USING *Caenorhabditis elegans* TO ELUCIDATE THE ROLE OF

O-GlcNAc IN INSULIN SIGNAL TRANSDUCTION

by

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Under the Direction of Lance Wells

ABSTRACT

O-GlcNAc is a regulatory carbohydrate post-translational modification found on serine and threonine residues on cytosolic and nuclear proteins in multicellular organisms. Global elevation of O-GlcNAc levels on intracellular proteins induces insulin resistance, the hallmark of type II diabetes, in mammalian systems. In *C. elegans*, attenuation of the insulin-like signal transduction pathway increases lifespan of the nematode. We demonstrate that the O-GlcNAc cycling enzymes, OGT that adds O-GlcNAc and OGA, which removes it, modulate lifespan in *C. elegans*. The median lifespan of the oga deletion strain increases ~25% while the median life span of the ogt deletion strain decreases ~25%. Furthermore, we demonstrate that the O-GlcNAc effect on nematode median lifespan is due to attenuation of insulin-like signal transduction above PDK1 in the pathway. These results highlight the conserved feature of the O-GlcNAc modification in attenuating insulin signaling and more finely define the point in the pathway in which elevated O-GlcNAc modification is inducing insulin resistance.

INDEX WORDS: O-GlcNAc, Insulin Signaling, *C. elegans* lifespan

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DEDICATION

This work is dedicated to my parents, Ivan and Jirina Stuchlik, whose love, wisdom, and kindness continue to amaze me.

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CHAPTER 1

INTRODUCTION

While characterizing terminal N-Acetylglucosamine residues on complex carbohydrates, in 1984, Torres and Hart first discovered the existence of an intracellular single β-N-Acetylglucosamine (O-GlcNAc) modification on proteins (*1*). Torres and Hart used a galactosyltransferase, which catalyzes the transfer of galactose to terminal GlcNAc residues, and a radioactive form of the donor for galactose modification, UDP-galactose, to map the GlcNAc residues. However they realized that much of the radioactive galactose was being incorporated in onto proteins located in the the cytosol (*1*). Following this discovery, the modification was shown to be an O-linked nuclear and cytosolic modification of serine and threonine amino acids on proteins (*2*) and many of the proteins found to be modified by O-GlcNAc are involved in transcription & translation, metabolism, stress, and signaling (*3*). Like phosphorylation, O-GlcNAc was shown to be dynamic and inducible in response to environmental stimuli (*4-8*).

A more focused study of the dynamic response has been in the area of elevation of global O-GlcNAc level in response to stress and elevation of global O-GlcNAc levels and its relation to insulin resistance (*5, 6, 9-15*). In regard to stress, it has been shown that stress stimuli such as heat shock, peroxide treatment, or ethanol treatment of COS-7 cells, increases global O-GlcNAc levels (*6*). Furthermore blocking or reducing global O-GlcNAc modification

pharmacologically causes cells to be more sensitive to the thermal, peroxide, or ethanol stress (*6*).

One of the more extensive studies of the functionality of the dynamic O-GlcNAc modification has been observed in its relation to insulin resistance. Initially it was the hexosamine biosynthetic pathway that led to the idea of O-GlcNAc function in insulin signaling. About 3-5 % of glucose that enters a cell is directed to the Hexosamine Biosynthetic Pathway (HBP). The end product of this pathway is UDP-GlcNAc, the donor sugar nucleotide for O-GlcNAc modification of nuclear and cytosolic proteins, as well as a donor for lipid and secretory protein complex glycosylation and glycosyl phosphatidylinositol (GPI) anchor synthesis (*16*). The HBP was suggested to serve as a nutrient sensor since the levels of UDP-GlcNAc are responsive to nutrient levels (*3*). It has been observed that inhibition of L-Glutamine: D-Fructose-6-phosphate Amido-transferase (GFAT), the rate-limiting enzyme of the HBP, resulted in reversal of the effects of hyperglycemia-induced insulin resistance (*16-18*). Over expressing GFAT in muscle, liver, fat and beta cells in transgenic mice, causes insulin resistance (hallmark of type 2 diabetes) in skeletal muscle and hyperinsulinemia resulting from excess insulin being secreted by beta cells (*19*). Further, over expression of GFAT in rat adipose cells results in decreased translocation of glucose transporter, GLUT4, to the plasma membrane (*20*).

Insulin resistance is the hallmark of type II diabetes. In type II diabetes, insulin is produced in response to food intake, however certain tissues have impaired insulin responsiveness. Most complications associated with diabetes

are caused by chronic hyperglycemia (*21*) which leads to the loss of sensitivity to insulin in tissues such as fat and muscle (*22, 23*). Although the biochemistry and molecular cell biology of diabetic complications from this disease is complex (*24*), "glucose toxicity" appears to be result of increased flux through the HBP (*25-28*).

The "glucose toxicity" associated with insulin resistance leads to a number of serious complications and diseases. Microvascular disease which results from insulin resistance is the leading cause of blindness, renal failure, and nerve damage (*29*). Diabetes associated atheroclerosis, the narrowing of arteries due to fatty buildup, leads to increased risk of stroke, myocardial infarction, and limb amputation (*29*). According to the American Diabetes Association, more than 65% of diabetic patients die from heart disease or stroke, having heart attacks occur earlier in life (*30*).

Following the finding that increased levels of the end-product of the HBP correlated with insulin resistance, studies were made in relation to global O-GlcNAc levels and insulin signaling as well as mapping various O-GlcNAc sites on insulin signaling proteins. Pharmacologically preventing the global removal of O-GlcNAc from proteins resulted in insulin resistance in 3T3-L1 adipocytes and inhibition of of Akt, a protein involved in insulin signaling, phosphorylation at Thr-308 (*5*). This pharmacological inhibition of the enzyme O-GlcNAcase, which catalyzes the removal of O-GlcNAc, in rat skeletal muscle also led to insulin resistance (*13*). Furthermore, overexpression of the enzyme, O-GlcNAc transferase (OGT), which catalyzes the addition of O-GlcNAc, in muscle and fat led to insulin resistance (*10*). A number of proteins in the insulin signal

transduction pathway have been shown to be O-GlcNAc modified, including IRS-1 (*5, 31-33*), the regulatory subunit of PI3-K (*34-36*), and AKT (*14, 15*) and when cells were treated with PUGNAc, an inhibitor of O-GlcNAcase, global elevations of the O-GlcNAc modification have been shown to impair phsophorylation of AKT, a component of the insulin signal transduction (*5, 14*).

In mammalian insulin signal transduction, insulin binds to the insulin receptor and the receptor autophosphorylates itself followed by recruitment of the insulin receptor substrates (IRSs), via SH2 domains that bind phosphorylated tyrosines. The IRS proteins become tyrosine phosphorylated and recruits phosphatidylinositol-3-kinase (PI3-K) to the plasma membrane. The PI3-K is then able to phosphorylate PIP2 to PIP3 followed by the recruitment of phosphatidyl-inositol dependent kinase (PDK1) and subsequent activation of the AKT. Activation of AKT is followed by the translocation of the GLUT4 glucose transporter to the plasma membrane which allows glucose to be taken up by the cell (*37*).

OGT, the enzyme which catalyzes the addition of O-GlcNAc was first characterized in 1992 followed by its cloning in 1997 (*38, 39*). After the O-GlcNAc Transferase cloning and characterization, the enzyme which catalyzes the removal of O-GlcNAc, O-GlcNAcase was cloned and characterized in 2001 (*40, 41*). Both of these cycling enzymes are highly evolutionarily conserved with only one gene of each found in mammals and *C. elegans* and two genes in plants and fish.

OGT consists of N-terminal tetratricopeptide repeats (TPR), which modulate protein-protein interaction, a flexible linker, and a catalytic C-terminus (*39, 42*). The number of TPR repeats varies from three to sixteen (*3*) with human nuclear and cytoplasmic OGT having about twelve repeats (*43*) and mitochondrial having nine TPR repeats (*3*). It therefore has been proposed, that OGT may use the TPR repeats to recognize its targets or to form complexes where interacting proteins dictate the specificity of targets (*3*). OGT was also shown to be essential for stem cell viability of mouse embryonic stem cells (*44*).

O-GlcNAcase has a N-terminal catalytic domain, a C-terminal histone acetyl transferase domain, and a caspase-3 cleavage site (*40, 41, 45-48*). However the histone acetyl transferase domain is not a complete acetyltransferase domain and may require additional proteins for the HAT activity (*3*). OGT has been reported to interact with a histone deacetylase complex by binding to the corepressor mSin3A (*49*) and therefore an appealing model has been proposed where both OGT and O-GlcNAcase participate in transcriptional control via chromatin remodeling (*3*).

A number of reports have also focused on animal models of O-GlcNAc and its relation to insulin resistance. In rats, a pharmacologically induced diabetic pancreas had increase in OGT activity and expression at the protein level and the pancreas showed 3.1 fold increase in O-GlcNAc modified proteins (*50*). When OGT was overexpressed transgenically in mice, the mice developed a type 2 diabetic phenotype (*10*) and elevated levels of O-GlcNAc modification were found in the corneas of diabetic Goto-Kakizaki rats (*51*). Furthermore, a

single nucleotide polymorphism (SNP) of the O-GlcNAcase gene correlates strongly with diabetes risk in Mexican Americans (*52*).

Interestingly mutations of insulin-like signaling in *Caenorhabditis elegans* increase its lifespan and dauer larvae formation (*53-58*). First published in 1993, mutations in the gene *daf-2* coding for the insulin-like receptor resulted in an adult that lives more than twice as long as wild type (*53*). The insulin signal transduction pathway negatively regulates *C. elegans* lifespan by suppression of the *daf-16* gene. The *daf-16* gene codes for the forkhead family of transcription factors, FOXO, (*59, 60*) that bind DNA (*61*), whose transcriptional targets include heat shock genes, oxidative stress response genes, and resistance to bacterial infection genes (*62-64*).

Recently, the *ogt-1* and oga-1 *C. elegans* null alleles have been characterized. The *ogt-1* null allele was shown to suppress the dauer larvae formation of a *daf-2* in a double mutant while the *oga-1* knockout allele enhanced the dauer larvae formation of *daf-2* in a double mutant. (*65, 66*). In the *ogt-1* null allele, although nuclear pore proteins lack O-GlcNAc, the transport of transcription factors appears normal. In addition, *ogt-1* has about 3-fold elevations in trehalose levels and glycogen stores and about a 3-fold decrease in triglyceride levels (*66*). Interestingly, the *oga-1* also has elevated stores of glycogen and trehalose and decreased lipid levels, but the elevation and decrease are not as prominent as with *ogt-1* null allele (*65*). Since lifespan and dauer larvae formation are related in C. elegans, we hypothesized that the *ogt-1* and *oga-1* null alleles would have altered lifespan.

If we are able to view different lifespans for the *ogt-1* and *oga-1* mutants, crossing them with known insulin signaling mutants that either enhance or shorten *C. elegans* lifespans may allow us to narrow down the point of functional O-GlcNAc modification in the insulin-like signaling in this nematode. Since insulin signaling is highly conserved, studying *C. elegans* insulin-like signaling may lead to discovery of a conserved mechanism where O-GlcNAc functionally regulates insulin signaling in mammals.

CHAPTER 2

RESULTS

O-GlcNAc cycling enzyme mutants modulate *C. elegans* lifespan. To examine whether *ogt-1* and *oga-1* modulate C. elegans lifespan, we allowed young adult nematodes to lay eggs and monitored the offspring lifespan. The medial lifespan, where half of the worms have died, of the *oga-1* strain is about 25% increased while the median life span of the *ogt-1* strain is about 25% decreased compared to wild type (Fig. 1A). Furthermore, the median lifespan of *ogt-1* and *oga-1* is statistically significant (Fig 1A). Because *C. elegans* insulin signaling is at the center of its lifespan regulation and previous studies suggest that O-GlcNAc is acting in the insulin pathway, this data suggests that O-GlcNAc is functionally involved in insulin signaling.

To correlate the genetic mutants with global O-GlcNAc levels, anti-O-GlcNAc westerns were performed. Wild type, *ogt-1*, and *oga-1* nematodes were lysed and whole cell extracts were analyzed by western analysis. The global O-GlcNAc levels for the strains were detected using anti-OGlcNAc antibody (RL-2). Correlating with the lifespan data, *oga-1* global O-GlcNAc levels were heavily increased compared to wild type while *ogt-1* global O-GlcNAc levels were undetected (Fig. 2B). Although published previously (*65, 66*), this data demonstrates that the *ogt-1* mutant is indeed a null allele and that the actual global O-GlcNAc levels correlate with the null allele phenotypes reported.

Since O-GlcNAcase has been reported to have histone acetyl transferase (HAT) activity (*47*) we generated *ogt-1; oga-1* double mutant strain to show that the increase in *oga-1* lifespan was due to the loss of the O-GlcNAcase activity rather than the loss of the HAT activity. The *ogt-1; oga-1* double mutant lifespan resembles the *ogt-1* lifespan (Fig. 2C) suggesting that it is the loss of O-GlcNAc modification which dictates lifespan in *ogt-1;oga-1* double mutant. Had the change in lifespan been HAT activity dependent, we would expect the *ogt-1;oga-1* double mutant lifespan to resemble that of *oga-1*.

O-GlcNAc cycling enzymes are acting in the *C. elegans* insulin signaling pathway. To examine whether the O-GlcNAc cycling enzymes were acting in the *C. elegans* insulin-like signaling pathway, we generated *ogt-1; daf-2* (e1370) and *oga-1; daf-16* (mu86) double mutants. The *daf-2* hypomorph has about 2.3 fold lifespan increase compared to wild type (*53*). The *ogt-1; daf-2* double mutant has a shortened lifespan compared to wild type; resembling *ogt-1* lifespan (Fig. 2A). The only other mutations which have been reported to alter the *daf-2* extended lifespan have been *daf-16*, the FOXO transcription homologue and the ultimate regulator of *C. elegans* lifespan (*53, 67*), and *daf-18*, the PTEN homologue, which negatively regulates insulin signaling downstream of *daf-2* and *age-2* (*67*).

Although the *ogt-1;daf-2* double mutant has been shown to suppress the dauer larvae formation of *daf-2* (*66*) it is not always the case that if a mutation alters dauer formation, it will be long lived. Not all long lived *C. elegans* mutants are dauer constitutive (*68*) and not all dauer constitutive mutants are long lived

(*67*). For example, the *age-1* (hx546) allele is long lived, but not dauer constitutive while the *age-1* (mg44) allele is dauer constitutive and long lived (*68*). The suppression of the *daf-2* extended lifespan by *ogt-1* null allele further suggests that *ogt-1* is acting in the *C. elegans* insulin-like pathway downstream of *daf-2* gene.

Since the insulin signaling pathway dependent increase in *C. elegans* lifespan requires the *daf-16* gene (*53, 56, 59, 69-72*) and *daf-16* is the ultimate regulator of the insulin signaling dependent life span increase, we monitored the lifespan of the *oga-1; daf*-16 double mutant. If *oga-1* is acting upstream of *daf-16*, the *daf-16* null allele will alter the *oga-1* extended lifespan. Our data shows that the *daf-16* mutation suppressed the increased lifespan of *oga-1*, further suggesting that *oga-1* is acting upstream of *daf-16* (Fig. 2B).

The *daf-2* hypomorph is developmentally delayed. Therefore, it is somewhat surprising that the *oga-1* mutant lives longer than wild type, but does not appear developmentally delayed. However, the *oga-1* lifespan increase is subtle compared to *daf-2* dramatic lifespan increase and more sophisticated analysis of the *oga-1* developmental delay may be required. We examining the developmental delay of *daf-2*, *ogt-1;daf-2* double mutant, and *oga-1; daf-2* double mutant, observed that all had developmental delay (Fig. 2C), but differing lifespan. *Daf-2 and oga-1; daf-2* lifespan was increased compared to wild type (data not shown for *oga-1; daf-2*) and virtually identical while *ogt-1;daf-2* lifespan was decreased compared to wild type resembling *ogt-1* lifespan (Fig 2A). This uncoupling of developmental delay and longevity is interesting because although

we have altered global O-GlcNAc levels of the whole organism, O-GlcNAc is likely acting in the insulin like signaling pathway only to alter lifespan, but is not affecting *C. elegans* development.

O-GlcNAc cycling enzymes are acting in the *C. elegans* insulin signaling pathway at or above daf-18. By use of *C. elegans* genetics, we showed in the previous section that *ogt-1* and *oga-1* are acting downstream of *daf-2* and upstream of *daf-16.* We therefore continued with the additional *C. elegans* mutants involved in insulin signaling to further narrow down the area in insulin signaling pathway of functional O-GlcNAc modification.

It has been demonstrated that *pdk1* activity is necessary and sufficient to transduce signaling from *age-1* to *akt* (*57*) and in mammalian cells, the O-GlcNAc dependent insulin resistance has been mapped to at or above *akt* since global increase in O-GlcNAc levels via pharmacological inhibition of O-GlcNAcase resulted in defective akt phosphorylation (*5*). We selected a *pdk1* (mg 142) allele that is a constitutively active mutant of the phosphoinositol dependent kinase and has slightly decreased lifespan compared to wild type. We generated *pdk1-CA* (mg142); *oga-1* double mutants and monitored their lifespan compared to *pdk1-CA*, wild type, and *oga-1*. We found that the constitutively active *pdk-1* mutation suppressed the *oga-1* extended lifespan and the *pdk1; oga-1* double mutant had lifespan similar to *pdk1* or wild type; suggesting that the *oga-1* is acting upstream of *pdk1* (Fig. 3A).

Since *pdk-1* is a PIP3 dependent kinase, it is negatively regulated by *daf-18*, a homologue to human tumor suppressor PTEN (*73*) which can

dephosphorylate PIP3 to PIP2 (*74*) (Fig. 4) and has also been shown to regulate longevity in *C. elegans* (*75, 76*). *Daf-18* is directly upstream of *pdk1* and was therefore the next step in the attempt to narrow down the functional O-GlcNAc modification in the insulin signal transduction. *Daf-18* has a decreased lifespan compared to wild type and has been shown to alter *daf-2* and *age-1*, both of which are upstream of *daf-18*, extended life spans (*67*). Therefore we used the *daf-18* hypomorph to test whether it would alter the *oga-1* extended life and provide evidence for functional O-GlcNAc modification upstream of *daf-18*. We found that *daf-18* (ok 480) indeed suppressed the extended lifespan of *oga-1* in the *daf-18 (ok 480); oga-1* double mutant (Fig. 3B). From this data, it appears that *ogt-1* and *oga-1* are acting at or above *daf-18* in the *C. elegans* insulin signaling pathway.

CHAPTER 3

DISCUSSION

In *C. elegans*, the insulin-like signaling pathway negatively regulates lifespan by suppression of the *daf-16* gene, which codes for the FOXO transcription factor. *Daf-2* gene mutants coding for the insulin-like receptor have lifespan more than double that of wild type (*53*) and mutations in the gene, *age-1*, coding for the catalytic subunit of PI3-K also have dramatically enhance lifespan (*55*). Both of the *daf-2* and *age-1* extended lifespan mutants have been shown to require both the activities of *daf-16*, the FOXO transcription factor, and *daf-18*, a PTEN tumor suppressor homologue. Since double mutants of *daf-2;age-1* did not live significantly longer than *daf-2,* the data suggests that both of these are acting in the same pathway (*56*).

The PTEN tumor suppressor can dephosphorylate the phosphatidylinositol 3,4,5-triphosphate, a substrate for PI3K and a secondary messanger necessary for PDK1 activity, and therefore negatively regulate the insulin signaling pathway. In C. elegans, the *daf-18* gene can completely suppress both of the extended lifespans of *daf-2* and *age-1* (*73*) which are upstream of *daf-18*, but this suppression can be reversed by genetic manipulation of a downstream gene. One example is the suppression of the *daf-18* effects on the *age-1; daf-18* and *daf-2; daf-18* double mutants by *akt-1* and *akt-2* RNAi. When *akt-1* and *akt-2* RNAi is introduced into *age-1;daf-18* and *daf-2;daf-18* double mutants that are

not dauer constitutive, the dauer formation is induced. Therefore the *C. elegans* lifespan and dauer formation can be used to tease apart which gene is acting upstream and which is likely acting downstream.

The *ogt-1* and *oga-1* null alleles of OGT and O-GlcNAcase respectively, not only modulate *C. elegans* lifespan, but the *ogt-1,* like *daf-18* and *daf-16*, is able to suppress the *daf-2* extended lifespan. The suppression of the *daf-2* extended lifespan by *ogt-1* and the suppression of the *oga-1* extended lifespan by *daf-16* further suggests that the O-GlcNAc cycling enzymes are acting downstream of *daf-2* and upstream of *daf-16*. However, the *ogt-1* null allele does not suppress the extended lifespan of all of the *daf-2* nematodes, but rather changes the median lifespan significantly. It therefore appears that the longevity and median lifespan can be uncoupled in *C. elegans*.

The suppression of the *oga-1* extended lifespan by both constitutively active *pdk1* and *daf-18* mutant allows us to further narrow the likelihood of the functional O-GlcNAc modification in the insulin signaling from at or above *akt* (*5*) to at or above *pdk1*. With this study we have therefore provided data that supports previous finding of O-GlcNAc acting at or above *akt* in the insulin pathway with new data that points farther up the insulin signaling to at or above *pdk1.*

In the *C. elegans* insulin like signaling pathway downstream of *daf-2* and upstream of *pdk1/ daf-18* signaling lie three genes. The catalytic subunit of PI3K, *age-1*, whose mutations have dramatically increased lifespan (*68*), the regulatory subunit of PI3K, *aap-1*, whose mutations also have dramatically increased

lifespan (*77*), and the IRS homologue, *ist-1*, which does not arrest at dauer larvae stage at high temperatures when ist-1 gene is inactivated by RNAi, suggesting that it would not have a dramatically extended lifespan. However, both *ist-1* and *aap-1* gene suppression by RNAi enhances dauer formation in *age-1* (hx546) mutant (*77*). Enhancement of a long lived mutant has also been seen with *oga-1* enhancing the *daf-2* dauer formation (*65*). Both IRS and the regulatory subunit of PI3K in mammalian systems have been reported to be O-GlcNAc modified and because both *ist-1* and *aap-1* also enhance dauer phenotype like *oga-1*, they are likely candidates for functional O-GlcNAc modification.

The insulin signaling pathway is highly conserved from worms to mammals and the insulin/IGF-1 pathway has been shown to influence lifespan in worms, flies, and mammals (*78, 79*). In Drosophila, the insulin/IGF-1 receptor mutations increase its lifespan by about 80% (*80*) and the Drosophila IRS-1 homologue, *chico*, mutant increases lifespan by about 40% (*81, 82*). Mammals have separate receptor for IGF-1 and insulin, yet both of these appear to contribute to lifespan regulation (*79*). Mice with IGF-1 heterozygous knockout live about 30% longer than wild type (*83*) and mice which lack insulin receptor in adipose tissue have extended life-span of about 18% than wild type (*84*).

Other pathways than the insulin-like signaling pathway have been shown to regulate *C. elegans* lifespan, but the insulin/IGF-1 pathway has been the most studied. Additional pathways which have been shown to modulate *C. elegans* lifespan are germline signaling, SIR2, and JNK signaling (*85*). Laser ablation of

germline precursor cells has been shown to increase *C. elegans* life span by about 60% (*86*), but the removal of the whole reproductive system has no affect on lifespan. Interestingly, the germline dependent increased lifespan is dependent on *daf-16* (*86*), but the germline ablation extends the *daf-2* already long-lived lifespan. Therefore it is believed that the germline ablation extends lifespan through a pathway which is working parallel to insulin signaling pathway (*85*). In *C. elegans*, silent information regulator (*sir2*) overexpression extends lifespan and is dependent on *daf-16* gene (*87*) and in mammals, the SIRT1 ortholog, has been reported to deacetylate FOXO (*88*). JNK overexpression has also been shown to increase lifespan in *C. elegans* in a *daf-16* dependent manner (*89*) and JNK has been shown to phosphorylate *daf-16* at sites different from AKT phosphorylation (*89*).

Interestingly, the *oga-1* gene is one of 17 genes that have a consensus binding sequence for DAF-16 within 1kb of the star codon both in *C elegans* and *Drosophila* {Lee, 2003 #42}. Therefore one possible model may be that increased O-GlcNAc levels inhibit insulin signaling and DAF-16 is then able to translocate to the nucleus and activate *oga-1* transcription. The O-GlcNAcase enzyme is then able to remove the O-GlcNac modifications and serve as a back regulator of the insulin signaling pathway; keeping the O-GlcNAc modification dynamic.

The future studies will focus on the genetic mutants of both the regulatory and catalytic subunits of PI3K, *aap-1* and *age-1,* respectively and the *ist-1* gene. IST-1, the regulatory subunit of PI3K, and the catalytic subunit of PI3K will be

examined for O-GlcNAc modification by tandem mass spectrometry based analysis. If sites of O-GlcNAc modification are found on any of these three proteins, site directed mutants that prevent glycosylation and wild type sequences will be generated and introduced into C. elegans null backgrounds for the proteins of interest to examine the impact of the various mutated sites.

CHAPTER 4

MATERIALS AND METHODS

GROWTH AND MAINTENANCE OF STRAINS

The *C. elegans* mutant strains *ogt-1* (ok430), wild type (N2), *oga-1* (T20B5.3), *daf-2* (e1370), *pdk1-CA* (mg142), *daf-16* (mu86), and *daf-18* (ok480) were generated and kindly provided by the *C. elegans* Gene Knockout Consortium (Oklahoma Medical Research Foundation, Oklahoma City). The nematodes were maintained on NGM agar medium seeded with Escherichia coli (strain OP50) at 17-21 degree Celsius depending on the strain.

DOUBLE MUTANT CROSSES

Males were generated for *ogt-1* and *oga-1* mutant strains by incubation of L4 stage nematodes in 30 deg C for 6 – 8 hours. The *ogt-1* or *oga-1* males were then crossed with *daf-2* (e1370), *daf-16* (mu86), *pdk1-CA* (mg142) and *daf-18* (ok480). F2 offspring were selected for double homozygous mutations of *ogt-1, oga-1, daf-16,* and *daf-18* via size difference of the gene versus wild type using polymerase chain reaction (PCR). *Daf-2* (e1370) homozygous mutants were selected by amplifying a section of the gene followed by DNA sequencing. *Pkd1* homozygous mutants were selected via PCR amplification of a portion of the gene and restriction cut using HpaII restriction enzyme.

PCR ANALYSIS

C. elegans genomic DNA was amplified using BioRad iTaq DNA polymerase Kit. Protocol was followed according to the manufacturer with the following primers. *Ogt-1* left (gccaaagaattgatttcgga), *ogt-1* right (tgctcttgcaccacaaccta), *oga-1* left (caatgtcgtcaatggctacg), *oga-1* right (gttgttgaaggtaagcccca), *daf-16* forward (agaccgttggtcaaatgcttgctg), *daf-16* reverse (tggctgatcgggaagtgtcagatt), *daf-2* forward (tggagatttcggaatggctcgtga), *daf-2* reverse (ttgtcgaatgactcgtcgtcgcat), *daf-18* left (cctccgactgctccagtaac), *daf-18* right (aaggaatggcttgaagcaga), *pdk1* forward (gagatgctagctgacgga), and *pdk1* reverse (caggccaaatgttagagta). For *ogt-1, oga-1, daf-18,* and *pdk1*, an annealing temperature of 55 deg C and thirty-five 4-minute amplifying cycles were used. For *daf-2*, an annealing temperature of 65 deg C and thirty-five 1-minute amplifying cycles were used.

WORM LYSIS FOR PCR ANALYSIS

Worms were suspended in 8 ul of 50 mM KCl, 20 mM Tris, pH 8.4 and frozen for 15 minutes at –80 deg C. Solution was thawed and 4 ul of lysis solution (10 mM Tris pH 8.4, 50 mM KCl, 25 mM MgCl2, 0.45% Tween-20, 0.05% gelatin, Proteinase K 60 ug/mL) were added and mixed by tapping the eppindorf. The solution was frozen for 10 minutes at –80 deg C, thawed, and topped with mineral oil. The eppindorf was then heated for 1 hour at 65 deg C followed by 20 minutes at 95 deg C. The DNA extract was then stored at –20.

LIFESPAN

For lifespan studies, adult hemaphrodites were allowed to lay eggs for about 6 hours. When eggs grew to adulthood, worms were moved every day for about 5

days. After nematodes finished laying eggs, they were maintained on NGM agar medium seeded with Escherichia coli (strain OP50) at 20-21 degree Celsius.

PROTEIN LYSIS

10-12 large plates of each worm strain were collected in 15 mL polystyrene tubes with M9 buffer (3g KH2PO4, 6g Na2HPO4, 5g NaCl, 1mL 1M MgSO4,to 1 liter). Nematodes were centrifuged for 1 minute at 2000 rpm and rinsed twice with PBS followed by 1 minute centrifugation at 2000 rpm. The worms were then washed twice with lysis washing buffer (50 mM HEPES (pH 7.8), 300 mM NaCl, 10% glycerol) followed by 1 minute centrifugation at 2000 rpm each time. The nematodes were then frozen at –80 deg C. Packed frozen worms were transferred to mortar pestle that had been pre-chilled with liquid nitrogen. Liquid nitrogen was poured on the worms as needed to keep them frozen while the packed worms were ground to flour consistency. The frozen ground worms were transferred to 15 mL polystyrene tubes and 10 mL of cold lysis buffer (50 mM HEPES pH 7.8, 300 mM NaCl, 10% glycerol, 0.2% Triton X-100, 2 mM DTT, 10 mM MgCl2, 100 ug/mL Dnase I, protease inhibitor cocktail, and phosphatase inhibitor cocktain) was immediately added to ground worms. The solution was allowed to incubate on ice and the lysate was sonicated six times for 8 seconds each with 30 second breaks on ice. The lysate was then alliquoted into 1.5 mL tubes and centrifuged at 16,000 rpm for 1 hour. The middle portion of each tube was collected. Bradford assay (Bio-Rad) was used to determine protein concentration.

WESTERN ANALYSIS

Equal μg loading of each lysate was separated on a 10% NuPaGE Bis-Tris gel (Invitrogen) followed by transfer onto a membrane (Millipore). The blot was blocked for one hour at room temperature with 3% BSA in TBST (1x TBS, 0.05% tween-20) and incubated with RL2 antibody (1:2000) in 3% BSA, TBST overnight at 4 deg C. Membrane was rinsed three times with TBST and incubated in anti-mouse IgG (1:2000) modified with horseradish peroxidase (Amersham Pharmacia) for 1.5 hours followed by three washes with TBST and one wash with TBS. The membrane was visualized using a chemiluminescent substrate (super signal west pico; Pierce). The membrane was then probed with alpha-tubulin (sigma) to determine loading concentrations. Blot was washed six times with TBST and blocked with 3% BSA in TBST. Membrane was incubated with anti-alpha-tubulin (1:2000) in 3% BSA/TBST for 1 hour at room temperature followed by six washings with TBST and an incubation with horseradish peroxidase modified anti-mouse-IgG (Pierce; 1:10,000) for 1 hour. Membrane was washed again six times with TBST and developed with super signal west pico (Pierce) chemiluminescent substrate.

DEVELOPMENTAL PICTURES

Adult hemaphrodites were allowed to lay eggs for about 1 hour. Eggs were incubated on NGM agar medium seeded with Escherichia coli (strain OP50) at 17 degree Celsius. Pictures were taken 76 hours after egg laying.

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FIGURE LEGENDS

Figure 1A. ogt-1 and oga-1 modulate *C. elegans* lifespan. Lifespans for O-GlcNAc transferase mutant (ogt-1) and O-GlcNAcase mutant (oga-1) are shown in comparison to wild type *C. elegans*. The median lifespan is shown with error bars.

Fig. 1B. Global O-GlcNAc levels of C. elegans lysates. Anti-O-GlcNAc (RL-2 antibody) western is shown for ogt-1 mutant, wild type, and oga-1 mutant. Anti-α-tubulin western (below) is used for loading control.

Fig. 1C. ogt-1; oga-1 double mutant lifespan is similar to ogt-1 lifespan. ogt-1; oga-1 double null allele lifespan is shown compared to ogt-1, wild type, and oga-1.

Fig. 2A. O-GlcNAc is acting downstream of daf-2. Lifespan of ogt-1; daf-2 double mutant showing ogt-1 suppression of daf-2 extended lifespan.

Fig. 2B. O-GlcNAc is acting upstream of daf-16. Lifespan of oga-1; daf-16 double mutant showing daf-16 suppression of oga-1 extended lifespan.

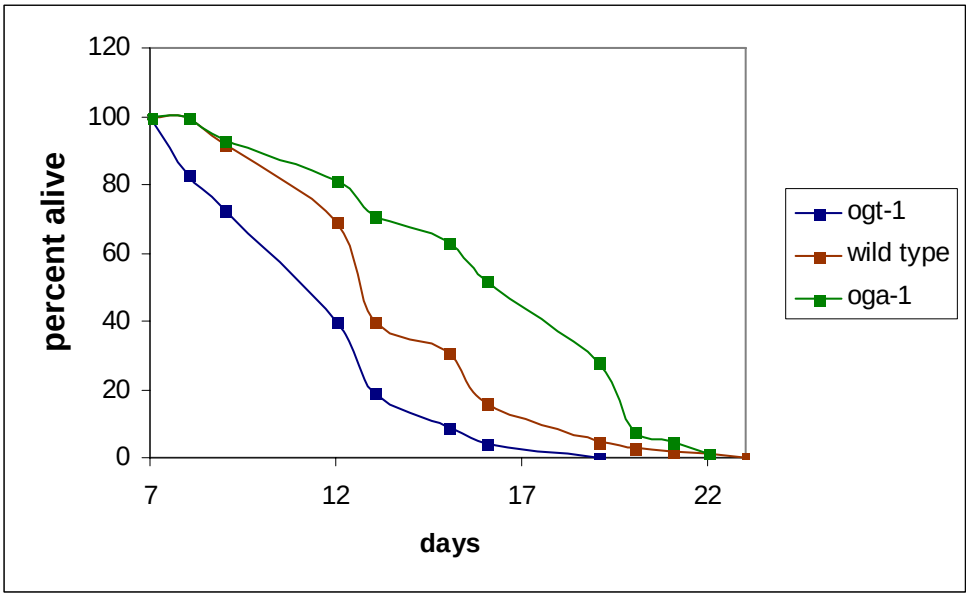
Fig. 2C. Ogt-1 does not suppress the developmental delay of daf-2 in the ogt-1;daf-2 double mutant. While ogt-1 suppresses daf-2 extended lifespan, it does not alter daf-2's delayed development. Pictures shown, a representative from 10 offspring worms taken under same magnification, are of ogt-1, wild type, oga-1, daf-2, ogt-1;daf-2, and oga-1;daf-1 (65 hours from egg laying at 17 deg C.). Ogt-1, wild type, and oga-1 appear to have similar development while daf-2, ogt-1;daf-2 and oga-1;daf-2 have delayed development compared to wild type.

Fig. 3A. O-GlcNAc is acting at or above pdk1. Lifespan of oga-1; pdk1 double mutant showing pdk1 suppression of oga-1 extended lifespan.

Fig. 3B. O-GlcNAc is acting at or above daf-18. Lifespan of oga-1; daf-18 double mutant showing daf-18 suppression of oga-1 extended lifespan.

Fig. 4. Mammalian and *C. elegans* insulin signaling pathway. Depicted are mammalian insulin signaling proteins and their C. eleg

FIGURE 1A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100
80
60
40
20
0
percent alive
7
12
17
22
days
ogt-1
wild type
oga-1

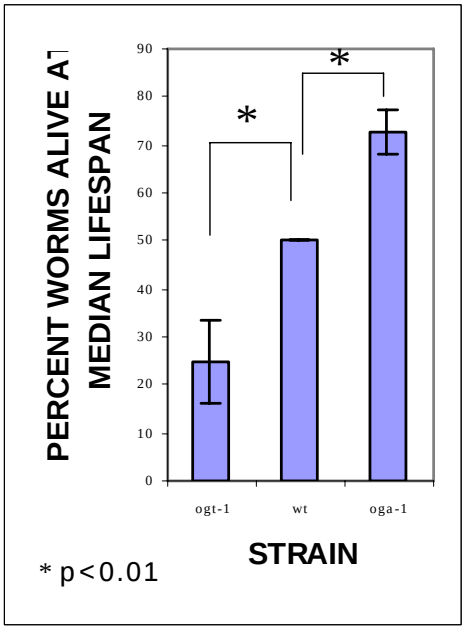

PERCENT WORMS ALIVE AT
MEDIAN LIFESPAN
90
80
70
60
50
40
30
20
10
0
*
*
ogt-1
wt
oga-1
STRAIN
* p<0.01

FIGURE 1B

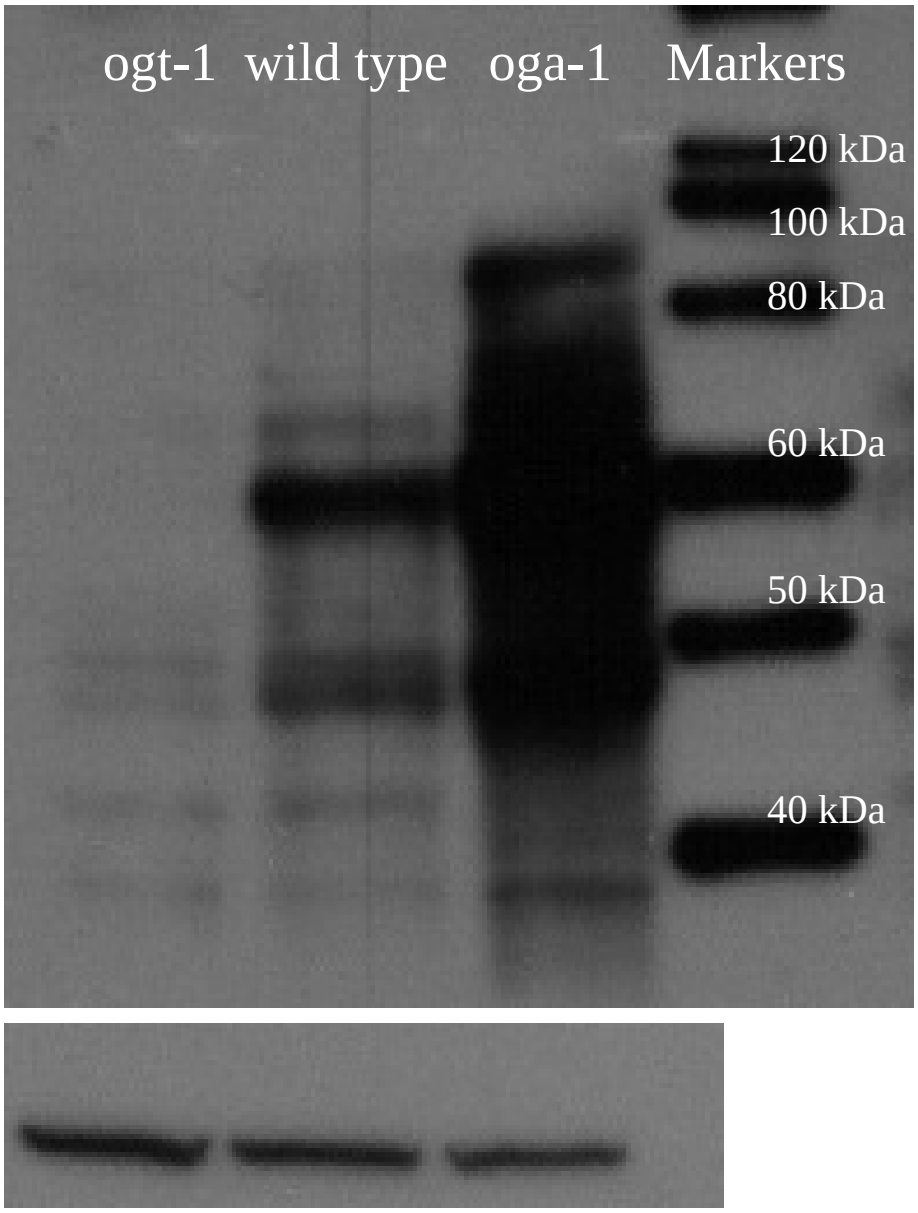
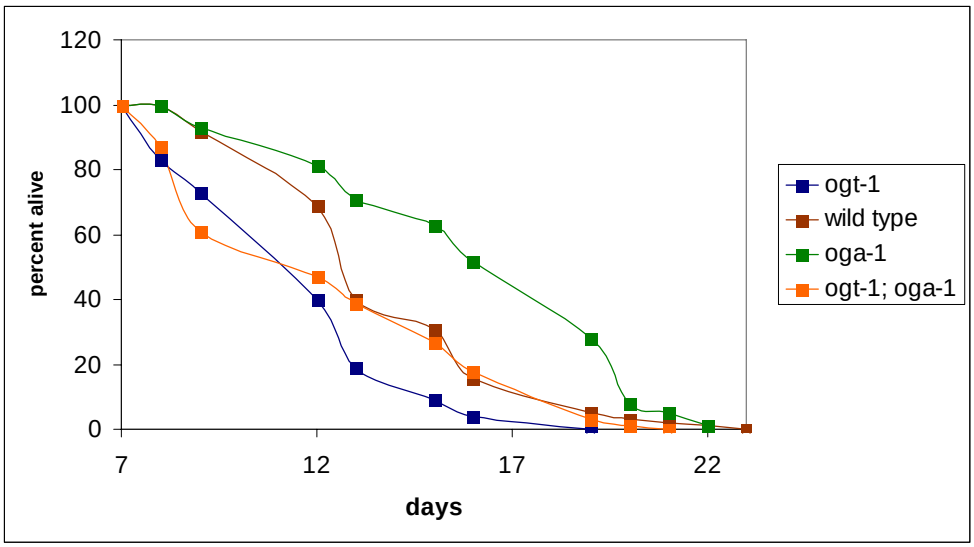

ogt-1
wild type
oga-1
Markers
120 kDa
100 kDa
80 kDa
60 kDa
50 kDa
40 kDa

FIGURE 1C


120
100
80
60
40
20
0
percent alive
7
12
17
22
days
ogt-1
wild type
oga-1
ogt-1; oga-1

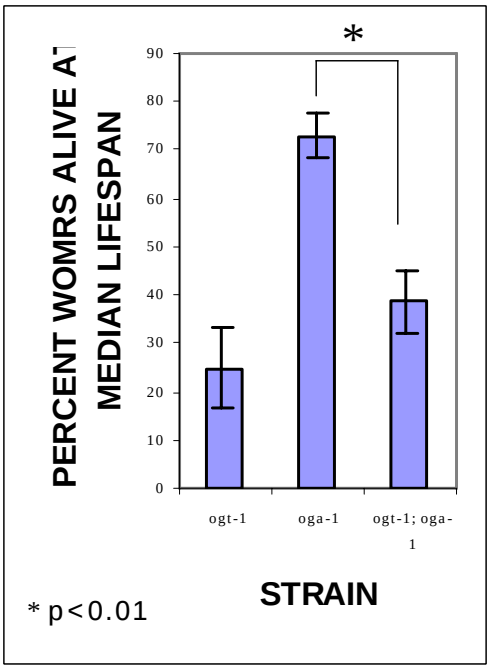
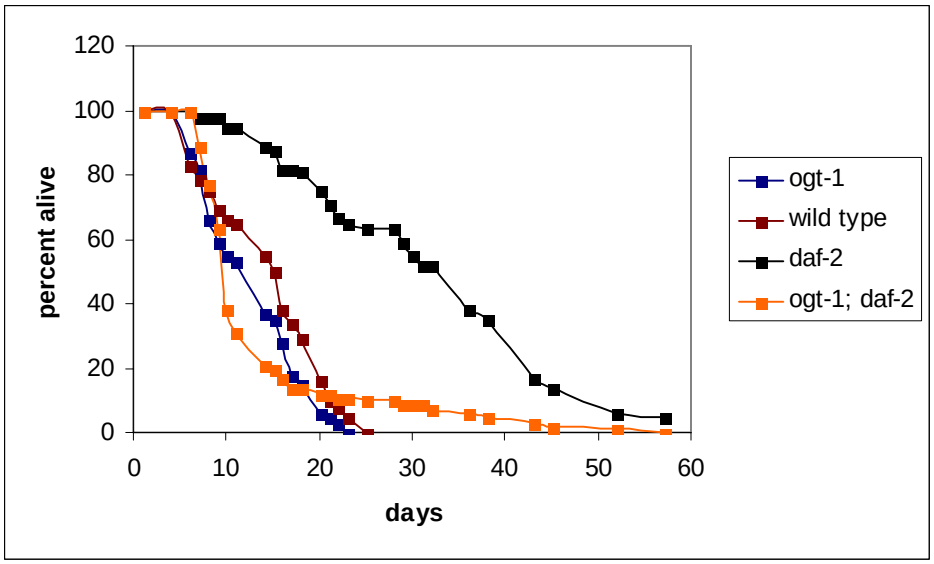

*
PERCENT WOMRS ALIVE AT
MEDIAN LIFESPAN
90
80
70
60
50
40
30
20
10
0
ogt-1
oga-1
ogt-1; oga-1
STRAIN
* p<0.01

FIGURE 2A


120
100
80
60
40
20
0
percent alive
0
10
20
30
40
50
60
days
ogt-1
wild type
daf-2
ogt-1; daf-2

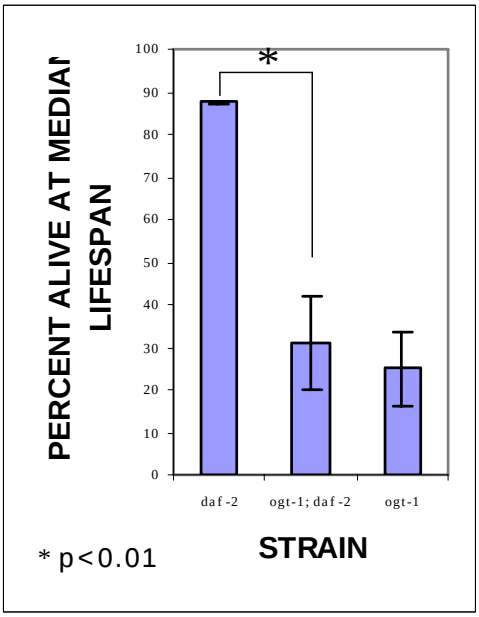
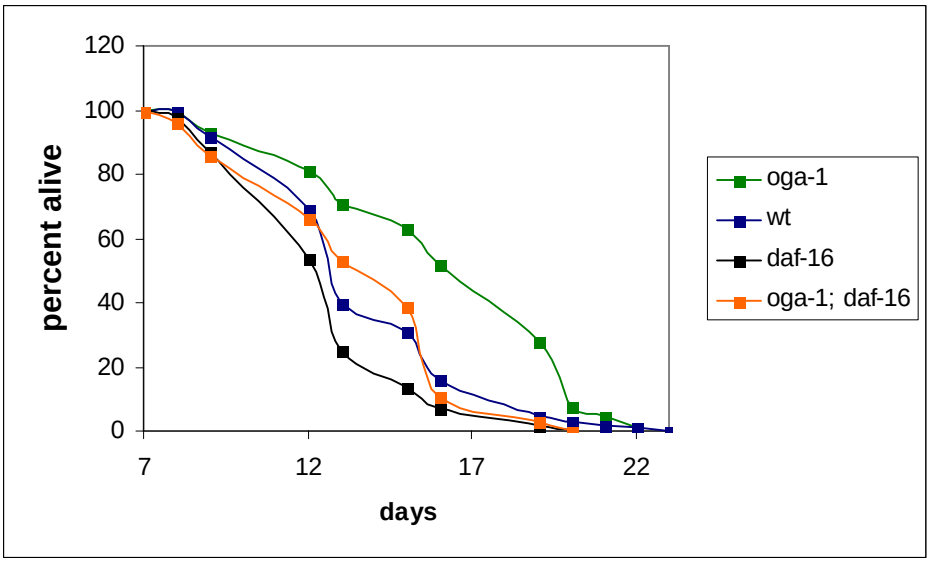

PERCENT ALIVE AT MEDIAN LIFESPAN
100
90
80
70
60
50
40
30
20
10
0
*
daf-2
ogt-1; daf-2
ogt-1
STRAIN
* p<0.01

FIGURE 2B


120
100
80
60
40
20
0
percent alive
7
12
17
22
days
oga-1
wt
daf-16
oga-1; daf-16

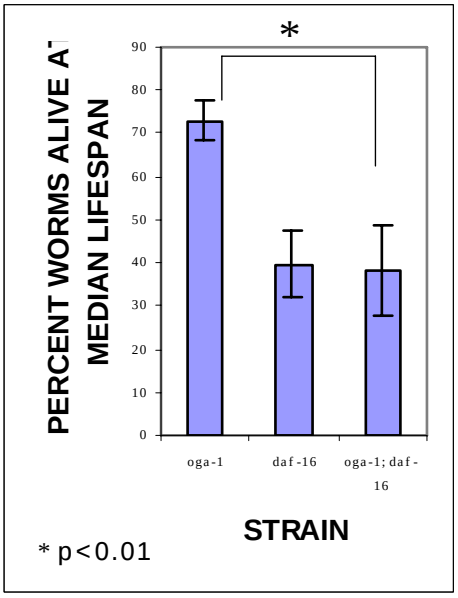
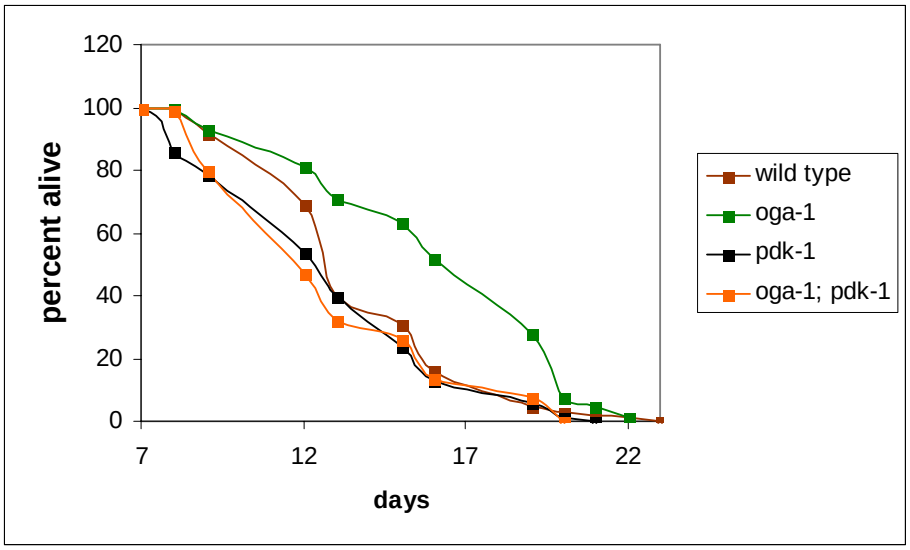

*
PERCENT WORMS ALIVE A
MEDIAN LIFESPAN
90
80
70
60
50
40
30
20
10
0
oga-1
daf-16
oga-1; daf-16
STRAIN
* p<0.01

FIGURE 2C

FIGURE 3A


120
100
80
60
40
20
0
percent alive
7
12
17
22
days
wild type
oga-1
pdk-1
oga-1; pdk-1

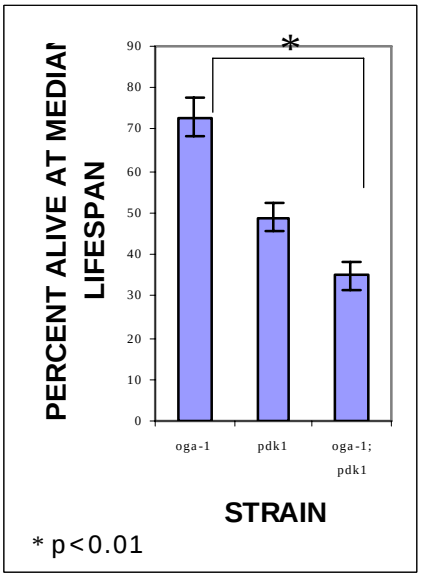
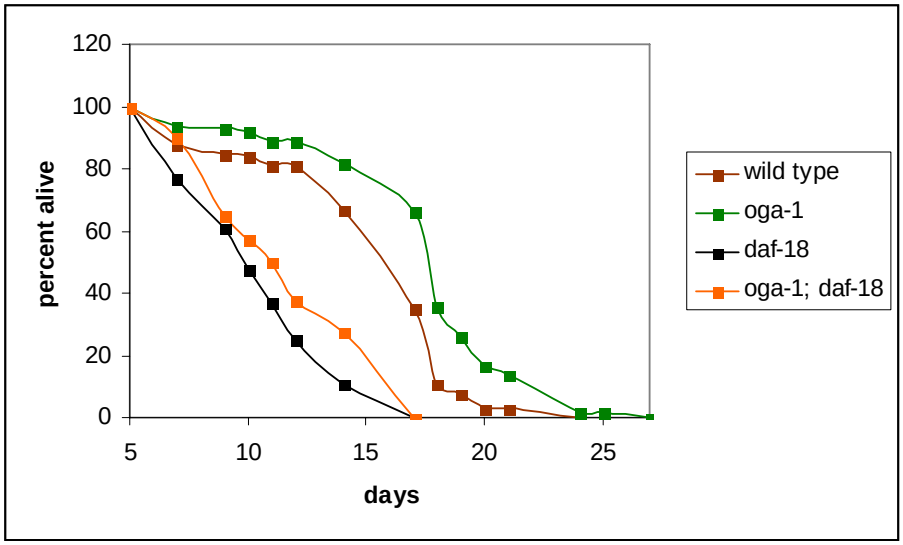

PERCENT ALIVE AT MEDIAN LIFESPAN
90
80
70
60
50
40
30
20
10
0
*
oga-1
pdk1
oga-1; pdk1
STRAIN
* p<0.01

FIGURE 3B


120
100
80
60
40
20
0
percent alive
5
10
15
20
25
days
wild type
oga-1
daf-18
oga-1; daf-18

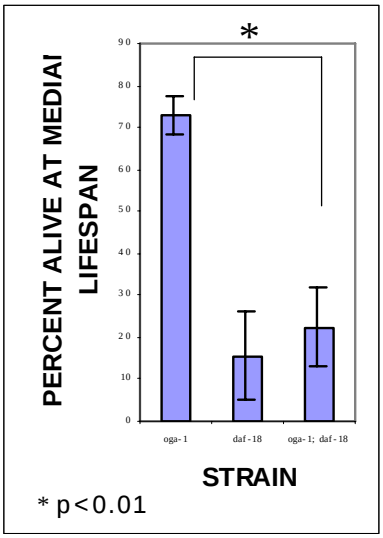
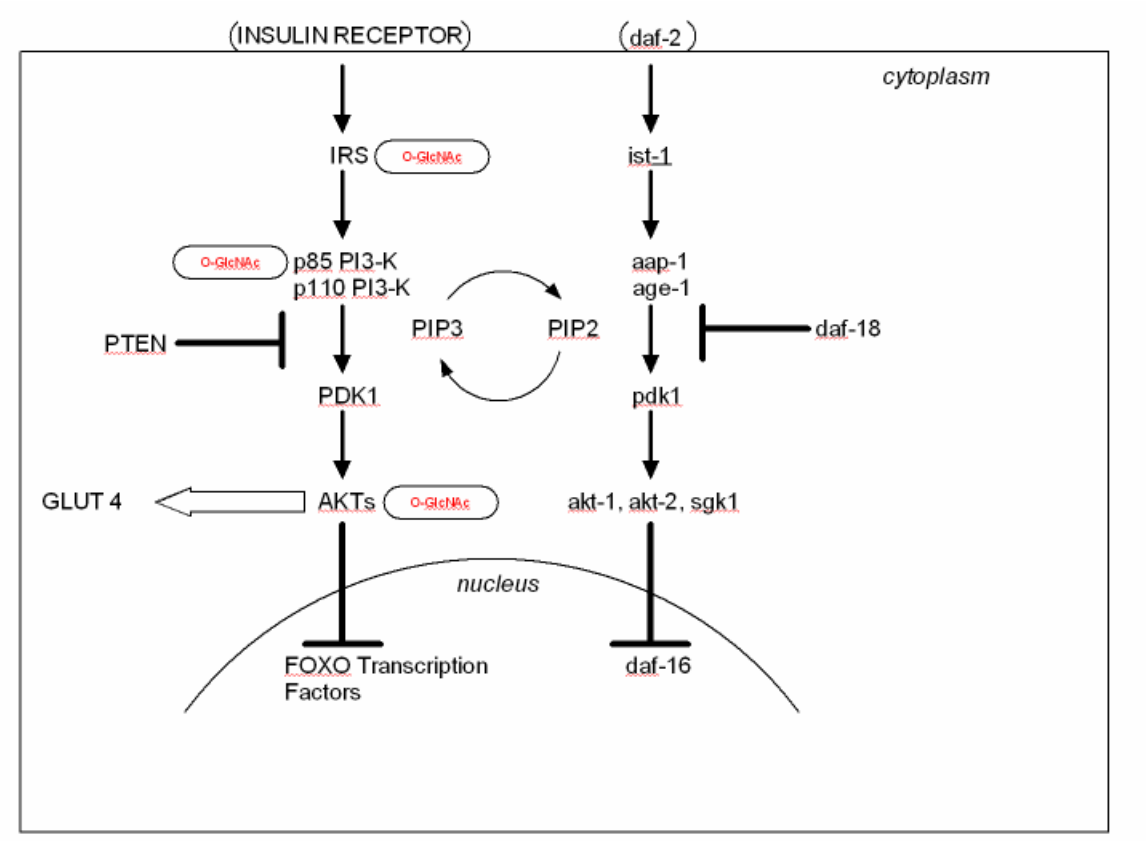

*
PERCENT ALIVE AT MEDIAI
LIFESPAN
90
80
70
60
50
40
30
20
10
0
oga-1
daf-18
oga-1; daf-18
STRAIN
* p<0.01

FIGURE 4


(INSULIN RECEPTOR)
(daf-2)
cytoplasm
IRS
O-GlcNAc
ist-1
O-GlcNAc
p85 PI3-K
p110 PI3-K
aap-1
age-1
PTEN
PIP3
PIP2
daf-18
PDK1
pdk1
GLUT 4
AKTs
O-GlcNAc
akt-1, akt-2, sgk1
nucleus
FOXO Transcription Factors
daf-16