

Protein *O*-GlcNAcylation regulates *Drosophila* growth through the insulin signaling pathway

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Abstract Modification of nuclear and cytosolic proteins by *O*-linked *N*-acetylglucosamine (*O*-GlcNAcylation) is ubiquitous in cells. The *in vivo* function of the protein *O*-GlcNAcylation, however, is not well understood. Here, we manipulated the cellular *O*-GlcNAcylation level in *Drosophila* and found that it promotes developmental growth by enhancing insulin signaling. This increase in growth is due mainly to cell growth and not to cell proliferation. Our data suggest that the increase in the insulin signaling activity is mediated, at least in part, through *O*-GlcNAcylation of Akt. These results indicate that *O*-GlcNAcylation is one of the crucial mechanisms involved in control of insulin signaling during *Drosophila* development.

Keywords OGT · *O*-GlcNAcase · Size control · Insulin · *Drosophila*

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Abbreviations

GPX1	Glutathione peroxidase 1
NButGT	1,2-Dideoxy-2'-propyl- α -D-glucopyranoside [2,1-D]- Δ 2'-thiazoline
<i>O</i> -GlcNAc	<i>O</i> -linked <i>N</i> -acetylglucosamine
OGT	<i>O</i> -linked GlcNAc transferase

Introduction

O-GlcNAcylation is a unique type of protein glycosylation in which a single GlcNAc residue is linked via an *O*-linked glycosidic bond to either serine or threonine of nuclear or cytoplasmic proteins. It is observed in most multicellular organisms and found on many proteins with diverse functions [1]. A large proportion of these *O*-GlcNAcylated proteins are also known to be modified by phosphorylation. In fact, *O*-GlcNAc modification and *O*-phosphate modification can occur dynamically reciprocal at the same or adjacent sites [1]. The biological functions of *O*-GlcNAcylation are diverse as is reflected by the large repertoire of target proteins [1].

Enzymes that add or remove *O*-GlcNAc are the *O*-linked GlcNAc transferase (OGT) and the *O*-GlcNAcase, respectively. Their *in vivo* functions have been investigated in *Caenorhabditis elegans* and mice. *C. elegans* null mutants for OGT or *O*-GlcNAcase are viable and fertile, but display defects in macronutrient storage, suggesting that insulin signaling is impaired [2, 3]. In fact, both mutants interact genetically with a temperature-sensitive allele of *daf-2*, the gene that encodes an insulin-like receptor, in a way that an elevated cellular *O*-GlcNAcylation level suppresses insulin signaling. In mice, OGT is an essential gene [4], and studies on conditional knockout

animals indicate that loss of *OGT* causes T-cell apoptosis, neuronal tau hyperphosphorylation, and fibroblast growth arrest [5]. Transgenic mice overexpressing *OGT* develop insulin resistance and hyperglycemia [6, 7]. Several components of the insulin signaling pathway in mammals are *O*-GlcNAcylated, such as Akt [8] and FOXO [9]. The mechanism by which *O*-GlcNAcylation changes their activity is currently not understood. Previous studies of Akt yielded controversial results as to whether *O*-GlcNAcylation of Akt increases or decreases its phosphorylation and influences its activity [7, 8, 10]. These discrepancies may be attributed to differences in cell type or experimental setup.

The *OGT* of *Drosophila* has recently been reported to be a protein encoded by the Polycomb group gene *super sex comb (sxc)* [11, 12]. The protein is involved in PcG-mediated transcriptional repression. A possible involvement of *sxc* in insulin signaling, however, is not known. To further analyze the *in vivo* function of *O*-GlcNAcylation in this regard, we used *Drosophila* as a model. We manipulated the cellular *O*-GlcNAcylation level either pharmacologically or genetically and found that *O*-GlcNAcylation regulates body growth of *Drosophila* by modulating insulin signaling. In contrast to findings in worm and mice, we found that *O*-GlcNAcylation in *Drosophila* enhances insulin signaling.

Materials and methods

Drosophila stocks

Act-GAL4, *en-GAL4*, *Act5C>CD2>GAL4*, *hsFLP*, and *UAS-GFP.nls* were obtained from the Bloomington Stock Center (Bloomington, IN). *UAS-OGA RNAi^X* (41823), *UAS-OGA RNAi^{II}* (41822), *UAS-OGT RNAi^{II}* (18610), and *UAS-OGT RNAi^{III}* (18611) were from Vienna *Drosophila* RNAi Center (Vienna, Austria), and *UAS-PTEN RNAi* (5671R-1) and *UAS-Akt RNAi* (4006R-3) were from the National Institute of Genetics (Shizuoka, Japan).

Antibodies

The following antibodies were used: rabbit anti-dFOXO (1:500) [13], rabbit anti-phospho-histone H3 (1:50; Cell Signaling, 9701), rabbit anti-active caspase 3 (1:50; Cell Signaling, 9661), goat anti-rabbit IgG-FITC (1:200; Jackson ImmunoResearch Labs, 111-096-047), goat anti-rabbit IgG-Texas Red (1:200; Jackson ImmunoResearch Labs, 111-076-047), rabbit anti- β -actin (1:1000; Cell Signaling, 4967), rabbit anti-*OGT* (1:1000; Santa Cruz Biotechnology, H-300), mouse anti-*O*-GlcNAc CTD 110.6 (1:1000; Covance, MMS-248R-500), mouse anti-*O*-GlcNAc RL2

(1:1000; ABR, MA1-072), rabbit anti-Akt (1:1000; Cell Signaling, 9272), rabbit anti-phospho-(Ser505)Akt (1:1000; Cell Signaling, 4054), rabbit anti-phospho-(Ser/Thr) Akt substrate (1:1000; Cell Signaling, 9611), mouse anti-V5 (1:5000; Invitrogen, 46-0705), and anti-FLAG M2 affinity gel (Sigma, A2220).

Immunohistochemistry, co-immunoprecipitation, and immunoblotting

Immunohistochemistry was performed as described previously [14]. For analysis of the fat body tissues, larvae were heat-shocked for 10 min at 34°C 48 h after egg deposition. After staining the tissue with phalloidin-rhodamine (Molecular Probes), the samples were mounted in Prolong Gold plus DAPI (Invitrogen, S36939) and observed with a AxioImager A1 microscope (Carl Zeiss) using a 10 × 0.3 NA Plan Neofluar lens (Carl Zeiss). For image acquisition, AxioCam HRc camera (Carl Zeiss) and AxioVision Rel 4.8 software were used. For imaging wing imaginal discs, samples were mounted in the same anti-fading medium and observed with a LSM 510 META confocal microscope (Carl Zeiss) using a Neo-Fluar 10 × NA 0.3 objective lens at room temperature. Image processing and analysis were performed with Imaris and Adobe Photoshop CS5.

Co-immunoprecipitation and immunoblotting were performed as described previously [15].

Synthesis of 1,2-dideoxy-2'-propyl- α -D-glucopyranosyl-[2,1-D]- Δ 2'-thiazoline (NButGT)

NButGT (9c compound) was synthesized according Macauley et al. [16].

Results

Reduction of *O*-GlcNAcase activity increases *Drosophila* body growth

To study the *in vivo* role of *O*-GlcNAcylation in fly development, we performed a drug-feeding experiment using the *O*-GlcNAcase inhibitor NButGT [16] to increase the level of protein *O*-GlcNAcylation. The optimal amount of NButGT that efficiently elevated *O*-GlcNAcylation was 200 μ l of a 10-mM NButGT solution added on the surface of solidified fly food in a vial (Supplementary Fig. 1a). The body weight of male and female adult flies fed with NButGT from the first to the third instar larval stages was increased by 10.4 and 7.9%, respectively, compared to the mock-fed control groups (Fig. 1a, b). This was due to increased growth of larval imaginal discs since the size of wing imaginal discs was increased by 10.7%, examined in

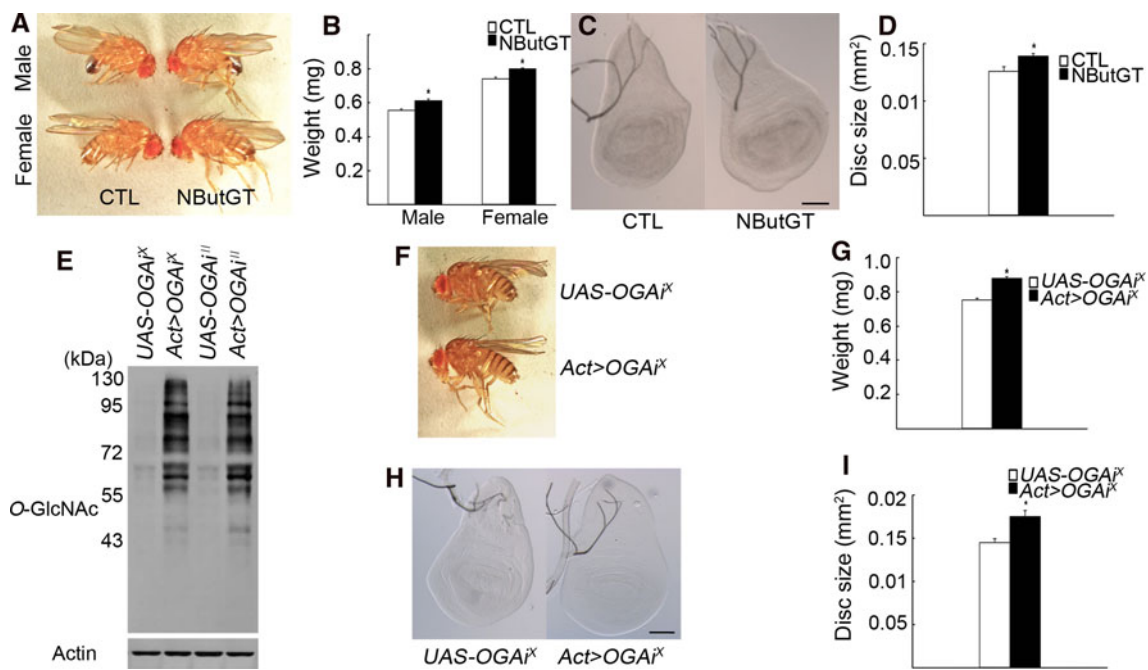


Fig. 1 Reduction of *O*-GlcNAc activity increases *Drosophila* body growth. **a** The size of NButGT-fed flies compared to that of mock-fed control (CTL) flies. **b** Body weights of control ($n = 293$ ♂ and $n = 297$ ♀) and drug-fed ($n = 278$ ♂ and $n = 313$ ♀) adult flies. Experiments were done in triplicate with >100 male and >100 female flies in each experiment. **c, d** The size of wing discs from mock-fed ($n = 15$) and NButGT-fed ($n = 17$) male wandering third instar larvae. **e** Knock-down efficiency of two *OGA* RNAi transgenes as analyzed by Western blotting using anti-*O*-GlcNAc RL2 antibody as a measure of cellular *O*-GlcNAcylation level in larvae of indicated

genotypes. β -actin was used as a loading control. **f, g** The size and weight of *Act>OGA RNAi^X* ($n = 277$) female flies compared to the *UAS*-only control flies ($n = 259$). Since the *UAS-OGA RNAi^X* stock contained the attached X chromosome, only females were analyzed. Experiments were done in triplicate with >100 animals analyzed in each experiment. **h, i** The sizes of the wing discs of *UAS*-only ($n = 13$) and *Act>OGA RNAi^X* ($n = 13$) female larvae. **b, d, g, i** Data are mean \pm SE * $p < 0.001$ by Student's *t* test. **c, h** Scale bars represent 100 μ m

male larvae, compared to control (Fig. 1c, d). This growth-promoting effect of NButGT was not due to its metabolic conversion in an energy source, since the same amount of glucose had no significant effect on growth (Supplementary Fig. 1b). Of note, NButGT-fed larvae eclosed faster, indicative of an accelerated developmental growth rate (Supplementary Fig. 1c).

To show specificity of the NButGT effect, we knocked down *O*-GlcNAcase using RNAi in vivo. From FlyBase (<http://flybase.org>), we identified a single *Drosophila melanogaster* gene, *CG5871*, that encodes a protein of 1019 amino acids with significant homology to human *O*-GlcNAcase (35.3% overall identity; 56.3% identity of the two *N*-terminal halves containing the *O*-GlcNAcase catalytic domain). No mutant for *CG5871* has been reported. Hereafter, we call *CG5871 Drosophila OGA*. For its knock-down, two RNAi strains were used, *UAS-OGA RNAi^X* and *UAS-OGA RNAi^{II}* (^X and ^{II} mark the transgene insertions). Both strains, which were crossed to *Actin-GAL4* flies (hereafter named *Act>OGA RNAi*), were viable and had significantly reduced *OGA* transcripts (29.8 and 23.2% of the *UAS*-only controls, respectively; see Supplementary Fig. 1d). Both strains also showed a marked

increase in cellular *O*-GlcNAcylation levels (Fig. 1e). The *OGA RNAi^X* was selected for further studies. The body weight of *Act>OGA RNAi^X* adult females was increased by 16.8% compared to the *UAS*-only control flies (Fig. 1f, g). The size of the larval wing discs was increased by 21.0% compared to the *UAS*-only control flies (Fig. 1h, i). Thus, increased *O*-GlcNAcylation level either due to *O*-GlcNAcase inhibition or RNAi knock-down results in increased body growth in *Drosophila*.

RNAi knock-down of *OGT* decreases *Drosophila* body growth

The effect of decreased *O*-GlcNAcylation on body growth of *Drosophila* was analyzed by RNAi knock-down of *OGT*. We tested two different RNAi transgenic strains, *UAS-OGT RNAi^{II}* and *UAS-OGT RNAi^{III}*. Only *OGT RNAi^{II}*, driven by *Act-GAL4*, resulted in nearly complete loss of *OGT* protein (Fig. 2a). *Act>OGT RNAi^{II}* flies were early pupal lethal, similar as reported for the amorphic allele of *sxc/OGT* [11] and had strongly reduced cellular *O*-GlcNAcylation level (Fig. 2a). The size of the wing imaginal discs of *Act>OGT RNAi^{II}* larvae was reduced by

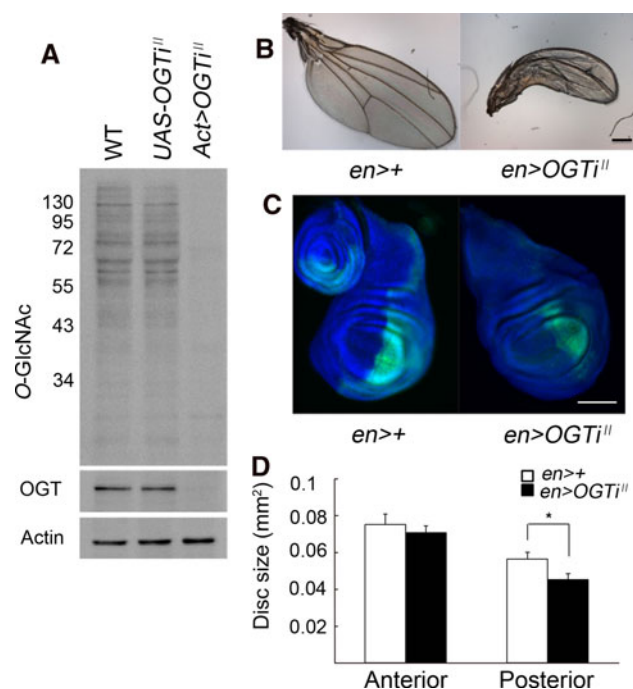


Fig. 2 RNAi knock-down of *OGT* decreases *Drosophila* body growth. **a** Knock-down efficiency of the *OGT RNAi^{II}* transgene was analyzed by Western blotting with anti-*O*-GlcNAc RL2 antibody. β -actin was used as a loading control. **b** Selective expression of *OGT RNAi^{II}* in the posterior compartment of the wing disc using the *en-GAL4* driver results in a shrunken, posteriorly bent adult wing. **c** Growth reduction in the posterior compartment of the wing disc of an *en>OGT RNAi^{II}* larva. The GFP-positive region is the Engrailed expression domain (*dorsal is up, anterior is left*). **d** Measurement of the anterior and the posterior compartments of the wing discs of *UAS*-only ($n = 13$) and *en>OGT RNAi^{II}* ($n = 15$) male larvae. Data are mean \pm SE * $p < 0.05$ by Student's *t* test. **b, c** Scale bars represent 100 μ m

36.5% compared to that of the *UAS*-only control larvae (Supplementary Fig. 2). Selective expression of *OGT RNAi^{II}* in the posterior half of the wing discs using *engrailed-GAL4* (*en-GAL4*) not only resulted in growth retardation in this domain, but also in a posteriorly bent wrinkled wing (Fig. 2b–d). Since the *OGT RNAi* phenotype is the reverse of the *OGA RNAi* phenotype, and the NButGT-fed flies are a phenocopy of the *OGA RNAi* flies, this indicates that the RNAi phenotypes of *OGA* and *OGT* are gene-specific and not due to an off-target effect. Thus, decreasing the cellular *O*-GlcNAcylation levels results in decreased body growth of *Drosophila*.

The effects of *O*-GlcNAcylation on body growth are mainly due to cell growth

The decrease in body growth in *OGT RNAi* flies may be due to decreased cell proliferation, decreased cell growth, increased cell death, or any combination of the three. We examined cell proliferation using anti-phospho-histone H3

antibody, which stains dividing cells in M phase [17]. When *OGA RNAi^X* or *OGT RNAi^{II}* was expressed in the posterior compartment of the wing disc, no significant changes in the phospho-histone H3 staining were detected between RNAi-expressing discs and controls or between the anterior and the posterior compartments of the *en>RNAi* discs (Fig. 3a, b; data not shown). To determine the apoptotic cell death rate, we analyzed active caspase-3 immunostaining in the wing discs [18]. Only a slight increase in apoptosis in the *OGT RNAi^{II}*-expressing wing disc domain was found (Fig. 3c, d). Thus, apoptosis may contribute to the altered body size, but it is likely not a major cause (see below). We then assessed cell growth by comparing cell numbers in a defined area of a particular lobe of the fat body from control and RNAi-expressing larvae. The average size of fat body cells of *Act>OGA RNAi^X* larvae was increased by 33.1% compared to the *UAS*-only control larvae, whereas that of *Act>OGT RNAi^{II}* larvae was reduced by 20.1% (Fig. 3e, f; also compare Fig. 4h, k to b, e, respectively). The number of wing hairs in a defined area of the *Act>OGA RNAi^X* flies was 87.9% of that in *UAS*-only control flies (Supplementary Fig. 3A, B), indicating that impairment of cell growth was a major factor for the decreased body size. To validate these findings further, we expressed *OGA RNAi^X* or *OGT RNAi^{II}* transgenes post-mitotically in a small patch of cells in an otherwise wild-type background using the FLP/FRT-based “flip-out” technique [19]. During the larval stage, most cells, including the fat body cells, grow in size through endoreplication but do not proliferate. By expressing the RNAi transgenes from early to mid-larval stages and simultaneously marking those cells with GFP, we could determine the effect of *O*-GlcNAcylation exclusively on cell growth. *OGA RNAi^X*-expressing cells were larger than neighboring wild-type cells (Fig. 3g; see Supplementary Fig. 3 for quantification), whereas *OGT RNAi^{II}*-expressing cells were markedly smaller than neighboring wild-type cells (Fig. 3h). Together, this demonstrates that the effect of *O*-GlcNAcylation on body growth is mainly through cell growth.

O-GlcNAcylation enhances insulin signaling activity

The changes in body growth and in eclosion time observed in *OGA* and *OGT RNAi* flies are highly reminiscent of the phenotypes of insulin signaling mutants [20]. We reasoned that the *O*-GlcNAcylation level may influence insulin signaling activity and analyzed it by examining dFOXO in the fat body. dFOXO is a negative regulator of the insulin signaling pathway and is translocated to the nucleus when insulin signaling is inactive [13]. Fat body cells of *UAS-OGA RNAi^X*-only (Fig. 4a–c) or *UAS-OGT RNAi^{II}*-only (Fig. 4d–f) control larvae showed stronger nuclear to

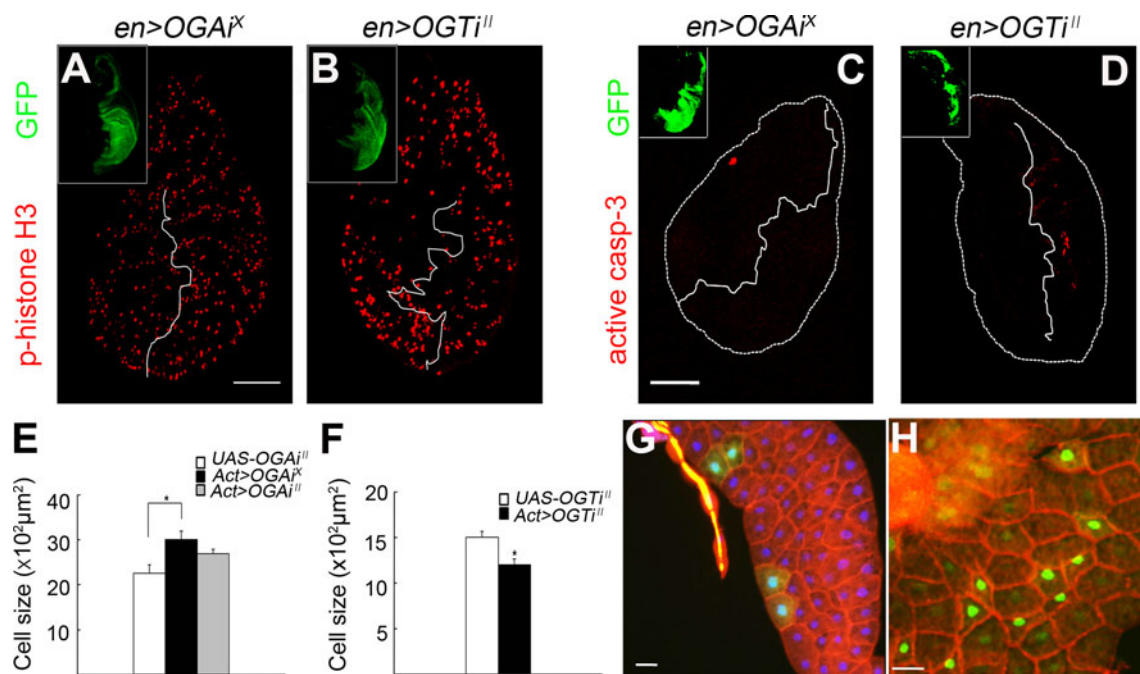


Fig. 3 The effect of *O*-GlcNAcylation on body growth is mainly due to cell growth and not proliferation. **a, b** Demonstration of proliferating cells in the larval wing discs using anti-phospho-histone H3 antibody (red fluorescence). The Engrailed expression domain was labeled with GFP (green in insets). **a** *en>OGA RNAi^X*. **b** *en>OGT RNAi^{fl}*. Scale bar represents 100 μm . **c, d** Demonstration of cells undergoing apoptosis in the larval wing discs using anti-active caspase-3 antibody (red fluorescence). The Engrailed expression domain was labeled with GFP (green in insets). **c** *en>OGA RNAi^X*. **d** *en>OGT RNAi^{fl}*. Scale bar represents 100 μm . **e, f** Fat body cell size of *Act>OGA RNAi* female and *Act>OGT RNAi^{fl}* male larvae. For a fair comparison, stage-matched third instar larvae were dissected,

and the fat body lobe located closest to the gonad was used for size comparison. Cell number was counted in a defined area of the lobe. Data are mean \pm SE **p* < 0.01. **g, h** Post-mitotic effects of *O*-GlcNAcylation on cell growth. *OGA* or *OGT* was knocked down using the flip-out method by applying a 10-min heat shock (34°C) 48 h after egg laying. **g** Genotype: *hsFLP¹²²/UAS-OGA RNAi^X; +/+; Act5C>CD2>GALA UAS-GFP.nls/+*. See Supplementary Fig. 3 for quantification. **h** Genotype: *hsFLP¹²²/+; UAS-OGT RNAi^{fl}/+; Act5C>CD2>GALA UAS-GFP.nls/+*. *OGA* or *OGT RNAi*-expressing cells were labeled with nuclear GFP (green), and the cell boundary was visualized using phalloidin-rhodamine (red). Scale bar represents 50 μm

cytosolic staining for dFOXO. In contrast, fat body cells of *Act>OGA RNAi^X* larvae showed nuclear dFOXO staining equal to cytosolic staining (Fig. 4g–i). This indicates enhanced insulin signaling activity in *OGA* knock-down larvae. Conversely, fat body cells of *Act>OGT RNAi^{fl}* larvae showed a marked increase in nuclear staining and almost no cytosolic staining of dFOXO (Fig. 4j–l; note also the decrease in cell size).

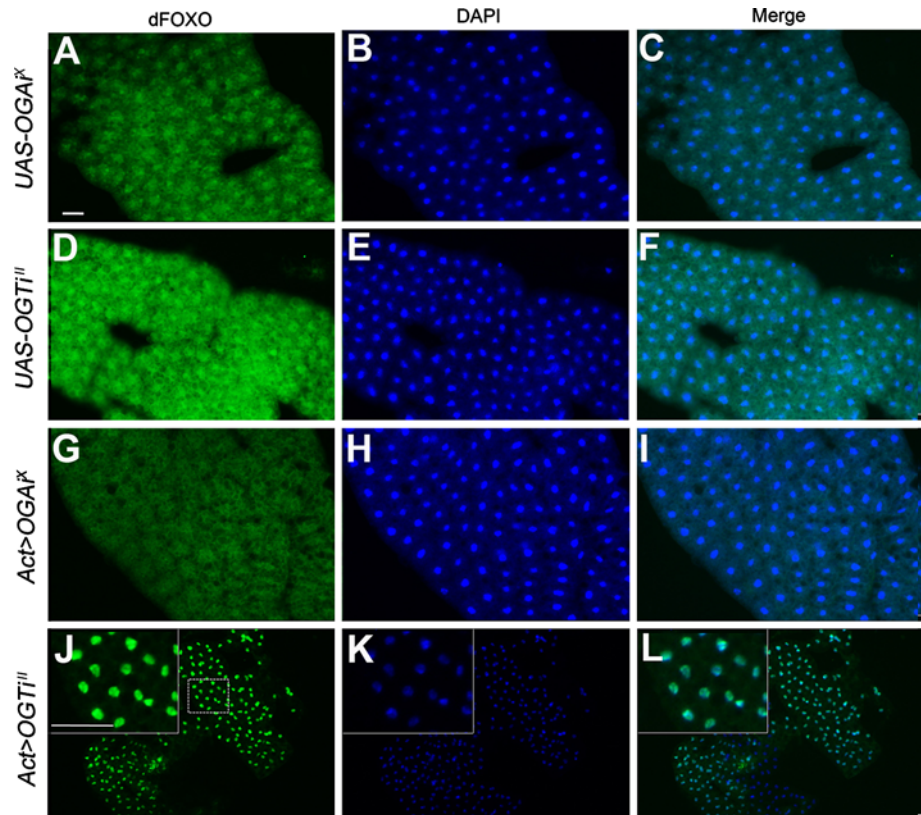
We next analyzed Akt phosphorylation using an antibody that detects phospho-Ser505, which corresponds to phospho-Ser473 of mammalian Akt. Compared to *UAS*-only controls, *Act>OGA RNAi^X* larvae had an increased ratio of phospho-Akt to total Akt (Fig. 5a, b). Conversely, *Act>OGT RNAi^{fl}* larvae had a substantially decreased ratio of phospho-Akt to total Akt. Thus, the activity of insulin signaling is modulated by the cellular *O*-GlcNAcylation level.

We then examined whether increased cellular *O*-GlcNAcylation enhances Akt kinase activity as a correlate to the phosphorylation state of Ser505 of Akt. Since no

antibody that effectively immunoprecipitates *Drosophila* Akt was available, we assessed Akt kinase activity using phospho-(Ser/Thr) Akt substrate antibody [21]. Phosphorylation of Akt substrate proteins was markedly increased in *Act>OGA RNAi^X* larvae and substantially decreased in *Act>OGT RNAi^{fl}* larvae (Fig. 5a). To verify that *O*-GlcNAcylation affects insulin signaling activity in a cell-autonomous manner, we overexpressed V5-tagged OGT in S2 cells. This increased insulin-induced Akt phosphorylation up to 2.2-fold (Fig. 5c, d).

Based on findings in mammals, we predicted Akt a candidate protein for *O*-GlcNAcylation [7, 8, 10, 22]. Indeed, FLAG-tagged *Drosophila* Akt was modified with *O*-GlcNAc, and its *O*-GlcNAcylation was substantially increased by OGT overexpression and inhibition of *O*-GlcNAcase (Fig. 5e). Thus, increased *O*-GlcNAcylation of Akt in vitro correlates not only with its phosphorylation state (Ser505) but also with its kinase activity in vivo, suggesting that modulation of insulin signaling and body growth occurs, at least in part, through *O*-GlcNAcylation of Akt.

Fig. 4 *O*-GlcNAcylation inhibits nuclear localization of dFOXO in the fat bodies. Fat bodies of third instar larvae of *UAS-OGA RNAi^X* only (a–c), *UAS-OGT RNAi^{II}* only (d–f), *Act>OGA RNAi^X* (g–i), and *Act>OGT RNAi^{II}* (j–l). a, d, g, and j dFOXO immunostaining (green). b, e, h, and k DAPI staining (blue) for nuclei. c, f, i, and l Merged images. The insets in j–l show the area marked with a dashed line in j at higher magnification. Scale bars represent 50 μ m



Discussion

Our data demonstrate that *O*-GlcNAcylation is a key post-translational protein modification that controls insulin signaling in *Drosophila*, which is in agreement with previous findings in *C. elegans* and mice [2, 3, 6, 7] and a report published after completion of our manuscript [23]. However, the mechanism of *O*-GlcNAcylation-mediated modulation of *Drosophila* insulin signaling is different from the mechanism observed in worm and mice. Whereas *O*-GlcNAcylation in *C. elegans* and mice suppresses insulin signaling [2, 7], *O*-GlcNAcylation in *Drosophila* enhances insulin signaling, indicating species-specific differences in this evolutionarily conserved mechanism.

How does *O*-GlcNAcylation affect insulin signaling activity and body growth in *Drosophila*? Our results suggest that *O*-GlcNAcylation of Akt and possibly of its upstream signaling components modulates the activity of insulin signaling and consequently of growth in *Drosophila* during development. This is based on the fact that increased cellular *O*-GlcNAcylation is paralleled by increased Akt phosphorylation and kinase activity and decreased nuclear localization of dFOXO and vice versa. We found that modulation of cellular *O*-GlcNAcylation by *OGT* or *OGA* RNAi knock-down increases *Drosophila* body size mainly through cell growth and perhaps apoptosis, but not proliferation. Mutations in different insulin

signaling components have been shown to influence growth by different mechanisms. For example, PI3K and the upstream components of the PI3K pathway are necessary for both cell growth and proliferation, whereas components downstream of PI3K are necessary for cell growth, but not for cell proliferation [20, 24]. Thus, regulation of Akt by *O*-GlcNAcylation appears to be a key component in the insulin signaling pathway of *Drosophila*.

Protein modification by *O*-GlcNAc and *O*-phosphate may have antagonistic effects on each other [1, 25]. However, the two modifications may not be mutually exclusive since they may occur simultaneously at different sites. Treatment with insulin-like growth factor-1 increases both phosphorylation and *O*-GlcNAcylation of Akt1 in SH-SY5Y neuroblastoma cells [8]. Studies on glutathione peroxidase 1 (GPX1) under hyperglycemic conditions suggest that *O*-GlcNAcylation on GPX1 increases GPX1 phosphorylation and consequently enhances the activity of the protein [26]. To better understand the significance of Akt *O*-GlcNAcylation, further investigations, including identification and manipulation of *O*-GlcNAc modification sites, are warranted.

While our present data suggest Akt *O*-GlcNAcylation is the cause of the growth change, it is also possible that the growth changes in *OGT* or *OGA* RNAi flies result from changes in the production of insulin-like growth factors or larval feeding behavior in a cell non-autonomous manner.

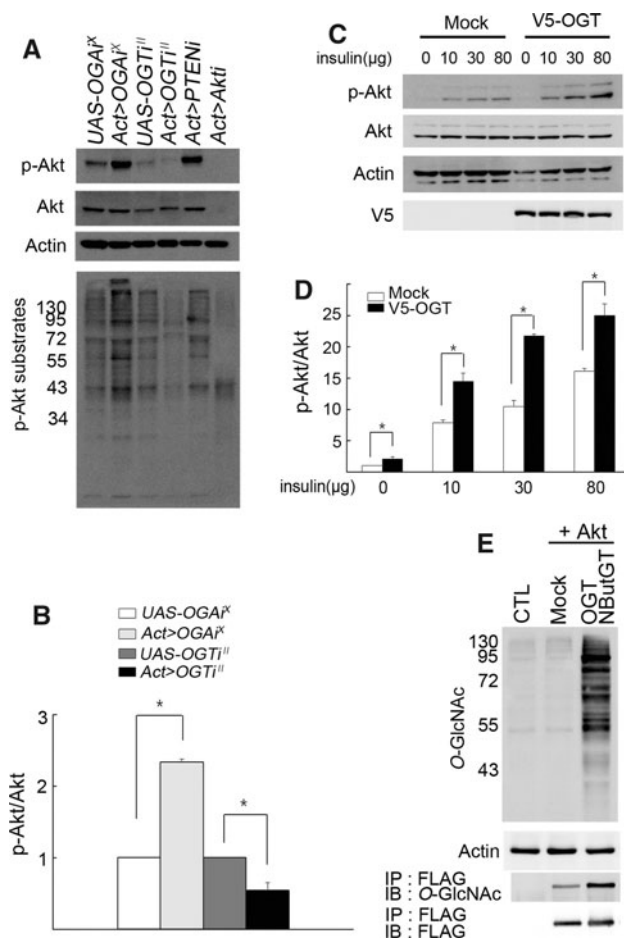


Fig. 5 O-GlcNAcylation increases the level of Akt phosphorylation and Akt is O-GlcNAcylated. **a** Phosphorylation and kinase activity of Akt in *OGA RNAi^{RX}* or *OGT RNAi^{II}* larvae as analyzed by Western blot. Akt phosphorylation was examined using anti-phospho-(Ser505) Akt and anti-Akt antibodies. β -actin was used as a loading control. To assess Akt kinase activity, phosphorylation state of Akt substrates in larval lysates of the indicated genotypes was analyzed using phospho-Akt substrate antibody. **b** Quantification of the ratio of phospho-Akt to total Akt. For reason of comparison with *Act>OGA^{RX}* and *Act>OGT^{II}*, the values of the *UAS*-only controls were arbitrarily set to 1. Experiments were performed in triplicate. Data are mean \pm SE $*p < 0.025$. **c** The effect of OGT overexpression on insulin-induced Akt phosphorylation in S2 cells. Five milliliters of *Drosophila* S2 cell culture was treated with 10, 30, or 80 μ g of insulin and incubated for 15 min before Western-blot analysis. β -actin was used as a loading control. **d** Quantification of the results in **c**. Experiments were done in triplicate. Data are mean \pm SE $*p < 0.05$. **e** O-GlcNAcylation of Akt. FLAG-tagged Akt was expressed in S2 cells either alone or together with *Drosophila* OGT and 50 μ M of NBuGT. Total O-GlcNAcylation level was examined using anti-O-GlcNAc RL2 antibody. β -actin was used as a loading control. To demonstrate O-GlcNAcylation of Akt, Akt was immunoprecipitated using anti-FLAG antibody followed by Western blotting with anti-O-GlcNAc antibody CTD110.6. For control, the same immunoprecipitates were blotted with anti-FLAG antibody

However, we believe that a direct modification of the insulin signaling component is the main cause. Namely, *en-GALA*-driven *UAS-OGT RNAi* decreased the growth of

the wing imaginal discs specifically in the Engrailed expression domain. Furthermore, expression of *OGA* or *OGT RNAi* in small cell patches resulted in cell-size changes in the RNAi-expressing cells only. The degree of increase of cell size in the fat body cells of *Act>OGA RNAi* animals was comparable to that in the cell-patch experiments and similar results were obtained with *OGT RNAi*. Last but not least, OGT overexpression increased insulin-induced Akt phosphorylation in S2 cells. Thus, the effects of *OGA* or *OGT RNAi* occur largely in a cell-autonomous manner. We also believe that the observed phenotypes are not a secondary consequence of abnormal epigenetic regulation, despite that both OGT and *OGA* may exert their actions as chromatin remodeling factors [11, 12, 27]. This is related to the known relationship between insulin signaling and growth, and that several components of the insulin signaling pathway are modified by O-GlcNAcylation [7–9, 28].

Our present findings point to the intriguing possibility that O-GlcNAcylation is regulated during *Drosophila* development, which would add another layer of complexity to the regulation of OGT [1]. Therefore, it will be interesting to investigate the regulation of the protein O-GlcNAcylation machinery, including the hexosamine signaling pathway, during development and in various diseased states.

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Conflict of interest The authors declare no conflict of interest.

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