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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