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FACILITATIVE GLUCOSE TRANSPORTER AND ITS REGULATION BY  
INSULIN/IGF-LIKE SIGNALING IN *CAENORHABDITIS ELEGANS*

A Dissertation Presented

by

Shun Kitaoka

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy  
Specializing in Animal, Nutrition and Food Sciences

October, 2015

Defense Date: April 29, 2015

Dissertation Examination Committee:

Feng-Qi Zhao, Ph.D., Advisor

Anthony D. Morielli, Ph.D., Chairperson

Tom Jetton, Ph.D.

Jana Kraft, Ph.D.

Cynthia J. Forehand, Ph.D., Dean of the Graduate College

## Abstract

In humans, the functional regulation of facilitative glucose transporters (GLUTs) by insulin plays a central role in the maintenance of glucose homeostasis. The insensitivity of tissues to this regulation results in diabetes mellitus, however, the underlying mechanisms remain largely unknown. To establish *Caenorhabditis elegans* (*C. elegans*) as a model system to study the mechanisms of insulin regulation of GLUTs because of the well-conserved insulin/IGF-like signaling (IIS) and many unique advantages of this organism, we functionally characterized 9 candidate genes of human GLUT homologues in *C. elegans* based on their sequence homologies to GLUTs. We found that FGT-1 is the only functional GLUT homologue with the ability to transport 2-deoxy-D-glucose (2DG) in *Xenopus* oocytes. FGT-1 mediated 2DG transport could be inhibited by the GLUT inhibitor phloretin and exhibited a Michaelis constant ( $K_m$ ) of 2.8 mM, which is smaller than the  $K_m$  values of human GLUT1 and GLUT4. In addition to glucose, FGT-1 could also transport mannose, galactose, and fructose. Using a FGT-1::GFP fusion construct under the control of the 5 kb promoter sequence of the *fgt-1a* gene, FGT-1 was shown to be ubiquitously expressed in *C. elegans* tissues and cells, including the digestive tract, neurons, and body wall muscle. Two FGT-1 alternative splicing isoforms, FGT-1A and FGT-1B, showed similar transport activity and tissue localization.

To study the function of FGT-1 and its regulation by IIS, the changes in several phenotypes that are known to be regulated by IIS were observed in FGT-1-knockdown worms or null strains in the presence or absence of IIS activity. FGT-1 knockdown resulted in fat accumulation but had no effects on dauer formation or brood size in both wild-type and *daf-2* (insulin receptor) gene mutant strains. However, the function of FGT-1 in animal growth and aging was dependent on the IIS background, suggesting IIS regulation of FGT-1 function. Consistently, FGT-1 mediated glucose uptake was almost completely defective in the *daf-2* and *age-1* (PI3 kinase) mutants, and phloretin could only marginally inhibit 2DG uptake in these strains. This defect was only partially related to the approximately 60% decrease in FGT-1 protein levels in these mutants, suggesting the involvements of both post-transcriptional and post-translational regulatory mechanisms. We also found that OGA-1, an O-GlcNAcase, is essential for the function of FGT-1, implying possible regulation of FGT-1 function by glycosylation.

In summary, our study has established *C. elegans* as a powerful model to study the mechanism by which insulin regulates glucose transporters and has provided insights into the mechanism of defective glucose uptake by tissues in patients with diabetes.

## Citations

Material from this dissertation has been published in the following form:

Kitaoka, S., Morielli, A. D. & Zhao, F.-Q.. (2013). FGT-1 is a mammalian GLUT2-like facilitative glucose transporter in *Caenorhabditis elegans* and its malfunction induces fat accumulation in intestinal cells. PLoS ONE, 8(6), e68475.

Material from this dissertation has been submitted for publication to Aging Cell on April 23, 2015 in the following form:

Kitaoka, S., Morielli, A. D. & Zhao, F.-Q.. Regulation of glucose transport by insulin/IGF-like signaling in *Caenorhabditis elegans*. Aging Cell.

## Acknowledgements

I would first like to express my gratitude to my advisor, Dr. Feng-Qi Zhao, for accepting me as his student and my *C. elegans* project proposal. He always patiently gave me chances to improve my scientific skills such as ways of critical thinking, writing, research designs, presentations, and many more. I also thank all of my lab mates working together over these years and department faculty members, staff, and graduate students for their continuous support. My sincere appreciation goes to my graduate committee members, Dr. Anthony D. Morielli, Dr. Tom Jetton, and Dr. Jana Kraft for giving me valuable comments and suggestions on my research. I must highlight Dr. Morielli's cooperation in my research as he provided access to critical equipment which were indispensable to complete this dissertation.

I would also like to show my appreciation to Wakunaga Pharmaceutical Co. Ltd., especially Mr. Hironobu Wakunaga and Dr. Takami Oka, for giving me a great opportunity to enhance my scientific expertise and career, and supporting my entire program at the University of Vermont.

My deepest gratitude goes to my wife, Mayumi, for her understanding and dedicated assistance in last four years. I definitely would not have been able to successfully complete my graduate program and also living in the US without her. I would finally like to thank my parents for their encouragement and support during these four years.

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# **Chapter 1: Literature Review**

## **1.1 Introduction**

The incidence of diabetes mellitus has increased in recent decades, particularly in economically affluent countries, and now is gradually becoming widespread worldwide. In 2010, approximately 347 million people had diabetes, and this number will increase to 438 million people by 2030 (Rawal et al. 2012; Wang et al. 2015). Diabetes severely affects the quality of life of patients and has high costs for both patients and society. The estimated direct and indirect medical expenditure for diabetes treatment was 132 billion dollars in 2002 in the US alone (Hogan et al. 2003). Many efforts have been made to develop effective drug treatments for diabetes. Although these attempts have shown some positive results, the current medications remain insufficient to overcome diabetes, and the number of diabetic patients is continuously increasing (Ginter and Simko 2010; Narayan 2005; Parikh et al. 2011).

Diabetes is a metabolic syndrome characterized by high blood glucose levels. Insulin is the major hormone that regulates blood glucose levels by stimulating glucose uptake and utilization in peripheral tissues, such as muscle and fat. In type 1 diabetes, pancreas  $\beta$ -cells is deteriorated because of autoimmune destruction, whereas in type 2 diabetes, the peripheral tissues establish insulin resistance and are not able to increase glucose uptake in response to insulin stimulation. Importantly, insulin secretion is defective in both conditions. Type 2 diabetes represents approximately 90% of total diabetes incidence (WHO 2014). In diabetes patients, the amount of insulin-sensitive

glucose transporters expressed on the cell surface is closely related to the level of insulin resistance (Garvey et al. 1998). Thus, understanding the regulatory mechanisms of glucose homeostasis mediated by insulin signaling is critical for developing the proper treatment for diabetes.

## **1.2 Regulation of glucose homeostasis in humans**

The essential energy source glucose is a substrate for macromolecule synthesis in most living organisms. Glucose is supplied primarily from the blood stream to most tissues, although the synthesis of glucose by gluconeogenesis and the breakdown of stored sugar by glycogenolysis can also meet glucose demands in some tissues, such as liver and muscle (Vissing and Haller 2012; Rui 2014). The blood glucose level is strictly regulated by endocrine hormones; excessively high or low blood glucose levels cause several metabolic disorders (Kalsbeek et al. 2014). Insulin and glucagon are the dominant hormones that control blood glucose levels, but several other hormones are also involved, including growth hormone, glucocorticoids, and thyroid hormones. Although most of these hormones are responsible for up-regulating blood glucose levels, insulin is the only known hormone that lowers blood glucose concentrations (Yki-Järvinen 1993; Noguchi et al. 2013). After food intake, ingested carbohydrates are digested, absorbed from the gastrointestinal tract, diffused to the blood stream, and circulated around the body. The increased blood glucose level stimulates the  $\beta$ -cells in the islets of Langerhans of the pancreas to secrete insulin (Lindsay et al. 2003). Insulin decreases blood glucose by

stimulating cellular glucose uptake, glycolysis, and glycogenesis (Probst and Unthan-Fechner 1985; Srivastava and Pandey 1998) and by inhibiting glucose production by gluconeogenesis and glycogenolysis in the liver, muscle, or adipose tissues (Sutherland et al. 1996; Bollen et al. 1998). The stimulation of glucose uptake primarily in muscle and fat tissues plays a key role in decreasing blood glucose levels; this stimulation is mediated by the activation of glucose transporter proteins. Because of the hydrophilic property of glucose, carrier proteins are essential for transporting glucose through the plasma membrane of cells. Insulin secretion disruption or tissue insulin sensitivity are the major causes of diabetes (Wang et al. 2015; Ma et al. 2015).

In contrast, when people are in a fasting state, decreased blood glucose levels induce the  $\alpha$ -cells of the islets of Langerhans of the pancreas to release glucagon. Glucagon inhibits glycolysis and activates gluconeogenesis and glycogenolysis in the liver to supply additional glucose to the blood stream (Bazotte et al. 1988; Exton et al. 1972). The primary control of glucagon secretion is mediated by glucose-sensitive neural stimulation. Pancreas  $\alpha$ -cells are innervated by the sympathetic nervous system, which stimulates glucagon secretion by the neurotransmitter norepinephrine, and this stimulation is effectively blocked by neuropeptide Y (Ahrén 2000; Pettersson et al. 1987). Unlike the function of insulin, no evidence indicating that glucagon directly regulates the activity of glucose transporters in any tissues have been found. Recently, glucagon excess was found to be as important as insulin deficiency in diabetes, thus, this hormone has also been targeted in therapeutic studies of diabetes (Unger and Cherrington 2012). Other hormones such as growth hormone and glucocorticoids also activate

anabolic glucose reactions in their target cells, however, the activation of these reactions accelerates metabolism of the target cell and does not supply glucose to the blood stream.

### **1.3 Mammalian glucose transporters**

Thus far, three families of glucose transporters have been identified in mammals. The facilitative glucose transporter (GLUT) family mediates glucose diffusion across the plasma membrane in passive manner along its concentration gradient (Thorens and Mueckler 2010; Zhao and Keating 2007). Glucose uptake in most tissues and cells is performed by this family of transporters. The Na<sup>+</sup>/glucose cotransporter (SGLT) family mediates active transport, which allows the transport of glucose against its concentration gradient (Castaneda-Sceppa and Castaneda 2011). The major roles of this family of transporters are to absorb monosaccharides from the intestinal lumen and to reabsorb glucose from the proximal tubules of the kidney. The SWEET (Sugars Will Eventually be Exported Transporters) family has recently been discovered and has a possible function in the cellular sugar efflux in human cells (Chen et al. 2010).

#### **1.3.1 Facilitative glucose transporters (GLUTs)**

Currently, 14 isoforms of GLUTs have been reported in humans (Table 1); these isoforms have high sequence similarity and share common structural characteristics such as 12 transmembrane domains, an N-glycosylation site on the first or fifth loop, and sugar transporter signatures (Zhao and Keating 2007; Sun et al. 2012). Despite the well conserved common structures among GLUT isoforms, individual transporters have

different transport kinetics, sugar specificities, and tissue distribution (Zhao and Keating 2007). Amino acid sequence differences roughly classify GLUT members into three groups: classes I, II, and III. Class I and II GLUTs contain a longer extracellular loop 1 with a conserved N-linked glycosylation site. Class III family members have a large loop with a glycosylation site on the fifth extracellular loop instead of the first loop, and their first loop is relatively short (Augustin 2010; Zhao and Keating 2007).

### **1.3.2 Class I**

The class I GLUT family consists of GLUT1-4 and GLUT14 (Thorens and Mueckler 2010; Mueckler and Thorens 2013). GLUT1 is the most ubiquitously expressed isoform and mediates glucose transport by itself or with other GLUT isoforms in individual tissues, making this isoform the “general” glucose transporter. GLUT1, which was originally isolated from erythrocytes, abundantly localizes in the red blood cells to enhance their capacity to carry glucose for circulation (Mueckler and Thorens 2013). GLUT1 is also expressed in high levels in the endothelial and epithelial-like barriers of the brain, eyes, brain astrocytes, peripheral nerve, and placenta (Illsley 2000; Takata et al. 1990; Pellerin et al. 2002). In a *Xenopus laevis* oocyte model, GLUT1 shows transport activity, with Michaelis constant ( $K_m$ ) values of 5-11 mM for 2-deoxy-D-glucose (2DG) (Vera and Rosen 1989; Bentley et al. 2012) and of 3 mM for glucose (Uldry et al. 2002). In addition to glucose, this transporter has the capacity to transport galactose, mannose, and glucosamine (Uldry et al. 2002; Carruthers et al. 2009; Bentley et al. 2012). N-linked glycosylation, which is a structural characteristic of the GLUT family, is indispensable for the high affinity of GLUT1 for glucose (Asano et al. 1991).

GLUT2 is a low-affinity, high capacity GLUT that has the highest  $K_m$  for glucose (~17 mM) among GLUT family members (Johnson et al. 1990). GLUT2 also transports galactose, mannose, and fructose with low affinity (Johnson et al. 1990). Interestingly, this transporter shows a high affinity for glucosamine ( $K_m = 0.8$  mM) (Uldry et al. 2002). The major site of GLUT2 localization is hepatocytes, where GLUT2 takes up glucose and fructose after feeding and releases glucose in a fasted state (Guillam et al. 1998). In intestine cells, GLUT2 primarily mediates glucose diffusion to the blood stream on the basolateral membrane (Fukumoto et al. 1988), however, GLUT2 can also be translocated to the apical membrane for glucose absorption, depending on the availability of glucose and fructose in the intestinal lumen (Kellett et al. 2008; Leturque et al. 2009). GLUT2 is also expressed in the basolateral membrane of the epithelial cells of proximal convoluted tubules in the kidney and is involved in glucose reabsorption mediated by SGLT transporters (Guillam et al. 1998).

GLUT3 is considered a neuron-specific glucose transporter because of its prominent localization in the brain (Shepherd et al. 1992; Haber et al. 1993; McCall et al. 1994). The high affinity of GLUT3 for glucose ( $K_m = 1.4$  mM for 2DG) (Manolescu et al. 2007) is essential for its expression in the brain because the glucose concentration in the brain is particularly lower compared to that in the blood. GLUT3 is also localized in the testis, placenta, embryo, and white blood cells, which are all tissues with high demands for glucose (Illsley 2000; Pantaleon et al. 1997; Simpson et al. 2008). The other substrates of GLUT3 are galactose, mannose, maltose, and xylose, but not fructose (Colville et al. 1993). GLUT14, another class I GLUT family member, was identified as a

gene duplication product of GLUT3 (95% identical to the nucleotide sequence of GLUT3) (Wu and Freeze 2002). GLUT14 is localized primarily in the testis.

GLUT4 is one of the most widely studied glucose transporters because it mediates insulin-regulated glucose uptake in muscle cells and adipocytes. In these cells, insulin stimulates GLUT4 translocation from the intracellular pool to the plasma membrane, thus increasing glucose uptake and utilization (Leto and Saltiel 2012; Saltiel and Kahn 2001). This regulation plays a critical role in maintaining whole body glucose homeostasis (Leto and Saltiel 2012). For GLUT4, N-glycosylation is important for the translocation machinery, however, it plays no major role in GLUT4 transport activity (Zaarour et al. 2012; Haga et al. 2011). Although the major localization of GLUT4 is in the muscle and adipose tissues, GLUT4 is also expressed in some CNS neurons together with GLUT3 to increase glucose uptake for high energy demand in specific neurons (Apelt et al. 1999). GLUT4 transport affinity for glucose is similar to that of GLUT1 ( $K_m = 5-6$  mM), and dehydroascorbic acid and glucosamine are also substrates of GLUT4 (Uldry et al. 2002; Kasahara and Kasahara 1997). The detailed mechanisms of GLUT4 regulation are discussed later.

### **1.3.3 Classes II and III**

GLUT5, 7, 9, and 11 are included in the class II GLUT family. This class is characterized by its fructose transport activity. Indeed, GLUT5 is the major fructose transporter in human. The hydrophobic NXV/NXI sequence motif in helix 7 is known to be important for fructose recognition, and this motif is conserved among class II isoforms (Manolescu et al. 2007). GLUT5 and 7 are localized primarily in the apical membrane of

the small intestine for fructose and glucose uptake (Li et al. 2004; Barone et al. 2009). GLUT9 functions in the kidney, liver, and pancreatic  $\beta$ -cells. GLUT9 knockdown by siRNA reduces glucose-stimulated insulin secretion in mice and rats (Evans et al. 2009). GLUT11 is a muscle-specific transporter that is dominantly expressed in the heart and skeletal muscles (Scheepers et al. 2005; Doege et al. 2001). Interestingly, GLUT9 and 11 have alternative splicing and promoter usage, which is unique in the GLUT family, resulting in splicing isoform-specific tissue distribution (Augustin et al. 2004; Scheepers et al. 2005).

The class III family members GLUT6, 8, 10, 12, and 13 have some unique structures. As above-described, class III GLUTs have an N-glycosylation site in extracellular loop 5 rather than in loop 1. In addition, all class III isoforms contain an internalization signal that sequesters transporters in intracellular vesicles. The consensus internalization motifs are a YSRI motif for GLUT10 (Dawson et al. 2001; Augustin 2010) and a dileucine motif for other members (Lisinski et al. 2001; Flessner and Moley 2009; Zhao and Keating 2007). Although one study showed that intracellular vesicles containing GLUT8 were successfully induced by insulin to translocate to the plasma membrane (Carayannopoulos et al. 2000), various studies have contradicted this result (Lisinski et al. 2001; Widmer et al. 2005; Augustin et al. 2005; Shin et al. 2004), suggesting that class III GLUTs are not insulin-regulated transporters but function in intracellular sugar trafficking. GLUT8 is localized in a late-endosomal/lysosomal compartment inside cells in the testis (Diril et al. 2009), synaptic dense core vesicles of nerve terminals, and secretory granules in the brain (Ibberson et al. 2002). GLUT12

contains a dileucine motif similar to that of GLUT8, however, this transporter localizes in the Golgi apparatus and plasma membrane (Flessner and Moley 2009). GLUT6 and 10 also show internal localization, although no detailed sub-cellular localization study has been performed. All these members have specificity for glucose (Zhao and Keating 2007; Mueckler and Thorens 2013). GLUT13, also known as HMIT, is a unique transporter that does not transport glucose despite its conserved protein structure containing all motifs known to be important for glucose transport activity. Instead, the major substrate of GLUT13 is myo-inositol since this isoform is a H<sup>+</sup>/myo-inositol co-transporter.

#### **1.3.4 Na<sup>+</sup>/glucose cotransporters (SGLTs)**

The SGLT family of proteins shares structural characteristics of 14 transmembrane domains and sodium:solute symporter family signature domains (Zhao and Keating 2007). These members localize primarily in the intestine or kidney. SGLTs expressed in the intestines mediate sugar absorption from the intestinal lumen, and transporters expressed in the kidneys function in the reabsorption of monosaccharides from the proximal convoluted tubules. SGLT1 is the major SGLT expressed in intestinal cells (Wright et al. 1992), whereas SGLT2 is the major kidney isoform. Specific inhibitors of SGLT2 have been developed for diabetes medication (Jung et al. 2014). Human SGLT3 does not show transport activity for glucose but functions as a glucose sensor (Diez-Sampedro et al. 2003). Interestingly, a single point mutation of SGLT3 on the sugar interaction site sufficiently recovered the glucose transport activity of SGLT3 (Bianchi and Díez-Sampedro 2010).

### **1.3.5 SWEETs**

A new family of sugar transporters called the SWEET family was recently identified in plants (Chen et al. 2010). This transporter family is found to be conserved in plants, bacteria, and metazoans, including humans (Chen et al. 2010; Xu et al. 2014). The SWEET family of proteins has only seven transmembrane domains and primarily mediates cellular sugar efflux because the expression of human SWEET1 in yeast and oocytes promotes only sugar efflux (Wright 2013). This transporter family is localized systemically in plants and in the oviduct, epididymis, intestine, and lactating mammary gland in mammals (Chen et al. 2010). Surprisingly, bacterial and fungal pathogens and symbiotes of plants hijack this transporter family to acquire glucose from their hosts (Chen et al. 2010). This new class of sugar transporters may have major glucose transport functions in plants but most likely supports GLUT and SGLT families in cellular sugar efflux in animals. The human GLUT, SGLT, and SWEET family transporters are summarized in Table 1.1.

## **1.4 Regulation of glucose transport by insulin and its role in type 2 diabetes**

### **1.4.1 Insulin secretion and signal transduction**

Insulin is a protein hormone that is secreted from pancreatic  $\beta$ -cells and that is encoded by a single-copy INS gene in humans. The INS gene first encodes a precursor of insulin known as preproinsulin. Preproinsulin is a 110 amino acid protein that consists of

a 21 amino acid A chain, a 30 amino acid B chain, a 31 amino acid C chain, and a signal peptide on the N-terminus. The signal peptide guides the translocation of preproinsulin into the endoplasmic reticulum lumen and is then cleaved by a signal peptidase to form proinsulin (Chan et al. 1976; Patzelt et al. 1978). Then, proinsulin forms a three dimensional structure with three disulfide bonds between its A and B chains (Huang and Arvan 1995). The folded proinsulin is transported to the Golgi apparatus where its C chain is cleaved to produce mature insulin and C-peptide (Huang and Arvan 1995). In contrast to insulin, the function of C-peptide has not been known for a long time, however, current studies have highlighted C-peptide as another therapeutic target for diseases including diabetes because this hormone appears to play important roles in anti-inflammatory, anti-oxidant, and cell protective mechanisms (Wahren and Larsson 2015).

Insulin secretion is dependent on the plasma glucose concentration (Schmitz et al. 2008).  $\beta$ -cells form clusters that localize along capillaries surrounding pancreas islets, allowing the rapid sensing of nutrients such as glucose in the blood and the diffusion of insulin into the circulation (Suckale and Solimena 2008).  $\beta$ -cells are activated by several nutrients, such as glucose, other monosaccharides, amino acids, and fatty acids, however, the amplitude of  $\beta$ -cell induction is much larger for glucose compared to amino acids or fatty acids (Chang and Goldberg 1978). GLUT2 participates in the first step of blood glucose sensing mechanisms. Because GLUT2 is the dominant glucose transporter expressed in  $\beta$ -cells, glucose diffusion into  $\beta$ -cells is largely dependent on GLUT2 function in humans. After glucose is taken up, it is metabolized inside cells and promotes closure of ATP-sensitive potassium channels by increasing the intracellular ATP/ADP

ratio, which subsequently opens voltage-dependent  $\text{Ca}^{2+}$  channels to promote the exocytosis of insulin-containing granules (Maechler and Wollheim 1999; Bender et al. 2006). Some amino acids can also be directly incorporated into the tricarboxylic acid (TCA) cycle to accelerate exocytosis (Sener and Malaisse 1980). In addition, dietary amino acid can also activate insulin secretion through gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) because these incretin hormones stimulate  $\beta$ -cells directly by binding to their specific receptors on these cells (MacDonald et al. 2002b, 2002a). Free fatty acids also stimulate insulin secretion *via* signaling from FFAR1 (free fatty acid receptor 1) activation (Feng et al. 2012; Kristinsson et al. 2013).

In insulin target cells, insulin binds to the extracellular domain of the insulin receptor (IR), which is the  $\alpha$ -subunit of IR, leading to the phosphorylation of its intracellular tyrosine kinase domain on its  $\beta$ -subunit, which allows IR to serve as a docking site for many proteins, including IR substrate (IRS) proteins (Lizcano and Alessi 2002). IRS proteins trigger the activation of phosphatidylinositol 3-kinase (PI3K) by assembling its regulatory and catalytic subunits. Active PI3K converts phosphatidylinositol (3,4)-bisphosphate (PIP<sub>2</sub>) lipids to phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>), which is the second messenger of this pathway. Phosphatase and tension homologue (PTEN) negatively regulates the insulin signaling cascade by counteracting this PI3K conversion (Gupta and Dey 2012). This modulation permits the fine-tuning of second messenger levels. PIP<sub>3</sub> recruits phosphoinositide-dependent kinase (PDK) 1 and 2 to the plasma membrane, and each isoform of PDK phosphorylates different sites of Akt/protein kinase B (PKB) (Cantley 2002; Hodgkinson et al. 2002).

mTORC2, which is a protein complex in the mTOR cascade, also phosphorylates the same phosphorylation site of Akt as PDK2 (Sarbasov et al. 2005; Chen and Sarbasov 2011). Akt activation phosphorylates AS160 and inhibits its Rab-GAP activity, promoting GLUT4 translocation to the plasma membrane (Lizcano and Alessi 2002; Hoffman and Elmendorf 2011; Smith et al. 2011). In addition, Akt phosphorylates FOXO (O class of forkhead box) 1 transcription factor to retain it in the cytosolic region as a transcriptionally inactive protein (Van Der Heide et al. 2004). Although a defect in Akt and AS160-mediated GLUT4 translocation is thought to be the dominant factor of insulin resistance, FOXO1 is also a key factor in diabetes because constitutively active FOXO1 expression in the liver is sufficient for causing insulin resistance (Zhang et al. 2002; Lu et al. 2012).

#### **1.4.2 Subcellular translocation of GLUT4 and its regulation by insulin**

GLUT4 is the major transporter that mediates insulin-regulated glucose uptake in tissues (Augustin 2010; Leney and Tavaré 2009). This transporter is normally located on intracellular microsomes in the absence of insulin stimulation, and once insulin signaling becomes active, GLUT4 storage vesicles (GSVs) translocate from intracellular compartments into the plasma membrane (Silverman et al. 2009), resulting in GLUT4 translocation to the cell surface to take up glucose. Because this step is one of the key steps that is compromised in insulin resistance observed in diabetes, great efforts have been made to reveal the regulatory mechanisms of GLUT4 translocation for over 20 years. Although the mechanisms remain far from clear, recent investigations have revealed several key molecular systems involved in this process. The cell surface

localization of GLUT4 is tightly regulated by balancing the levels of GSV exocytosis and endocytosis. Under conditions with no insulin, slow exocytosis and rapid endocytosis of GSVs retain surface transporter levels at less than 5% of total GLUT4 pools (Muretta et al. 2008; Slot et al. 1991), and the balance is switched by insulin stimulation to increase GLUT4 levels on the plasma membrane.

Upon insulin signaling, PI3K activates two different downstream cascades, Rac- and Akt-mediated pathways. Although many signaling molecules of GSV translocation are regulated by Akt, Rac1/Rho GTPase is also a key player for the mechanical mobilization of GSVs (Klip et al. 2014). Rac1 activation is required for actin remodeling. The activities of the actin branching complex Arp2/3 and the actin filament-depolymerizing enzyme cofilin are regulated by Rac1 for cell membrane remodeling by dynamic actin cycling (Chiu et al. 2010). How the actin cycling mechanisms interact with GLUT4 mobilization remains unclear, however, GSV polarization beneath the plasma membrane requires actin cycling (Tong et al. 2001; Sun et al. 2010; Talior-Volodarsky et al. 2008).

During GLUT4 translocation, the downstream target of Akt is AS160, a GTPase-activating protein (GAP) of the Rab family (Minea et al. 2005). AS160 is a regulatory protein that inhibits several Rab GTPases in the GLUT4 exocytosis pathway (Sano et al. 2007; Sun et al. 2010). AS160 is the central regulator of GLUT4 translocation because the expression of constitutively active AS160 in adipocytes and muscle cells prevents insulin-dependent GLUT4 translocation (Chen et al. 2012; Sun et al. 2010). In contrast, the siRNA-mediated inhibition of AS160 significantly increased

membrane GLUT4 localization without insulin stimulation (Ishikura et al. 2007). The identification of AS160-targeted Rab GTPases is being actively investigated because more than 60 Rab GTPases exist in the human genome (Stenmark and Olkkonen 2001). Recently, Rab8A and Rab13 have been identified as the targets of AS160 in muscle cells (Sun et al. 2010; Ishikura and Klip 2008). Additionally, Rab10 was found to be indispensable for GLUT4 translocation in adipocytes, although a direct interaction between AS160 and Rab10 has not been confirmed thus far (Sano et al. 2007). When insulin signaling is activated, AS160 GAP activity is blocked, and the downstream Rab proteins are ready to be activated. At this point, guanine nucleotide exchange factor (GEF) function is required for the activation of Rab GTPases. GEFs containing DENN (differentially expressed in normal and neoplastic cells) domain proteins DENND4A-C were found to interact with Rab10 (Yoshimura et al. 2010). DENND4C knockdown successfully inhibited the surface expression of GLUT4 under insulin activation (Sano et al. 2011). Rabin8/Sec2p, GRAB, and DEEND1C are the known GEFs of Rab8A, although which of these GEFs is incorporated in the insulin-regulated GLUT4 pathway is unknown (Guo et al. 2013; Blümer et al. 2013). The identification of GEFs for Rab13 remains under investigation. Activated Rab proteins are capable of driving the motor protein myosin 5a (Roland et al. 2009). While GLUT4 is translocated, GSVs are trafficked by this myosin molecule along the actin cytoskeleton since myosin can interact with actin filaments through their head groups (Semenova et al. 2008). After GSVs are intracellularly trafficked, they are tethered to the plasma membrane. Although no direct observations have been made, the molecular tracking of GSVs with total internal

reflection fluorescence (TIRF) microscopy in myocytes indicated that Rac1-regulated cortical actin filaments and myosin 5a-driven GSVs are tethered by myosin 1c and  $\alpha$ -actinin 4 function (Boguslavsky et al. 2012; Talior-Volodarsky et al. 2008). GSV trafficking and dynamic actin sorting at the cell periphery are both regulated by insulin signaling, while GSV membrane tethering with myosin 1c and  $\alpha$ -actinin 4 is thought to be a passive mechanism. The final step of GSV exocytosis is the membrane insertion of these compartments. SNARE proteins are present on both GSVs and the plasma membrane to promote the membrane fusion process. VAMP2 is a v-SNARE protein localized on GSVs, and the t-SNARE protein syntaxin 4 is localized on the plasma membrane with the plasma membrane-associated accessory protein synaptosomal-associated protein 23 (SNAP23) (Foster and Klip 2000; Randhawa et al. 2004; Kawaguchi et al. 2010). The regulation of this fusion step by insulin remains under investigation, however, the SNARE regulatory protein Munc18 has been implicated as a target of insulin (Khan et al. 2001; Bryant and Gould 2011).

Internalization by endocytosis is another key pathway of GLUT4 translocation, but this step has not been as well studied as exocytosis because insulin may have a minor effect or no effect on endocytosis (Foster et al. 2001; Leto and Saltiel 2012). GLUT4 is internalized in muscle cells by clathrin-mediated endocytosis and in adipose cells by both clathrin-mediated and cholesterol-dependent endocytosis. Clathrin-mediated endocytosis is not regulated by insulin signaling (Blot and McGraw 2006). In adipose tissues, insulin decreases the rate of GLUT4 endocytosis (Blot and McGraw 2006; Czech and Buxton 1993). The regulatory mechanism may be mediated by switching cholesterol-dependent

endocytosis to clathrin-mediated endocytosis to delay GLUT4 internalization under active insulin conditions (Blot and McGraw 2006).

### **1.4.3 Other mechanisms by which insulin regulates GLUTs**

The regulation of GLUT4 activity by insulin is not driven only by subcellular translocation. In type 2 diabetes, insulin-stimulated GLUT4 translocation decreases but the expression level of GLUT4 does not change in muscle tissues (Shepherd and Kahn 1999; Graham and Kahn 2007), however, both GLUT4 translocation and GLUT4 expression levels decrease in adipose tissue (Shepherd and Kahn 1999). Moreover, recent research has shown that insulin may also increase GLUT4 transcription in muscle cells (Moraes et al. 2014). GLUT4 expression is controlled by several different transcription factors including MyoD, MEF2A, NF $\kappa$ B1, TNF- $\alpha$ , SREBP-1c, O/E-1, and NF-1 (Im et al. 2007). O/E-1 and NF-1 bind to insulin response elements on the GLUT4 gene promoter and regulate GLUT4 expression levels in an insulin-responsive manner (Cooke and Lane 1999; Miura et al. 2004; Dowell and Cooke 2002). MEF2A and MyoD are known to be up-regulated by insulin and to bind to AT-rich and E-box elements in the GLUT4 promoter region, respectively, to increase GLUT4 transcription (Moraes et al. 2014). Zinc finger protein 407 (ZFP407) increases the transcription level, splicing efficiency, and mRNA stability of GLUT4 in adipose tissue (Buchner et al. 2015). Therefore, these studies have revealed that transcriptional regulation may also be an important component of the regulation of GLUT4 function by insulin.

GLUT3 function in white blood cells is partially regulated by insulin. GLUT3 is normally localized in intracellular vesicles in white blood cells but translocates to the

plasma membrane by various proliferative stimuli including insulin stimulation (Daneman et al. 1992; Simpson et al. 2008). These translocation stimuli function in a cell type-specific manner; for instance, insulin stimulation can trigger GLUT3 activation in B-lymphocytes and monocytes but not in neutrophils or T-lymphocytes (Daneman et al. 1992; Estrada et al. 1994; Maratou et al. 2007). This insulin- and other stimuli-activated translocation should play a role in immune responses rather than in blood glucose homeostasis regulation (Simpson et al. 2008).

GLUT2-mediated glucose efflux in the liver is indirectly regulated by insulin. GLUT2 is plentifully expressed on the hepatocyte plasma membrane under low blood glucose conditions primarily to release glucose produced in hepatocytes. When the blood insulin level increases in the fed state, glucose catabolism is accelerated in the liver (Saltiel and Kahn 2001), and receptor-bound insulin is endocytosed into hepatocytes for insulin clearance from the circulation (Di Guglielmo et al. 1998). GLUT2 is concurrently internalized inside the cell (Eisenberg et al. 2005). Intriguingly, IR and GLUT2 physically interact during internalization so that IR and GLUT2 form a receptor-transporter complex when they are endocytosed by insulin activation (Eisenberg et al. 2005). Thus, GLUT2-mediated glucose efflux is blocked in a high blood glucose state by insulin-regulated internalization mechanisms.

The insulin pathway is not the only GLUT regulatory mechanisms but there are several different expression control systems of GLUT genes. For instance, tumor cells require high glucose uptake which is mediated by GLUT function and the expression stimulation is triggered by several factors such as hypoxia, estradiol, and epidermal

growth factor (Macheda et al. 2005).

### **1.5 *Caenorhabditis elegans* (*C. elegans*)**

*C. elegans* is a small soil nematode, a multiple-organ animal that has been used as an *in vivo* laboratory model organism for biological and medical studies (Brenner 1974; Kaletta and Hengartner 2006). *C. elegans* have short reproductive cycles (3 days) and lifespans (3 weeks), with large brood sizes (300 progenies/adult) (Brenner 1974). In harsh environments, worms enter into an alternative developmental stage called the dauer stage; dauer larvae can survive more than three months without energy sources (Cassada and Russell 1975). The predominant sex of this worm is hermaphrodite, where the male population is less than 0.1% of the total population under normal conditions. Severe environmental conditions increase the male population to more than 1% for efficient mating to enhance genetic diversity. Their cell lineage was fully described in early studies (Sulston et al. 1983; Sulston and Horvitz 1977). The *C. elegans* hermaphrodite loses 131 cells by apoptosis during embryonic and early larval (L2) stages, and the adult hermaphrodite is finally composed of 959 cells (somatic nuclei) (Sulston et al. 1983; Sulston and Horvitz 1977). This worm has several organs and systems, including a nervous system, intestine, muscle, hypoderm, secretory-excretory system, gonads, and uterus. The neuronal connectivity of this organism is also fully understood, the nervous systems of hermaphrodites and males are composed of 302 and 391 neurons, respectively

(Sulston and Horvitz 1977; Sulston et al. 1983). The worm body is transparent throughout its lifespan.

## **1.6 *C. elegans* as a model system for biomedical research**

The *C. elegans* worm has several advantages for biological studies and for drug discovery. Their small body size (~1 mm in length) allows for easy maintenance in the laboratory and handling in 96- or 384-well plates for high throughput analysis. Their simple and short life cycle can accelerate experimental speeds (Brenner 1974). Their transparent body at all developmental stages allows the visualization of all cells under light and fluorescent microscopes. The knowledge of its complete cell lineage enhances detailed microscopic analyses. The completely sequenced *C. elegans* genome harbors ~20,000 predicted coding genes that show homology to 60-80% of human genes (Kuwabara and O'Neil 2001; *C. elegans* Sequencing Consortium 1998). Moreover, plentiful forward and reverse genetic approaches are available. Gene knockout and mapping methods are relatively easy in worms, and a large collection of mutant strains is stocked and readily accessible from the *Caenorhabditis* Genetic Center (<http://www.cbs.umn.edu/cgc>) and National Bioresource Project (<http://www.shigen.nig.ac.jp/c.elegans/>). RNAi techniques, which were discovered in *C. elegans* research (Fire et al. 1998), are easy to apply to *C. elegans*, and the whole genome collection of synthesized dsRNAs or feeding bacteria expressing specific dsRNAs are readily available (Tabara et al. 1998; Kamath et al. 2001).

The high conservation of disease pathways and genes between *C. elegans* and humans and the unique advantages described above make *C. elegans* an ideal and effective *in vivo* model for disease pathway identification and high-throughput compound screening. Of the current animal models, *C. elegans* is the most cost-effective high-throughput model (Kaletta and Hengartner 2006; Silverman et al. 2009). Although non-mammalian systems sometimes do not provide direct correspondence to human diseases, the effort- and cost-effectiveness of the worm model make it an efficient system to begin studying novel pathways and screening compounds in combination with later validation steps in mammalian models.

### **1.6.1 Genetic analysis of disease pathways**

*C. elegans* is widely used in basic studies of molecular pathways related to human diseases such as metabolic syndrome, oncology, and neurodegeneration, as well as several genetic diseases (Kaletta and Hengartner 2006). One of the advantages of *C. elegans* is its fully characterized cell lineage description. Apoptosis is known as a cellular response that is associated with several conditions including infection, tissue degeneration, malignancy, and immunity (Lockshin and Zakeri 2007; Wang 2014; Pasparakis and Vandenabeele 2015). Apoptotic cell characterization and their cell death timing are fully understood in *C. elegans*, making *C. elegans* a good model for the study of programmed cell death-associated disease pathways. To identify the genes involved in the apoptosis pathway, forward genetic screening using ethyl methane sulfonate (EMS) as a mutagen was employed, and mutant worms with disrupted programmed cell deaths were screened. This screening resulted in the identification of loss-of-function mutations

in *ced-3* and *ced-4*, which are homologues of human caspases and apoptotic protease activating factor-1 (APAF-1), respectively (Ellis and Horvitz 1986; Hedgecock et al. 1983; Conradt and Xue 2005). In the same manner, further genetic screening revealed the central apoptotic factors CED-9/Bcl2 and EGL-1/BH3-only (Bcl2 homology domain-3) proteins (Hengartner et al. 1992; Trent et al. 1983). Currently, these factors are considered central regulators of the programmed cell death pathway.

Parkinson's disease (PD) is another prominent example of a disease model using *C. elegans*. The loss of dopamine neurons and aggregation of proteins (Lewy bodies) are recognized as pathological characteristics of PD (Kuwahara et al. 2008). In *C. elegans*, hermaphrodites have only eight dopamine neurons, whereas thousands of dopamine neurons are present in the midbrain in mammals (Nass et al. 2002). The simplicity of *C. elegans* enables comprehensive analyses of basic molecular mechanisms. Moreover, transgenic worms expressing GFP specifically in dopamine neurons can allow the number of dopamine neurons in intact animals to be visualized because of their transparent bodies (Nass et al. 2001). A forward genetic analysis that used GFP transgenic worms to screen for mutants resistant to neurotoxin 6-hydroxydopamine (6-OHDA) identified several mutations in *dat-1*, a dopamine transporter gene (Nass et al. 2005). Another forward genetic screen was recently performed and revealed two novel alleles of *dat-1* and other mutants (Hardaway et al. 2012, 2014). Although the mutations have not yet been mapped, the mutated genes are possible drug targets for PD (Hardaway et al. 2012).

The combination of genetic screening with a humanized worm model has also been applied in PD studies. Although some human disease-contributed genes are not conserved in *C. elegans*, these disease-related genes can be expressed in these worms, and the transgenic worms can be used to study disease pathways associated with the expressed human genes. Familial PD was first discovered in a family with  $\alpha$ -synuclein gene defects (Polymeropoulos et al. 1997). Although the  $\alpha$ -synuclein gene does not have a strong homologue present in the *C. elegans* genome, human  $\alpha$ -synuclein overexpression in worms causes dopamine neuronal death and several other phenotypes that are characteristic of PD (Cooper et al. 2006; Lakso et al. 2003). Indeed, *C. elegans* microarray studies have shown that the expression of  $\alpha$ -synuclein causes alterations in proteosomal and mitochondrial pathways (Vartiainen et al. 2006). Reverse genetic screening using genome-wide RNAi techniques was employed to identify proteins involved in human  $\alpha$ -synuclein aggregation in worms, and 80 genes that may be involved in PD were isolated (Van Ham et al. 2008). In a recent study, glutaredoxin was identified as a protective protein of neurodegeneration in PD models including *C. elegans* (Johnson et al. 2014). The idea of humanized worm models has also been applied in Alzheimer's disease by expressing human  $\beta$ -amyloid peptide and TAU, the major proteins of extracellular senile plaques in Alzheimer's disease patients (Kraemer et al. 2006; Alexander et al. 2014; McColl et al. 2012). Thus, the overexpression strategies used in *C. elegans* allow human disease pathways, as well as disease-related genes that are not present in this organism, to be studied in this simple worm model.

### 1.6.2 Chemical genetic analysis

To study disease pathways in *C. elegans*, chemical genetic analysis is a useful approach that can identify molecular targets and mechanisms of already established drugs. For example, the antidepressant fluoxetine regulates serotonin (5-hydroxytryptamine, 5-HT) levels at the synapse cleft, however, the molecular mechanism of this drug was not known. Therefore, *C. elegans* was used to study its mechanism. In *C. elegans*, fluoxetine induces several behaviors including feeding, egg-laying and locomotion, which are regulated by 5-HT (Chase and Koelle 2007). The screening of fluoxetine-resistant mutants identified loss-of-function mutations in *mod-5*, which encodes a serotonin reuptake transporter (SERT) in *C. elegans* (Ranganathan et al. 2001). Further chemical genetic analysis revealed that fluoxetine could stimulate egg-laying through the Gq protein EGL-30, suggesting that fluoxetine acts on a G-protein coupled receptor (GPCR) signaling pathway (Dempsey et al. 2005).

Farnesyltransferase inhibitors (FTIs) block the post-translational modification of several proteins including Ras and are known as anti-cancer compounds (Karp et al. 2001). Some FTIs have shown pro-apoptotic activity in a Ras-independent manner, indicating the presence of additional targets. Both Ras-dependent and pro-apoptotic phenotypes were observed in FTI-treated worms, thus, *C. elegans* was confirmed to be useful in a chemical genetic study of FTIs (Lackner et al. 2005). Forward and reverse genetic screenings have identified several endosomal trafficking proteins including the enzyme Rab geranylgeranyl transferase (RabGGT). RabGGT was found to be overexpressed in some tumor tissues, and its knockdown induced apoptosis in

mammalian cancer cell lines (Lackner et al. 2005). Indeed, the chemical genetic analysis of FTIs identified RabGGT and endosomal trafficking as potential therapeutic targets for the modulation of apoptosis and cancers.

### **1.6.3 Compound screening using *C. elegans***

Currently, the efficiency of drug screening is of primary interest for pharmaceuticals because the cost of drug development is rapidly increasing. During drug screening, chemical compounds are tested in several screening steps. Hundreds of compounds are selected from large chemical libraries during the first high-throughput screening, which is usually performed using *in vitro* screening models, and those compounds that pass the initial screening are called “hit compounds”. Then, the hit compounds undergo the next screening processes to confirm whether they work under more physiological conditions, often in animal models. The selected compounds after the second screening are called “lead compounds”, which are the candidates for further drug development (Bleicher et al. 2003). During this process, many hit compounds fail to become lead compounds because of problems in absorption, metabolism stability, and/or toxicity, which are found during late *in vivo* testing (Giacomotto and Ségalat 2010; Gribbon and Andreas 2005). Although lead compounds may be modified to achieve a threshold of absorption or toxicity, these modifications often reduce their drug developability and activity (Hann et al. 2001; Oprea et al. 2001). The considerable loss of invalid compounds from *in vitro* screenings results in a waste of costs and efforts. These disadvantages can be solved directly in animal models. In addition, *in vivo* animal models allow the identification of compounds that may not be obtained from *in vitro* cell-based

models because such compounds only have effects on tissues and require integrated interactions of multiple cells. Thus, the use of intact animal screening models has a much higher possibility of obtaining physiologically active compounds. However, most animal models show much lower throughput than *in vitro* cell-based screening models, and screening large chemical libraries in such low-throughput models is time-consuming and expensive. To overcome the limitations of traditional animal models, small animal models including worm models have recently been spotlighted (Giacomotto and Ségalat 2010; O'Reilly et al. 2014).

The capacity of high-throughput screening applications in intact animal models is the prominent advantage of *C. elegans* for compound screenings. *C. elegans* can absorb chemical molecules from the intestine, skin, and sensory neuronal endings, although large compounds may not be efficiently absorbed (Choy et al. 2006; Smith and Campbell 1996). Worms can normally be maintained in liquid buffer containing 1-2% DMSO, which is a common solvent of chemical compounds, enabling efficient compound exposure for screening (Nass and Hamza 2007). For high-throughput screening, fluorescence-activated cell sorter (FACS)-like technology can be applied for worm sorting using a Complex Object Parametric Analyzer and Sorter (COPAS) system. This worm sorter is capable of sorting organisms ranging from 70 to 1300  $\mu\text{m}$  long, thus, this system can be applied for *C. elegans* and for other small organisms (Pulak 2006; Burns et al. 2006; Boyd et al. 2012). The automated and high-speed analysis of nematodes based on the size, OD, and intensity of fluorescent markers provides high-throughput analysis that does not differ from *in vitro* cell assays. Indeed, this technology has already been

applied in several large-scale compound screenings using *C. elegans* (Benson et al. 2014; Hunt et al. 2012; Squiban et al. 2012).

#### **1.6.4 Limitations of the *C. elegans* model and potential solutions**

*C. elegans* is a powerful model for both disease pathway studies and compound screenings. However, several limitations of this model have to be realized for proper analysis. First, some disease pathways and molecules are simply not available in worms, although the majority of human genes are conserved in *C. elegans*, and their functions show strong correlations with human counterparts (*C. elegans* Sequencing Consortium 1998). Indeed, *C. elegans* does not have some human organs and systems, such as the circulation system, lung, and bones. The simple body organization and relatively simple molecular pathways of *C. elegans* restrict worm models to limited disease targets. Moreover, although specific disease pathways are found in worms, they may not completely reflect the pathology of human disease. Thus, the validation of the molecular pathways and compounds discovered in worm models must be conducted in mammalian models. Another major potential pitfall of worm models is that some compounds do not work in the worm body even when the compounds are known human-optimized drugs. To demonstrate activity, compounds must be absorbed and delivered to the right target tissues. A compound's movement pattern is called pharmacokinetics. The pharmacokinetic property of a drug is not necessarily similar between humans and worms, thus, some chemical molecules do not work or act differently in worms. Importantly, one of the advantages of animal models is the assessment capacity of pharmacokinetics that cannot be analyzed by *in vitro* models.

Because of these drawbacks, careful preliminary studies are needed to validate *C. elegans* models, particularly during the construction of compound screening models. Results from worm studies should be always confirmed in mammalian models before they are applied for drug studies. This validation takes advantage of high-throughput screening in intact animals to obtain strong hit compounds during the initial screening step.

### **1.7 Insulin/IGF-1-like signaling in *C. elegans***

Insulin/IGF-1-like signaling (IIS), which is well conserved, has been rigorously studied in *C. elegans* (Fig. 1.1). The *C. elegans* genome contains more than 40 insulin-like peptides (ILPs) (Ritter et al. 2013) that are secreted from cells and tissues such as neurons or intestines, respectively, and that show autocrine, paracrine, and endocrine effects (Ritter et al. 2013; Kaletsky and Murphy 2010). Among these ILPs, some function as agonists of DAF-2, whereas other ILPs are known to antagonize the IIS pathway. The knockout of any single ILP or some combinations of these ILPs did not show any IIS phenotypes, indicating that the agonistic and antagonistic effects of ILPs show complicated redundancy rather than simple pairwise effects (Ritter et al. 2013). Many homologues of IIS molecules have been identified and studied because of their critical functions in the overall metabolism of *C. elegans*. AGE-1 was isolated as the first gene to increase *C. elegans* longevity by a single gene mutation (Friedman and Johnson 1988). The *age-1* gene encodes a homologue of mammalian PI3K, which is central to the insulin

signaling cascade in mammalian cells (Guarente and Kenyon 2000). DAF-18, a homologue of the human tumor suppressor PTEN, suppresses AGE-1 function to modulate IIS (Ogg and Ruvkun 1998; Liu and Chin-Sang 2014). *daf-2* encodes IIS receptor, and mutant *daf-2* animals show longer lifespans (Kenyon et al. 1993). DAF-2 is the only IR-like molecule in *C. elegans*, however, some splicing variants are expressed in a tissue-specific manner (Ohno et al. 2014). Unlike mammalian IR, the DAF-2 protein itself harbors an IRS-like domain. An independent IRS homologue IST-1, also exists, although IST-1 is not required for the IIS pathway (Wolkow et al. 2002). AKT-1 and -2 are homologues of Akt/PKB, that activate the downstream FOXO transcription factor DAF-16 which have shown to be indispensable for the IIS cascade in *C. elegans* (Paradis and Ruvkun 1998). SGK-1, which is a homologue of mammalian serum- and glucocorticoid-inducible kinases (SGKs), is activated by the mTORC2-like complex in *C. elegans* and can phosphorylate DAF-16 similar to AKT kinases (Mizunuma et al. 2014). *pdk-1* encodes a homolog of mammalian Akt/PKB kinase PDK and functions as an activator of AKT-1, AKT-2, and SGK-1 (Paradis et al. 1999). Finally, IIS in *C. elegans* is transduced to DAF-16, a homologue of the FOXO transcription factor. DAF-16 is normally suppressed by IIS by restricting its location to the cytoplasm. When IIS is active, which is assumed to be the normal condition in wild-type (wt) *C. elegans*, DAF-16 is phosphorylated by AKT-1, AKT-2, and/or SGK-1 kinases to stay in cytosolic compartments (Wolff and Dillin 2006). This phosphorylation prevents DAF-16 from nuclear localization and, therefore, inhibits transcription factor activity. When IIS is inactive, this inhibition is released, and DAF-16 translocates into the nucleus to regulate

downstream genes that cause lifespan extension and several other phenotypes. The fact that a *daf-16* mutation eliminates the knockdown phenotypes of other factors in the IIS pathway indicates that DAF-16 is the central regulator of IIS in *C. elegans* (Lin et al. 2001; Murphy et al. 2003; Lee et al. 2003; Tepper et al. 2013). Thus, the molecular pathway of *C. elegans* IIS represents high conservation with the mammalian insulin signaling cascade (Haigis and Yankner 2010; Zheng and Greenway 2012). In contrast, regulation of glucose transporters by insulin has not been reported in *C. elegans*. In addition, whether IIS regulates Rac1-stimulated actin remodeling and Akt-dependent exocytosis in *C. elegans* is not known. The Rac1 homologue CED-10 is found in *C. elegans* and is known to regulate intracellular actin remodeling (Shakir et al. 2008), however, no evidence of IIS regulation of this small GTPase molecule has been reported. No homologous gene of the Akt-targeted protein AS160 is found in the *C. elegans* genome. These observations imply that the insulin-induced GLUT4 translocation-type regulation mechanism may not be conserved in *C. elegans*.

### **1.7.1 IIS phenotypes**

IIS activity is known to be required for many biological processes in *C. elegans*. A long lifespan is a signature phenotype of IIS mutants. The relatively short lifespan of *C. elegans* is a considerable advantage for studies regarding longevity regulatory pathways including IIS (McCormick et al. 2012; Kenyon et al. 1993). DAF-16 is a known central regulator of the aging processes in *C. elegans*. DAF-16 activation by IIS or by other pathways leads to increased oxidative stress responses and autophagy activity, which are thought to be the major factors of lifespan extension (Munkácsy and Rea

2014). Oxidative stress is assumed to have central roles in aging and is closely related to mitochondrial activity. *daf-2* mutants have decreased intracellular glucose levels (Schulz et al. 2007). Under cellular glucose-deficient conditions, mitochondrial activity is up-regulated to counteract energy depletion, and the activated mitochondria produce additional reactive oxygen species (ROS), which are harmful to cells (Schulz et al. 2007). However, induced ROS activate several oxidative stress response genes that protect cells from ROS damage (Zarse et al. 2012; Schulz et al. 2007; Cypser et al. 2006). In *daf-2* mutants, the oxidative stress response increases, which positively affect longevity.

IIS also regulates reproductive ability and timing. The DAF-2 mutant shows a significantly decreased progeny number, which is thought to be caused by a defect in oocyte production (Gems et al. 1998). IIS is required for germline proliferation, which is critical for the complete development of gonads and the production of gametes (Michaelson et al. 2010). Interestingly, germline proliferation defects are not observed in adult-stage DAF-2 mutants, and other signaling cascades, such as Notch signaling, regulate germ cell differentiation throughout the adult stage (Austin and Kimble 1987).

IIS mutants show increased fat storage (Kimura et al. 1997; Horikawa and Sakamoto 2010). DAF-16 activates the expression of genes associated with lipid accumulation, such as fatty acid desaturase FAT-2, -6, and -7 and fatty acid elongase ELO-2 (Ashrafi et al. 2003; Horikawa and Sakamoto 2010). RNAi of these genes caused reduced fat accumulation because of the up-regulation of  $\beta$ -oxidation-related genes (Horikawa et al. 2008; Van Gilst et al. 2005). Thus, IIS mediates the activation of lipid synthesis and the inactivation of  $\beta$ -oxidation, leading to fat accumulation in IIS mutant

animals. In addition, fat accumulation phenotypes are observed in many dauer pathway mutants (Watts 2009). Because dauer larvae live an extremely long period without feeding, they require more fat storage to keep the energy supply from fatty acid  $\beta$ -oxidation and gluconeogenesis. Indeed, unlike mammals, *C. elegans* possesses a glyoxylate pathway, which converts acetyl-CoA into oxaloacetate to efficiently utilize oxidized fatty acids for gluconeogenesis (Fig. 1.2) (McElwee et al. 2006). Importantly, the glyoxylate cycle may be up-regulated in *daf-2* mutants (Depuydt et al. 2014).

### **1.7.2 Energy metabolism in *C. elegans***

Similar to other animals, glucose is an important energy source in *C. elegans* (Braeckman et al. 2009; Lu and Goetsch 1993). Environmental glucose availability affects cellular energy metabolism, which causes changes in several phenotypes in *C. elegans*. Glucose supplementation in culture medium alters reproduction, dauer formation, growth, and lifespan phenotypes, which are well known to be dependent on IIS (Mondoux et al. 2011; Schlotterer et al. 2009; Choi 2011; Lee et al. 2009). For instance, 0.1-2% of glucose supplementation decreases lifespan of wt worms and eliminates the extended lifespan of *daf-2* mutant strains (Lee et al. 2009; Schlotterer et al. 2009; Schulz et al. 2007). The glucose effect is specific to D-glucose, but not to L-glucose, indicating that the phenotype change is due to glucose metabolism because L-glucose is a non-metabolized enantiomer of glucose in *C. elegans* (Lee et al. 2009). In addition to glucose, glycerol production is also thought to have an important function in the aging process. Dietary glycerol can also decrease the lifespan of these worms. These dietary glucose and glycerol effects are dependent on the activity of AQP-1, a glycerol transporter protein

(Lee et al. 2009), implying that gluconeogenesis from glycerol may be an important source of glucose in *C. elegans*. Moreover, trehalose, a disaccharide form of glucose, may be important for glucose storage in *C. elegans* (Pellerone et al. 2003). Interestingly, supplementation with trehalose can increase the lifespan of *C. elegans*, although trehalose can be directly metabolized to produce glucose in these worms (Honda et al. 2010). Since trehalose itself has other physiological functions such as cell defense in dauer larvae (Erkut et al. 2011), the trehalose-induced lifespan extension may result from its functions beyond energy metabolism.

Transcriptomic, proteomic, and metabolomic studies have shown overwhelming metabolic shifts in IIS mutants, such as *daf-2* mutants. In energy metabolism, enzymes in glycolysis, gluconeogenesis, and the TCA cycle, including glyoxylate shunt enzymes, are thought to be up-regulated in *daf-2* mutants (Fuchs et al. 2010; Depuydt et al. 2014). In addition, the intracellular concentrations of several metabolites that are intermediate products of glucose metabolism cascades show significant changes in *daf-2* mutants (Fuchs et al. 2010; Depuydt et al. 2014). Elevated metabolite availability is thought to be one of the critical factors of the longer lifespan of IIS mutants because other long-lived strains share similar metabolite profiles (Fuchs et al. 2010; Depuydt et al. 2014). Furthermore, sugar uptake activity is significantly decreased in *daf-2* mutants (Zarse et al. 2012; Feng et al. 2013), although the intracellular glucose concentration is similar in *daf-2* mutants compared to wt (Fuchs et al. 2010). All of these observations indicate that energy metabolism in *C. elegans* is tightly regulated by IIS as in mammals.

## 1.8 Conclusion and the aims of this study

Understanding glucose homeostasis regulation is crucial for the development of medications to treat metabolic diseases including diabetes. Although the maintenance of glucose homeostasis involves various regulatory mechanisms, the regulation of glucose transporters is a critical mechanism. *C. elegans* has emerged as an ideal model system for studying the mechanisms by which insulin regulates glucose transporters because of its unique advantages and the conservation of the IIS pathway. The overwhelming evidence that energy metabolism is tightly regulated by IIS in *C. elegans* supports **our hypothesis that glucose transporter function is regulated by IIS in this organism.**

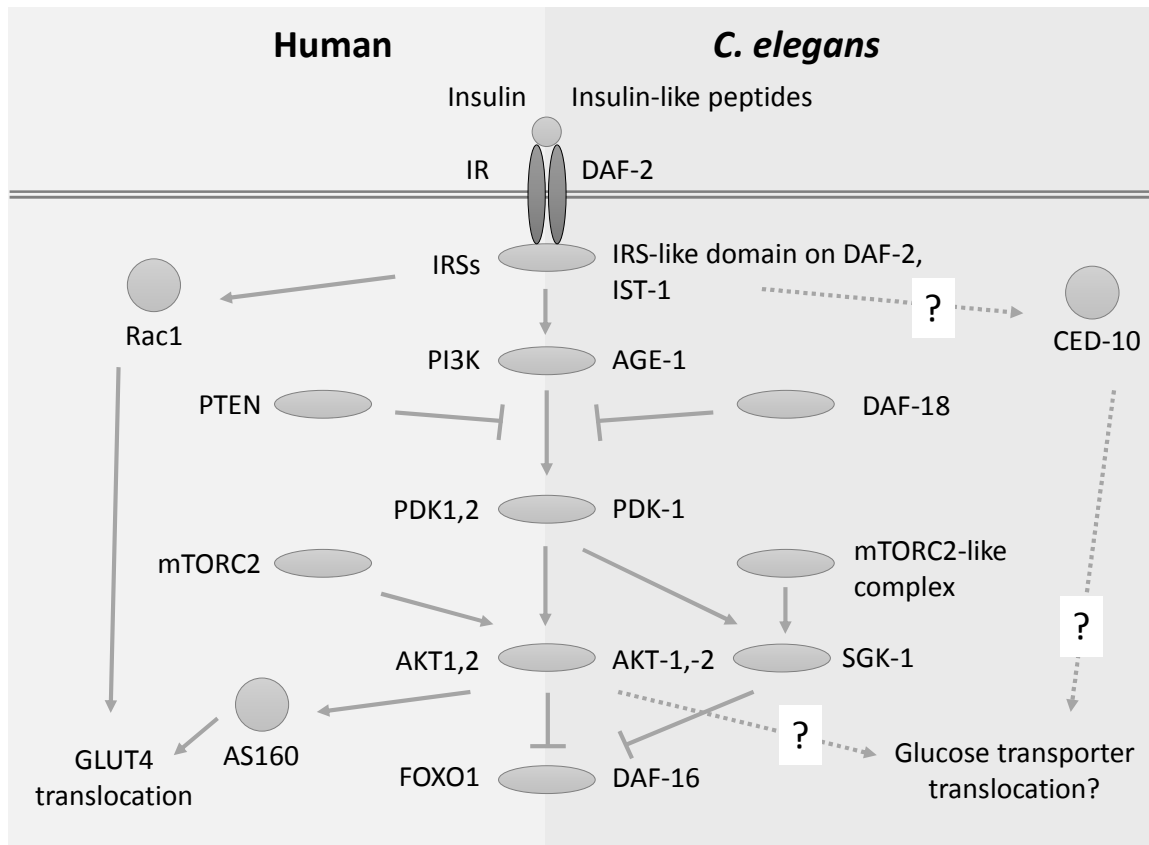
Thus, the objective of this study is to investigate whether and how IIS regulates glucose transporters in *C. elegans*. **Our specific aims are as follows: i) to identify facilitative glucose transporters in *C. elegans* (FGTs), ii) to study whether the functions of FGTs are regulated by IIS, and iii) to investigate how IIS regulates FGTs.** Our study will establish *C. elegans* as a powerful model to study the mechanism by which insulin regulates glucose transport and provide insights into the mechanism of defective glucose uptake by tissues in diabetes patients.

## 1.9 Table and Figures

**Table 1.1. Summary of the human sugar transporter families**

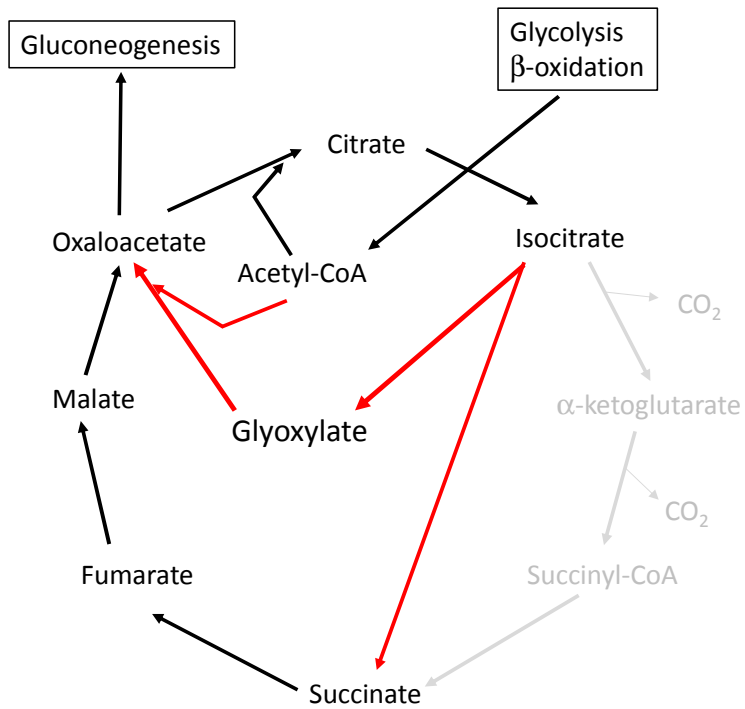
Human gene	Protein	Predominant substrates	Tissue distribution
<b>Facilitative glucose transporter family (GLUT)</b>			
SLC2A1	GLUT1	Glucose, galactose, mannose, glucosamine	Ubiquitous distribution
SLC2A2	GLUT2	Glucose, galactose, fructose, mannose, glucosamine	Liver, islets, kidney, intestine, brain
SLC2A3	GLUT3	Glucose, galactose, mannose, xylose	Brain, neurons, testis, leucocytes
SLC2A4	GLUT4	Glucose, glucosamine	Muscle, heart, fat
SLC2A5	GLUT5	Fructose	Intestine, kidney
SLC2A6	GLUT6	Glucose	Brain spleen, leucocytes
SLC2A7	GLUT7	Glucose, fructose	Intestine, colon, testis, prostate
SLC2A8	GLUT8	Glucose, fructose, galactose	Testis, blastocyst, brain, muscle, adipocytes
SLC2A9	GLUT9	Urate, glucose, fructose	Kidney, liver, intestine
SLC2A10	GLUT10	Glucose, galactose	Heart, muscle, liver, pancreas
SLC2A11	GLUT11	Glucose, fructose	Heart, muscle
SLC2A12	GLUT12	Glucose	Heart, prostate, mammary gland
SLC2A13	GLUT13/HMIT	myo-inositol	Brain, fat
SLC2A14	GLUT14		Testis
<b>Sodium-coupled glucose transporter family (SGLT)</b>			
SLC5A1	SGLT1	Glucose, galactose	Kidney, intestine
SLC5A2	SGLT2	Glucose	Kidney, intestine
SLC5A3	SGLT3	Sensing of glucose	Intestine, skeletal muscle
<b>SWEET family</b>			
SLC50A1	SWEET1	Glucose	Golgi apparatus

SLC: solute carrier gene super family, References: Modified tables from Zhao and Keating 2007, Mueckler and Thorens 2013



**Figure 1.1. Conservation of insulin signaling molecules between humans and *Caenorhabditis elegans***

Insulin signaling molecules and signaling cascades in humans and *Caenorhabditis elegans* (*C. elegans*) are shown. Activation is indicated with arrow-headed lines, and inhibition is indicated with bar-headed lines. Unknown regulatory mechanisms are indicated with a dashed line with a question mark. The double line indicates the plasma membrane of the cell.



**Figure 1.2. Overview of the glyoxylate cycle**

Substrates in tricarboxylic acid (TCA) cycle and glyoxylate cycle are shown. Glyoxylate cycle specific reactions are indicated by arrow-headed red lines. TCA cycle specific substrates and reactions are indicated with gray color.

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## **Chapter 2: FGT-1 is a mammalian GLUT2-like facilitative glucose transporter in *Caenorhabditis elegans* and its malfunction induces fat accumulation in intestinal cells**

Shun Kitaoka<sup>1</sup>, Anthony D. Morielli<sup>2</sup> and Feng-Qi Zhao<sup>1</sup>

<sup>1</sup> Laboratory of Lactation and Metabolic Physiology, Department of Animal Science, University of Vermont, Burlington, Vermont, United States of America

<sup>2</sup> Department of Pharmacology, College of Medicine, University of Vermont, Burlington, Vermont, United States of America

Key word: *Caenorhabditis elegans*, comparative genomics, glucose transporter, insulin signaling, lipid synthesis

This chapter has been published in the following form: Kitaoka, S., Morielli, A. D. & Zhao, F.-Q.. (2013). FGT-1 is a mammalian GLUT2-like facilitative glucose transporter in *Caenorhabditis elegans* and its malfunction induces fat accumulation in intestinal cells. PLoS ONE, 8(6), e68475.

## 2.1 Abstract

*Caenorhabditis elegans* (*C. elegans*) is an attractive animal model for biological and biomedical research because it permits relatively easy genetic dissection of cellular pathways, including insulin/IGF-like signaling (IIS), that are conserved in mammalian cells. To explore *C. elegans* as a model system to study regulation of the facilitative glucose transporter (GLUT), we have characterized the GLUT gene homologues in *C. elegans*: *fgt-1*, *R09B5.11*, *C35A11.4*, *F53H8.3*, *F48E3.2*, *F13B12.2*, *Y61A9LA.1*, *K08F9.1* and *Y37A1A.3*. Exogenous expression of these gene products in *Xenopus* oocyte showed transport activity to unmetabolized glucose analogue 2-deoxy-D-glucose only in FGT-1. The FGT-1 mediated transport activity was inhibited by the GLUT inhibitor phloretin and exhibited a Michaelis constant ( $K_m$ ) of 2.8 mM. Mannose, galactose and fructose were able to inhibit FGT-1 mediated 2-deoxy-D-glucose uptake ( $P < 0.01$ ), indicating that FGT-1 is also able to transport these hexose sugars. A GFP fusion protein of FGT-1 was seen only on the basolateral membrane of digestive tract epithelia in *C. elegans*, but not in other tissues. FGT-1::eGFP expression was seen from early embryonic stages. Knockdown or mutation of *fgt-1* resulted in increased fat staining in both wild-type and *daf-2* (mammalian insulin receptor homologue) mutant animals. Other known common phenotypes of IIS mutant animals, including dauer formation and brood size reduction, were not affected by *fgt-1* knockdown in wild-type or *daf-2* mutants. Our results indicated that in *C. elegans*, FGT-1 is mainly a mammalian GLUT2-like intestinal glucose transporter and is involved in lipid metabolism.

## 2.2 Introduction

Glucose is an essential energy source and substrate for macromolecule synthesis in most, if not all, living organisms. In mammalian cells, glucose is taken up by two families of glucose transporters located in the cell plasma membrane. The facilitated glucose transporters (GLUT, gene symbol: SLC2A) mediate passive glucose diffusion across the plasma membrane in most tissues while Na<sup>+</sup>/glucose cotransporters (SGLT, gene symbol: SLC5A) mediate Na<sup>+</sup>-dependent secondary active glucose transport mainly in the epithelial cells of the small intestine and kidney convoluted tubules [1,2].

The GLUT family contains 14 members which have high sequence similarities and share common structure characteristics, including 12 transmembrane domains and sugar transporter signatures [2,3]. However, individual transporters have different transport kinetics, tissue distribution and regulatory properties [2]. Among the 14 members, class I GLUTs (GLUT1-4) are well studied because of their physiological and pathophysiological roles in the cells. GLUT1 is ubiquitously expressed and mediates basic glucose uptake. GLUT2 is mainly localized in the plasma membrane of hepatic cells and the basolateral membrane of the luminal epithelial cells of the intestine and kidney convoluted tubules [4]. In intestinal and kidney cells, GLUT2 is responsible for releasing glucose absorbed or reabsorbed by SGLT1 or SGLT2 in the apical membrane into the blood stream [2,5,6]. GLUT3 is known as a neuron-specific glucose transporter and is mainly expressed in the brain [7,8]. GLUT4 mediates insulin-regulated glucose uptake in muscle cells and adipocytes. In these cells, insulin stimulates translocation of

GLUT4 from the intracellular pool to the plasma membrane and thus, increases glucose uptake and utilization [9,10]. This regulation plays a critical role in maintaining whole body glucose homeostasis. GLUTs belong to the major facilitator superfamily (MFS) consisting of members present ubiquitously in bacteria, archaea, cyanobacteria, fungi, protozoa, plants and animals.

*C. elegans* is an attractive animal model for biological and biomedical research because of its small size, simplicity, short lifespan (21 days) and quick turn over (3 days), ease of propagation and maintenance, routine genetic manipulations, and cost-effectiveness. It has been widely used as a model system for studying aging, reproduction, metabolism, and other physiological processes that are well known to be regulated by insulin/IGF-like signaling (IIS). IIS is well conserved in *C. elegans* and many mammalian insulin signaling molecule homologues have been identified (e.g., DAF-2 is the *C. elegans* counterpart of mammalian insulin receptor) and studied in *C. elegans*. However, there have been no GLUT homologues functionally identified in *C. elegans* and no studies have been published in IIS regulation of glucose uptake in *C. elegans* so far.

To explore *C. elegans* as a model system to study regulation of glucose transporter functions, especially by IIS, we have characterized potential *C. elegans* GLUT homologues, *fgt-1*, *R09B5.11*, *C35A11.4*, *F53H8.3*, *F48E3.2*, *F13B12.2*, *Y61A9LA.1*, *K08F9.1* and *Y37A1A.3*, which show high sequence homologies to human GLUTs.

## 2.3 Results

### 2.3.1 Bioinformatic analysis of GLUT homologues in *C. elegans* genome

BLASTP searches were performed using protein sequences of human GLUT1 through 12 genes against the *C. elegans* database (Wormbase: <http://www.wormbase.org/>). 47 genes were found to have higher scores than a cut off E-value of 1E-2 (Table S2.1). After removing those candidates with known functions other than sugar transport, the remaining candidates were analyzed for facilitative glucose transporter signatures, including 12 transmembrane domains [3], a N-glycosylation site either within the first or sixth extracellular loop and several highly conserved residues [2]. Nine genes, including *fgt-1*, *R09B5.11*, *C35A11.4*, *F53H8.3*, *F48E3.2*, *F13B12.2*, *Y61A9LA.1*, *K08F9.1*, and *Y37A1A.3*, passed these analyses and are considered initial *C. elegans* GLUT (ceGLUT) candidates (Figure 2.1). FGT-1 and R09B5.11 have the highest homologies to the class I family of human GLUTs (hGLUTs) compared to the other candidates. The BLASTP E-values of FGT-1 and R09B5.11 against hGLUT1 or hGLUT4 were 7E-82, 1E-66, 3E-84 and 6E-67, respectively, which were much lower than other candidates ( $> 2E-36$  and  $> 5E-31$  to hGLUT1 and hGLUT4, respectively) (Table S2.1). R09B5.11 was predicted to lack two potential transmembrane domains (Figure 2.1) but remained in the candidate list because of its high E-values in our BLASTP analysis.

The deduced amino acid sequences of all 9 ceGLUT candidates were aligned with the class I family of hGLUTs in Figure S2.1. Figure 2.2A shows the higher resolution

alignments of FGT-1 and R09B5.11 with hGLUT1-4. These alignments showed that many residues were well conserved in these sequences, including those in transmembrane domain (TM) 1 and 5 which were predicted to be absent in R09B5.11 and some residues that are known to be functionally important in hGLUTs, such as R92, E146, R153, E329, R333/4, W388, E393, R400, and W412 as reported by Schurmann *et al.* (2007) [11]. Moreover, they all contained deduced sugar transporter domains and major facilitator domains (PFAM: <http://pfam.sanger.ac.uk/>). A phylogenetic tree drawn by the alignment showed that hGLUT2 was the closest isoform for all ceGLUT candidates and FGT-1 and R09B5.11 were the closest homologues to human class I GLUTs (Figures 2.2B and S2.2).

Thus, we pursued functional characterizations of all of these 9 ceGLUT candidates. We first cloned the full length cDNAs of these *C. elegans* genes by PCR using specific primers which are located in 5'- or 3'-untranslated regions of corresponding genes based on the sequence information in Wormbase. In the database, *fgt-1* was predicted to have two mRNA variants, *fgt-1a* and *fgt-1b*, which are different in the first exon, resulting in different *N*-termini of the protein. We cloned *fgt-1a* cDNA for this study because the *fgt-1a* showed a slightly lower E-value than *fgt-1b* in our BLASTP search against class I family of hGLUTs (Table S2.1).

### **2.3.2 Glucose transport activity of ceGLUT candidates**

The functional glucose transport activities of ceGLUT candidates were assessed in *Xenopus laevis* oocytes. Synthesized cRNA of individual ceGLUT candidates was injected into oocytes and the oocytes were then incubated in 10 mM 2-deoxy-D-glucose

(2-DG) containing 1  $\mu\text{Ci}$  2-deoxy-D-[1- $^3\text{H}$ ]-glucose ( $^3\text{H}$ -2-DG) for 15 min. The hGLUT1 cRNA-injected oocytes were used as a positive control and the water-injected oocytes were used to determine the endogenous levels of glucose uptake in the oocytes. FGT-1 showed significant 2-DG transport activity although the activity appeared much lower than hGLUT1, while all other gene products examined showed no activity for 2-DG transport (Figure 2.3A). We confirmed the plasma membrane localizations of FGT-1 and R09B5.11 in the oocytes by injection of GFP fusion constructs of FGT-1 and R09B5.11 into oocytes. Both FGT-1::eGFP and R09B5.11::eGFP were localized in the plasma membrane of oocytes (Figure 2.3B, C). The eGFP fusion protein of FGT-1 showed the same 2-DG uptake activity as non-fusion FGT-1 while R09B5.11::eGFP fusion protein failed to show activity, similar to our results for untagged R09B5.11 (Figure 2.3D). These results indicated that although the R09B5.11 is localized to the plasma membrane, it has no transport activity to glucose, unlike FGT-1.

Since facilitative glucose transport is known to be inhibited by phloretin, we examined if the glucose transport activity of FGT-1 is inhibited by phloretin. The *fgt-1* cRNA-injected oocytes were incubated in 2-DG solution including increasing concentrations of phloretin from 0  $\mu\text{M}$  to 200  $\mu\text{M}$ . Phloretin significantly inhibited FGT-1 mediated 2-DG uptake with concentration of 50  $\mu\text{M}$  or higher ( $P < 0.01$ ,  $n = 10$ ) (Figure 2.3E). Non-linear one phase decay analysis estimated an inhibitory plateau at 277 pmols/oocyte/15min.

We further performed the transport kinetic analysis of FGT-1 to 2-DG with increasing concentrations of 2-DG (Figure 2.3F). The  $K_m$  value of FGT-1 for 2-DG was determined to be 2.8 mM.

### **2.3.3 Hexose substrate specificity of FGT-1**

The substrate specificity of FGT-1 to different hexose sugars was analyzed by the competitive inhibition of mannose, galactose and fructose, on 2-DG uptake of FGT-1 in oocytes (Figure 2.4). The inhibition effects of hexose sugars were compared with that of L-glucose, which is not transported by GLUTs [12]. Indeed, L-glucose showed no inhibition to the 2-DG uptake by FGT-1 as compared to the uptake without addition of any hexose sugar (Figure 2.4). The 2-DG uptake by FGT-1 was significantly inhibited by D-glucose (79 % inhibition), 3-*O*-methylglucose (77 %), D-mannose (79 %), D-galactose (33 %) or D-fructose (42 %) ( $P < 0.01$ ,  $n = 40$ , Figure 2.4). These results indicated that FGT-1 is able to transport D-mannose as well as D-glucose and is also able to transport D-fructose and D-galactose with lower activities.

### **2.3.4 Cellular and subcellular localizations of FGT-1 and R09B5.11 in *C. elegans***

To study the specific physiological functions of FGT-1, we built the *fgt-1::egfp* fusion construct under the control of 2 kb upstream promoter sequences of *fgt-1*. The plasmid was injected into *C. elegans*. The expression of FGT-1::eGFP fusion protein was observed in pharyngeal muscle and intestinal cells (Figure 2.5A). The expression started

from early embryonic stage (Figure 2.5B). In adult animals, FGT-1::eGFP was mainly localized in the plasma membrane of intestinal cells (Figure 2.5C). To determine the apico-basal polarity of FGT-1::eGFP subcellular localization, we immunostained the *fgt-1::egfp* animal embryos with an antibody to the apical membrane marker IFB-2 which is an intermediate filament protein and a component of the terminal web on the apical domain of intestinal cells [13,14]. FGT-1::eGFP was not co-localized with IFB-2, suggesting that FGT-1::eGFP was only expressed on the basolateral membrane but not on the apical membrane (Figure 2.5D-I). In addition, FGT-1::eGFP localization was not affected by fasting (data not shown).

Expression of R09B5.11::eGFP was also observed in *C. elegans*. R09B5.11::eGFP was expressed from the early embryonic stage through the L2 stage and appeared to be expressed in seam cells (Figure 2.6).

### **2.3.5 In vivo functional analysis of FGT-1 in *C. elegans***

Because FGT-1 is mainly located in intestinal cells, which are the major site of fat accumulation in *C. elegans*, we studied the role of *fgt-1* in fat accumulation. We injected into *C. elegans* 112 bp double stranded *fgt-1* RNA (dsRNA) synthesized from exon 6 of the *fgt-1* gene, which has limited sequence homologies to other GLUT homologues. The *fgt-1* mRNA levels measured by real-time RT-PCR did not show a significant reduction of *fgt-1* mRNA in the *C. elegans* injected with *fgt-1* dsRNA (data not shown). Due to the lack of the antibodies to FGT-1, we confirmed the RNAi effect on *fgt-1* protein levels by injecting *fgt-1* dsRNA into *fgt-1::egfp* transgenic animals. F1 progeny were analyzed by

Western blotting analysis using an anti-GFP antibody. In dsRNA-injected transgenic animals, FGT-1::eGFP protein level was decreased to 16% of that in the animals injected with buffer, confirming the knockdown effect of our RNAi (Figure 2.7A, B).

The *fgt-1* dsRNA was then injected into wild-type and *daf-2* mutant animals and fat was then stained in these animals with sudan black B. As shown in Figure 2.7C and 2.7D, fat staining intensity in the wild-type and *daf-2* mutant *C. elegans* injected with *fgt-1* RNAi was 139% and 109%, respectively, compared to the control animals ( $P < 0.001$ ,  $n > 47$ ). We further confirmed this result in a *fgt-1(tm3165)* mutant animal which showed 9% higher fat storage compared to wild-type animals ( $P < 0.01$ ,  $n = 41$ ) (Figure 2.7E).

In addition, we analyzed the effects of *fgt-1* RNAi on dauer formation and brood size. For both phenotypes, *fgt-1* RNAi had no significant effect in wild-type and *daf-2(e1370)* animals (Figure 2.7F, G). Increasing cultivation temperature to 25 °C from 20 °C, which led to strong dauer formation in *daf-2(e1370)*, also had no effects on dauer formation by *fgt-1* RNAi in both wild-type or *daf-2(e1370)* animals (Figure 2.7F).

## 2.4 Discussion

BLASTP searches of *C. elegans* genome with hGLUT cDNAs and structural analyses resulted in nine *C. elegans* GLUT candidate genes: *fgt-1*, *R09B5.11*, *C35A11.4*, *F53H8.3*, *F48E3.2*, *F13B12.2*, *Y61A9LA.1*, *K08F9.1*, and *Y37A1A.3*. Among them, *fgt-1* and *R09B5.11* displayed the highest sequence homologies to the class I family of hGLUTs. Thus, in this study, we aimed to characterize the glucose transport properties of

these proteins and study their physiological functions in *C. elegans*. The glucose transport properties of all 9 ceGLUT candidates were first assayed in *Xenopus* oocyte model, which is broadly used for measurement of glucose transporter activity because of its very low endogenous glucose transport level [15]. While FGT-1 showed significant glucose transport activity, no such activity was observed for all of other candidates. Since R09B5.11 was localized on the plasma membrane in oocytes, our results indicated that unlike FGT-1, R09B5.11 is not a facilitative glucose transporter in *C. elegans*. The inability of R09B5.11 to transport glucose may be explained by its structural analysis. Although it has high sequence homologies to GLUTs and FGT-1 (55 % to hGLUT1 and 62% to FGT-1), it is predicted to have only 10 TMs rather than 12 TMs predicted in all hGLUTs [2,3] and FGT-1. R09B5.11 may lack one or two TMs that are required for glucose transport function or all 12 TMs may be essential [3]. However, R09B5.11 showed expression in the early developmental stage and may play a role in development. Although it had no transport activity for glucose, it may have transport activity to other substrates. Similar to R09B5.11, all other 7 candidates showed no transport activity to glucose, however, we cannot completely exclude the possibility that these proteins transport glucose because we have not examined their subcellular localizations. If a protein is localized intracellularly in our experimental condition, its transport activity would not be revealed in our analysis.

Since FGT-1 showed glucose transport activity, we further analyzed its transport properties and kinetics. The transport activity of FGT-1 can be inhibited by a facilitative glucose transport inhibitor phloretin [16] in a dose dependent manner. Kinetic assays

revealed that FGT-1 has a  $K_m$  of 2.8 mM for 2-DG, an un-metabolized glucose analog. This  $K_m$  value is much lower than the  $K_m$  reported for hGLUT1 (11.7 mM) or hGLUT4 (4.6 mM) [17,18], which is not surprising because *C. elegans* lives in a low glucose soil environment and may require cells to have transporters with a high affinity for glucose for its acquisition. In addition, we showed that the 2-DG transport activity of FGT-1 can be inhibited by not only D-glucose and glucose analogs 3-OMG, but also by several other hexose sugars, including D-mannose, D-galactose and D-fructose. It appears that FGT-1 can transport D-mannose equally well as D-glucose, but has relative low activities for D-fructose and D-galactose.

To study the *in vivo* physiological functions of FGT-1 in *C. elegans*, we first investigated its cellular and subcellular localizations in whole animals using FGT-1::eGFP fusion protein controlled by the *fgt-1* promoter. Since the eGFP fusion protein of FGT-1 showed the same 2-DG uptake activity as non-fusion FGT-1 in our oocyte assay, FGT-1::eGFP should reflect FGT-1 native localization in *C. elegans*, although experiments to determine if FGT-1::eGFP can functionally rescue *fgt-1* mutant phenotypes are needed to fully support this conclusion. FGT-1 is seen to be mainly distributed to the basolateral membrane of intestinal cells. In mammals, intestinal glucose absorption is mainly mediated by the Na<sup>+</sup>/glucose cotransporter SGLT1 located on the apical membrane [19]. It is not known whether *C. elegans* uses the same mechanism and a SGLT1 homologue has not been reported in *C. elegans*. A BLASTP search using human SGLT sequences showed no SGLT homologous genes in *C. elegans*. Nevertheless, it is likely that glucose is also absorbed from the intestine lumen by an active glucose

transporter in *C. elegans*. FGT-1 may play a role similar to mammalian GLUT2 which is responsible for releasing absorbed glucose from the intestinal cells to interstitial fluid [2,5,6]. Supporting this idea, our phylogenetic tree analysis indicates that hGLUT2 is the closest human class I GLUT isoform to FGT-1.

We next investigated the effects of knockdown of *fgt-1* on multiple physiological phenotypes in *C. elegans*. We found that fat staining was increased in *fgt-1* knockdown and mutant worms. This is consistent with our observation that FGT-1 is an intestinal transporter because intestine is the major site of lipid storage in *C. elegans* [20,21]. This result also suggests a role for FGT-1 in lipid metabolism in *C. elegans*. However, it is surprising that knockdown or mutation of *fgt-1* resulted in an increased fat staining because it indicates that knockdown or mutation of *fgt-1* results in either an increased lipogenesis or a decreased lipolysis or a combination of both. Since glucose is a substrate and an energy source for lipid synthesis, increased fat staining in *fgt-1* knockdown and mutant animals implies that glucose is not limited in these cells. This is consistent with our hypothesis that FGT-1 may be involved in release of absorbed glucose from intestinal cells, not in glucose absorption. Malfunctions of FGT-1 may reduce glucose release and increase intracellular glucose levels in intestinal cells, leading to increased lipid synthesis.

We did not observe any effects of knockdown of *fgt-1* on dauer formation and brood size, indicating that FGT-1 does not play an essential role in overall energy metabolism and reproductive function in *C. elegans*. *C. elegans* undergoes dauer larva formation when nutrients become limited [22]. Thus, knockdown of *fgt-1* appears not to have a detrimental effect on energy metabolism in *C. elegans*, which is also consistent with our

observation that FGT-1 is local to intestine cells and may not be responsible for glucose uptake in other tissues and cells in *C. elegans*. Other glucose transporter isoforms may have to be expressed in other tissues and cells to mediate glucose uptake. Another explanation is that *C. elegans* cells may be able to efficiently use other energy sources to replace glucose, such as glycerol [23].

It is well studied that insulin stimulates GLUT4 translocation in mammalian adipose cells and muscle cells [9,10]. During fasting, GLUT4 is mainly located intracellularly while after feeding, the rising insulin levels in blood stimulate GLUT4 translocation to the plasma membrane which increases glucose uptake by the cells. We investigated the possibility that IIS may regulate FGT-1 subcellular localization in *C. elegans* by examining the subcellular location of FGT-1::eGFP in fasting condition. The localization of the fusion protein showed no changes compared to non-fasting condition. In addition, since dauer formation and reproduction as well as fat staining are well known to be regulated by IIS in *C. elegans* and none of them were affected by the knockdown of FGT-1 in both wt and *daf-2* mutant in this study, our data suggest that FGT-1 may not be involved in IIS regulation of these processes. However, our data in *daf-2(e1370)* was confounded by the non-null nature of the allele. Further studies with different IIS signaling mutant, such as *daf-16* (a mammalian FOXO homologue), and with different culture conditions (such as temperature and calorie restriction) are required to make this conclusion.

In summary, in this study we have identified FGT-1 as a mammalian GLUT counterpart in *C. elegans*. FGT-1 is a digestive tract specific isoform located in the

basolateral membrane of intestinal epithelial cells. Thus, FGT-1 may function like mammalian GLUT2 and serve to release glucose from intestinal cells to the interstitial fluid. Knockdown or mutation of *fgt-1* increases fat storage in intestinal cells, indicating a role of FGT-1 in lipid metabolism in *C. elegans*.

## 2.5 Materials and Methods

### 2.5.1 *C. elegans* strains and culture

*C. elegans* strains used in this study were obtained from the Caenorhabditis Genetics Center (<http://www.cbs.umn.edu/cgc>). The strains used were wild-type N2 and *daf-2(e1370)*. *C. elegans* strains were cultivated at 20 °C under standard conditions [24].

### 2.5.2 Plasmid constructions

PCR primers used for all plasmid constructions are listed in Table 2.1. PCR reactions in plasmid constructions were all carried out using the high fidelity Platinum *Pfx* DNA polymerase (Invitrogen) unless otherwise specified. The full length cDNA of individual genes was amplified from the cDNA library of wild-type worms with the following primer sets: *fgt1*-5utr and *fgt1*-3utr for *fgt-1*, *R09*-5utr and *R09*-3utr for *R09B5.11*, *C35*-5utr and *C35*-3utr for *C35A11.4*, *F53*-5utr and *F53*-3utr for *F53H8.3*, *F48*-5utr and *f48*-3utr for *F48E3.2*, *F13*-5utr and *F13*-3utr for *F13B12.2*, *Y61*-5utr and *Y61*-3utr for *Y61A9LA.1*, *K08*-5utr and *K08*-3utr for *K08F9.1* and *Y37*-5utr and *Y37*-3utr for

*Y37A1A.3*. The cDNA amplicons were inserted into the pCR-blunt II-TOPO vector (Invitrogen) to form pCR-*fgt1*, pCR-R09, pCR-C35, pCR-F53, pCR-F48, pCR-F13, pCR-Y61, pCR-K08, and pCR-Y37 plasmids, respectively.

*C. elegans* expression plasmids of *fgt-1* and *R09B5.11* were constructed in *C. elegans* expression vectors pPD95.77 and pPD95.79 (Addgene plasmids 1495 and 1496) which harbor eGFP gene for expressing FGT-1::eGFP or R09B5.11::eGFP fusion proteins. A 2 kb promoter sequence upstream of the transcription initiation site of each gene was also cloned to the vectors to direct fusion gene expression. The *fgt-1* promoter was amplified from *C. elegans* genomic DNA with the primer set of *fgt1up-5sph* and *fgt1up-3*, and the *fgt-1a* full length cDNA was amplified from the pCR-*fgt1* plasmid with the primer set of *fgt1cdna-5* and *fgt1cdna-3xba*. Both amplicons were digested with *Sph* I and *Xba* I, respectively, and ligated simultaneously into *Sph* I- and *Xba* I-digested pPD95.77 to form the *C. elegans* expression plasmid of *fgt-1* (pPD-*fgt1g*). The *R09B5.11* promoter sequence was amplified from the *C. elegans* genomic DNA with the primer set of *R09up-5sph* and *R09up-3*, and the full length cDNA sequence was amplified from the pCR-R09 plasmid with the primer set of *R09-5utr* and *R09cdna-3sma*. These amplicons were used as the templates for overlap extension PCR amplification of the DNA fragment containing both the promoter and cDNA by the primer set of *R09up-5sph* and *R09cdna-3sma*. This amplicon and the pPD95.79 vector were digested with *Sph* I and *Sma* I, and then ligated to form the *C. elegans* expression plasmid of *R09B5.11* (pPD-R09g).

To construct the *Xenopus* oocyte expression plasmid of individual genes, the full length cDNA of each gene was respectively inserted into the pSP64T vector, a plasmid

which contains 5'- and 3'-flanking sequences of *Xenopus*  $\beta$ -globin gene [25]. The *fgt-1* cDNA was excised from pCR-*fgt1* plasmid with *Kpn* I and *Xho* I. The *R09B5.11*, *F48E3.2*, and *Y37A1A.3* cDNAs were excised from the pCR-R09, pCR-F48, and pCR-Y37 plasmid by *EcoR* I, respectively. The *C35A11.4*, *F13B12.2* and *Y61A9LA.1* cDNAs were excised from pCR-C35, pCR-F13 and pCR-Y61 plasmids with *Sac* I and *Pst* I, respectively. The *F53H8.3* and *K08F9.1* cDNAs were excised from pCR-F53 and pCR-K08 plasmids with *Kpn* I and *Pst* I, respectively. The pSP64T vector was digested with *Bgl* II. These digested vector and individual cDNAs were blunted by T4 DNA polymerase (New England Biolabs) and then individually blunt-ligated to form the expression plasmids of *fgt-1* (pSP-*fgt1*), *R09B5.11* (pSP-R09), *C35A11.4* (pSP-C35), *F53H8.3* (pSP-F53), *F48E3.2* (pSP-F48), *F13B12.2* (pSP-F13), *Y61A9LA.1* (pSP-Y61), *K08F9.1* (pSP-K08) and *Y37A1A.3* (pSP-Y37), respectively. The human GLUT1 plasmid in pSP64T, pSP64T-hGLUT1, was a generous gift from Gwyn Gould [25].

To observe *fgt-1* and *R09B5.11* subcellular localizations in *Xenopus* oocytes, the *fgt-1::egfp* and *R09B5.11::egfp* fragments were also inserted into the pSP64T vector. These fragments were cloned from *fgt-1::egfp* or *R09B5.11::egfp* transgene-expressed worms by PCR with the primer set of *fgt1g-5bcl* and *ppd-3bcl* or *R09g-5bgl* and *ppd-3bgl*, respectively. The amplified *fgt-1::egfp* fragment was digested with *Bcl* I and *R09B5.11::egfp* fragment was digested with *Bgl* II. These fragments were then ligated into the *Bgl* II-digested pSP64T vector to form pSP-*fgt1g* and pSP-R09g.

### **2.5.3 Generation of transgenic worms**

Transgenic worms were generated as described previously [26]. The *rol-6d* pRF4 (generous gift of M. Koelle, Yale University) was used as a coinjection marker [27,28]. pPD-fgt1g plasmid was microinjected into the wild-type animals with pRF4 at 100 µg/ml. Animals were then fixed with 10 mM sodium azide. Transgenic animals bearing the *gfp* reporter were selected by the roller phenotype and observed under a confocal laser microscopy (Zeiss LSM 510 META Laser Scanning Microscope).

### **2.5.4 *Xenopus* oocyte harvest and injection of cRNA into oocytes**

cRNAs of *fgt-1*, *R09B5.11* and hGLUT1 were synthesized by *in vitro* transcription from the pSP-fgt1, pSP-R09 and pSP64T-hGLUT1, respectively, using the mMessage mMachine kit (Ambion). Oocytes were harvested from *Xenopus laevis*. The synthesized cRNA (2 µg/µL) or sterile water was injected into isolated mature oocytes (stage V and VI) following previously established procedures [17].

The use of *Xenopus laevis* in this study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont (#13-025).

### **2.5.5 Glucose uptake, kinetic and inhibition assays**

2-DG uptake and its kinetic and inhibition analyses of FGT-1 and R09B5.11 were carried out in *Xenopus* oocytes as described previously [17]. cRNA- or water-injected oocytes were incubated in Barth's media [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 0.82 mM MgSO<sub>4</sub>, 0.41 mM CaCl<sub>2</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5 mM HEPES, pH 7.6] with 10 µg/mL penicillin and 10 IU/mL streptomycin (Gibco) for three days and then subjected for uptake assay using 10 mM 2-DG containing 1 µCi <sup>3</sup>H-2-DG. For phloretin inhibition assay, 0, 10, 25, 50, 75, 100 or 200 µM of phloretin were added to the assays, respectively. For substrate specificity assay, 30 mM of each inhibitor sugar was added to the assays with L-glucose as a control. For the kinetic assays, increasing concentrations from 0 to 25 mM of 2-DG containing 3 µCi <sup>3</sup>H-2-DG were used. For all experiments, ten or more oocytes were used in each assay and the oocytes were incubated in uptake solutions for 15 min. Each experiment was carried out individually for at least three times.

### **2.5.6 Localization analysis of FGT-1 and R09B5.11 in oocytes**

cRNA of *fgt-1::eGFP* or *R09B5.11::eGFP* fusion construct *in vitro* transcribed from the plasmid pSP-fgt1g or pSP-R09g were injected into *Xenopus* oocytes. The plasma membrane localization of gfp fusion proteins were observed three days after the injection under a confocal laser microscopy (Zeiss LSM 510 META Laser Scanning Microscope).

### 2.5.7 RNAi in *C. elegans*

A 112 bp DNA fragment of *fgt-1* exon 6 was amplified from the pCR-*fgt1* plasmid with the primer set of *fgt1*-ds5 and *fgt1*ds-3 (Table 2.1). The amplicon was inserted into pCR-blunt II-TOPO vector to form pCR-*fgt1*ds. To make the dsRNA for RNAi, the DNA fragment was amplified from the pCR-*fgt1*ds plasmid with the M13 forward and reverse primers (Table 2.1) and the PCR product was then used as a template for transcribing the single stranded RNA (ssRNA) using Sp6 RNA polymerase (Fermentas) and T7 RNA polymerase (Promega), respectively. The double stranded RNA (dsRNA) was formed with the complementary ssRNAs and treated with Turbo DNase (Ambion).

RNAi experiments were carried out by injecting the *fgt-1* dsRNA into N2 *C. elegans* as described previously [29]. The synthesized *fgt-1* dsRNA was injected into young adult worms. Progeny from embryos laid during 12 through 24 hours after the injection were isolated and observed for various phenotypes described below.

### 2.5.8 Western blotting and immunostaining

RNAi efficiency was assessed by semi quantitative measurement of FGT-1::eGFP protein levels in *fgt-1::egfp* worms with *fgt-1* RNAi. Protein levels were analyzed using Western blotting procedures as described previously [17]. Ten worms were collected in sample buffer [62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) 2- $\beta$ -mercaptoethanol]. Samples were incubated in boiling water for 5 min, centrifuged, and the supernatant was applied for SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and incubated with an anti-GFP (B-2, 1:250, Santa Cruz

biotechnologies) or an anti-actin (C4, 1:500, MP Biomedicals) primary antibody for 2 hours at room temperature, and then with a horse-radish peroxidase-conjugated anti-mouse IgG secondary antibody (1:5000, Amersham) for 1 hour at room temperature. The membrane was finally washed and detected using the West-Pico chemiluminescent kit (Pierce).

For the whole-mount immunostaining, mixed staged worms were settled on Poly-Prep slides (Sigma), fixed and permeabilized using freeze-cracking followed by methanol treatment [30]. An anti-IFB-2 primary antibody (MH33, 1:20, DSHB) was applied overnight at 4 °C. The Alexa Fluor 555 conjugated anti-mouse IgG secondary antibody (1:250, Invitrogen) was then applied for 2 hours at room temperature. Immunostained animals were observed under a confocal laser microscopy (Zeiss LSM 510 META Laser Scanning Microscope).

### **2.5.9 Sudan black B staining**

Saturated solution of fat staining dye Sudan black B in 60% isopropanol was diluted to 50% saturated in 60% isopropanol. Wild-type or RNAi-treated *C. elegans* were collected and fixed for one hour in PBS and an equal volume of 2x MRWB buffer [160 mM KCl, 40 mM NaCl, 20 mM EDTA, 10 mM spermidine, 30 mM HEPES, pH 7.4, 50% Methanol] containing 1% paraformaldehyde. After the fixation, samples were washed with PBS and Sudan black B staining solution was added and incubated for 16 hours at room temperature. Worms were then washed by PBS containing 0.05% Tween-20 and observed with an optical microscope. Images taken by the microscopy were

analyzed with Image J software 1.44p (<http://rsbweb.nih.gov/ij/>) to quantify the levels of Sudan black B staining. The experiment was repeated at least three times.

#### **2.5.10 Brood size and dauer formation rate measurement**

For brood size assay, ten F1 progenies of dsRNA- or buffer- injected worms were individually put on NGM agar plate and transferred to new plates daily. The number of hatched progeny was counted daily. Counting was stopped when the worm produced less than ten progenies per 24 hours.

Dauer formation assay was performed as described previously [31]. Five dsRNA- or buffer-injected worms were put on individual NGM agar plates for 24 hours in 20 °C for laying eggs. After removing the parent worms, F1 progenies were incubated for 60–72 hours in 20 °C or 25 °C. Dauer worms were scored visually, and scoring was confirmed using SDS. Worms were considered dauers if they survived a several minute incubation in 1% SDS.

Each experiment was repeated individually at least three times.

#### **2.5.11 Statistical analysis**

For the uptake and kinetic analyses, any uptake in *fgt-1* cRNA-injected oocytes lower than three times the mean value of water-injected oocytes was considered to be injection failure and disregarded from the assay. 2-DG uptake in *fgt-1* cRNA-injected oocytes was corrected by subtraction of the mean 2-DG uptake of water-injected oocytes

at the corresponding concentration and incubation time. Statistical significance was determined by the student's *t*-test, Tukey-Kramer honestly significant difference (HSD) test, and Dunnett's one-way ANOVA, as indicated in individual figure legends, and the analyses were carried out using JMP 8.0 software (SAS Institute Inc.). Plots and curve fitting analysis for the Michaelis-Menten equation were carried out using GraphPad Prism 5.04 (GraphPad Software Inc.).

## **2.6 Acknowledgments**

We acknowledge Michael Koelle (Yale University) for providing us the pRF4 marker plasmid. We thank Gwyn Gould (University of Glasgow) for providing us the hGLUT1 construct and pSP64T vector. The MH33 antibody developed by Robert H. Waterston was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biology, University of Iowa. We also thank Ruth Blauwiel and Yong Shao (both at the University of Vermont) for technical assistance with *Xenopus laevis* surgery.

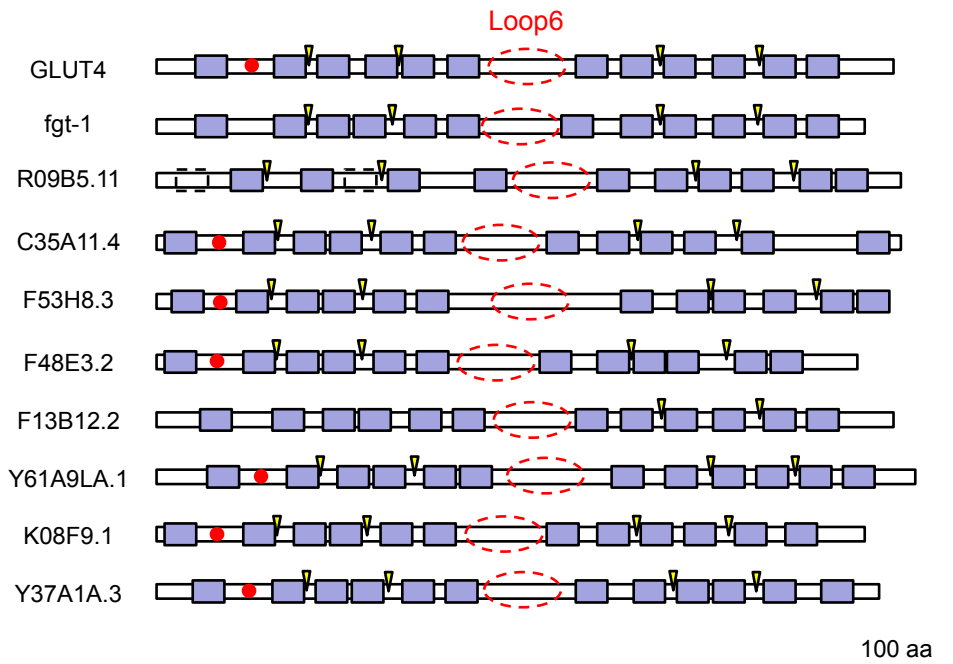
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## 2.8 Figures



**Figure 2.1. Structural schematic representation of GLUT candidate genes in *C. elegans*, comparing with human GLUT4.**

The amino acid sequence of individual *C. elegans* genes was obtained from Wormbase (<http://www.wormbase.org>). Blue boxes indicate the predicted transmembrane domains by Wormbase and the dashed boxes in *R09B5.11* indicate the missing predicted transmembrane domains. Red filled circles indicate potential N-glycosylation sites predicted by NetNGlyc (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Arrowheads indicate known functionally important residues found in human GLUT4: R92, R153, R333/4, and E393 [11]. The predicted conserved long loop 6 is indicated by red dashed circles.

**A**

```

*      20      40      60      80
-----TM1-----
FGT-1 : -----MGVNDHDVSVPLQEVQSRVTEG-----KLRKCLAFSAFVITLASFQFGYHIGCVNAPGGLITFEWIGS : 63
R09B5.11 : MNAVASQNKNDRSFNMESESSSNVEKSEKENHHQSLDPDENWPFLLFCISSIALASFDQDFQICCLINAPGPLIIDWIKKC : 82
GLUT1 : -----MEPSSKLLGRMLAVGGAVLGSLOFGYNTGVINAPQKVIIEFYNQI : 47
GLUT2 : -----MTEDKVTGLTVFTVITAVLGSFQFGYDVGVINAPQOVILSHYRHV : 45
GLUT3 : -----MGTQKVTGPAIFAITVATIGSFQFGYNTGVINAPQKVIIEFYNQI : 45
GLUT4 : -----MPSGFQQIGSEDEGPPQQRVITGLTVLAVFSAVLGSLOFGYNTGVINAPQKVIIEFYNQI : 59
          T L          l S QfGy G iNAP I

*      100     *      120     *      140     *      160
-----TM2----- F --
FGT-1 : HKDLFD-----KELSDRENADLWASVAVSVFAVGGMIGGLSSGNLADKVGRRGA : 111
R09B5.11 : HFELFG-----EVLQYQADFVSVAVSMFVGGMFGSFCGFLADKFGRKST : 130
GLUT1 : WVHRYG-----ESILPTTLTTLWLSVAIFSVGGMIGSFSVGLFVNRFGRRNS : 95
GLUT2 : LGVPLDDRRAINNYVINSTDELPTISYSMNPKPTPAWEEETVAAQQLITMLWLSVSSFAVGGMTASFFGGWGLDTLGRKA : 127
GLUT3 : LTDKGN-----APPSEVLLTSLWLSVAIFSVGGMIGSFSVGLFVNRFGRRNS : 93
GLUT4 : WLGRQP-----EGPSSIPPGLTTLTTLWLSVAIFSVGGMIGSFSVGLIGIISQWLGRKRA : 111
          Ws V F VGGM sf G GR

*      180     *      200     *      220     *      240
-----TM3----- F -----TM4----- F -----TM5-----
FGT-1 : LFYNNLLALAAALMCLASVGVAYPVLGRLLIIGLNGCFSSAIVPMFLTEISENNLRGMGLSLHOLLVITIAIVSQIFGLP : 193
R09B5.11 : LFYNNLLALAAVCLSTSILFNFPYIVVGRFLVGLNCGITSGVPMFLTEIAPANLRKCKGSPHQLNLSVAIVLSQALGLP : 212
GLUT1 : MIMMNLAFVSAVLMGFSKLGKSFELLLGRFLLGVYGLTTCFVPMVVGVEVSPALRGALGTLHQLGVVGLIAAOVGLD : 177
GLUT2 : MIVANLISLVGALLMGFSKLGPSHILLIAGRSISGLYGLTISGLVPMYIGETIAPTALRGALGTLHQLAVTGLIISCIIGLE : 209
GLUT3 : MIVNLLAVTGGCFMCLCKVAKSVEMLLGRLLVGLFGLCTCFVPMYIGETIAPTALRGALGTLHQLGVVGLIAAOVGLD : 175
GLUT4 : MIVNVLAVLGGSLMCLANAAASYEMLLGRLLIIGAYSGITSGVPMVVGVEIAPTALRGALGTLHQLAVTGLIISCIIGLE : 193
          l N La mg k m i GR g cG g VPM E P LRG G * QL i l l Q GL

*      260     *      280     *      300     *      320
-----TM6-----
FGT-1 : HLLGTGDRNPLIFAFVTPAVLQALMLCHESPKYTMAVRGQRNEAESAKKLRDTEIVSTEIEAMQBEATAAGVQE-KPK : 274
R09B5.11 : QIFGTQVGFYIFACVAIETFLQLATIPFCVESPKYISKLNDRERARRIEKLRGHTKVDELEHMVQETMVTVEPLHQP : 294
GLUT1 : SIMGNKDLWELLLSIIIFIPALLOQIVLPPFCESPRFLINRNEENRKRKSVLKKLRCTADVTHDLOEMKESRQMMREKQV : 259
GLUT2 : FLLGNVYDLWHILLGLSGVRILQLSLLFFCESPRYIYKLDDEVKAKQSLKRLRCYDDVTKDINEMREREEASSEQKVS : 291
GLUT3 : FLLGSEELWELLLGFTLIPALLOQIVLPPFCESPRFLINRNEENRKRKSVLKKLRCTADVTHDLOEMKESRQMMREKQV : 257
GLUT4 : SLLGTASLWELLLGLTVLPALLQVLVLPFCESPRYIYIIONLEGPARKSLKRLRCWADVSGVLAELKDEKRKLERERPLSL : 275
          G Wp pa LQ l fCpESP l A L L g dV m E

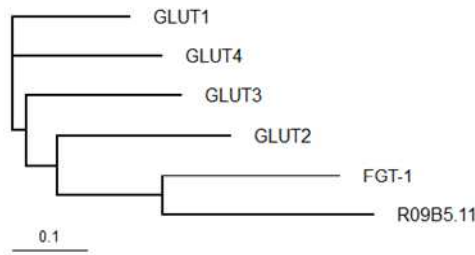
*      340     *      360     *      380     *      400     *
-----TM7----- F -----TM8----- F FF
FGT-1 : MGDMPFGALLWPMSTAIMMMLAQQLSGINAVMVFYSTVIFRGGAGLTGNEPFIYATIGMGAIVNIMTLISVVLVDHPKFGRRSLL : 356
R09B5.11 : YVSLFKGDNQWPMIVSILMMFSCQFSGISAVTFYSTLIFKRNGLSGNEPMYATVGFQCIKLIATPGCLFLIDHPKFGRRKRLH : 376
GLUT1 : LELFRSPAYRQPLIIAVVLQLSQQLSGINAVFYYSTSIFEKAGVQ--QPVYATIGSGIWNFTAFTVSVLFLVVER--AGRRTLH : 337
GLUT2 : IQLFTNSSYROPILVALMLHVAQFSCINGIFYYSTSIQTAGIS--KPVYATIGVGAIVNIMVFTAVSVFLVEK--AGRRSLF : 369
GLUT3 : LELFRVSSYROPILIIISIVLQLSQQLSGINAVFYYSTSIFKDGAGVQ--EPIYATIGAGVNTIFTVSVLFLVVER--AGRRTLH : 335
GLUT4 : LQLLGSRTHROPPLIIAVVLQLSQQLSGINAVFYYSTSIFETAGVQ--QPAIYATIGAGVNTVFTVSVLFLVVER--AGRRTLH : 353
          P QQ SGIn YST IF aG P YATiG G vn T s lv GRr L

*      420     *      440     *      460     *      480     *
-----TM9----- F -----TM10----- F F -----TM11----- F
FGT-1 : IAGLIGMVFVSTLILVGAITIQNSGGDKWASMSAIGFVLLFVISEATGPGALPWFVSEIFDSSARGNANSIIVMWNAAANLL : 438
R09B5.11 : IACLSGMCISSILIVITLITSNAG-YHWASMNVLFLSFWVTFAGGPGPIPWFTSELFDSATCRGAAVVSATSNWVANMM : 457
GLUT1 : IICLAGMAGCATLMTIALALLEQL--PWSMYSLSIVAIFGVVAFEVGPGPIPWFTIIVALEFSQGRPAALAVAGFSNWTSNFI : 417
GLUT2 : IICMGMFVCAIFMSVGLVLLNKF--SWMSYVSMIAIFLVSFSEIIGPGPIPWFTIIVALEFSQGRPAALAVAGFSNWTSNFI : 449
GLUT3 : IICLGMFVCAIFMSVGLVLLNKF--SWMSYVSMIAIFLVSFSEIIGPGPIPWFTIIVALEFSQGRPAALAVAGFSNWTSNFI : 415
GLUT4 : IICLAGMCGCATLMTVALLLLELV--PAMSVSIVAIFGVVAFEVGPGPIPWFTIIVALEFSQGRPAALAVAGFSNWTSNFI : 433
          Gl GM l L l Sy i FV F GPGpIPWF v E F R A a a sNW N

*      500     *      520     *      540     *      560     *
-----TM12-----
FGT-1 : VCLTFLPINNLMQOYSFFIFSGFIAFFIFYTWKVPETKKSIEIQAEFEKRK----- : 492
R09B5.11 : VCLTFLPINNIHQYAFLMFTFFFTFAIFITWKVVPETKKSIPSAIRKELAFMRKRICS----- : 516
GLUT1 : VCMCFQYVEQLCGPYVFIIFITVLLVLFIFITFYKVPETKKTREDEIASGRQG-GASQDKTPEELFHPGLGADSQV---- : 492
GLUT2 : VALCFQYIADFCGPYVFFLQAGVLLATLFFTFKVPETKKSIFEEIAAEFQKKSAGSARHPKAAAVEMKFLGATETV---- : 524
GLUT3 : VCLLFPSSAAHYLGAYVFIIFITGFLITFLAIFTFKVPETKKTREDEITRAFEQAHGADRSGKDVGMEMNSIEPAKETTTNV : 496
GLUT4 : ICMGFQYVAEAMGPYVVELLQAVLGLGFIIFITFLVVPETKKTREDEIQISAAEHRTPSLLEQEVKPSTELEYLGPDEND---- : 509
          vg F Y F F l F ft VPET G I f

```

**B**



**Figure 2.2. Amino acid sequence alignments of human GLUT1-4 and *C. elegans* FGT-1 and R09B5.11.**

A. Alignments of the deduced amino acid sequences of FGT-1, R09B5.11 and human GLUT1-4 were performed with the CLUSTAL W program with open gap penalty = 10 and gap extension penalty = 0.05. Residues that are highlighted by black shading background represent absolutely conserved amino acids and the gray shading indicates four or more conserved residues at that positions. Regions of presumed transmembrane domains (TM) [32] are indicated by the numbered dashed lines, and the functionally important residues for glucose uptake activity are given by the “F” letters at the top of the sequence alignments. In addition, the highly conserved amino acids are shown on the bottom of the sequence alignment. B. Phylogenetic tree of the aligned sequences in (A) by the CLUSTAL W. Scale bar indicates relative branch lengths obtained from CLUSTAL W alignment result.

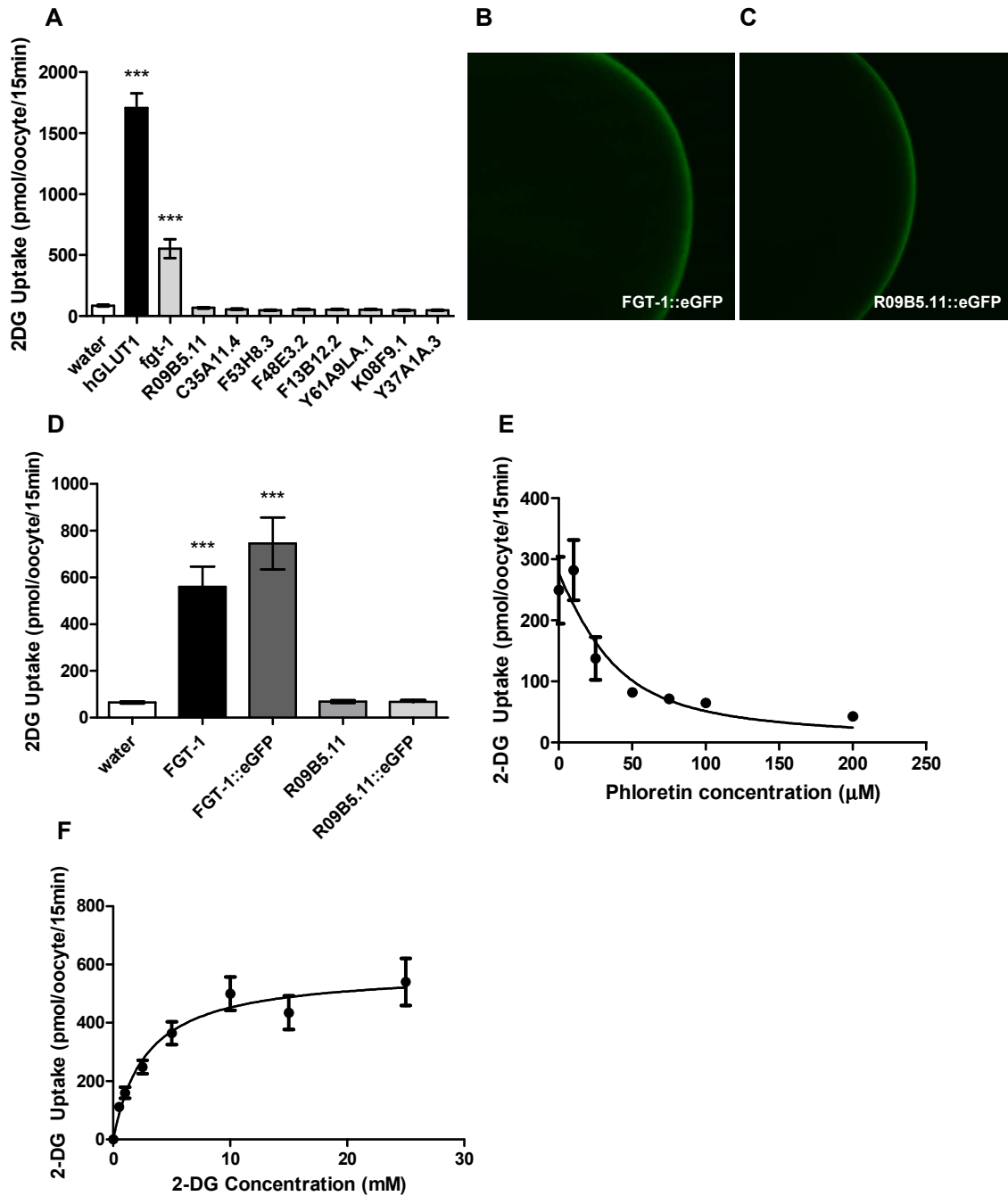
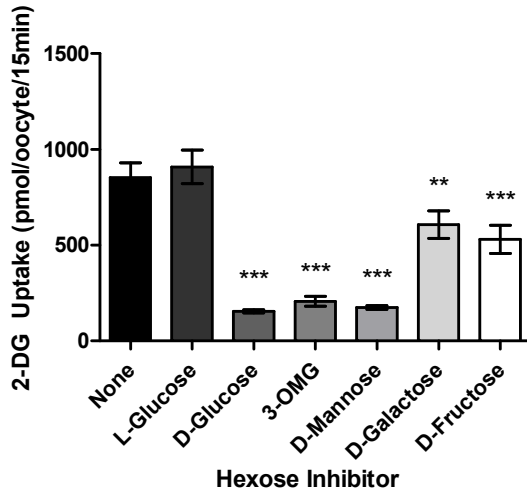


Figure 2.3. Glucose transport activity and kinetics of *C. elegans* GLUT candidates in *Xenopus* oocytes.

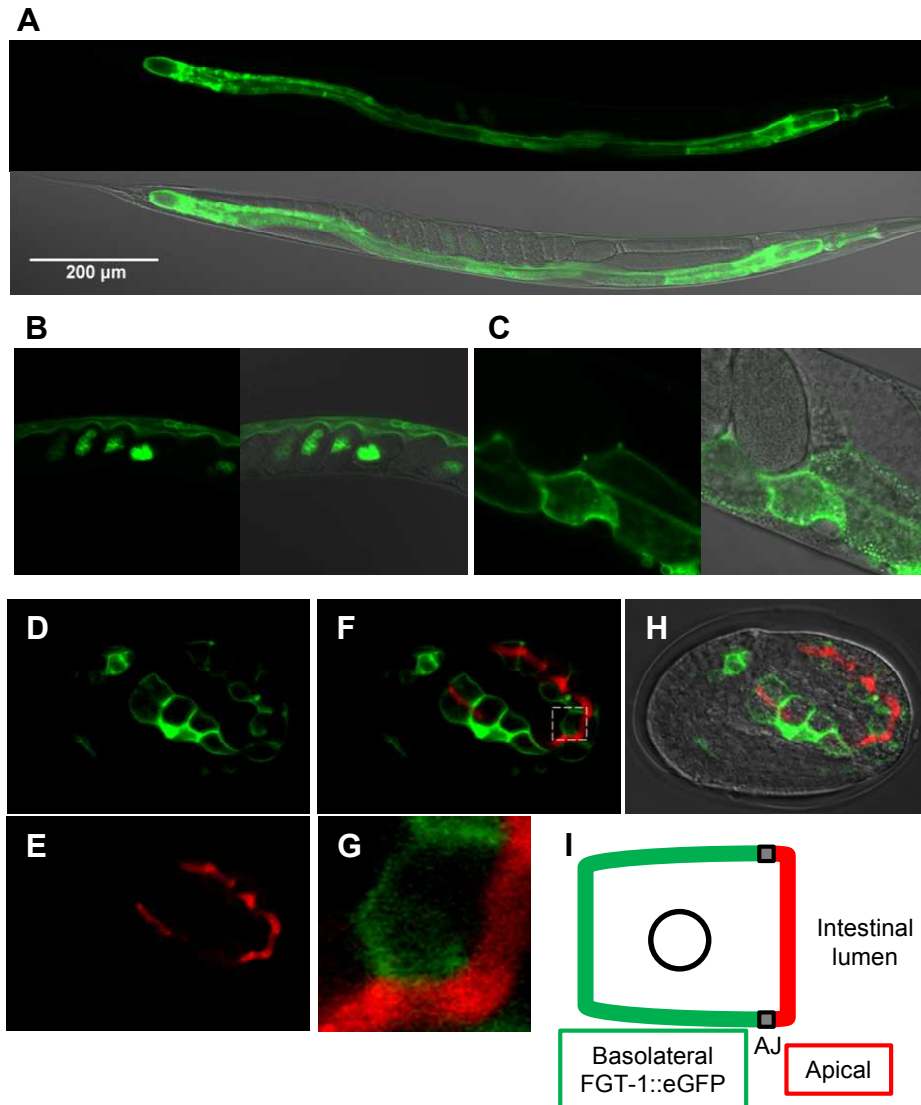
A. Analysis of 2-deoxy-D-glucose (2-DG) transport activities of FGT-1, R09B5.11, C35A11.4, F53H8.3, F48E3.2, F13B12.2, Y61A9LA.1, K08F9.1, and Y37A1A.3 in *Xenopus* oocytes. Water or the cRNA of *C. elegans* genes or hGLUT1 was injected into oocytes and the oocytes were incubated in 10 mM 2-DG containing 2-deoxy-D-[1-<sup>3</sup>H]-glucose (<sup>3</sup>H-2-DG) for 15 min before counted. Error bars represent SEM (n ≥ 31). Statistical analysis was conducted using Dunnett's one-way ANOVA before Tukey-Kramer HSD test, with water injected sample as the control group (\*\*\*P < 0.001). B. and C. Plasma membrane localization of FGT-1 and R09B5.11. eGFP fusion protein of FGT-1 (B) or R09B5.11 (C) were individually expressed in oocytes and observed under a confocal laser microscopy. D. Analysis of 2-DG transport activities of FGT-1::eGFP and R09B5.11::eGFP fusion proteins in *Xenopus* oocytes. Water or the cRNA of *fgt-1*, *fgt-1::egfp*, *R09B5.11* or *R09B5.11::egfp* was injected into oocytes and the oocytes were incubated in 10 mM 2-DG containing <sup>3</sup>H-2-DG for 15 min before counted. Error bars represent SEM (n ≥ 27). Statistical analysis was conducted using Dunnett's one-way ANOVA before Tukey-Kramer HSD test, with water injected sample as the control group (\*\*\*P < 0.001). E. Inhibition of glucose transport activity of FGT-1 by phloretin. The *fgt-1 cRNA-injected* oocytes were incubated in 10 mM 2-DG with <sup>3</sup>H-2-DG and increasing concentrations of phloretin from 0 mM to 200 uM for 15 min. The 2-DG uptake of *fgt-1 cRNA-injected* oocytes was corrected by the uptake of water-injected oocytes. Error bars represent SEM (n = 10). F. Kinetic analysis of 2-DG uptake by FGT-1. The *fgt-1 cRNA-* or water-injected oocytes were exposed to increasing concentrations of 2-DG (0-30 mM) containing <sup>3</sup>H-2-DG for 15 min. Points represent 2-DG uptake of *fgt-*

*I* cRNA-injected oocytes after correction for the uptake of water-injected oocytes. Error bars represent SEM ( $n \geq 13$ ). Michaelis-Menten nonlinear analysis was conducted in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).



**Figure 2.4. Hexose sugar substrate specificity of *fgt-1*.**

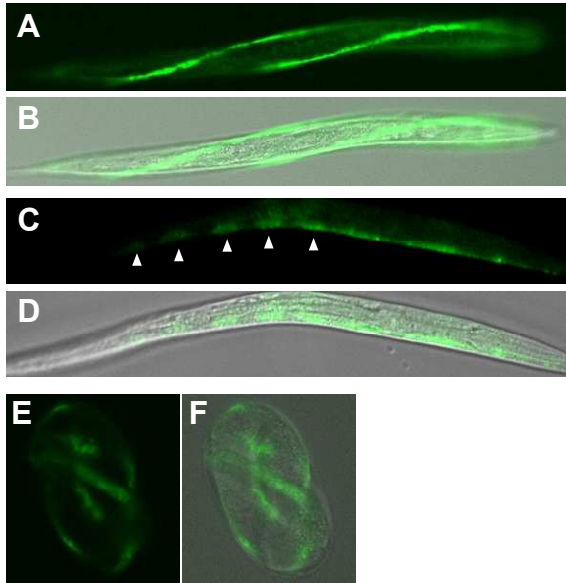
Oocytes injected with either *fgt-1* cRNA or water were exposed to 10 mM 2-DG containing  $^3\text{H}$ -2-DG and 30 mM concentrations of L-glucose, D-glucose, 3-O-methylglucose (3-OMG), D-mannose, D-galactose or D-fructose, for 15 min. 2-DG uptake from water-injected oocytes was subtracted from *fgt-1* cRNA-injected oocytes. Error bars represent SEM (n = 40). Statistical analysis was conducted using Dunnett's one-way ANOVA before Tukey-Kramer HSD test, with L-glucose as the control group (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).



**Figure 2.5. Cellular and subcellular localizations of FGT-1 in *C. elegans*.**

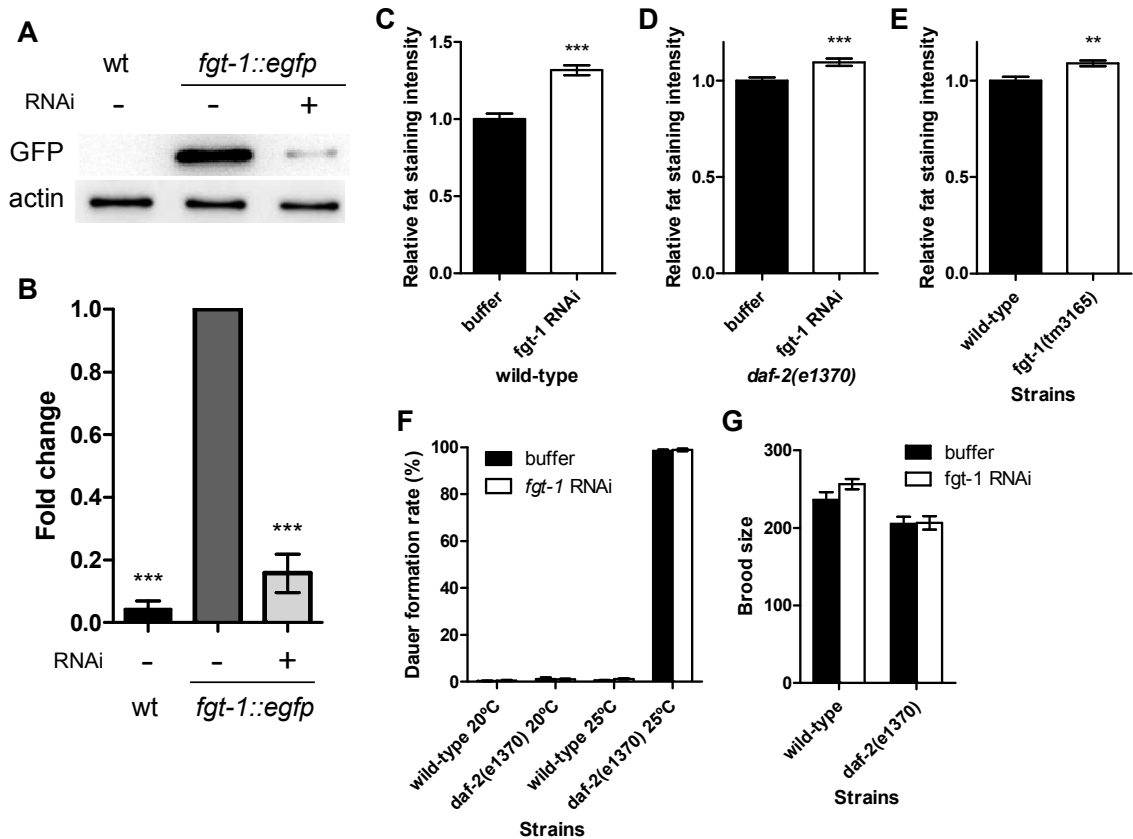
The *fgt-1::egfp* fusion construct under the control of *fgt-1* promoter was injected into *C. elegans* and expression of FGT-1::eGFP fusion protein was visualized with a confocal laser microscopy. A: whole animal, B: embryos inside of a parental worm, C: higher magnification image of mid-body. For each panel of A to C, GFP fluorescence was shown alone or as a merged picture with differential interference contrast (DIC) images.

D-H. Immunostaining of apical membrane marker IFB-2 for *fgt-1::egfp* embryos. Subcellular localizations of FGT-1::eGFP (green) and IFB-2 (red) were observed under a confocal laser microscopy. D: FGT-1::eGFP, E: IFB-1 immunostaining, F: Merged image of D and E. G: the higher magnification of the white box area in F. H: Merged image of eGFP fluorescence and DIC images. I. Schematic diagram of an intestinal cell with subcellular localizations of eGFP (green) and IFG-2 (red). The potential adherent junction (AJ) is indicated by dark box.



**Figure 2.6. Cellular localizations of R09B5.11 in *C. elegans*.**

The *R09B5.11::egfp* fusion construct under the control of *R09B5.11* promoter was injected into *C. elegans* and expression of R09B5.11::eGFP fusion protein was visualized with confocal laser microscopy. GFP image and merged picture of GFP with DIC image in L2 stage (A-D) or three-fold embryo (E and F) are shown. Left- and right-lateral strings of seam cells (A and B) and individual seam cells (C and D) are visualized. Arrowheads indicate each seam cell.



**Figure 2.7. Functional analyses of *fgt-1* on fat storage, dauer formation and brood size in *C. elegans*.**

A. The expression of the *fgt-1::egfp* fusion protein was detected by Western blotting analysis using an anti-GFP antibody in the wild-type worms (wt) injected with (+) or without (-) the *fgt-1* double stranded RNA (dsRNA, RNAi). B. The intensities of the fusion protein in A were quantified by Image Lab Software and normalized to  $\beta$ -actin levels. Error bars represent SEM (n = 4). C, D, and E. Fat accumulation in the *fgt-1* RNAi wt (C) and *daf-2(e1370)* mutant worms (D) as well as *fgt-1(tm3165)* mutant animals (E, no RNAi) was measured by sudan black B staining. Staining intensity was quantitated using Image J (NIH). Error bars represent SEM (n  $\geq$  41). Statistical analysis was

conducted using student's *t*-test (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). F and G. Comparison of the effects of *fgt-1* knockdown on dauer formation rate (F) and brook size (G). In F, wild-type and *daf-2(e1370)* worms were incubated in either 20 °C or 25 °C. Error bars represent SEM ( $n \geq 9$  and 25 for F and G, respectively). Statistical analysis was conducted using student's *t*-test.

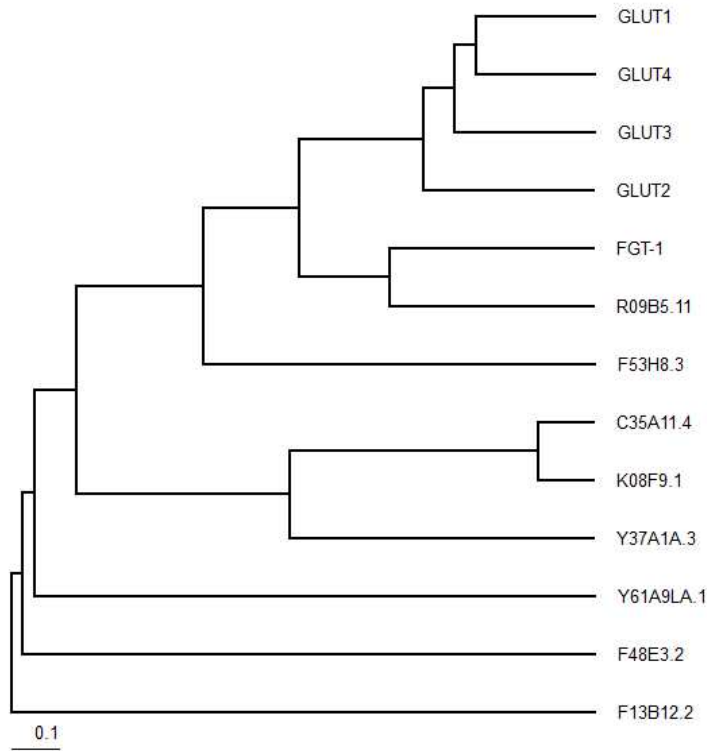
```

*      20      *      40      *      60      *      80
GLUT1 : -----MEPSSKLTGRMLAVGGAVLGSLOFNTNGVNAAPQKVIEEYNOTWVHRYG- : 53
GLUT2 : -----MTEDKVTGTVVFTVITAVLGSFQFDIGVNAAPQVLIISHYRHLVGLPDD : 52
GLUT3 : -----MOTGVNAPREKIEKEIKKLTTEKGN : 51
GLUT4 : -----MPSGQQIGSEDGEPQQRVGTITVLAVSAVLGSLQFQYINIGVNAAPQKVIEEQSYNETLGRQGP : 66
FGT-1 : -----MGVNDHDSVPLQEQVRSRTVEG-----KLTCLAFSAFVITLASFQYHIGCVNAAPGLITWELIGSHKDLDF : 69
R09B5.11 : MNAVVASQNKDRSFSNMESESSNVEKSEKENHHQSLPENWTPFFFCISSIALASFOQFQICINAPGLLIDWIKKCFELFG : 88
C35A11.4 : -----MVEAPSRMFCVAVITSIAGSFHFENLVLNPSQEAFLNEMOTLAKRFPD : 52
F53H8.3 : -----MVTSRYLITVTTVVLGGSAQFVSYGVVNAQVVTWIDWYOTYIDRYKA : 48
F48E3.2 : -----MQKLNWKLILSAVLAITAFQMGYNTNAYPTAIGSFRIELNESANEPYT : 50
F13B12.2 : -----MKQVTAAPVNAIFGQNSFMIRNVIEDQGVVFCILITILLSLILPVYVILLNVEKVFQARITFDHFNIFG : 72
Y61A9LA.1 : -----MPSHNSNGRGSIASDDSQPLLEADDAQRRRVMPVSLIHLISIALCLSSGFQYIIVASVILNPOYAOEQEINSSWIERTG : 81
K08F9.1 : -----MGETPSFRMLIVAVITSMAGSFHYQNLVLTNPSQDAFLSFMOTFAKRFDP : 52
Y37A1A.3 : -----MSSSGGNVAIIATQRAKKWPPRTSLMAIIVAFGSSPSFQFOLLITNPAQGAFIKELNASKHSNPPD : 67
          *      100      *      120      *      140      *      160      *      180
GLUT1 : -----ESLPTLTTLTALSIAISVVGMISSFSVGLFVNRFRNSMLMMNLDAFVSAVL : 109
GLUT2 : RKAINNVYINSTDELPTISYMNPKPTFWAEETVAAAQLITMLWLSVSSFAVGMATASFPGWLGDTLTKIKAMLVANILSLV : 141
GLUT3 : -----APPEVLLTSLWLSIAISVVGMISSFSVGLFVNRFRNSMLVNLVAVTGGCF : 107
GLUT4 : -----EGPSSIPPGLTTLTALSIAISVVGMISSFLIGISQMLSKRAMLVNVLAVLGGSL : 125
FGT-1 : -----KELSRNADLVAWVSVFAVGMICGLSSGWLADKVGKALFYNNLALAAAL : 125
R09B5.11 : -----EVLQYQADFTEVAWVSMVSGMFSFSCGFLADKVGKSTLLYNNLALLAAVC : 144
C35A11.4 : -----GLSDNTLQNTFVVAIFLFCALASFSIRLIADWIKKNGLYSIAVGVLAGG : 107
F53H8.3 : P-----LSLTVSNVDMFVSSIAICALLASFTRIIGEKYKRNMPFNGILNVFAALF : 103
F48E3.2 : -----LNSSEFWWAMLAIVFPAASVIVAGVADRQKWTFLGCTGSLLSLL : 104
F13B12.2 : -----LQLSKTEESLWELTSSQGVGALLCLIVSPISKGAKDVLMLRNVLVLAGSL : 128
Y61A9LA.1 : P-----ISDSTLHLKLLNVCFLATIPQFLAGMCSQPKKHTALIASFVYIPGALL : 136
K08F9.1 : -----GLSDHTLQNTFVVAIFLFCALASFSIPFAEGVKKNGLYSISVGVLAGG : 107
Y37A1A.3 : -----DNLSHLENRFIIVAFLESATFALIKTVAERFKKGVIVSLFAQVTSML : 122
          *      200      *      220      *      240      *      260
GLUT1 : -----MGSFKLKG-SPEMLLGGPIIIVYCGLTGQVPMYVGVSEPTALEALGLTLHQLGIVGILIAQVFLGDSIMNKDLP : 197
GLUT2 : MGSFKLGP-SHILLAGSISLYCGLISGLVPMYIGIAPTALRCAFGTLHQLAIVTGILISQIIGLEFILLNYDLHILLG : 229
GLUT3 : MGLCKVAK-SVEMLLDGLVLIPLCQLCTGFVPMYIGIISPTALRCAFGTLNQLAIVTGILIAQVFLGEPILSSEELP : 195
GLUT4 : MGLANAA-SYEMMLISGLVPMYVIGIAPTALRCAFGTLNQLAIVTGILIAQVFLGEPILSSEELPILLGLTVLPA : 213
FGT-1 : MGLKLSVG-APYVMLDGLIILNCGFSALAVPMPLTISPNLNLKMLGSLHQLLVTIALIVSQIFGLBHLITGDRP : 213
R09B5.11 : MSLTSLFN-PYPMVFGPLVLLNCGITISGLVPMYVGLLAPANLRKCCSFHQLNLSVAIVLSQALGKQIFQYQV : 232
C35A11.4 : SIASKFIP-LPELYIASVIVMWSVSVSLGLSALFSLASPKQNGCAIGMMTGTCTVQLTVCVGSVIAMPQIFTE : 195
F53H8.3 : ELVAKRFS-SPELLFGSFVYINMGLSSGLVPMYVGLITDVKYKSPAGTLHQLAIVAFSDWFSLLIGLPEVL : 191
F48E3.2 : ALFAIILK-MPLMFGFSLVMSLSAAISMNGLLILFQSSSHMGLISFNAEMAFVITNLGLGFGMOSILQNVG : 190
F13B12.2 : MFLSYNIT-PFIAPVGLHLLTIVYTLACAFALYLYQOIKENKIKSMSCFLHIAVCFSSLGAIFSLPFMSET : 216
Y61A9LA.1 : CAAAKWCFAPFELLVGLIISLAVNGVNTVATVWIVCAPPQIRMAAMQEFPMALGSLTQAVGVV-PSTDEL : 223
K08F9.1 : SIASKFIP-LPELYIASVIVMWSVSVSLGLSGLFSLASPKQNGCAIGMMTGTALQITVGVSVAMPQIFST : 195
Y37A1A.3 : TIVSFVIV-NHILFPAFLMVGITISMGIAAMFVITSSAYCQVSSSLINGVLLQLSITVGAVMAMPNVLNETH : 210
          *      280      *      300      *      320      *      340
GLUT1 : -----LLQCIVLEPCSSRFLLINRNE-----ENRKSVKLKLRTADVTHDLQEMKEESRQMREKVVITLELFRSP : 266
GLUT2 : LLQSLLEPCSSRFLLINKLDE-----EVRKQSLKRLRQYDDVTKDINEMRKEEASQKVSIIQLPTNS : 298
GLUT3 : LLQSAALEPCSSRFLLINKK-----EENKQILQLWGTQDVSQDIQEMKDESARMSQEKQVTVLELFRV : 264
GLUT4 : LLQLVLEPCSSRFLLIQLN-----EGPRKSLKRLTQWADVSGVLAELDEKRLERERPLSLLQLLGR : 282
FGT-1 : VLQALMLPCSSRFLLMAVRGQ-----RNEPESALKRDTEDVSTEIEAMQEAATAAGVE-KPKMGDMFKS : 281
R09B5.11 : FLQATLPCSSRFLLISKLND-----REPRRILEKLRGHTKVEEELHMVQETMTVTEPLHQPQVYSLFKG : 301
C35A11.4 : LFGAALPCSSRFLLIQRGA-----TEAPTKSIAYYNCIDEAEKHLNIEKEQRKNTKFKMMDIVRKK : 263
F53H8.3 : LALVILEPCSSRFLLGTKHD-----RDKLKDVELLIGKEQAPHPFISVIREVALD-EGDGTFRLELFRP : 258
F48E3.2 : SVACFLVPCSSRFLLKXHD-----ATPGRALQFQNIKDEEKMMVLDLKEEEMGHQKNGSLPDM : 259
F13B12.2 : LMLAASYLIDPNNLQMGKY-----TELEKTFVVDIIBDDEDELEKYEWMVPEMPQLSLCSAFSNS : 284
Y61A9LA.1 : VMSWMSVVALEIDIMEXYND-----YDEKKAQKHKVESEDDPSVESEILCSQIKKKEKKEKIESEHSGEIMFMP : 304
K08F9.1 : LVFGAALPCSSRFLLIQKGS-----LESITISFYKCEKDEADKHVKIKQKQNMSTKPKTMDLVLIQK : 263
Y37A1A.3 : TTVLVLEVWVHSSVYASQDKHHLHAMFKRIVASVFKYHISDPEAEVADNLETHQITRSQESIISVWKS : 287
          *      360      *      380      *      400      *      420      *      440
GLUT1 : -----AYQPIILIAVVLQSLQGLG-INAVFYYSSTIFEKAVQ-OPVYATIGSGVNTAFTVLSLFWVER-AGRR : 341
GLUT2 : -----SYQPIILVALMLHVAQFSG-INGFIYYSSTIFQTAIS-KPVYATIGVAVNMPVAVSVLVEK-AGRR : 373
GLUT3 : -----SYQPIIISIVLQSLQGLG-INAVFYYSSTIFKDAVQ-EPVYATIGAGVNTVFTVLSLFLVER-AGRR : 339
GLUT4 : -----THQPIILIAVVLQSLQGLG-INAVFYYSSTIFETAVG-OPVYATIGAGVNTVFTVLSLFLVER-AGRR : 357
FGT-1 : -----ALLMFMSTAIMMMLAQGLG-INVAMVYQTVFERGALIGNEPFTATIGMAVMMMLISVWLDHHPKGRS : 360
R09B5.11 : -----DYMGMVSLIMMFSQGLG-IGAVTYFADLVEKGLGNEPMTATIGKILAFQGLIDPDKKRAIAGG : 370
C35A11.4 : -----SLDKAFVGVVTFAMSPGCVAVINAFAPDLKDTLKVLEASLANDALSIVSMISSIAAVIVDR-NRRP : 340
F53H8.3 : -----DNLPIPLAVSIVMIAQQTFC-CTAVFAFSTDMELANLTPVIARFSTLAINVYLPACTSPPLIKH-V : 335
F48E3.2 : -----ANQPVRGFLGLATMQLTASVWVVFYSTDLMDAFSYELSESVSTGMFLSLSLTVGMFIVEK-YSR : 336
F13B12.2 : -----SIRGILLGMVVSATQIFG-SMVTISYSTEMEAVSFIDILVPLPALGSILSILLTIPALRWET-RRRP : 361
Y61A9LA.1 : WRAKDQTSRLISYCAWGVMMKIAVYVTCARSLRGYSTFLTYLTSHTFYQATWLSFATGLLRPLLPVFLVDR-L : 391
K08F9.1 : -----SLDKAFVGVVTFAMSPGCVAVISAFAPFLVNTLNVLQASLANDATTIVSIVSSVLAALIDK-NRRP : 340
Y37A1A.3 : -----FNRRITLGLMMVTFSMAGGCVITVINAFAPFLMNVKMDQATAINAAICFPFAGILVSTKIIDH-FRRP : 364
          *      460      *      480      *      500      *      520
GLUT1 : -----AGMACGAILMTIALALLEQLP-PMMSVLSLVAIFGVAFVFCPTIPWIVAFPSGQCPAALIAVAGFSNNTS : 428
GLUT2 : -----SGMFCVCAIFMSVGLVLLNKKF-SMMSYVSMIAFLFVSPFEICPPIPWIVAFPSGQCPAALIAVAGFSN : 460
GLUT3 : -----GMAFCCSTLMTVSLLLKDNV--NGMSFVCGIAGLVVAFVEICPPIPWIVAFPSGQCPAAMAVAGCSNNT : 426
GLUT4 : -----AGMCGAILMTVALLLRRV--PAMSYVSAIVAFGEVAFVEICPPIPWIVAFPSGQCPAAMAVAGFSNNT : 444
FGT-1 : -----TGMFVSTLLVGLALTIQNSGGDKWASYSAGFVLLFVISEATPCAPIPWIVAFPSDSSAGNANSIAVMM : 449
R09B5.11 : -----SGMCISSLIVITLTLNAG-YHWASVNVNVLFLSEVVTFAFGPPIWVFTSEFDSATSGRAAVSATSNNV : 468
C35A11.4 : -----AGILVCLNLIIFALMFTFYKFNHVLGFLIFCFICITFFPALGGLCYINNEVQGAASAAQSWASVIO : 429
F53H8.3 : -----ASCMVALMMLLSLFTLQNYENIENARVGTFSVLYMVCYGVG-SHIPWILASEFTQFQATVTVSVFV : 423
F48E3.2 : -----ASVNTAAIILFSLAISLSHY-WTWIYGCCILICLLHGYSVAMGIAWITSEVNPVNFSAASQSLVALA : 424
F13B12.2 : -----MFCILIANVLLIPLTSLSSD-SGWAAGFAAFMYGIGNLVGLVAVLPAVLPVPEAASASLGAVAVNMCIT : 449
Y61A9LA.1 : -----LVGSVSLVMIITAININGEL---KATPGLVLLVNTCCLSVSRVAREVLLLSLSSVSTLMPFALTKIGV : 475
K08F9.1 : -----AGILVCLNLIIFALMFTFYKFNHVLGFLIFCFICITFFPALGGLCYINNEVQGAASAAQSWASVIO : 429
Y37A1A.3 : -----GCLAVNNVVIVGLMYTFAETQNIQVYPLISACMNFLEPMLLSMLTGTGIVPQNCSSVNVNAVAVVRELL : 453
          *      540      *      560      *      580      *      600      *      620
GLUT1 : -----CPYV-FIIFVLLVLFIFTYFKVPEETGRTFDEIASGRFQG-GASQDKTPEELFHLGADSVQ : 492
GLUT2 : -----CPYV-FIIFAGVLLAFLTLFTFFKVPETKGSFEEIAAEFKKSGSAHRPKAAEMKFLGATETV : 524
GLUT3 : -----LQAYV-FIIFGFLITLAFITFFKVPETGRTFEDITRAFEQAGHADRSKGDVEMMNSIEPAKETT : 496
GLUT4 : -----MCPYV-FIIFAVLLLGFIFITFLRVPETGRTFDQISAAFHRTPSLLEQVVKPSTELEYLGPDE : 509
FGT-1 : -----MQQYS-FIIFSGLAFIFITYTKFVPEETKGSIEQIQAEFEKK----- : 492
R09B5.11 : -----LHQYA-FLMFPFTTALFTWKFVPEETKGSFSAERKELAFPKRKLQ----- : 516
C35A11.4 : -----LEAKWYLLIVAPVAVSLVLYFSIPETKSNKPFVEEAEIDLPKPLCKRNDVDRKQSEMVLSRQMLVDY : 515
F53H8.3 : -----VITFS-YFPEIGLAVGIFITYVLLPEETDRRMVETVEVHRTASLSAGRPMWASRPPSQEVQRLLD : 509
F48E3.2 : -----LPIITLVIFVPIGLICIMLILYVPEETKDHINVVEQLRETKAKRRESOKLETEESIGMKNL : 488
F13B12.2 : -----VQWS-YLISLIFTSIEMILWRLPEETKHYKIDPLEIRLLTDLGFSIAPNYGTLDDLEPTLF : 512
Y61A9LA.1 : -----LQAGS-LLLIPLTGVTVMCLYMLPEETSRMTVNEVLNVAAKKMDVFPF----- : 526
K08F9.1 : -----LEAWSYLLIVAPVAVSLVLYFSIPETKNSKPIEVEEAEELPKLPLCRSTRSNSIKPI : 490
Y37A1A.3 : -----TSEFMAYIFVPMVAVLVIFFLPEETGRNVEETREEYERKSL----- : 500

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**Figure S2.1. Amino acid sequence alignments of human GLUT1-4 and *C. elegans* GLUT candidates.**

Alignments of the deduced amino acid sequences of *C. elegans* genes of FGT-1, R09B5.11, C35A11.4, F53H8.3, F48E3.2, F13B12.2, Y61A9LA.1, K08F9.1, Y37A1A.3 and human GLUT1-4 were performed with the CLUSTAL W program with open gap penalty = 10 and gap extension penalty = 0.05. Residues that are highlighted by black shading background represent absolutely conserved amino acids and the gray shading indicates eight or more conserved residues at that positions. Regions of presumed transmembrane domains (TM) are indicated by the numbered dashed lines, and the functionally important residues for glucose uptake activity are given by the “F” letters at the top of the sequence alignments. In addition, the highly conserved amino acids are shown on the bottom of the sequence alignment.



**Figure S2.2. Phylogenetic analysis of human GLUT1-4 and *C. elegans* GLUT candidates.**

Human class I GLUTs and *C. elegans* GLUT candidates were aligned with CLUSTAL W and phylogenetic tree was drawn. Scale bar indicates relative branch lengths obtained from CLUSTAL W alignment result.

**Table 2.1. List of Primers.**

Primer Name	Sequences (5' - 3')
fgt1-5utr	AATGGGTGTCAACGACCATG
fgt1-3utr	AACCTATACGTTTCGCAGTG
R09-5utr	CAAAGGCTAGACATTCTATAC
R09-3utr	TGATTCTAATCCCGACTATG
fgt1up-5sph	CATGCATGCTGCGATTTGGAGCGAATCAG
fgt1up-3	TTCTGCAAAAAAAAAATTGATTTTTTAGGAG
fgt1cdna-5	ATGGGTGTCAACGACCATGATG
fgt1cdna-3xba	GCTCTAGACTTCCTCTTCTCGAATTCGGCTTG
R09up-5sph	CATGCATGCTTCCGGACTAGCTAGCATAAC
R09up-3	CTATCATTTTTATTTTGACTGGCAAC
R09cdna-3sma	GGGTGAACATATCCGTTTTCTCATG
fgt1g-5bcl	CGCTGATCAAATGGGTGTCAACGACCATG
ppd-3bcl	CGCTGATCACAGGGAGAAAGAGCATGTAG
R09g-5bgl	CGCAGATCTCAAAGGCTAGACATTCTATAC
ppd-3bgl	CGCAGATCTCAGGGAGAAAGAGCATGTAG
fgt1ds-5	GAAAGCGCGCTGAAAAAGCTC
fgt1ds-3	TATCTCCCATTTTTGGCTTCTCCTG
M13 forward	GTAAAACGACGGCCAGT
M13 reverse	GGAAACAGCTATGACCATG
C35-5utr	TAGGTTCGTCTATCACACC
C35-3utr	ACCCGCAAATAATTTGAATAG
F53-5utr	CAATACCTGAACCTATTTATTC
F53-3utr	AGAAGCATTAACCAACAACAG
F48-5utr	AGTTTCAGAACCAGTGACAAC
F48-3utr	TTATGCTGGCTGTCATTA
F13-5utr	TTTCGACTGGTGCATGTGGTG
F13-3utr	AGCGATTCAAAACAAAGTTGG
Y61-5utr	TTGCCTCAATAGACAGCCA
Y61-3utr	TGTCATACCCGCTTAAAATCG
K08-5utr	TGTACCTCCAGTAACTTTG
K08-3utr	TTAAATAGGTTTGATTGAATTC
Y37-5utr	TTACAGGCAACTGAATGTC
Y37-3utr	ACAAAAACGGGACGAGCC

**Table S2.1. Scores of BLASTP searches of human GLUTs against Wormbase.**

Genes	BLASTP scores against human GLUTs											
	class I				class II				class III			
	GLUT1	GLUT2	GLUT3	GLUT4	GLUT5	GLUT7	GLUT9	GLUT11	GLUT6	GLUT8	GLUT10	GLUT12
1 R09B5.11	3.00E-84	5.00E-75	6.00E-77	6.00E-67	2.00E-59	2.00E-61	2.00E-57	6.00E-45	2.00E-19	2.00E-23	1.00E-09	4.00E-18
2 H17B01.1a (fgt-1)	7.00E-82	1.00E-83	4.00E-83	1.00E-66	1.00E-61	4.00E-61	1.00E-61	4.00E-51	9.00E-34	3.00E-38	1.00E-12	5.00E-21
3 H17B01.1b (fgt-1)	9.00E-82	6.00E-83	6.00E-83	4.00E-66	1.00E-61	9.00E-61	2.00E-62	4.00E-51	2.00E-33	3.00E-38	2.00E-12	7.00E-21
4 C35A11.4	2.00E-36	3.00E-28	7.00E-40	5.00E-31	9.00E-23	1.00E-34	3.00E-35	7.00E-28	4.00E-18	6.00E-08	>1e-2	4.00E-16
5 F53H8.3	5.00E-35	8.00E-29	1.00E-34	3.00E-24	2.00E-22	>1e-2	1.00E-32	8.00E-22	1.00E-08	2.00E-08	7.00E-07	2.00E-05
6 K08F9.1	7.00E-34	2.00E-25	5.00E-36	3.00E-29	2.00E-24	2.00E-32	9.00E-36	1.00E-24	1.00E-13	3.00E-04	1.00E-03	7.00E-11
7 Y51A2D.5 (hmit-1.2)	4.00E-30	1.00E-27	3.00E-28	3.00E-17	1.00E-18	1.00E-19	3.00E-19	1.00E-16	7.00E-18	1.00E-20	9.00E-11	4.00E-32
8 F14E5.1	2.00E-29	1.00E-24	5.00E-29	1.00E-16	5.00E-22	6.00E-27	2.00E-29	4.00E-13	1.00E-11	2.00E-09	4.00E-06	8.00E-08
9 M01F1.5 (hmit-1.3)	2.00E-29	1.00E-33	7.00E-27	1.00E-25	5.00E-21	1.00E-20	7.00E-18	4.00E-13	2.00E-18	3.00E-23	4.00E-13	7.00E-38
10 F13B12.2	1.00E-27	2.00E-32	2.00E-27	9.00E-20	3.00E-26	1.00E-23	2.00E-29	5.00E-14	3.00E-12	1.00E-13	7.00E-03	3.00E-05
11 Y37A1A.3	9.00E-27	4.00E-20	2.00E-21	2.00E-22	4.00E-16	6.00E-22	4.00E-33	1.00E-21	1.00E-09	8.00E-09	8.00E-03	2.00E-06
12 F48E3.2	4.00E-25	4.00E-23	2.00E-23	4.00E-12	3.00E-18	5.00E-25	1.00E-18	7.00E-15	3.00E-08	6.00E-08	4.00E-06	1.00E-04
13 Y61A9LA.1	2.00E-24	7.00E-21	6.00E-24	7.00E-18	1.00E-20	4.00E-21	3.00E-27	7.00E-17	>1e-2	2.00E-04	>1e-2	>1e-2
14 Y51A2D.18	2.00E-21	>1e-2	>1e-2	3.00E-18	>1e-2	>1e-2	>1e-2	>1e-2	8.00E-05	1.00E-21	>1e-2	>1e-2
15 K08H10.6	4.00E-21	3.00E-17	3.00E-20	6.00E-19	3.00E-16	1.00E-25	1.00E-23	1.00E-20	2.00E-08	2.00E-08	1.00E-06	6.00E-08
16 ZK829.9	5.00E-20	4.00E-15	4.00E-19	2.00E-13	5.00E-11	3.00E-20	3.00E-20	3.00E-14	5.00E-11	1.00E-11	1.00E-03	2.00E-04
17 Y39B6A.41	1.00E-19	3.00E-20	3.00E-15	1.00E-13	2.00E-14	8.00E-22	5.00E-22	9.00E-05	>1e-2	>1e-2	>1e-2	>1e-2
18 F11D5.7	7.00E-12	7.00E-08	1.00E-07	5.00E-05	1.00E-06	2.00E-09	2.00E-09	3.00E-04	>1e-2	>1e-2	>1e-2	>1e-2
19 Y39E4B.5	4.00E-10	2.00E-07	4.00E-10	3.00E-04	9.00E-03	1.00E-05	1.00E-12	3.00E-08	4.00E-07	>1e-2	3.00E-04	>1e-2
20 ZK563.1	7.00E-10	3.00E-10	2.00E-11	6.00E-06	1.00E-06	5.00E-06	9.00E-14	8.00E-07	1.00E-05	5.00E-04	>1e-2	3.00E-06
21 ZK455.8a	8.00E-10	3.00E-06	1.00E-05	>1e-2	>1e-2	>1e-2	7.00E-06	>1e-2	>1e-2	7.00E-05	>1e-2	2.00E-08
22 Y66D12A.13	1.00E-09	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	4.00E-03	8.00E-03	>1e-2
23 K09C4.5	2.00E-09	3.00E-09	1.00E-09	>1e-2	9.00E-05	2.00E-06	3.00E-06	1.00E-04	>1e-2	>1e-2	>1e-2	6.00E-04
24 K09C4.1a	5.00E-09	2.00E-10	2.00E-06	3.00E-08	9.00E-05	9.00E-08	2.00E-09	6.00E-06	>1e-2	7.00E-06	>1e-2	>1e-2
25 ZK455.8b	1.00E-08	4.00E-05	5.00E-05	>1e-2	>1e-2	>1e-2	2.00E-05	>1e-2	>1e-2	4.00E-04	>1e-2	1.00E-07
26 C03B1.13	2.00E-07	5.00E-08	3.00E-04	6.00E-08	5.00E-03	3.00E-05	1.00E-07	4.00E-05	>1e-2	>1e-2	>1e-2	>1e-2
27 B0361.11	6.00E-05	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	9.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	3.00E-07
28 F11D5.5	9.00E-05	8.00E-09	3.00E-04	>1e-2	9.00E-03	5.00E-04	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
29 F17C11.12a	2.00E-04	>1e-2	7.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	1.00E-08
30 F17C11.12b	2.00E-04	>1e-2	9.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	2.00E-08
31 K09C4.4	2.00E-04	7.00E-04	5.00E-03	>1e-2	>1e-2	>1e-2	3.00E-04	>1e-2	>1e-2	6.00E-05	>1e-2	2.00E-04
32 F45E10.2b	1.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
33 Y57G11C.23	1.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	5.00E-03	>1e-2	>1e-2	>1e-2
34 C06H5.6	2.00E-03	4.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
35 F45E10.2a	2.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
36 B0252.3b	>1e-2	5.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
37 B0252.3c	>1e-2	8.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
38 C53B4.1	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	1.00E-06
39 F52F12.1a (oct-1)	>1e-2	>1e-2	6.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
40 F52F12.1b (oct-1)	>1e-2	>1e-2	6.00E-03	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2
41 K05F1.6a	>1e-2	>1e-2	>1e-2	>1e-2	4.00E-04	>1e-2	>1e-2	>1e-2	>1e-2	7.00E-08	>1e-2	>1e-2
42 K05F1.6b	>1e-2	>1e-2	>1e-2	>1e-2	4.00E-04	>1e-2	>1e-2	>1e-2	>1e-2	2.00E-07	>1e-2	>1e-2
43 T01B11.7 (oat-1)	>1e-2	5.00E-06	1.00E-03	>1e-2	>1e-2	>1e-2	3.00E-04	>1e-2	1.00E-11	>1e-2	>1e-2	4.00E-04
44 Y51A2D.4 (hmit-1.1)	>1e-2	1.00E-27	3.00E-21	>1e-2	3.00E-17	4.00E-18	3.00E-19	2.00E-16	5.00E-19		5.00E-14	3.00E-41
45 Y82E9BR.16a	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	8.00E-06	>1e-2	>1e-2	>1e-2	>1e-2
46 ZK637.1	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	4.00E-07
47 ZK892.3	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	>1e-2	1.00E-03

## **Chapter 3: Regulation of glucose transport by insulin/IGF-like signaling in *Caenorhabditis elegans***

Shun Kitaoka<sup>1</sup>, Anthony D. Morielli<sup>2</sup>, and Feng-Qi Zhao<sup>1</sup>

1 Laboratory of Lactation and Metabolic Physiology, Department of Animal and Veterinary Sciences,

2 Department of Pharmacology, College of Medicine, University of Vermont, Burlington, Vermont, United States of America

Running title: IIS regulation of FGT-1 in *C. elegans*

Key words: *Caenorhabditis elegans*, glucose transporter, glucose uptake, insulin/IGF-like signaling, lifespan, posttranscriptional regulation

This chapter has been submitted for publication to Aging Cell on April 23, 2015 in the following form: Kitaoka, S., Morielli, A. D. & Zhao, F.-Q.. Regulation of glucose transport by insulin/IGF-like signaling in *Caenorhabditis elegans*. Aging Cell.

### 3.1 Abstract

Insulin regulation of facilitated glucose transporter (GLUT) plays a key role in the maintenance of glucose homeostasis in humans. To establish *Caenorhabditis elegans* (*C. elegans*) as a research model to study the mechanism underlying insulin regulation of GLUT due to the well-conserved insulin/IGF-1-like signaling (IIS) pathway and relatively easy genetic manipulation, we functionally identified FGT-1 as the only GLUT homologue in *C. elegans* and showed that FGT-1 is ubiquitously expressed in neurons, intestine, and body-wall muscles. The function of FGT-1 in animal growth and aging was dependent on the IIS background, suggesting IIS regulation. Indeed, FGT-1 mediated glucose uptake was almost completely defective in *daf-2* and *age-1* mutants. This defect was only partially related to the approximately 60% decrease in FGT-1 protein and should also involve other post-translational mechanisms. Moreover, we found that OGA-1, an *O*-GlcNAcase, is essential for the function of FGT-1, implying the potential regulation of FGT-1 functions by glycosylation. In summary, our study establishes *C. elegans* as a powerful model to study the mechanism of insulin regulation of glucose transport and provides insights into the mechanism of defective tissue glucose uptake in patients with diabetes.

### 3.2 Introduction

Insulin is a central regulator for sustaining glucose homeostasis in human. When blood glucose levels increase after meals, insulin is secreted from the islets of Langerhans in the pancreas to activate glucose uptake and utilization in peripheral tissues, mainly muscle and adipose tissues (Leto and Saltiel 2012). This stimulation is primarily mediated by the insulin-induced subcellular translocation of the insulin-sensitive facilitated glucose transporter 4 (GLUT4) in muscle and fat cells. GLUT4 is stored in intracellular vesicles under unstimulated conditions, and following the activation of insulin signaling, GLUT4-containing vesicles translocate to the plasma membrane to facilitate glucose uptake from the blood (Saltiel and Kahn 2001; Leto and Saltiel 2012). In patients with type II diabetes, the regulation of GLUT4 by insulin is defective, and muscle and fat cells become unresponsive to insulin signals, which is termed insulin resistance (Pessin and Saltiel 2000; Saltiel and Kahn 2001). Although the mechanisms underlying insulin resistance have been under extensive investigation, the precise mechanisms remain largely unknown.

Insulin signaling is highly conserved in the animal kingdom, including *Caenorhabditis elegans* (*C. elegans*). In *C. elegans*, most of major signaling molecules in the insulin/IGF-1-like signaling (IIS) cascade are well conserved, and IIS has been known to play important functions in many physiological processes such as reproduction, growth, metabolism, and aging (Lapierre and Hansen 2012; Gems et al. 1998; Kimura et al. 1997; Tomioka et al. 2006; Zarse et al. 2012). For instance, knockdown of DAF-2, the

only homologue of the mammalian insulin/IGF-1 receptor, dramatically increases the lifespan of *C. elegans* (Gems et al. 1998; Friedman and Johnson 1988; Kenyon et al. 1993). This lifespan extension is dependent on DAF-16, the homologue of mammalian FOXO transcription factor; in the *daf-2;daf-16* double mutant animal, the longer lifespan is essentially eliminated (Kenyon et al. 1993). Because of its advantage of relatively easy genetic manipulation and the availability of a large collection of mutant strains, in addition to its many other unique advantages for study such as a short lifespan and quick turnover, *C. elegans* may be an attractive model system to study the mechanisms underlying insulin regulation of glucose transport.

To explore *C. elegans* as a model system for the study of insulin regulation of glucose transporter functions, we characterized 9 facilitated glucose transporter candidates in *C. elegans* (FGT) based on their high sequence homologies to human GLUTs in our previous study (Kitaoka et al. 2013). FGT-1 was identified as a sole functional counterpart of GLUT in *C. elegans* with the ability to transport glucose in *Xenopus* oocytes. It also showed broad transport activities for other monosaccharides, including mannose, galactose, and fructose (Kitaoka et al. 2013). The localization of FGT-1 was mainly restricted to the digestive tract when expressed under its 2 kb promoter sequence (Kitaoka et al. 2013), suggesting that there are other unidentified FGT isoforms in other tissues or that the 2 kb FGT-1 promoter sequence cannot faithfully represent its endogenous promoter function. In addition, in *C. elegans*, FGT-1 was found to be involved in lipid metabolism but not in dauer formation and reproduction (Kitaoka et al. 2013). Because all of these processes are known to be regulated by IIS (Gems et al.

1998; Kimura et al. 1997), our previous findings were not able to determine whether FGT-1 is regulated by IIS in *C. elegans*.

In the present study, we investigated the tissue localization of another FGT-1 alternative splicing isoform, FGT-1B, and re-examined the tissue localization of FGT-1A in response to a longer promoter sequence. We showed that FGT-1 is regulated by IIS by comparing the phenotypic differences in growth, lifespan, and glucose uptake between FGT-1 mutants in wild-type (wt) or IIS mutant backgrounds. We further investigated the regulatory mechanisms of IIS on FGT-1 function.

### **3.3 Results**

#### **3.3.1 Tissue localization and glucose transport activity of FGT-1A and -1B**

Because FGT-1(A) was identified as the sole functional GLUT homologue in *C. elegans* and its expression was mainly observed in the digestive tract in our previous study, we hypothesized that another FGT-1 splicing isoform, FGT-1B, is expressed in other tissues. FGT-1B utilizes a distinct exon 1 from FGT-1A and, therefore, has a slightly different promoter sequence (186 bp of extra sequence at the 3' end, Fig. 3.1A). To determine whether FGT-1A and -1B have isoform-specific tissue localizations, we expressed FGT-1A::GFP and FGT-1B::GFP under the corresponding 2 kb upstream promoter sequences of *fgt-1a* and *fgt-1b* in wt *C. elegans*. FGT-1B showed the same localization as FGT-1A: primarily in the pharynx and intestinal cells (Fig. 3.1B, C). The glucose transport activity of FGT-1B was also compared with FGT-1A in *Xenopus laevis* oocytes. The uptake

activity of FGT-1B for 2-deoxy-D-glucose (2DG, an un-metabolized glucose analogue) did not differ from that of FGT-1A (Fig. 3.1D), which indicated that the small structural difference between FGT-1A and FGT-1B at the N-terminus does not alter sugar transport activity.

To examine whether the 2 kb upstream sequence of FGT-1 faithfully represents the endogenous localization of FGT-1, we reanalyzed the tissue localization of FGT-1A::GFP driven by a longer 5 kb promoter sequence. As shown in Fig. 3.1E-H, the 5 kb promoter drove FGT-1A::GFP expression not only in the digestive tract like the 2 kb promoter but also throughout the body, including in the head neurons, vulva, and body wall muscle. This observation suggests that FGT-1 may be ubiquitously expressed in *C. elegans* and that the 2 kb promoter sequence may not be long enough to include all of the regulatory elements of the *fgt-1* promoter.

### **3.3.2 The developmental growth and lifespan of *fgt-1* mutants were dependent on the IIS background**

To study the functions of FGT-1 in *C. elegans* development and aging and the effects of IIS on FGT-1 functions, we first compared the developmental growth rates (growth times from L1 larvae to young adult) between wt and *fgt-1(tm3165)* mutant animals as well as between the *fgt-1* mutants in either wt or the *daf-2(e1370)* mutant background. These worms were grown on media supplemented with or without glucose or 2DG. Confirmation of the null function of FGT-1 in the *fgt-1(tm3165)* mutant is shown in Fig. S3.1. The null functional mutation of the *fgt-1* gene in the wt worm did not change the

growth rates of the animals on either normal or glucose-supplemented medium (Fig. 3.2A). However, the *fgt-1* mutation slightly slowed the growth of the *daf-2* mutant on glucose-supplemented medium ( $P < 0.05$ ) but not on normal medium (Fig. 3.2A). Supplementation of 2DG dramatically suppressed the development of wt worms but not of the *daf-2* mutant (Fig. 3.2A). Less than 50% of the wt animals could reach the adult stage when supplemented with 2DG, and thus their developmental growth rates could not be determined based on their average time to reach the adult stage from L1 larvae. Instead, we analyzed the percentages of wt and *fgt-1* mutant L1 larvae to reach the adult stage at the 120-hr time point in the 2DG condition (Fig. 3.2B). This experiment revealed that almost twice of the *fgt-1* mutant worms could reach the adult stage compared to wt worms at 120 hr ( $P < 0.05$ ), which suggested that the malfunction of FGT-1 reduced the toxic effect of 2DG on the development of *C. elegans*. Although supplementation with 2DG slightly slowed the development of the *daf-2* mutant strain, the *fgt-1* mutation did not affect the development rate of the *daf-2* mutant in the 2DG condition (Fig. 3.2A).

Next, we analyzed the lifespan of the *fgt-1(tm3165)* mutant and *fgt-1(tm3165);daf-2(e1370)* double mutant cultured on normal and on glucose- or 2DG-supplemented media. In the wt background, the *fgt-1* mutation had no effect on the lifespan of the worm when cultured under both normal and glucose-supplemented (5 and 30 mM) conditions (Fig. 3.2C and Fig. S3.2A). However, consistent with the above observations for animal development, the *fgt-1* mutant had a longer lifespan than the wt in the 2DG condition ( $P < 0.001$ ) (Fig. 3.2C). Both glucose and 2DG supplementation significantly shortened the lifespan of the wt and *fgt-1* mutant worms (Fig. S3.2B). In

contrast, in the *daf-2* mutant background, the *fgt-1* mutation [*fgt-1(tm3165);daf-2(e1370)*] significantly shortened the animal lifespan on normal medium ( $P < 0.001$ ) but not on glucose- or 2DG-supplemented medium (Fig. 3.2D). As has been well established in previous studies (Lee et al. 2009; Kenyon et al. 1993; Schulz et al. 2007), the *daf-2* mutant had a much longer lifespan than the wt (Fig. S3.2C). Glucose supplementation reduced the lifespan of both *daf-2* and *daf-2;fgt-1* mutants, however, 2DG supplementation increased the lifespan of the *daf-2;fgt-1* mutant but not the *daf-2* mutant (Fig. S3.2D).

### 3.3.3 The IIS mutant has impaired facilitated glucose transport activity

Because our above analyses showed that the *daf-2* background affects the functions of FGT-1 in animal growth and lifespan, we examined the activities of facilitated glucose transport and Na<sup>+</sup>-dependent glucose transport in wt and IIS mutant animals by measuring the uptake activities of tritium-labeled 2DG (<sup>3</sup>H-2DG) in wt and mutant strains of *fgt-1(tm3165)*, *daf-2(e1370)*, *daf-2(e1370);fgt-1(tm3165)*, *age-1(hx546)*, *akt-1(mg306)*, and *daf-2(e1370);daf-16(mgDf50)* in the presence or absence of phloretin, a facilitated glucose transport inhibitor, or phlorizin, a Na<sup>+</sup>-dependent glucose transport inhibitor (Fig. 3.3). First, glucose uptake activities in *fgt-1*, *daf-2*, *daf-2;fgt-1*, and *age-1* mutants were similar and approximately 40% lower than those in wt ( $P < 0.001$ ). The *akt-1* mutant also displayed lower glucose uptake than wt ( $P < 0.05$ ) but higher uptake than the *fgt-1*, *daf-2*, *daf-2;fgt-1*, and *age-1* mutants ( $P < 0.05$ ). In addition, glucose uptake activity in *daf-2;daf-16* did not differ from that in wt (Fig. 3.3A). Second, phloretin significantly

inhibited 2DG uptake in wt, *daf-2*, *akt-1*, and *daf-2;daf-16* ( $P < 0.05$ ) but not in the *fgt-1* and *daf-2;fgt-1* mutants. Phloretin tended to inhibit 2DG uptake in *age-1* ( $P = 0.058$ ) (Fig. 3.3B). Finally, phlorizin inhibited 2DG uptake in all of the tested strains ( $P < 0.05$ ) (Fig. 3.3C).

### 3.3.4 Overexpression of FGT-1 rescued the *daf-2(e1370);fgt-1(tm3165)* phenotypes

Because the *daf-2(e1370);fgt-1(tm3165)* double mutant had a significantly lower lifespan than the *daf-2(e1370)* single mutant when cultured on normal medium (Fig. 3.2D), we verified whether overexpression of FGT-1 in the *daf-2;fgt-1* mutant could rescue this phenotype. An FGT-1::GFP expression construct under the control of the 5 kb *fgt-1a* promoter sequence was introduced into the *daf-2(e1370)* and *daf-2(e1370);fgt-1(tm3165)* mutants to generate FGT-1 overexpression in the *daf-2* and *daf-2;fgt-1* mutant strains. Our previous study confirmed that the FGT-1::GFP fusion protein retains the normal sugar transport function of FGT-1 (Kitaoka et al. 2013). The expression vector without FGT-1 cDNA was also introduced into the *daf-2(e1370)* and *daf-2(e1370);fgt-1(tm3165)* mutant strains to generate the vehicle control strains. The lifespan of these transgenic strains was analyzed. As shown in Fig. 3.4A (upper panel), overexpression of FGT-1 in *daf-2;fgt-1* completely rescued the decreased lifespan ( $P = 0.017$ ) of *daf-2;fgt-1*, while the lifespan of the FGT-1::GFP-expressed *daf-2;fgt-1* mutant did not differ from that of the vehicle-introduced *daf-2* strain ( $P = 0.658$ ). However, overexpression of FGT-1 in the *daf-2* single mutant strain had no effect on the lifespan of *daf-2* (Fig. 3.4A, bottom panel). The FGT-1::GFP-mediated glucose transport activity was verified by phloretin-inhibited

2DG uptake in the FGT-1::GFP-overexpressed *fgt-1*, *daf-2*, and *daf-2;fgt-1* transgenic strains but not in the vehicle-introduced transgenic strains (Fig. 3.4B).

### 3.3.5 IIS regulates FGT-1 expression

To study the mechanisms underlying the impaired facilitated glucose transport activity in the IIS mutants, the mRNA level of FGT-1 was analyzed in wt and IIS mutant worms by real-time PCR. Whereas the FGT-1 mRNA level remained unchanged in *age-1(hx546)* and *akt-1(mg306)* compared to wt, it was approximately 50% higher in the *daf-2(e1370)* and *daf-2(e1370);daf-16(mgDf50)* mutants ( $P < 0.001$ ) (Fig. 3.5A).

Due to the lack of FGT-1 antibody, we analyzed the effect of IIS on FGT-1 protein expression in the transgenic worms expressing the FGT-1::GFP fusion protein using an antibody against GFP. The IIS in the transgenic worm was manipulated by feeding RNAi bacteria of *daf-2*, *age-1*, *akt-1*, or both *daf-2* and *daf-16* (*daf-2;daf-16*). Real-time PCR confirmed the effectiveness of each individual bacteria-mediated RNAi (Fig. 3.5B). Expression of the FGT-1::GFP fusion protein in the transgenic worm was decreased by more than 50% by *daf-2* and *age-1* RNAi ( $P < 0.05$ ) but not by *akt-1* and *daf-2;daf-16* RNAi (Fig. 3.5C, D).

### 3.3.6 IIS does not appear to regulate the subcellular localization of FGT-1

To assess whether the subcellular localization of FGT-1 is regulated by IIS in *C. elegans*, the FGT-1::GFP fusion protein was expressed in wt or *daf-2(e1370)* strains, representing

IIS active or inactive conditions, respectively. No visible differences in the subcellular localization of FGT-1::GFP were apparent in either strain, wherein it was located in the plasma membrane of, at least, the intestinal cells, which possess adipose cell-like functions in human (Fig. 3.6A). Next, we semiquantitatively examined the FGT-1::GFP distributions in the cytosolic and plasma membrane fractions of *C. elegans* and compared the distributions in wt and *daf-2* strains. There were no significant differences in the FGT-1::GFP distribution in the membrane fraction (more than 90%) in either strain (Fig. 3.6B, C).

### **3.3.7 The glycosylation gene OGA-1, an O-GlcNAcase of *C. elegans*, affects glucose uptake in animals**

In human, *N*- and *O*-linked glycosylation modifications are known to regulate the function of GLUT1 (Samih et al. 2003; Ahmed and Berridge 1999). The amino acid sequence of FGT-1 does not contain any predicted *N*-glycosylation sites (NetNGlyc 1.0 Server) (Spiro 2002) but have potential *O*-linked glycosylation sites. In *C. elegans*, OGT-1 and OGA-1 encode the sole *O*-GlcNAc transferase and *O*-GlcNAcase, respectively (Rahman et al. 2010). To study the potential regulation of these glycosylation genes in glucose uptake in *C. elegans*, we first assessed 2DG uptake activity in the *ogt-1(ok1474)* and *oga-1(ok1207)* mutant strains. Whereas the *ogt-1* mutant showed the same level of 2DG uptake activity as wt, the transport activity was sharply decreased in the *oga-1* mutant ( $P < 0.001$ ) (Fig. 3.7A). In addition, both phloretin and phlorizin inhibited 2DG uptake in the *ogt-1* mutant ( $P < 0.001$ ) but not in the *oga-1* mutant (Fig. 3.7B, C),

indicating that the *oga-1* mutant strain has impairments in both facilitated glucose transport and Na<sup>+</sup>-dependent transport activities. The double mutant of *oga-1(ok1207);fgt-1(tm3165)* showed the same level of uptake activity as the *oga-1(ok1207)* mutant, which suggested that the impaired 2DG uptake in the *oga-1* mutant resulted from the impaired function of FGT-1. To examine this possibility, the mRNA and protein levels of FGT-1 were analyzed in *oga-1* mutant or knockdown animals. There were no differences in FGT-1 mRNA levels between wt and *oga-1* mutant strains, as measured by real-time PCR (Fig. 3.7D). However, knockdown of OGA-1 by RNAi (Fig. 3.7E) reduced the expression of the FGT-1::GFP fusion protein by 37% ( $P < 0.001$ ) in FGT-1::GFP transgenic worms (Fig. 3.7F, G). Taken together, our data demonstrated that OGA-1 might be involved in the regulation of expression and function of FGT-1 in *C. elegans*.

## 3.4 Discussion

### 3.4.1 FGT-1 is the sole functional GLUT homologue and the existence of Na<sup>+</sup>-dependent glucose transport in *C. elegans*

In our previous study (Kitaoka et al. 2013), we investigated the glucose transport activity of 9 candidates of GLUT homologues in *C. elegans* based on their sequence homologies to GLUTs and found that FGT-1 was the only protein with transport activity for 2DG in *Xenopus* oocytes. Feng et al. conducted a similar study and reached the same conclusion (Feng et al. 2013). In the present study, we provided further evidence supporting that

FGT-1 is the only functional GLUT homologue in *C. elegans*: 1) In contrast to wt, the impaired 2DG uptake in the FGT-1 null function mutant *fgt-1(tm3165)* could not be further inhibited by phloretin, a GLUT inhibitor (Lefevre 1959; Zhao and Keating 2007; Ida-Yonemochi et al. 2012), whereas it could be further inhibited by phlorizin, a SGLT inhibitor (Ehrenkranz et al. 2005) (Fig. 3.3); 2) Under the control of the 5 kb *fgt-1* promoter, FGT-1 was ubiquitously expressed in *C. elegans* (Fig. 3.1) (Kitaoka et al. 2013), suggesting that FGT-1 may be ubiquitously involved in glucose transport in the tissues and cells of *C. elegans*. In addition, the current study showed that two FGT-1 splicing isoforms, FGT-1A and -1B, likely have the same or similar tissue distributions and glucose transport activity in *C. elegans* (Fig. 3.1). Furthermore, we also demonstrated the presence of Na<sup>+</sup>-dependent glucose transport in *C. elegans* based on the inhibition of 2DG uptake activity by phlorizin (Fig. 3.3). However, BLASTP searches using protein sequences of human SGLTs against the *C. elegans* database (Wormbase) did not reveal any proteins with significant sequence homology to SGLTs. Thus, the transporters responsible for Na<sup>+</sup>-dependent glucose transport in *C. elegans* remain to be identified.

### **3.4.2 The physiological roles of FGT-1 in *C. elegans***

Although FGT-1 is the only GLUT-type glucose transporter in *C. elegans*, the null function mutation of *fgt-1* gene had no effects on the growth and lifespan of animals cultured on both normal and glucose-supplemented media, as observed for the *fgt-1(tm3165)* strain (Fig. 3.2). In addition, our previous study showed that the dauer formation rate and brood size phenotypes were also not affected by *fgt-1* RNAi (Kitaoka

et al. 2013). These observations were surprising because it is well known based on previous studies that even a small amount of glucose supplementation is sufficient to alter the growth, fertility, and lifespan of *C. elegans* (Mondoux et al. 2011; Schlotterer et al. 2009; Choi 2011), which was also confirmed in the present study (Fig. 3.2). The insensitivity of *C. elegans* growth and lifespan to the function of FGT-1 may be explained by the following three hypotheses. 1) Under normal condition, gluconeogenesis may be a major source of glucose in *C. elegans*. In particular, unlike mammals, nematodes can convert acetyl-CoA into oxaloacetate via the glyoxylate pathway (McElwee et al. 2006; Depuydt et al. 2014). Therefore, nematodes have the ability to produce glucose from fatty acids, in addition to amino acids and glycerol. Thus, *C. elegans* cells may not rely on FGT-1 to take up glucose. 2) Under high glucose conditions, the residual glucose transport activity observed in the *fgt-1* mutant strain may still take up sufficient glucose to shorten the lifespan of the worm. Almost half of the 2DG uptake activity was retained in the *fgt-1* mutant, and it was not inhibited by phloretin (Fig. 3.3). Less than half of this residual activity could be further inhibited by phlorizin, indicating a Na<sup>+</sup>-dependent mechanism (Fig. 3.3). The remaining residual activity may include intestinal accumulation of the isotope, but other transport mechanisms cannot be ruled out. For example, the recently discovered SWEET system may also participate in this process. The *C. elegans* genome contains seven SWEET homologues (Chen et al. 2010). 3) The effect of glucose in *C. elegans* may be dominant in some cell types. For example, if the concentration of glucose in intestinal cells has a dominant effect on the growth and lifespan of the animal, FGT-1 would play a minimal role in this process because it is

located in the basolateral membrane of intestinal cells (Kitaoka et al. 2013) and should not be involved in glucose uptake from the intestinal lumen by these cells. The malfunction of FGT-1 would increase the intracellular glucose concentration and reinforce the effects of glucose, which is supported by the increased fat storage observed in our previous study (Kitaoka et al. 2013). However, the present study showed that the toxicity of 2DG in the growth and lifespan of *C. elegans* was affected by the function of FGT-1 (Fig. 3.2), indicating that *C. elegans* may be sensitive to the intracellular concentration of 2DG.

### **3.4.3 IIS mutants have impaired FGT-1 mediated glucose transport**

Although FGT-1 is not required for growth and aging in wt worms, it is required for animal aging in the *daf-2* mutant. The *daf-2(e1370)* mutant had a lifespan that was almost twice as long as the wt, but the null mutation of the *fgt-1* gene in the *daf-2* mutant [*daf-2(e1370);fgt-1(tm3165)*] significantly shortened the lifespan of animals cultured on normal medium (Fig. 3.2D). Importantly, this lifespan change could be rescued by the overexpression of FGT-1 (Fig. 3.3). The requirement for the function of FGT-1 in the *daf-2* mutant is not known. There are contradictory reports of gluconeogenesis in the *daf-2* mutant. Whereas the expression of gluconeogenesis enzymes in *daf-2* mutants increases (McElwee et al. 2006; Fuchs et al. 2010; Depuydt et al. 2014), implying an increase in gluconeogenesis, the enzyme activity of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis, is much lower (Depuydt et al. 2014). If gluconeogenesis is defective in the *daf-2* mutant, the cells may require FGT-1 to take up glucose. However,

there is no difference in the intracellular glucose concentration between *daf-2* and wt worms (Fuchs et al. 2010). Nevertheless, when the *daf-2* mutant was cultured on glucose-supplemented medium, its extended lifespan disappeared, and the mutation of *fgt-1* [*daf-2;fgt-1*] had no further effect on the shortened lifespan of the *daf-2* mutant (Fig. 3.2D). High glucose conditions may also result in sufficient intracellular glucose, which enhances the aging process in *daf-2* and *fgt-1* mutants due to the residual glucose transport activity (Fig. 3.3). Intriguingly, the *daf-2* mutant could completely tolerate the toxic effects of 2DG on animal growth and aging, and this tolerance was not affected by the function of FGT-1 (Fig. 3.2). The mechanism underlying this tolerance and the reason why 2DG provides a protective effect of the *fgt-1* mutation in the *daf-2* mutant are not known. Nevertheless, our data clearly showed that the function of FGT-1 is dependent on the *daf-2* background.

Our above observations for the function of FGT-1 in wt and *daf-2* mutant are contradictory to the results reported by Feng *et al.*, who showed that wt worms with FGT-1 knockdown by RNAi had an extended lifespan and that this extended lifespan was reduced in the *daf-2* mutant background (Feng et al. 2013). These different observations might be a result of the differences in the lifespan analysis conditions employed in the two studies. First, Feng *et al.* used the RNAi method to knock down FGT-1, whereas we used the *fgt-1(tm3165)* mutant, a confirmed FGT-1 null function mutant strain. The RNAi used in the study by Feng *et al.* was delivered by feeding bacteria of the HT115 strain, which is known to affect the metabolic rate and lifespan of the worms to a greater extent than the more popularly used bacterial strain OP50 (Reinke et al. 2010). In addition,

RNAi may have potential off-target effects. Second, the lifespan analysis conducted in the study by Feng *et al.* utilized FUdR, which may have very little effect on the lifespan of the wt worm but can have a significant effect on the lifespan of some mutant strains (van Raamsdonk and Hekimi 2011; Davies et al. 2012). The present lifespan assay was performed under general laboratory culturing conditions for *C. elegans* without any additional treatment, which should reflect more natural responses of the worms.

Although there were differences in the effects of FGT-1 knockdown or mutation on lifespan between the present study and that by Feng *et al.*, both studies showed that the function of FGT-1 is dependent on the *daf-2* background, suggesting the potential regulation of FGT-1 function by IIS. Indeed, both studies demonstrated a dramatic decrease in 2DG uptake in the *daf-2* mutant. Our study further showed that in *daf-2* mutant animals, 2DG uptake activity was decreased to a level similar to that in *fgt-1* mutant animals. The activity did not further decrease in the *daf-2;fgt-1* double mutant and could be inhibited only slightly by phloretin. These results indicated that FGT-1 mediated glucose uptake was almost completely impaired in the *daf-2* mutant. In addition, our data showed that the impaired function of FGT-1 in the *daf-2* mutant was dependent on the DAF-16 transcription factor because the DAF-16 mutation the *daf-2* mutant completely recovered the decreased 2DG uptake (Fig. 3.3B). Furthermore, our study showed that 2DG uptake was also significantly impaired in other IIS mutants, including *age-1* and *akt-1*. The uptake observed in the *age-1* mutant was similar to that in the *daf-2* mutant, whereas the uptake in the *akt-1* mutant was between that of the wt and the *daf-2* and *age-1* mutants (Fig. 3.3B). These observations suggest that AGE-1 may mediate the

stimulation of FGT-1 function by DAF-2, whereas AKT-1 was only partially involved in this process. In the IIS cascade in *C. elegans*, there are at least two additional signaling molecules downstream of AGE-1: AKT-2 and SGK-1 (Hertweck et al. 2004; Evans et al. 2008). These signaling pathways may also cooperatively function with AKT-1 to mediate DAF-2 regulation of FGT-1 function. Because 2DG uptake could be inhibited by phlorizin in all of the tested strains to similar degrees (Fig. 3.3C), Na<sup>+</sup>-dependent glucose transport may not be regulated by IIS in *C. elegans*, which is similar to the phenomenon observed in mammals.

#### **3.4.4 Regulation of FGT-1 expression by IIS**

To study the mechanism underlying the serious defect in FGT-1 mediated glucose uptake in IIS mutants, we first studied the expression of FGT-1 in IIS mutants. The mRNA expression of FGT-1 was not decreased in IIS mutants but was even increased in *daf-2* and *daf-2;daf-16* mutants (Fig. 3.5A), excluding the transcriptional inhibition of FGT-1 expression by IIS. However, the protein level of FGT-1 was substantially lower in DAF-2 and AGE-1 knockdown worms compared to control worms (Fig. 3.5C, D). This decreased protein expression should at least partially contribute to the impaired FGT-1 mediated glucose uptake in the *daf-2* and *age-1* mutants. At present, it is not known whether this decreased protein expression of FGT-1 was due to a decrease in mRNA stability or translational efficiency, or an increase in protein degradation. It is noteworthy that the FGT-1 protein level did not decrease in the AKT-1 knockdown animals (Fig. 3.5C, D), indicating that the depressed FGT-1 protein expression was not the only mechanism responsible for the impaired glucose uptake in the IIS mutants.

### 3.4.5 Post-translational regulation of FGT-1

Additional evidence that IIS does not regulate the function of FGT-1 solely by reducing FGT-1 protein expression in *C. elegans* is provided by our observation that FGT-1 mediated glucose uptake in the *daf-2* mutant was almost completely abrogated (Fig. 3.3B), whereas the protein level of FGT-1 was only reduced to 40% (Fig. 3.5D). Other mechanisms could account to the function of the remaining 40% of FGT-1 protein. One possible mechanism is the regulation of the FGT-1 subcellular localization by IIS. In human, regulation of the intracellular localization of GLUT4 plays a central role in insulin regulation of glucose homeostasis (Saltiel and Kahn 2001; Leto and Saltiel 2012). However, we did not observe significant differences in the subcellular localization of the FGT-1::GFP fusion protein or in its distribution in the plasma membrane fraction between wt and *daf-2* mutant animals (Fig. 3.6). These observations do not support the occurrence of IIS-induced FGT-1 intracellular translocation in *C. elegans*, which is consistent with our preliminary Wormbase search showing no conserved homologues in the *C. elegans* genome of some of the key molecules involved in GLUT4 translocation such as AS160 (Bogan et al. 2003; Sano et al. 2007). However, we must note that our observations had technical limitations. First, the fluorescence intensity of the fusion protein in the worms might be overly saturated and thus preclude the discrimination of any visual differences in subcellular localization. Second, because our plasma membrane fractions were prepared from the whole animal and because subcellular translocation may only occur in specific tissues such as muscles, as in human, our membrane fractions from the whole animal might mask any changes in the plasma membrane of specific tissues.

Third, our plasma membrane fraction may have contained intracellular membrane vesicles containing intracellular FGT-1.

Another potential mechanism is the regulation of FGT-1 glycosylation by IIS. In mammals, the function of GLUT1, a GLUT that is systemically expressed throughout the body and is responsible for basic cellular glucose uptake, is fine-tuned by tissue-specific glycosylation modifications (Samih et al. 2000, 2003; Ahmed and Berridge 1999; Pyla et al. 2013). In the present study, we could only examine the effects of the mutation of genes involved in glycosylation in *C. elegans* on the function of FGT-1. Because FGT-1 does not have any predicted *N*-linked glycosylation sites, we investigated the effects of mutation of the *O*-linked glycosylation enzymes OGT-1 and OGA-1. Intriguingly, OGA-1, a homologue of *O*-GlcNAcase, was found to be essential for the function of FGT-1 as well as Na<sup>+</sup>-dependent glucose uptake (Fig. 3.7). The total defect in FGT-1 mediated glucose uptake in the *oga-1* mutant could not be solely due to the approximately 30% decrease in FGT-1 protein and is likely related to modification of the FGT-1 glycosylation status. However, surprisingly, the mutation of OGT-1, an *O*-GlcNAc transferase, showed no effect on glucose uptake in *C. elegans* (Fig. 3.7). This finding may be explained by the observation that *O*-linked glycosylation of FGT-1 is not dependent on the function of OGT-1 but on other factors. Nevertheless, further studies are required to investigate FGT-1 glycosylation and its potential regulation by IIS.

### **3.4.6 Concluding remarks**

In this study, we demonstrated that in *C. elegans*, FGT-1 mediated facilitated glucose uptake is severely defective in IIS mutants and that DAF-2, AGE-1, and AKT-1 are

involved in the control of FGT-1 function by regulating its protein expression and by other unidentified mechanisms. The results of our study are important in at least three research fronts. 1) We establish *C. elegans* as a powerful and unique model to study the mechanisms of insulin regulation of glucose transport because of its relative ease of genetic manipulation. 2) We provide insight into the impaired glucose uptake in patients with diabetes. Our results suggested the possibility of a largely depressed expression of GLUT proteins in the tissues of patients with diabetes, who have defective insulin signaling. 3) *C. elegans* may be used as a drug-screening model to search for compounds that stimulate glucose uptake in patients with diabetes.

### **3.5 Materials and Methods**

#### **3.5.1 *C. elegans* strains and culture**

*C. elegans* strains wt (N2), *daf-2(e1370)*, *age-1(hx546)*, *akt-1(mg306)*, *daf-16(mgDf50)*, *daf-2(e1370);daf-16(mgDf50)*, *oga-1(ok1207)*, and *ogt-1(ok1474)* were obtained from the Caenorhabditis Genetics Center (University of Minnesota), and *fgt-1(tm3165)* was provided by the National BioResource Project (Tokyo Women's Medical University School of Medicine). The double mutant of *daf-2(e1370);fgt-1(tm3165)* was constructed by crossing *daf-2(e1370)* and *fgt-1(tm3165)*, and the *daf-2(e1370)* and *fgt-1(tm3165)* alleles were confirmed by allele-specific PCR using the primer sets 'e1370chk' and 'tm3165chk', respectively (Zhang 2011). The double mutant of *oga-1(ok1207);fgt-1(tm3165)* was constructed by crossing *oga(ok1207)* and *fgt-1(tm3165)*, in which the *fgt-*

*l(tm3165)* allele was confirmed by allele-specific PCR with the primer set ‘tm3165chk’, and the *oga-1(ok1207)* allele was verified with two sets of primers: ‘ok1207chk’ and ‘ok1207WT’. All of the primer sequences used herein are listed in Table S3.1. The *C. elegans* strains were cultivated at 20 °C under standard conditions unless otherwise specified [24].

### 3.5.2 Plasmid constructions

All of the PCR reactions used for the plasmid constructions were conducted using high-fidelity Platinum *Pfx* DNA polymerase (Life technologies) unless otherwise specified.

To construct the *C. elegans* expression vector of FGT-1B::GFP under the control of the 2 kb *fgt-1b* 5’ upstream promoter sequence, FGT-1B cDNA was amplified from the cDNA library of the wt worm with the primer set ‘fgt1bcDNA’, and the amplicon was inserted into the *Sal* I site of pPD95.79 (Addgene plasmids #1496). The 2 kb 5’ upstream region of the *fgt-1b* gene was amplified from the genomic DNA of wt with the primer pair ‘fgt1bup2k’, and the amplicon was inserted into the *Sph* I and *Sal* I sites of pPD95.79 harboring the FGT-1B cDNA to form pPD-fgt1bg2k. For the expression vector of FGT-1A::GFP containing 5 kb of the *fgt-1a* 5’ upstream promoter sequence, the FGT-1A cDNA was amplified from the cDNA library of wt with the primer set ‘fgt1acDNA’, and the amplicon was inserted into the *Sma* I and *Sal* I sites of pPD95.79. The 5 kb 5’ upstream sequence of the *fgt-1a* gene was amplified from the genomic DNA of wt with the primer pair ‘fgt1bup5k’ and the Qiagen LongRange PCR Kit (Qiagen). The amplicon

was inserted into the *Sph* I and *Sal* I sites of pPD95.79 harboring FGT-1A cDNA to form pPD-fgt1b5k.

To construct the *Xenopus* oocyte expression plasmid, the full-length cDNAs of FGT-1B and mutated FGT-1A and -1B were amplified from the cDNA library of wt or *fgt-1(tm3165)* worms, respectively. The primer set ‘fgt1bcDNAX’ was used to clone the wt and mutated FGT-1B cDNAs, and ‘fgt1acDNAX’ was used for the mutated FGT-1A cDNA. The cDNA amplicons of FGT-1B and mutated FGT-1A and -1B were inserted into the *Bgl* I site of pSP64T (obtained from Gwyn Gould, University of Glasgow) (Gould et al. 1991) to form pSP-fgt1b, pSP-fgt1atm, and pSP-fgt1btm, respectively.

### 3.5.3 Generation of transgenic worms

Transgenic worms were generated as described previously (Takashima et al. 2012). The *rol-6d* pRF4 (obtained from M. Koelle, Yale University) was used as a co-injection marker (Kramer et al. 1990; Mello and Fire 1995). To generate the transgenic worm harboring the FGT-1B::GFP expression vector under the 2 kb FGT-1B promoter, the pPD-fgt1bg2k plasmid was microinjected into the wt worm. A transgenic line expressing FGT-1A::GFP under the 2 kb FGT-1A promoter was constructed in our previous study (Kitaoka et al. 2013). To observe FGT-1A::GFP localization under the 5 kb FGT-1A promoter, the pPD-fgt1g5k plasmid was microinjected into wt or *daf-2(e1370)* animals. For the rescue experiments, pPD-fgt1g5k was microinjected into wt, *fgt-1(tm3165)*, *daf-2(e1370)*, and *daf-2(e1370);fgt-1(tm3165)* strains without pRF4. All of the microinjected DNA solutions were prepared at a final concentration of 100 µg/mL. Transgenic animals

bearing the pRF4 marker construct were selected based on the roller phenotype, and other strains were isolated by GFP reporter fluorescence observed under a confocal laser microscope (Zeiss LSM 510 META Laser Scanning Microscope).

### **3.5.4 Glucose transport assay in *Xenopus* oocytes**

The cRNAs of *fgt-1a* were generated in our previous study (Kitaoka et al. 2013). cRNAs of *fgt-1b*, *fgt-1a(tm3165)*, and *fgt-1b(tm3165)* were synthesized by *in vitro* transcription from pSP-*fgt1b*, pSP-*fgt1atm*, and pSP-*fgt1btm*, respectively, using the mMessage mMachine kit (Ambion). The synthesized cRNA (2 µg/µL) or sterile water was injected into isolated mature oocytes from *Xenopus laevis* (stage V and VI) following previously established procedures (Bentley et al. 2012). The use of *Xenopus laevis* in the present study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont (#13-025).

The 2DG uptake analysis of wt or mutated FGT-1A and FGT-1B was performed in *Xenopus* oocytes as described previously (Kitaoka et al. 2013; Bentley et al. 2012). Briefly, cRNA- or water-injected oocytes were incubated in Barth's medium with 10 µg/mL penicillin and 10 IU/mL streptomycin (Gibco) for three days and then subjected to the uptake assay using 10 mM 2DG containing 1 µCi <sup>3</sup>H-2DG. Ten or more oocytes were used in each assay, and the oocytes were incubated in uptake solutions for 15 min. Each experiment was performed independently three times.

### **3.5.5 Developmental growth and lifespan assay**

The times (hr) required to grow from L1 larvae to young adults were recorded in *C. elegans* cultured on normal medium or medium supplemented with 5 mM glucose or 5 mM 2DG. The percentages of L1 larvae of wt and *fgt-1(tm3165)* on 2DG-supplemented medium that developed into young adult worms were counted at the 120-hr time point. For each condition, at least 100 worms were used.

The lifespan was observed in worms cultured on normal medium or medium supplemented with 30 mM glucose or 5 mM 2DG. Synchronized, normal medium-cultured L4 worms were transferred to each culture condition (day 0), and the surviving worms were then counted every two days. On each counting day, the worms were transferred to new plates until completion of their propagation and then transferred to new plates every four days unless the food bacteria were depleted before that cycle. Worms that had dried on the plate wall were censored from the analysis. The initial number of 60 worms was observed in each assay, and each assay was repeated twice. The survival fractions are expressed by Kaplan-Meier survival curves.

### **3.5.6 Glucose uptake assay of intact worms**

Synchronized young adult worms were washed out from the culture plates and incubated in M9 saline [22 mM  $\text{KH}_2\text{PO}_4$ , 22 mM  $\text{Na}_2\text{HPO}_4$ , 85 mM NaCl, and 1 mM  $\text{MgSO}_4$ ] for 1 hr to rinse off the food bacteria. A volume of worms of 10% was collected for protein

quantification for normalization of the uptake activity. The remaining worms were subjected to the uptake assay using 5 mM 2DG containing 3  $\mu\text{Ci}$   $^3\text{H}$ -2DG in M9 saline in the presence or absence of 100  $\mu\text{M}$  phloretin or phlorizin. The worms were incubated in the uptake solutions for 2 hr at 20 °C and then washed thoroughly three times with ice-cold PBS containing 0.5% Tween-20 prior to lysis in 0.5% SDS containing 60  $\mu\text{g}/\text{mL}$  proteinase K for 1 hr at 55 °C. The radioactivity of the lysed worms was counted using a Tri-Carb Liquid Scintillation Counter 2900TR (Perkin Elmer Inc.). Each experiment was performed independently four times.

### **3.5.7 Feeding RNAi**

RNAi feeder plasmids of DAF-2 and DAF-16 were obtained from Addgene (Addgene plasmid #34833 and #34834, respectively) (Dillin et al. 2002). An RNAi feeder plasmid of AGE-1 was obtained from Source Bioscience. RNAi feeder plasmids of AKT-1 and OGA-1 were obtained from GE Healthcare Dharmacon Inc. These plasmids were transformed into HT115 (DE3) bacteria, and RNAi was performed by culturing the worms on plates together with these feeding bacteria.

### **3.5.8 mRNA quantification**

The mRNA levels of FGT-1, DAF-2, AGE-1, AKT-1, DAF-16, and OGA-1 were measured by reverse-transcription quantitative PCR (RT-qPCR) with primer pairs to amplify FGT1q, DAF2q, AGE1q, AKT1q, DAF16q, and OGA1q, respectively (Table

S3.1) (Jin et al. 2011). The mRNA levels of CDC-42 and PMP-3, which were analyzed with primer sets for CDC42q and PMP3q, respectively (Table S3.1), served as internal controls to normalize the expression of the other mRNAs (Hoogewijs et al. 2008). To perform the RT-qPCR, the cDNA library was synthesized from the total RNA of individual strains with poly-T primer and SuperScript III Reverse Transcriptase (Life technologies), and the cDNA samples were amplified with SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.) using the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc.). The output data were then analyzed with CFX manager software version 3.0 (Bio-Rad Laboratories, Inc.).

### **3.5.9 Plasma membrane fractionation**

Membrane fractionation of the worms was conducted as described previously (Gally et al. 2004). Synchronized young adult worms were dissolved in homogenization buffer [20 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA, protease inhibitor cocktail (P8340, Sigma)] and underwent freezing and thawing in liquid nitrogen three times. Lysed worms were homogenized with the sonic dismembrator 100 (Fisher Scientific). Sucrose was added (final concentration of 0.25 M), and the solution was centrifuged twice at 5,000 g to remove worm debris. A portion of the supernatant was collected as worm lysate, and the rest was centrifuged at 150,000 g for 1 hr. The resulting supernatant was collected as the cytosolic fraction, and the pellet was dissolved by homogenization in homogenization buffer with 0.5% NP-40 and 0.5% Tween-20 and used as the plasma membrane fraction.

### **3.5.10 Western blot analysis**

Western blotting procedures were conducted as described previously (Kitaoka et al. 2013). Briefly, synchronized young adult worms were homogenized and prepared with NuPAGE LDS sample buffer (Life technologies) containing 100 mM dithiothreitol. The samples were incubated in boiling water for 5 min and centrifuged. The supernatants were subjected to SDS-PAGE, and the proteins were transferred to PVDF membranes. The membranes were incubated with anti-GFP (B-2, Santa Cruz biotechnologies; 1:500), anti-RME1 (DSHB; 1:40), or anti-actin (C4, MP Biomedicals; 1:5000) primary antibody for 2 hr to overnight at room temperature and then with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Amersham; 1:5000) for 2 hr at room temperature. The membranes were finally washed and detected using the West-Pico chemiluminescent kit (Pierce). The resulting bands were quantified using Image Lab software version 3.0 (Bio-Rad Laboratories, Inc.).

### **3.5.11 Statistical analysis**

For the 2DG transport assay in oocytes, any uptake in *fgt-1a* or *fgt-1b* cRNA-injected oocytes less than three times the mean value of water-injected oocytes was considered as injection failure and discarded from the analysis. The 2DG uptake in *fgt-1a* or *fgt-1b* cRNA-injected oocytes was corrected by subtraction of the mean 2DG uptake of water-injected oocytes. To quantify the subcellular distribution of FGT-1::GFP, the level of FGT-1::GFP in whole lysate was normalized to the actin level, and then the ratios of the

cytosolic and membrane fractions of FGT-1::GFP to the normalized FGT-1::GFP level in the lysate were calculated.

The statistical analysis of the individual experiments is indicated in the figure legends, and the analyses were conducted using GraphPad Prism 6.03 (GraphPad Software) and JMP Pro 11.2 (SAS Institute Inc.).

### **3.6 Acknowledgments**

We acknowledge Dr. Michael Koelle (Yale University) for providing the pRF4 marker plasmid and Dr. Gwyn Gould (University of Glasgow) for the pSP64T vector. The RME1 antibody developed by Hadwiger *et al.* (Washington University School of Medicine) was obtained from the Developmental Studies Hybridoma Bank created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

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### 3.8 Figure legends

#### **Figure 3.1. Tissue localization and glucose transport activity of the FGT-1 isoforms**

##### **A and B**

A. Gene structures of *fgt-1a* and *fgt-1b* (adapted from Wormbase). The filled boxes represent exons, and the lines connecting the boxes represent introns. B, C. Tissue localization of FGT-1B::GFP (B) and FGT-1A::GFP (C) in L2 worms, driven by the corresponding 2 kb upstream promoter sequences of *fgt-1a* and *fgt-1b*. D. 2-deoxy-D-glucose (2DG) uptake activity of FGT-1A and -1B in *Xenopus* oocytes injected with the corresponding FGT-1A or -1B cRNA or water. Error bars represent SD ( $N = 3$ ). The statistical analysis was conducted using one-way ANOVA with Tukey's HSD test, and significant differences are indicated by the different alphabetical letters on each bar ( $P < 0.001$ ). E-H. Tissue localization of the FGT-1A::GFP fusion protein in L2 worms, driven by the 5 kb upstream promoter sequence of *fgt-1a*. GFP images of the whole body at the L2 stage (E), head (F) and mid-gut (G) of the young adult, and embryo (H).

#### **Figure 3.2. Developmental growth rate and lifespan of the *fgt-1*-null mutant**

A. Growth times (hr) from L1 larvae to young adult were measured in wild-type and mutant worms of *fgt-1(tm3165)*, *daf-2(e1370)*, and *daf-2(e1370);fgt-1(tm3165)* cultured on normal medium or medium supplemented with 5 mM glucose or 5 mM 2-deoxy-D-glucose (2DG). Error bars represent the SD ( $N = 3$ ). The statistical analysis was

conducted using one-way ANOVA with Tukey's HSD test, and significant differences are indicated by the different alphabetical letters on each bar ( $P < 0.05$ ). B. The percentages of L1 larvae that developed into young adult worms at the 120-hr time point were measured in wild-type and *fgt-1(tm3165)* in conditions containing 5 mM 2DG. Error bars represent SD ( $N = 3$ ). The statistical analysis was conducted using the student's *t*-test ( $*P < 0.05$ ). C, D. The lifespan of wild-type (N2) and *fgt-1(tm3165)* (C) as well as *daf-2(e1370)* and *daf-2(e1370);fgt-1(tm3165)* worm strains (D) cultured on normal medium or medium supplemented with either 30 mM glucose or 5 mM 2-deoxy-D-glucose (2DG). The survival fractions for each condition are presented as Kaplan-Meier survival curves. The statistical analysis was conducted using the log-rank (Mantel-Cox) test.

**Figure 3.3. Glucose uptake activity in *fgt-1(tm3165)* and insulin/IGF-like signaling in mutant worms**

A. Intact worms of wild-type and mutants of *fgt-1(tm3165)*, *daf-2(e1370)*, *fgt-1(tm3165);daf-2(e1370)*, *age-1(hx546)*, *akt-1(mg306)*, and *daf-2(e1370);daf-16(mgDf50)* were incubated with tritium-labeled 2-deoxy-D-glucose ( $^3\text{H}$ -2DG) for 2 hr and evaluated for 2DG uptake. Uptake in the wild-type worm was set at 1. Error bars represent the SD ( $N = 4$ ). The statistical analysis was conducted using one-way ANOVA with Tukey's HSD test, and significant differences are indicated by the different alphabetical letters on each bar ( $P < 0.05$ ). B-D. Intact worms of wild-type and mutants were incubated with  $^3\text{H}$ -2DG in the presence or absence of phloretin (B), phlorizin (C), or both inhibitors (D) for

2 hr and evaluated for 2DG uptake. Uptake in wild-type worms in the absence of any inhibitors was set at 1. Error bars represent SD ( $N = 4$ ). The statistical analysis was conducted using a paired student's  $t$ -test ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ).

**Figure 3.4. Lifespan and 2-deoxy-D-glucose (2DG) uptake rescue in the *daf-2;fgt-1* mutant by overexpression of FGT-1**

A. Lifespan assay of *daf-2(e1370)* and *daf-2(e1370);fgt-1(tm3165)* transgenic worms introduced with the FGT-1::GFP expression vector (oe) or control vector (vehicle) cultured on medium without sugar supplementation. The survival fractions of each strain are presented as Kaplan-Meier survival curves. The statistical analysis was conducted using the log-rank (Mantel-Cox) test. B. The 2-deoxy-D-glucose (2DG) uptake assay in the presence or absence (control) of phloretin in wild-type (N2) or *fgt-1(tm3165)*, *daf-2(e1370)*, and *daf-2(e1370);fgt-1(tm3165)* transgenic worms introduced with the FGT-1::GFP expression vector (oe) or the control vector (vehicle). Error bars represent the SD ( $N = 4$ ). The statistical analysis was conducted using the paired student's  $t$ -test ( $**P < 0.01$ ,  $***P < 0.001$ ).

**Figure 3.5. Effects of insulin/IGF-like signaling on FGT-1 mRNA and protein expression**

A. The mRNA levels of FGT-1 in wild-type and *daf-2(e1370)*, *age-1(hx546)*, *akt-1(mg306)*, and *daf-2;daf-16(mgDf50)* mutants were measured by real-time reverse transcription PCR and normalized to the mRNA levels of CDC-42 and PMP-3. The

mRNA level of FGT-1 in wild-type worms was set at 1. Error bars represent the SD ( $N = 3$ ). The statistical analysis was conducted using one-way ANOVA with Tukey's HSD test, and significant differences are indicated by the different alphabetical letters on each bar ( $P < 0.001$ ). B. Transgenic *C. elegans* expressing the FGT-1::GFP fusion protein were fed individual RNAi bacteria of *daf-2*, *age-1*, *akt-1*, or both *daf-2* and *daf-16* and analyzed for the relative mRNA levels of DAF-2, AGE-1, AKT-1, or DAF-16 by real-time PCR. Error bars represent the SD ( $N = 3$ ). The statistical analysis was conducted using the paired student's *t*-test ( $*P < 0.05$ ,  $***P < 0.001$ ). C, D. Expression of the FGT-1::GFP fusion protein in FGT-1::GFP transgenic worms that were fed control RNAi bacteria (vehicle) or individual RNAi bacteria of *daf-2*, *age-1*, *akt-1*, and both *daf-2* and *daf-16* were analyzed by Western blot analysis with an antibody against GFP (C). Actin served as an internal control. The relative FGT-1::GFP levels were quantitated (D). Error bars represent SD ( $N = 3$ ). The statistical analysis was conducted by one-way ANOVA with Tukey's HSD test, and significant differences are indicated by the different alphabetical letters on each bar ( $P < 0.001$ ).

**Figure 3.6. Subcellular localization of FGT-1 in wild-type and *daf-2* mutant worms**

A. Localization of FGT-1::GFP under the 5 kb *fgt-1a* promoter during the L2 stage in wild-type and *daf-2(e1370)* worms. B. Western blot analysis of FGT-1::GFP in cytosolic (Cyt) and plasma membrane (Mem) fractions and whole lysate (Lys) prepared from wild-type and *daf-2(e1370)* worms with GFP antibody. RME1 is a plasma membrane marker, and actin was used as an internal control. C. The percentages of FGT-1::GFP in the

cytosolic and plasma membrane fractions detected in B. Error bars represent the SD ( $N = 3$ ).

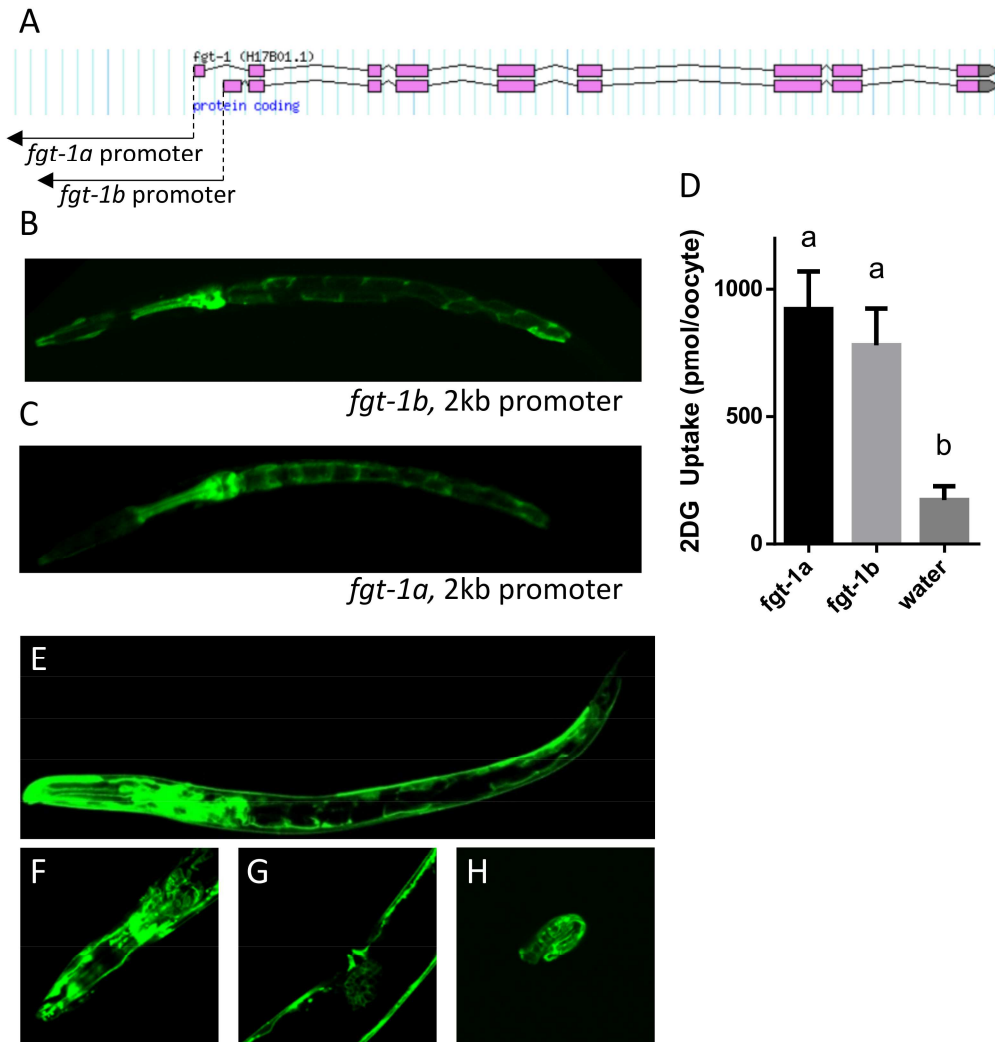
**Figure 3.7. Effect of the mutation of glycosylation genes on glucose uptake and FGT-1 expression**

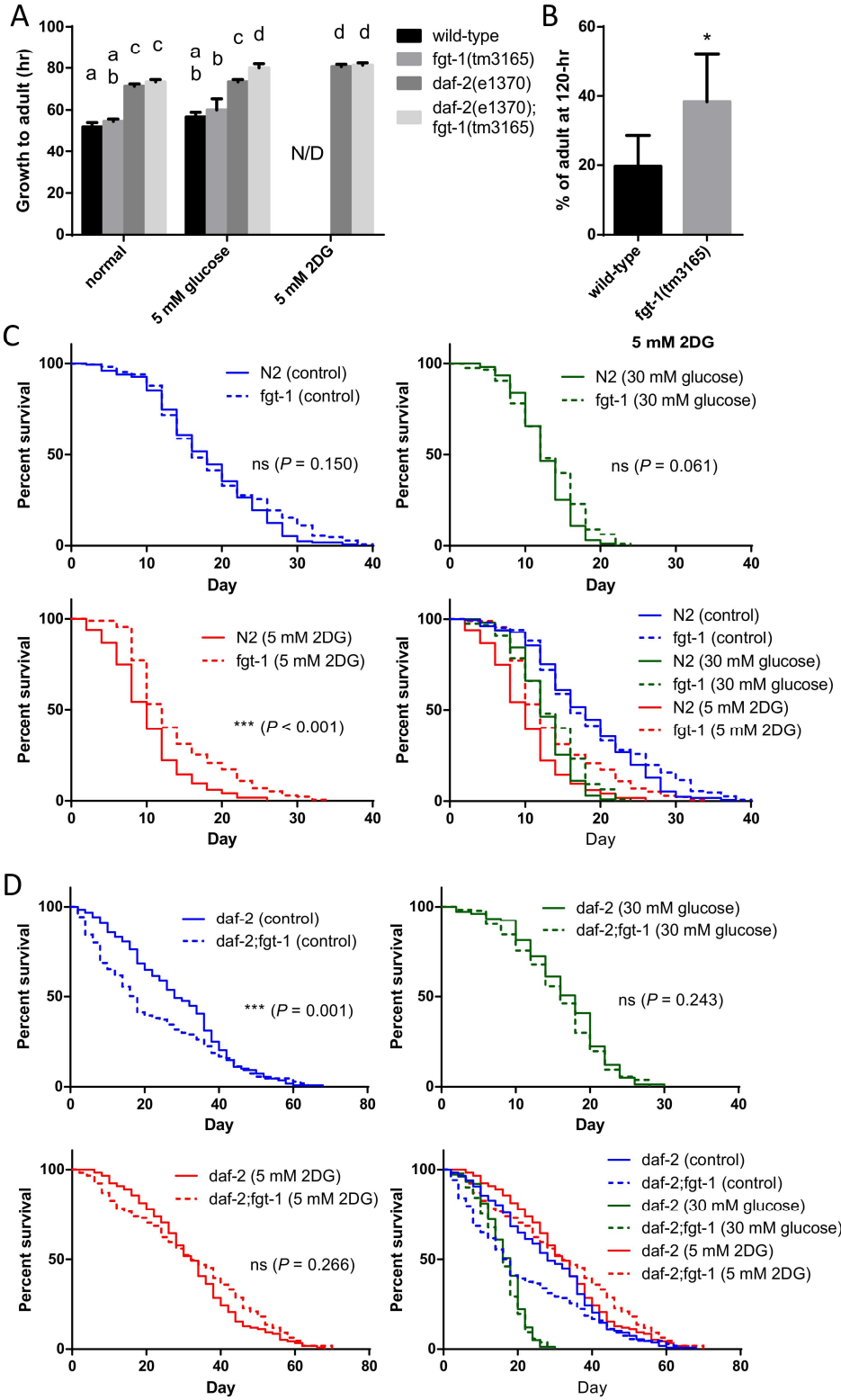
A-C. The 2-deoxy-D-glucose (2DG) uptake activity in wild-type, *ogt-1(ok1474)*, *oga-1(ok1207)*, and *oga-1(ok1207);fgt-1(tm3165)* was analyzed in the presence of no inhibitor (A), phloretin (B), or phlorizin (C). Worms were incubated with tritium-labeled 2-DG ( $^3\text{H}$ -2DG) for 2 hr and assessed for the uptake of 2DG. Uptake in wild-type worms was set at 1. Error bars represent the SD ( $N = 4$ ). D. mRNA levels of FGT-1 in wild-type and *oga-1(ok1207)* were analyzed by real-time PCR and normalized to the mRNA levels of CDC-42 and PMP-3. The mRNA level of FGT-1 in wild-type worms was set at 1. Error bars represent the SD ( $N = 3$ ). E. Transgenic *C. elegans* expressing the FGT-1::GFP fusion protein were fed RNAi bacteria of *oga-1* or the vector control (vehicle) and analyzed for the mRNA level of OGA-1 by real-time PCR. Error bars represent the SD ( $N = 3$ ). F, G. The expression of the FGT-1::GFP fusion protein in FGT-1::GFP transgenic worms fed either control RNAi bacteria (vehicle) or RNAi bacteria of *oga-1* were analyzed by Western blot analysis using an antibody against GFP (F). ACT-1 served as an internal control for normalization. The relative FGT-1::GFP levels were quantitated (G). Error bars represent the SD ( $N = 3$ ). In A, the statistical analysis was conducted using one-way ANOVA with Tukey's HSD test, and significant differences

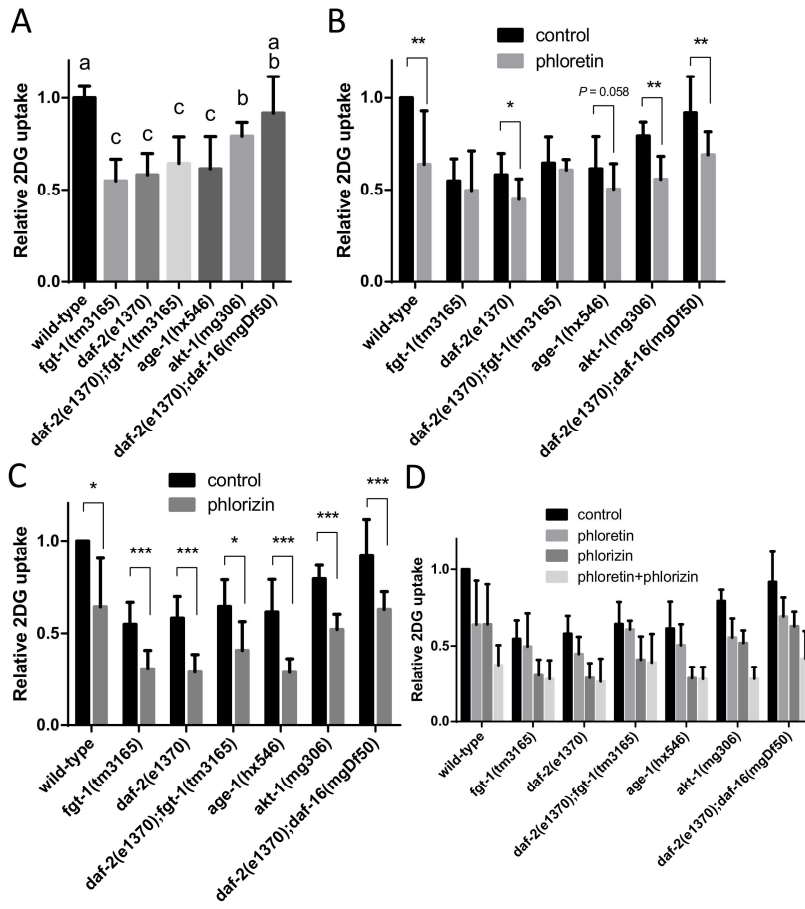
are indicated by the different alphabetical letters on each bar ( $P < 0.001$ ). In B-E and G, the statistical analysis was performed using the paired student's  $t$ -test (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### 3.9 Figures

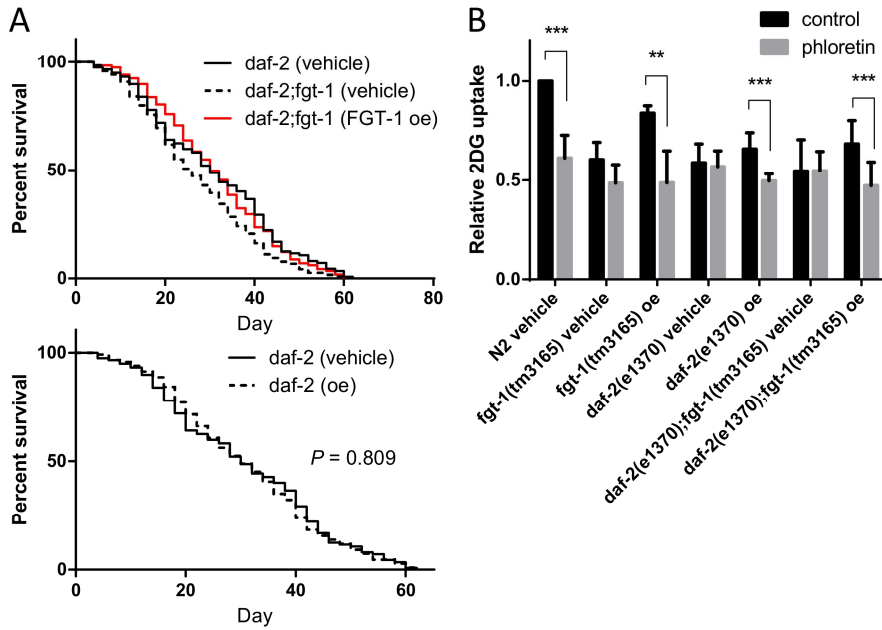
Kitaoka\_Fig1



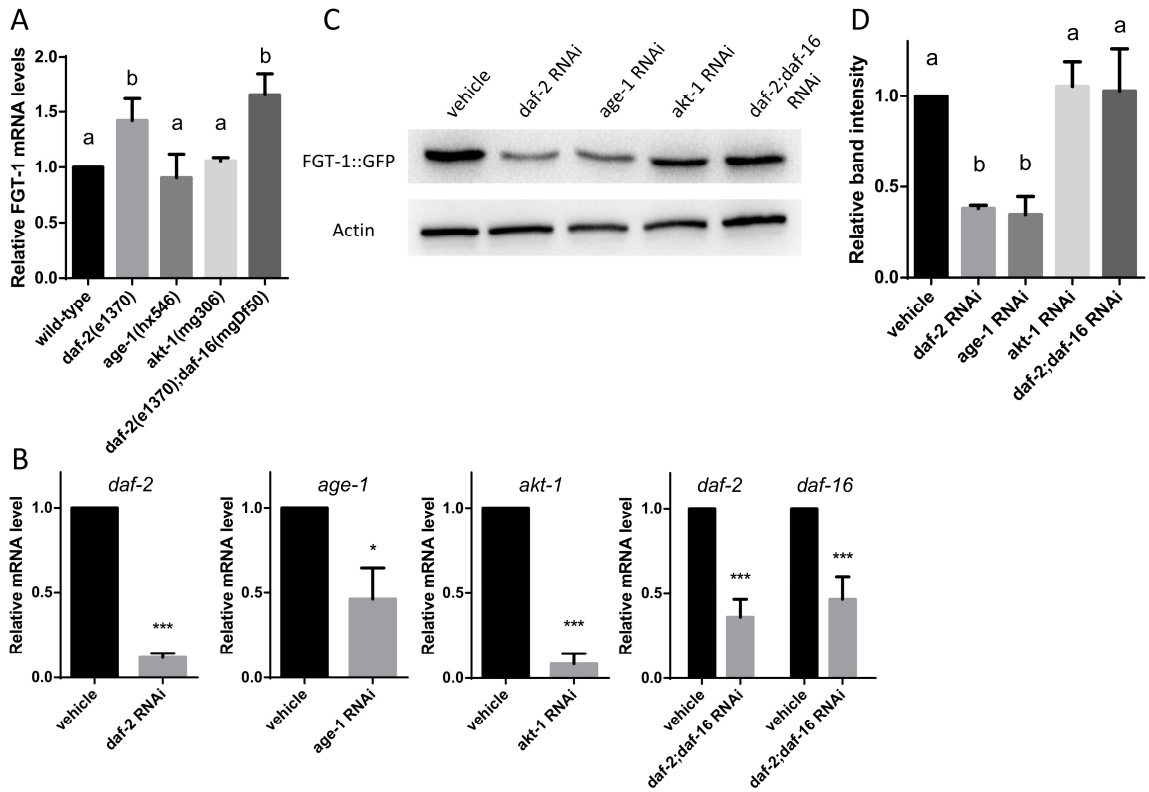


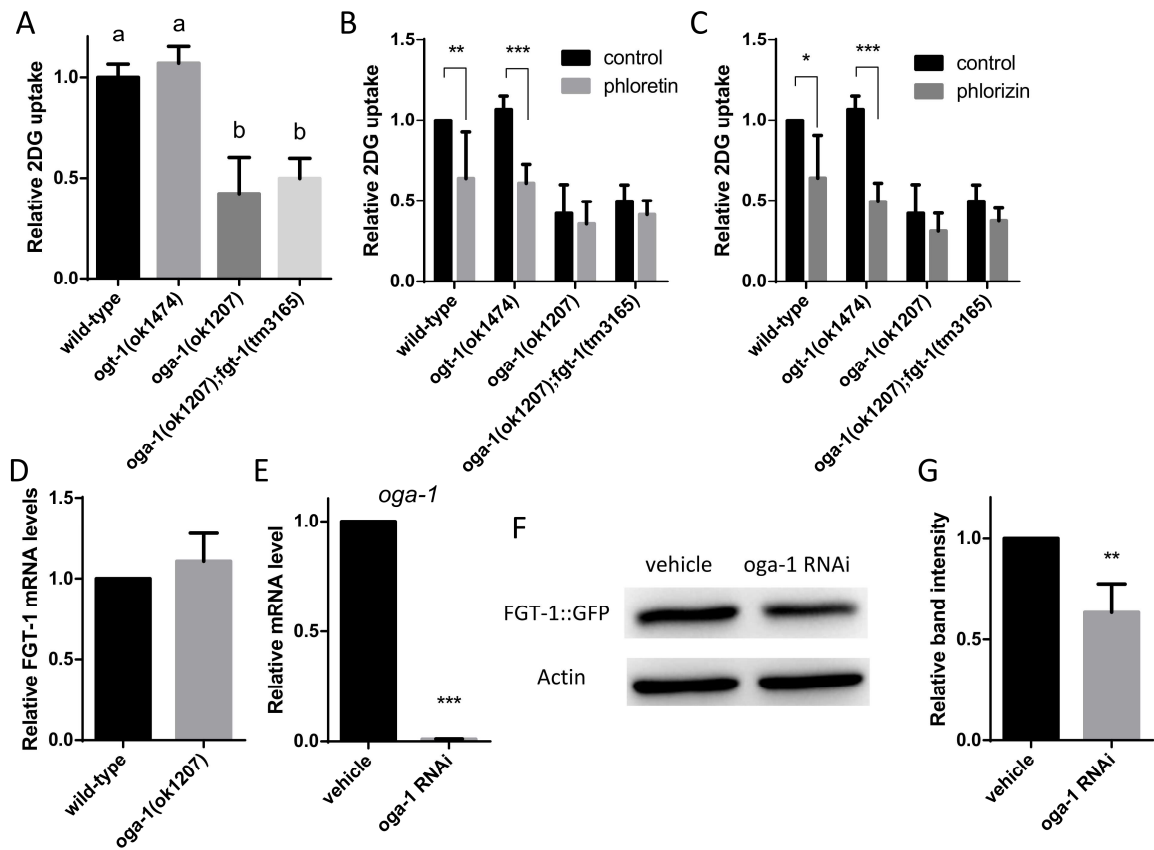
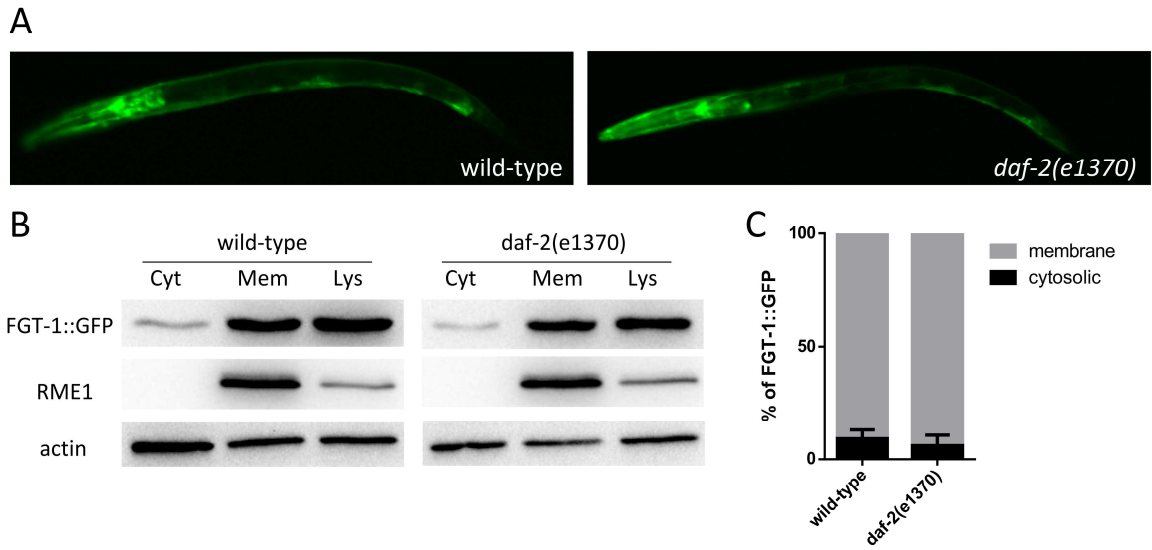


Kitaoka\_Fig4

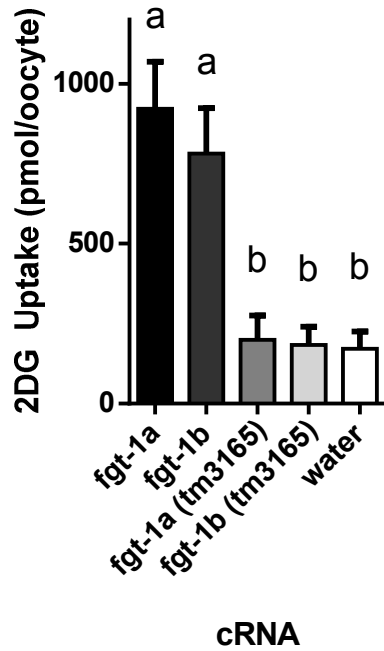


Kitaoka\_Fig5



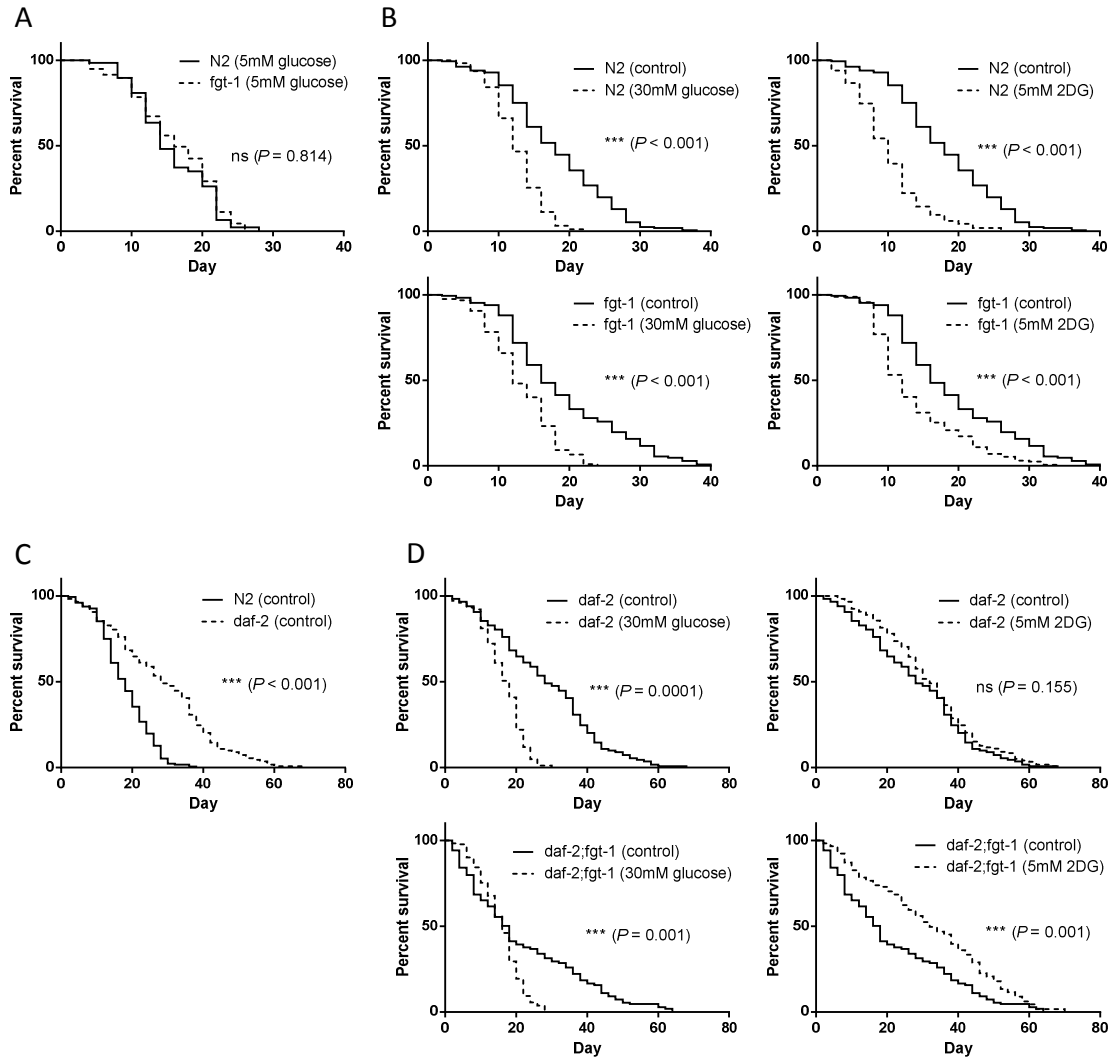


### 3.10 Supplementary information



**Figure S3.1. Functional analysis of the mutated *fgt-1* gene in the *fgt-1(tm3165)* strain**

FGT-1A and -1B cDNAs were cloned from the *fgt-1(tm3165)* mutant and transcribed into their corresponding cRNAs. The cRNAs were individually injected into *Xenopus* oocytes, and the oocytes were measured for the uptake activity of 2-deoxy-D-glucose (2DG) in comparisons with those injected with the cRNAs of wild-type FGT-1A or -1B. Water was used as a negative control. Error bars represent the SD. The statistical analysis was conducted using one-way ANOVA with Tukey's HSD test, and significant differences are indicated with the different alphabetical letters on each bar ( $P < 0.001$ ).



**Figure S3.2. Lifespan comparisons between wild-type (N2), *fgt-1(tm3165)*, *daf-2(e1370)*, and *daf-2(e1370);fgt-1(tm3165)* worms cultured on normal medium (control) or medium supplemented with 30 mM glucose or 5 mM 2-deoxy-D-glucose (2DG)**

A. Lifespan comparison between wild-type and *fgt-1(tm3165)* worms on glucose medium.  
 B. Lifespan comparisons of wild-type and *fgt-1(tm3165)* worms on normal glucose or 2DG media.  
 C. Lifespan comparison between wild-type and *daf-2(e1370)* on normal

medium. D. Lifespan comparisons of *daf-2(e1370)* and *daf-2(e1370);fgt-1(tm3165)* worms on normal, glucose-, or 2DG-supplemented medium. The lifespan is presented as a Kaplan-Meier survival curve. The statistical analysis was conducted using a log-rank (Mantel-Cox) test.

**Table S3.1. List of primer sets**

Name	Forward primer	Reverse primer
e1370chk	CGGGATGAGACTGTCAAGATTGGAGATTTCCG	CAACACCTCATCATTACTCAAACCAATCCATG
tm3165chk	GCGGGGGTTCAGCTGTGTAA	TATGATACGGAGTTTCGCCA
ok1207chk	GCAAACAGAAAGGTGAGCTAG	AACTCAGAAGGCACAGGCTC
ok1207WT	ACGAGCGGTTCCGACGTTAG	TTCCGACGCATTACACTTCC
FGT1q	TCCAGGAGGGCTTATCAC	AATACGGATACTGCGACGGAC
DAF2q	GTGGCGTGAGAATGAAGTGAG	GGAATTTCTGAGAATCCGTTG
AGE1q	CGGAAAGACCAAACCTGGGATC	CGTAGGCTTCGACGCATAACG
AKT1q	CAAAGCCTAAGGAAGGACAACC	CATGAATCCAACGCTGACGAAC
DAF16q	TCGTCGTCTCGTGTCTTCTCCA	TTCCATAGGCACCCGGTAGTG
OGA1q	ACATTTCCGATTAAGTATTGACTGTG	AGATGCTTGCCTGTTCTGG
CDC42q	CTGCTGGACAGGAAGATTACG	CTCGGACATTCTCGAATGAAG
PMP3q	GTTCCCGTTCATCACTCAT	ACACCGTCGAGAAGCTGTAGA
fgt1bcDNA	AAGTCGACGAAGAAAATTCATTTAAAATAATGTCGGA	CTTCCTCTTCTCGAATTCG
fgt1b2kbup	CATGCATGCATTTGGAGCGAATCAGGC	AAGTCGACGAAATAAATATATATTTTCTAGATTTAGACG
fgt1acDNA	AAGTCGACAGAAATGGGTGTCAACGACCATG	CTTCCTCTTCTCGAATTCG
fgt1b5kbup	CATGCATGCAGACGGCTGAAAGTGCCTCCAG	AAGTCGACGAAATAAATATATATTTTCTAGATTTAGACG
fgt1bcDNAX	TACTGATCAGAAGAAAATTCATTTAAAATAATGTCGGA	TACTGATCAAACCTATACGTTTCGCAGTG
fgt1acDNAX	TACTGATCAAATGGGTGTCAACGACCATG	TACTGATCAAACCTATACGTTTCGCAGTG

## Chapter 4: General Discussion

### 4.1 Energy metabolism and sugar transport in *C. elegans*

In *C. elegans*, energy metabolism has been widely studied, particularly in aging research. Because of its short lifespan, *C. elegans* has been used as a primary model system for aging studies, and these studies have established a close relationship between the energy metabolism rate and lifespan (Newgard and Pessin 2014). Calorie restriction can extend the lifespan of *C. elegans*, whereas high energy supply, such as glucose supplementation, can dramatically shorten the lifespan of *C. elegans* (Lee et al. 2009; Schlotterer et al. 2009; Schulz et al. 2007). Several independent long-lived strains share some common metabolic profiles, including elevated gene expression and metabolite levels in energy metabolism pathways such as glycolysis and gluconeogenesis (Fuchs et al. 2010; Depuydt et al. 2014; Patti et al. 2014). Several regulatory pathways, such as IIS and TOR signaling, are known to play essential roles in energy metabolism and aging in *C. elegans* (Hansen et al. 2013; Rockenfeller and Madeo 2010). Although glucose is an important energy source in *C. elegans*, as in other animals, the glucose transporter system and its regulation by IIS had not been investigated previously. Knowledge in these areas would help us to use *C. elegans* as a powerful model system to study the mechanisms by which insulin regulates glucose transporters because of the many unique advantages of *C. elegans*, including relatively easy genetic manipulation and largely available mutant strains.

Our studies have identified FGT-1 as the only functional GLUT homologue (Kitaoka et al. 2013), however, the reproduction, development, and aging processes appear to be independent of FGT-1 function in wt worms. This independence can be explained by compensatory functions of other glucose transporter families, such as SGLT and SWEET, and by the unique ability of gluconeogenesis to proceed from substrates such as fatty acids (by the glyoxylate cycle). However, functioning FGT-1 is required in IIS mutants because FGT-1 knockout reduced the lifespan of the *daf-2* mutant worm. Consistently, IIS mutants have severely defective FGT-1 function and substantially decreased FGT-1 protein expression. Although why IIS mutants require functioning FGT-1 in contrast to the wt worms remains unknown, our work established the regulation of FGT-1 expression and function by IIS in *C. elegans*.

Incorporating the results of this study into the accumulated knowledge regarding the energy metabolism of *C. elegans*, we propose a general model of FGT-1 function and IIS regulation in sugar metabolism of *C. elegans* (Fig. 4.1). IIS is normally active in wt worms, sustaining high FGT-1 expression and possibly stimulating glucose synthesis rates from glycerol, amino acids, and fatty acids. IIS also inhibits trehalose synthesis from glucose. In addition, SGLT- and SWEET-types of glucose transporters may be involved in glucose uptake or efflux, at least in some cell types. Glucose availability is high in wt worms. Environmental stresses, such as starvation and heat stress, inhibit the IIS signaling cascades, which activate several stress response mechanisms (Baumeister et al. 2006). One of these responses is to alter energy metabolism rates including glucose metabolism pathways. Under weak IIS conditions, FGT-1 expression is low, resulting in

low glucose uptake. We hypothesize that when IIS is inhibited, the gluconeogenesis rate is low due to the inhibited enzyme activity of PEPCK (Depuydt et al., 2014), trehalose synthesis is increased (Honda et al. 2010), and the glycolysis rate is high (Depuydt et al., 2014). These effects further decrease glucose availability but increase metabolite levels in the cells, resulting in a longer lifespan by unknown mechanisms (Fuchs et al. 2010; Depuydt et al. 2014).

#### **4.2 *C. elegans* as a model for medical research and drug screening**

Diabetes is the most prevalent disease with GLUT dysfunction. Our FGT study in *C. elegans* provides a powerful model of diabetes study because we observed a severe glucose uptake defect in IIS mutants. Further study of the mechanisms of this defect may provide insights into the defective glucose uptake in the peripheral tissues of patients with diabetes. Although no evidence of an IIS-dependent subcellular translocation mechanism of FGT-1 function has been observed unlike human GLUT4, IIS mutants with inhibited FGT-1 expression still remain good models to study the mechanism of reduced GLUT expression observed in patients with diabetes (Moraes et al. 2014; Shepherd and Kahn 1999). In addition, even if IIS does not regulate FGT-1 subcellular translocation in *C. elegans*, this organism can still be reconstructed to study GLUT4 translocation by taking advantage of its transparent body and by humanizing it with the expression of GLUT4 and the key genes that are involved in the process of subcellular translocation and in its regulation by insulin but that are absent in *C. elegans*. This type of

humanization of *C. elegans* has been widely performed and has greatly advanced our understanding of some human diseases (Sin et al. 2014).

Furthermore, the defective glucose uptake in IIS mutants also provides an extremely attractive model of drug screening for diabetes treatment. The high-throughput and cost-effective nature of *C. elegans* enhances the efficiency of the first chemical screening step in an intact animal. Chemical compounds that stimulate the glucose uptake activity of IIS mutant worms, such as the *daf-2* mutant, would be strong drug candidates for patients with both type 1 and type 2 diabetes because the candidate chemical compounds should increase the insulin-independent glucose uptake. Thus, developing new technology to measure glucose uptake activity with a small amount of worms and non-radioactive labeled glucose is important. The fluorescent-labeled 2DG analog 2NBDG (Zou et al. 2005) is an ideal substrate for quantitative, high-throughput screening using the COPAS system. The recently developed enzymatic 2DG measurement technique (Zhao et al. 2014) may also be applied in high-throughput drug screening in *C. elegans*.

GLUT dysfunction in humans results in several diseases other than diabetes. GLUT1 deficiency syndrome is characterized by neurological defective symptoms during development, such as infantile seizures, developmental delay, acquired microcephaly, and ataxia. Several types of heterozygous mutations of the GLUT1 gene were found to cause defective glucose transport across the blood-brain barrier (Seidner et al. 1998; Pascual et al. 2004). Thus, the symptoms of GLUT1 deficiency syndrome may be due to low glucose concentrations in the cerebrospinal fluid. This syndrome is treated with a

ketogenic diet, however, no effective drug has been developed thus far (Gras et al. 2014). Because of the heterozygous dysfunction nature of the GLUT1 gene in this syndrome, functional GLUT1 remains expressed and capable of transporting glucose in patients. Therefore, medicines that can stimulate GLUT1 expression levels can enhance glucose transport activity and alleviate GLUT1 deficiency syndrome symptoms. GLUT2 deficiency syndrome is known as Fanconi-Bickel syndrome, which is caused by homozygous or compound heterozygous mutations of the GLUT2 gene (Santer et al. 1997; Grünert et al. 2012). The symptoms are hepatomegaly with glycogen accumulation, fasting hypoglycemia, dwarfism, and renal syndrome with glucosuria and aminoaciduria (Santer et al. 1998; Grünert et al. 2012). These symptoms are due to a lack of GLUT2 function during glucose diffusion in hepatocytes and glucose reabsorption in the kidney. GLUT2 knockout mice show similar phenotypes (Mueckler and Thorens 2013). GLUT2 is also expressed in pancreatic  $\beta$ -cells and hypothalamus, and GLUT2 knockout mice have a diabetic phenotype. However, patients suffering from Fanconi-Bickel syndrome rarely present diabetes because of GLUT1 and GLUT3 expression in human  $\beta$ -cells (De Vos et al. 1995). Thus far, no specific drug treatment exists for Fanconi-Bickel syndrome, where the stabilization of glucose homeostasis and compensation for renal losses by dietary supplementation are the primary current treatments for this syndrome. Because functional GLUT2 is not usually found in these patients, medical treatment could focus on targeting the up-regulation of other glucose transporters such as GLUT9 in hepatocytes and SGLT2 in the kidney. Artery tortuosity syndrome is also associated

with GLUT10 homozygous mutations (Coucke et al. 2006), although the physiological relevance of GLUT10 defects in this syndrome is not yet known.

Pharmaceutical companies are unwilling to develop medicines for rare diseases such as Fanconi-Bickel syndrome and artery tortuosity syndrome because of the low probability of investment-return (Kontoghiorghie 2014). The establishment of a new cost-effective drug development strategy should encourage drug development for these rare diseases. *C. elegans* models offer this advantage. In addition, *C. elegans* has some molecular mechanisms that are identical to those of humans and that can be humanized for individual diseases. This organism can be used as a drug screening model for compounds that increase glucose uptake in specific tissues. One possible target is O-linked glycosylation of GLUTs. Our study has shown that the function of FGT-1 is strongly affected by the function of the glycosylation gene OGA-1, implying regulation of FGT-1 by glycosylation in *C. elegans*. Thus, *C. elegans* can be an attractive model to study the glycosylation of GLUTs for new medical approaches.

Finally, our study also showed that FGT-1 knockdown is linked to fat accumulation in *C. elegans*. The physiology of elevated fat storage in the absence of FGT-1 function is intriguing. Extended studies of the underlying mechanisms may reveal novel ideas of obesity mechanisms and medications for treatment. Thus, *C. elegans* can be a model system for obesity studies that target FGT-1 function.

### 4.3 Future directions

1) Further investigation of the mechanisms by which IIS regulates FGT-1 expression. In our studies, we found that IIS stimulates FGT-1 protein expression by a post-transcriptional mechanism, however, the specific mechanism remains unknown. Further studies should focus on the regulation of FGT-1 mRNA stability, translational efficiency, and protein degradation by IIS. Specifically, because we showed that IIS regulation of FGT-1 expression is dependent on DAF-16 activity, the regulation of FGT-1 mRNA stability, translational efficiency, and protein degradation by DAF-16 should be investigated.

2) The study of post-translational regulation of FGT-1 by IIS. Because the reduced FGT-1 protein levels could not explain the severely defective FGT-1 mediated glucose uptake observed in IIS mutants (*daf-2* and *age-1*) in our study, IIS should also regulate FGT-1 function by post-translational mechanisms. One such mechanism may be through glycosylation. This study showed that OGA-1, but not OGT-1, is required for FGT-1 function. Since OGA-1 is an O-GlcNAcase, which removes glycan from proteins, whereas OGT-1 is an O-GlcNAc transferase, which adds glycan to proteins, analyzing the relationship between FGT-1 function and its glycosylation states as well as the enzymes involved in FGT-1 glycosylation will be important. In addition, glycosylation states should be analyzed in wt and IIS mutants to establish the mechanism by which IIS regulates FGT-1 glycosylation. Moreover, glucose is an important substrate for the glycosylation of proteins because glucose can be metabolized through the hexosamine biosynthesis pathway (HBP) to synthesize uridine diphosphate N-acetyl-glucosamine

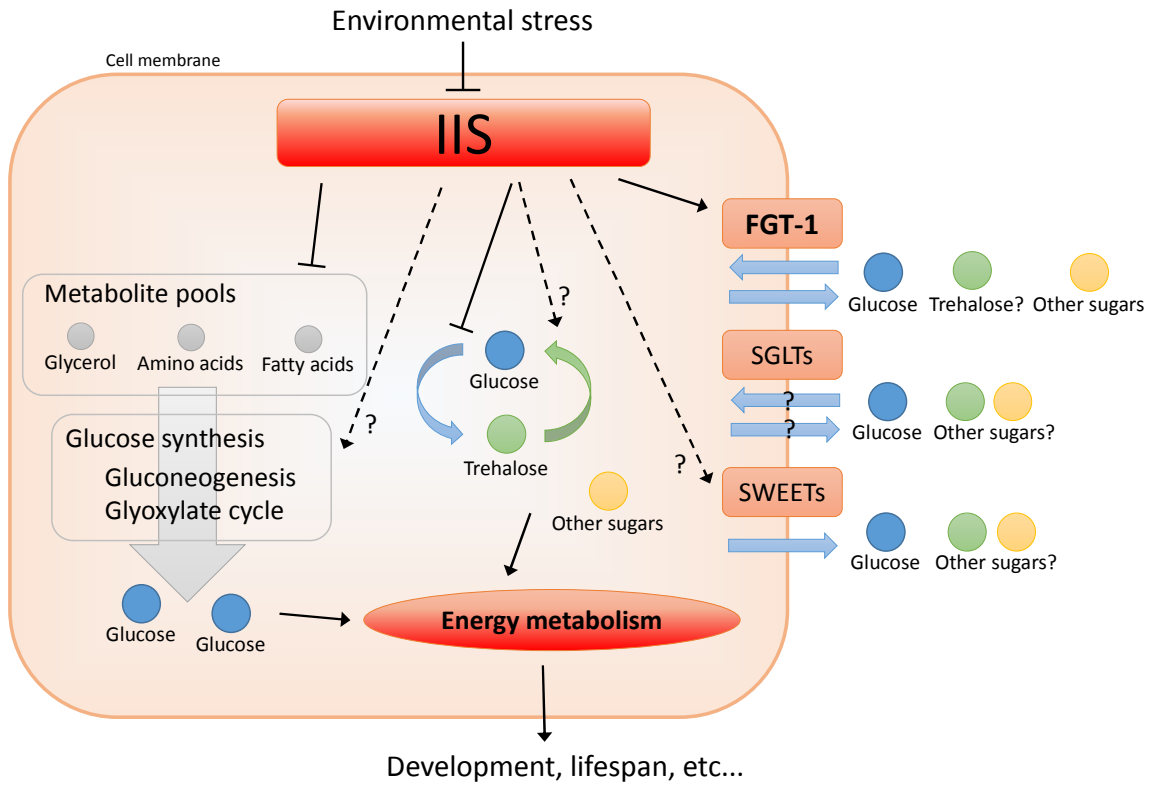
(UDP-GlcNAc), which is a direct substrate of O-glycosylation (Chatham et al. 2008). Thus, the glycosylation of FGT-1 may provide mutual feedback regulation between glucose transport activity and O-glycosylation levels. Another possible post-translational mechanism is changes in subcellular localization. Although our study did not show a localization difference between FGT-1 in wt and *daf-2* mutant worms, our results were not exclusive because of technical limitations as discussed in chapter 3. Techniques that are more sensitive and tissue-specific should be developed for this study.

3) The study of Na<sup>+</sup>-dependent glucose transport in *C. elegans*. Despite the absence of SGLT homologous genes in the *C. elegans* genome in our BLASTP search, phlorizin showed significant inhibition of the glucose uptake activity in *C. elegans* in this study, suggesting the existence of Na<sup>+</sup>-dependent glucose transport. Identifying the proteins that are responsible for this activity is important and may establish *C. elegans* as a model to study glucose absorption from intestinal lumen. A domain search of the sodium:solute symporter family (SSF; PF00474) in the *C. elegans* genome identified some homologues of human sodium-coupled monocarboxylate transporters (SMCTs), which transport several small monocarboxylic acids including lactate, pyruvate, short chain fatty acids, and ketone bodies, but not glucose (Miyachi et al. 2004; Coady et al. 2004; Yanase et al. 2008). These SMCT homologues may have broader substrate specificity including glucose transport activity in *C. elegans*.

4) Last but not least, the study of IIS regulation of gluconeogenesis. Based on the results of this study, we hypothesize that gluconeogenesis is inhibited in IIS mutants, resulting in their dependence on FGT-1 activity. This hypothesis can be tested by

measuring glucose synthesis rates from radiolabeled substrates in wt and IIS mutant worms. Such observation may reveal that *C. elegans* can be an excellent model system to study the mechanism by which insulin regulates gluconeogenesis.

## 4.4 Figures



**Figure 4.1. A proposed model of insulin/IGF-like signaling (IIS) regulation of sugar metabolism in *Caenorhabditis elegans***

Activation is indicated with arrow-headed lines, and inhibition is indicated with bar-headed lines. Hypothesized regulation and transport activities are indicated with question marks and dash lines.

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