FGT-1-mediated glucose uptake is defective in insulin/IGF-like signaling mutants in *Caenorhabditis elegans*

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Insulin signaling plays a central role in the regulation of facilitative glucose transporters (GLUTs) in humans. To establish *Caenorhabditis elegans* as a model to study the mechanism underlying insulin regulation of GLUT, we identified that FGT-1 is most likely the only functional GLUT homolog in *C. elegans* and is ubiquitously expressed. The FGT-1-mediated glucose uptake was almost completely defective in insulin/IGF-like signaling (IIS) mutants *daf-2* and *age-1*, and this defect mainly resulted from the down-regulated FGT-1 protein expression. However, glycosylation may also be involved because OGA-1, an O-GlcNAcase, was essential for the function of FGT-1. Thus, our study showed that *C. elegans* can be a new powerful model system to study insulin regulation of GLUT.

Insulin is a central regulator for sustaining glucose homeostasis in humans. When blood glucose levels increase after meals, insulin is secreted to activate glucose uptake and utilization in peripheral tissues, mainly muscle and adipose tissues [1]. This stimulation is primarily mediated by the insulin-induced subcellular translocation of the insulin-sensitive facilitative glucose transporter (GLUT) 4. 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 as insulin resistance [2,3]. The mechanisms underlying insulin resistance still remain largely unknown.

Insulin signaling is highly conserved in the animal kingdom, including *Caenorhabditis elegans* (*C. elegans*). In *C. elegans*, most of the major signaling molecules in the insulin cascade are well conserved, although insulin-like growth factors (IGF) may

Abbreviations

2DG, 2-deoxy-D-glucose; 3H-2DG, tritium-labeled 2DG; *C. elegans*, *Caenorhabditis elegans*; FGT, facilitative glucose transporter in *C. elegans*; GLUT, facilitative glucose transporter; IGF, insulin-like growth factor; IIS, insulin/IGF-like signaling; SGLT, Na+/glucose cotransporter; wt, wild-type.
function through the same cascade [termed insulin/IGF-like signaling (IIS)] unlike in humans, and IIS has been known to play important functions in many physiological processes, such as reproduction, growth, metabolism, and aging [4–8]. For instance, knockout of DAF-2, the only homolog of the mammalian insulin/IGF-1 receptor, dramatically increases the lifespan of C. elegans [5,9,10]. This lifespan extension is dependent on DAF-16, the homolog of mammalian FOXO transcription factor [10]. Importantly, the presence of glucose impairs lifespan extension of IIS mutants by downregulating DAF-16 [11]. 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 GLUT functions, we characterized nine GLUT candidates in C. elegans (FGT) based on their high sequence homologies to human GLUTs in our previous study [12]. FGT-1 was identified as the sole counterpart of GLUT with the ability to transport glucose in Xenopus oocytes. The localization of FGT-1 was mainly restricted to the digestive tract when expressed under its 2 kb promoter sequence [12], 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. However, our previous findings were not able to determine whether FGT-1 is regulated by IIS in C. elegans.

In this 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 also showed that FGT-1 is regulated by IIS by comparing the differences in 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.

Materials and methods

Plasmid constructions and C. elegans strains and culture

All plasmids and C. elegans strains used in this study are described in Data S1. The C. elegans strains were cultivated at 20 °C under standard conditions unless otherwise specified [13].

Glucose transport assay in Xenopus oocytes

The cRNAs of gft-1a were generated in our previous study [12]. cRNAs of gft-1b, gft-1a(tm3165), and gft-1b(tm3165) were synthesized by in vitro transcription from pSP-gft1b, pSP-gft1atm, and pSP-gft1btm, respectively, using the mMessage mMachine kit (Ambion, Austin, TX, USA). The 2DG uptake analysis of wt or mutated FGT-1A and FGT-1B was performed in Xenopus oocytes as described previously [12,14].

Glucose uptake assay of intact worms

Synchronized young adult worms were washed out from the culture plates and incubated in M9 saline for 1 h. A 10% volume of worms was collected for protein quantitation. The remaining worms were subjected to the uptake assay using 0.5 mm 2DG containing 3 μCi 3H-2DG in M9 saline in the presence or absence of 100 μM phlorizin. The worms were incubated in the uptake solutions for 2 h 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 μg·mL⁻¹ proteinase K for 1 h at 55 °C. The radioactivity of the lysed worms was counted using a Tri-Carb Liquid Scintillation Counter 2900TR (Perkin Elmer Inc., Waltham, MA, USA). Each experiment was performed independently four times.

Feeding RNAi

RNAi feeder plasmids of DAF-2 and DAF-16 were obtained from Addgene (Cambridge, MA, USA; plasmid #34833 and #34834). An RNAi feeder plasmid of AGE-1 was obtained from Source Bioscience (Kennesaw, GA, USA). RNAi feeder plasmids of AKT-1 and OGA-1 were obtained from GE Healthcare Dharmacon Inc (Pittsburgh, PA, USA). These plasmids were transformed into HT115 (DE3) bacteria, and RNAi was performed by culturing the worms on plates together with these feeding bacteria.

mRNA quantitation and western blot analysis

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 FGT1q, DAF2q, AGE1q, AKT1q, DAF16q, and OGA1q, respectively (Table S1) [15]. The mRNA levels of CDC-42 and PMP-3, which were analyzed with primer sets CDC42q and PMP3q, respectively (Table S1), served as internal controls to normalize the expression of the other mRNA [16]. RT-qPCR and western blot analysis were performed as described previously [12]. Band intensity of the western blot was quantified with IMAGE LAB 4.1.
(BioRad Laboratory, Hercules, CA, USA) and normalized to the level of β-actin.

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.

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, La Jolla, CA, USA) and JMP PRO 11.2 (SAS Institute Inc., Cary, NC, USA).

Results

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

Because FGT-1(A) was identified as the sole GLUT homolog in C. elegans with glucose transport activity and its expression was mainly observed in the digestive tract in our previous study [12], 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. 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.

Fig. 1. Tissue localization and glucose transport activity of the FGT-1 isoforms A and B. (A) Gene structures of fgt-1a and fgt-1b. The 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 wild-type FGT-1A and -1B or mutated FGT-1A and -1B which were cloned from fgt-1(tm3165) mutant animals in Xenopus oocytes injected with the corresponding wild-type or mutated FGT-1A and -1B cRNA or water. Data are represented as means with SD from three independent experiments with 10 or more oocytes for each analyses. 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 midgut (G) of the young adult, and embryo (H). Scale bars indicate 50 μm.
and fgt-1b in wt C. elegans. FGT-1B showed the same localization as FGT-1A: primarily in the pharynx and intestinal cells (Fig. 1B,C). The glucose transport activity of FGT-1B was also compared with FGT-1A in Xenopus laevis oocytes (Fig. 1D). The uptake activity of FGT-1B for 2-deoxy-D-glucose (2DG) did not differ from that of FGT-1A as reported previously [17], 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. 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.

The IIS mutant has impaired facilitated glucose transport activity

To study whether IIS regulates glucose transport activity in C. elegans, we examined the activities of facilitated glucose transport and Na⁺-dependent glucose transport in wt and IIS mutant animals by measuring the uptake activities of tritium-labeled 2DG (³H-2DG) in wt and mutant strains of fgt-1(tm3165), daf-2(e1370), gkt-1(tm3165); daf-2(e1370), age-1(hx546), akt-1(mg306), and daf-16(mgDf50); daf-2(e1370) in the presence or absence of phloretin, a facilitated glucose transport inhibitor, or phlorizin, a Na⁺-dependent glucose transport inhibitor (Fig. 2). The null function of FGT-1 in fgt-1(tm3165) mutant was confirmed in Fig. 1D. First, glucose uptake activities in fgt-1, daf-2, gkt-1; daf-2, 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, gkt-1; daf-2, and age-1 mutants (P < 0.05). In addition, glucose uptake activity in daf-16; daf-2 did not differ from that in wt (Fig. 2A). Second, phloretin significantly inhibited 2DG uptake in wt, daf-2, akt-1, and daf-16; daf-2 (P < 0.05) but not in the fgt-1 and gkt-1; daf-2 mutants. Phloretin tended to inhibit 2DG uptake in age-1 (P = 0.058) (Fig. 2B). Finally, phlorizin inhibited 2DG uptake in all of the tested strains (P < 0.05) (Fig. 2C).

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 RT-qPCR. Although 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-16(mgDf50); daf-2(e1370) mutants (P < 0.001) (Fig. 3A).

Due to the lack of FGT-1 antibody, we analyzed the effect of IIS on FGT-1 protein expression in the transgenic worm 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-16 and daf-2. Real-time RT-qPCR confirmed the effectiveness of each individual bacteria-mediated RNAi (Fig. 3B). 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-16; daf-2 RNAi (Fig. 3C,D).

The glycosylation gene OGA-1, an O-GlCNACase of C. elegans, affects glucose uptake in animals

In humans, N- and O-linked glycosylation modifications are known to regulate the function of GLUT1 [18,19]. The amino acid sequence of FGT-1 does not contain any predicted N-glycosylation sites (NetNGlyc 1.0 Server) [20] but have potential O-linked glycosylation sites (NetOGlyc 4.0 Server). In C. elegans, OGT-1 and OGA-1 encode the sole O-GlcNAc transferase and O-GlCNACase, respectively [21]. 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(ok1207) and oga-1(ok1207) mutant strains. Although 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. 4A). 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. 4B,C), indicating that the oga-1 mutant strain has impairments in both facilitated glucose transport and Na⁺-dependent transport activities. The double mutant of fgt-1 (tm3165); oga-1(ok1207) 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 RT-qPCR (Fig. 4D). However, OGA-1 knockdown by RNAi (Fig. 4E) reduced the expression of the FGT-1::GFP fusion protein by 37% ($P < 0.001$) in FGT-1::GFP transgenic worms (Fig. 4F,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*.

**Discussion**

**FGT-1 is the sole functional GLUT homolog, and the existence of Na$^+$-dependent glucose transport in *C. elegans***

In our previous study [12], we investigated the glucose transport activity of nine candidates of GLUT homologs 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. [17] conducted a similar study and reached the same conclusion. In the present study, we provided further evidence supporting that FGT-1 is the only functional GLUT homolog in *C. elegans*: (a) 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 [22], whereas it could be further inhibited by phlorizin, a Na$^+$/glucose cotransporter (SGLT) inhibitor [23] (Fig. 2); (b) Under the control of the 5 kb *fgt-1* promoter, FGT-1 was ubiquitously expressed in *C. elegans* (Fig. 1) [12], 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. 1). Furthermore, we also demonstrated the presence of Na⁺-dependent glucose transport in *C. elegans* based on the inhibition of 2DG uptake activity by phlorizin (Fig. 2). However, BLASTP searches using protein sequences of human SGLTs against the *C. elegans* database (www.wormbase.org) did not reveal any proteins with significant sequence homology to SGLTs. Thus, the transporters responsible for Na⁺-dependent glucose transport in *C. elegans* remain to be identified.

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

Consistent with the findings of Feng *et al.* [17], our study showed the decreased glucose uptake and FGT-1 activity in IIS mutants. 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 because the activity did not further decrease in the fgt-1; daf-2 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 in the *daf-2* mutant completely recovered the decreased 2DG uptake (Fig. 2B). 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. 2B). In addition, FGT-1 protein level was dramatically decreased in *AGE-1 RNAi*, but not in *AKT-1 RNAi* (Fig. 3D). 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 [24,25]. 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. 2C), Na⁺-dependent glucose transport may not be regulated by IIS in *C. elegans*, which is similar to the phenomenon observed in mammals [26,27].

**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-16: daf-2* mutants (Fig. 3A), 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 IIS. However, the protein level of FGT-1 was decreased in IIS mutants, but was even increased in mutants. The mRNA expression of FGT-1 was not influenced by IIS regulation of FGT-1 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 homolog of O-GlcNAcase, was found to be essential for the function of FGT-1 as well as Na⁺-dependent glucose uptake (Fig. 4). This observation is consistent with the finding of Rahman et al. [21] that DAF-16 is required for the longevity of *oga-1* mutant because this study showed that IIS regulation of FGT-1 function is DAF-16 dependent. However, 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 the modification of the FGT-1 glycosylation status. Surprisingly, the mutation of OGT-1, an O-GlcNAc transferase, showed no effect on glucose uptake in *C. elegans* (Fig. 4). 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.

**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. 2B), whereas the protein level of FGT-1 was only reduced to 40% (Fig. 3D). 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, however, our preliminary Wormbase search showed no conserved homologs in the *C. elegans* genome of some of the key molecules involved in GLUT4 translocation such as AS160 [28,29].

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 [18,19,30,31]. 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 homolog of O-GlcNAcase, was found to be essential for the function of FGT-1 as well as Na⁺-dependent glucose uptake (Fig. 4). This observation is consistent with the finding of Rahman et al. [21] that DAF-16 is required for the longevity of *oga-1* mutant because this study showed that IIS regulation of FGT-1 function is DAF-16 dependent. However, 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 the modification of the FGT-1 glycosylation status. Surprisingly, the mutation of OGT-1, an O-GlcNAc transferase, showed no effect on glucose uptake in *C. elegans* (Fig. 4). 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.

**Conclusions**

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 two research fronts: (a) We establish *C. elegans* as a potential unique model to study the mechanisms of insulin regulation of glucose transport because of its relative ease of genetic manipulation and (b) *C. elegans* may be used as a drug-screening model to search for compounds that stimulate glucose uptake in patients with diabetes.

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**Author contributions**

SK designed and performed experiments and wrote the manuscript; ADM provided equipment and revised the
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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1. Materials and methods.

Table S1. List of primer sets.