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FBXW10 is negatively regulated in transcription and expression level by protein *O*-GlcNAcylation

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ABSTRACT

Intricate cross-talks exist among multiple post-translational modifications that play critical roles in various cellular events, such as the control of gene expression and regulation of protein function. Here, the cross-talk between *O*-GlcNAcylation and ubiquitination was investigated in HEK293T cells. By PCR array, 84 ubiquitination-related genes were explored in transcription level in response to the elevation of total protein *O*-GlcNAcylation due to over-expression of OGT, inhibition of OGA or GlcN treatment. Varied genes were transcriptionally regulated by using different method. But FBXW10, an F-box protein targeting specific proteins for ubiquitination, could be negatively regulated in all ways, suggesting its regulation by protein *O*-GlcNAcylation. By RT-PCR and Western blot analysis, it was found that FBXW10 could be sharply down-regulated in mRNA and protein level in GlcN-treated cells in a time-dependent way, in line with the enhancement of protein *O*-GlcNAcylation. It was also found that endogenous FBXW10 was modified by *O*-GlcNAc in HEK293T cells, implying *O*-GlcNAcylation might regulate FBXW10 in multiple levels. These findings indicate that *O*-GlcNAcylation is involved in the regulation of ubiquitination-related genes, and help us understand the cross-talk between *O*-GlcNAcylation and ubiquitination.

1. Introduction

Ubiquitination plays a critical role in maintenance of cellular homeostasis via the balance of protein synthesis and degradation or functional regulation of some client proteins. It is a 3-step enzymatic cascade involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3), which ultimately attaches ubiquitin to lysine residues of the target protein. While there is one conserved gene encoding two E1 isoforms in human, there are more than 50 genes encoding E2s and 500 genes encoding E3s [1]. E3 is considered to be the key factor determining the specificity of substrates. Based on domain structures, E3s are divided into three families: RING-figure type, HECT type and SCF complex type. The SCF complex consists of a Cullin-based scaffold and an adaptor protein (e.g., F-box proteins) that determines the substrate specificity of the ligase. Ubiquitins can also be removed from ubiquitinated proteins by specific enzymes termed as de-ubiquitinating enzyme (DUB) [2].

Ubiquitin-like proteins (UBLs) are similar to ubiquitin, including SUMO, NEDD8, ISG15 and FAT10 [3], whose attachment on client proteins could affect their subcellular localization, transportation or protein–protein interaction. UBLs are conjugated to target proteins by an analogous enzymatic cascade as in ubiquitination. For examples, AOS1/Uba2 is used as E1-like protein and Ubc9 as E2-like protein in SUMOylation, While APPBP1/Uba3 serves as E1-like protein and Ubc12 as E2-like protein in NEDDylation [2].

O-linked β-N-acetyl Glucosamine modification, namely *O*-GlcNAcylation, plays important roles in various cellular events, including the process of ubiquitin-dependent protein degradation. It was found that the elevation of total protein *O*-GlcNAcylation was in line with enhanced total protein ubiquitination due to GlcN treatment and E1 was *O*-GlcNAcylated, suggesting E1 should be a common link between *O*-GlcNAcylation and ubiquitination [4]. Though functional inhibition of proteasome might also participated in the GlcN-triggered elevation of protein ubiquitination [4,5]. The *O*-GlcNAcylation of P53 at Ser 149 could indirectly compete with ubiquitination via an intermediate phosphorylation process at Thr 155 [6,7], while the *O*-GlcNAcylation of histone H2B could promote its mono-ubiquitination at K120 [8], indicating the complexity of the cross-talk between *O*-GlcNAcylation and ubiquitination. More over, many enzymes involved in the enzymatic cascades, such as NEDD4–1 [9], RBP2 [10], RING1, RNF2 [7] and some DUBs [11,12], were modified by *O*-GlcNAc. *O*-GlcNAcylation was also involved in transcriptional regulation of some E3s, such as Skp2 and Skp1, via the modification of their specific transcription factors [13,14].

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In the present work, various ubiquitination-related genes were tested in transcription level in HEK293T cells in response to elevated total protein *O*-GlcNAcylation. It was indicated that FBXW10 was negatively regulated in mRNA and protein level due to enhanced protein *O*-GlcNAcylation. The endogenous FBXW10 was also modified by *O*-GlcNAc in HEK293T cells. It was the first work reporting the regulation of F-box protein by *O*-GlcNAc modification.

2. Materials and methods

2.1. Cell culture, transfection, and treatment

Two plasmids (pEGFP-ncOGT and pEGFP-sOGT) were constructed from the plasmid pET43.1–ncOGT kindly gifted by Prof. Hanover (NIDDK, National Institutes of Health) and used for over-expression of human sOGT and ncOGT. HEK293T cells were grown in DMEM-high glucose (Hyclon) supplemented with 10% fetal bovine serum (FBS, PAA), 1% penicillin/streptomycin (PS, Gibco) at 37 °C, 5% CO_2.

To elevate total protein *O*-GlcNAcylation, HEK293T cells were transfected with indicated constructs (pEGFP-sOGT or pEGFP-ncOGT), or treated with PUGNAc and/or GlcN (purchased from Alfa Aesar). Prior to GlcN treatment, the cells were starved in DMEM-no glucose (Gibco) for 24 h. All transfected cells were sorted out with flow cytometry (BD FACS Aria) after routine culture of 48 h.

2.2. PCR array analysis

Total RNA was extracted from the above cell samples using RNeasy MiNi Kit (QIAGEN), and treated with DNAase I (RNase-Free DNase Set, QIAGEN) to eliminate genomic DNA, following the provided protocols. After assessing RNA yield and quality, reverse transcription was performed using RT^2 First Strand Kit (QIAGEN) and the resultant cDNA was tested by RT^2 Profiler™ PCR Array Human Ubiquitination (Ubiquitylation) Pathway (PAHS-079A, QIAGEN). The integrated web-based software package was used for data analysis (www.SABiosciences.com/pcrarraydataanalysis.php).

2.3. Quantitative RT-PCR analysis

Starved HEK293T cells were treated with 20 mM of GlcN for 0, 0.5, 1, 2, 4, 6, 8 h. Total RNA was extracted from these cells, subsequent with reverse transcription to produce cDNA. The transcription level were analyzed via quantitative RT-PCR (qRT-PCR) using following primers: FBXW10 sense, 5′-TCAGGGCTCAATCAAGAC-3′; FBXW10 antisense, 5′-GGACAAAGGAAGGGATGT-3′; FBXO4 sense, 5′-AGGTCACTGATGGTGCAT-3′; FBXO4 antisense, 5′-ACATAGG ACGGCTGGATT-3′; β-actin sense, 5′-CTGGAACGGTGAAGGTGACA-3′ β-actin antisense, 5′- AAGGGACTTCCTGTAACAATGCA-3′. qRT-PCR was performed on a mastercycler ep realplex (Eppendorf) using the following thermal cycling conditions: 95 °C for 2 min; 40 cycles of 95 °C for 15 s, 55 °C for 15sec and 68 °C for 20 s. The results were analyzed by the ΔΔCt method.

2.4. Immunopreciptation

Anti-FBXW10 antibody T14 (sc-164383, Santa Cruz) was bound to Protein NHS Mag Sepharose (GE Healthcare) following the manufacturer's directions. Cell lysates from HEK293T cells or PUGNAc-treated HEK293T cells were incubated with the immobilized antibody at 4 °C overnight. The resulted complex was washed three times with washing buffer (50 mM Tris–HCl, 150 mM NaCl, 2 M Urea, pH 7.5) and eluted with elution buffer (0.1 M Glycine–HCl, 2 M urea, pH 2.9). The collected samples were analyzed by Western blot.

2.5. Western blot analysis and silver staining

Total protein concentration of the cell lysate was assayed by Bicinchoninic Acid (BCA) Method. 20 μg of each sample was separated on a 10% SDS–PAGE. Western blot was performed to determine total protein *O*-GlcNAcylation and the expression of FBXW10, using anti-*O*-GlcNAc antibody RL2 (ab2739; Abcam) and anti-FBXW10 antibody T-14, respectively. β-actin was used as the internal control and detected with anti-β-actin antibody (49671, Cell Signaling). Silver staining was carried out using the silver stain kit (Thermo Scientific Pierce) following the manufacturer's directions.

2.6. Statistical analysis

All experimental results and measurements were presented as means ± standard deviations. Unless otherwise indicated, comparisons were performed using Student's *t* test and statistically significant differences between groups were defined as P values $\leqslant 0.05$ and indicated in the legends to the figures.

3. Results

3.1. Enhancement of total protein O-GlcNAcylation

O-GlcNAcylation is a dynamic protein modification orchestrated by three determinant factors: *N*-acetylglucosaminyl transferase (OGT), β-*N*-Acetylglucosamindase (OGA) and UDP-GlcNAc. OGT and OGA are responsible for the sugar addition and removal, respectively. While UDP-GlcNAc is the only sugar donor involved in the glycosylation. Total cellular protein *O*-GlcNAcylation was routinely elevated by OGA inhibition or GlcN treatment [15–19], while it was decreased by the inhibition of OGT with either chemical inhibitors or siRNA [20–22].

Considering of the low level of protein *O*-GlcNAcylation in HEK293T cells, the elevation of total protein *O*-GlcNAcylation was achieved by (1) over-expression of sOGT or ncOGT, (2) inhibition of OGA with PUGNAc, or (3) GlcN treatment. The HEK293T cells transfected with pEGFP, pEGFP-sOGT or pEGFP-ncOGT were sorted out, respectively lysed and analyzed with Western blot. In comparison with the control, total protein *O*-GlcNAcylation was greatly enhanced in sOGT or ncOGT-expressing cells (Fig. 1A), indicating both these isoforms worked well in HEK293T cells. Surprisingly, sOGT was more efficient in triggering protein *O*-GlcNAcylation in comparison with ncOGT. Since *O*-GlcNAcylation could expressionally regulate the abundancy of some proteins [13,14]. Is this discrepancy caused by the differential expression of substrate proteins? To address this question, the samples were analyzed by silver stain. Similar protein bands were revealed on SDS–PAGE within the area where differential *O*-GlcNAcylation existed, suggesting that sOGT and ncOGT *O*-GlcNAcylate different substrate proteins in HEK293T cells. But the expression of sOGT and ncOGT led to varied protein abundancy in the molecular weight range of 27 to 34.6 kD in contrast to the control. And the expression of ncOGT led to differential protein expression in a molecular weight range >212 kDa in comparison with other two samples (Fig. 1B), indicating the over-expression of OGT resulted in differential expression of specific proteins in HEK293T cells. In addition, the inhibition of OGA and GlcN treatment could both dramatically enhance the total protein *O*-GlcNAcylation in HEK293T cells in a dosage-dependent manner. It was obvious that 0.1 mM of PUGNAC could dramatically elevate the protein *O*-GlcNAcylation level. Under GlcN treatment, the optimal

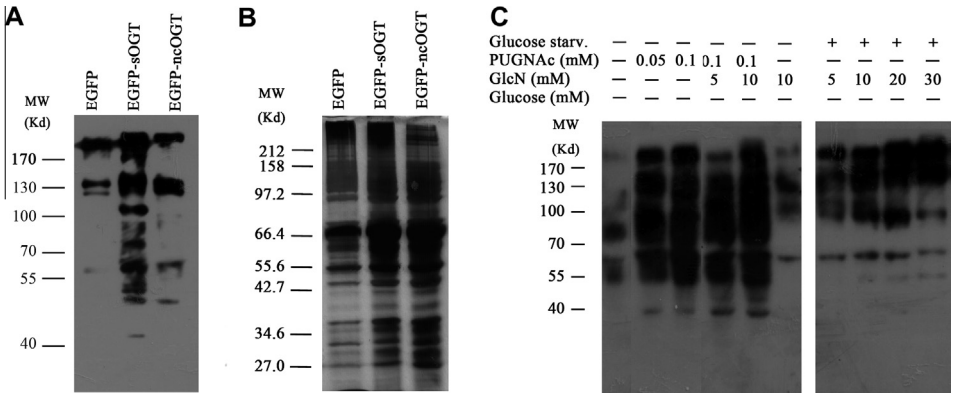

Fig. 1. Elevation of the total protein *O*-GlcNAcylation in HEK293T cells. (A) Western blot analysis of the cell lysate from HEK293T cells transfected with pEGFP, pEGFP-sOGT or pEGFP-ncOGT. (B) Silver staining analysis of the cell lysate from HEK293T cells transfected with pEGFP, pEGFP-sOGT or pEGFP-ncOGT. (C) Western blot analysis of cell lysate from HEK293T cells treated by PUGNAc and/or GlcN. For Western blot analysis, cell lysates were immunoblotted with anti-*O*-GlcNAc antibody (RL2) in a 1:2500 dilution.

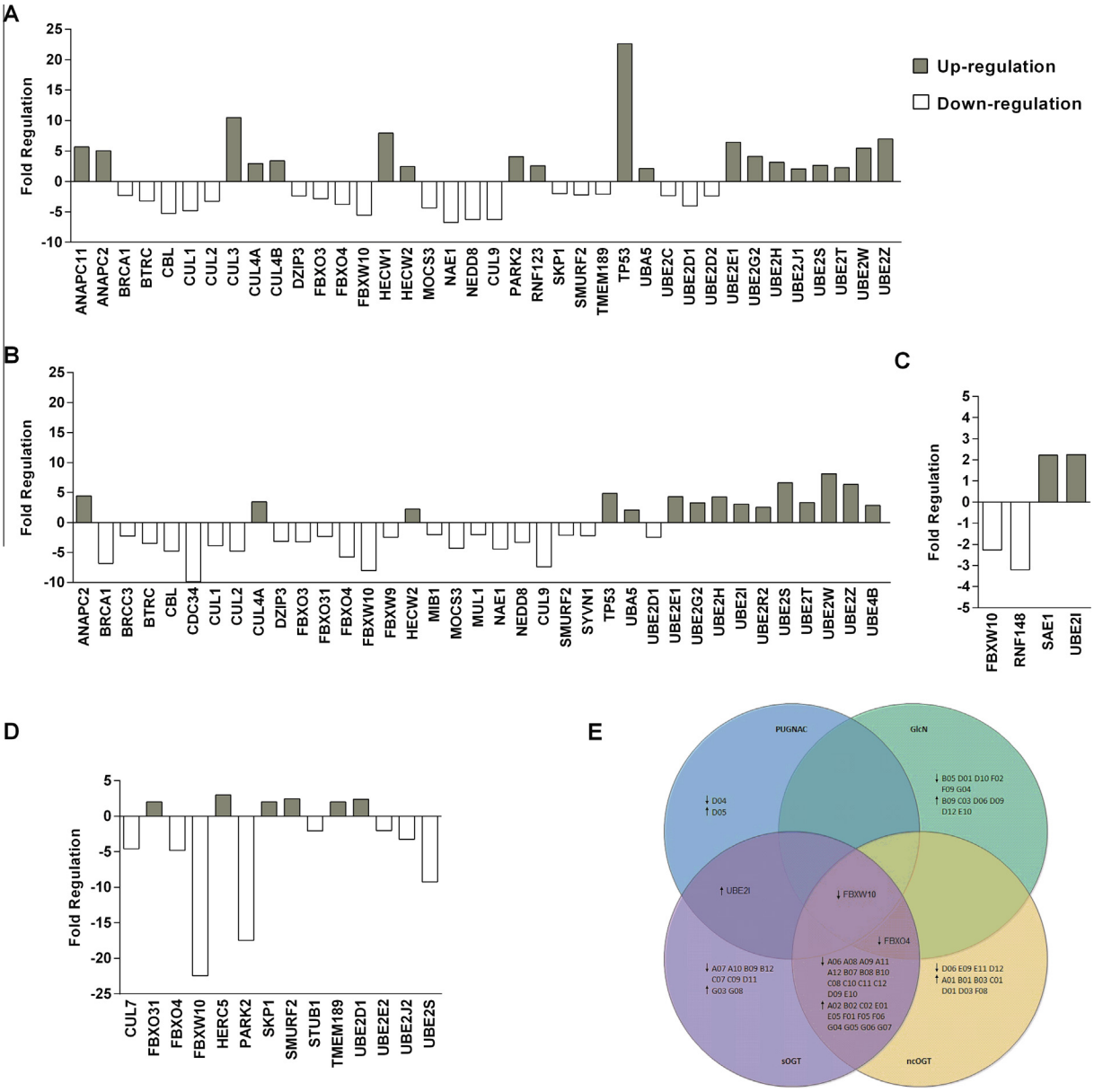

Fig. 2. PCR array analysis. 84 genes related to human ubiquitination were tested in response to elevated total protein *O*-GlcNAcylation. Total protein *O*-GlcNAcylation was elevated by (A) over expression of ncOGT; (B) over expression of sOGT; (C) treatment with PUGNAc; (D) treatment with GlcN. (E) Comprehensive analysis of the regulation of ubiquitination-related genes (↑, up-regulation; ↓, down-regulation).

Table 1
Transcriptional regulation of F-box genes in response to varied treatments.

Position*	Symbol	Fold change**			
		GlcN	PUGNAc	sOGT	ncOGT
B08	FBXO3	1.0446	1.8540	−3.2376	−2.8519
B09	FBXO31	2.0180	1.1334	−2.3053	−1.4661
B10	FBXO4	−4.8135	1.2751	−5.7555	−3.7632
B11	FBXW10	−22.4257	−2.2648	−8.0274	−5.5479
B12	FBXW9	1.4073	−1.3008	−2.4537	−1.7314

* Indicates the position in the PCR array.
** It is valid if the value of the fold change $\geqslant 2$ or $\leqslant -2$ (according to the manufacturer's instruction).

concentration was at 20 mM. The combination of these two methods revealed more potent. It was also indicated that the starvation of cells prior to GlcN treatment contributed to the elevation of total protein *O*-GlcNAcylation (Fig. 1C).

3.2. Regulation of ubiquitination-related genes

In response to the expression of sOGT or ncOGT, the ubiquitination-related genes were variously regulated. Among these genes, 28 were similarly regulated by both the isoforms of OGT. For example, CUL1/2, FBXO3/4 and FBXW10 were all down-regulated, while P53 and HECW2 were up-regulated. Others genes were regulated by either sOGT or ncOGT (Fig. 2A, B). For example, CUL3 was up-regulated by ncOGT, while CDC34 was down-regulated by sOGT. Totally, ncOGT could regulate much more ubiquination-related genes in comparison with sOGT, though less *O*-GlcNAcylation it initiated. To convince the genes regulated by the elevation of total protein *O*-GlcNAcylation, other two treatment strategies were performed. It was found that 14 genes were regulated by GlcN treatment, while only 4 genes were regulated by PUGNAc (Fig. 2C, D). A comparative analysis of these results indicated that FBXW10 and FBXO4 were negatively regulated by almost all used methods, suggesting that these genes should be negatively regulated by the elevation of protein *O*-GlcNAcylation, though little was known about the mechanism (Fig. 2E). Interestingly, both the genes belong to F-box gene family that encoding the determinant factors in SCF complex E3 ligases. In addition, other three F-box genes in the PCR array (FBXO3, FBXO31, and FBXW9) were all down-regulated in response to the expression of sOGT (Table 1).

3.3. FBXW10 is regulated in a time-dependent way in response to GlcN treatment

GlcN treatment led to dramatic elevation of total protein *O*-GlcNAcylation (Fig. 1C) and down-regulated the transcription of FBXW10 and FBXO4 by 22 and 4.8 folds, respectively (Fig. 2D), indicating GlcN treatment should be an efficient method to investigate the regulation of these two genes. To confirm the PCR array result and clarify the time dependence of the regulation, HEK293T cells were treated with GlcN for various time spans. The transcription and expression of these genes were determined by qRT-PCR and Western blot, respectively. In response to GlcN treatment, the transcription level of FBXW10 and FBXO4 was dramatically down-regulated with time extension (Fig. 3A). Both of them were down-regulated by 2-folds in 1 h and the transcription level of FBXW10 was declined to the bottom in 2 h. Total protein *O*-GlcNAcylation was gradually enhanced with time extension, while FBXW10 was obviously down-regulated and disappeared at 8 h (Fig. 3B). Considering of the transcription level at 1 h and the diminished protein level at 4 and 8 h, the half life of FBXW10 was presumed to be 3–4 h.

3.4. O-GlcNAcylation of FBXW10

To reveal the *O*-GlcNAcylation status of FBXW10, a program in dbOGAP (http://cbsb.lombardi.georgetown.edu/OGAP.html) was

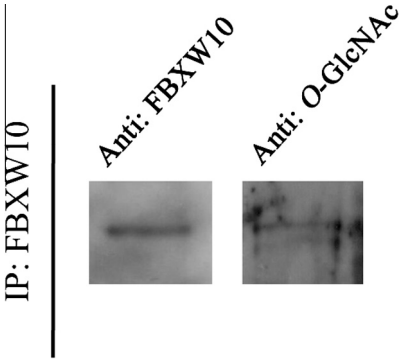

Fig. 4. *O*-GlcNAcylation status of FBXW10. Immunoprecipited FBXW10 was devided into two halves and immunobloted with anti-FBXW10 antibody in a 1:50 dilution and RL2 in a 1:1000 dilution.

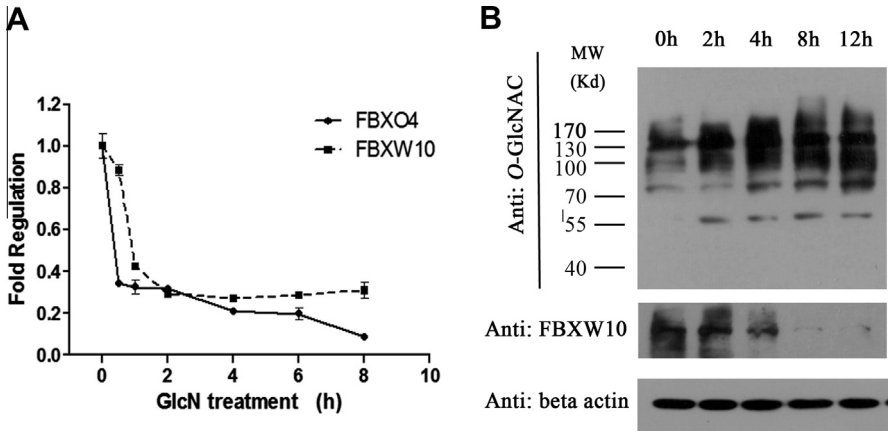

Fig. 3. Time-dependent regulation of FBXW10/FBXO4 in response to GlcN treatment. (A) Transcriptional regulation of FBXO4/FBXW10 by GlcN treatment for 0, 0.5, 1, 2, 4, 6, 8 h. Beta-actin gene was used as the internal control. Expression levels were analyzed using the ΔΔCt method, and data were expressed as the mean ± SD from three independent experiments ($p < 0.05$). (B) Changes of the total protein *O*-GlcNAcylaiton and the expression of FBXW10 in HEK293T cells treated by GlcN for 0, 2, 4, 8, 12 h, Cell lysate (20 μg) was imunnobloted with RL2 (1:2500), anti-FBXW10 antibody (1:100), and anti-β-actin antibody (1:1000) (control).

used to find the putative modification sites. Twelve *O*-GlcNAcylation sites were predicted, some of which were featured by the core "VSS" amino acid sequence that usually appeared in *O*-GlcNAcylated proteins (data not shown), implying that FBXW10 should be modified by *O*-GlcNAc. To convince the result, endogenous FBXW10 was immunoprecipitated from HEK293T cells and determined by Western Blot. An *O*-GlcANcylated protein band was revealed at the same site as FBXW10, proving the *O*-GlcNAcylation of FBXW10 (Fig. 4). GlcN-treated cells was also tried to enhance the *O*-GlcNAcylation of FBXW10 but in vain, due to the elimination of target protein (data not shown). In consideration of the influence on numerous proteins, such as on Rpt2 and P53, *O*-GlcNAcylation might have a role in functional regulation of FBXW10.

4. Discussion

Several post-translational modifications are involved in regulating proteins' stability or function, among which exist intricate cross-talks. Certain reviews have described the cross-talk between protein phosphorylation and *O*-GlcNAcylation in signaling pathway, transcription and chronic diseases [23]. Plenty of works also documented the influence of *O*-GlcNAcylation on ubiquitination-related processes [5,6,8,14]. Here, we further demonstrated the cross-talk between *O*-GlcNAcylation and ubiquitination.

To reach a reliable conclusion on the genes regulated by protein *O*-GlcNcyaltion, three methods were used to enhance the total protein *O*-GlcNAcylation in HEK293T cells, including the over-expression of sOGT/ncOGT, inhibition of OGA and GlcN treatment. Each could drastically enhance total protein *O*-GlcNAcylation and transcriptionally regulate some of the ubiquitination-related genes. The differential regulation might be ascribed to different *O*-GlcNAcylation status of specific proteins. The down-regulation of FBXW10 by all used methods suggests its negative regulation by enhanced protein *O*-GlcANcylation. Considering of the efficiency and convenience, the GlcN treatment method was performed to evaluate the time-dependent regulation of FBXW10 and FBXO4 in response to the elevation of protein *O*-GlcNAcylation. The decreased FBXW10 in transcription and expression level in line with the rising protein *O*-GlcNAcylation confirmed the transcriptional regulation of FBXW10, though it is unknown whether FBXW10 was regulated in translation level.

Over-expression of sOGT and ncOGT could result in different protein *O*-GlcNAcylation and varied regulation of ubiquitination-related genes in HEK293T cells. Since the expression of Skp2 and Skp1 could be regulated via the *O*-GlcNAcylation of their transcription factors[13,14], we hypothesize that the ubiquitination-related genes might be variously regulated because of the functional shift of some transcription factors, due to their different *O*-GlcNAcylation status on account of the expression of different isoforms of OGT.

There are 69 human F-box proteins, most of which remains uncharacterized. F-box proteins are classified into three families according to their homology domains: FBXWs (WD40 repeats), FBXLs (leucine-rich repeats), or FBXOs (variable or no homology domains) [24]. FBXW10 and FBXO4 belong to FBXWs and FBXOs, respectively. There is a lectin-like FBA domain in some FBXO proteins which can bind various glycans, such as high mannose glycans or sulfated glycans [25]. FBXW proteins participate in various biological processes via their interaction with different substrates [24]. For example, FBXW10 is involved in lamins-induced proteasomal degradation of certain HP1 isoforms [26]. To date, little is known on transcriptional regulation of F-box genes. In this work, it was indicated that enhanced protein *O*-GlcNAcylation could transcriptionally down-regulate two F-box genes and the expression of sOGT could also down-regulate other three F-box genes. We hypothesize that enhanced *O*-GlcNAcylation might down-regulate some F-box genes, if not all, via common transcriptional factors.

In summary, the data in this work proves that protein *O*-GlcNAcylation is involved in the regulation of ubiquitination-related genes, especially the negative regulation of some F-box genes. FBXW10 could be *O*-GlcNAcylated and down-regulated in both transcription and expression level in response to GlcN treatment. Further investigations should be performed to clarify the regulation mechanism of these genes and the functional regulation of FBXW10 by protein *O*-GlcNAcylation.

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