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氏名（本籍）	ｼｭｲ　ﾁｳｪｲ 徐　　志偉（中国）
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論文審査委員	主査　　教 授　井ノ口　仁 一
	副査　　教 授　関　政 幸
	副査　　教 授　顧　　建 国

Importance of *O*-GlcNAcylation in cell adhesion and migration

東北医科薬科大学大学院薬学研究科

徐　　志偉

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Importance of *O*-GlcNAcylation in cell adhesion and migration

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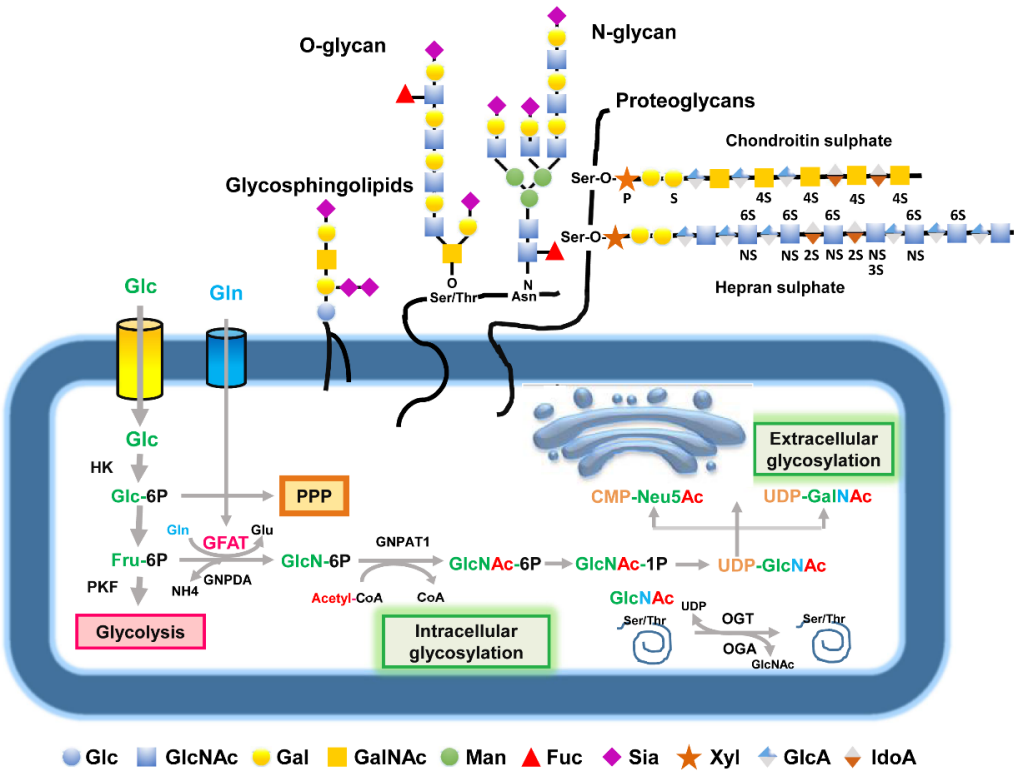
1. Introduction

Glycosylation participates in many important biological processes including cell growth, migration, adhesion, differentiation, signal transduction and receptor activation (1). Notable among the emerging glycosylations, *O*-GlcNAcylation has garnered considerable interest during the last few decades (2). *O*-GlcNAcylation is the product of glucose influx through the hexosamine biosynthetic pathway (HBP), which integrates glucose, amino acid, fatty acid and nucleotide to serve as the donor substrate for *O*-GlcNAcylation, uridine diphosphate GlcNAc (UDP-GlcNAc) (3). *O*-GlcNAcylation is controlled by *O*-GlcNAc transferase (OGT) and is a specific type of post-translational modification that consists of the covalent attachment of single GlcNAc to the nucleus and

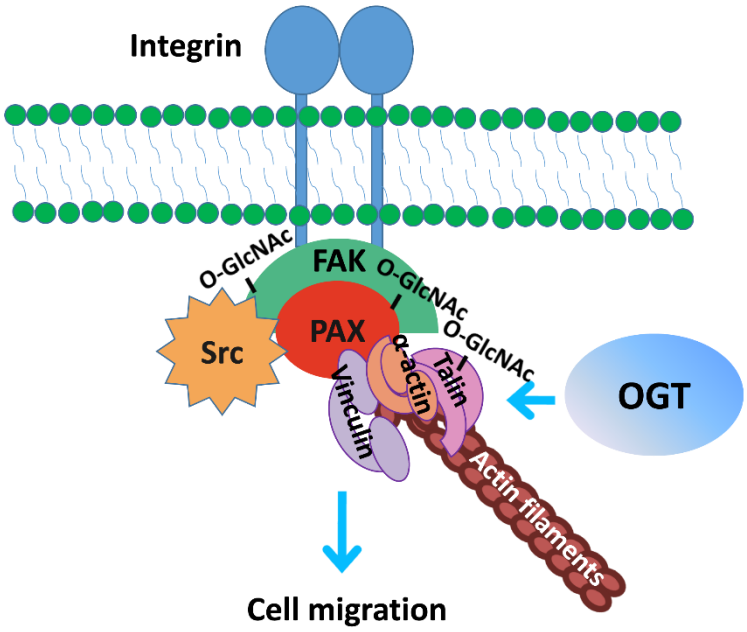


The synthesis of *O*-GlcNAcylation
(Refered to Vasconcelos-Dos-Santos A. et al. Front Oncol, 2015, 00138.)

cytoplasm of the serine or threonine residue of an extremely large family of target proteins (4,5). This post-translational modification is essential for cell survival and division (6), and aberrant *O*-GlcNAcylation provokes tumorigenesis, diabetes, and Alzheimer's disease (AD) by regulating cell signaling, transcription, metabolism, and cytoskeletal formation (7-10). The increased *O*-GlcNAcylation seems to be a general characteristic of cancer cells. For example, higher levels of *O*-GlcNAcylation expression have been observed in cancers of the liver (11), lung, colon (12), and breast (13). Furthermore, numerous breast cancer cell lines have shown higher levels of *O*-GlcNAcylation, and the levels of OGT expression in aggressive breast cancer cell lines are much higher than those seen in less aggressive breast cancer cell lines (14). *O*-GlcNAc modifications have also been observed in important target proteins such as p53(15), HIF-1α (16), β-catenin (13), and G6PD (17), which are involved in the regulation of malignant cancer characteristics by controlling cellular metabolism and proliferation. On the other hand, the suppression of OGT expression in breast or liver cancer cell lines decreases cell motility, which suggests that *O*-GlcNAcylation could be involved in cell migration (13,18).

Cell migration is a highly integrated multistep process that includes the development of cytoplasmic protrusions, attachment and spreading (19). The migratory capacity of cancer cells is initially mediated by alterations in the expression of cell-surface molecules known as integrins (20). It is becoming increasingly clear that integrins are crucial for cell migration in the tumor microenvironment (21). Following ligand binding, integrins cluster into focal contacts that contain different focal adhesion (FA)-associated proteins such as α-actinin, vinculin, talin, focal adhesion kinase (FAK), and paxillin, which link the integrins to the actin cytoskeleton (22). The processes of adhesion formation and disassembly drive the migration cycle through ligand binding, which in turn regulates

integrin activity and cytoskeletal complex formation, as well as adhesion dynamics (23). *O*-GlcNAcylation appears to occur in actin cytoskeletal regulatory proteins such as paxillin (24) and talin (25), as well as in microtubule-assembly proteins such as tubulin (26) and in microtubule-associated proteins (27). However, whether and how *O*-GlcNAcylation impacts cell migration remains unclear.



Proposed molecular mechanism of FA complex regulated by O-GlcNAc

In the present study, we used the doxycycline shRNA-inducible system to knockdown the OGT gene to identify the biological functions of *O*-GlcNAcylation and its regulatory mechanisms in cell adhesion and migration. We found that the knockdown of OGT aberrantly increased cell adhesion, FA formation, and integrin β1 activation, which in turn decreased cell migration. Thus, our findings may provide new insight into integrin-mediated cell migration and explain why *O*-GlcNAcylation is usually highly expressed in some malignant cancers.

2. Materials and methods

2.1 Antibodies and reagents

Experiments were performed using the following antibodies: mAb against *O*-GlcNAc (CTD110.6, 9875S) and peroxidase-conjugated secondary antibody against rabbit (7074S) from Cell Signaling Technology; the rabbit polyclonal antibody against OGT (O0164), mAb against α-tubulin (T6199) and VSV (V5507) from Sigma; mAb against integrin β1 (610468) and paxillin (610052) from BD Biosciences; mAb against active integrin β1 (HUTS-4; 2079Z) and peroxidase-conjugated secondary antibodies against mouse (AP124P) and goat (AB324P) from Millipore; Alexa Fluor 488 conjugated anti-mouse (A11029) from Invitrogen; TO-PRO-3 (T3605) from Molecular Probe; GFP-agarose (MBL, D153-8) and goat antibody against GFP (Rockland, 600-101-215). The mAb against human β1 (P5D2) was obtained from Developmental Studies Hybridoma Bank, University of Iowa, USA. Human fibronectin (FN) and doxycycline hyclate (D9891) was from Sigma-Aldrich. An ABC kit was acquired from Vector Laboratories, and Ab-Capcher Mag was from ProteNova (Takamatsu, Japan).

2.2 Cell culture and expression plasmids

HeLa and 293T cell lines (RIKEN, Japan) were maintained at 37 °C in DMEM high glucose (Invitrogen) supplemented with 10% FBS under a humidified atmosphere that contained 5% CO_2. To express GFP-tagged talin (28) and 2X VSV-tagged FAK, expression vector pEGFP-N1-talin-GFP (Addgene 26724) and pRKVSV-FAK were

kindly provided from Dr. Anna Huttenlocher (28) and Dr. Kenneth Yamada (29), respectively. The pcDNA3.1/myc-his expression vector containing human OGT was kindly provided by Dr. Yuanyuan Ruan (School of basic medical sciences, Fudan University, China). Transfection was performed using PEI MAX (molecular weight, 40 kDa; Polysciences Inc., PA) and following the dictates of the U.S. patent document (US20110020927A1) with minor modifications. Briefly, 24 h prior to transfections cells were seeded on a 10 cm dish, and expression vectors with PEI MAX (1 mg/ml in 0.2 M hydrochloric acid) were preincubated for 15 min at a 1:3 ratio in 2,000 µl of a solution that contained 20 mM CH_3COONa buffer, pH 4.0, and 150 mM NaCl. Cells and DNA complexes were further incubated for 24 h with 10 ml normal culture media to promote expression.

2.3 Establishment of doxycycline-inducible OGT knockdown cells

We used CS-RfA-ETBsd doxycycline (DOX)-dependent inducible RNA interference mediated by a single lentivirus vector (RIKEN) for the knockdown experiment (30). The following oligonucleotides were inserted into pENTR/H1/TO (sense, CACCGCTGAGCAGTATTCCGAGAAACTCGAGTTTCTCGGAATACTGCTCAGC C, antisense, AAAAGGCTGAGCAGTATTCCGAGAAACTCGAGTTTCTCGGAATACTGCTCAG C) with minor modification from a procedure established in a previous report (16). Using LR clonase, inserted oligo was then transferred to CS-RfA-ETBsd, which encodes tetracycline-dependent trans-activators for shRNA expression. To prepare the viruses, PEI MAX was used to transfect the resultant vector into 293T cells with packaging plasmids. HeLa and 293T cells were then infected by the obtained viruses and selected for stable

integration with 10 μg/ml of blasticidin. The shRNA-mediated silencing of OGT was induced by the addition of DOX in the established cell line, and the cells cultured by DOX-free medium were used as the control in the present study.

2.4 Immunoprecipitation and Western Blot

The cells were washed with PBS, and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) with protease and phosphatase inhibitors (Nacalai Tesque, Japan). The supernatants were collected, and the protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, IL). Equal amounts of proteins were subjected to SDS-PAGE and then transferred to PVDF membranes. To detect active integrin β1, we prepared samples under non-reducing conditions. The membranes were blocked either with 5% nonfat milk in TBST or with 3% BSA for 2 h at room temperature, and then the proteins were probed with antibodies against *O*-GlcNAc, OGT, α-tubulin, active integrin β1 (HUTS-4) (31,32), integrin β1 (Millipore), paxillin, VSV, and GFP. After being washed, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Detection was accomplished using an HRP substrate (Millipore) according to the manufacturer's instructions. For immunoprecipitation, the supernatant (500 μg of protein) was incubated with an anti-VSV or an anti-paxillin with an Ab-Capcher Mag. GFP-talin was immunoprecipitated with GFP-conjugated beads. The immunoprecipitates were washed with lysis buffer and subjected to SDS-PAGE. The immunocomplexes then were detected using the indicated antibodies. A mAb against α-tubulin was used as the loading control.

2.5 Cell adhesion Assay

Cell adhesion assays were performed in a 96-well CellCarrier (PerkinElmer, Japan) coated with FN (5 μg/ml) overnight. HeLa cells were pre-treated with or without DOX (0.1μg/ml) for 24 hours. Cells were re-plated at a density of 10^4 cells/well in plates using serum-free DMEM with 0.1% BSA, followed by incubation at 37 °C for 20 min. Non-adherent cells were removed by washing 3 times with PBS. Cells were fixed with 4% formaldehyde and stained DAPI (Invitrogen), and were then imaged by fluorescent microscopy using an Operetta CLS (PerkinElmer, Japan). To count the number of nuclei in the each well, images were analyzed using Harmony software (PerkinElmer).

2.6 Immunofluorescence

Cells were plated onto FN-coated glass coverslips (MatTek Corporation, Ashland, MA) for 1 h, washed with PBS, and fixed with 4% PFA. For permeabilization, the cells were treated with 0.1% Triton X-100 in PBS. The cells were blocked with 0.1% Tween 20 and 3% BSA in PBS and then stained with paxillin, active β1 (HUTS-4), total β1 (P5D2), and OGT antibodies overnight at 4 °C. The samples were followed by incubation with anti-mouse Alexa Fluor 488-conjugated secondary antibody, and were then incubated with TO-PRO-3. Images were acquired by sequential excitation using an Olympus FV1000 laser scanning confocal microscope with an UPlanSApo 60X/1.35 Oil objective and high sensitivity Gallium arsenide phosphide detector units operated by F10-ASW ver. 4.02 software. In order to count the number of FAs, we followed a protocol previously described using ImageJ (33), excluding focal adhesions that were less than 0.2 μm^2, because these disappeared quickly (34). OGT-overexpressing cells were identified via co-immunostaining with OGT. The relative fluorescence intensities of active integrin β1 and total integrin β1 were quantified using ImageJ software.

2.7 Video microscope

Glass-bottom dishes (Asahi Glass, Shizuoka, Japan) were pre-coated with FN (10 μg/ml) in PBS, let stand at 4 °C overnight, and were then blocked with 1% BSA. Ten Thousand cells were suspended in 2 ml of DMEM containing 3% FBS medium, which was then added to each FN-coated glass-bottom dish and monitored for 12 h using AxioVision equipment (Carl Zeiss, Germany). Images were acquired using an inverted microscope (Axio Observer.D1; Carl Zeiss) every 10 min with 5% CO_2 at 37 °C in a heated chamber equipped with temperature and CO_2 controllers (Onpu-4 and CO_2; AR BROWN, Tokyo, Japan) during time-lapse imaging. Cell motility was evaluated using an AxioVision Tracking module (Carl Zeiss).

2.8 Chemoenzymatic labeling assay

Chemoenzymatic labeling and biotinylation of proteins in cell lysates was carried out using the Click-iT *O*-GlcNAc Enzymatic Labeling System (Invitrogen). Briefly, the whole-cell lysate of 293T cells transfected with an expression plasmid for VSV-FAK or GFP-talin (500 μg), and HeLa cells was immunoprecipitated and then labeled with labeling enzyme GalT and UDP-GalNAz according to Click-iT *O*-GlcNAc Enzymatic Labeling System protocol (Invitrogen). Labeled proteins were conjugated with an alkyne-biotin compound following the Click-iT Protein Analysis Detection Kit protocol (Invitrogen). Control experiments were performed in the absence of GalT and UDP-GalNAz. Biotinylated and control samples were then subjected to SDS-PAGE and transferred to a PVDF membrane for further detection using an ABC kit (Vector Laboratories).

2.9 Statistics

All results shown are the results of at least two independent experiments and are shown as representative data. The values represent the mean ± S.E.M. P-values were calculated using a Welch's correction t-test using GraphPad Prism version 5. (* represents *P*-value < 0.05, ** represents *P*-value < 0.01).

3. Results

3.1 Established OGT knockdown (KD) cells

A growing number of studies have shown that *O*-GlcNAcylation plays a critical role in the regulation of tumor cell growth (14) and cancer metastasis (35,36). To investigate the effects of *O*-GlcNAc expression on cell adhesion and migration, we used the DOX-dependent inducible shRNA KD system to establish OGT KD HeLa cells. In this cellular system, OGT and *O*-GlcNAc were expressed at normal levels in the absence of DOX, whereas both expressions were drastically suppressed in the presence of DOX in the culture media at indicated concentrations, as shown in Fig. 1A. Furthermore, similar suppression levels were observed even following incubation at the lowest concentration of 0.1 μg/ml after 24 h (Fig. 1B), suggesting an effective KD of OGT and a rapid turnover of *O*-GlcNAc levels in HeLa cells. After culture for 48 hours, elongated cell shapes were converted to a more-rounded morphology, and the KD cells showed significantly increased cell spreading areas compared with those in the control cells (Fig. 1C). These observations suggest the impact that *O*-GlcNAcylation exerts on cell morphology.

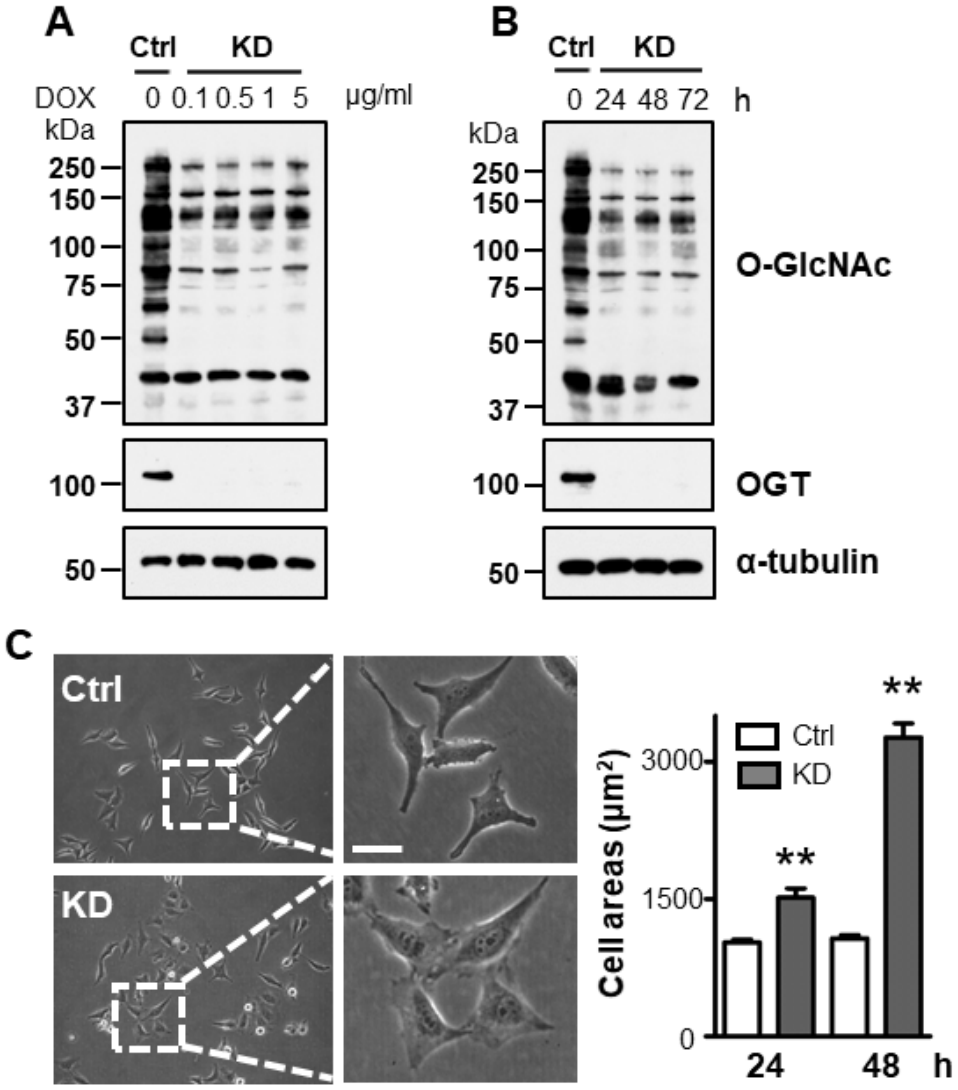


Fig. 1 Knockdown of OGT suppressed *O*-GlcNAcylation and enhanced cell spreading in HeLa cells

A,*B,* The expression levels of OGT and *O*-GlcNAcylation from cell lysates of doxycycline (DOX)-controlled OGT knockdown HeLa (KD) cells were verified with concentrations of DOX at 0, 0.1, 0.5, 1.0, and 5.0 μg/ml for 72 h (A); or at indicated time with 0.1 μg/ml DOX (B). The control (Ctrl) indicates the cells treated without DOX. Cell lysates from the indicated cells were subjected to WB with the *O*-GlcNAc (CTD110.6), OGT and α-tubulin antibodies. *C*, Representative images of cell spreading were shown after incubation for 48 h. Cells were incubated with (KD) or without (Ctrl) 0.1 μg/ml DOX for 24 or 48 h on a normal culture dish, after which the cell areas were measured. Values represent the mean ± S.E.M. (n=50). $^{**}P < 0.01$ (Welch's correction t-test). Scale bars,

15 μm. Experiments were independently repeated at least 2 times

3.2 Knockdown of *O*-GlcNAcylation enhanced cell adhesion and FA formation and suppressed cell motility

Next, we used a fibronectin (FN)-coated dish to investigate the effects of OGT KD on cell adhesion, FA formation, and cell motility. To verify the initial stage of cell adhesion, we performed a 20-min cell adhesion assay on FN. Interestingly, the number of adhered cells was drastically increased in the KD cells compared with that in the control cells (Fig. 2A). During cell adhesion, integrins and cytoplasmic proteins such as paxillin, talin and FAK become clustered in the plane of the cell membrane and in well-developed aggregates, the so-called FA plaque, which can be detected by immunofluorescence microscopy (37). Consistent with their enhancement of cell adhesion, in the present study OGT KD cells also promoted an increase in FA formation, by comparison with the activity in control cells (Fig. 2B). By contrast, the KD cells showed a significant reduction in cell motility, as observed by video microscopy (Fig. 2C). These data indicate that a loss of *O*-GlcNAcylation promotes cell adhesion and focal contact formation, while suppressing cell migration.

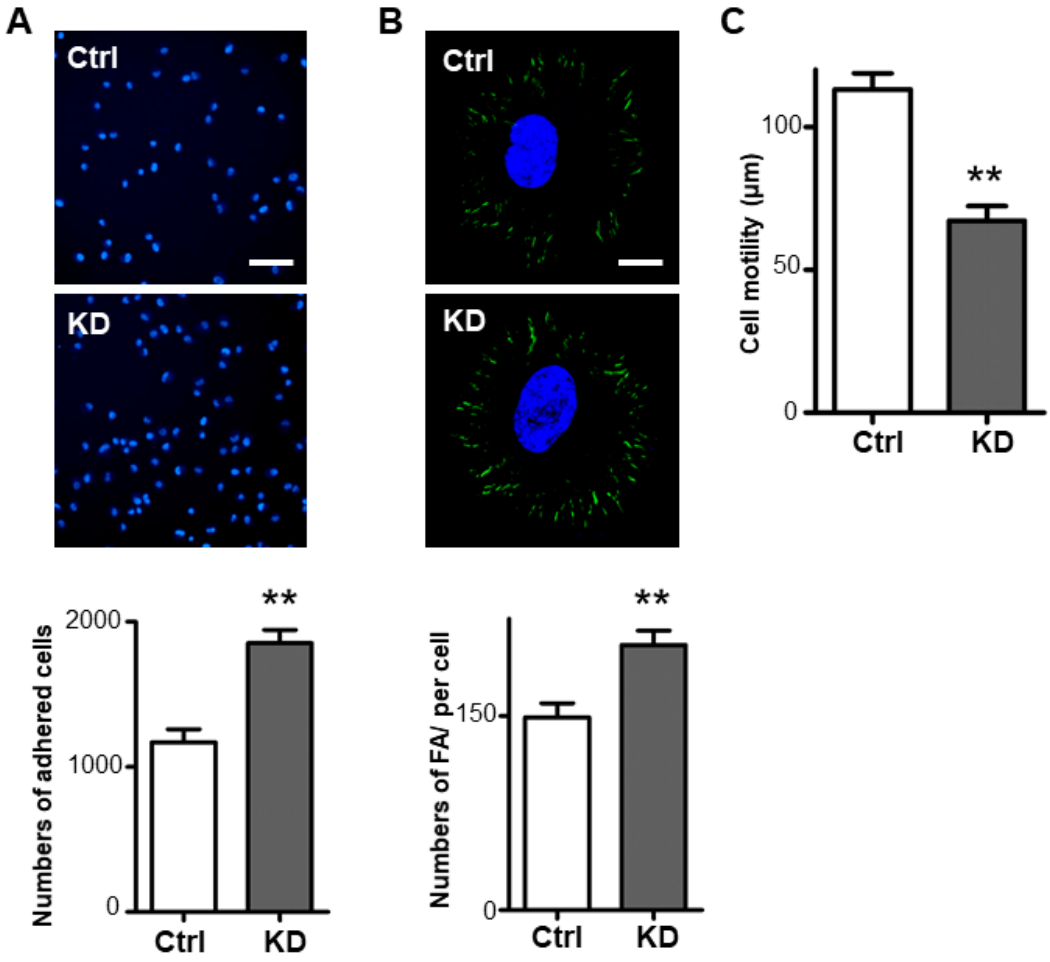


Fig. 2 Reduced *O*-GlcNAcylation promoted cell adhesion and FA formation, but decreased cell migration

HeLa cells were cultured in the presence (KD) or absence (Ctrl) of DOX for 24 h. *A*; Twenty minutes after replating cells onto FN-coated 96-well plates, the attached cells were fixed, and then the nuclei were stained and counted. Representative fields were photographed via fluorescent microscopy. Scale bars, 30 μm. Values represent the mean ± S.E.M. (n=11). ** $P < 0.01$ (Welch's correction t-test). *B*, Cells were allowed to spread on FN-coated coverslips for 1 hour. Cells were then stained with anti-paxillin antibody (green) and TO-PRO-3 (blue). The numbers of focal adhesion were quantified by ImageJ software. Scale bars, 5 μm. Values represent the mean ± S.E.M. (n=11). **$P < 0.01$ (Welch's correction t-test). *C*; Cell motility was observed by time-lapse video microscopy. Values represent the mean ± S.E.M. (n=30). ** $P < 0.01$ (Welch's correction t-test). Experiments were independently repeated at least 2 times.

3.3 Talin, FAK and paxillin were *O*-GlcNAc-modified proteins

Previous studies have revealed that some forms of protein FA plaque such as paxillin and talin are modified by *O*-GlcNAc (24,25). In the present study we investigated whether *O*-GlcNAc modification of those target proteins also occurred in HeLa cells in this system. Consistent with previous studies, *O*-GlcNAc modifications were detected on both paxillin and talin, while *O*-GlcNAcylation levels for both proteins were significantly decreased in KD cells, compared with that seen in control cells (Fig. 3A,B). The post-translational modifications of FAK might control the protein–protein-interaction adaptor functions in cell attachment (38). Importantly, we also found that FAK, a key molecule for integrin-mediated signaling, was also a target protein for *O*-GlcNAcylation, which was decreased in the KD cells (Fig. 3C). The suppression of *O*-GlcNAcylation on FAK and talin was also confirmed in DOX-induced OGT KD 293T cells (data not shown). To further establish the occurrence of *O*-GlcNAcylation in these proteins, we conducted a chemoenzymatic labeling assay using an azido-*N*-acetylgalactosamine sugar, as described in Experimental procedures. Clearly, talin, FAK and paxillin were labeled, which proved they are *O*-GlcNAcylated proteins (Fig. 4 A-C). These results suggest that *O*-GlcNAcylation may affect both integrin β1-mediated complex formation and FA formation, which confirms this process as a regulator of cell adhesion and migration.

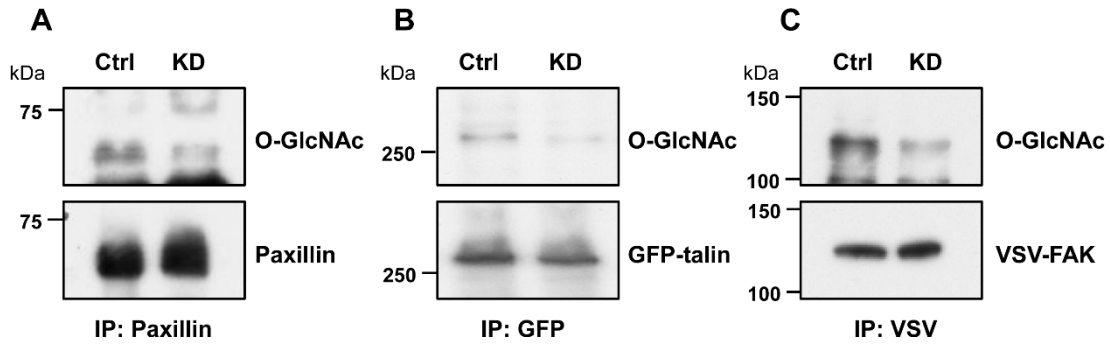


Fig. 3 Decreased *O*-GlcNAcylation levels of paxillin, talin and FAK in OGT-KD cells.

HeLa cells (A) or cells transfected with talin (B) or FAK (C) were incubated without (Ctrl) or with (KD) DOX. The cell extracts were immunoprecipitated with indicated antibodies, and western blotted with anti-*O*-GlcNAc or indicated antibodies, respectively. Experiments were independently repeated at least 3 times.

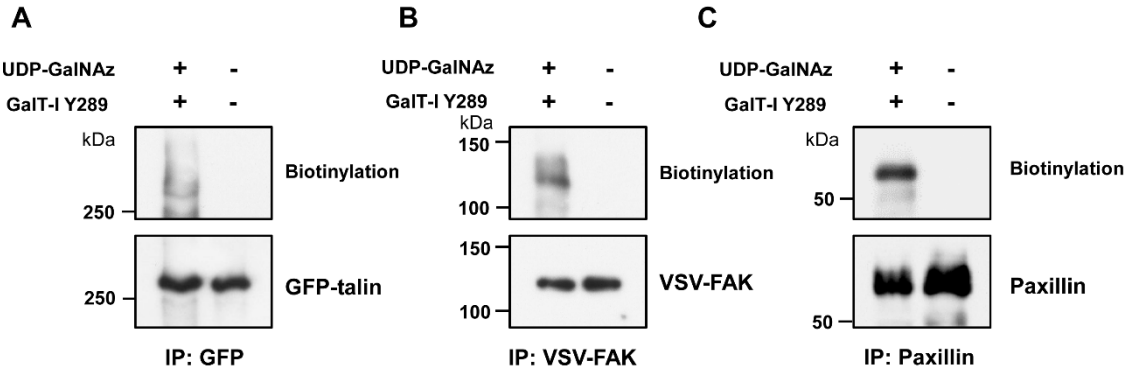


Fig. 4 Confirmation of *O*-GlcNAcylation on talin, FAK and paxillin.

Cell lysates of 293T cells transfected with talin (A), FAK (B) or wild-type HeLa cells (C) were immunoprecipitated with anti-GFP, anti-VSV or anti-paxillin antibodies, respectively, followed by click chemistry labeling of *O*-GlcNAc residues with (+) or without (-) GalT and UDP-GalNAz, and were detected using an ABC kit, as described in "Experimental procedures". Experiments were independently repeated at least 2 times.

3.4 Reduction of *O*-GlcNAcylation promoted complex formation

FAK is a key component of the signal transduction pathways triggered by integrins. When cells bind to the extracellular matrix (ECM), FAK is usually recruited to integrin-mediated nascent FA, because it interacts directly through the cytoskeletal proteins talin and paxillin, with the cytoplasmic tail of integrin β1 (39). Therefore, we compared the ability of control and KD cells to form FA complexes. As shown in Fig. 5A, the complexes immunoprecipitated with anti-FAK antibody showed higher levels of paxillin in KD cells than in control cells. Consistently, KD cells demonstrated a greater number of complex formations composed of both β1 integrin and talin (Fig. 5B) and talin and FAK (Fig. 5C). A similar phenomenon was also confirmed in OGT-KD 293T cells (data not shown).

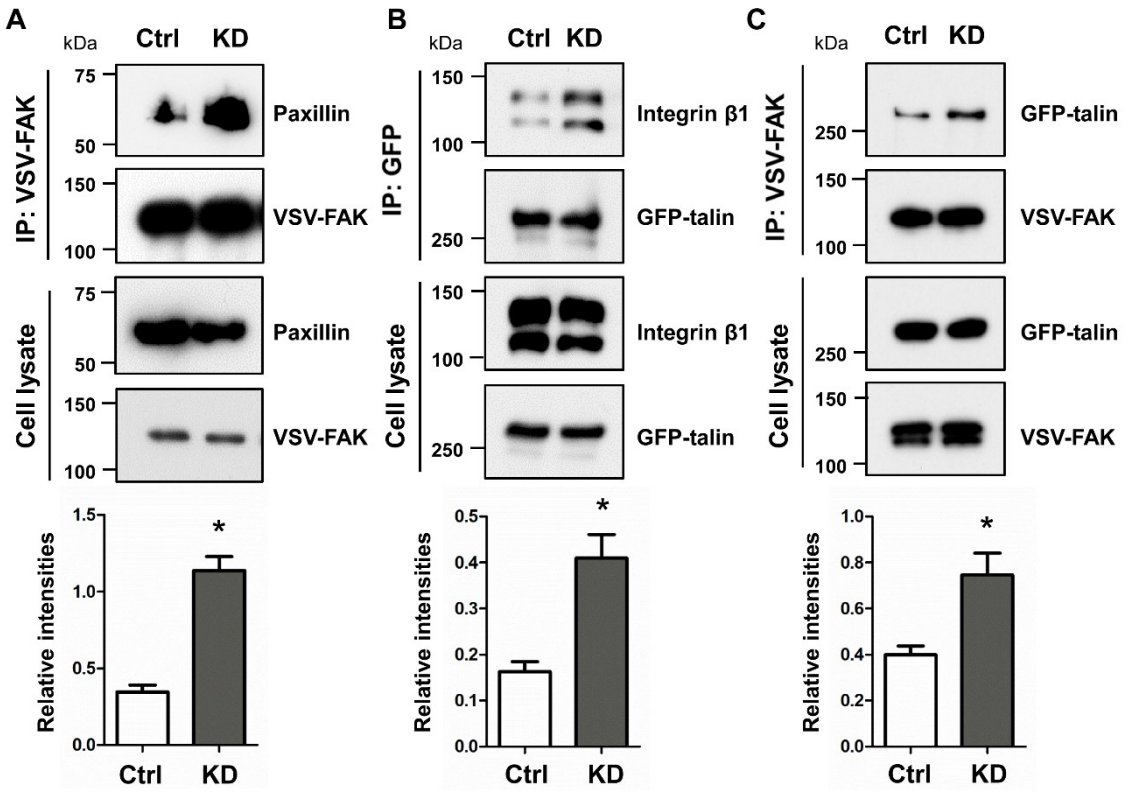


Fig. 5 Increased focal adhesion complex formation in OGT KD cells

Cell lysates from the Ctrl and KD HeLa cells that were transfected with expression plasmids of FAK (A), talin (B) or both FAK and talin (C), were immunoprecipitated by indicated antibodies and then subjected to Western blotting as described in "Experimental procedures". The relative ratios are shown at the bottom (n=3 individual experiments). Values represent the mean ± S.E.M. *$P < 0.05$ (Welch's correction t-test). Cell lysates were used as input. Experiments were independently repeated at least 3 times.

3.5 Knockdown of *O*-GlcNAcylation activated integrin β1

Given the increase in FA complex formation in KD cells, it is reasonable to speculate that OGT-KD may affect integrin activation. Integrin β1 whose conformation promotes ligand binding and appropriate affinity are said to be in active state. Integrin-mediated adhesion can recruit FA proteins to form FA plaque, and then trigger conformational activation, so-called inside-out signaling, of integrin β1 in the ectodomain, which then can be recognized by a specific antibody (40,41) that we used to examine the expression levels of active integrin β1 in both control and KD cells. The expression levels of active β1 in immunostaining (Fig. 6A) or cell lysates (Fig. 6B) were clearly upregulated in the KD cells compared with control cells. In contrast to KD cells, the expression levels of active β1 were suppressed in the OGT-overexpressing HeLa cells, which further suggested that *O*-GlcNAcylation negatively regulates integrin-mediated inside-out signaling. Thus, we were convinced that OGT could be a novel regulator for FA complex formation and integrin activation by dynamically regulating cell adhesion and migration.

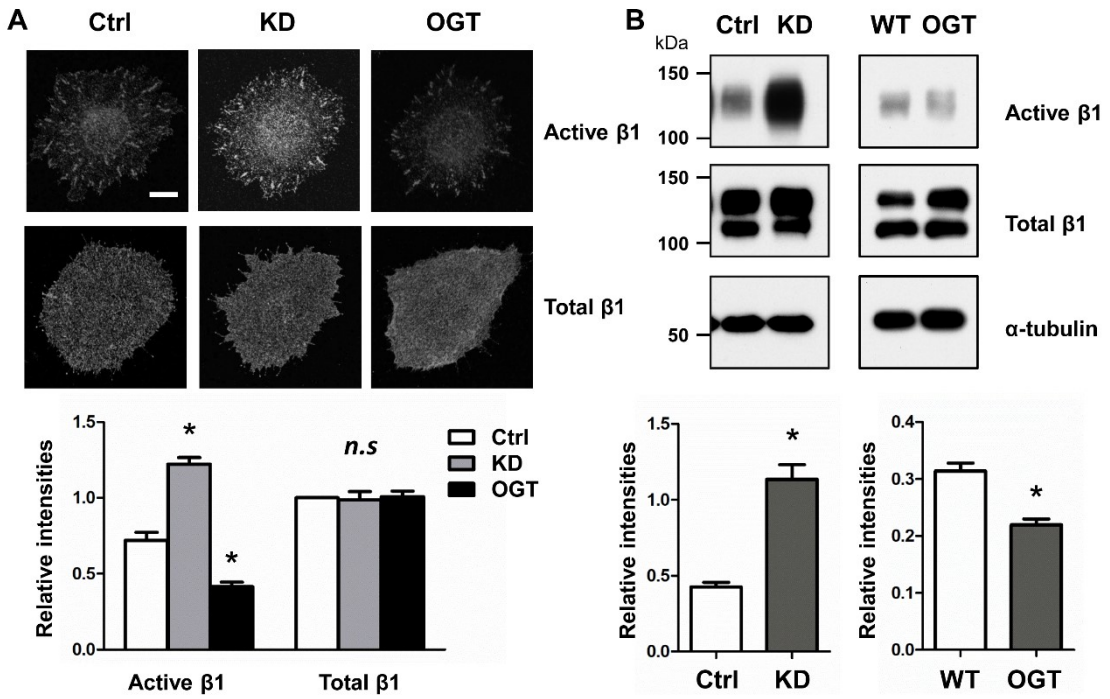


Fig. 6 Comparison of the expression levels of active integrin β1 among the Ctrl, KD and OGT-overexpressing cells

A; A representative immunostaining pattern with anti-active β1 or anti-β1 antibodies in the Ctrl, KD, and OGT-overexpressing (OGT) HeLa cells. Cells were cultured on FN-coated coverslips for 1 h and then subjected to immunostaining analyses. The relative fluorescence intensities of KD and OGT-overexpressing cells were compared with the control, and relative fluorescence intensity was 1.0 for the control cells. Scale bar, 5 μm. *B,* The expression levels of active and total integrin β1 were verified by immunoblotting with the indicated antibodies in Ctrl and KD HeLa cells, or parent (WT) or transiently OGT-overexpressing (OGT) HeLa cells. The relative ratios are shown at the bottom (n=10 random fields of view). Values represent the mean ± S.E.M. *$P < 0.05$ (Welch's correction t-test), n.s, not significant ($P > 0.05$), experiments were independently repeated at least 2 times.

4. Discussion

In the present study, we clearly showed that *O*-GlcNAcylation negatively regulates integrin-mediated cell adhesion and FA complex formation as well as integrin activation, which results in the control of cell migration on the ECM (Fig.7). Our findings are the first to demonstrate that OGT may function as a key regulator of FA complex formation during cell-ECM adhesion. These results provide clues to understanding the roles of *O*-GlcNAcylation in cell migration.

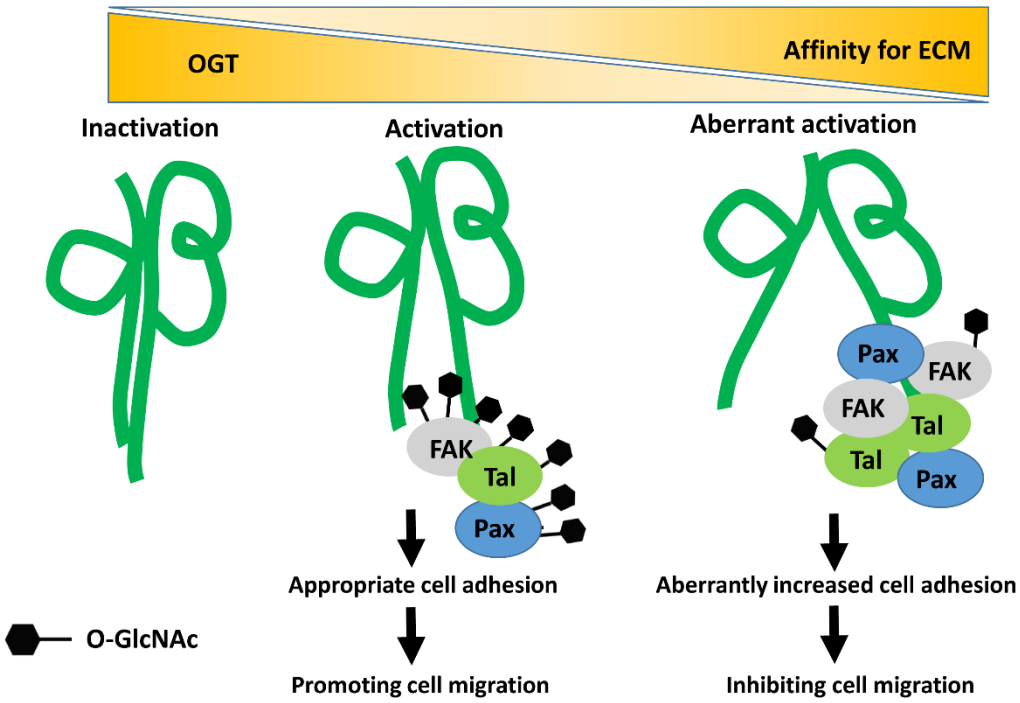


Fig. 7 Proposed molecular mechanism for the regulation of cell adhesion and migration by *O*-GlcNAcylation

Cell migration is a central process in the development and maintenance of multicellular organisms (19). Although the detailed mechanisms underlying cell migration remain unclear, it is reasonable to postulate that integrin-mediated cell adhesion could regulate

migration, which would allow communication between cell-ECM contact and the actin cytoskeleton through focal adhesions (42). The dynamic balance between adhesion receptors and the binding of ECM ligands provides FA turnover that regulates adhesion formation and disassembly (43). In the framework of this model, an imbalance in the processes of attachment and detachment leads to conformational changes that mediate abnormal adhesion (22). In the present study, we clearly demonstrated that the suppression of *O*-GlcNAcylation inhibited HeLa cell migration while it enhanced cell-ECM adhesion (Fig. 2), which indicated that *O*-GlcNAcylation is involved in the regulation of integrin-mediated cell adhesion. Consistently, FAK serves as a key regulator of FA assembly and disassembly processes that are fundamental for efficient cell migration (44). Indeed, there were more stress fibers and focal adhesions in FAK-deficient cells, while cell motility was inhibited (45). Aberrant cell-ECM adhesiveness is likely to suppress cell migration, and proper cell adhesion is an important determinant for cell migration (46). Thus, our data are reasonable in that the knockdown of *O*-GlcNAcylation aberrantly increased cell adhesion, as well as spreading and FA complex formation, which in turn decreased cell migration.

We were intrigued as to why a knockdown of OGT would enhance integrin activation. Integrins are the major cell surface receptors used to assemble and recognize a functional ECM, and to facilitate cell signaling and migration (47). The organization of cell adhesions is complex, and includes a number of cytoplasmic proteins such as paxillin, talin, FAK, vinculin, and α-actinin (40). Integrin activation is associated with an array of biological and pathological conditions involving both outside-in and inside-out signaling (48). Accumulating data have indicated that the cytoplasmic domain of the integrin β1 subunit cooperatively promotes integrin activation through the binding of talin (49).

Consequently, our results clearly showed the interaction of integrin β1 with talin, and the association of FAK, paxillin and/or talin both were greatly increased in the KD cells, which suggests that the KD of OGT promotes inside-out signaling (Fig. 5). A reciprocal relationship between *O*-GlcNAcylation and *O*-phosphorylation has been observed in the specific serine or threonine residue of particular proteins (50,51), Consistently, a loss of paxillin phosphorylation at Ser-250 markedly inhibits focal adhesion turnover and cell migration (52). Therefore, how *O*-GlcNAcylation affects the *O*-phosphorylation of FA complex proteins is worthy of clarification.

The *O*-GlcNAcylation of FAK is noteworthy. Integrins do not possess enzymatic activity; rather, they associate with a number of cytoplasmic protein kinases such as FAK and Src. Tyrosine-phosphorylated FAK is well known to be a promoter of interactions with various Src-homology 2- and 3-containing proteins, and to initiate enzymatic cascades via these associated kinases that ultimately leads to changes in cell behavior (53). By contrast, serine- or threonine-phosphorylation on FAK is not well understood. FAK phosphorylation at either Ser-732 or Ser-722 has recently been recognized as important for microtubule organization, nuclear movement, and neuronal migration during cell adhesion (54,55). Interestingly, phosphorylation of both Ser-843 and Ser-910 on FAK exhibited synchronized phosphorylation during cell mitosis (56), which may be related to *O*-GlcNAcylation since expression levels of OGT change during mitosis (57). Furthermore, a cluster of serine phosphorylation sites was recently identified at the initiation of the FA targeting domain in FAK (58), which may suggest some of those sites could be modified by *O*-GlcNAcylation. Thus, to elucidate the roles of OGT in cell biology, it is necessary to identify the specific sites and functions of *O*-GlcNAcylation in FAK.

Our results indicate that *O*-GlcNAcylation plays important roles in regulating cell adhesion, FA complex formation, and cell migration. Emerging data has already established that *O*-GlcNAc modification has a critical role in the progress of human diseases, and particularly diseases such as cancer, diabetes and Alzheimer's (10). Intriguingly, FAK has been associated with insulin resistance in adipocytes in the early stages of type II diabetes (59,60), and has also been implicated in the deposition of β-amyloid plaque (61,62). It would be reasonable to assume that dynamic regulation of FAK *O*-GlcNAcylation with phosphorylation may partially serve as a possible explanation for a number of diseases.

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Abbreviations

OGT: *O*-GlcNAc transferase;

KD: knockdown;

DOX: doxycycline;

shRNA: short hairpin RNA;

FAK: focal adhesion kinase;

FA: focal adhesion;

AD: Alzheimer's disease;

ECM: Extracellular matrix;

VSV: Vesicular Stomatitis Virus glycoprotein.

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