CYTOCHROME P450 GENE EXPRESSION MODULATES ANOXIA SENSITIVITY IN *Caenorhabditis elegans*

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With an increasing population suffering from obesity or diabetes mellitus (DM), it is more pertinent than ever to understand how physiological changes impact cellular processes. Patients with DM often suffer from obesity, hyperglycemia, altered fatty acids that contribute to vascular dysfunction, and increased risk to ischemia. *Caenorhabditis elegans* is a model system used to study the conserved insulin signaling pathway, cellular responses in whole organisms and the impact a glucose diet has on oxygen deprivation (anoxia) responses. RNA-sequencing (RNA-Seq) was used to analyze the expression of genes in the anoxia sensitive populations of N2 (wild-type) fed glucose and *hyl-2(tm2031),* a mutant with altered ceramide metabolism. Comparison of the altered transcripts in the anoxia sensitive populations revealed 199 common transcripts- 192 upregulated and 7 downregulated. One of the gene families that have altered expression in the anoxia sensitive populations encode for Cytochrome P450 (CYP). CYPs are located both in the mitochondria and endoplasmic reticulum (ER), but the CYPs of interest are all predicted to be mainly subcellularly localized to the ER. Here, I determined that knock-down of specific *cyp* genes, using RNA interference (RNAi), increased anoxia survival in N2 animals fed a standard diet. Anoxia sensitivity of the *hyl-2(tm2031)* animals was suppressed by RNAi of *cyp-25A1* or *cyp-33C8* genes. These studies provide evidence that the CYP detoxification system impacts oxygen deprivation responses. Using *hsp-4::GFP* animals, a transcriptional reporter for ER unfolded protein response (UPR), I further investigated the impact of *cyp* knock-down,

glucose, and anoxia on ER^{UPR} due to the prediction of CYP-33C8 localization to the ER. Glucose significantly increased ER^{UPR} and *cyp* knock-down non-significantly increased ER^{UPR}. Measurements of ER^{UPR} due to anoxia were made difficult, but representative images show an increase in ER stress post 9-hour anoxia exposure. This study provides evidence that glucose affects ER stress and that ER stress is involved in oxygen deprivation responses.

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INTRODUCTION AND BACKGROUND

Diabetes and Ischemia

Over the past three decades, there has been an increase in the prevalence of obesity and Diabetes Mellitus (DM). From 1980 to 2008, fasting plasma glucose has increased globally in men by 0.07 mmol/L per decade and in women by 0.09 mmol/L per decade. In the same time scale, it was also found DM prevalence to increase globally from ~8% to 9.8% in men and 9.2% in women (Danaei *et al.* 2011). The World Health Organization found that there were 347 million people with DM worldwide in 2013 and in 2014 600 million adults over the age of 17 were obese, accounting for 13% of the adult population (World Health Organization 2016). Obesity and DM are known to be more common in patients with high intakes of food, and subsequent increases in basal serum glucose and altered lipid levels. DM has been characterized as either damage to the pancreatic beta islet cells that secrete insulin (Type 1 DM, T1DM) or the decreased sensitivity to insulin (Type 2 DM, T2DM) (CDC - National Center for Health Statistics 2015) that leads to the inability to properly regulate serum glucose.

Patients with either obesity or DM also suffer from increased risk to ischemia due to vascular complications (i.e. atherosclerosis and varicose veins) (World Health Organization 2016). Ischemia is the blockage of blood flow which results in the interruption of O_2 delivery to tissue and waste removal, leading to cell death of effected tissues. In varicose veins, the inability to deliver O_2 to tissue is due to venous insufficiency syndrome, reverse venous flow, and can lead to loss of a limb (World Health Organization 2016). A source of ischemic damage is by the body's natural

reaction to restore blood flow after ischemia known as reperfusion (hyperfusion). Once blood flow is restored, there is an increased production of free radicals and reactive oxygen species (ROS) as well as an exaggeration of inflammation which can cause white blood cells to destroy cells (Eltzschig and Eckle 2011). While response to oxygen deprivation has been studied for many years, the negative consequences are still not fully understood and additional procedures to reduce tissue damage remain to be discovered (Hajeri *et al.* 2005; Mendenhall *et al.* 2006; Ghose *et al.* 2013; Flibotte *et al.* 2014; Cao *et al.* 2015, 2016; Garcia *et al.* 2015). With the increase in DM and obese patients leading to a larger population with risk of ischemia, it is of interest to determine the impact of genetic alterations that could result in susceptibility to oxygen deprivation and resulting ischemic damage. *C. elegans* stands as a genetic model system to be able to study cell response to oxygen deprivation.

C. elegans as a Model System

C. elegans was first introduced as a model system by Sydney Brenner. Each *C. elegans* is a small transparent nematode that is about 1 mm in length as an adult and has a life cycle of about 55 hours at 22°C as shown in Figure 1 (Brenner 1974). The nematode also has conserved pathways including insulin-like signaling and was the first multicellular organism to have its genome completely sequenced in 1998 (The C. elegans Sequencing Consortium 1998). Along with all of these attributes of a good model system, many molecular tools have been developed to elucidate mechanisms of biological processes in *C. elegans*. Two of the tools that exist to analyze gene expression changes are green fluorescent protein (GFP) and RNA-sequencing (RNA-

Seq). GFP-tagged genes are commonly used to qualitatively see a change in expression (Chalfie *et al.* 1994). One common method of quantitatively obtaining expression data is RNA-Seq. RNA from a control animal and a treatment animal is isolated, cDNA library is made and sequenced using next generation sequencing methods. The sequencing data is analyzed using bioinformatics, against a reference genome, and fold change is computed, thus allowing transcriptomic comparisons between two experimental sets (Mortazavi *et al.* 2008). Another tool routinely used in *C. elegans* to study gene function is RNA interference (RNAi). Andrew Fire and Craig Mello demonstrated the use of RNAi and developed the method for RNAi of specific gene expression by use of injection, soaking or feeding of complementary double-stranded RNA (Fire *et al.* 1998). This technique was expanded by Julie Ahringher's lab which made the RNAi library to knock-down *C. elegans* genes (Kamath and Ahringer 2003; Kamath *et al.* 2003). *C. elegans* is a powerful model system and holds the potential for understanding the mechanism of biological processes such as a whole animal response to oxygen deprivation.

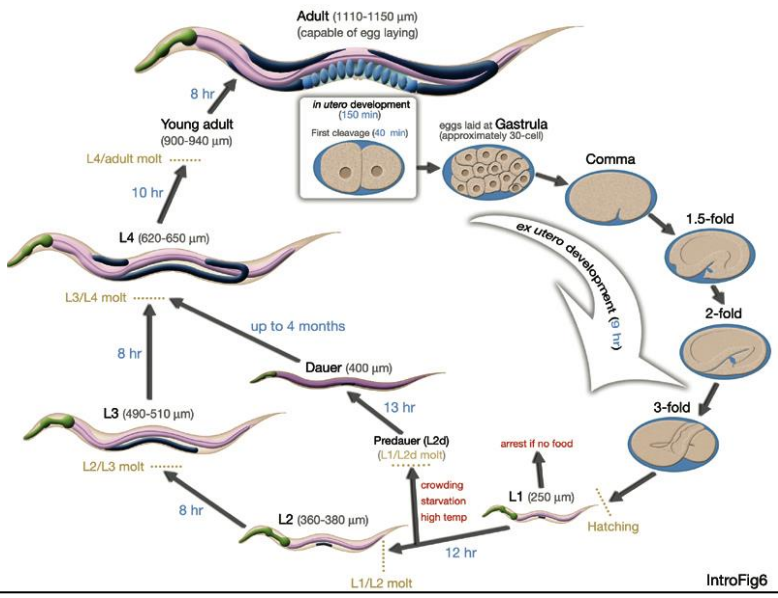

Figure 1: Life Cycle of C. elegans. There are four larval stages (L1-L4) leading up to adulthood after embryonic development. If stressors are encountered before progression to L2, the animal may enter into the alternative, stress resistant life stage known as the dauer state. Adapted from (WormAtlas 2012).

Oxygen Deprivation and Diet in *C. elegans*

C. elegans is a well-established model for studying oxygen deprivation since it is capable of surviving a broad range of oxygen levels. The animal enters into a state of suspended animation when exposed to anoxia (<0.001 kPa O_2) and will exit the state once returned to an oxygenated environment. The anoxia-induced suspended state is characterized by developmental arrest seen as cessation of microscopically observable movement (Padilla *et al.* 2002). All stages of the life cycle will enter into an anoxia-induced suspended state, however survival decreases with longer exposure to anoxia as seen in Figure 2. Not all surviving nematodes will regain full biological function and instead will irreversibly become impaired and display morphological changes including fluid accumulation, and kinking of the pharynx and intestinal lumen (Mendenhall *et al.* 2006, 2009; LaRue and Padilla 2011).

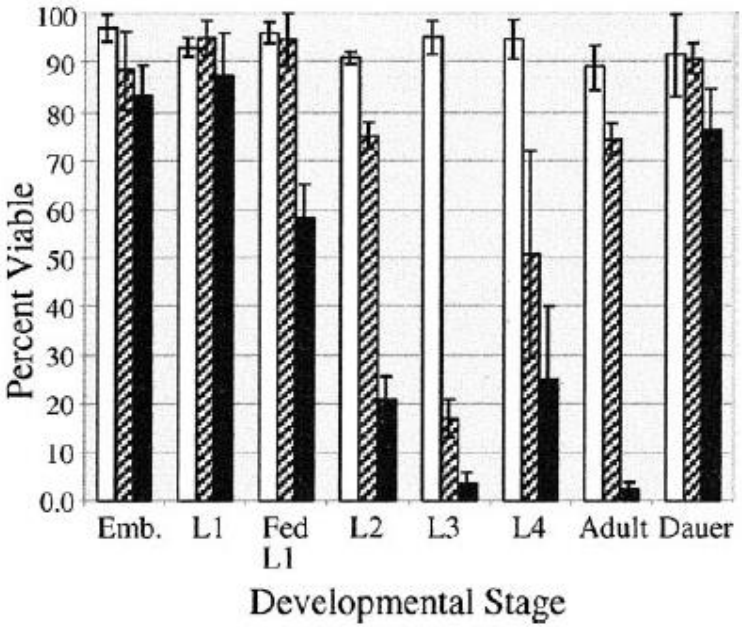

Figure 2: Viability of C. elegans Post-Anoxia Exposure. Viability of *C. elegans* after exposure to anoxia for 24 hours (white bars), 48 hours (slashed bars), or 72 hours (black bars) at each developmental stage. Adapted from (Padilla *et al.* 2002).

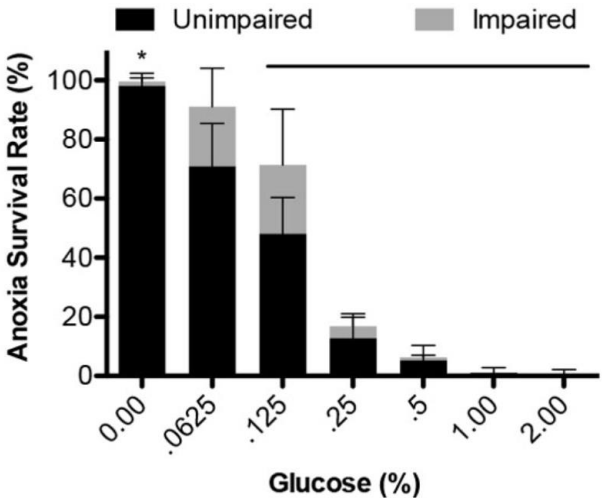

Figure 3: Impact of Glucose Diet on Anoxia Sensitivity. Survival rate of N2 day-1 adult *C. elegans* when exposed to 24 hours of anoxia and increasing concentrations of glucose in the diet. Adapted from (Garcia *et al.* 2015).

Anoxia sensitivity increases in *C. elegans* fed a glucose or fructose diet (Figure 3) (Garcia *et al.* 2015). Although the increase in anoxia sensitivity observed in glucose-fed *C. elegans* is not a direct reflection of ischemia in obese or DM patients, there is a rationale as to why *C. elegans* is a good model for these human health issues. First, the insulin-signaling pathway and lipid biosynthetic pathways are highly conserved. Second, a glucose diet increases lipid levels in *C. elegans* and is thus thought of as an obesity mimetic (Garcia *et al.* 2015). Third, a glucose diet increases levels of glucose within the worm and glycosylation of proteins (Mondoux *et al.* 2011). Finally, our previous RNA-Seq results show that a glucose diet induces similar genes observed to be altered in patients with DM (Lee *et al.* 2009; Garcia *et al.* 2015).

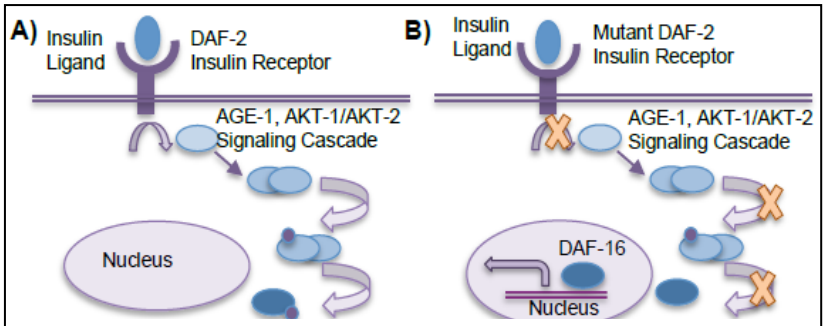

Figure 4: DAF-2 to DAF-16 Insulin Signaling. A) Binding of insulin to DAF-2 leads to the inhibition by phosphorylation of DAF-16 B) Mutated DAF-2 leads to the inability to phosphorylate DAF-16, allowing it to freely enter the nucleus to regulate transcription.

The conserved insulin-signaling pathway has been studied in great detail in *C. elegans* due to its role in many biological processes including longevity, stress

responses, and development. The *daf-2* (abnormal dauer formation) codes for an insulin receptor that, when activated, leads to the phosphorylation of the transcription factor DAF-16, not allowing DAF-16 to cross the nuclear membrane (Figure 4A). Lack of phosphorylated DAF-16, by mutation in the *daf-2* gene or absence of insulin ligand, results in the ability for DAF-16 to enter the nucleus and regulate expression of genes (Figure 4B) (Li and Zhang 2013; Lopez *et al.* 2013; Zigdon *et al.* 2013; Xia *et al.* 2014). The *daf-2(e1370)* mutant is resistant to many stresses (i.e. anoxia, heat, etc.), increased lifespan, and increased innate immunity (Gulbins and Li 2006; Gartner *et al.* 2008; Garcia *et al.* 2015). Despite *daf-2(e1370)* being an anoxia tolerant mutant, a glucose diet also increases anoxia sensitivity (Garcia *et al.* 2015).

In contrast to the anoxia tolerant *daf-2(e1370)* animal, the *hyl-2(tm2031)* mutant is sensitive to 48-hour anoxia exposure. The *hyl-2* gene is one of three ceramide synthase genes in *C. elegans* and codes for an enzyme that is required for the efficient synthesis of $C_{20\text{-}22}$ ceramides and sphingomyelin species (Menuz *et al.* 2009). Ceramides are intermediates for sphingolipids, a major component of cell membranes, and have been suggested to also act as signaling molecules for biological processes including inflammation and cellular stress responses in mammalian cells (Chalfant and Spiegel 2005; Jana *et al.* 2009; Novgorodov and Gudz 2009). Anoxia sensitivity in the *hyl-2(tm2031)* animal supports the idea that ceramides have a functional role in regulating stress response to oxygen deprivation.

In order to further understand how diet affects response to oxygen deprivation, gene expression data on control animals and animals fed glucose was obtained via RNA-Seq. In comparison to N2 animals, N2 fed glucose had 1,850 genes upregulated

and 520 genes downregulated. Amongst the 2,370 genes that had altered expression, seven of those genes code for a Cytochrome P450 with one of them having previously been seen to be involved with response to oxygen deprivation (Ma *et al.* 2013; Garcia *et al.* 2015).

Cytochrome P450

Cytochrome P450 (CYP) are monooxygenases that catalyze reactions involved in drug metabolism and synthesis of cholesterol, steroids, and lipids. CYPs are widely known for their involvement in phase I detoxification via oxidation and have been specifically seen to metabolize long chain fatty acids like arachidonic acid (NCBI 2002; Coon 2005). While largely studied for their metabolic properties, CYPs have been seen to be involved in the immune system and inflammation due to their metabolism of long chain fatty acids like arachidonic acid. Inflammatory cytokines regulate individual CYPs and control the release of substrates like arachidonic acid. CYP epoxygenases convert arachidonic acid into anti-inflammatory epoxyeicosatrienoic acids and CYP hydroxylases proinflammatory 20-hydroxyeicosatetraenoic acid (Christmas 2015). Acknowledging CYPs effect on inflammation, some CYPs may be involved in response to oxygen deprivation through modulation of inflammation. In mice, CYP2E1 expression has been seen to be induced by a high fat diet and hyperlipidemia. It was also shown that a synergistic effect on ischemic brain damage occurs when exposed to ischemia and reperfusion along with a high fat diet (Cao *et al.* 2015). Some CYPs have an effect on response to oxygen deprivation and *C. elegans* is a good model organism to understand an individual CYP's effect on response to oxygen deprivation.

There are 82 CYP genes in *C. elegans* and *cyp-13A12* is one of the eleven CYP genes that had altered expression in response to a glucose diet (Ma *et al.* 2013; Garcia *et al.* 2015). Previously *cyp-13A12* was seen to induce the sustained response to reoxygenation post oxygen deprivation (Ma *et al.* 2013). CYPs are located in the ER and mitochondria inner membrane (Coon 2005). Although little is known about the ER targeting sequences of *C. elegans*, *cyp-33E1* has been seen to be localized to the ER (Rolls *et al.* 2002). Mitochondrial function has been seen to play a role in reoxygenation (Novgorodov and Gudz 2009; Ghose *et al.* 2013), but ER function disruption by ER stress has not been investigated in respect to reoxygenation in *C. elegans*.

Induction of ER Stress by Oxygen Deprivation and Glucose

The inability for a cell to deal with misfolded proteins in the ER leads to a buildup of misfolded proteins causing ER stress, and leads to the signaling of the unfolded protein response (UPR) and the altering of gene expression in order to restore protein homeostasis or to promote apoptosis (Oslowski and Urano 2013). Many genes and their resulting proteins have been previously found to be involved with ER stress response, but two proteins are particularly important to the UPR in the ER (ER^{UPR}). The IRE1 protein is a stress-activated nuclease and PERK protein are two proteins are responsible for two pathways sensing ER stress and signaling the UPR (Harding *et al.* 2000; Scheuner *et al.* 2001). ER stress is commonly measured through an increase in phosphorylated IRE1 (p-IRE1) and phosphorylated PERK (p-PERK). Some of the targets of these proteins promote restoration of homeostasis (i.e. heat shock proteins), but other proteins induced by ER stress promote apoptosis (Cao *et al.* 2016). Numerous

studies have been done measuring ER stress with IRE1 and PERK, but few involve the impact of oxygen deprivation and glucose exposure.

Oxygen, glucose, and serum deprivation (OGD) resulted in measured ER stress through an increase in p-PERK in pancreatic cells (He *et al.* 2016). Although it was a combination of factors that lead to p-PERK induction in pancreatic cells rather than oxygen deprivation alone, oxygen deprivation has been seen to induce ER stress in multiple tissues. The turtle *Trachemys scripta elegans*, which can survive weeks without oxygen when submerged in cold water, shows an increase in p-PERK in heart, kidney, and liver tissue (Krivoruchko and Storey 2013). Glucose and oxygen deprivation induce ER stress individually, but glucose causes ER stress differently. High glucose and free fatty acids induce ER stress via increase in p-IREα and p-PERK in mammalian pancreatic cells (Oslowski and Urano 2013; Kooptiwut *et al.* 2014). The *hsp-4(SJ4005)* strain of *C. elegans* allows us to visually study ER stress induction from oxygen deprivation or a glucose diet in a whole organism.

hsp-4::GFP, A Marker for ER Stress

The *hsp-4* gene codes for a heat shock protein that functions as a chaperone and requires IRE-1 during ER stress for induction and aids in degradation of misfolded proteins. The HSP-4 protein binds to a misfolded protein to promote ubiquitination. While *pek-1* (ortholog of human PERK) is not required for *hsp-4* induction, it is still required for maximal UPR (Shen *et al.* 2001). ER stress was first shown to induce *hsp-4* transcription by dithiothreitol, a disruptor of disulfide bond formation in the ER, confirmed by Northorn blot and Reverse Transcriptase Polymerase Chain Reaction

(RT-PCR) analysis. *hsp-3* has compensatory regulation with *hsp-4* (Kapulkin *et al.* 2005), but it does not work as a marker because it has a relatively high basal expression and increase in expression only 2-fold in response to tunicamycin (Calfon *et al.* 2002). Figure 5 shows the *hsp-4::GFP,* transcriptional reporter, at a low basal GFP expression (Fig 5a) and with an increase in intestinal expression 9-fold when exposed to the ER stress inducer tunicamycin (Fig 5b), which acts by inhibiting N-linked glycosylation (Shen *et al.* 2001; Calfon *et al.* 2002). Exposed to dithiothreitol and tunicamycin, proteins are misfolded leading to ER stress and causing the UPR resulting in a significant increase in the expression of *hsp-4*. The *hsp-4::GFP* strain serves as a good reporter gene for ER stress allowing visualization of ER stress in *C. elegans*.

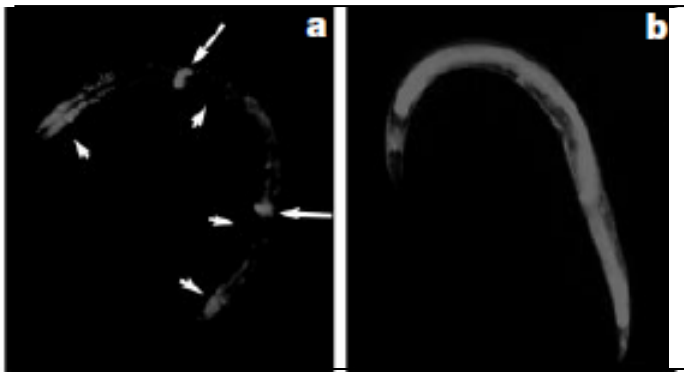

Figure 5: hsp-4::GFP in vivo. Fluorescent photomicrograph. White arrowheads track the outline of the body. **a)** *C. elegans* untreated adult *hsp-4::gfp(zcls4)V.* **b)** *C. elegans* adult *hsp-4::gfp(zcls4)V* treated with tunicamycin. Adapted from (Calfon *et al.* 2002).

OBJECTIVE AND HYPOTHESIS

In this study I investigate if a glucose diet or a mutation in the *hyl-2* ceramide synthase gene reduces anoxia survival. My objectives were to 1) determine if knock-down of specific *cyp* genes impacted anoxia survival and 2) use *the hsp-4::GFP* reporter strain to examine ER stress in the context of a glucose-supplemented diet and anoxia exposure. My hypotheses are that 1) knock-down of specific *cyp* genes impact anoxia survival in N2 animals fed a standard and glucose diet and *hyl-2(tm2031)* animals fed a standard diet and that 2) ER^{UPR} is induced in animals fed a glucose diet or exposed to anoxia. Use of RNAi and fluorescent microscopy techniques allowed me to test my hypotheses.

METHODS

Strains and Culture Conditions

The N2, *hyl-2(tm2031)*, *daf-2(e1370*), and *hsp-4::GFP* strains were maintained on nematode growth media (NGM) plates seeded with *E. coli* (OP50) at 20° (Brenner 1974). The hours post L4-adult molt or gonad morphology and other anatomical markers were used to identify adults. In the glucose-supplemented diet experiments, N2 animals were fed a glucose diet as per Garcia *et al.* (2015). Briefly, 370µl of 0.75M glucose stock was used to cover the entire plate surface of NGM plates (60mmx15mm petri dishes containing IPTG, ampicillin, and tetracycline). The 0.75M glucose solution was made by dissolving 13.512 g of appropriate glucose (D-(+)-Glucose, Sigma) in 100 ml of double-distilled water; solution was filter sterilized and kept at 4°C. After the glucose plates dried, they were seeded with 300µl of the appropriate bacteria (OD_{600nm}, 0.6≤0.9); the bacterial lawn covered the entire plate surface (Garcia *et al.* 2015).

Anoxia Exposure

Animals were placed into BD Biobag type A anaerobic environmental chambers (BD Biosciences, Rockville MD) at 20°C (Padilla *et al.* 2002; LaRue and Padilla 2011). Resazurin indicators (<.001kPa of O_2) confirm that anoxic conditions are reached by remaining white. To minimize deviations in anoxia exposure the trials that failed to reach anoxic conditions within 1.5 hours were nulled. The animals remained in anoxia for 24-48 hours, as indicated for each experiment, and were allowed to recover in air for 1 day before being scored as unimpaired, impaired, or dead. Animals that dried out by crawling up the side of the plate were nulled and marked as unscored.

RNAi

Animals were grown on the specified RNAi food from embryo to adult stage as previously described (LaRue and Padilla 2011). Briefly, ~10 N2 or *hyl-2(tm2031)* adults were allowed to lay eggs for a minimum of 2 hours onto a plate seeded with the *E. coli* RNAi food specific for a gene knock-down of interest (Kamath and Ahringer 2003; Kamath *et al.* 2003). After there are at least 50 eggs laid, the adults were picked off and the eggs were allowed to mature into adulthood. 50 1-day old adults were moved to a new plate, containing the same *E. coli* RNAi food, and then exposed to anoxia. In experiments focusing on F1 embryos, 1-day old adults were temporarily moved to new plates to egg lay for a minimum of 2 hours. The HT115 bacterial strain (bacteria containing an empty vector) was used as a control and the RNAi food to knock-down the *par-6* gene was used as a positive control for efficient RNAi knock-down. The phenotype observed in *par-6(RNAi)* P0 and F1 animals is 70% and 100% sterility, respectively.

Quantitative RT-PCR

Real-time Polymerase Chain Reaction (qPCR) was performed in a similar manner as previously reported (Garcia *et al.* 2015). Animals were collected as young adults for mRNA isolation and RNA extraction was performed as described above. Reverse transcription to generate cDNA was performed using SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, cat #18080-51). Quantitative RT-PCR was carried out using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) and SsoFast EvaGreen Supermix (Bio-Rad). Following primer validation, results were

analyzed using the $2^{-\Delta\Delta CT}$ Method (Livak and Schmittgen 2001). The mRNA level of Y45F10D.4 was used for normalization (Hoogewijs *et al.* 2008). The average of at least three technical replicates was used for each independent experiment. Primer sequences are available upon request. Statistical analysis was conducted using one-way ANOVA, Bonferroni multiple comparisons. Note that we included this section because it was used to validate the RNA-Seq data for some genes; however, the experiment was performed by lab colleague Mary Ladage as part of a collaborative publication (Ladage *et al. in review*).

Prediction of Subcellular Localization

In order to understand the effect of *cyp-33C8* on response to oxygen deprivation I used EuLoc, a web-server for predicting eukaryotic protein subcellular localization (Chang *et al.* 2013). The FASTA sequence for the *cyp-33E1* transcript as well as the *cyp-33C8* transcript was entered into the program. FASTA sequences used were according to the gene transcript information on the WormBase Database (http://www.wormbase.org/). The *cyp-33E1* transcript was entered to support the accuracy of the prediction from EuLoc it is a *C. elegans* CYP that is known to be located subcellularly to the ER (Rolls *et al.* 2002). EuLoc predicted *cyp-33E1* to be located within the Endoplasmic-Reticulum and Plasma-membrane.

Epifluorescent Microscopy

The *hsp-4::GFP* fluorescence was analyzed using a 509 nm filter on a Zeiss Mot II Plus epifluorescent microscope, and images were collected using an Axiocam

camera, and Zeiss software. *C. elegans* animals were placed onto a slide with a thin layer of 3% agarose gel and were paralyzed with a drop of 0.5% tricaine/0.05% tetramisole. Pictures of multiple animals were all taken with 48 ms of exposure to fluorescent light and pictures of single animals, used for measuring, were all taken with 150ms of exposure to fluorescent light. Some animals were exposed to 9-hour anoxia prior to being placed onto a slide. Animals were picked at 3 hours post 9-hour anoxia exposure and imaged. Corrected total fluorescence (CTCF) was calculated from values obtained by use of ImageJ. A minimum of 3 background readings were done in order to properly calculate CTCF as follows: CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

Statistics

For each experiment a minimum of three independent trials were conducted. For experiments assaying anoxia survival, each independent trial included two plates with a minimum of 50 animals per plate. After anoxia exposure animals were allowed a 24-hour recovery time before survivors were classified as impaired or unimpaired, as previously described (LaRue and Padilla 2011; Garcia *et al.* 2015). For the GFP experiments a minimum of 5 individual animals were measured per trial. The error bars in graphs indicate standard deviation. Statistical analyses were conducted for each experiment using one-way ANOVA, Bonferroni multiple comparisons using Prism 6.0.

RESULTS

RNA-Seq Analysis

As previously stated, there were 2,370 genes that had altered expression between N2 and N2 fed glucose (Garcia *et al.* 2015). While I focused on CYP gene characterization, a class of genes that were effected by a glucose diet, we also decided to further analyze the RNA-Seq data. In addition to obtaining expression analysis from N2 and N2 fed glucose, expression analysis was also performed via RNA-Seq on the anoxia sensitive mutant *hyl-2(tm2031)*. We identified 582 genes with altered expression in the *hyl-2(tm2031)* mutant relative to the N2 controls. Since we are interested in

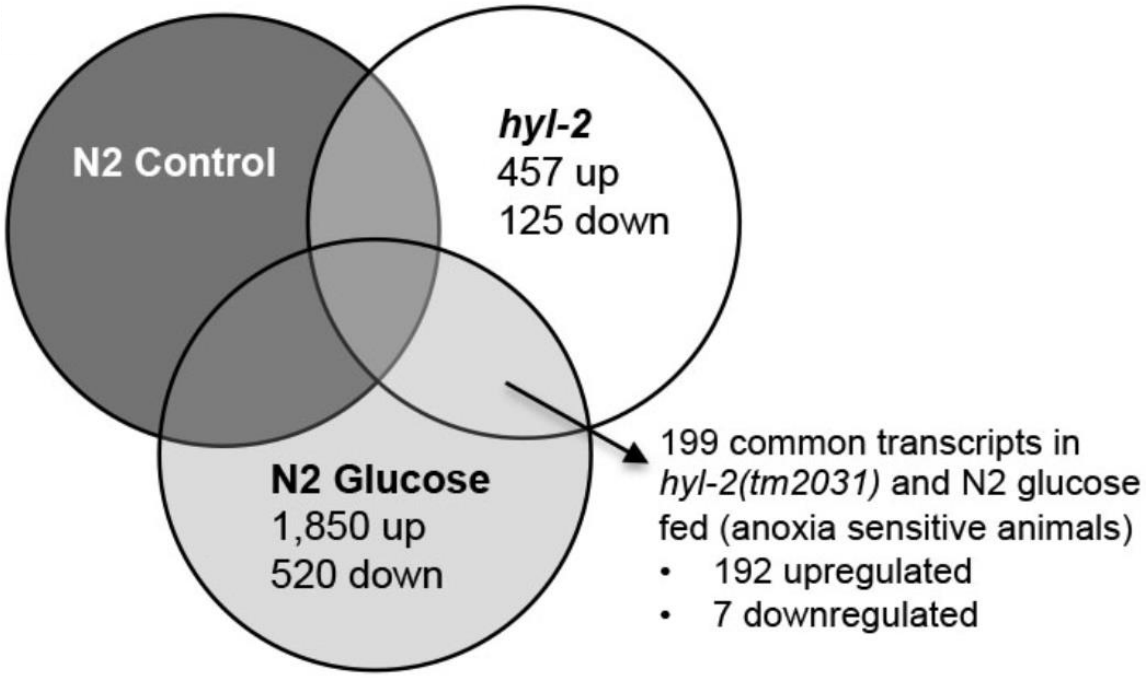

Figure 6: Analyzing RNA-Seq Data. Identifying that there are many gene expression changes between the anoxia sensitive populations (*hyl-2(tm2031)* and N2 fed glucose) and control, we compared the genes that were upregulated and downregulated similarly in the anoxia sensitive populations. Of the genes that had expression changes, 192 genes were similarly upregulated and 7 were similarly downregulated. Adapted from (Ladage *et al. in review*).

identifying genes that affect oxygen deprivation responses, we compared the transcript profiles of the anoxia sensitive populations N2 fed a glucose diet and the *hyl-2(tm2031)* mutant. This analysis narrowed the 2,370 genes from N2 fed glucose and 582 genes from *hyl-2(tm2031)* to 192 similarly upregulated genes and 7 similarly downregulated genes (Figure 6). Among these 199 genes, *cyp-13A12* and *cyp-33C8* were similarly upregulated, and *cyp-25A1* was similarly downregulated. Confirmation of downregulation of *cyp-25A1* was confirmed by qPCR (Ladage *et al. in review*). These *cyp* genes being similarly regulated in the anoxia sensitive populations supports the rationale to further investigate if the CYPs play a role in oxygen deprivation responses.

cyp Knock-Down Increased 48-hour Anoxia Survival

In order to understand the role of *cyp* genes, RNAi knock-down was performed on N2 animals with 48-hour anoxia exposure (Figure 7). Based on anoxia survival data of N2 on OP50 (Padilla *et al.* 2002), 48-hour anoxia exposure is a good range to test whether knock-down of the *cyp* genes of interest would increase or decrease anoxia survival. N2 animals, fed

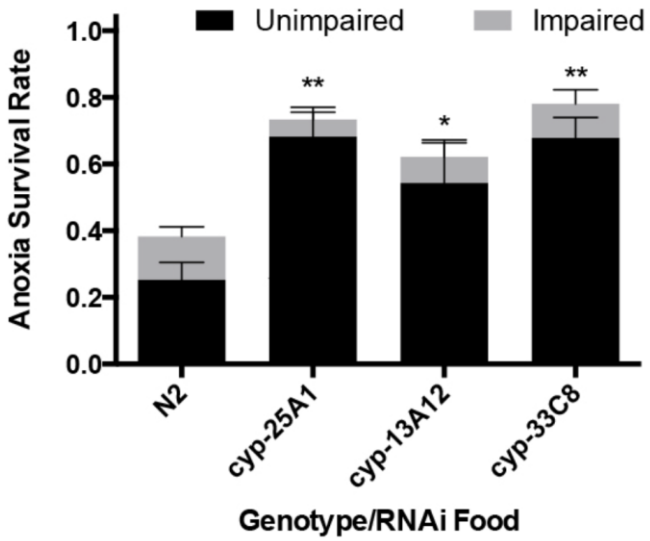

Figure 7: cyp Knock-Down in N2 day-1 Adults with 48-Hour Anoxia Exposure. Each RNAi knock-down of a *cyp* in the N2 animals resulted in significant increase in survival in the knock-down animals was in comparison to N2 control animals *(*$p<0.05$ denoted by *, $p<0.001$ denoted by ** One-way ANOVA). Error bars indicate standard deviation.

HT115 *E. coli*, with 48-hour anoxia exposure have a survival of 0.38±0.08. *cyp-25A1(RNAi)* animals increased in 48-hour anoxia survival to 0.73±0.17 significantly, compared to control animals ($p<0.001$). *cyp-13A12(RNAi)* and *cyp-33C8(RNAi)* animals increased in 48-hour anoxia survival significantly compared to control animals at 0.62±0.21 ($p<0.05$) and 0.78±0.11 ($p<0.001$), respectively.

cyp Knock-Down Suppresses *hyl-2(tm2031)* Anoxia Sensitivity

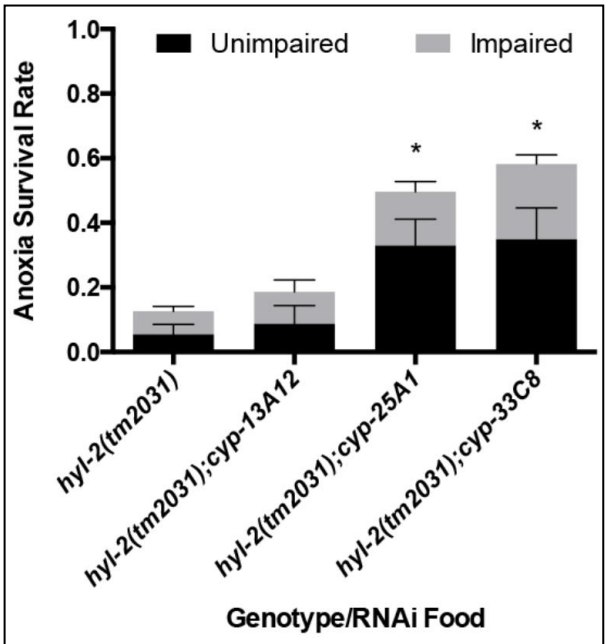

Figure 8: cyp Knock-Down in hyl-2(tm2031) Day-1 Adults with 48-Hour Anoxia Exposure. Each RNAi knock-down suppressed *hyl-2(tm2031)* 48-hour anoxia sensitivity, but *cyp-13A12* did not suppress at a significant level. Significant difference when compared to *hyl-2(tm2031)* controls $p<0.05$ denoted by * (One-way ANOVA). Error bars indicate standard deviation.

Since *cyp-25A1(RNAi)*, *cyp-13A12(RNAi)*, and *cyp-33C8(RNAi)* animals increased anoxia survival we tested if *cyp* knock-down could also suppress the anoxia sensitivity observed in the *hyl-2* mutant. *cyp* knock-down was performed in the 48-hour anoxia sensitive mutant *hyl-2(tm2031)* to see if *cyp* knock-down suppressed *hyl-2(tm2031)* anoxia sensitivity (Figure 8). *hyl-2(tm2031)* on HT115 survives at a rate of 0.13±0.08. *cyp-25A1* and *cyp-33C8* knock-down suppresses *hyl-2(tm2031)* anoxia sensitivity significantly to a survival rate of 0.50±0.22 ($p<0.05$) and 0.58±0.25 ($p<0.05$), respectively. *cyp-13A12* suppressed *hyl-*

2(tm2031) anoxia sensitivity, but *cyp-13A12* did not suppress the *hyl-2(tm2031)* sensitivity at a significant level.

cyp-33C8 Knock-Down Does Not Enhance Anoxia Survival in Glucose-Fed Adults

Due to the suppression of *hyl-2(tm2031)* anoxia sensitivity via *cyp* knock-down, the next step was to see how *cyp* knock-down effected glucose-induced 24-hour anoxia sensitivity. However, *cyp* knock-down was narrowed to *cyp-33C8* since *cyp-13A12* did not have significant suppression of *hyl-2(tm2031)* anoxia sensitivity and *cyp-25A1* RNAi has four off-target sites (*cyp-25A2*, *cyp-25A3*, *cyp-25A4*, and *cyp-25A5*) (Ladage *et al. in review*). Glucose-induced anoxia sensitivity was not significantly suppressed by *cyp-33C8* knockdown at any concentration tested (Figure 9A).

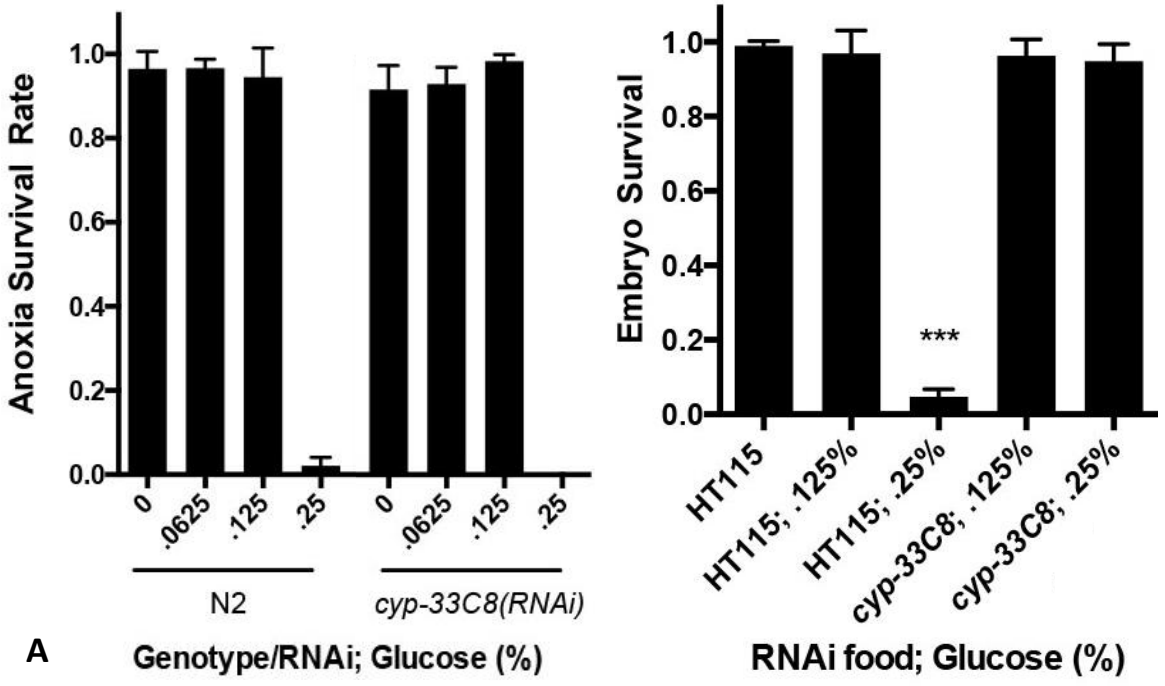

Figure 9: cyp-33C8 Knock-Down Does Not Enhance Anoxia Survival in Glucose-Fed Day-1 Adults, but it does in F1 Embryos at 0.25% Glucose. (A) P0 day-1 adults were exposed to a certain concentration of glucose and 24 hours of anoxia. (B) F1 embryos were exposed to a certain concentration of glucose and 24 hours of anoxia. Significant difference $p<0.0001$ by One-way ANOVA denoted by *** when comparing control animals fed glucose to knock-down animals.

cyp-33C8 Knock-Down Suppresses Glucose-Induced Anoxia Sensitivity in F1 Embryos

The embryos of N2 hermaphrodites fed a 0.25% glucose-supplemented diet were very sensitive to anoxia indicating that diet can impact the next generation's capacity to respond to stress (Figure 9B). Although *cyp-33C8* knockdown did not suppress glucose-induced 24-hour anoxia sensitivity in P0 day-1 adults, *cyp-33C8* knockdown does suppress glucose-induced anoxia sensitivity in F1 embryos (Figure 9B). *cyp-33C8* knockdown suppressed glucose-induced anoxia sensitivity in F1 embryos when the P0 hermaphrodite was grown on a glucose diet of 0.25% ($p<0.0001$) (Figure 9B).

CYP-33C8 Predicted to Localize to the ER

In order to understand the effect of *cyp-33C8* knockdown on *C. elegans,* Euloc was used to predict the subcellular localization of CYP-33C8 since CYPs are located in the ER or the mitochondria (Chang *et al.* 2013). The FASTA sequence for the *cyp-33E1* transcript as well as the *cyp-33C8* transcript was entered into the program in order to support the accuracy of the prediction from EuLoc since *cyp-33E1* is a *C. elegans* CYP that is known to be localized to the ER (Rolls *et al.* 2002). EuLoc predicted CYP-33E1 and CYP-33C8 to be localized to the Endoplasmic-Reticulum or Plasma-membrane. Additionally, CYP-25A1 was predicted to be localized to the Endoplasmic-Reticulum or Plasma-membrane and CYP-13A12 is predicted to be localized to the Plasma-membrane.

Glucose Increases ER Stress

Glucose exposure in mammalian pancreatic cells induces ER stress (Oslowski and Urano 2013; Kooptiwut *et al.* 2014), but how a glucose diet impacts the ER has not been fully studied in a whole animal. The *hsp-4::GFP* strain is often used to assay the ER unfolded protein response, caused by stress such as heat or tunicamycin, of whole animals (Calfon *et al.* 2002; Bertucci *et al.* 2009; Helmcke and Aschner 2010; Ient *et al.* 2012; Shore *et al.* 2012). We found that the *hsp-4::GFP* animals fed 0.5% glucose had a significant increase in *hsp-4::GFP* fluorescence relative to control animals (Figure 10). However, *cyp-33C8* knockdown did not suppress the *hsp-4::GFP* expression in glucose fed animals (Figure 10).

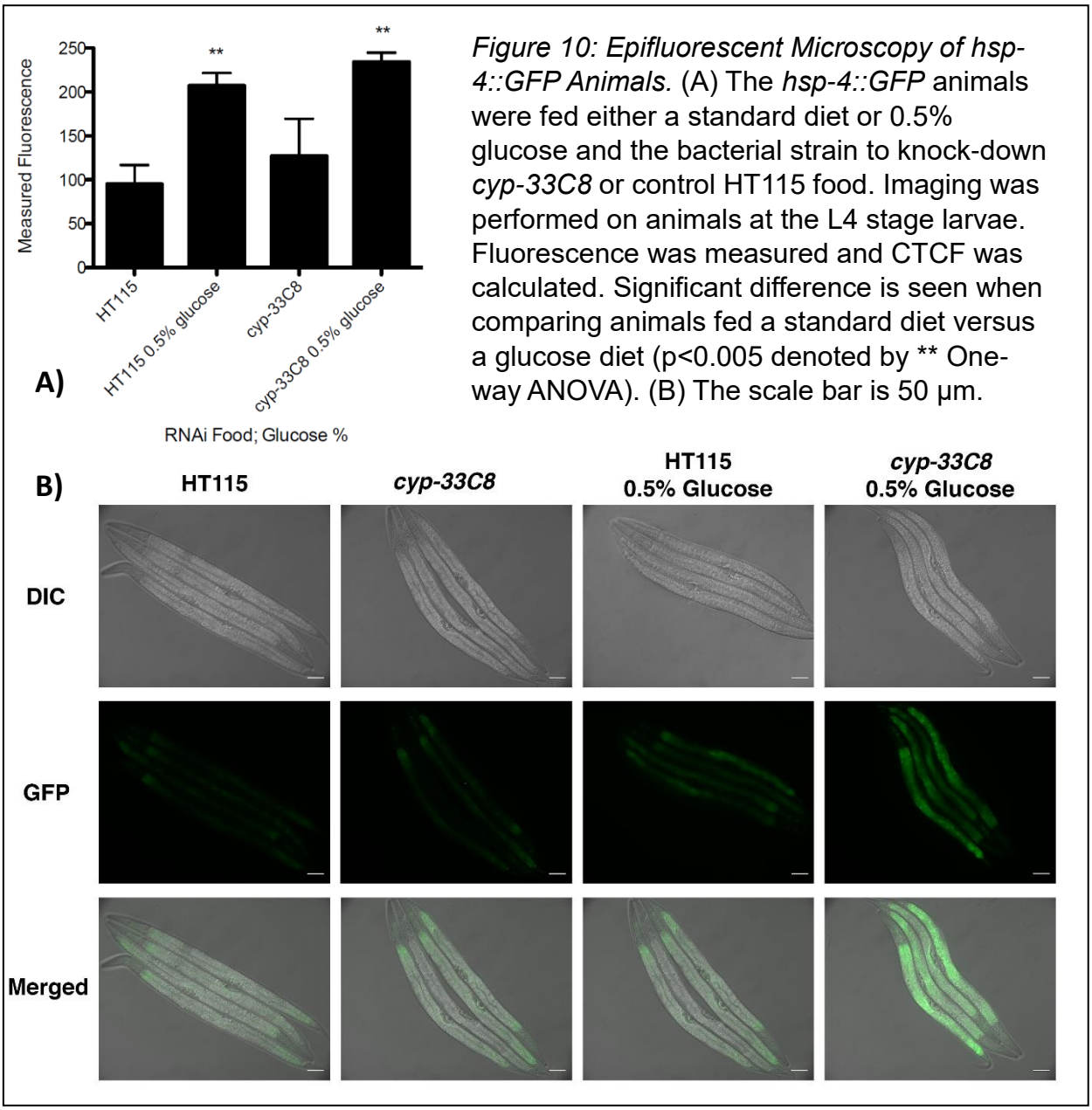

Figure 10: Epifluorescent Microscopy of hsp-4::GFP Animals. (A) The *hsp-4::GFP* animals were fed either a standard diet or 0.5% glucose and the bacterial strain to knock-down *cyp-33C8* or control HT115 food. Imaging was performed on animals at the L4 stage larvae. Fluorescence was measured and CTCF was calculated. Significant difference is seen when comparing animals fed a standard diet versus a glucose diet ($p<0.005$ denoted by ** One-way ANOVA). (B) The scale bar is 50 μm.

Anoxia Increases ER Stress

Anoxia exposure induces ER stress in heart, liver, and kidney of *Trachemys scripta elegans* (Krivoruchko and Storey 2013), however anoxia-induced ER stress has not been fully studied in a whole animal. The *hsp-4::GFP* strain was used to examine the impact anoxia has on ER stress. We determined that anoxia exposure coupled with

a glucose diet significantly increased the *hsp-4::GFP* fluorescence in day-1 adults 9-hour anoxia exposure (Figure 11, 12). However, since we examined adult animals an accurate measurement of fluorescence was a challenge due to potential interference of fluorescence within the intestine due to egg load within the uterus of day-1 adults. For future studies we recommend analysis of L4 animals or using RT-PCR to quantify the amount of GFP expressed. In the process of conducting this experiment I used exposed glucose fed *cyp-33C8(RNAi)* animals with 9-hour anoxia exposure; 9-hour exposure is a stress that glucose fed animals can survive (Garcia *et al.* 2015). Surprisingly, all of the glucose fed *cyp-33C8(RNAi)* animals died with 9-hour anoxia exposure (data not shown).

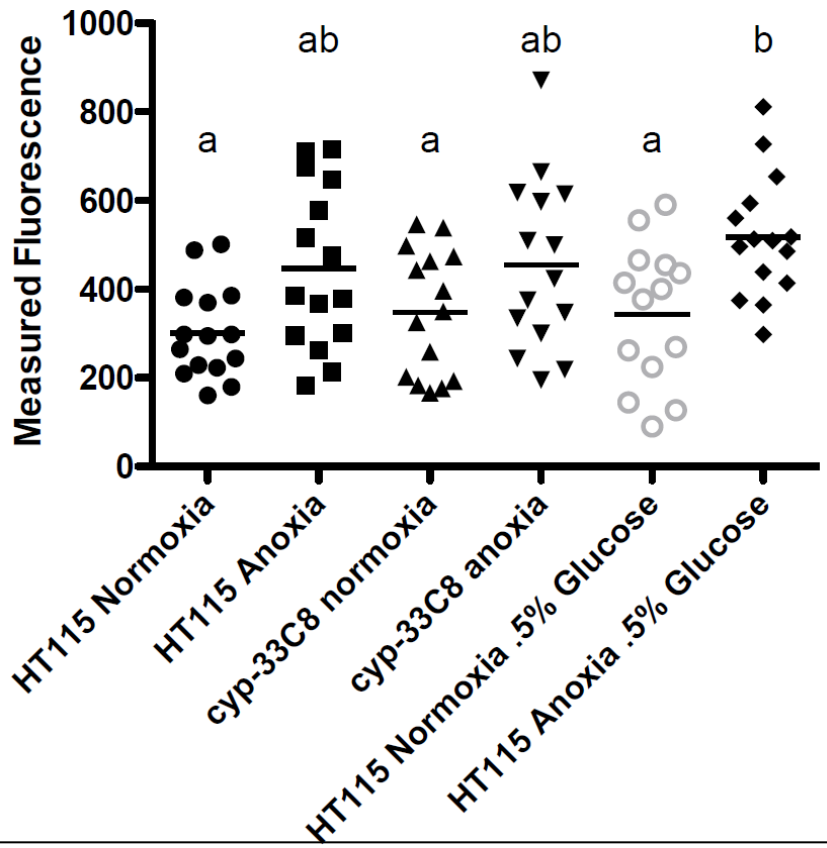

Figure 11: Measuring of Epifluorescent Microscopy in Day-1 Adults. The *hsp-4::GFP* animals were fed either a standard diet or 0.5% glucose and the bacterial strain to knock-down *cyp-33C8* or control HT115. Imaging was performed on animals at the day-1 adult stage. Anoxia animals had 9-hour anoxia exposure and allowed to recover for 3 hours before imaging. Significant difference is denoted by “a” and “b” ($p<0.05$, by One-way ANOVA).

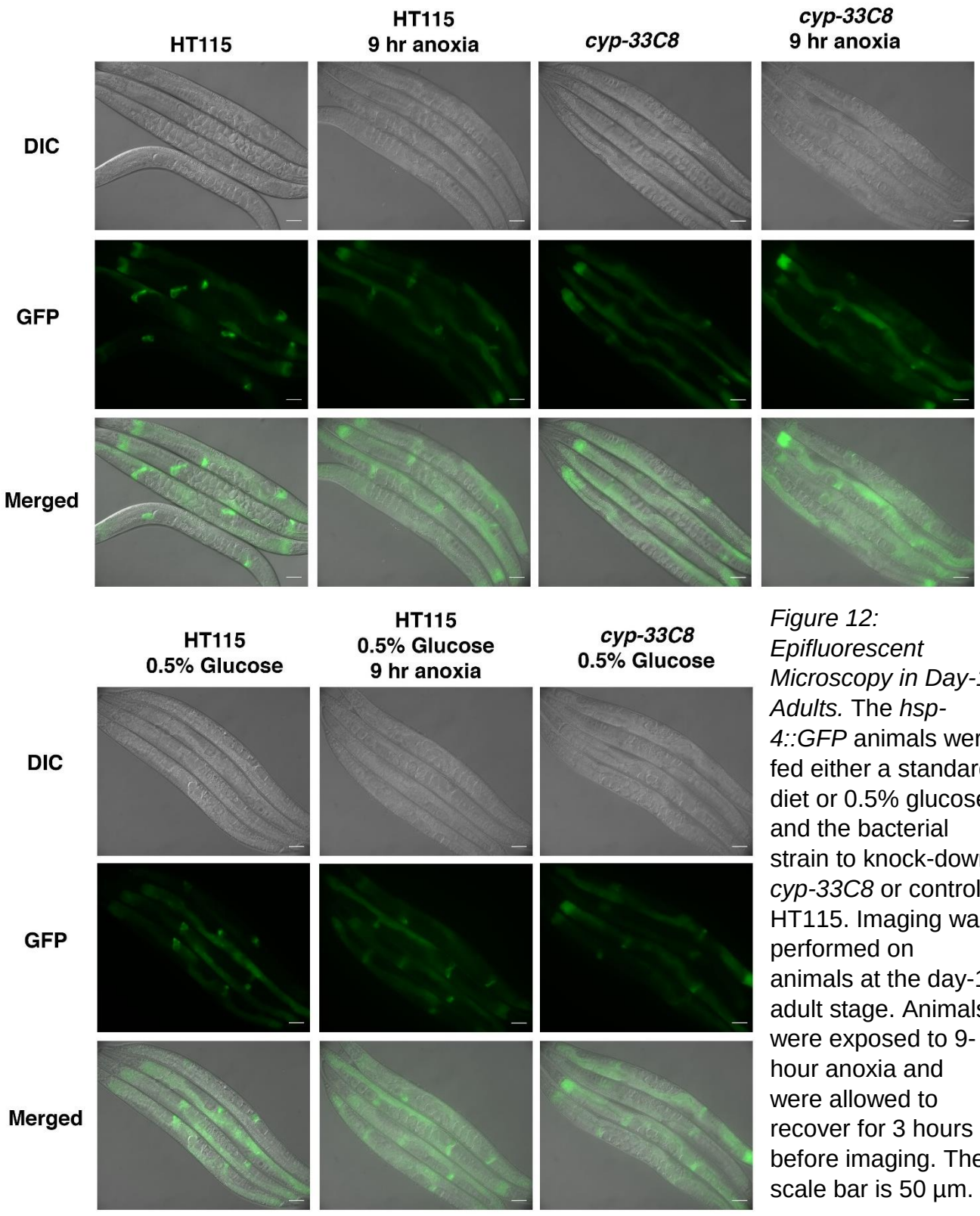

Figure 12: Epifluorescent Microscopy in Day-1 Adults. The *hsp-4::GFP* animals were fed either a standard diet or 0.5% glucose and the bacterial strain to knock-down *cyp-33C8* or control HT115. Imaging was performed on animals at the day-1 adult stage. Animals were exposed to 9-hour anoxia and were allowed to recover for 3 hours before imaging. The scale bar is 50 µm.

DISCUSSION AND CONCLUSION

Similar to DM co-morbidities a glucose diet in *C. elegans* affects the expression of a large number of genes (Garcia *et al.* 2015), increased lipid droplets, and sensitivity to oxygen deprivation. The analysis of RNA-Seq transcriptomic data of anoxia sensitive populations, N2 fed glucose and *hyl-2(tm2031)*, served as a guide to identify genes and gene classes that are involved in oxygen deprivation responses (Ladage *et al. in review*; Garcia *et al.* 2015). Since both populations are anoxia sensitive, the overlaps in genes with altered expression could include genes that are important for response to oxygen deprivation. 192 transcripts are similarly upregulated in the anoxia sensitive populations and 7 transcripts are similarly downregulated in the anoxia sensitive populations. Transcripts for the class of phase I detoxification Cytochrome P450 proteins are among the 7 transcripts that are similarly downregulated as well as the 192 transcripts that are similarly upregulated. Anoxia exposure was performed in *cyp* knock-down animals via RNAi to understand the impact of *cyp* expression on oxygen deprivation survival.

cyp-25A1(RNAi), cyp-13A12(RNAi), and *cyp-33C8(RNAi)* animals had an increase in 48-hour anoxia survival relative to controls indicating that these genes have a role in modulating oxygen deprivation survival. A possible explanation for these results is that *cyp* knock-down causes a shift in signaling molecule production like ceramides. While CYP do not act directly on ceramides, CYP function could alter the ER and cause a shift in ceramide metabolism to balance the oxygen deprivation response provided by $C_{20\text{-}22}$ ceramides action (Menuz *et al.* 2009; Hannun and Obeid 2011). An additional explanation for why *cyp* knock-down increased oxygen deprivation survival is that lower expression of CYP, a protein that uses oxygen as a cofactor (NCBI

2002), would result in less use of oxygen in cells and tissues during times of stress. Unlike *cyp-25A1* and *cyp-33C8*, *cyp-13A12* knock-down did not suppress *hyl-2(tm2031)* 48-hour anoxia sensitivity suggesting that increased oxygen deprivation survival is not as simple as improved oxygen conservation. Others have found that *cyp-13A12* is important for inducing the sustained response to reoxygenation post oxygen deprivation (Ma *et al.* 2013). Since RNAi of *cyp-13A12* did not suppress *hyl-2(tm2031)* anoxia sensitivity the decrease of the sustained response to reoxygenation may not be involved in anoxia survival per se.

Because *cyp-13A12* knock-down did not suppress *hyl-2(tm2031)* sensitivity and the RNAi *cyp-25A1* food has four potential off-target sites (Ladage *et al. in review*), further investigation was focused on the impact of *cyp-33C8* RNAi. Although *cyp-33C8* knock-down suppressed *hyl-2(tm2031)* anoxia sensitivity, it did not suppress glucose-induced 24-hour anoxia sensitivity in adult animals. A possible explanation for this phenomenon is that a high glucose diet may alter the levels of signaling molecules to a degree that *cyp-33C8* knock-down alone would not be sufficient to suppress the anoxia sensitive phenotype. Another possibility is that a glucose diet may induce anoxia sensitivity through additional routes other than the mechanism by which the *hyl-2(tm2031)* mutation induces anoxia sensitivity. The *cyp-33C8* RNAi did not suppress glucose-induced anoxia sensitivity in adult animals; however, it suppressed glucose-induced anoxia sensitivity in F1 embryos where the P0 animals were fed a 0.25% glucose diet. An explanation of why embryos have an altered response to *cyp-33C8* knock-down because mitochondria are being affected. CYP-33C8 may effect signaling

molecules that alter mitochondria dynamics and embryonic mitochondria are inherited from the parent animals (Lemire 2005).

Glucose fed animals are anoxia sensitive (Garcia *et al.* 2015) and here we show that they likely have an increase in ER stress as determined by examination of the ER^{UPR}. Thus suggesting that ER stress is one facet by which a glucose diet causes anoxia sensitivity and could potentially impact oxygen deprivation responses in a whole animal. Glucose-induced ER stress causing anoxia sensitivity and impacting oxygen deprivation responses would be consistent with previously seen data that a high glucose and fat diet causes an increase in ER stress in mammalian pancreatic cells and ischemic damage in neuronal cells of mice (Oslowski and Urano 2013; Kooptiwut *et al.* 2014; Cao *et al.* 2015). A glucose diet inducing ER stress may cause sensitivity to ischemia and oxygen deprivation and genes that impact ER stress may ultimately affect oxygen deprivation responses.

Despite prediction of CYP-33C8 localization to the ER, *cyp-33C8* knock-down in *hsp-4::GFP* animals did not significantly reduce the GFP signal, used as an indicator of ER^{UPR}, in glucose-fed animals. This suggests that the increased N2 anoxia survival and suppression of *hyl-2(tm2031)* anoxia sensitivity from *cyp-33C8* knock-down is not through modulation of ER stress by the UPR. Surprisingly, while analyzing the *hsp-4::GFP;cyp-33C8(RNAi)* I observed that these animals fed a 0.5% glucose diet with 9-hour anoxia exposure resulted in complete death. The death in the 0.5% glucose fed *hsp-4::GFP;cyp-33C8(RNAi)* animals with 9-hour anoxia exposure further supports the idea that improved survival in *cyp-33C8(RNAi)* animals is not due to modulation of ER stress. One possibility for the *cyp-33C8* knock-down decreasing survival in *hsp-4::GFP*

animals fed 0.5% glucose is because *cyp-33C8* may have a specific function in short-term oxygen deprivation responses since *cyp-13A12* is more associated with long sustained response to oxygen deprivation (Ma *et al.* 2013). I conclude that *cyp-33C8* knock-down impacts oxygen deprivation responses, but *cyp-33C8* does not make this impact by modulating ER stress. Possibly *cyp-33C8* knock-down impacts oxygen deprivation responses through altering mitochondrial dynamics via metabolizing signaling molecules.

Using the methods described above, a glucose diet failed to significantly increase ER stress in day-1 adults suggesting that either adults react differently than L4 larvae to the diet or that the methodologies need to be improved for examination of the *hsp-4::GFP* reporter in gravid animals. The intestine was a major portion of the animal that has changes in fluorescence and eggs produced in the day-1 adults likely caused interference. Despite the interference in *hsp-4::GFP* day-1 adults, the combination of a 0.5% glucose diet and 9 hours of anoxia still lead to a significant increase in *hsp-4::GFP* fluorescence. This shows that oxygen deprivation could possibly cause significant increase ER stress in a whole animal and warrants further investigation. Correctly analyzing *hsp-4::GFP* fluorescence post anoxia exposure will require epifluorescent microscopy with L4 *hsp-4::GFP* animals or quantifying GFP by use of RT-PCR.

A severe limitation to these studies is relying on characterization of *cyp* by RNAi knock-down alone. Analysis using *cyp* mutants would confirm the results of these studies. Currently, no loss-of-function mutants are available for the specific *cyp* genes investigated here. Studies presented show that *cyp* expression is involved in oxygen deprivation responses and adds value to creating loss-of-function *cyp* mutants. In order

to further understand oxygen deprivation responses, much more work needs to be done to understand the 199 common transcripts from the anoxia sensitive populations. Future studies need to understand their effects on survival post oxygen deprivation and how they affect ER stress or mitochondrial stress in a whole organism.

Ischemia and subsequent oxygen deprivation is still a very large concern in many different disease states such as DM and obesity. Patients with DM lack the ability to properly control glycemic levels and can lead to periods of hyperglycemia when the diet has high glucose content (CDC - National Center for Health Statistics 2015; World Health Organization 2016). A high glucose diet alters gene expression and impacts oxygen deprivation responses. Gene expression changes may be one mean in which a high glucose diet impacts the increased risk of ischemia and subsequent oxygen deprivation responses. A glucose diet may negatively alter *cyp* expression, but simple changes in diet can lead to changes in *cyp* expression to reverse the negative impacts (Menzel *et al.* 2007; Cho and Yoon 2015; He *et al.* 2015; Qiu *et al.* 2015). Understanding the effect of gene expression changes due to a high glucose diet can possibly reveal alterations that ultimately lead to increase in ischemic damage, but also provide alternate avenues to aid in preventing or lower ischemic damage in more susceptible individuals with DM or obesity.

REFERENCES

Bertucci A., Pocock R. D. J., Randers-Pehrson G., Brenner D. J., 2009 Microbeam irradiation of the C. elegans nematode. J. Radiat. Res. **50 Suppl A**: A49–A54.

Brenner S., 1974 The genetics of Caenorhabditis elegans. Genetics **77**: 71–94.

Calfon M., Zeng H., Urano F., Till J. H., Hubbard S. R., Harding H. P., Clark S. G., Ron D., 2002 IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature **415**: 92–96.

Cao X.-L., Du J., Zhang Y., Yan J.-T., Hu X.-M., 2015 Hyperlipidemia exacerbates cerebral injury through oxidative stress, inflammation neuronal apoptosis in MCAO/reperfusion rats. Exp Brain Res **233**: 2753–2765.

Cao G., Zhou H., Jiang N., Han Y., Hu Y., Zhang Y., Qi J., Kou J., Yu B., 2016 YiQiFuMai Powder Injection Ameliorates Cerebral Ischemia by Inhibiting Endoplasmic Reticulum Stress-Mediated Neuronal Apoptosis. Oxid. Med. Cell. Longev. **2016**.

CDC - National Center for Health Statistics, 2015 Basics | Diabetes | CDC.

Chalfant C. E., Spiegel S., 2005 Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. J. Cell Sci. **118**: 4605–4612.

Chalfie M., Tu Y., Euskirchen G., Ward W. W., Prasher D. C., 1994 Green fluorescent protein as a marker for gene expression. Sci. **263** : 802–805.

Chang T.-H., Wu L.-C., Lee T.-Y., Chen S.-P., Huang H.-D., Horng J.-T., 2013 EuLoc: a web-server for accurately predict protein subcellular localization in eukaryotes by incorporating various features of sequence segments into the general form of Chou's PseAAC. J. Comput. Aided. Mol. Des. **27**: 91–103.

Cho H.-J., Yoon I.-S., 2015 Pharmacokinetic interactions of herbs with cytochrome P450 and P-Glycoprotein. Evidence-Based Complement. Altern. Med. **2015**: 1–10.

Christmas P., 2015 Role of Cytochrome P450s in Inflammation. Adv. Pharmacol. **74**.

Coon M. J., 2005 CYTOCHROME P450: Nature's Most Versatile Biological Catalyst. Annu. Rev. Pharmacol. Toxicol. **45**: 1–25.

Danaei G., Finucane M. M., Lu Y., Singh G. M., Cowan M. J., Paciorek C. J., Lin J. K., Farzadfar F., Khang Y.-H., Stevens G. A., Rao M., Ali M. K., Riley L. M., Robinson C. A., Ezzati M., 2011 National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. Lancet **378**: 31–40.

Eltzschig H., Eckle T., 2011 Ischemia and reperfusion--from mechanism to translation. Nat Med. **17**: 1391–401.

Fire A., Xu S., Montgomery M. K., Kostas S. A., Driver S. E., Mello C. C., 1998 Potent and specific genetic interference by double-stranded RNA in Caenorhabditis

elegans. Nature **391**: 806–811.

Flibotte J. J., Jablonski A. M., Kalb R. G., 2014 Oxygen Sensing Neurons and Neuropeptides Regulate Survival after Anoxia in Developing C. elegans. PLoS One **9**: e101102.

Garcia a. M., Ladage M. L., Dumesnil D. R., Zaman K., Shulaev V., Azad R. K., Padilla P. a., 2015 Glucose Induces Sensitivity to Oxygen Deprivation and Modulates Insulin/IGF-1 Signaling and Lipid Biosynthesis in Caenorhabditis elegans. Genetics **200**: 167–184.

Gartner A., Boag P. R., Blackwell T. K., 2008 Germline survival and apoptosis. WormBook: 1–20.

Ghose P., Park E. C., Tabakin A., Salazar-Vasquez N., Rongo C., 2013 Anoxia-reoxygenation regulates mitochondrial dynamics through the hypoxia response pathway, SKN-1/Nrf, and stomatin-like protein STL-1/SLP-2. PLoS Genet. **9**: e1004063.

Gulbins E., Li P. L., 2006 Physiological and pathophysiological aspects of diving. Am J Physiol Regul Integr Comp Physiol **290**: R11–R26.

Hajeri V. a, Trejo J., Padilla P. a, 2005 Characterization of sub-nuclear changes in Caenorhabditis elegans embryos exposed to brief, intermediate and long-term anoxia to analyze anoxia-induced cell cycle arrest. BMC Cell Biol. **6**: 47.

Hannun Y. a, Obeid L. M., 2011 Many ceramides. J. Biol. Chem. **286**: 27855–62.

Harding H. P., Novoa, Isabel, Zhang Y., Zeng H., Wek R., Schapira M., Ron D., 2000 Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. Mol. Cell **6**: 1099–1108.

He Q., Li J. K., Li F., Li R. G., Zhan G. Q., Li G., Du W. X., Tan H. B., 2015 Mechanism of action of gypenosides on type 2 diabetes and non-alcoholic fatty liver disease in rats. World J Gastroenterol **21**: 2058–2066.

He J., Wang C., Sun Y., Lu B. O., Cui J., Dong N., Zhang M., Liu Y., Yu B. O., 2016 Exendin-4 protects bone marrow-derived mesenchymal stem cells against oxygen / glucose and serum deprivation-induced apoptosis through the activation of the cAMP / PKA signaling pathway and the attenuation of ER stress. Int. J. Mol. Med. **37**: 889–900.

Helmcke K. J., Aschner M., 2010 Hormetic effect of methylmercury on Caenorhabditis elegans. Toxicol. Appl. Pharmacol. **248**: 156–164.

Hoogewijs D., Houthoofd K., Matthijssens F., Vandesompele J., Vanfleteren J. R., 2008 Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. BMC Mol. Biol. **9**: 9.

Ient B., Edwards R., Mould R., Hannah M., Holden-Dye L., O'Connor V., 2012 HSP-4 endoplasmic reticulum (ER) stress pathway is not activated in a C. elegans model of ethanol intoxication and withdrawal. Invert. Neurosci. **12**: 93–102.

Jana A., Hogan E. L., Pahan K., 2009 Ceramide and neurodegeneration: Susceptibility of neurons and oligodendrocytes to cell damage and death. J. Neurol. **278**: 5–15.

Kamath R. S., Ahringer J., 2003 Genome-wide RNAi screening in Caenorhabditis elegans. Methods **30**: 313–321.

Kamath R. S., Fraser A. G., Dong Y., Poulin G., Durbin R., Gotta M., Kanapin A., Bot N. Le, Moreno S., Sohrmann M., Welchman D. P., Zipperlen P., Ahringer J., 2003 Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature **421**: 231–7.

Kapulkin W. J., Kapulkin V., Hiester B. G., Link C. D., 2005 Compensatory regulation among ER chaperones in C. elegans. FEBS Lett. **579**: 3063–8.

Kooptiwut S., Mahawong P., Hanchang W., 2014 Journal of Steroid Biochemistry and Molecular Biology Estrogen reduces endoplasmic reticulum stress to protect against glucotoxicity induced-pancreatic $^{N}_{L}$ -cell death. J. Steroid Biochem. Mol. Biol. **139**: 25–32.

Krivoruchko A., Storey K. B., 2013 Activation of the unfolded protein response during anoxia exposure in the turtle Trachemys scripta elegans. **374**: 91–103.

Ladage M. L., King S. D., Burks D., Quan D. L., Garcia A. M., Azad R. K., Padilla P. A., Glucose or Altered Ceramide Biosynthesis Mediate Oxygen Deprivation Sensitivity Through Novel Pathways Revealed by Transcriptome Analysis in Caenorhabditis elegans. Genes, Genomes, Genet.

LaRue B. L., Padilla P. A., 2011 Environmental and Genetic Preconditioning for Long-Term Anoxia Responses Requires AMPK in Caenorhabditis elegans. PLoS One **6**: e16790.

Lee S., Murphy C. T., Kenyon C., 2009 Glucose Shortens the Lifespan of Caenorhabditis elegans by Down-Regulating Aquaporin Gene Expression. Cell Metab. **10**: 379–391.

Lemire B., 2005 Mitochondrial genetics. WormBook: 1–10.

Li P.-L., Zhang Y., 2013 Cross Talk Between Ceramide and Redox Signaling: Implications for Endothelial Dysfunction and Renal Disease. **216**: 171–197.

Livak K. J., Schmittgen T. D., 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**: 402–8.

Lopez X., Goldfine A. B., Holland W. L., Gordillo R., Scherer P. E., 2013 Plasma ceramides are elevated in female children and adolescents with type 2 diabetes. J. Pediatr. Endocrinol. Metab. **26**: 995.

Ma D. K., Rothe M., Zheng S., Bhatla N., Pender C. L., Menzel R., Horvitz H. R., 2013 Cytochrome P450 drives a HIF-regulated behavioral response to reoxygenation by C. elegans. Science **341**: 554–8.

Mendenhall A. R., LaRue B., Padilla P. A., 2006 Glyceraldehyde-3-phosphate dehydrogenase mediates anoxia response and survival in Caenorhabditis elegans.

Genetics **174**: 1173–87.

Mendenhall A. R., LeBlanc M. G., Mohan D. P., Padilla P. a, 2009 Reduction in ovulation or male sex phenotype increases long-term anoxia survival in a daf-16-independent manner in Caenorhabditis elegans. Physiol. Genomics **36**: 167–178.

Menuz V., Howell K. S., Gentina S., Epstein S., Riezman I., Fornallaz-Mulhauser M., Hengartner M. O., Gomez M., Riezman H., Martinou J.-C., 2009 Protection of C. elegans from anoxia by HYL-2 ceramide synthase. Science **324**: 381–384.

Menzel R., Yeo H. L., Rienau S., Li S., Steinberg C. E. W., Stürzenbaum S. R., 2007 Cytochrome P450s and short-chain dehydrogenases mediate the toxicogenomic response of PCB52 in the nematode Caenorhabditis elegans. J. Mol. Biol. **370**: 1–13.

Mondoux M. A., Love D. C., Ghosh S. K., Fukushige T., Bond M., Weerasinghe G. R., Hanover J. A., Krause M. W., 2011 O-linked-N-acetylglucosamine cycling and insulin signaling are required for the glucose stress response in Caenorhabditis elegans. Genetics **188**: 369–382.

Mortazavi A., Williams B. A., McCue K., Schaeffer L., Wold B., 2008 Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Meth **5**: 621–628.

NCBI, 2002 The Reference Sequence (RefSeq) Project - Chapter 18.

Novgorodov S., Gudz T. I., 2009 Ceramide and Mitochondria in Ischemia/Reperfusion. J. Cardiovasc. Pharmacol. **53**: 198–208.

Oslowski C. M., Urano F., 2013 Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol. **490**: 71–92.

Padilla P. A., Nystul T. G., Zager R. A., Johnson A. C. M., Roth M. B., 2002 Dephosphorylation of Cell Cycle-regulated Proteins Correlates with Anoxia-induced Ssuspended Animation in Caenorhabditis elegans. Mol. Biol. Cell **13**: 1473–1483.

Qiu J.-X., Zhou S.-F., Zhou Z.-W., He Z., Zhang X., Zhu S., 2015 Estimation of the binding modes with important human cytochrome P450 enzymes, drug interaction potential, pharmacokinetics, and hepatotoxicity of ginger components using molecular docking, computational, and pharmacokinetic modeling studies. Drug Des. Devel. Ther. **9**: 841–866.

Rolls M. M., Hall D. H., Victor M., Stelzer E. H. K., Rapoport T. A., 2002 Targeting of Rough Endoplasmic Reticulum Membrane Proteins and Ribosomes in Invertebrate Neurons. Mol. Biol. Cell **13**: 1778–1791.

Scheuner D., Song B., McEwen E., Liu C., Laybutt R., Gillespie P., Saunders T., Bonner-Weir S., Kaufman R. J., 2001 Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol. Cell **7**: 1165–76.

Shen X., Ellis R. E., Lee K., Liu C. Y., Yang K., Solomon A., Yoshida H., Morimoto R., Kurnit D. M., Mori K., Kaufman R. J., 2001 Complementary signaling pathways regulate the unfolded protein response and are required for C. elegans development. Cell **107**: 893–903.

Shore D. E., Carr C. E., Ruvkun G., 2012 Induction of cytoprotective pathways is central to the extension of lifespan conferred by multiple longevity pathways. PLoS Genet. **8**.

The C. elegans Sequencing Consortium, 1998 Genome sequence of the nematode C. elegans: a platform for investigating biology. Science **282**: 2012–2018.

World Health Organization, 2016 WHO.

WormAtlas, 2012 Handbook - Hermaphrodite.

Xia J. Y., Morley T. S., Scherer P. E., 2014 The adipokine/ceramide axis: Key aspects of insulin sensitization. Biochimie **96**: 130–139.

Zigdon H., Kogot-Levin A., Park J. W., Goldschmidt R., Kelly S., Merrill A. H., Scherz A., Pewzner-Jung Y., Saada A., Futerman A. H., 2013 Ablation of ceramide synthase 2 causes chronic oxidative stress due to disruption of the mitochondrial respiratory chain. J. Biol. Chem. **288**: 4947–4956.