::GFP expression is increased by bacterial deprivation but unaffected by genetic models of DR

Previous work from another lab has shown that in response to a specific liquid DR protocol (iDR), *skn-1b* expression in ASI neurons is increased (Bishop and Guarente, 2007b). We sought to establish a simple, solid media assay in order to test the interaction between the withdrawal of food and *skn-1b* expression levels. Since the ASI neurons are chemosensory, we reasoned that external signals relating to food abundance may be involved in the regulation of *skn-1b* in these cells. There is precedent for this in the literature, and *daf-7* levels in ASI neurons respond to simple removal of food (Fletcher and Kim, 2017). We adapted this assay and tested whether SKN-1B::GFP levels respond to bacterial deprivation (BD). Animals were placed onto NGM plates containing no bacteria at D1 adult and imaged after 16 hours. We observed a significant increase in ASI specific SKN-1B::GFP fluorescence in BD conditions (Figure 3.2 A). Indeed, we saw a similar level of induction in BD conditions (~1.4x control) as we did when applying the Bishop & Guarente DR protocol (which uses HT115 *(de3) E.coli* killed with erythromycin) to animals carrying this transgene (Jennifer Tullet, Data not shown).

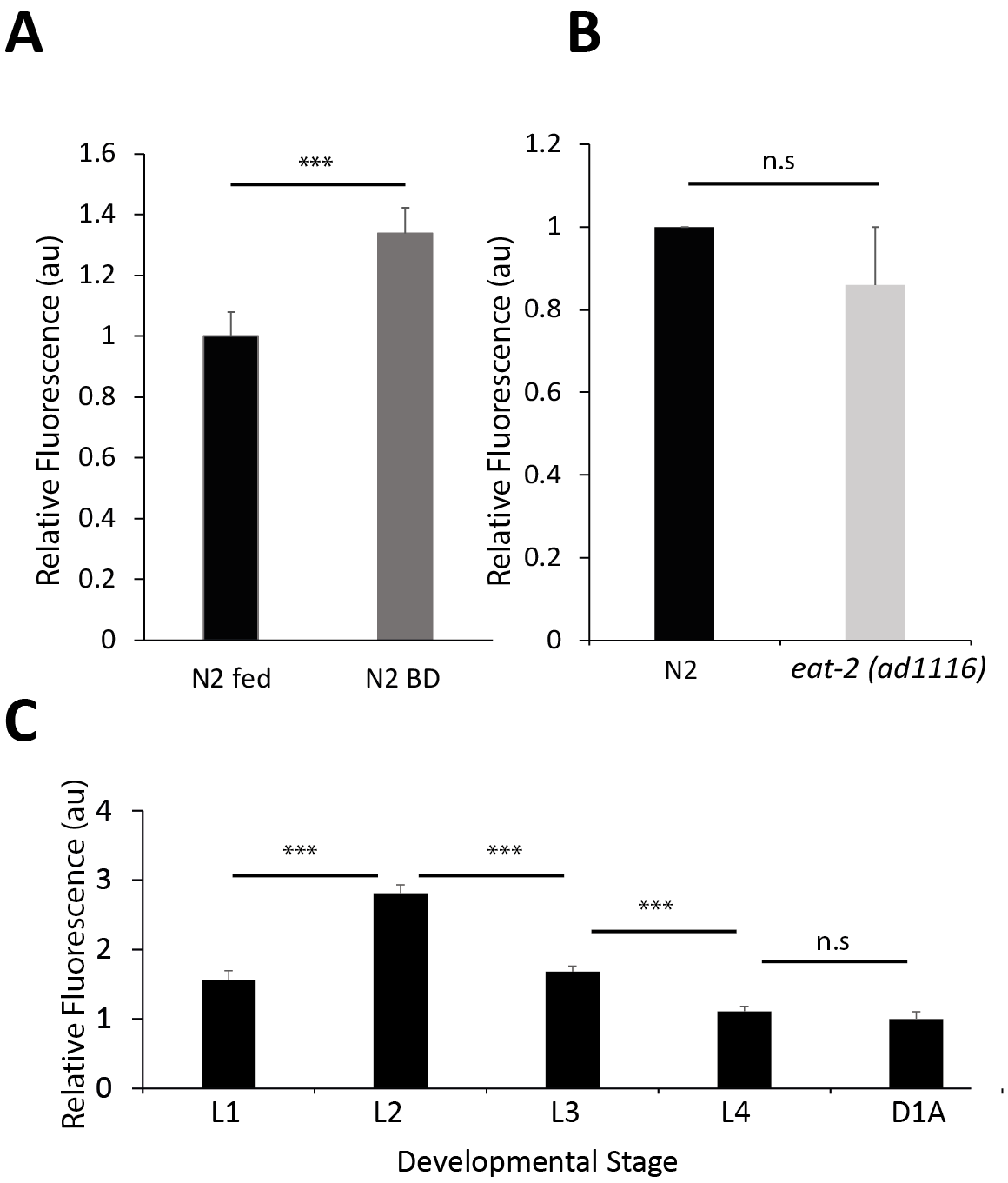

Figure 3.2: Expression of SKN-1B::GFP is modulated by bacterial deprivation and developmental stage. A: 16h BD increases SKN-1B::GFP fluorescence. (5 trials of same result, representative trial shown, students t test p=0.0047,n: fed = 32 neurons, BD = 32 neurons). **B:** There is no difference in SKN-1B::GFP between *eat-2(ad1116)* and N2 animals. Pooled data shown (individual trials N=4, of which 2 n.s., 1 *eat-2* up (p=0.03) and 1 *eat-2* down(p=0.001) by students t test). **C:** Expression of SKN-1B::GFP throughout larval development - expression peaks at L2. (N=3, representative data shown, all sequential e.g. L1-L2 differences >0.05 apart from L4-D1A, students t test). n for L1= 53, L2 = 72, L3 = 75, L4 = 37, D1A = 36. See methods 2.5.1 for procedure.

It is important to note that there is a clear difference between dietary restriction and the chemosensory perception of a lack of food in the area. *skn-1b* is required for dietary restriction longevity in at least one protocol, but the localisation to *skn-1b* to a specifically chemosensory neuron pair provides circumstantial evidence that *skn-1b* is responding to chemosensory signals. The *eat-2(ad1116)* mutation is said to be a model of dietary restriction due to defective pharyngeal pumping in these animals and is widely used to study DR in *C. elegans* (Lakowski and Hekimi, 1998). Since the source of *eat-2(ad1116)* longevity is defective pharyngeal pumping, mutant animals are not subjected to bacterial dilution as the abundance of food is not the limiting factor in food intake. When the *Pskn-1b::skn-1b::gfp* transgene was crossed into an *eat-2(ad1116)* background there was not a significant difference in fluorescence levels between *eat-2(ad1116)* and N2 animals (Figure 3.2 B). This is a particularly interesting result since it is compatible with a model in which *skn-1b* is responding to the external abundance of food rather than internal energy balance. However, given that different DR protocols based upon some form of bacterial dilution seem to activate different pathways to achieve longevity it is also possible that *eat-2* and BD differ in their requirement for *skn-1b* as a function of differences in their effects unrelated to chemosensation. We did not test whether subjecting *eat*-2 animals to BD could induce *skn-1b::GFP* which would allow more solid conclusion to be drawn. As noted in the discussion however, there remains some controversy about the validity of *eat-2* mutants as DR models and thus our hypothesis would benefit from further testing.

3.3. Expression of SKN-1B::GFP peaks at larval stage 2

As part of the characterisation of *skn-1b* expression patterns, it was decided to investigate how *skn-1b* is temporally regulated throughout development to adulthood. Other *skn-1*

isoforms are known to have both crucial developmental roles and an important role in adult organisms (see intro). Likewise, we wondered whether *skn-1b* is likely to be expressed during development, adulthood or both. Therefore an assay was performed where *WuEx217[Pskn-1b::skn-1b::GFP]* expression was quantified by measuring SKN-1B::GFP levels in each larval stage during development through to day 1 adult animals. Loosely synchronous populations were generated by a timed egg laying period of 5 hours, and animals from the resulting population were visually identified as L1, L2, L3 or L4 over the next 48 hours and imaged as described previously. Day 1 adult animals were obtained by placing synchronous L4 animals onto a new NGM plate and imaging after 16h. The resulting quantitative fluorescence data shows *skn-1b* expression is maintained throughout development but peaks in L2. This is in fitting with the known role of *skn-1b* in the DR response of adult worms, however the clear peak at L2 is strongly suggestive of a further role for *skn-1b* in organismal development. Since the *skn-1b (tm4241)* strain is viable, this developmental role seems likely to be non-essential to viability although other processes could be impacted. One hypothesis would be that *skn-1b* is involved in the decision to enter dauer since the L2 stage is when this decision is made and ASI neurons are involved in the secretion of signalling molecules which regulate dauer, notably *daf-28* and *daf-7.* We therefore tested whether *skn-1b(tm4241)* animals are predisposed to dauer entry at 25°C as many *daf-2* mutants are, however, we found no evidence that this is the case (Table S1). Thus, it is likely that *skn-1b* is involved in a non-dauer developmental process during or around the L2 stage of development, and we have not yet been able to identify this process.

3.4. SKN-1B::GFP is additionally expressed in ADL neurons in response to bacterial deprivation

As previously discussed, we examined the effect of 16h bacterial deprivation on *skn-1b* expression (Figure 3.2 A). While conducting this assay, we observed an additional interesting phenotype – SKN-1B::GFP seemed to be expressed in two additional neurons in BD conditions. However, this was not a fully penetrant phenotype and animals in each group could be identified with varying numbers of SKN-1B::GFP containing neurons. We therefore decided to quantify this phenomenon by scoring animals based upon the number of GFP expressing neurons visible in both bacteria deprived and fed conditions. Animals were picked at L4 stage and placed onto either seeded or unseeded NGM plates for 16-20 hours prior to imaging. Figure 3.3 A shows the resulting distribution for animals in unstarved conditions on a normal OP50 food source. The majority of animals under these conditions express *skn-1b* in the previously identified two ASI neurons. However, in figure 3.3 B it can be seen that under bacterial deprivation the majority of animals now visibly express *skn-1b* in 4 neurons, with two of these being adjacent to and anterior to the ASI neurons. In order to identify these neurons a dye filling assay was performed. N2 *[Pskn-1b::skn-1b::gfp]* animals were subjected to 16 hours of BD as previously, and subsequently incubated in 1:1000 DiI in M9 for 1.5 hours, transferred to unseeded NGM plates and imaged immediately. By colocalization of DiI and SKN-1B::GFP it was possible to identify these neurons as the ADL neurons (Figure 3.3 C). This may provide important insights about *skn-1b* function (see discussion). In a small number of animals we detected up to 6 neurons with some GFP expression in a neuron pair more anterior than ASI or ADL, however due to the low penetrance of this effect we did not further investigate these neurons.

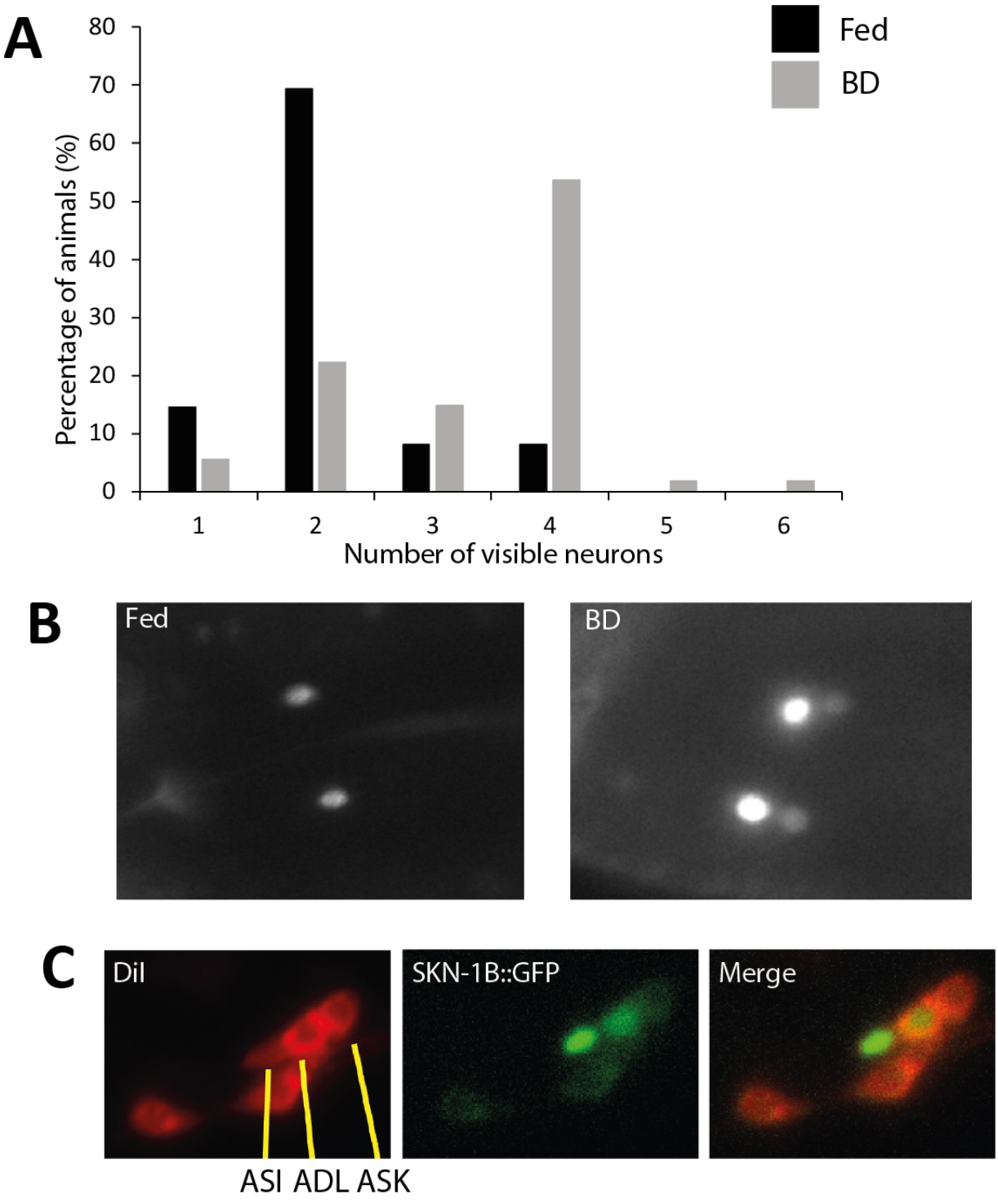

Figure 3.3: SKN-1B is expressed in ADL neurons in response to BD A: SKN-1B::GFP expression patterns in fed and starved conditions. N=3, pooled data (fed = 62 animals, BD = 54 animals). **B:** Representative images of starved and unstarved N2 [*Pskn-1b::skn-1b::GFP]* animals. **C:** DiI visualisation of amphid neurons, SKN-1B::GFP and colocalization.

Since *skn-1b* is required for DR longevity under some circumstances and we have shown an increase in *skn-1b* expression with our bacterial deprivation assay. Based on this data we can conclude that the withdrawal of food induces a switch in the expression pattern of *skn-1b* resulting in expression in ADL neurons, implicating these neurons in the BD response of *C. elegans* and demonstrating that *skn-1b* is likely to be expressed in ADL neurons as well as ASI neurons as previously reported.

3.5. Investigating the transcription factor activity of *skn-1b*.

The protein encoded by *skn-1b* differs significantly from that encoded by either *skn-1a* or *skn-1c*, which are similar except for the addition of a membrane localisation domain at the N-terminus of SKN-1A. Figure 3.4 A shows a comparison between SKN-1B and SKN-1C structure. SKN-1B lacks all described domains which are required for SKN-1C activation apart from the DNA binding domain, including the highly conserved DIDLID motif. During the initial characterisation of *skn-1* as encoding a transcription factor it was shown that deleting these domains significantly reduces the efficacy of *skn-1c* as a transcription factor (Walker *et al.*, 2000). Since *skn-1b* is lacking these domains and was never directly demonstrated to be capable of initiating transcription, there is reason to doubt whether SKN-1B is actually capable of initiating transcription. A luciferase reporter assay was therefore designed to measure the ability of SKN-1B to drive transcription. A luciferase reporter was created using the Promega PGL3 plasmid with three copies of the *skn-1* consensus target sequence upstream of firefly luciferase, as well as mammalian expression vectors (PCI-neo) containing the *skn-1b* and *skn-1c* cDNA.

These plasmids (and a renilla control to normalise for variation in transfection efficiency or pipetting error/sample handling) were then transiently transfected into HEK293T cells in the combinations shown in figure 3.4B and the resulting cells were processed using the Promega Dual-GLO luciferase kit. This should lead to the expression of SKN-1B or SKN-1C protein in these cells and the luciferase reporter plasmid will express luciferase if the protein activates transcription from the *skn*-1 binding site. Notably, in both cells expressing SKN-1B and SKN-1C increasing the amount of the expression vector added produces a dose dependent increase in fluorescence. There was no significant difference in luciferase fluorescence between SKN-1B and SKN-1C expressing cells the same expression vector concentrations, however in the absence of direct confirmation that expression levels are similar in these cells caution is required in interpreting relative activity. While not entirely unexpected, this result suggests that *skn-1b* is likely to function as an active transcription factor. One other possible confounding factor in this assay is that since untransformed PCI-neo was used to normalise the total amount of DNA transfected, in samples with less expression vector added (such as the lower concentrations of SKN-1B and SKN-1C expression vectors) that there could be a promoter dilution effect occurring where additional copies of the promoter expressing these constructs are now present and could artificially reduce expression of these constructs. Furthermore, a control with PGL3 without a SKN-1 binding site and the expression vectors would be useful to demonstrate that a functional binding site is required.

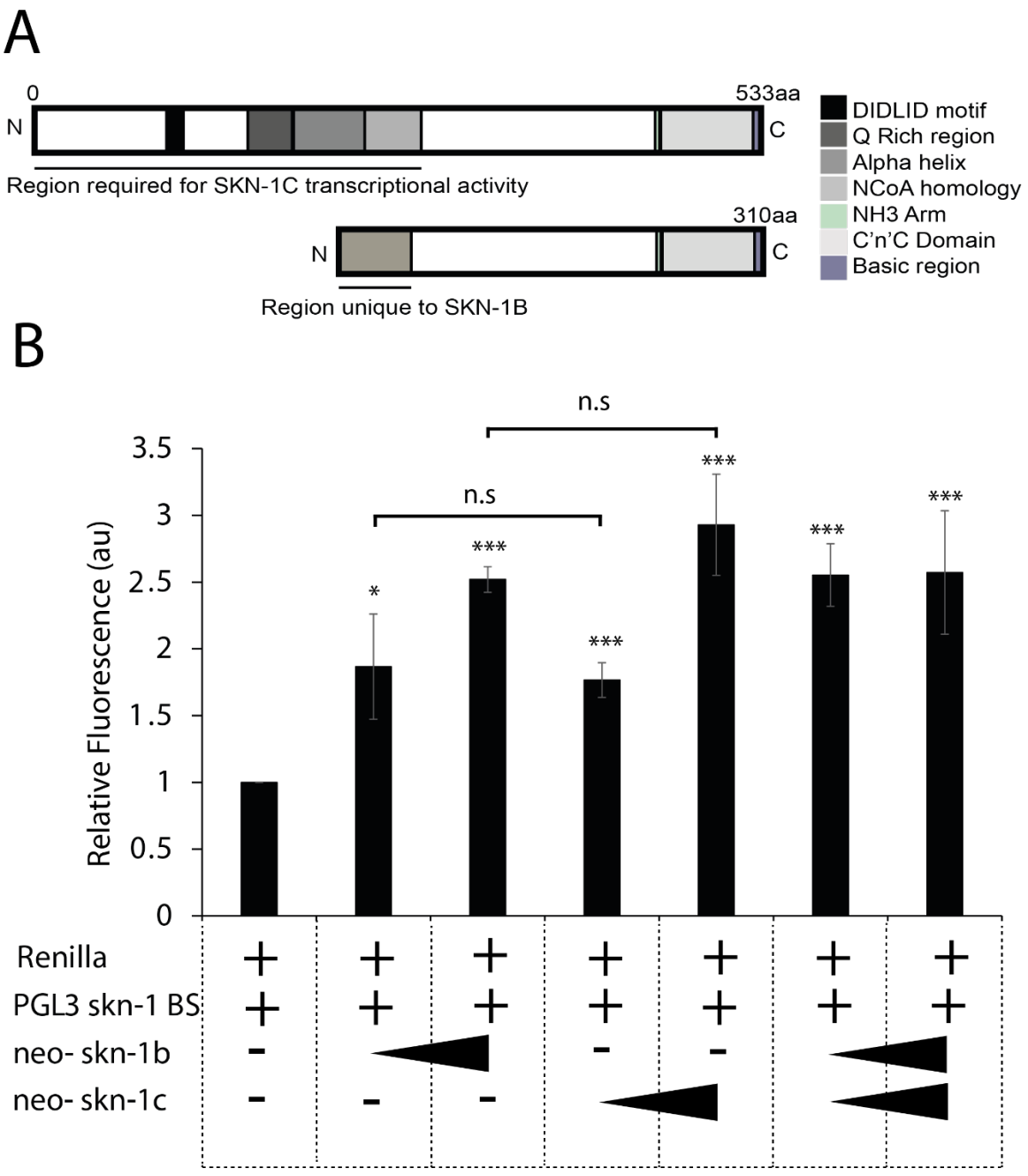

Figure 3.4: SKN-1B is capable of initiating transcription *in vivo*. A: A comparison between SKN-1C and SKN-1B. Notably no functional domains aside from the DNA binding domain are conserved in SKN-1B from SKN-1C. **B:** Relative fluorescence of HEK293T cells transiently transfected with a *skn-1* reporter plasmid (PGL-3 *skn-1 binding site*) and an expression vector (PCI-NEO). Dose increases in PCI-NEO are from 500ng to 2000ng of plasmid. N=3, data represents average fold difference from 3-5 trials. Significance relative to control unless otherwise indicated.

3.6. The mutation *tm4241* specifically knocks out *skn-1* isoform b.

One important aspect of this study is the use of the strain GA1058, which contains a the *skn-1b(tm4241)* mutation, an 294bp deletion of the sole unique exon in *skn-1b* which is not conserved in *skn-1a, c* or *d* (Figure 3.5A). By using this strain, it is possible to separate phenotypes associated with *skn-1b* from those of more studied *skn-1* isoforms. However, before conclusions could be drawn about *skn-1b* function from these animals it was clearly necessary to confirm the presence of the deletion and the lack of corresponding mRNA. The presence of the *skn-1b(tm4241)* mutation was confirmed by a three primer PCR strategy where an internal forwards primer is deleted by the *tm4241* deletion, giving a 400bp PCR product in the wild type and an 913bp product in animals carrying the *tm4241* deletion (Figure 3.5B, see methods table 2.2) While the *tm4241* deletion falls in an intronic region of other *skn-1* isoforms, we sought to exclude the possibility that proper transcription of *skn-1a/c* is somehow constrained by the presence of this mutation. We therefore examined levels of both *skn-1b* mRNA and levels of mRNAs corresponding to other *skn-1* isoform by RT-qPCR in both N2 and *skn-1b(tm4241)* animals. Animals were synchronised by isolating embryos using via a bleach egg prep as discussed in the methods section, and RNA was harvested from them at day 1 of adulthood. We then performed an RT-qPCR assay using primers specific for; *skn-1b, skn-1a, skn-1a/c* and all *skn-1* isoforms (Figure 3.5C). This clearly showed that there is almost no *skn-1b* RNA present in *skn-1b(tm4241)* compared to wild type, while levels of the other tested *skn-1* isoforms are not significantly different from those of *skn-1b(tm4241)*. From this we can conclude that the knockdown of *skn-1b* is both effective and specific to isoform b in this strain. One notable caveat here is that the lack of

any unique features on the predicted (but not observed *in vivo*) *skn-1d* isoform mean that RT-qPCR analysis is not possible and therefore the effect of *tm4241* on *skn-1d* is unknown, if indeed *skn-1d* is present *in vivo* at all. To further illustrate the lack of *skn-1b*, we ran out the qPCR products from N2 and *skn-1b(tm4241)* animals using *skn-1b* specific primers on a 2% agarose gel (figure 3.5 D) which demonstrates that there is no band present at the expected 115bp.

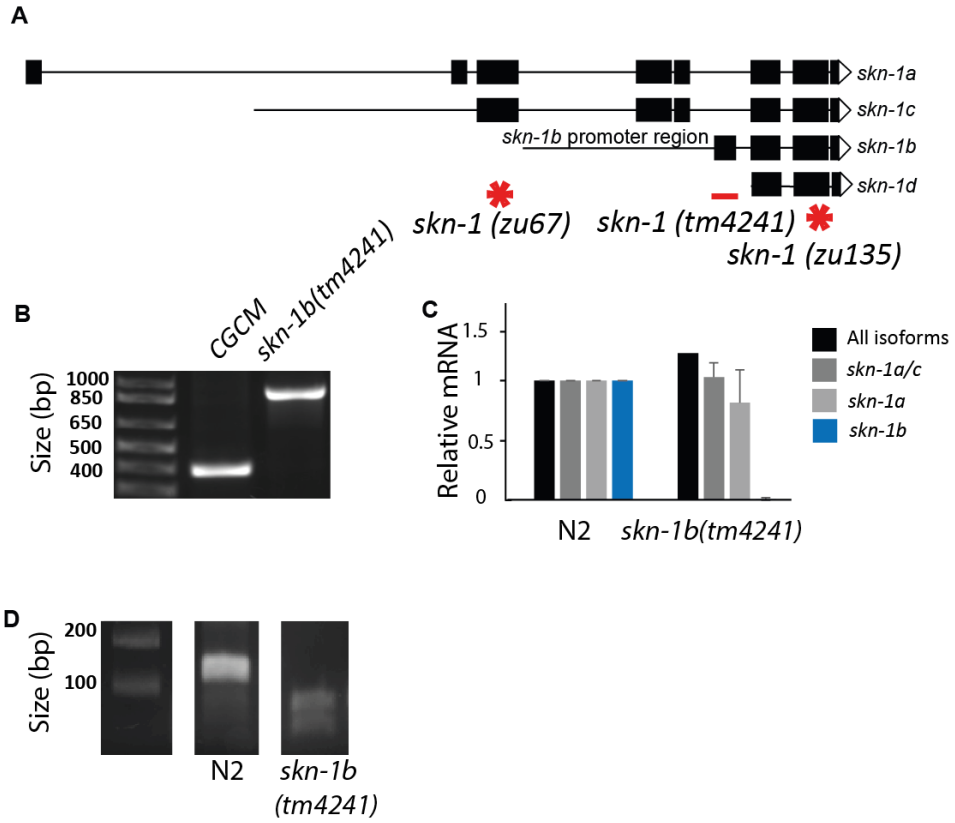

Figure 3.5: Confirmation of *skn-1b (tm4241)* mutation: A: Exon map of *skn-1* gene, showing the deleted *skn-1b* unique exon in *skn-1b (tm4241)* as well as *zu67* and *zu135* mutations. **B:** Confirmation of *skn-1b(tm4241)* mutation by three primer PCR strategy. **C:** RT-qPCR data of *skn*-1 isoforms (N2 cDNA vs *skn-1b* cDNA with *skn-1b* primers; p=0.003. All other primer-sets did not significantly vary from N2. 6 biological replicates per condition). **D:** A region of skn-1b specific exon amplified from N2 and *skn-1b* mutant worm cDNA.

3.7. Loss of *skn-1b* has no effect on fecundity.

Having confirmed our specific knockdown, we examined *skn-1b (tm4241)* for phenotypes resulting from its loss. One important finding with regards to *skn-1b* mutation is that homozygote *skn1b(tm4241)* animals are viable and can be easily maintained in the lab without special procedures (data not shown/not quantified). This contrasts starkly with mutations in other *skn-1* isoforms – notably with the commonly used *zu67* and *zu135* mutations which are indel mutations which result in loss of function of either *skn-1a* and *skn-1c* in *skn-1(zu67)*, or of all isoforms in *skn-1(zu135)* via the introduction of premature stop codons (Fig 3.5A) (Bowerman, Eaton and Priess, 1992; Bishop and Guarente, 2007a). Both of these mutations are embryonic lethal due to important roles of *skn-1* in early development (see introduction) and thus genetic balancers must be used to maintain these strains (Bowerman, Eaton and Priess, 1992). This is clearly not the case with *skn-1b*, suggesting that since *skn-1b* animals can develop normally, this developmental role is not conserved between *skn-1* isoforms. Mutations which inhibit the activity of growth processes such as translation often also reduce brood size (Hansen *et al.*, 2007). One such process is insulin signalling, and downregulation of this process generally reduces brood size or delays egg laying (Gems *et al.*, 1998). This extends to some *skn-1* mutant strains which are reported to have reduced brood size (Tang, Dodd, & Choe, 2015) or delayed onset of egg-laying (J. M. a Tullet et al., 2009). Since *skn-1c* is a key component of insulin signalling and *skn-1b* in ASI neurons is required for DR which regulates numerous growth processes we reasoned it would be informative to check for differences in brood size resulting from *skn-1b* mutation. We investigated the brood size of N2 and *skn-1b (tm4241)* animals with regard to both the total brood size of each and the timing of egg laying. Animals were picked at L4

stage each onto individual NGM plates and moved every day. Resulting offspring on each plate were counted, giving both total brood sizes and counts for each day from L4 (Figure 3.6 A &B). The results show no difference in brood size between control and *skn-1b(tm4241)* animals either in their total brood size, or in the days on which the offspring are laid. This supports the observation that *skn-1b(tm4241)* is viable to the same extent as controls, since offspring were counted after hatching and no difference in total brood was observed. It is also a notable difference from other *skn-1* mutant alleles that *skn-1b* does not influence brood size and is not required for embryonic viability (Tang, Dodd and Choe, 2016), suggesting divergent function of *skn-1b.*

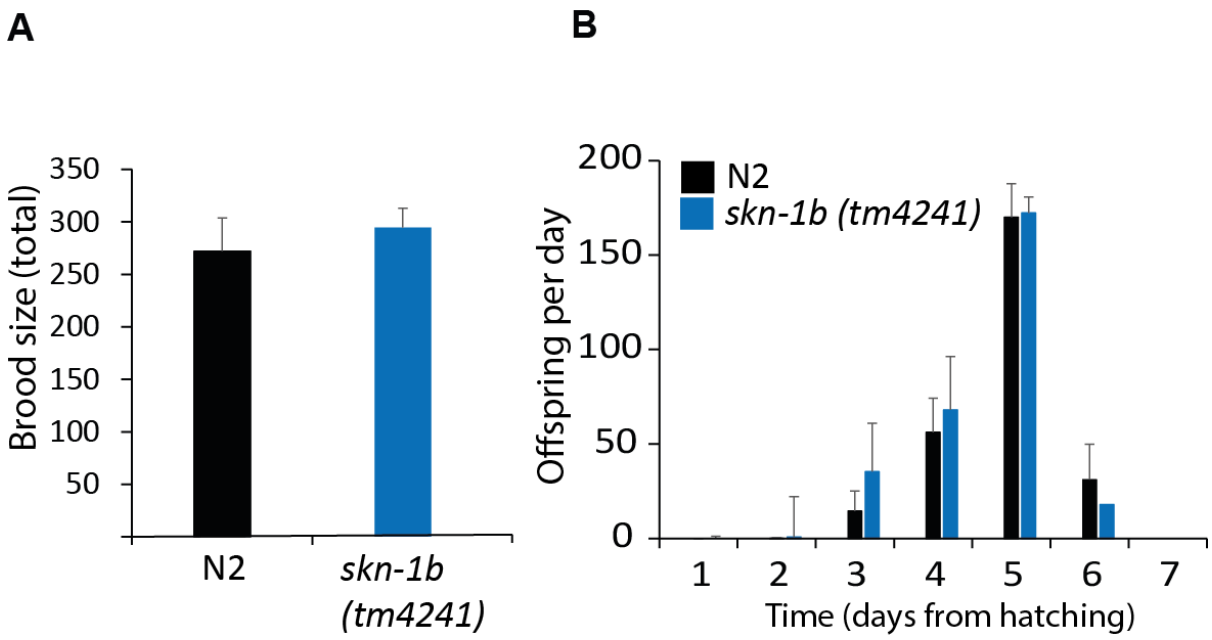

Figure 3.6: Brood size is unaffected by *skn-1b(tm4241)*: A: Total brood size of N2 and *skn-1b(tm4241).* There is no significant difference in total brood size between N2 and *skn-1b(tm4241)* (p=0.15 students t-test, N=26/20 over three trials) **B:** There is no trend to earlier or later egg laying in *skn-1b* animals.

3.8. *skn-1b(tm4241)* does not contribute to wild type *C. elegans* lifespan.

Loss of function mutations affecting either all *skn-1* isoforms or *skn-1a* and *skn-1c* are known to reduce normal lifespan relative to wild-type *C. elegans* (An, 2003), and proper *skn-1* function is required for additional longevity downstream of various longevity pathways (Blackwell *et al.*, 2015). We therefore set out to investigate the effect of specific *skn-1b* knockdown in our *skn-1b(tm4241)* animals on normal lifespan. In this assay animals were picked onto NGM plates at the L4 stage with OP50 *E.coli* as a food source. These animals were scored three times per week as alive or dead based upon responsiveness to light touch. At 20°C there was no significant difference in the normal lifespan of *skn-1b(tm4241)* animals compared to control animals (Figure 3.7). Because *skn-1b* is not required for normal longevity but other isoforms are, this implies a difference in function from better studied isoforms, with the mechanism by which other isoforms are required for normal lifespan not being conserved in *skn-1b*. There are numerous candidates for these processes since *skn-1* has many known target processes including stress response, detoxification, UPR, proper production of collagens and others (Blackwell *et al.*, 2015).

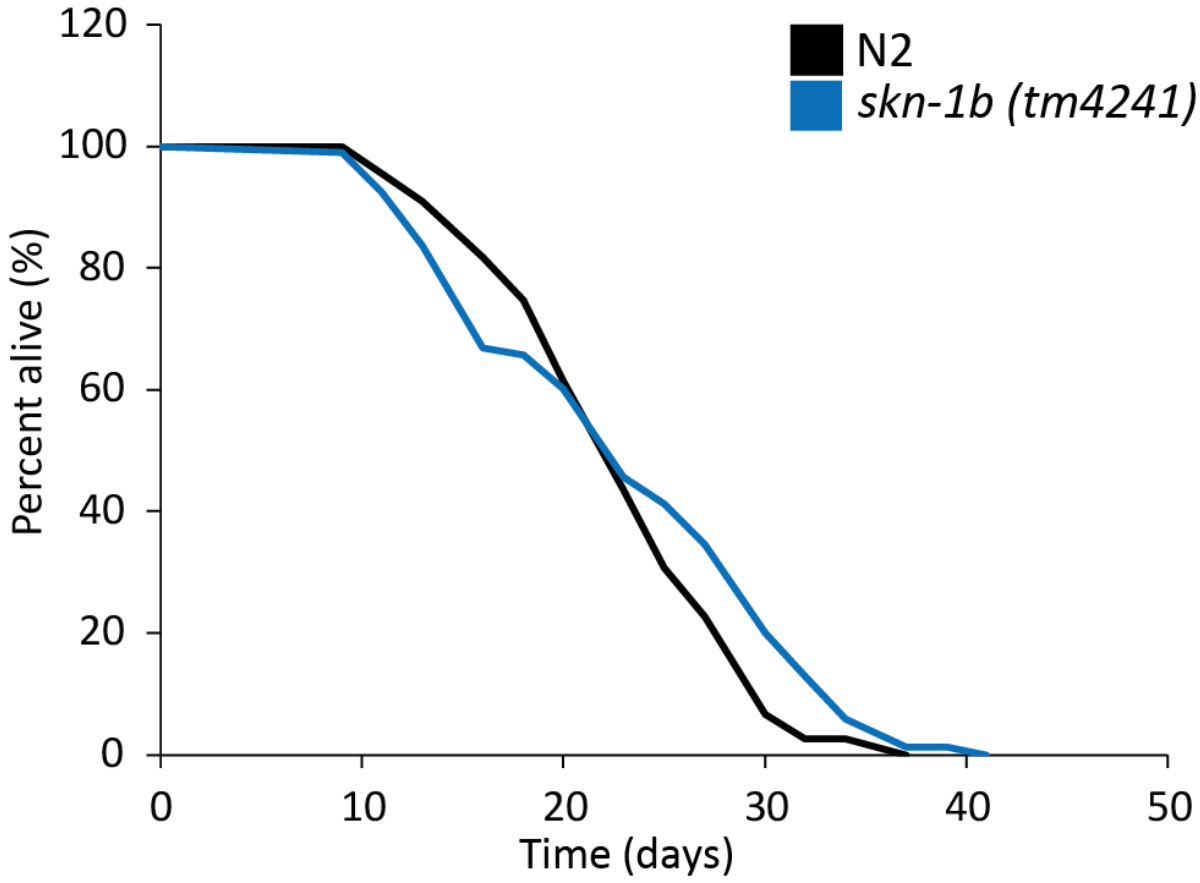

Figure 3.7: *skn-1b(tm4241)* has no effect on wildtype lifespan: A: Lifespan chart showing representative trials of *skn-1b(tm4241)* lifespan. N=3 (approx. 100 animals per condition), p=0.17; significance by log-rank test. 10mM FuDR was used in standard NGM plates.

3.9. *skn-1b* is induced by, but not required for resistance to oxidative stress

Loss of *skn-1c* sensitises animals to sodium arsenite induced oxidative stress (Blackwell *et al.*, 2015). These effects appear separable from the pro-longevity effects of *skn-1c* (Tullet *et al.*, 2017). In order to test for a possible interaction between *skn-1b* and oxidative stress, the induction of SKN-1B::GFP by our *Pskn-1b::skn-1b::GFP* transgene by 2.5mM sodium arsenite treatment was examined. Animals were synchronised to day 1 adulthood by picking L4s the day before. Animals were then placed into a liquid solution of either M9 + OP50 or 2.5mM sodium arsenite (in m9) and OP50 for one hour then moved to NGM plates for approximately 10 minutes while the liquid dried. In animals treated with sodium arsenite there was a strong induction of SKN-1B::GFP, suggesting a transcriptional upregulation of *skn-1b* is part of the animals response to oxidative stress (figure 3.8 A). Since SKN-1B::GFP is increased by oxidative stress, we next sought to test whether the whole worm is sensitised to oxidative stress by loss of *skn-1b.* To this end a stress assay was performed in which day 1 adult animals were placed into 2.5mM sodium arsenite and scored at regular intervals (8h/16hr timepoints) (Figure 3.8 B). There was no difference in survival between N2 and *skn-1b* animals. This demonstrates that *skn-1b* is not required for survival in response to oxidative stress challenge in *C. elegans*, in contrast to *skn-1c*. One possible reason for this is that *skn-1b* and *skn-1c* may actually be responding to oxidative stress in a similar way in both tissues, but since *skn-1b* is only present in ASI neurons in normal conditions and ASI ablated animals are do not die (Alcedo and Kenyon, 2004; Bishop and Guarente, 2007b), even serious unanswered oxidative stress in these cells may not be fatal for the animals.

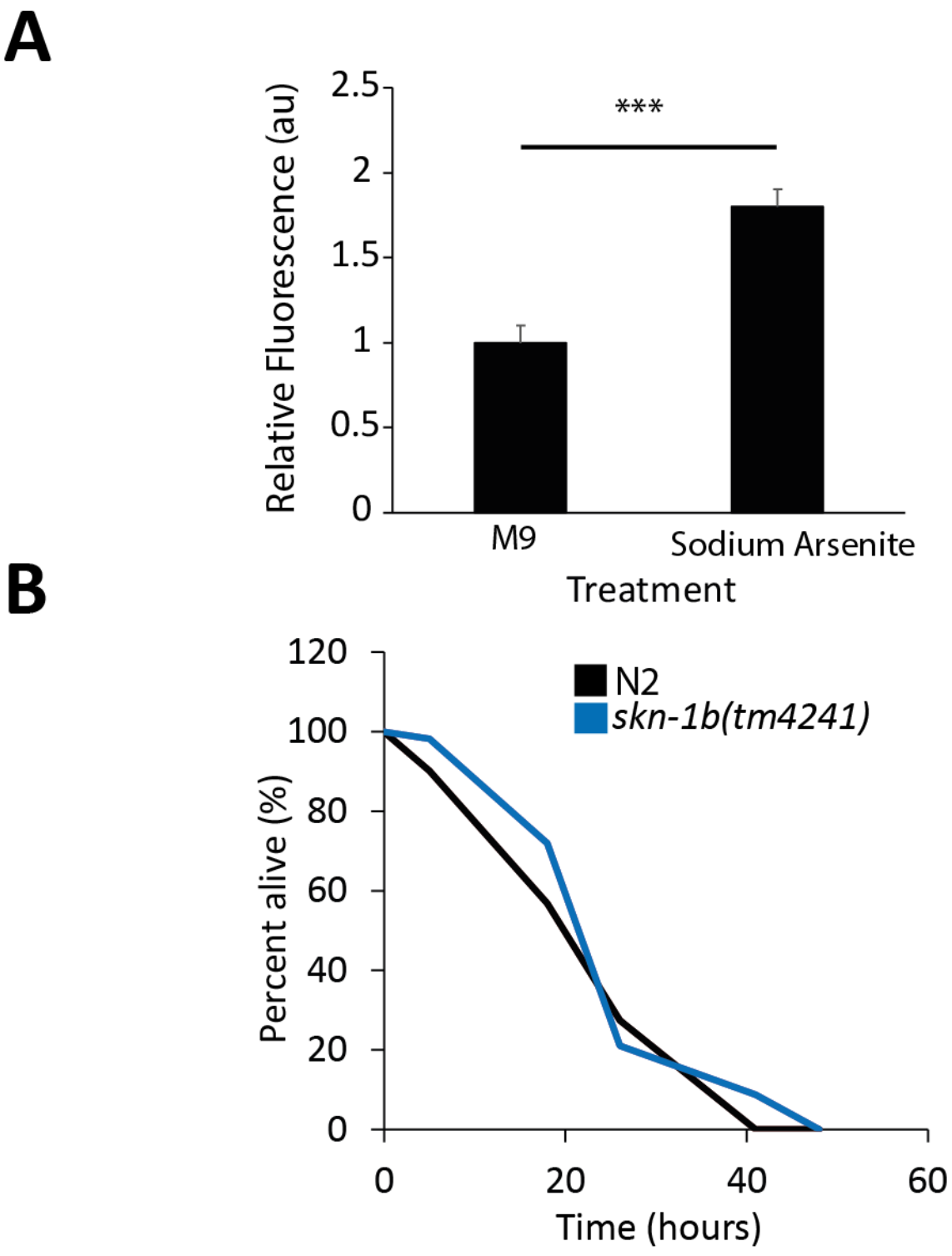

Figure 3.8: SKN-1B::GFP is induced by oxidative stress, but *skn-1b* mutation does not sensitise these animals to oxidative stress systemically. A: SKN-1B::GFP levels in M9 or 5mM sodium arsenite treated day 1 adults. $P=1x10^{-6}$ by students t-test. N= 3, representative trial shown (Control n = 29, Arsenite n = 27). **B:** Stress assay where survival of N2 and *skn-1b(tm4241)* animals was recorded in 2.5mM sodium arsenite.

Previous work on this gene performed by Jennifer Tullet shows that, in normal conditions, the expression of canonical *skn-1c* target *gst-4* (see 1.6.5, (Oliveira *et al.*, 2009)) does not change in a *skn-1b* background. However, we did not test whether this remains the case conditions of oxidative stress. Since *skn-1b* levels are increased by sodium arsenite, it could still be the case that under stressed conditions signalling from ASIs to intestinal insulin signalling is changed and we therefore have not rule out an indirect role of *skn-1b* in intestinal oxidative stress resistance.

Chapter 3 Discussion

In Bishop & Guarente (2007) the authors predicted that *skn-1b* can be expressed from its own promoter based on 5` RACE. In figure 3.1 we demonstrate that this is indeed the case – our *Pskn-1b::skn-1b::GFP* transgene produces strong fluorescence in ASI neurons. This strongly suggests differential regulation of *skn-1b* from other studied isoforms which rely on the *skn-1* operon promoter. This in an important finding since it provides a foundational rationale for many other findings here and in chapter four – it explains in part the capacity of *skn-1b* to respond to various stimuli independently of other isoforms of *skn-1*.

Figure 3.2 shows that *skn-1b* expression in ASI neurons responds to bacterial deprivation but not *eat-2(ad1116)*. The fact that *skn-1b* responds to the withdrawal of bacteria but not genetic induction of DR presents the possibility that *skn-1b* is responding to chemosensory information about food availability rather than reduced calorie intake. ASI neurons are chemosensory neurons which express 99 G-protein coupled receptors (Vidal *et al.*, 2018) and thus can likely internalise complex information about the external environment. The requirement of *skn-1b* for DR and its responsiveness to external food availability, but apparent lack of a response to DR induced genetically in a food-rich environment may suggest a role for *skn-1b* in integrating information about external food levels into the decision to pursue the DR related longevity response. Further work could be done to investigate this, with the logical follow on experiment being to test whether *skn-1b::GFP* is induced by the withdrawal of food from *eat-2(ad1116)* animals. Without knowing the result of this experiment it remains an open question whether the difference in *skn-1b::GFP* induction between BD and *eat-2* is a consequence of food levels or pathway activation

differences between the two DR protocols more generally. The link between chemosensation and DR is supported by data from the literature which shows that food-deprived *C. elegans* lifespan (which is extended relative to fed controls) is shortened when these animals are treated with the supernatant of *E.coli* cultures (Artan *et al.*, 2016). The data in this chapter suggests that *skn-1b* may be an important link between food derived sensory cues and longevity. Another possible explanation for this difference between BD and *eat-2(ad1116)* revolves around the viability of *eat-2* mutants as a model for DR at all. A recent paper from the Gems lab makes the suggestion that *eat-2* longevity results from reduced mechanical damage to the pharynx and less resulting bacterial invasion via the damaged pharynx (Zhao *et al.*, 2017). These findings seem to demonstrate that *eat-2* mutants are not a model of DR at all, calling into question many published findings based upon the use of these strains. Our data can be read as supporting this conclusion since *skn-1* is not induced in these mutants but is with DR and BD treatment. An alternative interpretation of our data, then, is simply that no DR is present in *eat-2(ad1116).* Supporting the finding of SKN-1B::GFP upregulation in response to BD, in figure 3.3 we demonstrate that SKN-1B::GFP expression patterns are changed in response to these conditions, with SKN-1B::GFP visible in ADL neurons. There is no published link (yet) between ADL neurons and dietary restriction longevity and ADL neurons are generally less well studied than the ASI neurons. The rescue of *skn-1b* in ASI neurons is capable of sensitising *skn*-1 mutant animals to DR (Bishop and Guarente, 2007b), however if *skn-1b* in ASI neurons is regulating signals which trigger ADL neurons to express *skn-1b* then rescuing *skn-1b* in ASIs would indirectly rescue ADL expression. This is clearly an interesting avenue of inquiry which is being followed up in our lab. One notable piece of corroborating data comes from Nikolaos Tataridas-Pallas in our lab who has demonstrated that chemotaxis to NaCl in *skn-*

1b(tm4241) animals is severely impaired. One known role of ADL neurons is in NaCl chemotaxis, supporting the notion that *skn-1b* is active in these cells. It has also been reported in a screen for G-protein coupled receptors (GPCRs) which regulate lifespan that *gpa-11* in ASH and ADL neurons regulates lifespan in a (probably) *daf-2* independent manner – thus signalling from ASH and/ or ADL neurons regulates longevity more generally, suggesting a role for the ADL in the DR response mediated by *skn-1b* is at least possible (Lans and Jansen, 2007). It is also interesting to note that information about external NaCl levels may give information about external food levels or quality, although this needs to be explored further before conclusions can be drawn. Furthermore, data recently put online (uncorrected proof) from the Zhang lab shows that killing of ASI and ADL neurons has a similar effect on food leaving behaviour of the animals subject to this treatment (Harris *et al.*, 2019) to those observed in our lab in *skn-1b* animals.

In figure 3.5 we also report that *skn-1b* expression peaks in the L2 stage and is expressed at higher levels in larvae compare to young adults. Since *skn-1b* is not required for larval viability (Figure 3.6) to any significant extent, we can rule out a requirement in the same essential processes that *skn-1a/c* are involved in during early development. The increased expression during development shown in figure 3.3 seems to suggest a role for *skn-1b* in developmental processes which have not yet been identified. We have examined the dauer entry of *skn-1b* animals in figure S1 but do not see any difference in this process, which is one major developmental decision which occurs in L2. We therefore hypothesise that *skn-1b* is likely involved in another developmental process around the L2 stage, perhaps of specifically ASI neurons but this demands further investigation. Data generated in our lab by other researchers indicates that *skn-1b* mutants display a series of chemosensory deficits and we are currently investigating whether ASI neuron development is involved in this.

Figure 3.9 summarises the results of our investigations into the expression patterns of SKN-1B::GFP.

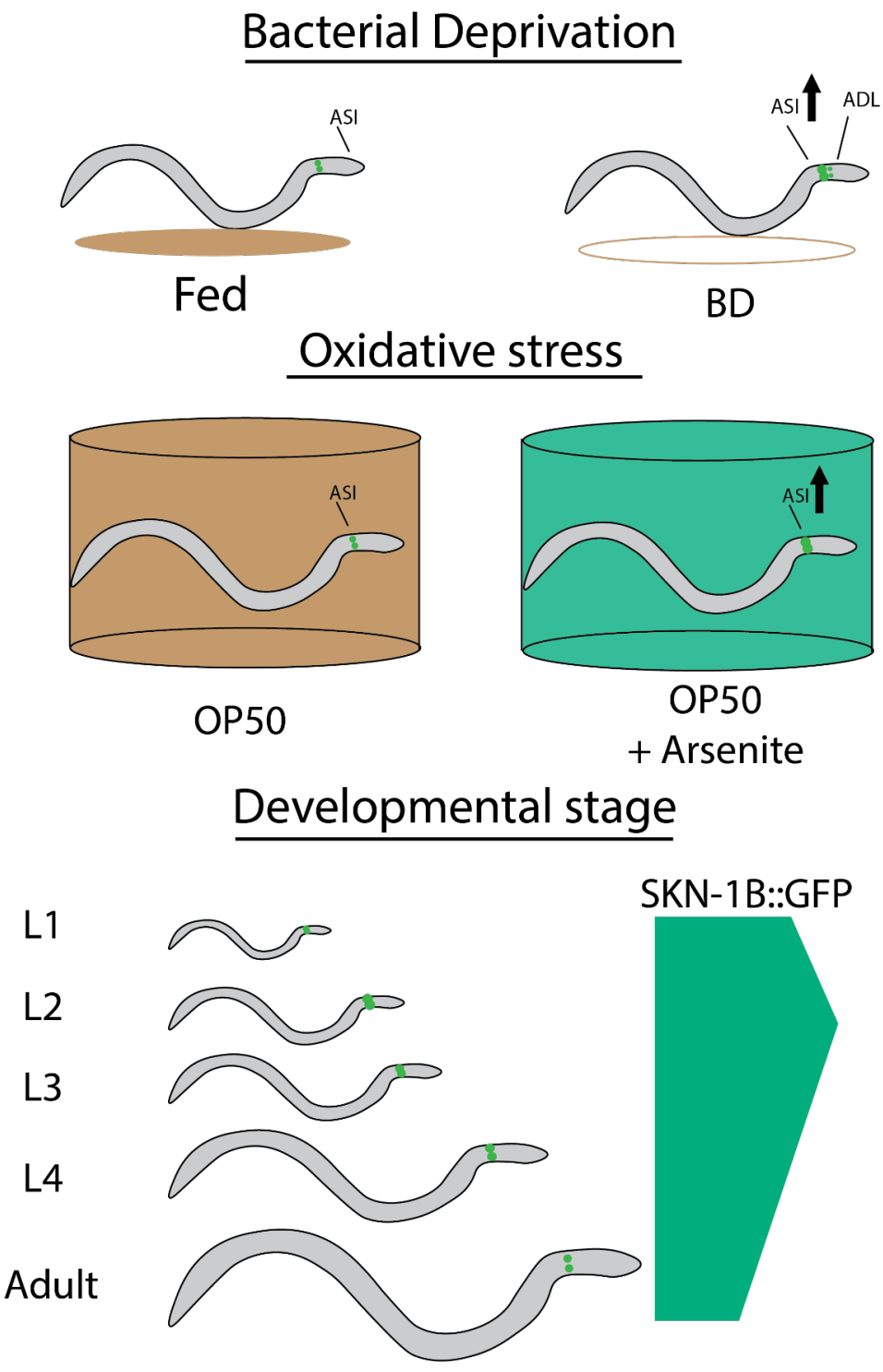

Figure 3.9: Model of expression. Model of *pskn-1b::skn-1b::GFP* expression based upon the results show in figures 3.1, 3.2, 3.3 & 3.8. This suggests that the expression pattern of endogenous *skn-1b* may be similar.

There has been a degree of uncertainty about the functionality of SKN-1B as a transcription factor. It has been correctly noted in the literature (e.g. (Tang and Choe, 2015)) that *skn-1b* is missing key functional domains which are essential for the proper activity of *skn-1c* (Walker *et al.*, 2000) including the DIDLID motif which all the way through to mammalian NRFs which differ significantly from SKN-1 in numerous other ways terms of their interaction with DNA, formation of a dimer and other interactions (Blackwell *et al.*, 2015). Figure 3.4 goes some way to resolving this, providing circumstantial evidence that SKN-1B protein is capable of initiating transcription from the canonical *skn-1* binding site in a luciferase assay, albeit with several limitations to the experimental design which could prove confounding as discussed in section 3.5. This supports a model where SKN-1B enacts a specific transcriptional program in response to certain stimuli, notably bacterial deprivation and some DR protocols in order to adapt to changes circumstances. Since *skn-1b* is expressed in only 4 neurons under these conditions, we reason that *skn-1b* is achieving its effect on the whole worm (by permitting DR longevity for example) via cell non-autonomous mechanisms.

Figure 3.5 shows that *skn-1b (tm4241)* animals specifically lack the *skn-1b* isoform. This gives us access to a unique tool in studying *skn-1b* since previous studies have relied on strains lacking all or several *skn-1* isoforms combined with transgenes which rescue *skn-1b* or neuronal *skn-1* expression (Bishop and Guarente, 2007b; Vanduyn *et al.*, 2010; Wilson *et al.*, 2017). There is an important qualification to add here however – *skn-1d* is an isoform predicted by the presence of an SL1 splice site which has not been directly confirmed *in vivo*. Since *skn-1d* does not contain any unique exons it has not been accessible to our methods of monitoring gene expression which have been primer-based strategies such as RT-qPCR. If *skn-1d* is expressed *in vivo* the *tm4241* deletion could influence its expression

particularly if the predicted *skn-1b* promoter drives expression of *skn-1d* as well, a reasonably likely scenario based upon the fact that all *skn-1d* exons are upstream of the predicted *skn-1b* promoter region and the *skn-1b* promoter is the nearest known promoter to it. This failure to exclude *skn-1d* as a potential confounding influence also extends to other published work, notably in the use of *skn-1b* transgenes in rescue experiments (e.g (Bishop and Guarente, 2007b)) due to the fact *skn-1d* was predicted after much of the initial work on *skn-1b* was performed.

Viability, brood size (figure 3.6) and normal lifespan (figure 3.7) are unaffected by loss of *skn-1b*, all of which are severely affected by loss of function the full *skn-1* gene. With regards to viability, *skn-1* is required for the formation of the earliest stages of the digestive system in the EMS blastomere and embryos lacking *skn-1* are not viable (Bowerman, Eaton and Priess, 1992). The *zu67* mutation which causes loss of function in *skn-1a* and *skn-1c* is sufficient to fully recapitulate this, with this mutation being embryonic lethal (Bowerman, Eaton and Priess, 1992). We show here that loss of *skn-1b* does not impact viability and thus that this crucial role in early embryonic development is not conserved in *skn-1b.* Taken together this shows that the developmental role of *skn-1* in mesodermal specification is solely attributable to *skn-1a/c* and not to *skn-1b*. Additionally in normal conditions the transcriptional regulation of the canonical *skn-1* target *gst4* is not changed by loss of *skn-1b*, suggesting that at least in normal conditions proper activity of intestinal *skn-1* isoforms is not contingent on the activity of neuronal *skn-1b* (Figure S3).

When challenged with oxidative stress, N2 animals expressing SKN-1B::GFP from the *skn-1b* promoter upregulate SKN-1B::GFP, but *skn-1b(tm4241)* animals are not short-lived in the same conditions (Figure 3.8). From this we conclude that *skn-1b* is responding to oxidative

stress, but that this response is not necessary for survival. This is not surprising since ASI neurons can be laser ablated (Alcedo and Kenyon, 2004; Bishop and Guarente, 2007b) or tetanus-killed (Chisnell and Kenyon, 2018) and animals do not die. They do, however display phenotypes consistent with loss of ASI specific functions (Harris *et al.*, 2019), so perhaps *skn-1b* is acting to protect these important functions in this context.

The data shown here establishes the activity of SKN-1B as a transcription factor and suggests that its role is likely distinct from that of SKN-1C. It shows that levels of external food regulate our SKN-1B::GFP transgene, including by inducing its expression in the ADL neurons. In the next chapter these themes are expanded on and we attempt to define pathways in which *skn-1b* sits to achieve this.

Investigation of *skn-1b* mediated signalling from ASI neurons.

In chapter three, we established that *skn-1b* is a functional transcription factor which is highly responsive to bacterial deprivation. Next, we sought to identify mechanisms by which *skn-1b* may be signalling to other parts of the animal in order to achieve the major role it plays in dietary restriction longevity. An important series of findings which informs the work done here is that work performed in our lab by Nikolaos Tataridas-Pallas which demonstrates that loss of *skn-1b* is associated with a range of behavioural phenotypes including a lack of salt chemotaxis and reduced exploratory behaviour (Fig S4, S6). This suggested that skn-1b may be involved in regulating signalling molecules implicated in these processes such as ILPs, TGF-β ligand or other neurotransmitters. My own work on this topic was pursued in parallel and can be divided into 3 main aims.

1. To identify candidate signalling molecules which are regulated directly or indirectly by *skn-1b* and act to communicate with distant tissues.
2. To identify candidate receptors on ASI neurons which regulate *skn-1b* activity.
3. To identify downstream processes which *skn-1b* regulates.

4.1. *Pdaf-28::GFP* expression requires *skn-1b*.

We reasoned that since *skn-1b* is influencing at least one systemic process by its requirement for DR longevity in the worm, there must be a mechanism via which *skn-1b* is able to communicate with other tissues - notably the intestine which is a major site of longevity promoting processes. In an effort to identify likely candidates we cross referenced a list of 23 neuropeptides secreted from ASI neurons (Wormatlas, ASI neurons) with data from the MODENCODE project (Celniker *et al.*, 2009; Niu *et al.*, 2011), checking for *skn-1* binding events reported in the 2kb upstream of the genes encoding these peptides. We identified 3 neuropeptides with reported *skn-1* binding events: *ins-9, nlp-1* and *daf-28* (Table 4.1) occurring at larval stage 2, which is when we previously observed *skn-1b* expression peaks. Our attention was immediately drawn to *daf-28* - it is the closest ILP (Insulin Like Peptide) to human insulin in terms of structure and its loss is associated with downregulated insulin signalling (Li, Kennedy and Ruvkun, 2003). We therefore acquired the strain GR1455 which expresses the *mgIs40 [Pdaf-28::GFP]* transcriptional reporter, and crossed it into our *skn-1b(tm4241)* background. We conducted a quantitative fluorescence microscopy experiment where levels of *Pdaf-28::GFP* were to be measured in an N2 and *skn-1b* background. To our surprise, we observed total suppression of the expression of this reporter in a *skn-1b* background but not N2 background (Figure 4.1.A). In order to confirm the presence of the *mgIs40* reporter we tested the non-fluorescing *skn-1b(tm4241) [Pdaf-28::GFP]* animals for GFP DNA by a two primer strategy. This demonstrated that transgene DNA was indeed present in these animals, implying that the lack of expression in these worms is due to total abrogation of *Pdaf-28* activity (Figure 4.1). Since *daf-28* is an ILP signalling molecule and a *daf-2* ligand, this implies that *skn-1b* is required for *daf-28*

expression, which is known to regulate insulin signalling (Li, Kennedy and Ruvkun, 2003). This was surprising due to the lack of a dauer phenotype at 25 °C (Figure S1) or 27°C (work by Jennifer Tullet, data not shown) in *skn-1b(tm4241)* animals but highly penetrant dauer phenotypes at these temperatures in *daf-28* deletion mutants (Klabonski *et al.*, 2016). We attempted qPCR for *daf-28* mRNA levels to support our transgene data but did not observe a total lack of *daf-28* mRNA, however we were unable to determine if there is a more modest change in mRNA levels due to high levels of internal variability (Figure S2) – see discussion for detail.

INS	BS	NLPs	BS	Misc	BS
INS-1		NLP-1		Daf-28	
INS-3		NLP-5		FLP-2	
INS-4		NLP-6		FLP-10	
INS-6		NLP-7		FLP-21	
INS-7		NLP-9		PDF-1	
INS-9		NLP-14			
INS-22		NLP-18			
INS-26		NLP-24			
INS-32					

Table 4.1: ASI expressed neuropeptides with a MODENCODE SKN-1::GFP CHIP peak in the 2kb region upstream of the transcriptional start site. All shown neuropeptides are expressed in ASI neurons, all of which have a predicted *skn-1* binding event in their promoter region are marked with green. Those with no reported binding event are marked in red.

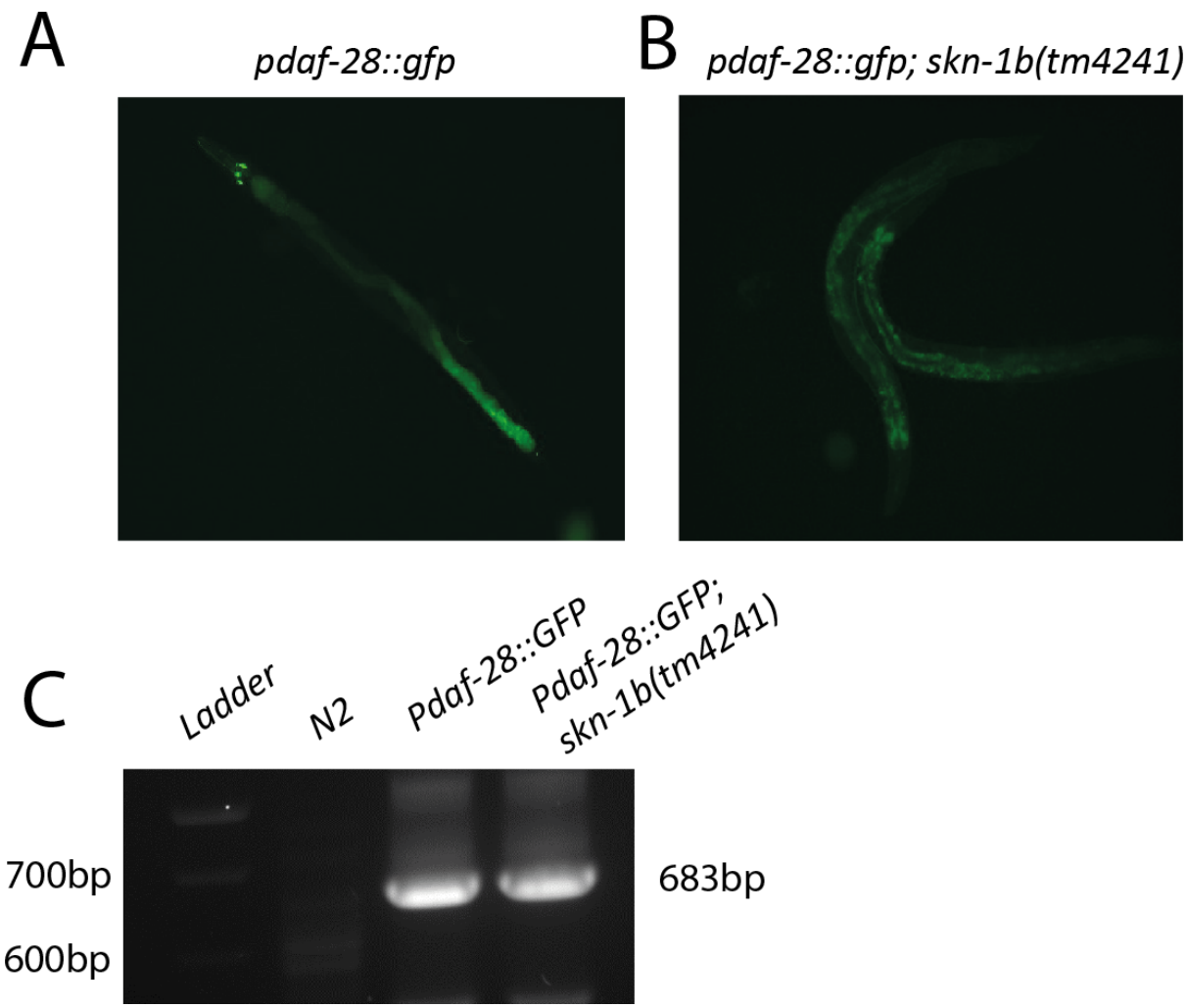

Figure 4.1: *Pdaf-28::GFP* expression is totally abrogated by loss of *skn-1b*. A: Expression patterns of *Pdaf-28::GFP* in a normal N2 genetic background, 100ms exposure B: Expression pattern in a *skn-1b(tm4241)* genetic background, 500ms exposure. All visible fluorescence is autofluorescence confirmed visually using the i3 cube (see 2.6.1). C: PCR for *gfp* in N2, N2 [*Pdaf-28::GFP]*, *skn-1b (tm4241) [Pdaf-28::GFP]*. Primers in table 2.2. Cross performed twice, for microscopy 25 animals per condition were examined.

4.2. *skn-1b* opposes *ins-7* expression in a cell non-autonomous manner.

There are 40 insulin like peptides in the *C. elegans* genome. We had been interested in *ins-7* due to its expression in ASI neurons (and others) and role in the longevity downstream of rIIS previously (Murphy *et al.*, 2003). *ins-7* is not likely to be a direct *skn-1b* target due to the lack of any suggestion of an interaction between *skn-1* and the *ins-7* promoter in the modencode data (table 4.1) (Niu *et al.*, 2011). Our rationale in this case for examining *ins-7* was to explore the possibility of indirect neuronal regulation of *ins-7*. There has been significant interest in *ins-7* in the regulation of *daf-2* signalling and our finding that *daf-28* expression is regulated by *skn-1b* raised the possibility that *skn-1b* may be involved in insulin signalling by regulation of at least one ILP. The *wwEx66 [Pins-7::GFP]* transgene was crossed into a *skn-1b(tm4241)* background. In WT worms this transgene is visible in a number of neurons and we therefore quantified its expression in the most prominent of these - likely the ASK based on published data (Z. Chen *et al.*, 2013) - using the FIJI distro of imageJ (Figure 4.2.C). We found a small but significant increase in *Pins-7::GFP* expression in our pooled data and in one of our trials, however in another two trials we did not detect a difference in neuronal GFP from this transgene. When pooled, these trials show a small but significant difference in neuronal GFP. We also quantified upper intestinal activation of this reporter and observed a much more significant change in this tissue, with *skn-1b(tm4241)* animals displaying a strong increase in *Pins-7::GFP* fluorescence which was reproducible in all three trials and robust (Figure 4.2.D). These data seem to suggest that while there may be small perturbations in neuronal *ins-7* expression, there is a major effect on intestinal *ins-7* expression resulting from loss of *skn-1b.*

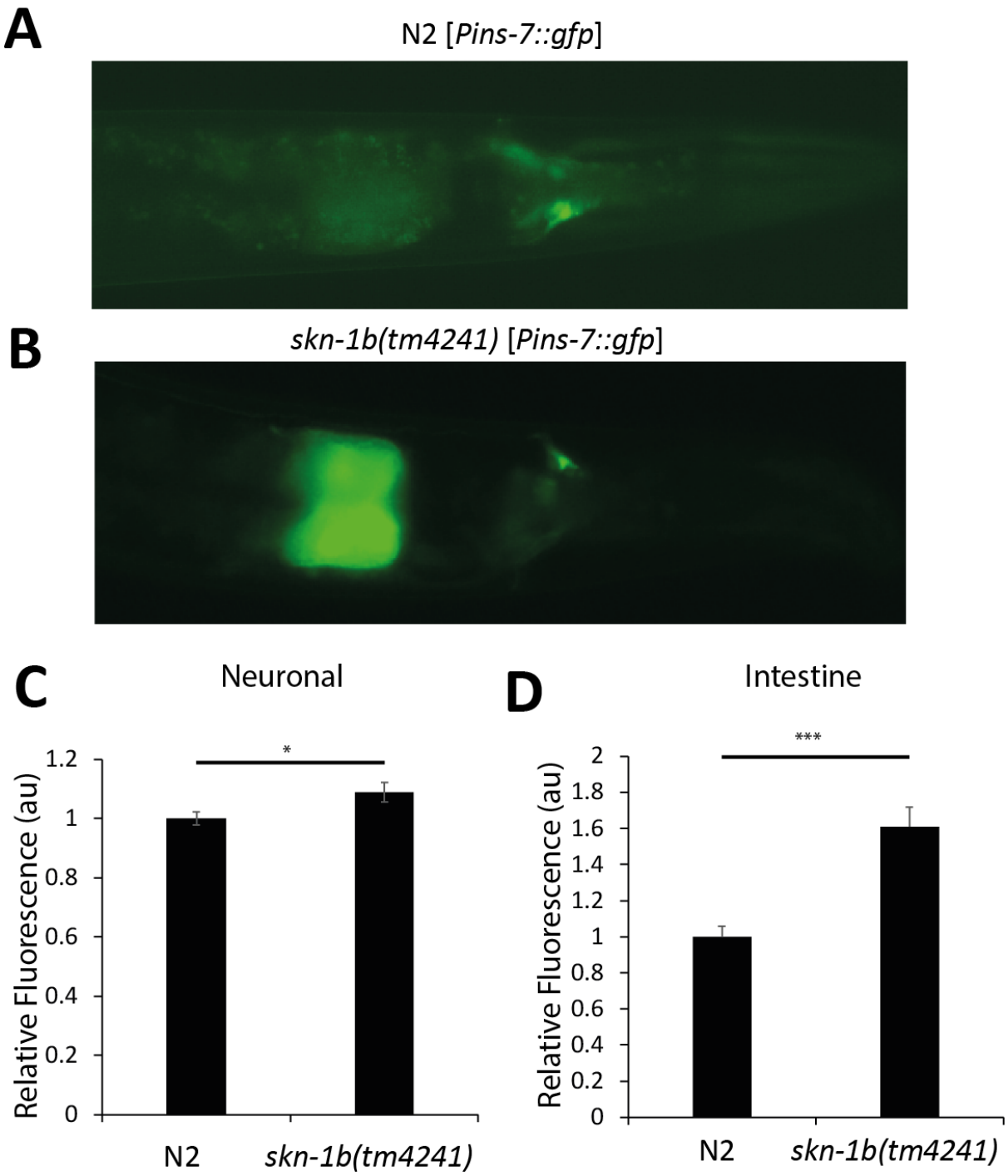

Figure 4.2: Intestinal *Pins-7::GFP* expression is altered cell non-autonomously by loss of neuronal *skn-1b*. A: *Pins-7::GFP* in a normal N2 genetic background. **B:** *Pins-7::GFP* in a *skn-1b(tm4241)* genetic background. **C:** Neuronal *Pins-7::GFP* fluorescence (pooled data from three trials p= 0.02 N= 121,102). **D:** Upper intestinal *Pins-7::GFP* fluorescence (pooled data from three trials p=2.5x10^{-6-}, N=119, 107).

Notably, none of these effects are localised to the ASI neuron suggesting a cell non-autonomous regulation of ILPs in distant tissues resulting from *skn-1b* loss. Since *ins-7* is a *daf-2* agonist this would have the effect of inhibiting the activity of *daf-16* and *skn-1c* in the intestine. It has previously been demonstrated that increased *daf-2* activity results in an upregulation of *ins-7* as part of a positive feedback effect which would amplify the *ins-7* signal (Murphy *et al.*, 2003). Interestingly, *ins-7* expression in the intestine – but not neurons – is increased with age, suggesting that intestinal *ins-7* is important for ageing (Murphy, Lee and Kenyon, 2007). In order to gain a more complete understanding in the changes of ILP regulation resulting from *skn-1b*, we sent RNA samples from N2 and *skn-1b(tm4241)* animals to a qPCR company (qstandard) in order to examine levels of all 40 ILPs. Unfortunately, internal variation in the results meant that even tenfold differences did not achieve statistical significance (Table S3).

4.3. *Pdaf-7::GFP* expression is increased in skn-1b mutant worms.

Our interest in *daf-7* stemmed from an overlap in the function of *daf-7* and *skn-1b. daf-7* is expressed in ASI neurons and is required for dietary restriction longevity by the sDR protocol (Fletcher and Kim, 2017), as is *skn-1b* albeit by a different DR protocol (Bishop and Guarente, 2007b) (for DR protocols see Figure 1.1). We wondered whether the transcriptional program enacted by *skn-1b* in response to reduced food levels signals to other tissues via *daf-7*. To examine the effect of skn-1b on daf-7 expression, the *drcSi7 [Pdaf-7::venus]* transcriptional *daf-7* reporter was crossed into the *skn-1b(tm4241)* background and fluorescence levels compared with N2 animals carrying the same reporter.

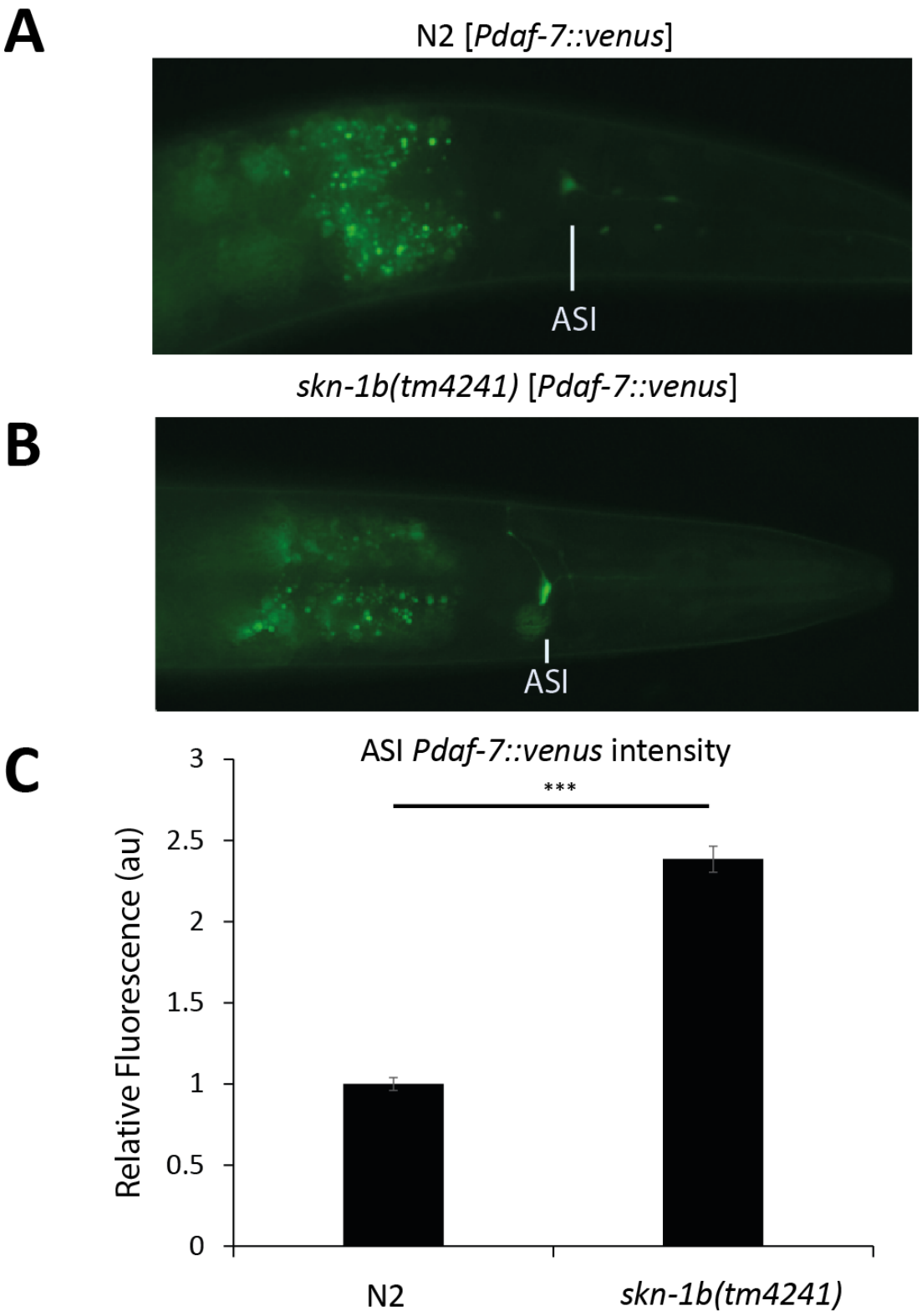

Figure 4.3: The *daf-7* promoter is inhibited by *skn-1b* expression. A: *Pdaf-7::venus* representative image in an N2 background. B: *Pdaf-7::venus* in a *skn-1b(tm4241)* background. C: Relative fluorescence of *Pdaf-7::venus* in an N2 and *skn-1b(tm4241)* background (Pooled data from three trials, N2 vs *skn-1b(tm4241)* $p=1.11x10^{-30}$. N2 n=230, *skn-1b* n = 237).

Animals were synchronised at day 1 adult by picking L4s on day 0 and imaged using a leica DMR microscope. ASI neuron fluorescence can then be compared to detect changes in the activity of the *daf-7* promoter. Perhaps surprisingly, we detected an increase in *Pdaf-7* activity in *skn-1b(tm4241)* animals where fluorescence was more than doubled in these animals (Figure 4.3). This was highly reproducible and significant. Since both *skn-1b* and *daf-7* levels increase with dietary restriction, the apparent inference that *skn-1b* is negatively regulating *daf-7* may seem contradictory. Nonetheless, this data still suggests that at least under some circumstances SKN-1B can act to suppress daf-7 transcription. There is no reported *skn-1* binding event in the modencode data in the *daf-7* promoter (Celniker *et al.*, 2009; Niu *et al.*, 2011), so any interaction is likely to be indirect.

4.4. Longevity incurred by reduced insulin signalling is independent of *skn-1b*.

Both *daf-28* and *ins-7* are insulin like peptides whose main function is as agonists of the insulin receptor (*daf-2*). Reduction in *daf-28* would act against IIS and is associated with rIIS longevity (Malone, Inoue and Thomas, 1996). An upregulation of intestinal *ins-7* would suggest the opposite since *ins-7* feeds back to stimulate *daf-2* and it has been demonstrated that *ins-7* is upregulated in response to IIS signalling and this enacts a form of positive feedback which propagates the *ins-7* signal (Murphy *et al.*, 2003). Therefore, both of these observations could support a model where insulin signalling is changed in *skn-1b(tm4241)* animals, although the downstream result on general levels of *daf-2* activation is unclear. Since reduced insulin signalling is one of the best studied pro-longevity interventions in numerous model organisms, we sought to establish whether *skn-1b* is required not just for dietary restriction longevity but for the longevity resulting for downregulation of insulin

signalling. Initially, an epistasis analysis assay was performed in which *skn-1b(tm4241)* was crossed into the *daf-2(e1370)* background. The initial lifespan analyses showed a total requirement for *skn-1b(tm4241)* (Table S1) for *daf-2(e1370)* longevity. There is considerable divergence between different classes of *daf-2* mutations (Gems *et al.*, 1998), so we therefore decided to examine different alleles, including class 1 and class 2 alleles. We chose the *daf-2* alleles *e1370, e1368 and e1391*. Of these three alleles, *e1368* is a class 1 allele with a less extreme lifespan extension and less severe inhibition of insulin signalling. The best studied allele of *daf-2* is *e1370* which is a class two allele with more severe phenotypes and *e1391* is our strongest allele, with a lifespan of approximately 90 days in our hands and even more severe *daf-2* associated phenotypes. We were surprised to observe that both *daf-2(e1368)* and *daf-2(e1391)* did not show a requirement for *skn-1b* (Figure 4.4). Mutants in *daf-2* are predisposed to dauer entry at 25°C, and there is evidence in the literature that the requirement for rIIS effectors downstream from *daf-2* varies at dauer-inducing (25°C) and non-inducing temperatures (15°C). Specifically, *skn-1* is not essential for the full *daf-2* longevity at 25°C but is required at 15°C (Ewald *et al.*, 2014). Lifespan analyses of the strains were performed at these temperatures in order to test whether some requirement for *skn-1b* was being masked by dauer-dependent longevity mechanisms. When tested at these temperatures we did not observe a change in the requirement for *skn-1b* in any of the three strains (Table S1). This led to the suspicion that a background mutation in our *daf-2(e1370)* animals could be responsible, and a backcross was performed on the *daf-2(e1370)* strain present in the lab. After the backcross the requirement for *skn-1b* disappeared, confirming this (Figure 4.4A). In all three *daf-2* alleles, therefore, we did not observe any requirement for *skn-1b*. In figure 4 .4 A, B and C we show representative lifespans at 20°C for each of these *daf-2* alleles.

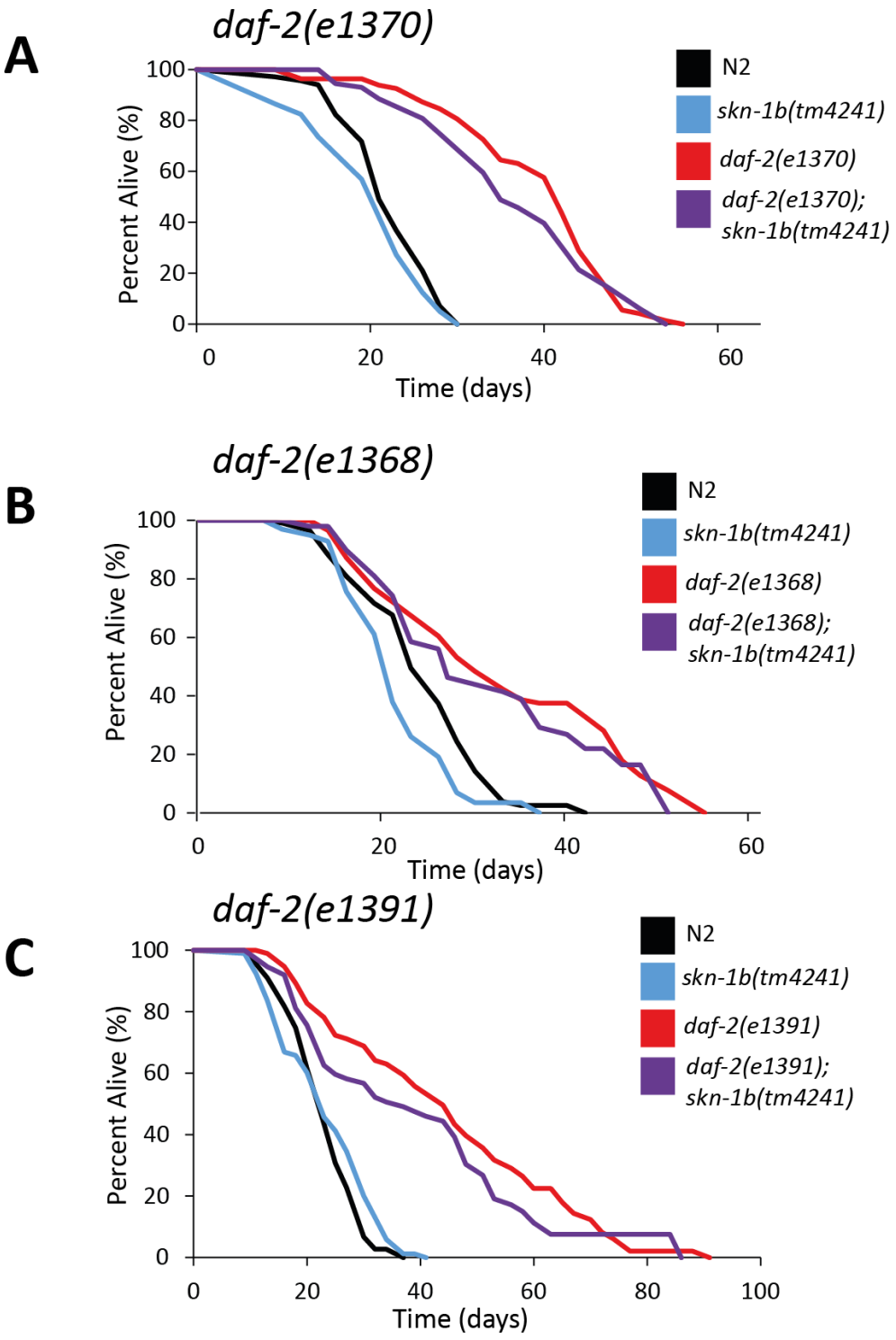

Figure 4.4: *skn-1b* is not required for *daf-2* longevity: A: Lifespan analysis of epistatic interaction between *skn-1b(tm4241)* and *daf-2(e1370)*. B: Lifespan analysis of epistatic interaction between *skn-1b(tm4241)* and *daf-2(e1368)*. C: Lifespan analysis of epistatic interaction between *skn-1b(tm4241)* and *daf-2(e1391)*. In all cases *daf-2(x)* vs *daf-2(x);skn-1b(tm4241)* p=>0.05. See table S1 for full lifespan data and statistical analysis. Representative trials (non-pooled) shown, N=3 with each condition starting with 100 animals

In table S1, we show lifespans at 15°C and 25°C which also no do not show any requirement for *skn-1b*. Therefore, both dauer dependent and independent rIIS longevity seems to be independent of *skn-1b.*

This is more consistent with a model where *skn-1b* is acting in by integrating chemosensory information into the decision to enact the DR longevity response but is not acting via the regulation of insulin signalling to do so.

4.5. The guanylate cyclase *daf-11* is required for *skn-1b* activity.

We reasoned that if *skn-1b* is responding to chemosensory information, then one or several of the chemosensory receptors expressed in the cilia of the ASI neurons is likely to be involved in the internalisation of this information. ASI neurons express more G-Protein Coupled Receptor (GPRC) genes than any other cell in the animal (Vidal *et al.*, 2018), as well as numerous other cell surface receptors (ASI neuron Wormatlas) suggesting that they are a major site of chemosensation. From the literature, it is known that *daf-11* mutants display a range of deficits in chemotaxis which are similar to those observed by other in our lab in *skn-1b(tm4241).* Additionally, *daf-11* is implicated in the positive regulation of *daf-7* (Murakami, Koga and Ohshima, 2001) and *daf-28* (Li, Kennedy and Ruvkun, 2003), which caused us to investigate whether there is an interaction between *daf-11* and *skn-1b* regulation. Data from chapter 3, figure 3.5 demonstrated that SKN-1B::GFP is induced in response to the removal of food in our bacterial deprivation protocol, where animals are placed onto plates with no food for 16h. Therefore, an epistasis experiment was designed to test the requirement of *daf-11* for *skn-1b* expression.

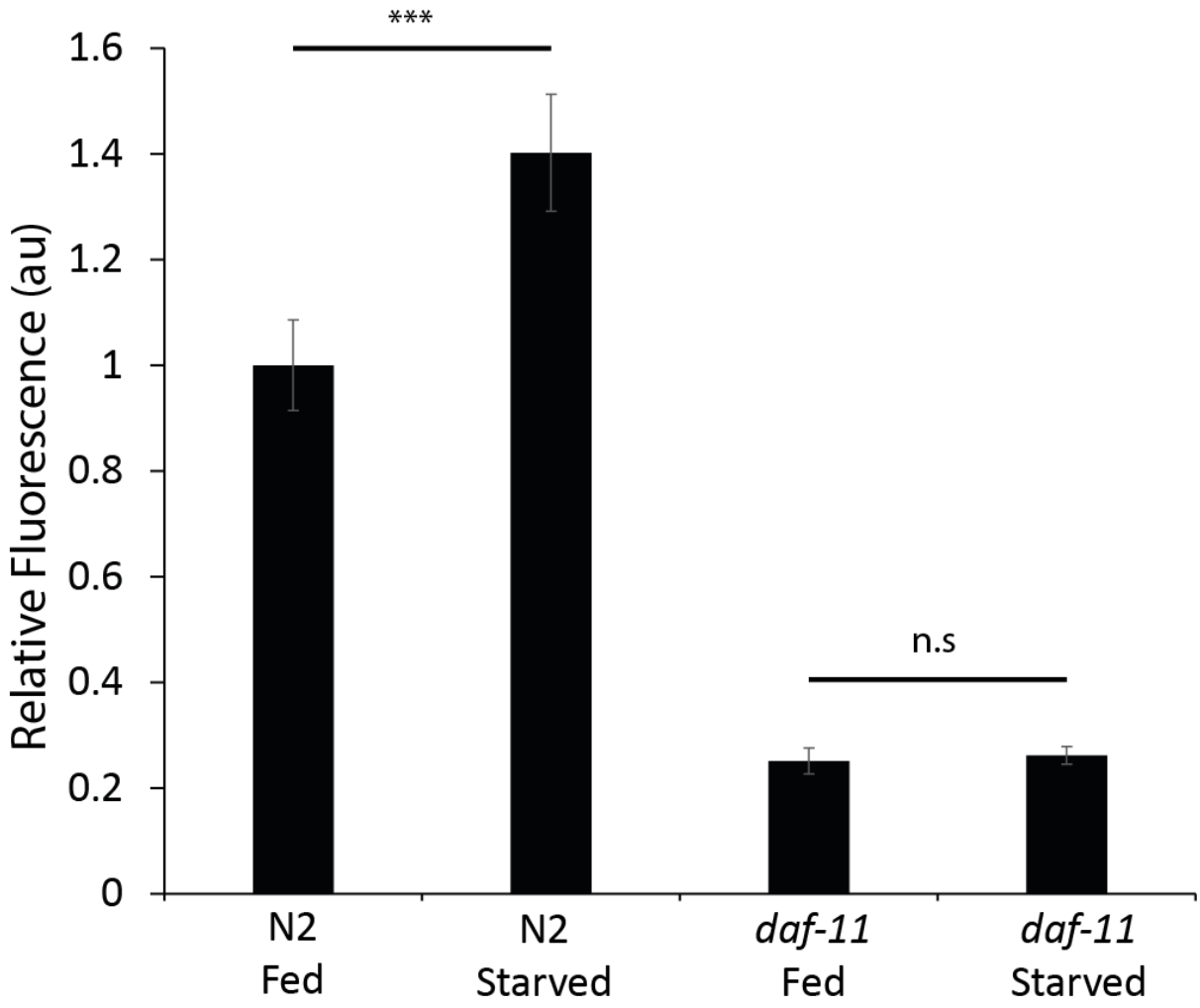

Figure 4.5: *daf-11* promotes *skn-1b::GFP* expression in ASI neurons: Relative fluorescence of *SKN-1B::GFP* in ASI neurons (N2 fed vs N2 starved p= 0.0075; *daf-11* fed vs *daf-11* starved p= 0.81; N2 fed vs *daf-11* fed p = 6.15×10^{-6}). Representative trial shown, N=3 (~35 worms per group).

Our *WuEx217 [Pskn-1b::skn-1b::GFP]* reporter was crossed into *daf-11 (m47)* mutants and the resulting strain compared with an N2 control using quantitative fluorescence microscopy. In a wild type background, the bacterial deprivation protocol increased SKN-1B::GFP levels by ~1.4 fold, as in figure 3.5. In the *daf-11(m47); WuEx217 [Pskn-1b::skn-1b::GFP]* strain in fed conditions there was a radical reduction in SKN-1B::GFP levels (Figure 4.5). Importantly, when subjected to BD these animals totally failed to respond by increasing SKN-1B::GFP. This suggests that not only does normal *skn-1b* expression depend upon the action of *daf-11*, but that the BD dependent induction of *skn-1b* is also dependent on *daf-11*. This implicates *daf-11* very strongly in the internalisation of information about reduced food density via its regulation of *skn-1b*.

4.6. The ER unfolded protein response requires *skn-1b*

One outstanding question is which processes *skn-1b* regulates in the wider organism. Data from other labs has previously demonstrated that neuronal *xbp-1* is sufficient to activate the UPR^{ER} response in the whole animal via cell non-autonomous mechanisms (Taylor and Dillin, 2013). In *D. melanogaster*, IRE-1 - a key component of the UPR^{ER} - is required for longer lifespan under DR in a manner reminiscent of *skn-1b* in *C. elegans.* Furthermore, *xbp-1* is a known *skn-1* target with a strong binding event demonstrated in MODENCODE and *skn-1* has been demonstrated (in the context of *skn-1c*) to directly regulate numerous UPR^{ER} genes (*xbp-1, ire-1, hsp-4, pek-1, atf-6)* (Glover-Cutter, Lin and Blackwell, 2013). In addition, data generated by Nikolaos Tataridas-Pallas in our lab showed that *skn-1b* mutants are defective in plate exploration (Fig S4), and that this effect is shared by and not additive with *daf-28 (sa191)* animals but not *daf-28(tm2308).* The difference between these strains is that *daf-28(sa191)* is a dominant negative mutation which results in large quantities of unfolded

DAF-28 accumulating in ASI neurons, while *daf-28(tm2308)* is a deletion of a large portion of the *daf-28* gene resulting in a complete lack of expression (Klabonski *et al.*, 2016). The *daf-28(sa191)* strain has been shown to be subject to a bystander effect where the accumulation of unfolded DAF-28 leads to the induction of UPR stress and results in dysregulation of *daf-7*, with the likely implication that other signalling peptides secreted from the ASI could also be affected (Klabonski *et al.*, 2016). This raised the possibility that the reason for the difference in plate exploration phenotypes between these two *daf-28* mutant strains in our investigations was that the UPR^{ER} in these animals is defective in ASI neurons or more generally, and that unanswered UPR^{ER} stress in our *skn-1b* animals rather than loss of *daf-28* was leading to altered behaviour and regulation of secreted signalling molecules. Therefore the *zcIs4[Phsp-4::GFP]* transcriptional reporter for *hsp-4*, a marker of UPR^{ER} activation was used to test the interaction between *skn-1b* and the UPR^{ER}. This reporter was crossed into our *skn-1b (tm4241)* strain and normal basal levels of *Phsp-4* activation were compared between N2 and *skn-1b(tm4241)* animals, as were activation levels upon induction of UPR^{ER} stress by tunicamycin treatment. Animals were synchronised by picking L4s and imaged at D1 adulthood, either after 4h of treatment with 25ng/ul of tunicamycin in M9 or after treatment with M9 + the same concentration of DMSO as results from addition of the tunicamycin in DMSO stock. Fluorescence was measured in the whole worm by simply selecting the entire animal as the ROI for quantification. In normal conditions, loss of *skn-1b* reduces GFP levels to 80.5% of control with strong significance and reproducibility suggesting that even in normal conditions the UPR^{ER} response is weakened by loss of *skn-1b.*

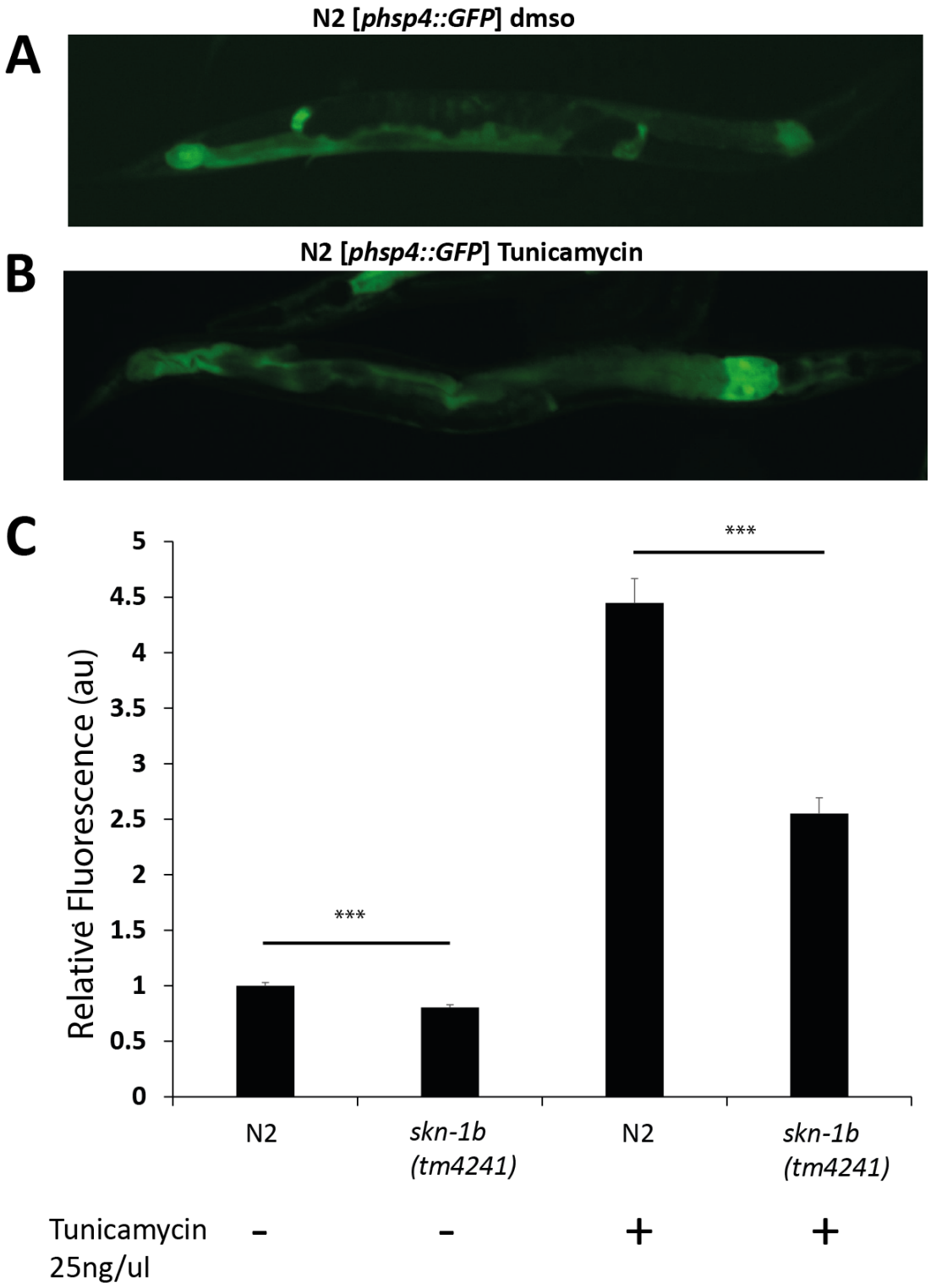

Figure 4.6: *skn-1b* is required for *Phsp-4::GFP* induction in normal conditions and in response to UPR^{ER} stress. A&B: *Phsp-4::GFP* expression in N2 animals with and without tunicamycin. C: *Phsp-4::GFP* levels in N2 and *skn-1b(tm4241)* animals (N2 – tunicamycin vs *skn-1b(tm4241)* – tunicamycin , $p=6.5\times10^{-7}$; N2 + tun vs *skn-1b(tm4241)* + tunicamycin, $p=4.17\times10^{-11}$). Whole worm fluorescence (see 2.6.1), significance by students t test.

When treated with tunicamycin there is a striking failure to respond to UPR^{ER} stress to the same extent as control animals do, with N2 background animals showing a 4.4 fold increase in *Phsp-4::GFP* expression and *skn-1b(tm4241)* animals showing a 2.5 fold induction (Figure 4.6). There are two interesting conclusions which result from this data; firstly we show that UPR^{ER} is reduced in *skn-1b(tm4241)* animals in normal conditions. We do not see a statistically significant shortening of lifespan in these animals, and this may be indicative of metabolic changes resulting from loss of *skn-1b* dependent gene expression. Secondly, we show that the UPR^{ER} is not able to respond to induction of the UPR by tunicamycin to the same extent when *skn-1b* expression is lost, suggesting that proper *skn-1b* function is required for the UPR^{ER} response increasingly when unfolded protein stress is higher. Since we are measuring whole worm fluorescence, both of these results suggest that this effect is cell non-autonomous, suggesting that our initial interest in the neuronal control of global UPR^{ER} was justified and that *skn-1b* is cell non-autonomously signalling to the UPR^{ER}.

Chapter 4 Discussion

Our data of *daf-28* regulation using our transgene (figure 4.1) is striking and seems to suggest that *skn-1b* is required for *daf-28* expression. This would place *daf-28* as a key signalling molecule downstream of *skn-1b* and implicate *skn-1b* in the direct regulation of insulin signalling in the rest of the animal. However, efforts in our lab are ongoing to confirm whether there is indeed a total loss of *daf-28* in *skn-1b(tm4241)* animals. Notably, the lack of a dauer phenotype at 25°C or an effect of wild-type lifespan in *skn-1b(tm4241)* animals cause some degree of uncertainty in the safety of this result (Figure 3.7, supplemental Figure 1) The original *daf-28(sa191)* mutant is a dominant negative mutation which causes the ASI neurons to become overloaded with misfolded DAF-28 which results in a bystander effect on *daf-7* which promotes dauer entry, so the radical effect on dauer at 20°C seen in the *sa191* strain would not necessarily be expected (Klabonski *et al.*, 2016). However, the *daf-28(tm2308)* strain (which should approximate the result of *skn-1b(tm4241)* if our transgene in this case is accurate) does show dauer phenotypes at 25°C and is reported to be 60-80% penetrant at 27°C (Fernandes de Abreu *et al.*, 2014). The propensity of *skn-1b(tm4241)* to enter dauer has been checked in our lab at 25°C and 27°C, with no dauer phenotype at 25°C (Figure S1) and very low levels of dauer at 27°C (unpublished data, Jennifer Tullet). Additionally, there is a weak lifespan extension at some temperatures with *daf-28* (Zhang *et al.*, 2018) which we did not observe with our *skn-1b(tm4241)* animals. In short, the phenotypes of *skn-1b(tm4241)* and *daf-28(tm2308)* do not match, and the transgenic data suggests that they should. We undertook efforts to confirm this by qPCR but were largely frustrated by a high degree of variability between samples although the data

we did get was not supportive of the conclusion that *daf-28* mRNA is totally absent (Figure S2). High variability when measuring ILPs seems to be not uncommon since other labs often use SM-FISH to measure ILP expression levels e.g (Meisel *et al.*, 2014), or sort for ASI neurons by FACS and perform qPCR on these cells only (communications with Taylor lab at LMB). Unfortunately, neither of these techniques were immediately available at Kent during the time of this study. In whole worm qPCR, there was not a statistically significant difference between N2 and *skn-1b(tm4241)* in *daf-28* mRNA levels and establishing whether our data demonstrates a confounding, unexpected transgene issue or actual biological *daf-28* regulation is a matter of urgency before this study can be written up into a paper. One possible way to reconcile our data with the published data on *daf-28(tm2308)* is that we also observe an increase in *Pdaf-7::venus* expression in *skn-1b(tm4241)* animals – an increase in DAF-7 would act against dauer via TGF-β signalling and potentially mask the pro-dauer effects of reduced DAF-28. We hope to resolve this by the use of translational reporter strains and/or by sorting for ASI neurons via FACS to perform qPCR directly enriched populations of those cells.

In figure 4.2 we show an increase in intestinal *Pins-7::GFP* in a *skn-1b(tm4241)* genetic background, and a very weak increase in neuronal *Pins-7::GFP* in the same background. This would have the effect of upregulating insulin signalling in these animals, acting against longevity by rIIS, which would suggest that in normal animals *skn-1b* is acting indirectly to dampen insulin signalling. Increased insulin signalling in the intestine is known to increase *ins-7* expression in a positive feedback interaction whereby *daf-16* inhibition of *ins-7* is released (Murphy *et al.*, 2003). This data supports a model where insulin signalling is opposed by *skn-1b* in normal conditions in some tissues. The activity of this transgene has

previously been shown to increase with old age, suggesting a relationship between intestinal *ins-7* and ageing (Murphy, Lee and Kenyon, 2007).

In a recent review, Lin *et al* (Lin *et al.*, 2017) note that several lines of evidence point towards a model in which the longevity of *C. elegans* is regulated in part by neuronal signals. They specifically speculate that *skn-1* in ASI neurons may act against the secretion of signals which oppose lifespan from ASI neurons. In figure 4.3 we show that a candidate for such a secreted signal is *daf-7*, the expression of our *daf-7* reporter increases more than two-fold upon the introduction of the *skn-1b(tm4241)* deletion. The published data on *daf-7* is complex – *daf-7* is induced by, and required for, the longevity downstream of dietary restriction (Fletcher and Kim, 2017) which demonstrates that *daf-7* acts to promote (rather than oppose) DR longevity. However, various loss-of-function mutants in TGF-β pathway components, including *daf-7* loss-of function mutants *daf-7(e1372)* and *daf-7(m62)* extend lifespan in a *daf-16* dependent manner (Shaw *et al.*, 2007). Therefore, there is strong published data which demonstrate that both upregulation of *daf*-7 and loss of function of *daf-7* are capable of extending lifespan in certain contexts, due to dual regulation of IIS and DR effectors with opposing effects on longevity. While the simultaneous induction of *skn-1b* and *daf-7* by DR in the same cell seems to argue against a model where *daf-7* expression is inhibited by *skn-1b*, our transgene data is quite clear that *Pdaf-7::GFP* expression is increased by the loss of *skn-1b.* One intriguing possibility which we have not had the time to adequately explore is that *skn-1b* acts to oppose *daf-7* not as a binary "off" switch on *daf-7*, but as a mechanism by which the *daf-7* signal is modulated, since *daf-7* downstream targets are diverse and exert opposing effects on lifespan. Indeed, *skn-1b* could be induced in ASI neurons in an indirect negative feedback interaction with *daf-7* expression, where increased *daf-7* expression is detected and *skn-1b* expression is induced to prevent a situation where

daf-7 escapes *skn-1b* regulation. Since *daf-7* signals to distant receptors and functions largely as an intercellular signalling molecule, one way to achieve this would be via common regulation by an upstream gene product. Indeed, *daf-11* has been shown to regulate *daf-7* (Murakami, Koga and Ohshima, 2001) and we show here that it positively regulates *skn-1b*. Very recent data from our lab shows phenotypes supportive of a model where *daf-7* expression is increased in *skn-1b(tm4241)* since these animals show increased quiescence, a phenotype associated with *daf-7* expression (You *et al.*, 2008). Further exploration of this possibility is warranted and may be undertaken in our lab in the future.

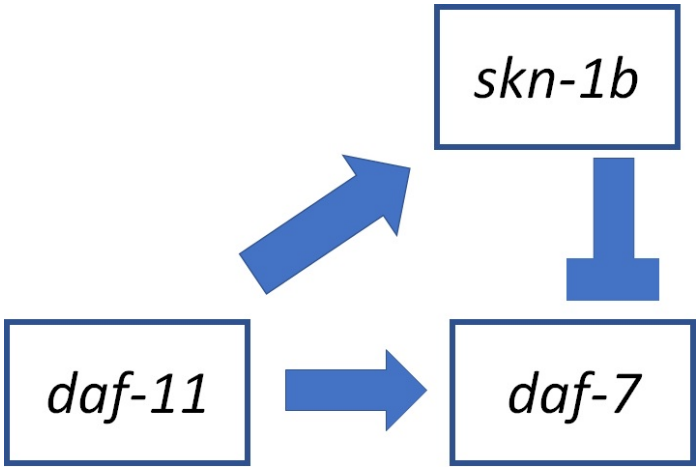

Figure 4.7: Model of suggested *skn-1b* interaction with *daf-7* and *daf-11*.

The data presented here also shows that the guanylate cyclase encoding gene *daf-11* which is involved in the sensation of the external environment and satiety behaviour (Birnby *et al.*, 2000; You *et al.*, 2008) is required for normal *skn-1b* expression, and that animals which have lost *daf-11* can no longer upregulate *skn-1b* in response to BD (Figure 4.5). This is particularly interesting since mutants in *daf-11* were shown to suppress *Pdaf-7::GFP* and to regulate dauer via this interaction. As discussed previously, we show that *skn-1b* inhibits *Pdaf-7::venus* transgene expression but both are apparently upregulated in response to BD.

One way to reconcile this is that *skn-1b* and *daf-7* are simultaneously induced downstream of *daf-11* activity, with *skn-1b* opposing *daf-7* expression in a negative feedback interaction. Notably, the regulation of both *daf-7* and *skn-1b* by *daf-11* (both of which are required for DR longevity) (Bishop and Guarente, 2007b; Fletcher and Kim, 2017), combined with the insensitivity of *skn-1b(tm4241)* mutants to BD suggests that *daf-11* mutant strains will be unable to be long lived in response to dietary restriction, however this remains to be tested.

The data shown here demonstrates that loss of *skn-1b* causes the UPR to fail to respond to UPR^{ER} stress adequately as measured by whole worm induction of *Phsp-4::GFP* (a phenomenon which is also present in aged animals (Taylor and Dillin, 2013)), linking chemosensory signals to the regulation of lifespan promoting processes such as the UPR^{ER} (Figure 4.6). Constitutive activation of the UPR^{ER} is capable of extending lifespan (Taylor and Dillin, 2013) and in Drosophila IRE-1 is required for dietary restriction longevity (Li *et al.*, 2016). The work shows that *skn-1b* is required (reproducibly and with high significance) to maintain normal levels of UPR^{ER} activation in the whole animals. There are two possible explanations for this observation -firstly, that *skn-1b* via regulation of the UPR^{ER} in ASI neurons is able to cell non-autonomously regulate the UPR^{ER} in other tissues. Constitutive expression of *xbp-1s* in neuronal tissue is capable of achieving this effect and *skn-1* is known to regulate numerous UPR^{ER} genes (*xbp-1, ire-1, hsp-4, pek-1, atf-6)* via direct interaction with the promoters of these genes (Glover-Cutter, Lin and Blackwell, 2013). There is therefore a clear logical pathway to suggest that *skn-1b* activation one or some of these genes in neuronal tissues could regulate the UPR^{ER} response in whole animals. The implication of this is that in *skn-1b(tm4241)* animals unfolded protein stress in the ER would not be adequately dealt with in ASI neurons and other tissues. Notably, this could result in the improper processing of secreted signals from ASI neurons which are mostly prepared for

secretion in the ER, including ILPs and *daf-7*. Another interpretation of the data relating to unstressed animals is that loss of *skn-1b* results in fundamental changes to the metabolism of the animal which lead to reduced protein synthetic load and thus lessen the UPR^{ER} response due to reduced levels of unfolded proteins. One might expect to see a lifespan extension in *skn-1b* animals were this the case (which we do not), but one obvious candidate for a process which could achieve this fundamental metabolic change is insulin signalling – we show dysregulation of ILPs results from *skn-1b* loss in figures 4.1 and 4.2, and *daf-7* and figure 4.3 which also interacts with insulin signalling. When challenged with tunicamycin, an inducer of unfolded protein stress, *skn-1b(tm4241)* animals dramatically fail to respond, as measured by whole worm induction of *Phsp-4::GFP*. This would suggest that these animals are more vulnerable to unfolded protein stress. Unfolded protein stress is considered a progeric process by some in the field, and It has been suggested that the loss effective protein folding and UPR machinery results in much of the pathological effects of ageing (Ben-Zvi, Miller and Morimoto, 2009; Taylor and Dillin, 2011). The activation of the UPR is also thought to be one mechanism by which hormesis (where mild stresses often extend lifespan) may be working (Cabreiro *et al.*, 2011).

Nrf-2 is thought to be the mammalian orthologue of *skn-1b/c*, however in our investigations of the role of *skn-1b* in *C. elegans* we have largely been by the data away from canonical *skn-1c* roles and towards roles in chemosensation and behaviour (Blackwell *et al.*, 2015). Nrf-2 seems to more closely approximate the physiological roles of *skn-1c* based upon the data which we have seen (reveiwed in Silva-Palacios *et al.*, 2018), leaving open the possibility of similar roles of hypothalamic Nrf-2, or divergent regulation of *skn-1b* roles in mammals.

RNA Polymerase III limits lifespan in *C. elegans*

Aims

In this study we set out to investigate the role of RNA polymerase III, if any, in the regulation of adult lifespan. Our reasoning was that since inhibition of TOR pathway activity extends lifespan in all tested organisms, and Pol III is known to be positively regulated by TOR activity, there was therefore a possibility that Pol III may be involved in limiting adult lifespan downstream of TORC1. The worm aspect of this study, which comprises the work which I have performed, was informed by findings in the Alic lab in UCL that in *D. melanogaster* Pol III inhibition was indeed capable of extending lifespan, and we sought to establish whether this was a conserved mechanism which is more broadly applicable or whether this was confined to *D. melanogaster*. Our aims at the outset were to;

- Develop a reliable assay for Pol III inhibition in a temporally controlled manner
- Identify phenotypes resulting from Pol III inhibition
- Determine how inhibition of Pol III interacts with lifespan
- Identify relevant physiological phenotypes which are influenced by Pol III inhibition. and
- To perform epistasis experiments in order to better understand the position of Pol III in the relevant longevity mediating pathways.

5.1. Knockdown of *rpc-1* by RNAi feeding delays development

First, we sought to develop a reliable methodology for post-developmentally reducing Pol III activity *in vivo*. We identified *rpc-1*, which is predicted to encode the *C. elegans* homolog of human RPC-1 (yeast C160), as a potential target. *rpc-1* is unique to RNA polymerase III and encodes the largest subunit of Pol III (Kishore & Done, 2018). We targeted *C. elegans rpc-1* by RNAi feeding, an effective method of administering RNAi to *C. elegans* in a temporal manner (Timmons & Fire, 1998). We examined whether *rpc-1* RNAi caused any phenotypes which might confirm that *rpc-1* RNAi treatment would reduce RNA Polymerase III activity *in vivo*. To this end, we assayed the development of animals treated with *rpc-1* RNAi over three generations. In this assay, animals were placed on *rpc*-1 RNAi bacteria and grown over three generations from eggs laid on the RNAi bacteria so that all generations had experienced *rpc-1* RNAi all through development. While the effect was subtle at first, after three generations growth is radically slowed relative to control animals (Figure 5.1 A). Similar developmental delays have been observed by others in interventions which reduce mRNA translation such is mutations in *rsks-1* (*C. elegans* S6K) and *ifg-1* (*C. elegans* eIFG4G) (Pan *et al.*, 2007a). Since numerous Pol III products are required for protein synthesis both directly as with tRNAs and via their requirement for ribosome biogenesis as with 5S rRNA, and that inventions against pol III reduce protein synthesis in *D. melongaster* (Filer *et al.*, 2017), it is reasonable to speculate that this delay is due to reduced translation resulting from Pol III inhibition. Furthermore, these data show that it is important to separate the

effects of *rpc-1* on adult lifespan from those it exerts on development since these developmental effects could complicate interpretation of data otherwise.

5.2. *rpc-1* RNAi treatment during adulthood results in reduced *rpc-1* mRNA levels.

Next we set out to directly examine mRNA levels of *rpc-1* by RT-qPCR. Here, animals were synchronised by allowing egg laying for a timed period of 5 hours, and offspring were selected at L4 stage and placed onto NGM plates seeded with either control RNAi or *rpc-1* RNAi or control bacteria for 4 days. To confirm that this treatment was effective at reducing transcript levels *in vivo* worms were harvested from the RNAi treatments and *rpc-1* mRNA levels measured using quantitative RT-PCR. This showed that *rpc-1* RNAi successfully reduced *rpc-1* mRNA levels by approximately 60% relative to control (Figure 5.1 B). Therefore, it is possible to downregulate *rpc-1* mRNA levels in adult animals.

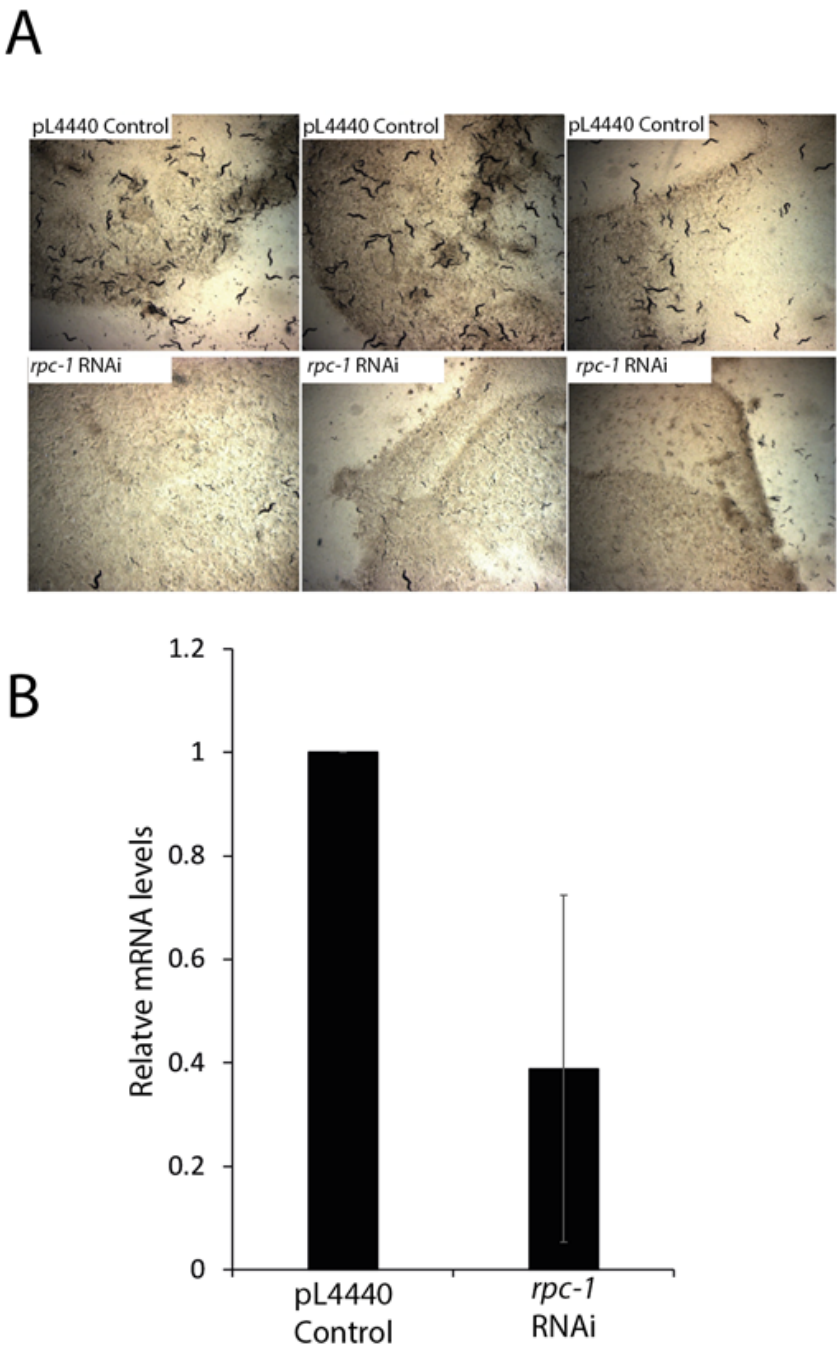

Figure 5.1: *rpc-1* RNAi reduces *rpc*-1 mRNA levels in adult worms and influences development in *C. elegans*. A) Animals grown on *rpc-1* RNAi bacteria for three generations experience severely slowed growth. Empty Vector (EV) and *rpc-1* RNAi animals were initially synchronised and grown for two generations. B) Animals grown on *E.coli* HT115(de3) containing pL4440 *rpc-1* at larval stage 4 have reduced *rpc-1* transcript levels when compared to animals on pL4440 control. Transcript levels determined by RT-qPCR. N=8, Reduction of 61%, $p=9.36x10^{-5}$. Detailed method in 2.4.4 and primers used in table 2.2.

5.3. *rpc-1* RNAi treatment extends lifespan of *C. elegans*

Our collaborating lab (at UCL) in this project were interesting in examining the role of RNA Pol III knockdown on lifespan. They observed that reducing RNA Pol III by inhibition of *d*C160, the *D. melanogaster* homolog of *rpc-1,* increased the lifespan of these animals. They could also achieve a similar lifespan extension using a strain of flies carrying a heterozygous mutation in *d*C53 (another specific subunit of RNA Pol III). Since RNA Pol III is itself highly conserved, we sought to establish whether this lifespan extension was conserved in *C. elegans*. If so, this would be suggestive of a broadly conserved role for Pol III in the regulation of lifespan. To further strengthen this analysis, our collaborators in The University of Groningen performed a similar analysis in yeast. We therefore performed a lifespan analysis of animals treated with *rpc-1* RNAi from L4 stage compared to the relevant controls. Initially this analysis was performed at 20°C (Figure 5.2 A) and showed a significant increase in mean lifespan relative to control at this temperature (Table S2). However, in the course of this analysis we identified an unexpected and confounding phenotype – animals treated with *rpc-1* RNAi showed a highly penetrant "gut explosion" phenotype which meant that ~70% of animals in the *rpc-1* treated group were ultimately censored (Figure 5.2 B). The gut explosion phenotype causes extrusion of the intestine through the vulva which means that these animals must be censored it from the analysis since this phenotype is clearly life-limiting. Such high occurrence of gut explosions could in theory result in a selection effect where a pre-existing subset of long-lived animals are present on both control and RNAi plates, but when challenged with the *rpc-1* RNAi this subset disproportionately survives, increasing the mean lifespan of the non-censored population. In order to ascertain whether

rpc-1 RNAi was extending lifespan or whether the censoring itself was the issue, we set out to reduce censoring.

5.4. Inhibition of *rpc-1* at 25°C extends lifespan with reduced loss of vulval integrity

It had been previously observed (Leiser, Begun and Kaeberlein, 2011) that shifting animals to 25°C was sufficient to overcome the loss of vulval integrity seen in *hif-1* mutants at 15°C and 20°C. Since the confounding phenotype this study encountered was basically the same as that which we observed, we wondered whether we could achieve lower censoring by using the same strategy. We placed L4 animals onto NGM plates contain *rpc-1* RNAi and control bacteria and stored the animals in a 25°C incubator for the duration of the study. Under these conditions, we were able to reproduce the lifespan extension which we had previously observed (Figure 5.2 C). Additionally, at 25°C censoring was significantly reduced, with ~20% censoring in *rpc-1* treated worms at this temperature (Figure 5.2 D). We therefore concluded that RNAi against *rpc-1* is indeed capable of extending the lifespan of *C. elegans* and that this is not a function of a selection process caused by the high rate of censoring. The reason for the difference in censoring at 20°C and 25°C remains elusive and further study in this area may well lead to useful insights about the effects of Pol III inhibition on the organism. For the purposes of exploring adult lifespan in the context of ageing, we adopted 25°C as the standard condition for our lifespans from this point onwards.

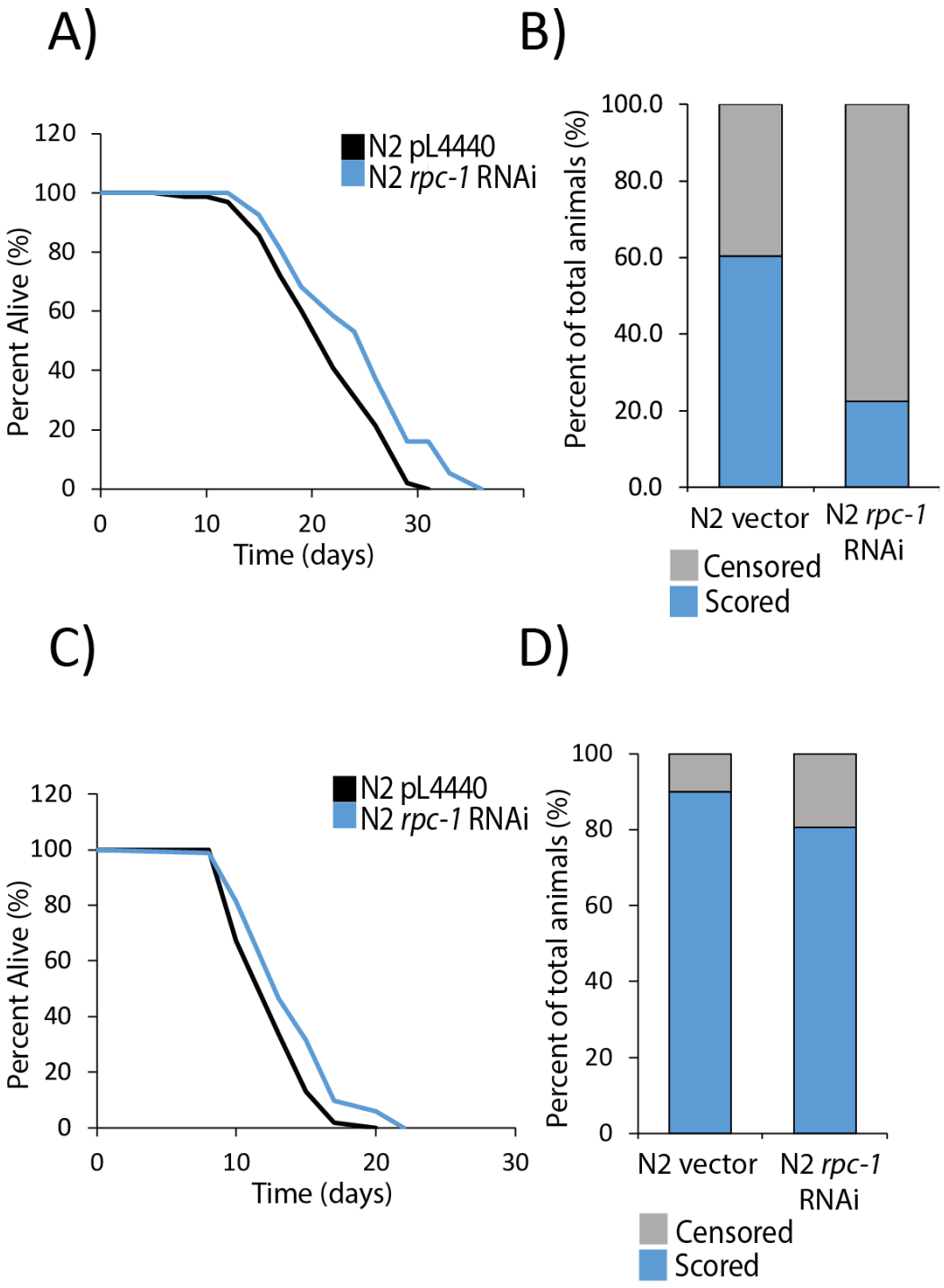

Figure 5.2: *rpc-1* RNAi extends lifespan Ai) *rpc-1* RNAi increased lifespan at 20°C (N=4, representative trial shown. Significance for this trial determined by log-rank test p=0.032. Aii) At 20°C, animals treated with *rpc-1* RNAi displayed a highly penetrant lethal gut explosion phenotype with ~70% of such animals censored due to this. Bi) At 25°C *rpc-1* RNAi extends lifespan (N=3, representative trial shown, Significance determined by log-rank test p=0.009) Bii) At 25°C the gut explosion phenotype is less penetrant, with 19.5% of *rpc-1* RNAi worms censored under these conditions. For lifespan extension and censoring data from all trials, see table 5.1 (lifespan table).

5.5. Inhibition of *rpc-1* in the intestine is sufficient to extend lifespan

Many life extending interventions in *C. elegans* exert their effects in a tissue specific manner, and often the intestine is the key tissue in mediating these effects (Libina, Berman and Kenyon, 2003; Piper *et al.*, 2008). In order to determine if this is also the case with inhibition of RNA Pol III we used the strain VP303, which lacks *rde-1*, an essential gene for the RNAi response to exogenous dsRNA which is required for the processing of this dsRNA into siRNA (Tabara *et al.*, 1999).To examine the effect of gene knockdown specifically in the gut, *rde-1* is then rescued in the intestine by reintroducing *rde-1* under the control of the intestinal specific *nhx-2* promoter, (Espelt *et al.*, 2005; Qadota *et al.*, 2007). To test whether Pol III activity was important in the gut we fed *rpc-1* RNAi to VP303 animals and them to control fed animals. In VP303 animals fed *rpc-1* RNAi, the mean lifespan of these animals is increased by 14% (Figure 5.3, Table 5.1). This is a similar extension as that seen in N2 (WT) animals (Figure 5.2, Table 5.1). This suggests that the major tissue in which RNA pol III exerts its effect on lifespan is the intestine, since tissue specific inhibition in the intestine can recapitulate the majority of the effect seen in whole worm inhibition of *rpc-1*. The same is also true in *D. melanogaster*, where our collaborators at UCL observed that tissue specific inhibition of *d*C160 in the gut was capable of recapitulating this lifespan extension seen with whole fly inhibition.

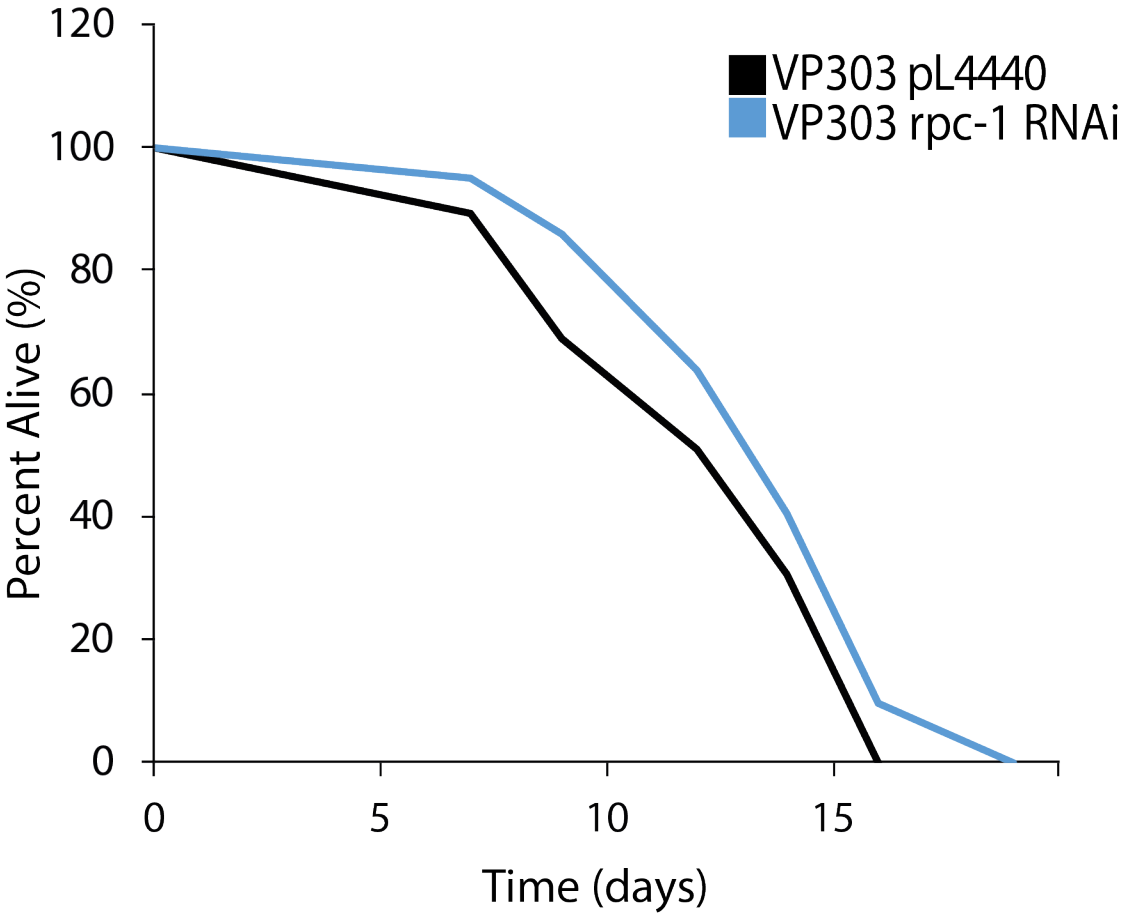

Figure 5.3: *rpc-1* RNAi in the intestine is sufficient to extend lifespan. A) *rpc-1* RNAi extends lifespan of VP303 *rde-1(ne219)* V; *kbIs7 [nhx-2p::rde-1 + rol-6(su1006)]* animals which lack the ability to process RNAi in any tissue except for the intestine. Representative experiment shown, N = (vector = 90; *rpc-1* = 67extension = 11%, p=0.0094 significance determined by log-rank test.

Strain	*rpc-1 RNAi*	Temp	Mean extension (%)	Mean Censoring (%)
N2	Yes	20C	23	77
N2	Yes	25C	10	20
VP303	Yes	20C	14	56
VP303	Yes	25C	12	4
RB1206	No	25C	10	2
RB1206	Yes	25C	25	5

Table 5.1: Mean lifespan extension relative to control for lifespan experiments in this chapter. Full full data including p values and individual experiment see lifespan table Fig. S2.

5.6. Inhibition of *rpc-1* protects the *C. elegans* gut barrier.

Intestinal barrier function is decreased with age in *C. elegans* and interventions which extend lifespan such as DR can also protect the function of this barrier. Since we have identified the intestine as a key tissue in the longevity resulting from inhibition of Pol III we wondered whether intestinal barrier function of ageing *C. elegans* is also protected by *rpc-1* RNAi. Additionally, we sought to identify *rpc-1* RNAi related phenotypes in the gut to support our finding that *rpc-1* is active in the gut during ageing. To that end we performed a "smurf assay" based upon published assays (Gelino *et al.*, 2016). Briefly, animals were fed blue dye for 3 hours at the desired age and scored for the degree to which the blue dye had invaded the body cavity (i.e passed through the gut barrier). Worms were imaged at 20X magnification on a light microscope and scored by a semi-quantative scale in which individual animals were rated from 0 to 4 for "smurfness", with 0 being no loss of gut integrity and 4 being the whole body cavity stained (Figure 5.4 A). As previously observed, we confirmed that in WT worms, the severity of the gut barrier dysfunction increased with time (Gelino *et al.*,2016). Interestingly, however, we observed that the gut barrier of worms treated with *rpc-1* responded differently to age with reduced susceptibility to total gut dysfunction (Figure 5.4 B). While this effect was modest, it demonstrates that *rpc-1* treatment of the whole animal protects against gut barrier dysfunction, a known pathology associated with ageing and lifespan. This was also the case in *D. melanogaster* (Filer *et al.*, 2017).

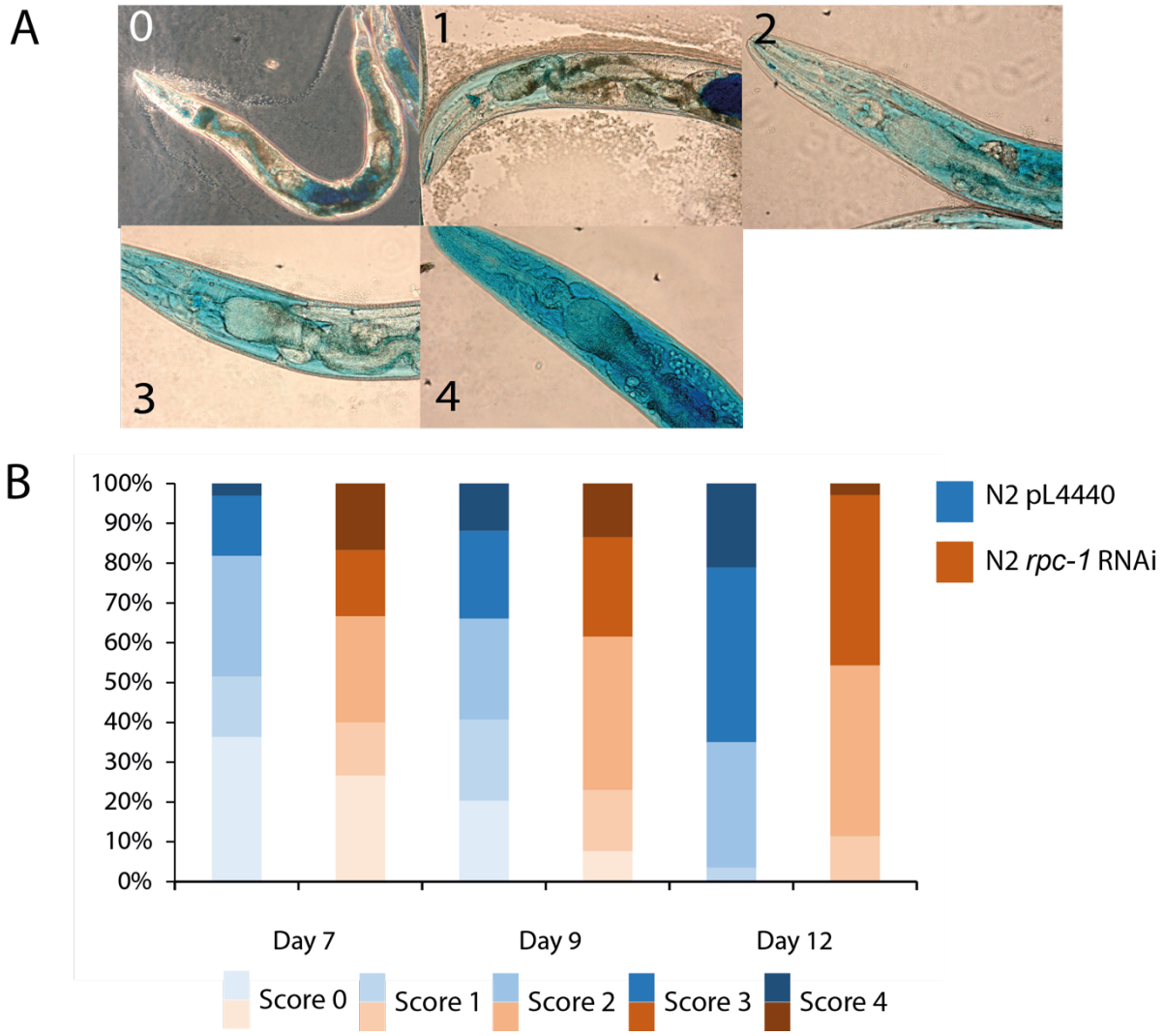

Figure 5.4: Gut barrier function is protected by *rpc-1* RNAi A) Representative images of the scoring system used to analyse the severity of the invasion of blue dye via the loss of gut barrier function. B) Pooled data of the population distribution of smurfness at day7, 9 and 12. RNAi against *rpc-1* reduces the severity of gut barrier dysfunction with age. (OLR; effect of age, P < 10−4; rpc-1 RNAi, P = 0.51; interaction, P = 0.01; left to right, c.i. = 5.0–31%, 16–50%, 24–48%, 25–51%, 53–78% and 34–66%, percentage of Smurf grades 3 and 4)

5.7. Inhibition of RNA polymerase III extends lifespan via a mechanism separate from S6K.

RNA polymerase III is known to be regulated by TORC1 by a process where TORC1 physically localises to Pol III target genes and activates transcription by Pol III via phosphorylation of components of the Pol III complex. In *D. melanogaster,* as part of this study, Danny Filer of the Alic lab at UCL has performed work which demonstrates that the longevity resulting from rapamycin treatment is not additive with that of Pol III inhibition, placing Pol III downstream of TOR in longevity. Ribosomal protein S6 kinase beta-1 (S6K1) is a serine threonine kinase which upon phosphorylation by TOR can phosphorylate eIF4B, recruiting it to the preinitiation complex (Holz *et al.*, 2005) and thus is required for initiation of translation. In *C. elegans* and mice, S6K1 orthologues are capable of extending lifespan when inhibited (Kapahi *et al.*, 2004; Selman *et al.*, 2009). Since S6K also limits lifespan downstream of TORC1, we sought to determine whether Pol III is involved in this same longevity regulating pathway, or whether it branches before S6K. In other words, we sought to establish whether S6K mediated life extension is separable from Pol III mediated longevity. *C. elegans* offers a good system for performing simple epistasis experiments through the wealth of readily available pre-existing mutants and RNAi clones. We performed an epistasis assay designed to determine the relationship between S6K and Pol III in the context of lifespan. We acquired the long-lived *C. elegans* S6 Kinase mutant strain *rsks-1(ok1255)* and treated these animals with either control or *rpc-1* RNAi as described previously. The results show that, alone, treatment with *rpc-1* RNAi and mutation of *rsks-1* extend lifespan by a similar degree (table 5.1, Fig 5.5). However, in *rsks-1* worms also treated with *rpc-1* RNAi, we see an additive lifespan extension roughly double that of either

treatment alone. This suggests that these interventions are likely to impact on different mechanisms of lifespan extension. Since previously published data implicates TORC1 in the regulation of both pathways (Holz *et al.*, 2005; Moir and Willis, 2013), these data suggest that S6K and Pol III influence lifespan through pathways downstream of TORC1 but separate from each other, as shown in the model in Fig 5.6. However, it is worth noting that while interpreting data based on partial knockdowns as with RNAi and partial loss of function mutation (e.g *rsks-1(ok1255)*) that effects being additive is not definite proof of separateness. Indeed, one could equally conclude that if both TOR and Pol III inhibit translation, that these interventions may be inducing incrementally more severe restriction of protein synthesis and producing an additive effect for that reason. Additionally, it remains to be demonstrated in *C. elegans* whether or not the observed relationship between TORC1 and Pol III is conserved from *D. melanogaster*.

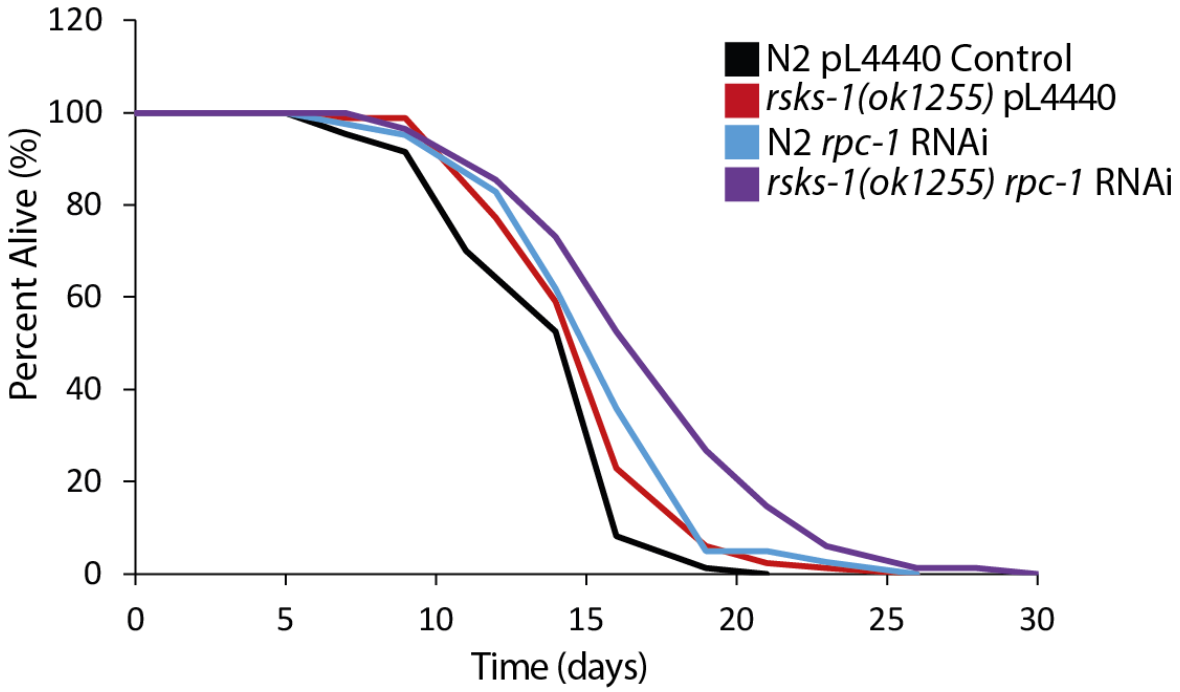

Figure 5.5: S6K and Pol III inhibition additively extend lifespan. A) *rsks-1(ok1255)* and *rpc-1* RNAi extend lifespan in an additive manner. L4 animals moved onto *rpc*-1 RNAi HT115(*de3)* or control HT115(*de3)* on day 0. Significant differences in all conditions except between N2 *rpc-1* and *rsks-1(ok1255)*. Significance by log rank test. Extensions = *rsks-1 (ok1255)* 9.96%, p=0.013; *rpc-1* RNAi 12.31%, p=0.0004; *rsks-1* + *rpc-1* 25.08%, $p=1x10^{-7}$. N=3, 2x repeats performed by Isabel Goncalves-Silva (data not shown).

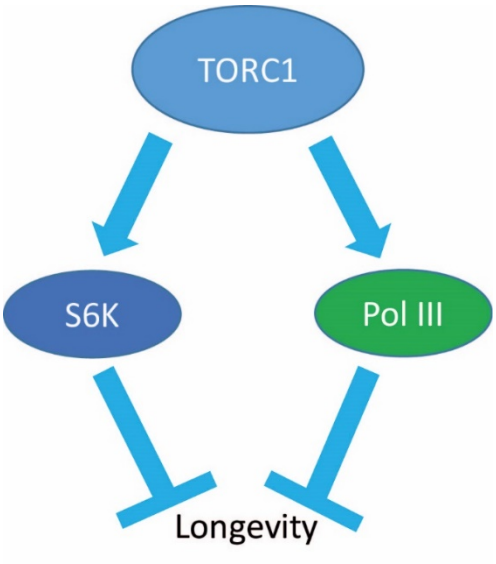

Figure 5.6: Model of the proposed relationship between TORC1, S6K and Pol III in limiting longevity.

5.8. Chapter 5 Discussion

We show here that inhibition of RNA Polymerase III is capable of extending the lifespan of wild-type N2 *C. elegans* at 20°C and 25°C (Figure 5.6). As detailed in the brief introduction section of this chapter, RNA polymerase III activity is essential for normal organismal function, with pol III products being required for numerous essential processes including the requirement for tRNAs in protein synthesis (Crick, 1968). It is therefore perhaps surprising that reducing ability of Pol III to manufacture these products would have a beneficial effect on organismal health and longevity since this intervention would appear to frustrate the efforts of the cell to perform its normal and essential functions. One explanation for this can be found in the concept of antagonistic pleiotropy, whereby genes which have a beneficial effect on development (specifically, which increase the chance of an organism successfully reproducing) then have a deleterious effect on fitness at later ages (Williams, 1957). RNA polymerase III is an attractive candidate for a gene which may be exerting antagonistically pleiotropic effects during ageing for several reasons. First, pol III is required for growth (Grewal, 2015), and pol III products are required for the prompt execution of protein synthesis, with tRNAs being essential for the process and reductions in the levels of 5s rRNA being associated with reduced protein synthesis (Dohme and Nierhaus, 1976) . A low level of pol III during growth would then render pol III rate limiting and as a result slow the development of the organism, exactly as we observed in Figure 5.5 and supported by the reduced protein levels seen in *D. melanogaster* with reduced Pol III activity (Filer *et al.*, 2017). This means that the organism is reaching reproductive maturity more slowly, and therefore is less likely to reproduce at all since these animals are subject to age independent

hazard for longer prior to reproduction - a particularly serious disadvantage when age independent hazard is high as it often is for wild organisms. It therefore seems likely that evolutionary pressure would seek to avoid this scenario. Secondly, it is already well known that interventions which reduce levels of translation/protein synthesis are often capable of extending mean lifespan (Hansen *et al.*, 2007), indeed Insulin/IGF1 activation, TOR pathway activation, the consumption of non-DR diets are all generally pro-growth activities which limit lifespan in model organisms and direct interventions against translation (e.g. by targeting S6K1 or EIF4a) also extend lifespan (Jia, 2004; Hansen *et al.*, 2007; Zid *et al.*, 2009). In support of translation being involved in our observations of Pol III limiting lifespan, our collaborators working with *D. melanogaster* observed a reduction in protein synthesis was observed as a result of Pol III inhibition, which is assumed to be the case in *C. elegans* as well. This sets up the two components of a gene which is antagonistically pleiotropic – optimising Pol III for rapid early growth and development is likely to exert a life limiting effect by hyperactive translation in later life. One possible mechanism of the lifespan extension observed in worms could therefore be reduced stress on the protein folding machinery leading to reduced proteotoxic stress. Indeed, *d*C52$^{-/+}$ animals are more resistant to tunicamycin – an inducer of proteotoxic stress which may suggest that the protein folding machinery and UPR less stressed in these animals (Filer *et al.*, 2017). Whether this is true in *C. elegans* remains to be demonstrated, and work on this project is ongoing in our lab.

Our data adds other work which highlights the *C. elegans* intestine as a key tissue during ageing of these animals. For example, the *C. elegans* intestine is particularly crucial in the life-extending activities of *daf-16* downstream of reduced insulin signalling, with *daf-16* expression in this tissue being capable of recapitulating much of the *daf-16* dependent aspect of *daf-2* longevity (Libina, Berman and Kenyon, 2003). Here we show a similar result,

that tissue specific inhibition of RNA polymerase III in the intestine is also capable of recapitulating much of the longevity seen with whole animal inhibition. Our data also supports that finding the *C. elegans* gut integrity declines with age and that at least some pro-longevity interventions in the gut protect gut barrier function (Gelino *et al.*, 2016).

We demonstrate here that in *C. elegans* inhibition of Pol III and the partial loss of function mutation of S6K result in an additive effect on longevity. This data can be interpreted as circumstantial evidence that these two molecules are separately regulating longevity downstream TORC1 as shown in the model in Figure 5.6. This is an attractive model since the mechanism of the regulation of S6K and Pol III by TORC1 are quite well characterised, and occur through separate mechanisms (Holz *et al.*, 2005; Michels *et al.*, 2010; Tsang, Liu and Zheng, 2010; D. Chen *et al.*, 2013; Moir and Willis, 2013). However, caution is needed in drawing significant conclusions from this experiment pending the results of further research taking place in our lab for two reasons. Firstly, both *rsks-1(ok1255)* and *rpc-1* RNAi treatment are partial knockdowns which do not fully abrogate the function of *rpc-1* or *rsks-1* products. Therefore, the cumulative effect observed could simply be due to incrementally more serious inhibition of the same pathway resulting in an increased response. This is exemplified by the fact that, for example, treating a *daf-2(1368)* partial loss of function mutant for the *C. elegans* insulin receptor with RNAi against the mRNA of the same *daf-2* gene can further increase the lifespan of the mutant and result in further decreased insulin signalling, phenocopying more severe mutants of *daf-2* (Yamawaki *et al.*, 2008). In the aspect of our study which was performed in *D. melanogaster*, however, the Alic Lab were able to exclude this possibility by demonstrating that S6K phosphorylation was reduced by rapamycin treatment but not by heterozygous deletion of Pol III. Furthermore, Pol III inhibition in *C. elegans* has not yet been found to be downstream of TORC1 as it is in flies.

Given the high conservation of TOR, S6K and Pol III, it is a reasonable hypothesis that it may be, but pending more data caution is required when drawing major conclusions from the epistasis experiments which we were able to perform with the time available.

Finally, taken together with the work performed in flies for this project the data shown here forms part of a larger picture in which reduced TORC1 signalling regulates S6K and Pol III separately in order to extend lifespan. One major issue with TORC1 inhibition as a strategy for treating diseases of ageing is the possibility of side effects given the central role of TORC1 in the regulation of metabolism. In *C. elegans* and *D. melanogaster* reduced TORC1 function has serious side effects; in *C. elegans* increased developmental arrest and intestinal atrophy result from severe TOR inhibition (Long *et al.*, 2002) and rapamycin treatment of *D. melanogaster* reduces fecundity (Bjedov *et al.*, 2010). However, inhibition of Pol III in flies does not have an effect of fertility, suggesting that at least some of the toxicity associated with inhibition of TORC1 may be absent with inhibition of Pol III. This study suggests that Pol III or downstream processes may be a useful target for interventions against the diseases of ageing and contributed to our understanding of how longevity is determined.

6.1. Concluding Remarks

In the first two results chapters, various aspects of *skn-1b* function are examined. This is of relevance in ageing research due to the total requirement of *skn-1b* for some DR protocols (Bishop and Guarente, 2007b). The data shown here contributes useful datapoints and conclusions to the field. Firstly, we shed more light on the activity and expression of *skn-1b*. It has been noted by ourselves and other that SKN-1B protein is lacking certain key domains (Figure 3.4), including all three transactivation domains identified in the initial characterisation of SKN-1C (Walker *et al.*, 2000). In Chapter three we provide some evidence that SKN-1B protein is a functional transcription factor capable of driving transcription from the consensus SKN-1 binding site (TTATCAT) (Figure 3.4) (Blackwell *et al.*, 1994; Walker *et al.*, 2000). Also in this chapter we confirm previous predictions about the regulation of *skn-1b* by demonstrating that the unique *skn-1b* promoter predicted by the 5' RACE is indeed active in the ASIs in normal conditions, with our *wuEx217(Pskn-1b::skn-1b::GFP)* transgene being expressed in ASI neurons under the control of this promoter, and we confirm this by CRISPR tagging of endogenous *skn-1b* with *wrmScarlet* (Figure 3.1). Supporting and expanding on the conclusion that *skn-1b* can be separately regulated from other isoforms, these data show a surprising lack of overlap of function between *skn-1b* and *skn-1a/c*. In particular, *skn-1b* is not required for development while other isoforms are (Figure 3.6), *skn-1b* does not affect brood size while other isoforms do (Figure 3.6) and *skn-1b* does not regulate normal lifespan, while other isoforms do (Figure 3.7). With regards to the role of *skn-1* in the oxidative stress response we show that *skn-1b* appears transcriptionally upregulated in response to this stress (like other isoforms) but that this is dispensable for

lifespan (unlike other isoforms) (Figure 3.8). We also observe that expression patterns of *skn-1b* change in response to BD (Figure 3.2), which we show causes a 1.4 fold induction of SKN-1B::GFP (similar to that achieved by IDR – Jennifer Tullet unpublished data) in these animals. Significantly, *skn-1b* expression is also induced in the ADL neurons in response to BD (Figure 3.3) – the ADL neurons have not previously been implicated in a DR response but have been implicated in the regulation of longevity by *daf-2* independent mechanisms (Lans and Jansen, 2007).

The observation that *skn-1b* is responsive to the withdrawal of food is further developed by data which supports a model of where *skn-1b* regulation is mediated by external signals about food abundance rather than internal signals about DR such as energy or nutrient levels. Data shown here demonstrates that while BD causes an induction of SKN-1B::GFP, the genetic model of DR *eat-2(ad1116)* does not (Figure 3.2), albeit with further work required to ascertain whether DR induces *skn-1b::*GFP in an *eat-2* background. Since *eat-2(ad1116)* animals are grown on ample bacteria, this suggests that sensation of food is what induces SKN-1B::GFP. This is also intuitive since the ASI and ADL neurons are part of the amphid sensilla and each have known roles in chemosensation (Bargmann and Horvitz, 1991; Hukema *et al.*, 2006; Lans and Jansen, 2007). Due to a confluence of phenotypes between *daf-11* and *skn-1b*, this guanylate cyclase was investigated as a possible upstream regulator of *skn-1b.* We demonstrate that *daf-11* is required for normal SKN-1B::GFP levels and that loss of *daf-11* prevents transcriptional upregulation of *skn-1b* resulting from the withdrawal of food (Figure 4.5). This indicates that *daf-11* is required for internalisation of external signals in the regulation of *skn-1b.* This is supported by data on plate exploration from Nikolaos Tataridas-Pallas which shows that *daf-11* animals and *skn-1b* animals each experience similar and non-additive defects in plate exploration. Possible downstream *skn-*

1b targets were investigated and we report here changes in the expression transcriptional reporters for the ILPs *daf-28 and ins-7* and of the TGF-β ligand *daf-7* resulting from *skn-1b* deletion (Figure 4.1, 4.2 and 4.3 respectively). With regards to the *daf-28* data, our transcriptional reporter showed a total lack of fluorescence in a *skn-1b* background (Figure 4.1) but we have been unable to reproduce this by qPCR (albeit with high variation as discussed) and we additionally do not observe the *daf-c* phenotype which this would seem to predict. Therefore, this data is to be treated with a degree of caution although what could cause such a radical change this reporter's activity in our *skn-1b* animals is unclear. The changes in *ins-7* expression in the intestine seem to suggest a cell non-autonomous, positive regulation of insulin signalling in the intestine of these animals (Figure 4.2). A more unbiased approach to ILP expression data would be preferable and unfortunately our attempts at this were unsuccessful due to high internal variation in our samples, however RNA-Seq is being undertaken in our lab to resolve this. In contrast to our *daf-28* data, the increase in *daf-7* expression which we report (Figure 4.3) is supported by phenotypes. First, increased *daf-7* levels are known to increase quiescence (You *et al.*, 2008) and recent data from Nikolaos Tataridas-Pallas demonstrates that this is indeed the case. Further data of Nikolaos's demonstrates that *daf-7(e1372)* and *skn-1b(tm4241)* animals experience severe defects in exploratory behaviour and that this effect is non-additive (Fig S5). This data implies that *skn-1b* negatively regulates *daf-7* in what is likely an indirect effect, and we suggest a negative feedback model for *skn-1b* regulation of *daf-7.*

Taken together, these data suggest a model where *skn-1b* integrates information about the external food environment into the DR longevity response by regulation of secreted neuropeptides.

In the third results chapter we examine the role played by RNA polymerase III in ageing. This study grew out of the initial observation in the Alic lab that inhibition of RNA polymerase III was capable of extending lifespan in *D. melanogaster*, and we were able to demonstrate that the same is true in *C. elegans* (Figure 5.2) while the Heinemann lab showed this same in yeast. This effect is reproducible by tissue specific RNAi of Pol III in the intestine (Figure 5.3), and gut integrity is preserved in animals experiencing Pol III inhibition suggesting that this lifespan extension is mediated by the effect of Pol III inhibition in the intestine (Figure 5.4). Pol III inhibition and S6K mutation (partial loss of function) produce an additive lifespan extension (Figure 5.5), and at least in *D. melanogaster* Pol III inhibition extends lifespan downstream of TORC1, suggesting that Pol III limiting lifespan via a separate mechanism from S6K downstream of TORC1 (Figure 5.6). This work suggests that Pol III may be a potential drug target for intervening against diseases of ageing, and highlights Pol III activity as an important progeric process downstream of TORC1 activity. It is too early to say whether any treatments targeting Pol III in mammals could be effective, but worthy of note is the fact that Pol III has been highlighted recently as a cancer drug target, so Pol III inhibiting drugs which are safe for human consumption are likely to be increasingly available.

These projects attempt to shed more light on the ageing process in *C. elegans*, with both *skn-1b* and reduced TOR signalling (which regulates RNA Polymerase III) both being required for the response to dietary restriction in *C. elegans*. While relatively distinct from each other, both aspects of this project contribute to our understanding of how key molecules in the ageing process are able to achieve their effects, first by describing in more detail the function and regulation of *skn-1b* and how it responds to reduced food levels, and second by identifying RNA Polymerase III as an important mediator of longevity (Grewal, 2015).

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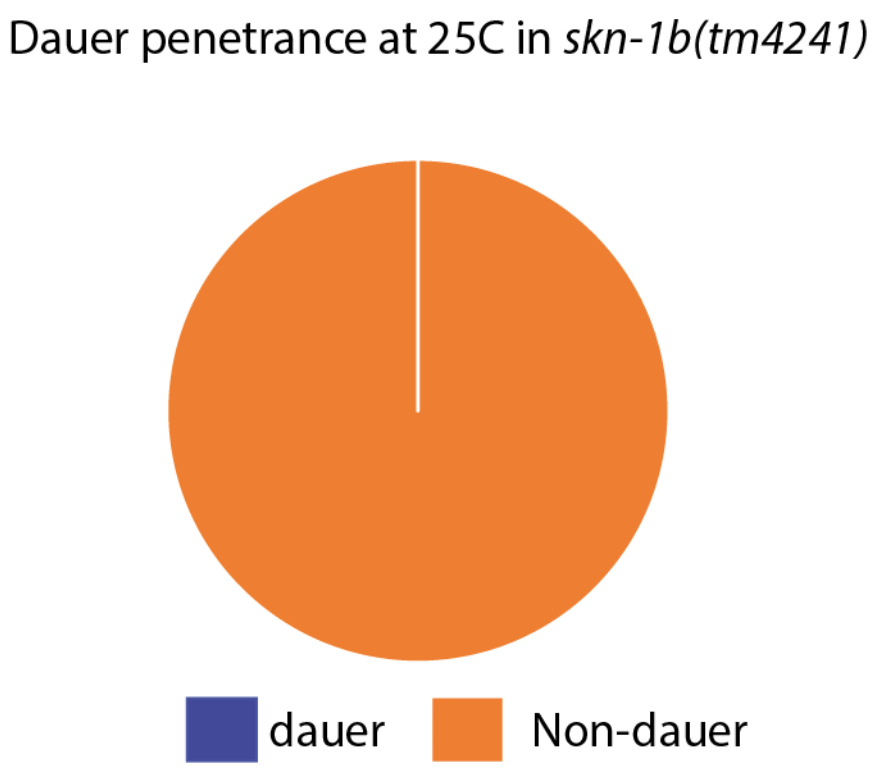
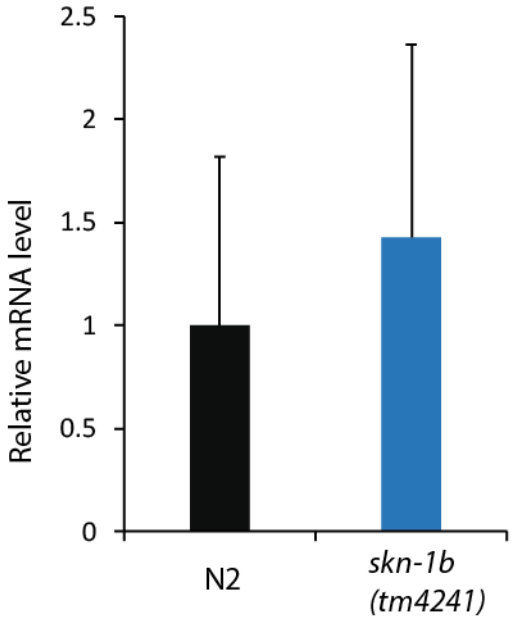
Appendices

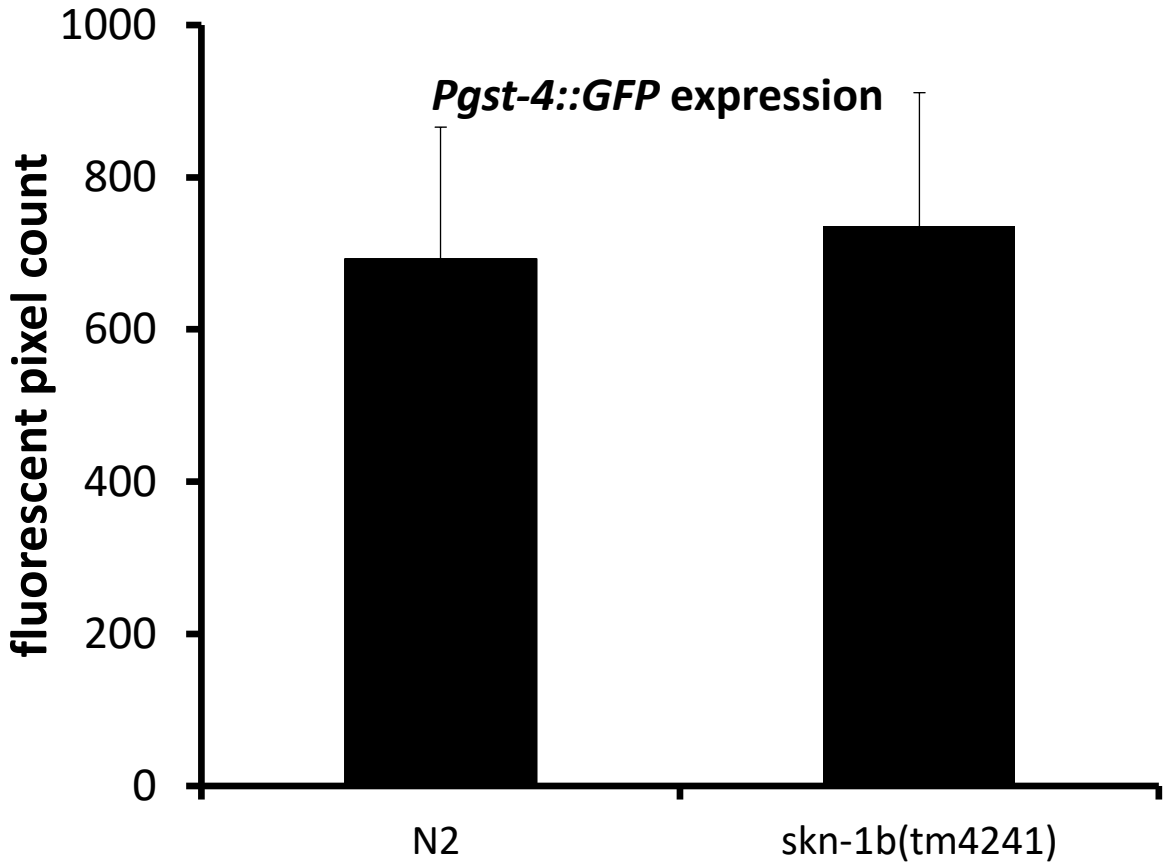


Supplemental Figure 1: Dauer penetrance at 25C in the *skn-1b(tm4241)* mutant strain: Animals were grown from eggs at 25C, N=3, 25 animals per trial.

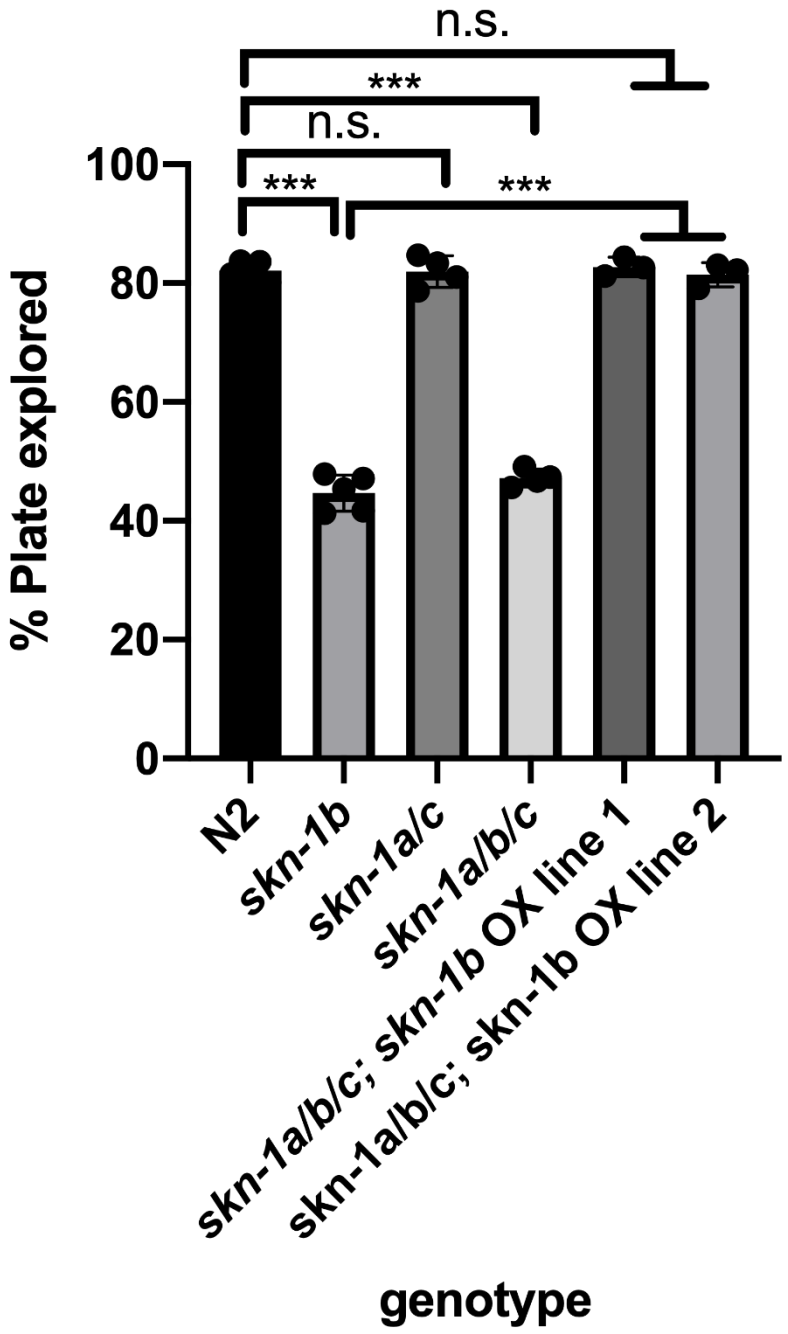


	N2	N2 Standard Dev	*skn-1b(tm4241)*	*skn-1b(tm4241)* Standard Dev	Significance (p=)
daf-28	1	0.81	1.43	0.93	0.29

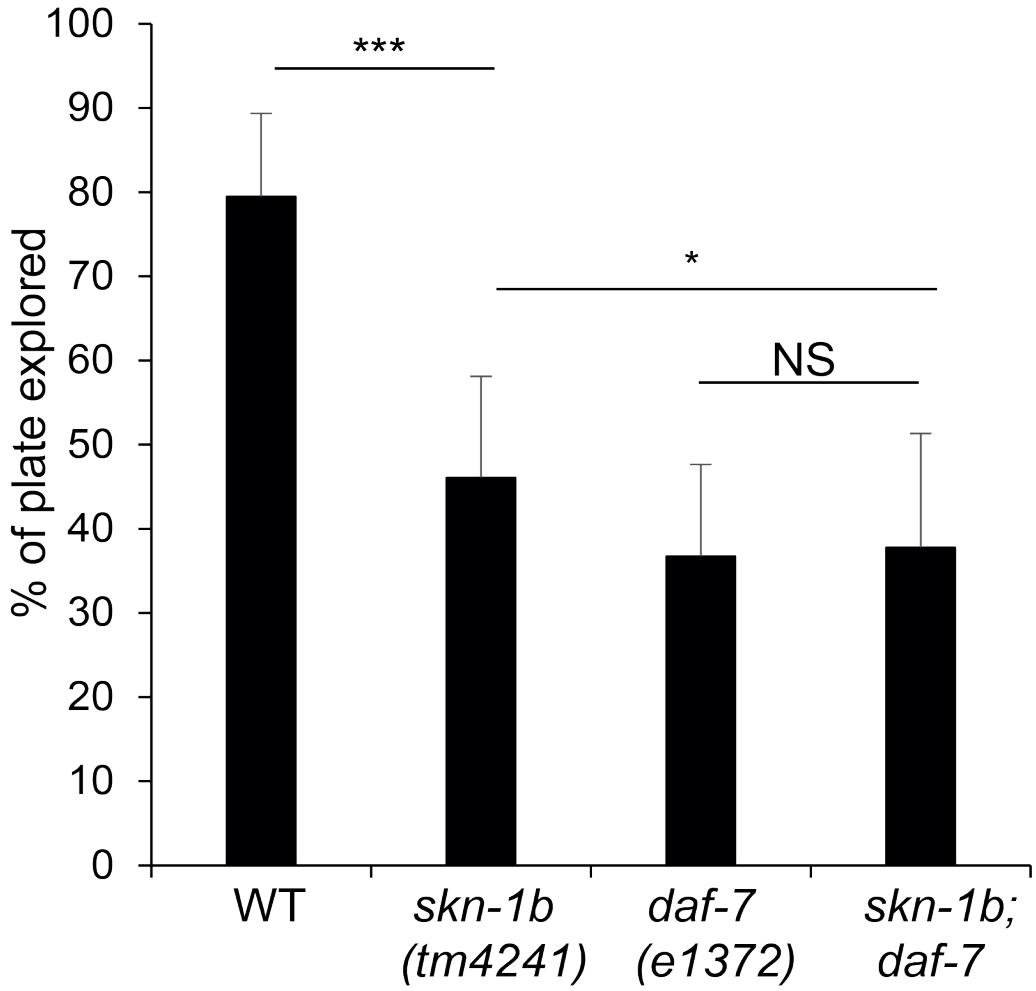
Supplemental Figure 2: *daf-28* qPCR. qPCR showing *daf-28* mRNA levels in N2 and *skn-1b(tm4241)* animals. N2 = 11 samples, *skn-1b(tm4241)* = 10 samples. However, variation remains high.



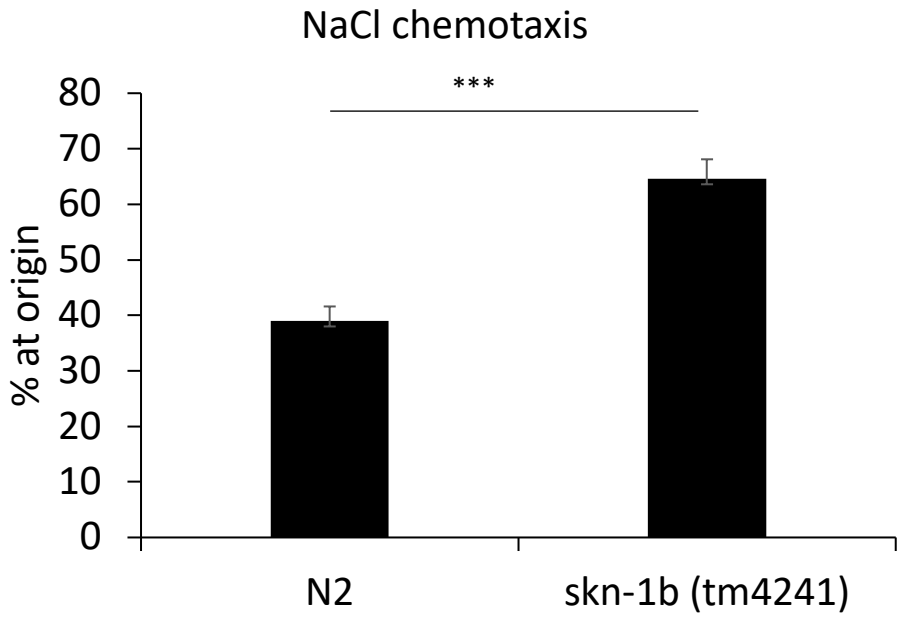
Supplemental Figure 3: Effect of skn-1b on a canonical skn-1 target: *Pgst-4::GFP* intensity is not changed in a *skn-1b(tm4241)* genetic background. N=3, total number of animals N=24, *skn-1b* = 34 n.s. by unpaired t-test.



Supplemental Figure 4: *skn-1b* plate exploration phenotype: Plate exploration data for *skn-1* mutants recorded by Nikolaos Tataridas-Pallas. Loss of *skn-1b* markedly decreases plate exploration while loss of *skn-1a/c* does not. Furthermore, loss of all *skn-1* isoforms recapitulates the phenotype and this can then be rescued by introducing the WuEx252 *pskn-1b::skn-1b::GFP* transgene. N=3/4 for each condition and each trial contains 15-25 animals.



Supplemental Figure 5: *daf-7* and *skn-1b* plate exploration epistasis. Plate exploration data for *skn-1* and *daf-7* mutants recorded by Nikolaos Tataridas-Pallas. Defects in plate exploration are similar in *skn-1b* and *daf-7* animals, and these effects are not additive in *skn-1b(tm4241); daf-7(e1372)*.



Supplemental figure 6: Salt chemotaxis assay of *skn-1b*. Salt chemotaxis data for N2 and *skn-1b* animals recorded by Nikolaos Tataridas-Pallas. Animals carrying the *skn-1b* mutation remain at the origin at a higher rate that N2 animals when a spot of 5mM NaCl is added to the periphery of the plate. N=3, each trial consists of approximately 100 animals on 3 plates. Significance by students t test (p=0.0007).

Trial	Strain	Genotype	Mean Lifespan (days)	Temp (°C)	Extension (%)	Significance (p) Log rank test vs X	n dead (total)
1	N2		24.93	15	0	GA1058: 0.14 DR1572: 0.1	38 (76)
1	GA1058	*skn-1b(tm4241)*	22.24	15	-8.22	DR1572: 2E-05 GA1060: 4E-05	68 (86)
1	DR1572	*daf-2(e1368)*	28.02	15	12.4	GA1060: 0.35	69 (102)
1	GA1060	*daf-2; skn-1b*	27.27	15	9.3	N2: 0.052	63 (84)
1	N2		20.66	20	0	GA1058: 0.64 DR1572: 9.7E-06	44 (50)
1	GA1058	*skn-1b(tm4241)*	19.95	20	-3.43659	DR1572: 3.6E-06 GA1060: 5.6E-09	53 (72)
1	DR1572	*daf-2(e1368)*	30.45	20	47.38625	GA1060: 0.58	45 (92)
1	GA1060	*daf-2; skn-1b*	29.57	20	43.12682	N2: 7.7E-07	57 (73)
2	N2		25.29	20		GA1058: 0.01 DR1572: 5E-07	88 (125)
2	GA1058	*skn-1b(tm4241)*	22.46	20	-11.2	DR1572: 0 GA1060: 6E-07	78 (112)
2	DR1572	*daf-2(e1368)*	34.04	20	34.6	GA1060: 0.46	68 (94)
2	GA1060	*daf-2; skn-1b*	32.47	20	28.4	N2: 0.0001	38 (59)
1	N2		12.99	25		GA1058: 0.97 DR1572: 0	68 (79)
1	GA1058	*skn-1b(tm4241)*	13.26	25	2.1	DR1572:0 GA1060: 0	96 (100)
1	DR1572	*daf-2(e1368)*	21.32	25	64.1	GA1060: 0.0093	68 (94)
1	GA1060	*daf-2; skn-1b*	20.02	25	54.1	N2: 0	72 (98)
2	N2		13.07	25		GA1058: 0.055 DR1572: 6.1E-09	81 (88)
2	GA1058	*skn-1b(tm4241)*	13.63	25	4.3	DR1572: 8.6E-09 GA1060: 6.00E-09	107 (119)
2	DR1572	*daf-2(e1368)*	17.47	25	33.7	GA1060: 0.11	36 (38)
2	GA1060	*daf-2; skn-1b*	16.31	25	24.8	N2: 0	52 (57)

Trial	Strain	Genotype	Mean Lifespan (days)	Temp (°C)	Extension (%)	Significance (p)	n dead (total)
1	N2		28.87	15	0	GA1058: 0.0015 DR1574: 1.8E-06	120(127)
1	GA1058	skn-1b(tm4241)	24.99	15	-13.4	DR1574: 5.2E-09 JMT5: 3.6E-08	80 (98)
1	DR1574	daf-2(e1391)	40.54	15	40.4	JMT5: 0.15	45(57)
1	JMT5	daf-2; skn-1b	36.57	15	26.67	N2: 2.5E-05	82(100)
2	N2		28.05	15	0	GA1058: 0.26 DR1574: <1E-10	79 (105)
2	GA1058	skn-1b(tm4241)	26.51	15	-5.5	DR1574: <1E-10 JMT5: <1E-10	66 (87)
2	DR1574	daf-2(e1391)	41.55	15	48.1	JMT5: 0.03	99 (123)
2	JMT5	daf-2; skn-1b	49.04	15	74.8	N2: <1E-10	28 (38)
1	N2		23.05	20	0	GA1058: 0.17 DR1574: <1E-10	79 (92)
1	GA1058	skn-1b(tm4241)	23.46	20	1.8	DR1574: <1E-10 JMT5: <1E-10	89 (96)
1	DR1574	daf-2(e1391)	44.35	20	92.4	JMT5: 0.10	77 (102)
1	JMT5	daf-2; skn-1b	39.12	20	69.7	N2: <1E-10	58 (78)
2	N2		23.77	20	0.0	GA1058: 3.8E-06 DR1574: <1E-10	89 (93)
2	GA1058	skn-1b(tm4241)	19.8	20	-16.7	DR1574: <1E-10 JMT5: <1E-10	53 (57)
2	DR1574	daf-2(e1391)	39.73	20	67.1	JMT5: 0.006	89 (139)
2	JMT5	daf-2; skn-1b	33.54	20	41.1	N2: 3.2E-08	60 (82)
4*	N2		28.87	15	0	GA1058: 0.02 GA82: 3E-04	120 (127)
4*	GA1058	*skn-1b(tm4241)*	24.99	15	-13.4	GA82: 1.4E-07 GA1059: 0.2	80 (98)
4*	GA82	*daf-2(e1370)*	34.98	15	21.2	GA1059: 1.4E-07	39 (66)
4*	GA1059	*daf-2;skn-1b*	26.31	15	-8.9	N2: 0.08	58 (86)
3*	N2		23.05	20	0	GA1058: 0.17 GA82: <1E-10	79(92)
3*	GA1058	*skn-1b(tm4241)*	23.46	20	1.8	GA82: <1E-10 GA1059: <1E-10	89 (96)
3*	GA82	*daf-2(e1370)*	37.28	20	61.7	GA1059: <1E-10	100 (157)
3*	GA1059	*daf-2;skn-1b*	21.38	20	-7.2	N2: 0.31	85 (88)

Trial	Strain	Genotype	Mean Lifespan (days)	Temp (°C)	Extension (%)	Significance (p)	n dead (total)
1	N2		14.92	25	0	GA1058: 2E-04 GA82: <1E-10	104 (106)
1	GA1058	*skn-1b(tm4241)*	16.49	25	10.5	GA82: <1E-10 GA1059: <1E-10	86 (95)
1	GA82	*daf-2(e1370)*	33.2	25	122.5	GA1059: <1E-10	41 (46)
1	GA1059	*daf-2;skn-1b*	22.15	25	48.5	N2: <1E-10	64 (69)
2	N2		12.68	25	0	GA1058: 0.16 GA82: <1E-10	89 (101)
2	GA1058	*skn-1b(tm4241)*	13.05	25	2.9	GA82: <1E-10 GA1059: 0.34	85 (93)
2	GA82	*daf-2(e1370)*	29.49	25	132.6	GA1059: <1E-10	51 (61)
2	GA1059	*daf-2;skn-1b*	13.52	25	6.6	N2: 0.12	91 (98)
1	N2		30.42	15	0	JMT31: 4.2E-09 JMT32: 0.006	79 (115)
1	GA1058	*skn-1b(tm4241)*	-	15	-	discontinued	0
1	JMT31	*daf-2(e1370)*	45.94	15	51.0	JMT32: 2E-04	33 (48)
1	JMT32	*daf-2; skn-1b*	35.81	15	35.81		55 (75)
2	N2		30.67	15	0	GA1058: 0.08 JMT31: 2.3E-06	31 (43)
2	GA1058	*skn-1b(tm4241)*	27.63	15	-9.9	JMT31: <1E-10 JMT32: <1E-10	51 (82)
2	JMT31	*daf-2(e1370)*	40.86	15	33.2	JMT32: 0.2	49 (64)
2	JMT32	*daf-2; skn-1b*	43.42	15	41.2	N2: 2.5E-09	57 (85)
3	N2		31.49	15	0	GA1058: 0.19 JMT31: 3.6E-06	61 (73)
3	GA1058	*skn-1b(tm4241)*	27.49	15	-12.8	JMT31: 6.2E-07 JMT32: 1.5E-05	33 (44)
3	JMT31	*daf-2(e1370)*	44.50	15	41.3	JMT32: 0.12	24 (38)
3	JMT32	*daf-2; skn-1b*	39.32	15	24.9	N2: 3E-03	54 (79)
1	N2		22.27	20	0	GA1058: 0.1 JMT31: <1E-10	77 (105)
1	GA1058	*skn-1b(tm4241)*	22.64	20	1.7	JMT31: <1E-10 JMT32: <1E-10	78 (93)
1	JMT31	*daf-2(e1370)*	40.25	20	80.7	JMT32: 0.3	71 (88)
1	JMT32	*daf-2; skn-1b*	36.97	20	66	N2: <1E-10	104 (116)
2	N2		22.25	20	0	GA1058: 0.1 JMT31: <1E-10	58 (69)
2	GA1058	*skn-1b(tm4241)*	19.86	20	-1.7	JMT31: <1E-10 JMT32: <1E-10	43 (53)
2	JMT31	*daf-2(e1370)*	39.26	20	76.4	JMT32: 0.26	74 (85)
2	JMT32	*daf-2; skn-1b*	36.48	20	64	N2: <1E-10	62 (69)

*: as preliminary data for this project Jennifer Tullet performed 3 x 15°C *daf-2(e1370);skn-1b(tm4241)* assays and 2x 20°C *daf-2(e1370);skn-1b(tm4241)* assays with the pre-backcrossed strains and the same patterns of results, and these lifespans were considered together with my own in our observations. JMT31/JMT32 are the backcrossed lines, GA82 and GA1059 are pre-backcross.

Trial	Strain	Genotype	Mean Lifespan (days)	Temp (°C)	Extension (%)	Significance (p)	n dead (total)
1	N2		13.09	25	0	GA1058: 0.11 JMT31: <1E-10	73 (85)
1	GA1058	*skn-1b(tm4241)*	12.82	25	-2.1	JMT31: <1E-10 JMT32: 7.8E-08	86 (90)
1	JMT31	*daf-2(e1370)*	25.97	25	98.4	JMT32: 0.015	93 (100)
1	JMT32	*daf-2; skn-1b*	21.9	25	67.3	N2: <1E-10	78 (89)

Table S1: Epistasis analysis of *daf-2* and *skn-1b*: All lifespans for *daf-2(e1368), daf-2(e1370) and daf-2(e1391)* epistasis with *skn-1b(tm4241)*. Notably the *daf-2(e1370)* strain GA82 and the *daf-2(e1370);skn-1b(tm4241)*. All statistics by Log-Rank test.

Trial number	Strain	Treatment	Mean life span	Temp (°C)	Extension (%)	p value	n dead (total)	n censored due to gut explosions
1	N2	Control RNAi	13.01	25°C			54 (60)	0
1	N2	*rpc-1* RNAi	14.31	25°C	10.0	0.009	62 (77)	2
2	N2	Control RNAi	14.09	25°C			85 (127)	4
2	N2	*rpc-1* RNAi	15.55	25°C	10.4	0.002	101 (143)	2
1	N2	Control RNAi	20.72	20°C			59 (100)	
1	N2	*rpc-1* RNAi	28.14	20°C	35.81	<0.001	22 (100)	
2	N2	Control RNAi	21.86	20°C			48 (100)	
2	N2	*rpc-1* RNAi	27.10	20°C	23.97	<0.001	29 (100)	
3	N2	Control RNAi	21.27	20°C			107 (200)	
3	N2	*rpc-1* RNAi	27.55	20°C	29.52	<0.001	51 (200)	
4	N2	Control RNAi	21.96	20°C			52 (86)	28
4	N2	*rpc-1* RNAi	24.67	20°C	12.3	0.032	21 (94)	61
1	N2	Control RNAi no FUDR	24.71	20°C			42 (100)	
1	N2	*rpc-1* RNAi no FUDR	27.77	20°C	12.38	<0.001	35 (100)	
2	N2	Control RNAi no FUDR	24.67	20°C			26 (100)	
2	N2	*rpc-1* RNAi no FUDR	29.43	20°C	19.29	0.002	23 (100)	
1	VP303	Control RNAi	16.887	20°C			52 (90)	31
1	VP303	*rpc-1* RNAi	18.92	20°C	12.1	0.024	29 (67)	27
2	VP303	Control RNAi	16.36	20°C			57 (84)	20

2	VP303	*rpc-1* RNAi	19.06	20°C	16.5	<0.001	50 (106)	45
1	VP303	Control RNAi	12.48	25°C			78 (84)	6
1	VP303	*rpc-1* RNAi	13.86	25°C	11.05	0.009	97 (103)	6
2	VP303	Control RNAi	12.42	25°C			79 (79)	0
2	VP303	*rpc-1* RNAi	13.99	25°C	12.64	0.001	80 (83)	3
1	N2	Control RNAi	14.15	25°C		*rpc-1: 0.0004*	75 (87)	7
1	N2	*rpc-1* RNAi	15.56	25°C	9.96	*rsks-1 rpc-1: 0.002*	83 (85)	
1	*rsks-1(ok1266)*	Control RNAi	16.05	25°C	13.42	N2: 0.013	79 (85)	3
1	*rsks-1(ok1266)*	*rpc-1* RNAi	17.70	25°C	25.08	*rsks-1: 0.0001*	82 (86)	4

Table S2: RNA polymerase III lifespans: All lifespans in the Pol III section. Three additional repeats of the *rpc-1* RNAi vs *rsks-1* epistasis experiment were performed by Isabel Goncalves-Silva.

Neuropeptide	N2	*skn-1b(tm4241)*	Fold change (*skn-1b/N2*)	Significance (p=)
daf-28	0.0070	0.0203	2.9175	0.0789
ins-1	0.0037	0.0083	2.2284	0.2596
ins-2	0.0429	0.0578	1.3465	0.6365
ins-3	0.0844	0.0676	0.8000	0.7083
ins-4	0.0751	0.0686	0.9146	0.8962
ins-5	0.0751	0.0686	0.9146	0.8962
ins-6	0.0044	0.0085	1.926	0.1761
ins-7	0.0053	0.0059	1.1070	0.780
ins-8	0.0074	0.0013	0.1750	0.4208
ins-9	0.0205	0.0107	0.5207	0.5076
ins-10	0.0410	0.0940	2.2790	0.2250
ins-11	failed			
ins-12	0.0729	0.1130	1.5500	0.4400
ins-13	0.1133	0.0524	0.4631	0.4840
ins-14	0.0016	0.0010	0.6171	0.5567
ins-15	0.0510	0.0133	0.2603	0.4205
ins-16	0.1508	0.0370	0.2456	0.4206
ins-17	0.4263	0.9606	2.2536	0.2570
ins-18	0.0298	0.0721	2.4154	0.2601
ins-19	failed			
ins-20	0.0019	0.0007	0.3622	0.4490
ins-21	0.2069	0.0281	0.1360	0.3403
ins-22	0.0809	0.1508	1.8633	0.1840
ins-23	0.1015	0.0268	0.2639	0.3747
ins-24	0.3615	0.2656	0.7347	0.6959
ins-25	0.0064	0.0033	0.5198	0.2685
ins-26	0.0105	0.0161	1.5355	0.1269
ins-27	1.8978	3.8186	2.0121	0.3768
ins-28	0.1374	0.2732	1.9883	0.0863
ins-29	0.2464	0.4530	1.8387	0.3875
ins-30	0.0011	0.0022	2.0741	0.1629
ins-31	0.0099	0.0024	0.2439	0.2651
ins-32	0.0409	0.0176	0.4304	0.5343
ins-33	0.0630	0.1232	1.9563	0.1128
ins-34	0.0751	0.0505	0.6732	0.5904
ins-35	0.3839	0.5580	1.4535	0.5753
ins-36	0.0057	0.0330	5.8002	0.1068
ins-37	0.5521	1.2812	2.3208	0.1362
ins-38	0.0585	0.0180	0.3073	0.4564
ins-39	0.0027	0.0032	1.1798	0.7626
daf-7	0.0438	0.0265	0.6054	0.3790

Table S3: qPCR performed by qStandard screening for ILP changes. 6 RNA samples per strain were analysed, no changes were significant, however even tenfold changes here do not achieve statistical significance, likely due to low levels of expression, sometimes in only two cells. We were not able to draw conclusions either way from this data.