

# Characterization of *pha-4* and *skn-1b* as possible targets of the E3 ubiquitin ligase WWP-1

Amrit Sareen

## Abstract

WWP-1 is a HECT domain E3 ubiquitin ligase that has been previously shown to be a positive regulator of lifespan in the tiny nematode *Caenorhabditis elegans* (Carrano et al, unpublished data). WWP-1 acts specifically in the diet restriction mediated longevity pathway and is not dependent on the transcription factor DAF-16. Two studies show that the transcription factor genes *skn-1* and *pha-4* mediate dietary restriction induced longevity leading us to characterize these as possible targets of WWP-1. Using lifespan analysis we find that *skn-1b* RNAi does not suppress *eat-2* based (DR equivalent) longevity in worms as we had originally anticipated. To directly determine the role of *pha-4* and *skn-1b*, we will establish an in vitro ubiquitination assay and continue lifespan studies on *pha-4* RNAi.

## Introduction

### AGING AND DIETARY RESTRICTION

Human aging is a vastly complex phenomenon governed by various molecular pathways and biochemical events. In 1935, McCay and colleagues observed that rats which were given less food lived approximately 30% longer<sup>1</sup>. In the years that followed, this type of diet –restricted–induced longevity has been studied in many organisms including yeast, worms, flies, rodents, and possibly primates. Today, it is common knowledge that calorie restriction, without malnutrition not only extends the maximum lifespan of the mentioned organisms, but also delays the onset and progression of age-related diseases such as cancer, autoimmune disorders and diabetes.

The nematode *Caenorhabditis elegans* is well suited for studying aging processes due to its short lifespan, small size, genetic tractability, ease of availability and easy knockout of genes using a

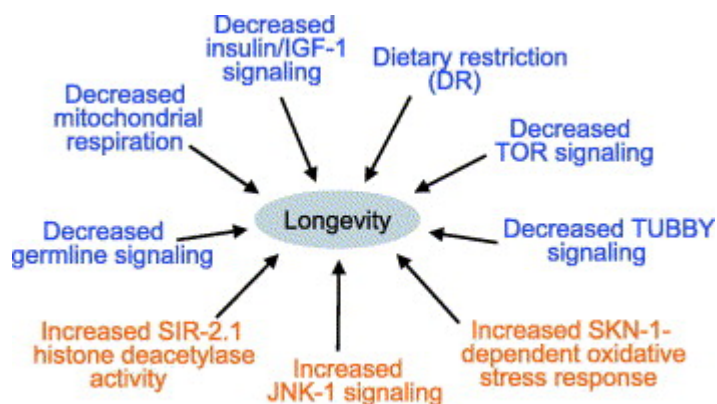


Figure 1: Mechanisms contributing to the lifespan of *C.elegans*.

Image courtesy: Schaffitzel et al. doi:10.1016/j.exger.2006.02.008  
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fully sequenced genome. Besides dietary restriction (DR), several other evolutionary conserved mechanisms have been found to contribute to the cascade of events that regulate the life span of *C.elegans* (Figure 1). Of all these regulatory systems, the well characterized insulin-/IGF-1-like signaling (IIS) pathway is a major determinant of life span and this pathway is heavily dependent on DAF-16<sup>2</sup>, a FOXO transcription factor. DAF-16 also influences the expression of a variety of genes involved in the germline-, TOR-, TUBBY-, JNK-1 signaling mechanisms as well as in SIR2 activity<sup>3,4,5</sup>.

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Unlike the IIS signaling mechanism, however, DR and the mitochondrial respiration pathway are not dependent on DAF-16.

DR requires another forkhead transcription factor in the FOXA family, PHA-4<sup>6</sup> and also requires *skn-1* which is an NF-E2 transcription factor<sup>7</sup>. The mitochondrial respiration pathway does not require DAF-16, but, unlike DR, which affects lifespan during adulthood, the effects of respiratory chain inhibition are seen during early development<sup>8</sup>.

### WWP-1 MEDIATES DR LONGEVITY

The cell has an intricate “recycling machinery”. If proteins are misfolded, or are no longer needed by the cell, they are tagged with ubiquitin using a cascade of enzymes called E1, E2, and E3. E1 is the ubiquitin activating enzyme and activates free ubiquitin monomers. E1 then transfers ubiquitin to the active site cysteine of E2 – the ubiquitin conjugating enzyme. The final step of the ubiquitylation cascade, in general, requires the activity of one of the hundreds of E3 ubiquitin-protein ligases. E3 enzymes possess one of two domains: The **HECT** (Homologous to the E6-AP Carboxyl Terminus) domain or the **RING** (Really Interesting New Gene) domain. If the E3 ligase possesses a HECT domain, E2 transfers the ubiquitin to E3 and E3 subsequently transfers it to the target substrate.

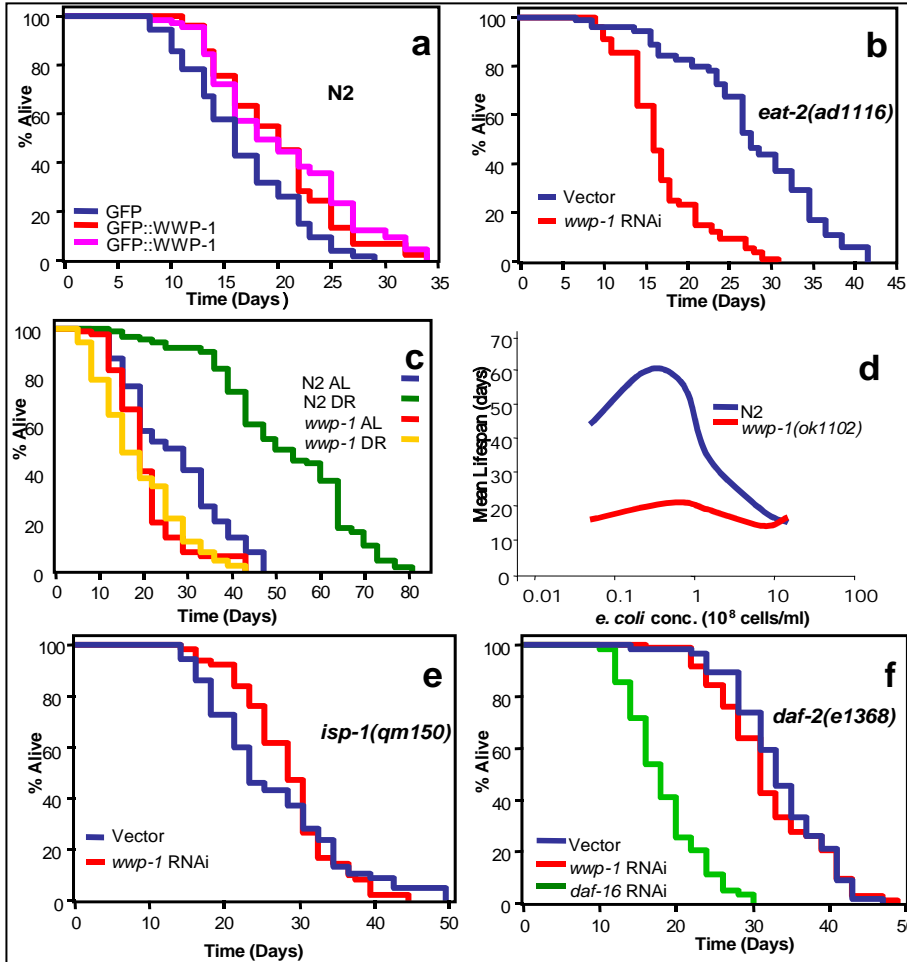
Wwp-1, is part of a family of HECT E3 ligases that contain a WW domain which bind tightly to polypeptides containing the amino acid motif PPxY and certain other proline rich motifs.

Sometimes, however, ubiquitination of a protein can increase the activity of proteins or can change its localization in cells. Thus, it remains possible that *wwp-1* may target a positive regulator of longevity, with ubiquitination leading to increased activity of the substrate. Thus RNAi knockdown of this substrate would then suppress the long lifespan of WWP-1 overexpressing worms.

Andrea Carrano, in collaboration with Andrew Dillin at the Salk Institute<sup>9</sup> have found an HECT E3 ligase, *wwp-1* that regulates lifespan in *C.elegans*. They found that overexpression of *wwp-1* in worms can extend lifespan by 20% compared with the control (Fig. 1a), indicating that *wwp-1* is a positive regulator of lifespan.

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**Figure 1. *wwp-1* is required and specific for the extension of lifespan by dietary restriction (DR).** The percentage of animals remaining alive is plotted against animal age. Lifespan values are given in Tables 1 and 2. **a**, *wwp-1* is a positive regulator of lifespan. Two independent *wwp-1* overexpressing strains (GFP::WWP-1) can extend longevity compared to control worms expressing GFP. **b**, *wwp-1* is essential for increased longevity in *eat-2* mutant worms. Lifespan analysis of *eat-2(ad1116)* mutant animals fed bacteria expressing *wwp-1* dsRNA or control vector. **c**, *wwp-1* is required for the increased longevity of true DR. Lifespan analysis of N2 and *wwp-1(ok1102)* mutant worms grown in DR or AL (*ad libitum*) *E. coli* concentrations. **d**, The longevity of *wwp-1* mutant worms does not change in response to varying bacterial concentrations. Lifespans of N2 and *wwp-1(ok1102)* mutant worms grown in S basal buffer with different *E. coli* concentrations. The slight decrease in longevity seen at the extreme ends of the curve of *wwp-1* mutant animals is likely due to increased stress by starvation (at the lowest concentrations) or hypoxia (at higher concentrations). Lifespan curves were plotted from a representative experiment of three independent experiments using 40-60 worms per dilution per experiment. **e,f** Loss of *wwp-1* does not affect other longevity pathways. **e**, *wwp-1* RNAi cannot suppress the extended longevity of *isp-1(qm150)* mitochondrial mutant worms. **f**, *wwp-1* is not required for increased longevity of DAF-2 signaling. Lifespan analysis of *daf-2(e1368)* fed bacteria expressing *wwp-1* dsRNA or control vector.

DR can be reproduced genetically by using *eat-2* mutant worms<sup>7</sup>. The control in this experiment was *eat-2* worms expressing *wwp-1* dsRNA which is essentially an empty vector. As seen in Fig 1b, reduced levels of *wwp-1* completely suppressed the extended longevity of *eat-2* mutant animals.

In the worm, dietary restriction can also be achieved by limiting the concentration of bacteria fed to worms in culture by bacterial dietary restriction, or bacterial dilution. Loss of *wwp-1* could also suppress the extended longevity of animals subjected to DR by bacterial dilution. This is seen in Figure 1d, where N2 (normal) animals exhibited a bell-shaped curve for lifespan in response to varying bacterial concentrations but the lifespan of *wwp-1* did not significantly change when fed the same bacterial concentrations.

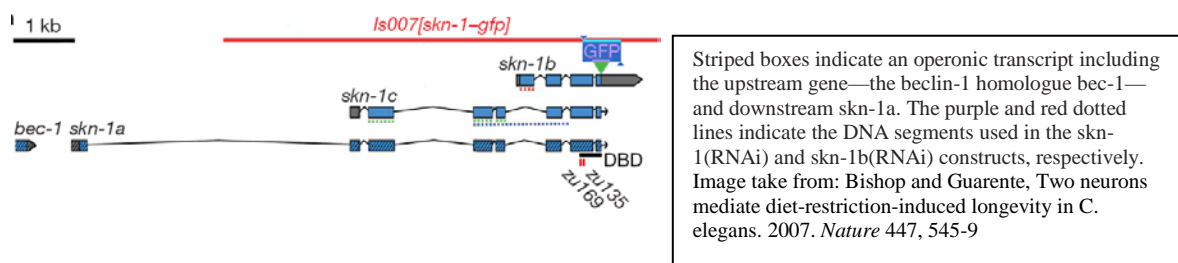
To determine whether *wwp-1* was acting specifically to affect DR longevity, they examined the effect of loss of *wwp-1* function on other pathways that influence longevity. Mutations in the iron sulfur component of complex III *isp-1* increase longevity by decreasing oxygen consumption<sup>8</sup> and therefore reduce mitochondrial respiration leading to longevity. They found that RNAi of *wwp-1* did not suppress the extended lifespan of *isp-1(qm150)* mutant animals (Fig 1e). Partial loss of function mutations in the insulin/IGF-1 receptor homolog DAF-2 also

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increase lifespan in a *daf-16* dependent manner<sup>10</sup>. While RNAi knockdown of *daf-16* completely suppressed the long lifespan of the *daf-2(e1368)* mutant animals, these animals fed *wwp-1* dsRNA lived as long as animals cultured on vector control bacteria (Fig. 1f). These results indicate that loss of *wwp-1* does not merely make animals sick but rather specifically regulates the response to DR that results in extended longevity.

Previous research has shown that the transcription factor gene *skn-1* mediates dietary restriction in two ASI neurons through transcriptional regulation<sup>7</sup>. The *skn-1* gene encodes three protein isoforms—SKN-1A, SKN-1B and SKN-1C—which have different amino termini but a common carboxy terminus<sup>7</sup>. According to the researchers, the *skn-1b* isoform specifically mediates dietary restriction longevity in the ASI neurons while the *skn-1c* isoform confers resistance to oxidative stress in the intestine. This discovery led us to pursue *skn-1b* as a potential target for *wwp-1*.



In other research, Siler Panowski, a graduate student in Andrew Dillin's laboratory discovered that the transcription factor PHA-4 has an adult-specific function in the regulation of diet-restriction-mediated longevity<sup>6</sup>. They found that a loss of *pha-4* fully blocked the entire response of lifespan to dietary restriction, as would be expected of a gene essential for diet restriction-mediated longevity. They also found that *pha-4* is specific to diet-restriction-induced longevity because it did not affect other longevity pathways, such as reduced IIS (by mutating *daf-2*) and reduced mitochondrial ETC activity (mutated *isp-1*)<sup>6</sup>. These results again led us to use *pha-4* as a possible candidate.

Thus, our goal is to identify a substrate that is positively regulated by ubiquitination. We will determine if loss of both substrate and WWP-1 will further suppress longevity.

### Methods Summary

#### LIFESPAN ANALYSIS

*skn-1b* colonies were obtained from Dr. Guarente's laboratory. RNAi cultures were then prepared in LB Amp TET. Each lifespan consisted of 10 NG plates (with Amp resistance) using 100 $\mu$ L RNAi culture, 100 $\mu$ L IPTG and 10 worms per plate. Lifespans were scored and adult worms were moved as long as they were reproducing (roughly 5-6 days). RNAi was initiated from hatching. JMP IN 5.1 software will be used to produce the lifespan graphs as seen in the results section

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## INDUCTION

BL21 bacteria expressing his tagged SKN-1B or PHA-4 protein was grown to an optical density (at wavelength 600 nm) between the range of 0.4 and 0.6. 0.1M IPTG was added to each of the induced samples to induce the expression of the T7 promoter. SDS-PAGE was then performed on the samples to check if the proteins were expressed and the gel was coomassie stained.

## Results and Discussion

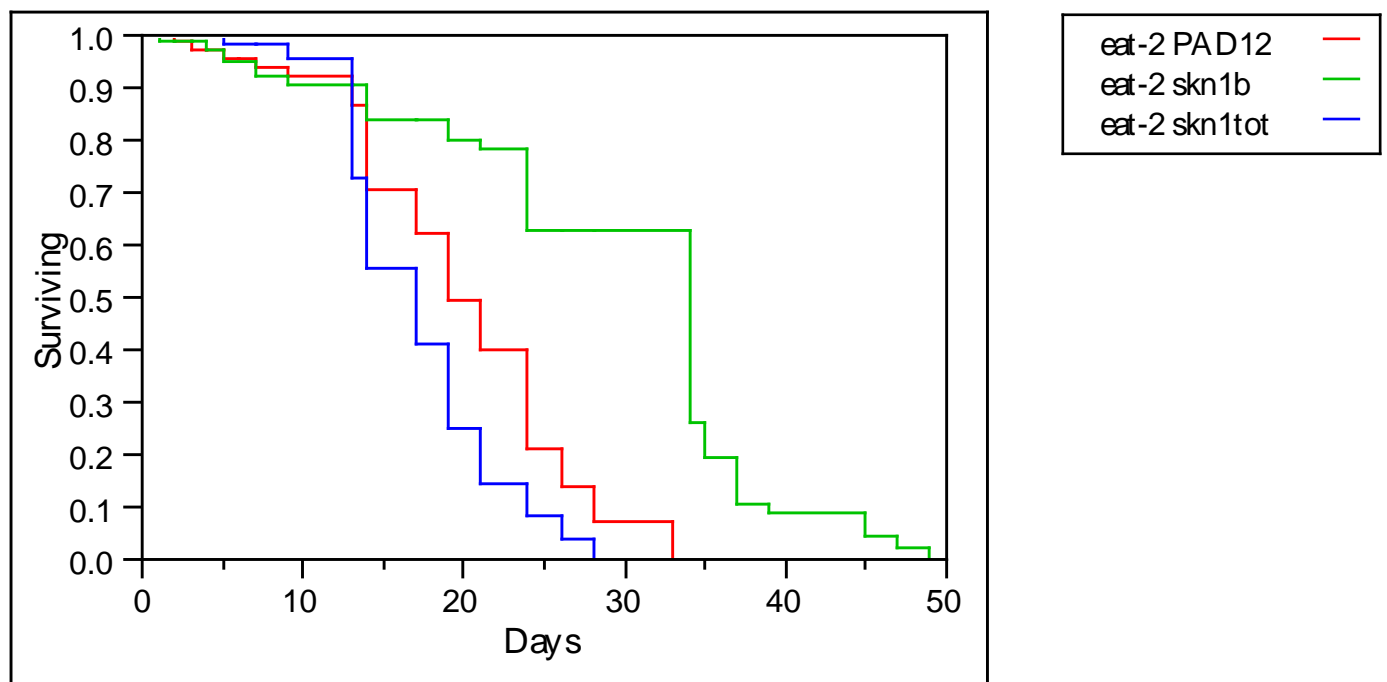


Fig 2: Lifespan analysis of *skn-1b* RNAi and *skn-1total* RNAi. PAD12 is a control.

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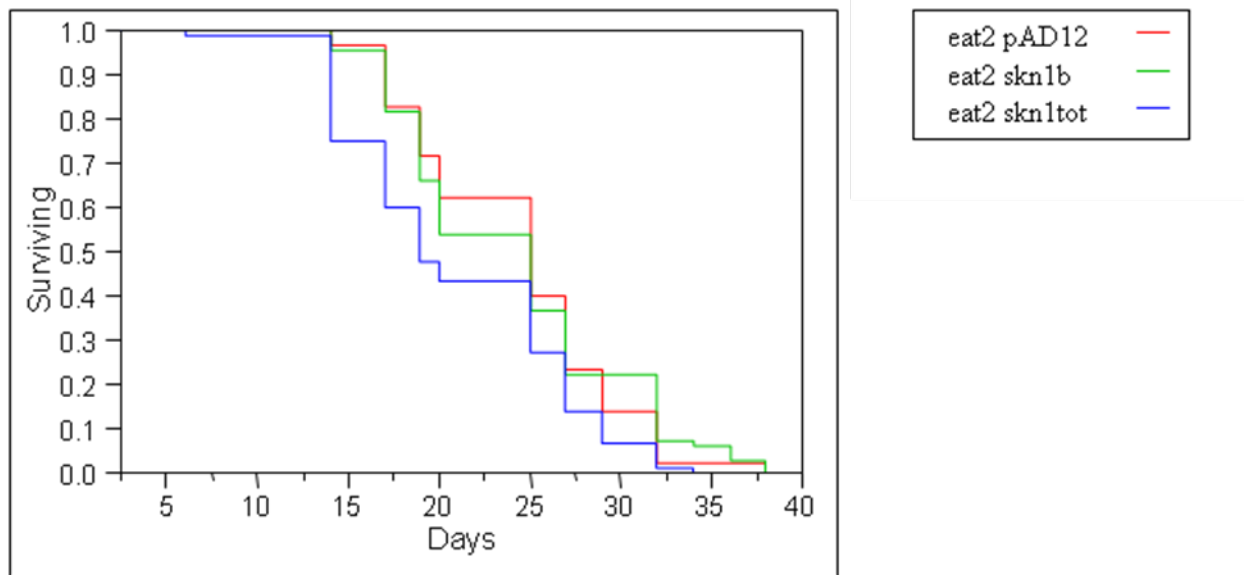
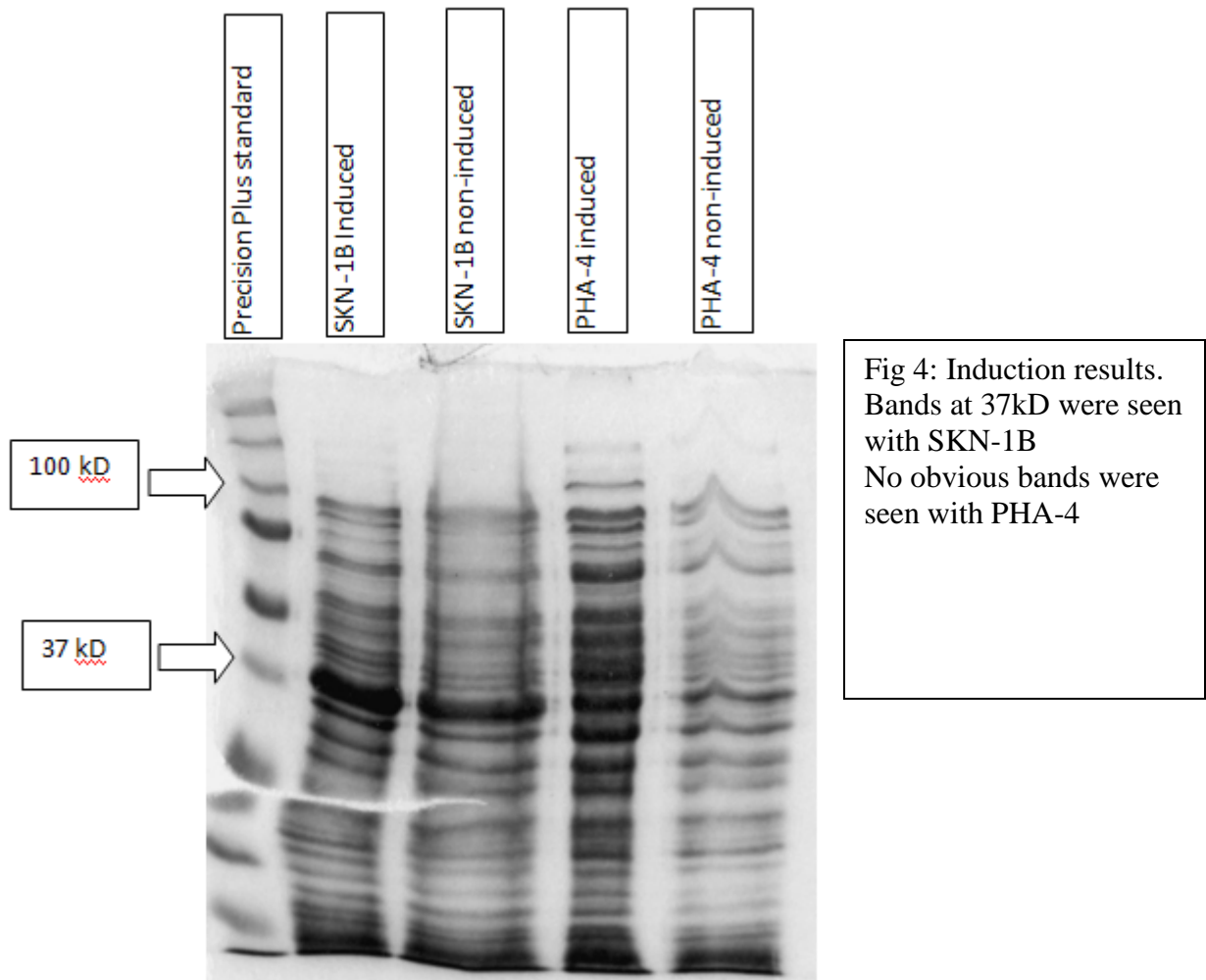


Fig 3: Lifespan analysis of *skn-1b* RNAi and *skn-1total* RNAi. PAD12 is a control. The bacteria grew differently the second time

*skn-1b* RNAi does not suppress *eat-2* longevity, as we had originally expected. *skn-1b* is activated, through DR, in ASI neurons (a pair of neurons in the *C.elegans* head) and these neurons signal peripheral tissues to increase metabolic activity<sup>7</sup>. RNAi may not have been very effective in affecting a transcriptional factor gene involved with these neurons in the central neuronal system.

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Although bands were seen for SKN-1B expression, no bands were seen for PHA-4. This does not, however rule out the possibility that there is no expression.

### **Further Experimentation**

We will need to purify his tagged *pha-4* and *skn-1b* and then establish an in vitro ubiquitination assay with purified components: E1 + ubc18 (an E2 conjugating enzyme) + WWP-1 and Ub.

Additionally, lifespan analysis of *pha-4* RNAi will need to be done.

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## References

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  - <sup>9</sup> Carrano et al, unpublished data