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DEVELOPING *C. ELEGANS* AS A MODEL TO STUDY TYPE 2 DIABETES
MELLITUS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science in Biochemistry at Virginia Commonwealth University.

by

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List of Abbreviations

AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
cAMP	3'-5'-cyclic adenosine monophosphate
AVs	Autophagic vesicles
ETC	Electron transport chain
IGF-1	Insulin-like growth factor 1
LROs	Lysosome-related organelles
L1s	First larvae stage
OCT	Organic cation transporter
Pi	Inorganic phosphate
T2DM	Type 2 diabetes mellitus

Abstract

DEVELOPING *C. ELEGANS* AS A MODEL TO STUDY TYPE 2 DIABETES MELLITUS

By Jheesoo Ahn, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2014.

Director: Young-Jai You, Ph.D.

Caenorhabditis elegans has been studied as a model organism in various areas of biomedical research because it shares many conserved functions at molecular and genetic levels with humans. Specifically, it is an ideal organism to study heterogeneous metabolic syndromes such as Type 2 Diabetes Mellitus (T2DM) as *C. elegans* can be used to delineate molecular pathways that are at the core of its problems. A growing number of populations worldwide are faced with chronic T2DM, which also manifests several complications, such as blindness, neuropathy and cardiovascular diseases. Currently, metformin is the first-line drug of choice administered to treat T2DM. While the mechanism by which it alleviates the symptoms of diabetes is unknown, it has been found to reduce metabolic rate by partially inhibiting the mitochondrial complex I in mammals. Using *C. elegans* as a genetic model organism, we show that metformin reduces the

mitochondrial activity through endosomal Na^+/H^+ exchanger, which a previous lab member has found to be a potential target of metformin. Furthermore, we show that high glucose diet—known to reduce the worm’s lifespan—alter the endosomal-lysosomal system and autophagy, providing insights to using *C. elegans* as a diabetic model. Based on these results, we propose that *C. elegans* can serve as a model organism to study T2DM as well as provide new ways to further investigate the pathophysiology of this disease.

1. Introduction

1.1 Type 2 Diabetes Mellitus

Diabetes is one of the most prevalent diseases today affecting people worldwide, where an estimated 382 million people have been diagnosed with diabetes in 2013. The number is expected to reach 592 million people by the year 2035¹. Its economic burden has also been increasing, as the total estimated cost of diagnosed diabetes in 2012 was \$245 billion, which was a 41% increase from the estimated cost of \$174 billion in 2007. These factors are two of many which highlight the burden it creates on our society in addition to reduced quality of life, suffering of people with diabetes, their families and friends². Type 2 diabetes mellitus (T2DM) is the most common of the two types. T2DM is characterized by insulin resistance and glucose intolerance³. As a result, individuals with T2DM display both hyperinsulinemia and hyperglycemia³. Throughout the years, there have been many advances in T2DM research, which have elucidated pathways like translocation of GLUT4 transporters in presence of insulin and subsequent glucose uptake⁴. Even though we have made significant numbers of discoveries on the pathophysiology of this disease, we are still left with no known cure. Hence, we aim to generate a simple genetic model to further characterize its pathophysiology using *Caenorhabditis elegans*.

1.2 Metformin

Metformin (1,1-dimethylbiguanide) is a biguanide that is used as a first-line drug of choice for the treatment of type 2 diabetes mellitus⁶. Its main function is to suppress

glucose production by the liver and restore insulin sensitivity in the peripheral tissues, perhaps secondarily to the reduction in glucose concentration. Yet, this has added attention to studying metformin's beneficial effect on other insulin-related diseases such as polycystic ovary syndrome, certain cancers and neurological disorders^{7,3}. The current view of its actions is that once it is taken up via organic cation transporter (OCT) 1 or 2 (mainly expressed in the liver and kidney, respectively), it then targets the mitochondrion, where it partially inhibits complex I of the electron transport chain (ETC). It is known that the drug inhibits complex I without affecting subsequent steps of the mitochondrial machinery and induce a decrease in NADH oxidation, proton pumping across the inner mitochondrial membrane and oxygen consumption rate. These sequential effects lead to the reduction of the proton gradient and proton-driven synthesis of ATP from ADP and inorganic phosphate (Pi)⁸. Consequently, the cell's AMP/ATP ratio increases leading to the activation of AMP-activated protein kinase (AMPK) pathway, reducing gluconeogenic gene expression and energy consuming processes such as lipogenesis and fatty acid and protein synthesis⁷. Metformin has also shown to work in a mitochondria-independent manner through antagonistic action on the glucagon signaling pathway. This mechanism involves AMP build-up and inhibition of adenylate cyclase activity, leading to a reduction in cyclic adenosine monophosphate (cAMP) levels, thereby, inhibiting protein kinase A (PKA) activity and glucagon-dependent glucose production in mice⁷. Despite these two compelling lines of evidence of metformin's actions, its mode of action still remains unclear due to its pleotropic effects⁷. This brought up questions about how metformin mediates its actions in two completely distinct pathways and what its molecular targets are.

Our previous work with metformin has directed our attention on the role of the endocytic pathway upstream of the mitochondrion. Our lab has discovered that metformin targets an endosomal Na^+/H^+ exchanger (NHE) in *C. elegans* and similar results have been shown with *Drosophila melanogaster* and mammalian cells.

1.3 Na^+/H^+ exchanger

In *C. elegans*, metformin (100mM) induces increase in median lifespan under fed conditions due to changes in folate and methionine metabolism in the bacterial food^{9,10}; however, it decreases the first stage larva's (L1's) lifespan in the absence of food, reducing their survival from approximately 14 days to 7 days at 20°C (Figure 1). Using this metformin sensitive phenotype, our lab performed an unbiased genetic screen for mutants resistant to metformin and identified a mutation in the *nhx-5* gene. The *C. elegans* genome contains nine NHEs, NHX-1 through NHX-9, all of which exchange vesicular or extracellular sodium for an intracellular proton¹¹. Some are expressed on the plasma membrane of cells, while others are expressed on the membranes of intracellular organelle. NHX-5 is expressed on intracellular endosomes¹¹. Our finding suggested that metformin could potentially target NHX-5 and could be regulating the endocytic cycle. Proper regulation of the endocytic cycle is essential for the cell's pH homeostasis as well as for metabolic processes such as mTOR activation and life span^{12,13}. Putting these pieces of evidence together, we further investigated the role of NHX-5 in the regulation of the endocytic cycle in *C. elegans*.

Figure 1

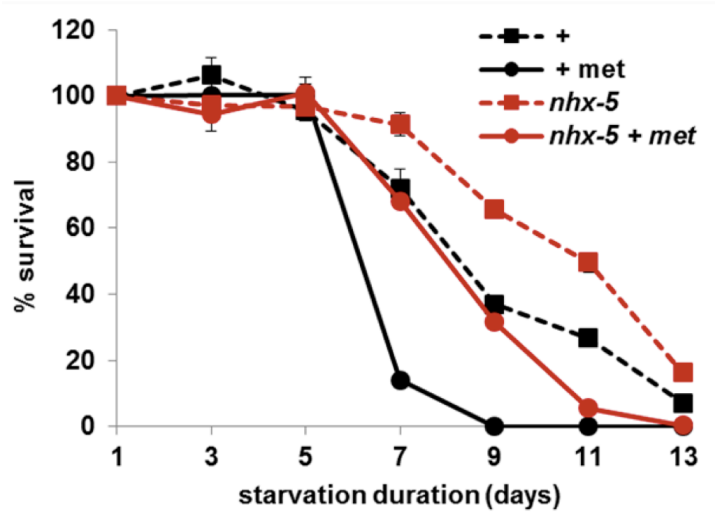


Figure 1. Metformin reduces L1 longevity in an *nhx-5* dependent manner.

Metformin (100 mM, ●) reduces L1 longevity in wild type. The *nhx-5* mutant is resistant to metformin (●). $p < 0.001$ between metformin treated wild type and metformin treated *nhx-5* mutant by log rank test.

1.4 The endocytic cycle in *C. elegans*

NHX-5, as a potential target of metformin, led us to investigate the role of NHX-5 in the endocytic cycle. The best-studied endocytosis pathway in worms is the clathrin-coated pit pathway¹⁴. Once clathrin-coated pits are formed and pinched off from the plasma membrane, these vesicles are uncoated and fused with early endosomes in a reaction requiring the small GTPase Rab 5. There are recycling endosomes, which transport receptors back to the plasma membrane, whereas some materials from the extracellular space are transported from early to late endosomes and eventually to lysosomes for degradation (Figure 2)¹⁴. Lysosome-related organelles (LROs), also known as gut granules in *C. elegans*, are acidified compartments that contain acid hydrolases and share the functions of the classical lysosome¹⁵.

Autophagy involves induction, sequestering of cytoplasmic cargoes, nucleation and elongation of a double-membrane organelle, the autophagosome, followed by degradation of internalized products³. Autophagosomes, which are crucial compartments in catabolism, are an interrelated part of the endosomal-lysosomal system because autophagosomes fuse with the lysosome for degradation at the end of the autophagy process. In the absence of lysosomal degradation, autophagy does not progress normally and results in pathologic accumulation of autophagic vesicles (AVs), misfolded proteins and abnormal organelles³. With these known facts and conserved pathways in *C. elegans*, we further investigated the major compartments of the endocytic cycle as well as autophagy to study their roles in diabetes, in addition to the role of NHX-5 in the regulation of the endocytic cycle.

Figure 2

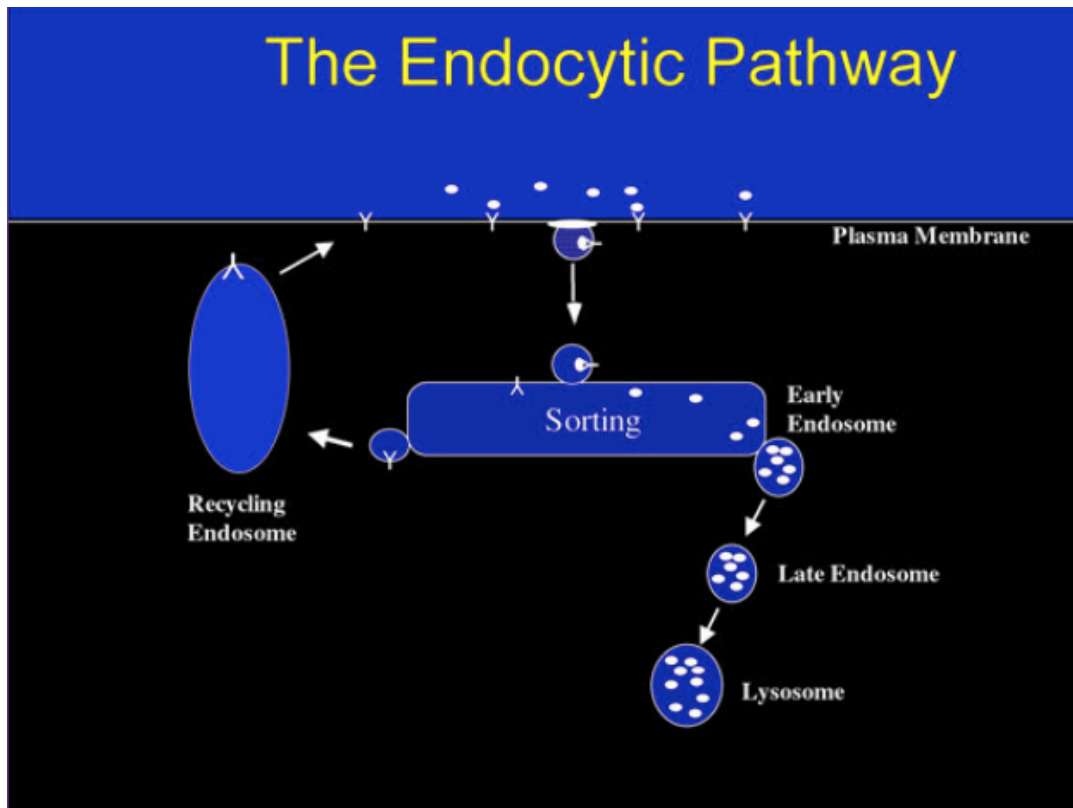


Figure 2. General model of the endocytic pathway in *C. elegans* (from Grant et al, 2006).

“Cargo molecules are endocytosed and targeted to early endosomes. Some cargos are further transported to lysosomes through late endosomes. Others are recycled back to the plasma membrane via the recycling pathway.”

1.5 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans was first proposed as a model organism back in the 1960s by Sydney Brenner as an ideal organism to study genetic and molecular mechanisms fundamental to more complex nervous and behavioral systems of higher organisms¹⁶. Thereafter, *C. elegans* has been further used for studying metabolism, reproduction, aging and other physiological processes, providing useful knowledge of the biology and molecular pathways underpinning human diseases. With this in mind, we aim to establish a new diabetes model in *C. elegans* to further characterize the pathophysiology of the disease. To be used as a model, *C. elegans* has to have conserved cellular processes and pathways that can be targeted by metformin. Indeed, our studies with metformin and others' studies have shown that there are known human-to-worm conserved pathways and cellular processes such as insulin signaling (insulin/IGF-1), AMPK/mTOR pathways and the endocytic cycle¹⁷. More importantly, this organism will enable us to test the drug's effect *in vivo*, which could be a challenge in mammalian systems. We first demonstrate that *C. elegans* displays the same physiological effect with treatment of metformin as it does in the mammalian systems. Furthermore, we show that glucose can mimic a diabetic condition in *C. elegans*. Finally, we developed transgenic worms to study the major components of the endocytic cycle to investigate whether misregulation of the endocytic cycle is a key pathophysiology of diabetes. My study shows that *C. elegans* can be a valid model to study T2DM to advance towards finding a cure for this disease.

2. Results

2.1 Metformin disrupts mitochondrial function in an *nhx-5* dependent manner.

When hatched in the absence of food, the first stage larvae (L1s) of *C. elegans* survive starvation for approximately 14 days. Our lab previously showed that metformin (100mM) reduces L1 longevity to 7 days (Figure 1). Metformin has been known to inhibit mitochondrial complex I in mice and decrease lifespan in *D. melanogaster*⁶. Hence, to better understand what effect metformin has on *C. elegans*, we used a whole-worm oxygen consumption assay. First, we followed an established protocol for preparing eggs for this experiment to synchronize the developmental stages of worms²⁷. Synchronizing worms was critical for the later step when we needed thousands of young adults in the same stage for the assay. We then incubated the eggs overnight at 20°C on a rocker to allow the eggs to hatch in nutrient-free medium and arrest at L1. We recorded the oxygen level for 7 min and the rate of consumption was quantified as the slope of the best-fit-line between 30 sec and 5 min 30 sec (Figure 3). The oxygen consumption rate decreased approximately 1.5 fold in wild type with the metformin treatment (Figure 4). This indicated that metformin reduced the respiration rate significantly by reducing the mitochondrial activity¹⁸. We then measured the oxygen consumption levels of the *nhx-5(ok644)* to see if metformin's lowering effect on respiration rate was mediated in part through NHX-5. Metformin treatment did not change the oxygen consumption rate of *nhx-5* (Figure 5); hence, we failed to detect metformin's effect on *nhx-5*.

Figure 3

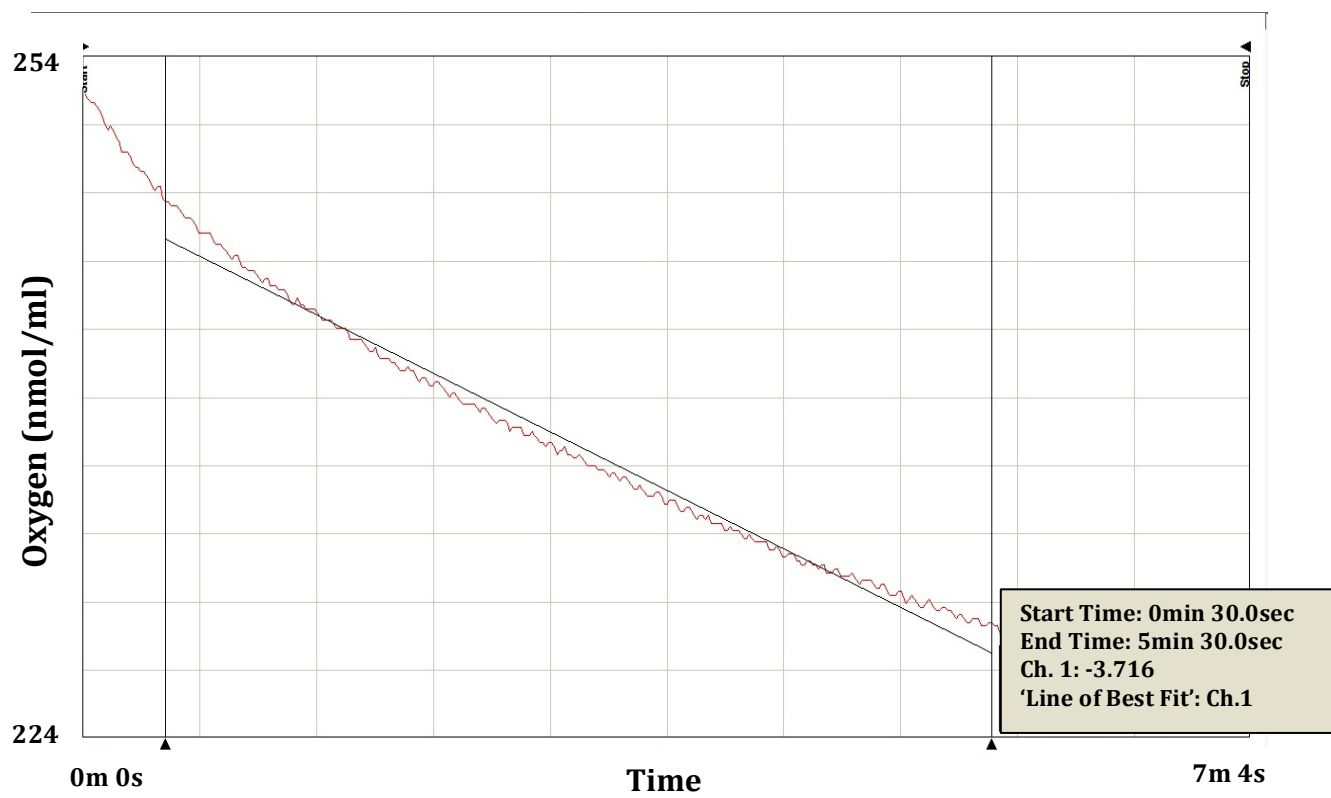


Figure 3. Representative graph of O₂ view and best line-of-fit.

One representative graph of one wild-type sample shows how the rate was measured. The negative slope represents worms' oxygen consumption as a function of time. The slope gives the rate in nmol/ml/min. The slope is always negative; therefore, we took the absolute values of the rates and used these values to make comparisons among the samples.

Figure 4

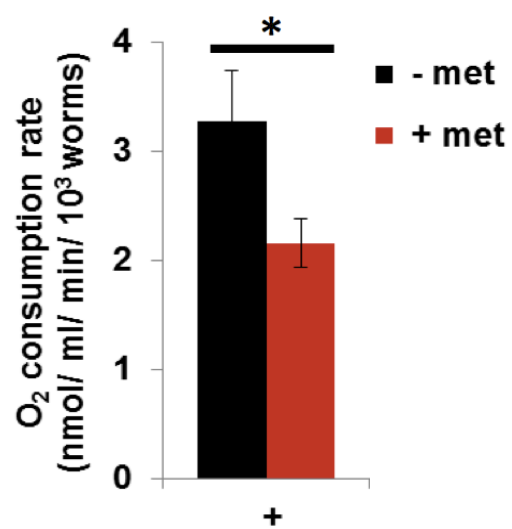


Figure 4. Metformin affects mitochondrial function in wild type.

In wild type, the oxygen consumption rate decreased significantly by approximately 1.5 fold with metformin treatment. Approximately 3000 worms were treated with 100 mM metformin for 30 min. Each experiment was repeated at least three times. Error bars indicate standard error of the mean. * $p < 0.05$ by paired student's t-test.

Figure 5

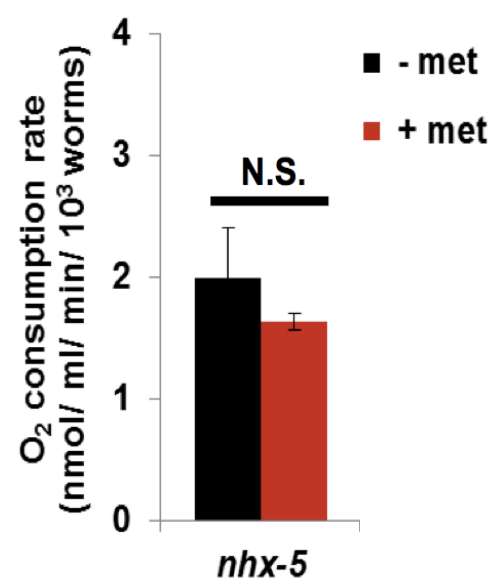


Figure 5. Metformin has no effect on *nhx-5* deletion mutant.

In the *nhx-5* mutant, oxygen consumption rate did not change with metformin treatment.

Approximately 3000 worms were treated with 100 mM metformin for 30 min. Each experiment was repeated at least three times. Error bars indicate standard error of the mean. N.S.= Not Significant by paired student's t-test.

Interestingly, the basal rate of oxygen consumption in the *nhx-5* mutant was much lower than in the wild type. This may be due to the nature of the *nhx-5* mutation, where the deletion itself caused a decrease in oxygen consumption by altering other cellular processes. Nonetheless, results from the assay suggested that NHX-5, at least in part, was responsible for mediating metformin's action on the mitochondrial activity and, therefore, respiration rate in *C. elegans*.

2.2 Metformin disrupts autophagy in *C. elegans*.

Next, we asked how metformin reduces L1 longevity under starvation. We hypothesized that metformin reduces L1 longevity through autophagy mainly due to misregulation of endocytic cycle. Autophagy is a tightly controlled mechanism that provides energy essential for survival of the organism. We examined autophagy as a potential target of metformin's harmful effects for two reasons. (1) We have shown that during L1 starvation, an optimum level of autophagy is critical for the worm's survival¹⁹(Figure 6) (2) Our previous results suggest that metformin interferes with the endocytic cycle through NHX-5. As mentioned above in the introduction, autophagosomes fuse with lysosomes to degrade their contents. Thus, altering the endocytic cycle will also alter lysosomal function and potentially affect autophagy. To test our hypothesis, we used a transgenic worm that carries an integrated line of GFP::LGG-1. LGG-1 encodes the *C. elegans* ortholog of mammalian LC3 and yeast Atg8, which are proteins required for transport and maturation of autophagosomes and degradation of cellular components by autophagy^{20,21}. Under normal growth conditions, it displays diffuse cytoplasmic localization.

Figure 6

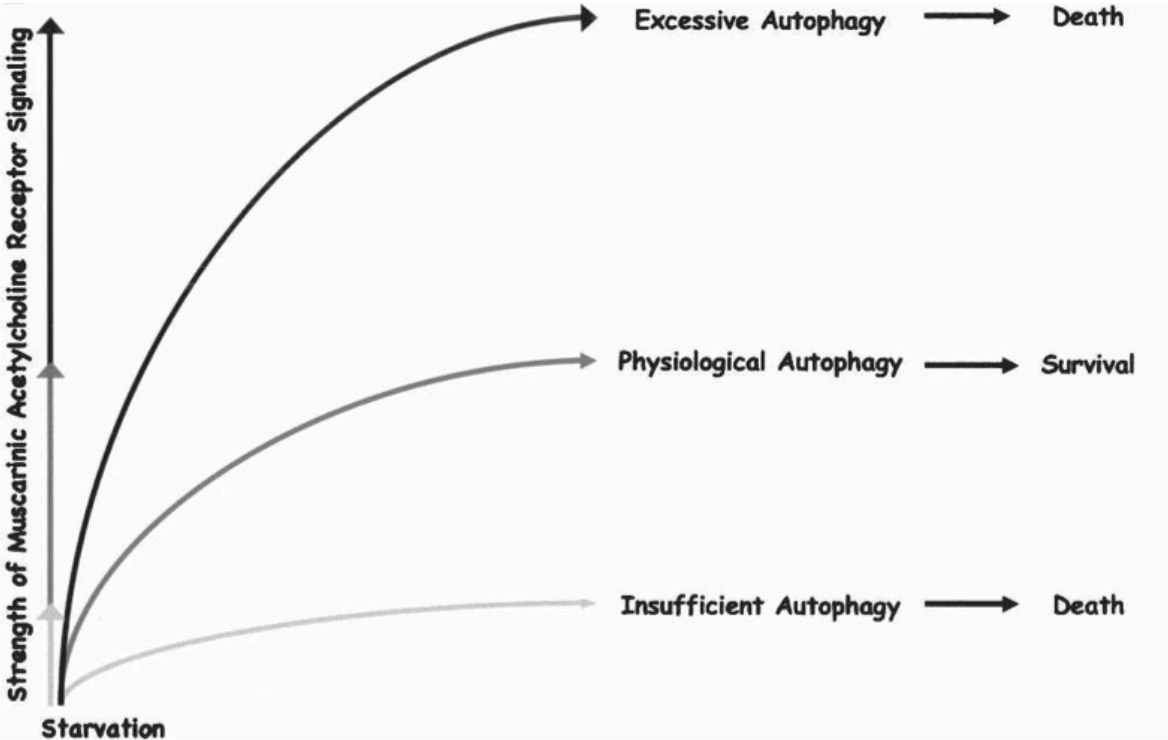


Figure 6. Model of dual autophagy in the survival of *C. elegans* (from Kang et al, 2008)

This model suggests that physiological levels of autophagy are critical in the survival of *C. elegans*. If there are insufficient or excessive levels of autophagy, then it could contribute to the organism's death¹⁹.

During starvation, however, GFP::LGG-1 shows an increase in punctate structures in the pharyngeal muscles and hypodermal cells. We counted puncta in the pharyngeal muscle, one of the easiest places to locate the structures in *C. elegans*²¹ (Figure 7A and B). The number of puncta steadily increased as starvation progressed. This indicated an increase in autophagy level to break down more of the cellular components to replenish energy. When we compared metformin treated and non-treated worms, the difference in the number of puncta started appearing from day 5 of starvation. Worms starved for 4 days did have punctate staining, but did not show any significant difference in the number regardless of metformin (Figure 8). Metformin treatment decreased the number of puncta staining, which suggested an inhibitory effect of the treatment. As the starvation day progressed to 7 days, an increasing number of worms looked disfigured, indicating the toxic effect of metformin on L1 longevity shown previously in the L1 lifespan study (Figure 1). Intriguingly, another study showed that metformin shortened the lifespan of *C. elegans* cultured in the absence of *E. coli* strain OP50, suggesting metformin's harmful effect in the absence of food regardless of developmental stages¹⁰. This study, in conjunction with our data, suggests that metformin disrupts the physiological state of autophagy leading to shortened lifespan.

Figure 7

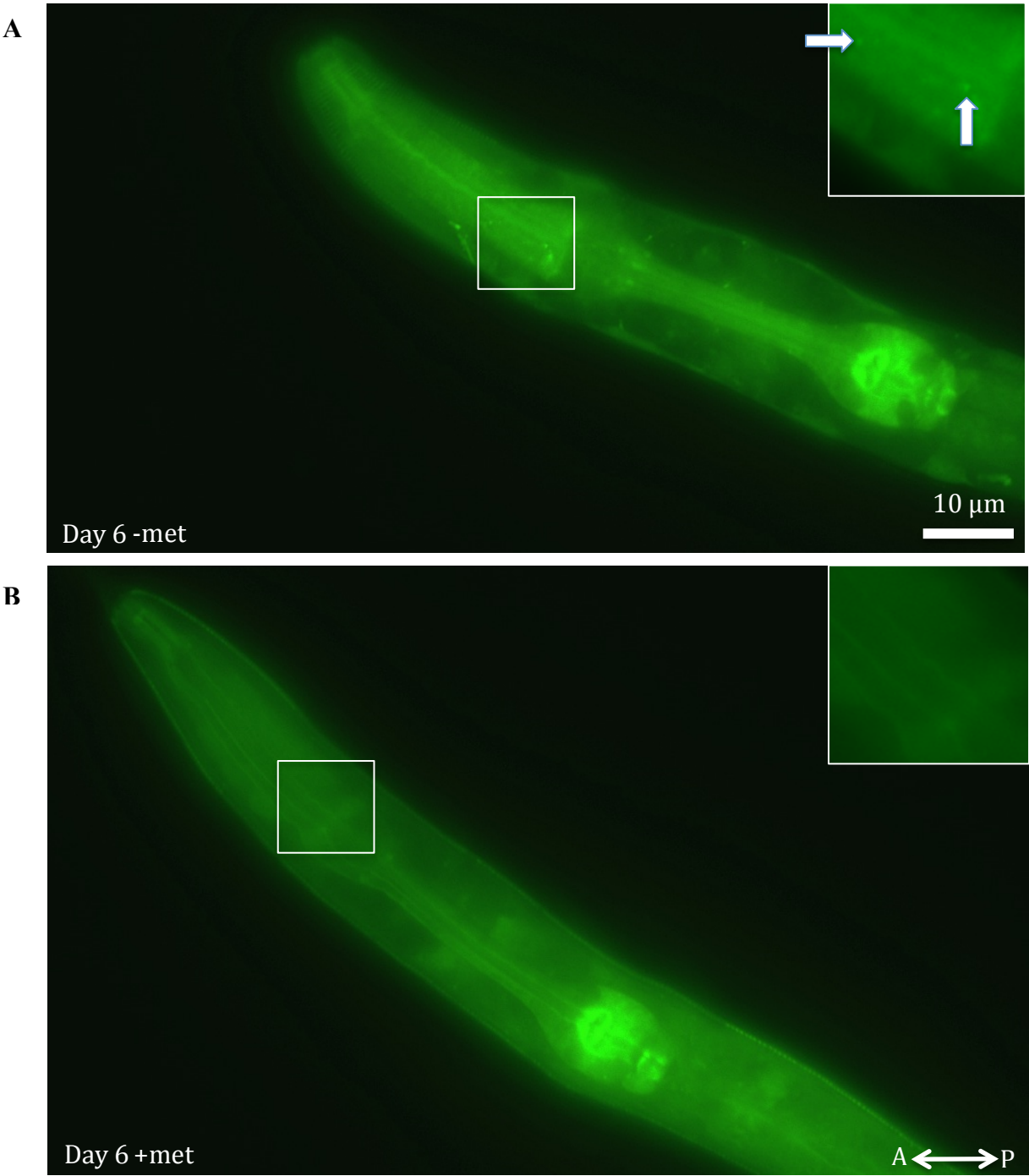


Figure 7. Worms show punctate structures under starvation.

These are representative pictures of worms starved for 6 days in the absence (A) and presence of metformin (B). The arrow shows representative GFP::LGG-1 positive punctate structures, which label both preautophagosomal and autophagosomal structures. These were taken with a fluorescence microscope in the green fluorescence channel. The color and brightness of both images were equally adjusted for a better representation using Adobe Photoshop CS5 and Microsoft© Powerpoint 2011. A=Anterior, P=Posterior.

Figure 8

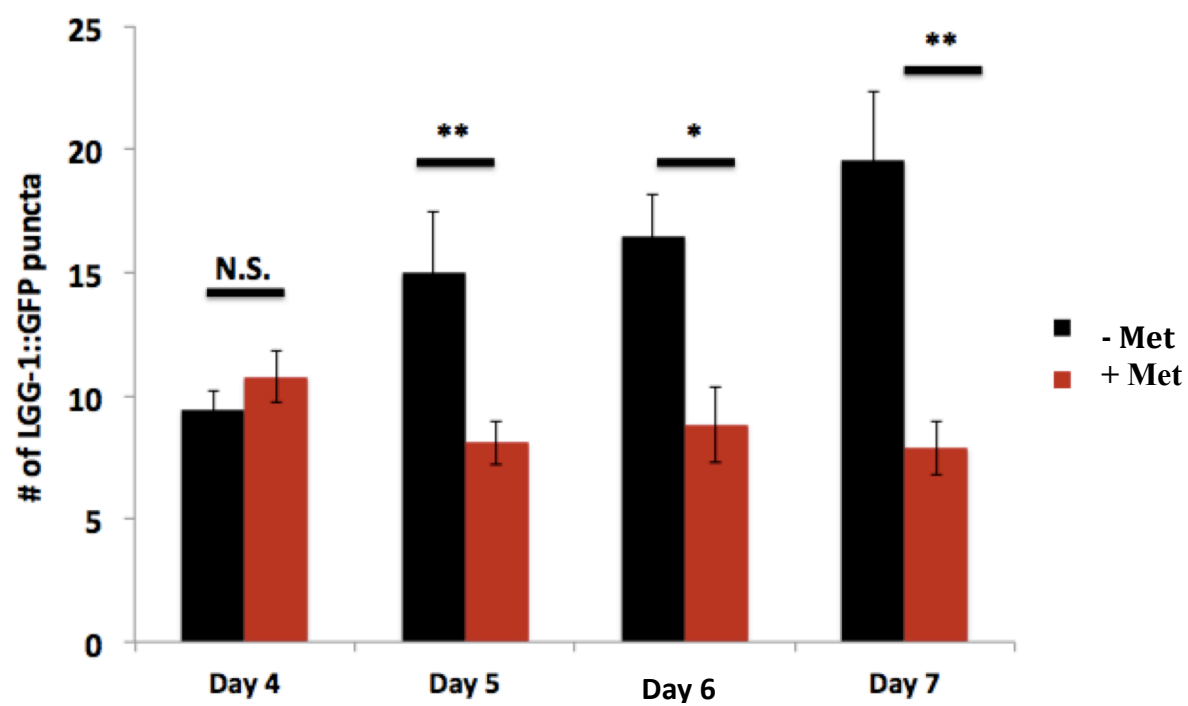


Figure 8. Metformin decreases the level of autophagy.

Punctate structures were counted using pictures like those shown above in Figure 7. Each bar represents the average of at least 5 worms. Error bars indicate standard error of the mean. N.S.= Not Significant; * $p < 0.05$; ** $p < 0.01$ by paired student's t-test.

2.3 Developing genetic tools to study changes in the endocytic cycle.

C. elegans responds to metformin with reduced oxygen consumption rates and autophagy levels. Next, we searched for a treatment under which *C. elegans* we could develop a diabetic-mimetic condition. T2DM is induced mostly by excess of nutrition disrupting glucose and insulin homeostasis. High D-glucose diet in *C. elegans* has been shown to reduce the lifespan of adult worms (Figure 9). Another study also showed that glucose restriction increased lifespan in worms by inducing mitochondrial respiration and oxidative stress²². Based on these studies, we hypothesize that treating worms with high D-glucose could mimic a diabetic state in *C. elegans*. We chose to examine changes in the endocytic cycle after high glucose diet for two reasons: (1) Our genetic screens suggested metformin targets a compartment of the endocytic cycle, which mediates traffic between late endosomes and lysosomes and (2) my result showed that during L1 starvation, metformin disrupted the level of autophagy, leading to poor survival of worms. These two results suggested that one of the main pathophysiological features of diabetes may arise due to an abnormal endocytic cycle.

We chose to use Nile red to stain the lysosome and its related organelles. Nile red, a lipophilic dye, is a convenient way to visualize LROs easily, as it can be taken up along with the nutrient source, *E. coli*¹⁵. Synchronized L1s were mounted on a plate treated with Nile red, with or without glucose. Pictures were taken at young adult stage two days (48-50 hours) after plating. The stage of the worms was critical because LRO staining increases dramatically after the young adult stage due to production of eggs as well as an increase in number of LROs. LROs also have been known to stain age-related organelles¹⁵.

Figure 9

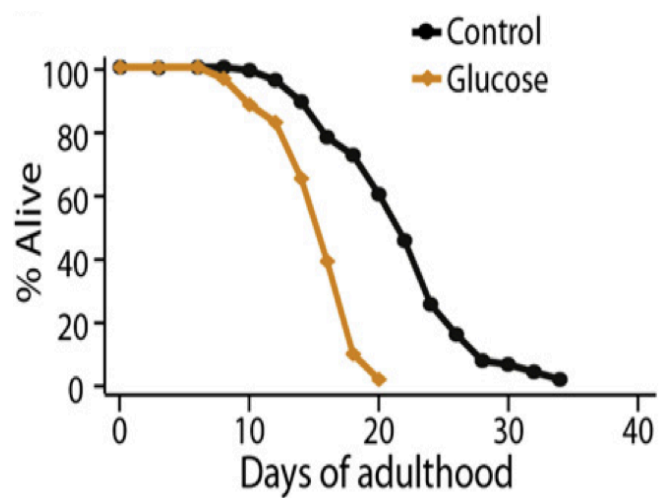


Figure 9. High glucose is harmful to worms (from Lee et al, 2009).

2% D-glucose treatment reduces adult lifespan in *C. elegans*. There was approximately 20% decrease in lifespan in parallel with ~50% increase in glucose levels inside the worms. 1%, 0.5%, 0.25% and 0.05% glucose decreased lifespan while 0.025% did not²³.

We saw a significant increase in the staining of wild type treated with glucose both in the images (Figure 10A and B) as well as in quantification (Figure 11). This suggested that LROs were altered morphologically and/or functionally.

Our previous results and the oxygen consumption data suggested that metformin mediated its action through NHX-5. So, we tested whether *nhx-5* displayed detectable differences in Nile red staining. Interestingly, the *nhx-5* showed a reduction in Nile red staining at its basal level compared to wild type (Figure 12). This suggested that the basal level and integrity of LROs in *nhx-5* mutants were lower than in the wild type. A possible explanation for this reduction in *nhx-5* mutant LROs is that it could be due to in part endosomal misregulation. Following these results, we generated molecular tools to elucidate differences in morphology and/or rates in the endocytic cycle between the wild type and *nhx-5* mutant (Table 1). These tools will be used for future studies.

Based on our findings, including oxygen consumption and studying the components of the endocytic cycle, I propose a diabetes model, which suggests a misregulation, which could in part explain the pathophysiology of diabetes (Figure 13).

Normal regulation of the endocytic cycle leads to normal degradation and recycling of materials in a normal state; however, in a diabetic state, there is misregulation in the endosomal-lysosomal system. Two explanations for this are: (1) the endosome is misregulated away from the lysosome due to pH mis-regulation, which can be caused by the *nhx-5* mutant and (2) the endosome fuses with the lysosome, but fusion does not cause degradation because the lysosomal pH is not optimized for proper enzymatic breakdown.

Figure 10

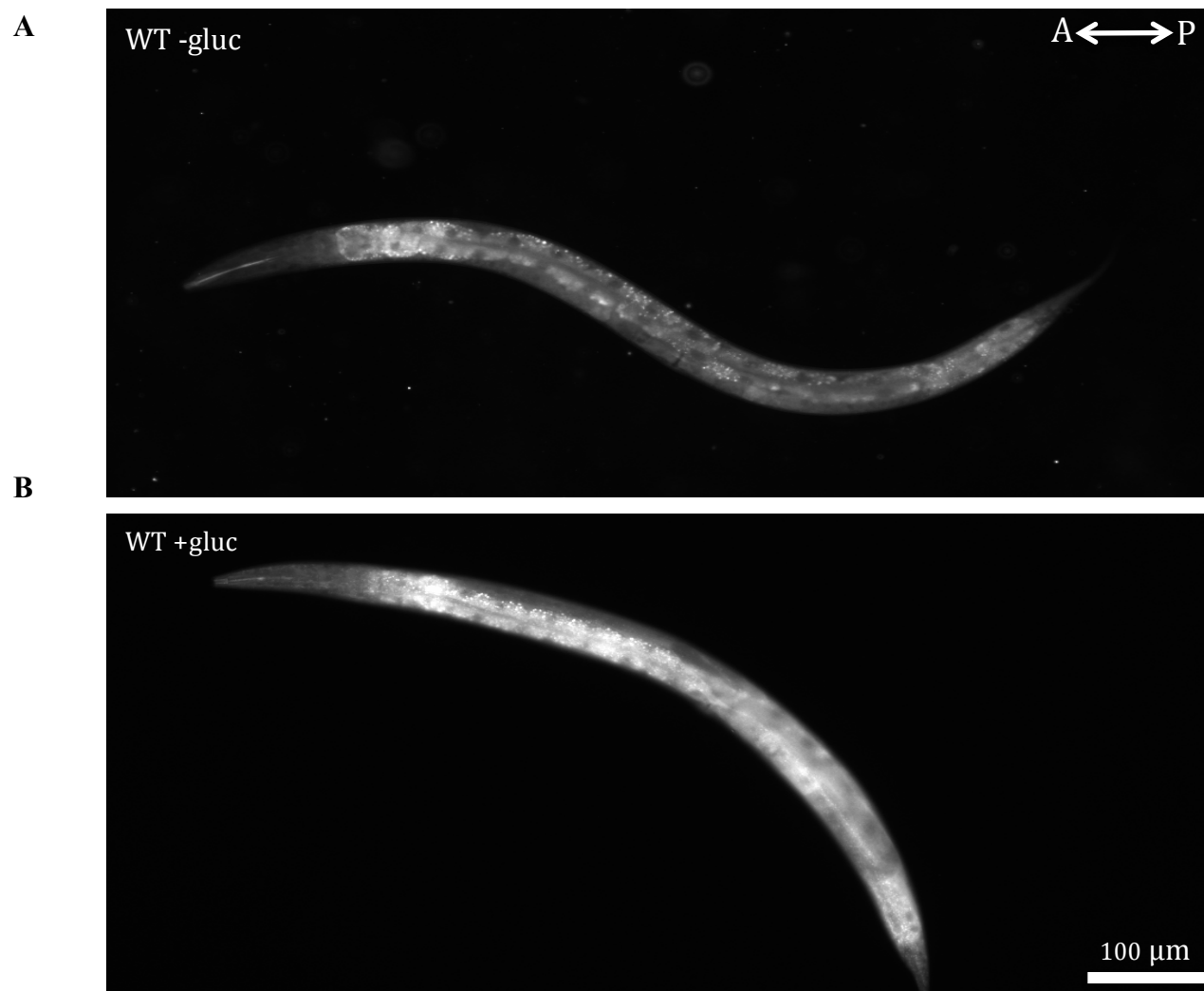


Figure 10. Nile Red stains lysosome-related organelles.

Representative pictures of Nile red stained young adult worms in the absence (A) and presence of glucose (B). The Materials and Methods section contains a detailed explanations of how Nile red staining was done. A=Anterior; P=Posterior

Figure 11

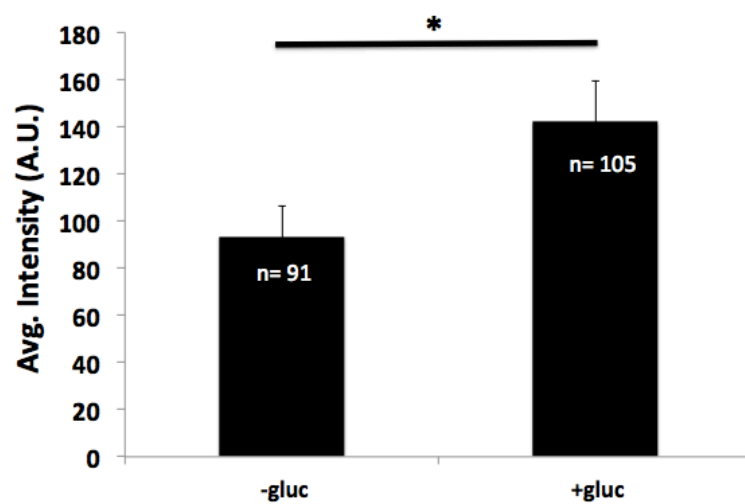


Figure 11. Wild-type worms with glucose treatment show increase in LROs.

Quantification of intensities was performed using pictures like those shown above in Figure 10. This is gathered data from six independent experiments. The total number of worms for each condition is indicated above. Error bars indicate standard error of the mean. $*p < 0.05$ by paired student's t-test. A.U.= Arbitrary Unit.

Figure 12

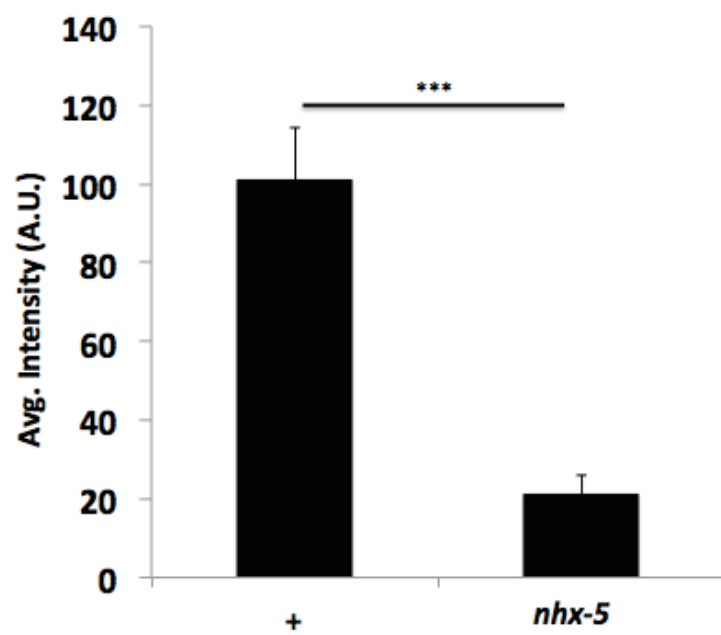


Figure 12. *nhx-5* mutants show lower basal level of LROs compared to the wild-type.

There was a decrease in Nile red intensities in the *nhx-5* deletion mutant compared to the wild-type. Approximately 20-30 worms were quantified for each strain and the experiment was repeated at least three times. Error bars indicate standard error of the mean. *** $p < 0.001$ by paired student's t-test. A.U.= Arbitrary Unit.

Figure 13

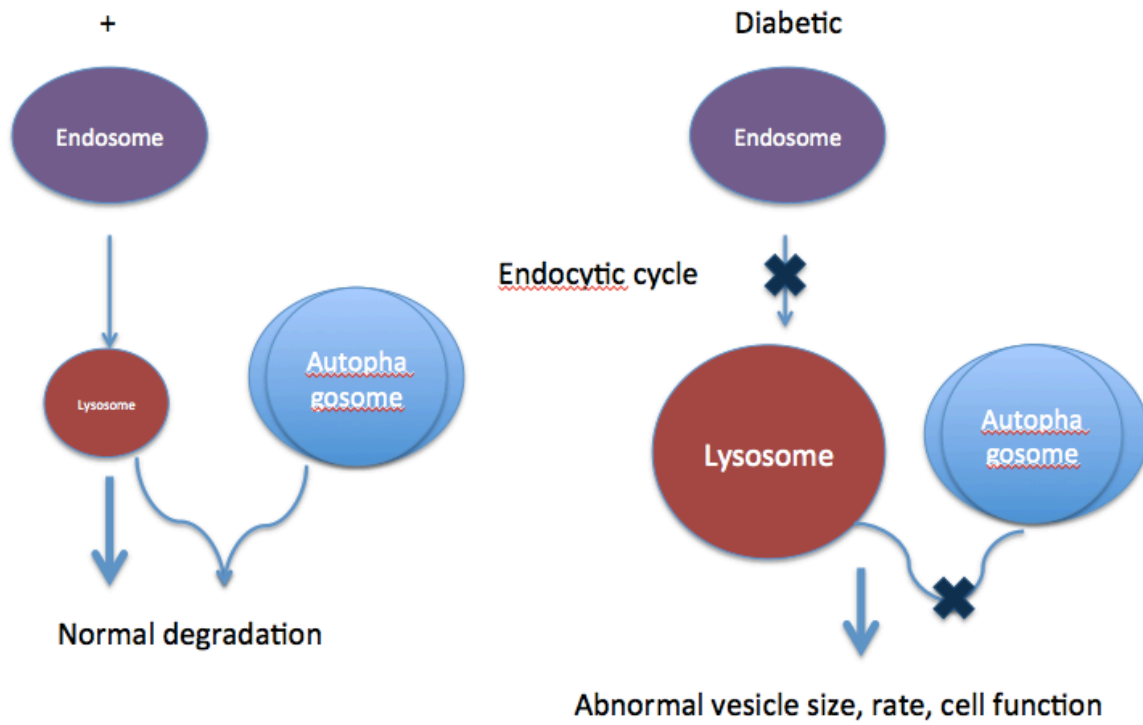


Figure 13. A proposed diabetes model.

This model shows a simplified version of a diabetic model from our studies. In wild type, there is a normal regulation of the endocytic cycle and autophagy, leading to orderly degradation of materials. In a diabetic state, there is misregulation of the endocytic cycle and autophagy. We speculate that 1) Endosomal pH is not modulated to its optimal pH leading to its mistrafficking away from the lysosome. 2) The endosome fuses with the lysosome, but the lysosome cannot degrade materials because pH is not optimal for protease activation. When the endosome-lysosome network starts to be misregulated, autophagy will be affected because degradation of materials in the autophagosome requires fusion with lysosome in the final step.

This leads to an enlargement and/or increase in number of lysosomal vesicles, which is also in part explained by our glucose treatment experiment. While the autophagosome is not directly regulated by endosome, the final degradative process is dependent on the endosomal-lysosomal system. If this system breaks down, then it is most likely that autophagy will also be impeded from performing its normal functions.

3. Materials and Methods

***C. elegans* Strains and Maintenance**

C. elegans variant Bristol, strain N2 was used as the wild-type. All strains were stored at 20°C and maintained on NGM plates seeded with *Escherichia coli* strain HB101 unless noted otherwise. Four strains were obtained from the *Caenorhabditis* Genetic Center (CGC, University of Minnesota): GH383 *glo-3* (*zu446*), GH403 *glo-3*(*kx94*), JJ1271 *glo-1*(*zu391*), CB1003 *flu-2*(*e1003*). Other strains used were YJ53 *nhx-5*(*ok661*) *X* and DA2123 *adIs2122*[*lgg-1::gfp;rol-6(gf)*]. YJ53 was derived from RB836 *nhx-5*(*ok661*) by outcrossing 3 times against N2. RB836 obtained from the CGC. Transgenic worms carrying extra-chromosomal array were made by microinjection into N2: YJ201 [*rab-5::mcherry*], YJ90 [*ges-1p::snb-1::pHluorin*] and YJ92 [*rol-6p::snb-1::pHluorin*].

Nile Red Staining

Prior to L1 transferring, NGM plates were layered with 600 µL of a 500 µg/mL Nile red stock in acetone, which was made previously. Nile Red staining was quantified using the ImageJ and WormFluorescent1 programs, in which the intensities were calculated by subtracting the background intensity from the total intensity, multiplying the area by the intensity above threshold, and averaging final calculation for each condition. Nile red quantification data compiled from 20–30 per experiment.

Glucose Treatment

The NGM plates were treated with 100 mM D-glucose on the lawn of bacteria. Egg-prepped L1 worms were plated and exposed to glucose plates for 48-50 hours before the pictures were taken.

Quantification of Oxygen Consumption Rates

Wild type and the *nhx-5* mutant YJ53 were prepared and synchronized by egg preparation²⁷. Synchronized L1s were grown to young adulthood for 48~50 hours at 20°C. Young adults were collected with M9 and were resuspended in either 2 mL of 100 mM metformin in M9 or 2 mL of buffer only (control) for 30 minutes in liquid. Oxygen consumption rates were measured for seven minutes and normalized to the number of worms, counted after the recording. Approximately 2000-3000 worms were used. The experiments were performed in triplicate for each treatment and every experiment was repeated at least three times. Oxygen consumption rates were measured using the Hansatech Instrument Oxytherm system with S1/MINI electrode and Peltier Electrode Chamber. The data was acquired using O₂ view data acquisition and system configuration software. A 1M stock solution of metformin (Aldrich, Catalog # D150959-5G) was prepared prior to experiments.

Markers and Microinjections

The DNA construct of *synaptobrevin-1::pHluorin* was expressed in the desired location in the worm by fusing it to specific promoters from the *ges-1* and *rol-6* genes using PCR primers design by Dr. Jeongho Kim. DNA constructs were constructed prior to injection. Microinjection needles were pulled using the laser micropipette puller system Model P-2000 (Sutter Instrument). Injection was performed using an Axio Observer.A1 Zeiss

compound microscope, eppendorf TransferMan® NK2 micromanipulator and FemtoJet® microinjector (Fisher Scientific).

Microscopy and Image Analysis

For Nile red analysis by differential interference microscopy (DIC) and epifluorescence, live worms were mounted on 3% agarose pads with 50 mM Sodium Azide (NaN_3). The pictures were taken with an Axioplan2 Zeiss Imager compound microscope and AxioCam camera with Openlab software (Improvision). Exposure time: 100 msec. Magnification: 10x. Confocal images were taken with a Zeiss LSM 700 with a 63x/1.40 Oil DIC M27 objective in single or multi-track mode using a single excitation wavelength of 488 nm for GFP. Additional image processing was done using LSM Zen software (Carl Zeiss).

Autophagosome Count and Analysis

Worms carrying an integrated line expressing LGG-1::GFP were egg-prepped and either incubated either in the absence or the presence of metformin (100 mM) for varying times. They were incubated at 20°C on a rocker, and a drop of worms was mounted on an agar pad for pictures. Approximately 5-8 images were taken per worm at different focal planes to get a whole-worm count of the punctate structures in the pharyngeal muscles. Exposure Time: Auto-live. Magnification: 100x Oil.

4. Conclusions and Future Directions

C. elegans is a simple organism, yet it has provided us with great amount of knowledge in the basic sciences, which are still critical to our understanding of the world, ourselves and many other related phenomena. Additionally, important contributions were made through *C. elegans* such as RNA interference, green fluorescent protein (GFP) and cell death. On top of everything it has already given to the scientific community, it still continues to be an ideal organism to study more complex issues related to human diseases in metabolism, neurology, reproduction and behavior. Nonetheless, there is, unequivocally, room for exploration of subjects that have not yet been studied in *C. elegans*.

Today, we live in a world where excessive food consumption is a commonplace. It comes at a cost with an epidemic public health problem such as Type 2 Diabetes Mellitus (T2DM). It is not curable to this day, but there have been significant advances in treatment options. One of the most widely prescribed and efficient treatments is metformin. Since its discovery, metformin has been studied extensively to elucidate its molecular target. Understanding its target is essential to cater to the needs of patients, as T2DM is complicated with other diseases and polymorphisms of important cellular components such as OCT1⁷. One of the best-understood molecular mechanisms of action of metformin is that it partially inhibits mitochondrial complex I, thereby activating the AMPK pathway⁶. To find out if this activation was conserved throughout the phyla, we wanted to test whether metformin also inhibited respiration in *C. elegans*. Indeed, we saw a decrease in oxygen consumption, which gave us a lead to subsequent experiments.

More importantly, a broader understanding we wanted to gain from this study with *C. elegans* was to examine if we could use *C. elegans* as a model system to study T2DM. We know that T2DM is a complex disease influenced by environmental and genetic factors; therefore, narrowing down on the cause of this disease to one mechanism could answer what is really crucial in the pathogenesis of T2DM. We used metformin as a tool to understand what it does in *C. elegans* both in physiological and non-physiological states. We found that metformin decreases L1 lifespan in the starved state and significantly inhibits the mitochondrial activity in an *nhx-5* dependent manner. After these two findings, we looked for an answer as to how metformin decreased L1 lifespan. We found that metformin inhibited autophagy levels, which explained, in part, the detrimental affect it had on starved L1s. Autophagy has been shown to be beneficial at its physiological level, but in excess or insufficient levels it could cause death of an organism. The resulting studies showed that metformin disrupted the level of autophagy. We speculate that the inhibitory effect is due to misregulation of the endosomal-lysosomal system, since the degradation of materials in the autophagosome requires fusion with the lysosome in its final step. This raises the next question: is regulation of the endocytic cycle important for the pathogenesis of T2DM and can we use *C. elegans* to test this?

Our previous work with metformin in *C. elegans* led us to discovering endosomal NHX-5. Since then, we found that it could be an important player in mediating metformin's action as well as modulating the endocytic pathway. Thereafter, studies with glucose and lysosome-related organelles showed that LROs might be dysfunctional in the presence of high glucose. The exact mechanism by which this occurs is not clearly defined

by our results, but we propose that it is due to the misregulation of endosomal vesicles and/or lysosomal degradation. There is continuous cross-talk between endosomes and lysosomes; therefore, it is important that they are regulated properly. Autophagy is also essential to our understanding because it will indirectly depend on the integrity of the endosomal-lysosomal system. Understanding these dynamic processes of autophagy and endosomal-lysosomal systems is critical as many maladaptive processes of the cycle could lead to shorter lifespan and disease states^{24,25,26}. Endocytic vesicles as well as autophagosomes constitute a continuous system, connecting one destination to another. Studying these processes in a live system both intracellularly and in the whole-organism is a challenge, but our study sheds some light on this dynamic process.

In this study, we examined a conserved cellular pathway in *C. elegans* to further characterize the pathophysiology of T2DM. Our results suggest that the endocytic cycle and autophagy may be an area of research for future studies related to T2DM. Such studies could be plausibly be conducted in *C. elegans*; however, there are challenges that need some insights to go beyond what we learned from this study. First and foremost, future studies should focus on how autophagy is regulated in *nhx-5* mutants. This will give us a better understanding of autophagy regulation in relation to endosome and NHX-5. Moreover, further studies should characterize LRO functions in the endocytic cycle to elucidate what happens in enlarged and non-functional LROs. Last but not least, finding solutions to monitor pH modulation in endosomes and to overcome autofluorescence will help link these dynamic processes of endosomes to the lysosome as well as to autophagy and mitochondria.

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Literature Cited

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APPENDIX-A

Genotype	Expression	Purpose	Experiments
<i>act-5p:: rab-5::mCherry</i>	Early endosome (Intestine)	GTPase marker for early endosomes	✓
<i>ges-1p::snb-1::pHluourin</i>	Endosome (Intestine)	Neutralization of pH in endosomes	✓
<i>rol-6p::snb-1::pHluourin</i>	Endosome (Hypodermis)	Neutralization of pH in endosomes	✓

Table 1. Tools to monitor endocytic cycle and pH changes in its compartments.

Transgenic worms were made and used for detecting organelles expressed at designated sites. Many fluorescence pictures of these transgenic worms were taken, but since the results were inconclusive, they were not included in this study.

APPENDIX-B

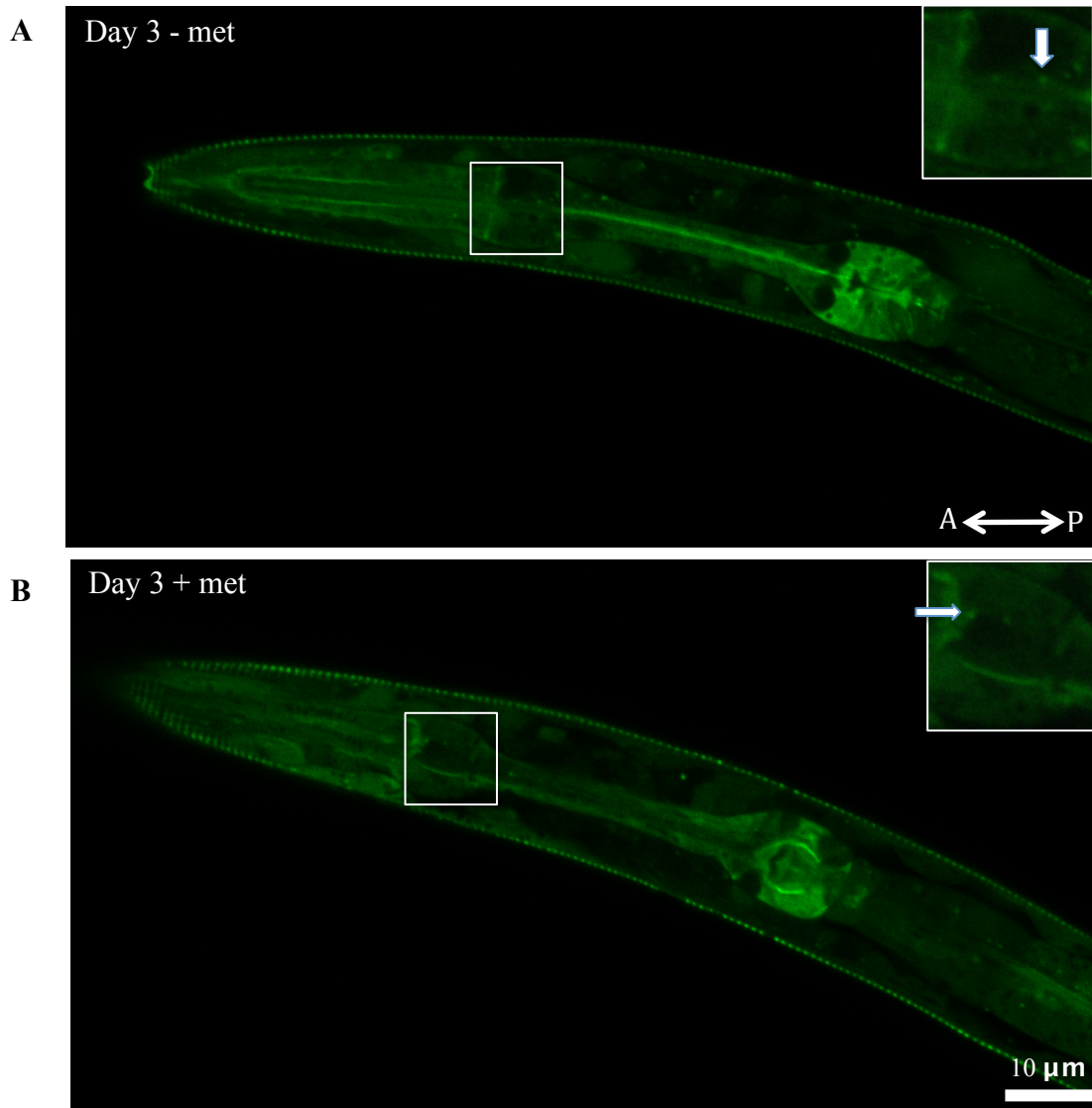


Figure 14. Punctate structures were detectable in L1s starved for 3 days.

These are confocal images of 3 days starved L1s in absence (A) and presence of metformin (B). They show clearer punctate structures as well as clear outlines of pharyngeal muscles compared to the previous fluorescence images. A=Anterior; P=Posterior

VITA

Jheesoo Ahn was born in Anyang, South Korea. She grew up in Korea until she was nine years old and immigrated to the United States with her parents and sister. Jheesoo graduated from Walt Whitman High School, Bethesda, Maryland in 2008 with the President's Award for Educational Excellence and went on to University of Maryland, College Park, receiving a Bachelor of Science in Biological Sciences specialized in Physiology and Neurobiology and a Minor in Violin Performance in 2012. Following her graduation, she enrolled in the CERT program and continued her graduate studies upon joining the lab of Dr. Young-Jai You in June 2013.