**Investigating regulation of aging by transcription factors DAF 16 and SKN-1 in the IIS pathway of C Elegans**

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1. Introduction

 Aging in eukaryotic organisms is a deleterious process that can result in changes in metabolism, reproduction and physiology. Mutations and accretion of damaged proteins that lead to different diseases eventually lead to a decreased lifespan (Eleftherianos and Castillo 2012). Observing the disproportionate use of healthcare resources by the elderly in comparison to their rapidly rising population in most countries, it is an issue of increasing relevance. Comprehending molecular pathways of aging and regulation can lead to developing methods to decrease effects of age-linked diseases, therefore improving human health and lifespan. There have been many studies conducted on model invertebrate organisms such as Caenorhabditis elegans (C. elegans) and Drosophilia melanogaster that have aided in finding genes that have a significant effect on longevity that can be used to understand the parallel mechanisms in humans (Eleftherianos and Castillo 2012). Most of these genes were found in pathways that regulate metabolic functions or respond to external factors.

 Furthermore, the Insulin/insulin like growth factor (IGF)-1 signaling (IIS) pathway has been shown to regulate aging in many organisms. The findings from invertebrate models propagated research into its role in mammalian aging, showing that it affected aging in humans (Heemst 2012). The IIS pathway is related to the Target of Rapamycin (TOR) pathway, which is a protein kinase that controls metabolism and protein synthesis to regulate homeostasis (Eleftherianos and Castillo 2012). This pathway has been found to have a significant play in human diseases such as cancer, heart disease, and diabetes all of which potentially decrease longevity. The IIS pathway interacts considerably with the TOR pathway; specifically, limiting the IIS pathway blocks TOR activity that results in lifespan expansion (Heemst 2012). Additionally the role of the IIS pathway encompasses connecting functions like reproduction and aging to nutrition sensing is highly conserved, which gives it increasing importance to study.

IIS pathway in C. elegans:

 Caenorhabditis Elegans, also known as the roundworm is an ideal invertebrate model to study aging as it has a short lifespan, along with a low cost. It is noted that a single mutation that inhibits the IIS pathway is capable of doubling the lifespan of the C. elegans, along it to stay active for a significantly longer period of time. The highly conserved pathway begins with the secretion of insulin like peptides in response to food, which act as ligands (Murphy 2013). They bind to an IGF-1 like receptor known as DAF-2/IGFR. This results in activation of AGE-1/PI3K, which activates the serine and threonine kinases AKT-1, AKT-2, and PDK-1. Figure 1: IIS pathway in C. elegans (Murphy 2013)

Consequently, DAF-16 is phosphorylated, which is a homolog of the FoxO transcription factors in humans (Murphy 2013). This causes inactivation of DAF-16, which then translocates from the nucleus to the cytosol. Inversely, when IIS is down regulated DAF-16 is activated and goes from the cytoplasm to the nucleus. In the nucleus it is able to promote the expression of longevity promoting genes (Murphy 2013).

IIS pathway targets:

 The three downstream longevity-regulating transcription factors that have been recognized in the IIS pathway are DAF-16/FoxO, SKN-1/nuclear factor erythroid 2(NRF2) and heat shock transcription factor 1 (HSF-1). DAF-16 is regulated by many protein kinases, and as previously mentioned it affects aging by activating downstream target genes that augment cellular maintenance (Altintas et al 2016). An oxidative stress responsive transcription factor known as SKN-1 also promotes longevity by down regulating IIS. SKN-1 is also isolated to the cytoplasm through phosphorylation by AKT-1/-2 and other protein kinases (Altintas et al 2016). This transcription factor controls the expression of genes that are a part of stress response and detoxification. When SKN-1 is overexpressed it is able to extend the lifespan of C. elegans by promoting protein homeostasis via proteasome production and rearranging the extracellular matrix (Altintas et al 2016). Studies into SKN-1 give scientists insight into how the human ortholog Nrf/CNC may have an influence on mammalian lifespan. HSF-1 or heat shock transcription factor decreases proteotoxicity along with enhancing longevity. When it is inhibited, lifespan is reduced due to increased tissue aging. Both SKN-1 and HSF-1 promote longevity through an augmented resistance to various stressors (Altintas et al 2016).

Experimental Focus:

 It has been concluded that DAF-16 has a major role in increasing the lifespan of C. elegans through the IIS pathway (Uno and Nishida 2016). However it has been speculated that it does not act alone, because just an overexpression of DAF-16 scarcely increases lifespan. Mutations in the other transcription factors also affect the lifespan phenotype, suggesting that HSF-1 and SKN-1 assist DAF-16 in regulating intersecting longevity genes (Uno and Nishida 2016). It has been noted that SKIN-1 can be activated by MPK-1 to affect DAF-16 activity, however this mechanism is not fully understood. Longevity phenotypes produced by DAF-16 and SKN-1 mutants are similar, which suggests that they might function together to increase the lifespan of C. elegans (Uno and Nishida 2016). Furthermore, it is speculated that IIS inhibits SKN-1 similar to the inhibition of DAF-16. In an experiment that tested how IIS inhibited SKN-1, it was shown that SKN-1 was able to accumulate in intestinal nuclei independently of DAF-16 activity and activate target genes for longevity (Tullet et al 2008). Despite this, their similarities in regulation suggest that DAF-16 and SKN-1 might cooperate together to promote longevity. This experiment will test this hypothesis by investigating how the transcription factors affect each other by using DAF-2 mutants and observing the aging phenotypes as a result of downstream gene expression.

1. Experiment

 The objective of this experiment is to determine if SKN-1 and DAF-16 act together to promote longevity in C. elegans, through a reduction in the IIS pathway. In DAF-2 mutants, the transcription factors are able to bind to the regulatory regions of their target genes and activate expression. DAF-2 mutants assure that the IIS pathway is down regulated, allowing for both SKN-1 and DAF-16 to translocate to the nucleus of cells to promote transcription of their respective target genes. Testing a DAF-2 mutant for longevity without altering expression of any other factors will serve as the control. This will show normal aging patterns when the IIS pathway is down regulated. Then DAF-2/SKN-1 double mutants will be used to determine what phenotypes are expressed and if the lifespan can be altered by DAF-16 without SKN-1. Succeeding this, the opposite will be done using a DAF-2/DAF-16 double mutant. If the longevity phenotypes are different in one of the double mutant combinations, it shows that the transcription factors do regulate each other and work together to increase lifespan of C. elegans. To achieve this, the target genes will be cloned and amplified by PCR from the genomic DNA of the desired strains of C. elegans (Tullet et al 2008). Mating two individual mutant strains creates the double mutant strains and mutations will be confirmed by sequencing. The following methods will be used to complete the experiment and subsequent analysis to the aforementioned process.

RNA interference:

RNA interference also known as Post-Transcriptional gene silencing is a process that can regulate the expression of protein coding genes. It is used to study the loss of function involved with specific genes by utilizing double stranded RNA oligonucleotides (Przybysz et al 2009). When the dsRNA is introduced, it is able to turn the genes off using a natural process of silencing. In this experiment the C. elegans will be fed worms of E. coli, which are genetically modified to induce transcription of dsRNA that is homologous to a target gene (Przybysz et al 2009. This method will be used to turn off genes to study age-dependent oxidative stress response. A control will be used that is non homologous to any C. elegans genes to ensure that the RNAi (RNA interference) is functional. The survival in response to stress will be relative to the control, which involves placing the worms in a plate seeded with E. coli expressing the plasmid pPD129.36. For each gene that is being tested, multiple independent RNAi experiments using the appropriate plasmid-expressing bacteria will be conducted to ensure that the longevity response is due to the silencing of the particular gene (Przybysz et al 2009).

 Figure 2: Overview of RNAi (Meins 2000)

To ensure that the RNAi has worked properly, RT-qPCR will be conducted, to make a measurement of the genes at the mRNA level (Wang 2000). It will examine the small interfering RNA (siRNA) by real-time quantitative PCR detection of mRNA levels. This method has been shown to be more accurate in measuring RNAi accuracy when compared to a western blot to show decrease in protein secretion (Holmes et al 2010). A cell culture of intestinal cells will be taken from the C. elegans treated with RNAi. The cells will then be transfected with siRNA duplexes and placed in serum. The RNA will be extracted to synthesize cDNA with reverse transcriptase and appropriate primers (Holmes et al 2010). The primers will be inspected using BLAST to ensure specificity. Following this the PCR products will be ran on an agarose gel for identification (Holmes et al 2010). This process will confirm that the RNAi was successful.

Oxidative stress response (Juglone exposure):

The stage 4 larvae that have been manipulated with RNA interference will be exposed to varying concentrations of 5-hydroxy-p-napthoquinone (Juglone) for one hour and then transferred back into their plates. This exposure will mimic oxidative stress and is able to form damaging proteins that the C. elegans need to recover from (Przybysz et al 2009). The worms will be given 24 hours to recover and the worms from each mutant combination strain will be tested, along with the control group for comparison to test for relative survival. A microarray analysis will be used to measure the survival of the sub-populations as done by Przybysz et al in 2009. The mutants cellular mRNA will be used to make the DNA copy to apply to a microarray, and the gene expression will be measured after Juglone exposure to compare to the control group (Przybysz et al 2009).

1. Discussion

 The objective of this experiment is to determine how SKN-1 and DAF-16 affect each other in terms of promoting longevity in C. elegans. This will be done in the experiment using the aforementioned methods, which involve using RNA interference methods to silence certain genes. Through this, we can gain insight into whether the two transcription factors regulate each other and or affect the ability to increase lifespan. The hypothesis of this experiment was that they do in fact affect each other and regulate one another. If the DAF-2/DAF-16 mutant shows decreased signs of longevity, then this will indicate that SKN-1 is related to DAF-16 in some way that affects its ability to increase lifespan. First it is of important to ensure that the RT-qPCR produces the expected results to ensure that the RNAi has silenced the appropriate genes. Furthermore, experimental results that would indicate dependency are if the SKN-1 is unable to reach the nucleus as efficiently, and if the mutant is not able to react to the oxidative stress as well as the control group. The C. elegans lifespan assay will also show a shorter result in this case. All of these results can also apply to the DAF-2/SKN-1 mutant in a similar way when taking into account the resultant activity levels of DAF-16.

 Results that show that the two transcription factors are related will provide insight into how similar homologs in humans and other mammals also act together to further the lifespan and healthspan. Further experiments can also be done to narrow down the exact pathways and genes that are affected to see the overlap between the transcription factors to shed light on how diseases that affect the IIS or SKN-1 and DAF-16 in particular could be treated. This could then be used to further research in mammals.

 In the case that the two transcription factors are not related to each other, experiments with different methods could be used to confirm these results. Results that show DAF-16 acting to extend lifespan independently would be contrary to current research (Uno and Nishida 2016). This subject could be further studied to understand how the similar phenotypes occur when the transcription factors do not have an overlap. The increase in lifespan for DAF-16 and SKN-1 can be studied independently if it is known that they do not affect each other. Finally, similar experiments could be conducted with the other transcription factors that are speculated to act along with DAF-16, the pivotal transcription factor associated with an increased lifespan in C. elegans.

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