Activity-based proteasome profiling

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O-GlcNAcylation and hHR23B functions

7.1 Introduction

Protein O-linked GlcNAcylation (O-GlcNAc) is a post-translational modification (PTM) characterized by the covalent and reversible bonding of a N-acetylglucosamine moiety to the γ-hydroxyl of serine or threonine residues of a protein via a β-C2 linkage. It bears resemblance to protein phosphorylation and O-GlcNAcylation levels can respond rapidly to intracellular or environmental cues(*1*). The level of O-GlcNAc is regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), and is highly dependent on the level of its donor substrate, UDP-N-acetylglucosamine (UDP-GlcNAc)(*1, 2*). UDP-GlcNAc levels are regulated by an interplay of the hexosamine biosynthetic pathway (HBP) and the metabolic pathways of amino acid, nucleotide, fatty acid, and glucose(*3*). Therefore, protein O-GlcNAcylation levels can vary significantly according to the cell or tissue type, and the nutrition state of the cell. Hundreds of proteins are found to be O-GlcNAcylated, and the list is still increasing(*4, 5*). They belong to diverse families, including metabolic enzymes, transcription factors, heat-shock proteins, and architectural proteins.

The consequence of protein O-GlcNAcylation is complicated and highly dependent on the specific biological network that the protein is being involved in. The physical presence of O-GlcNAcylation alone could affect protein-protein interaction (PPI). For example, O-GlcNAcylation on transcription factor SP1 affects its binding to NF-Y, which is another important transcription factor in complex with SP1 to synergistically activate transcription of many genes, and further interrupts the transcription regulated by SP1 and NF-Y(*6*). Oligomerization of tau is a key process contributing to the progressive death of neurons in Alzheimer's disease. Treatment of tau transgenic mice with an O-GlcNAcase inhibitor increased O-GlcNAcylation level of tau in vivo and decreased neuronal cell loss. It has been proven that O-GlcNAcylation on Tau prevents it from aggregation(*7*). Alternatively, O-GlcNAcylation can affect the PTM state of a given protein. O-GlcNAcylation might show overlapping modification sites with protein phosphorylation on the same or adjacent amino acid residues. In the case of transcription regulation,

phosphorylation activates δ-lactoferrin and P53 but also makes them susceptible to ubiquitination. O-GlcNAcylation inhibits δ-lactoferrin and P53 phosphorylation under normal conditions, thus suppressing them from activation and protecting them from degradation by the proteasome(*8*, *9*). O-GlcNAcylation has also been shown to reduce ubiquitination through the recruitment of deubiquitin enzymes (DUBs) on the target proteins, as shown in the case of Bmal1/Clock in the regulation of the circadian clock(*10*, *11*). The lectin property of heat-shock protein Hsp70 was proposed to allow Hsp70 bind to O-GlcNAcs on the damaged protein, thus prevent them from ubiquitination and degradation under hyperthermia and UV stress(*12*, *13*). Due to the diverse outcomes of protein O-GlcNAcylation, the effect of O-GlcNAcylation on a given protein should be examined by the change in interaction between that protein and its binding partners, directly or indirectly through the involvement of the regulation of other PTMs.

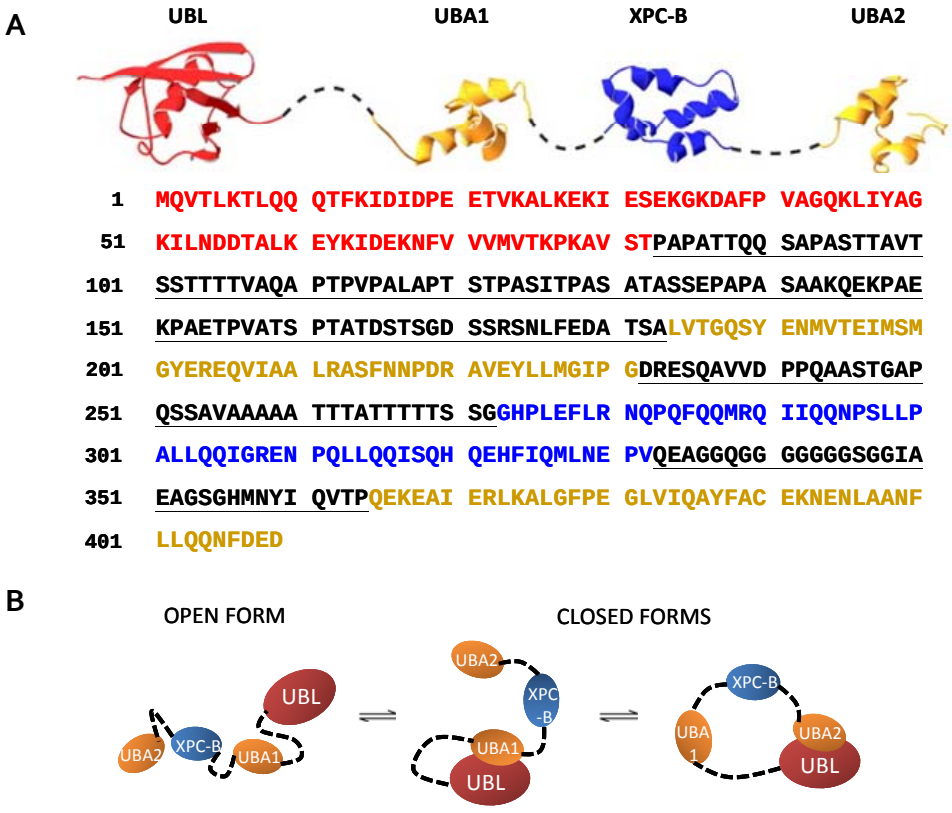

Figure 1: Schematic presentation of hHR23B protein.

(A) The upper panel is a scheme of the hHR23B functional domains, UBL (ubiquitin like domain), UBA1(ubiquitin associate domain 1), XPC-B (XPC binding domain) and UBA2 (ubiquitin associate domain 2). The lower panel is protein sequence of hHR23B. The linker sequences are shown underlined.

(B) Models of open or closed conformations of hHR23B. The UBL domain can interact with UBA1 or UBA2 domains intra-molecularly. When they do not interact with each other, the protein shows an open form. Or, it shows the closed form.

In previous O-GlcNAcylation profiling experiments using a metabolic labeling method(*4*), one of the modified proteins identified was the ubiquitin receptor protein hHR23B. hHR23B is a multi-functional scaffold protein involved in two important biological pathways, the global genomic nucleotide excision repair (GG-NER) and the ubiquitin proteasome system (UPS)(*15*). Functionally, it is often referred to as "NER accessory protein" for its role in stabilizing XPC during the recognition of nucleotide lesions, or "ubiquitin shuttle protein" for its role in trafficking Lys-48 linked polyubiquitinated proteins to the proteasome for their controlled degradation. hHR23B is evolutionarily conserved, as it shares an overall structural and functional similarity to its human paralog hHR23A, and the yeast ortholog Rad23(*15*). Starting from the N-terminus, hHR23B consists of four intra-molecular domains, namely the UBL (ubiquitin like), UBA1 (ubiquitin associate 1), XPC binding, and UBA2 (ubiquitin associate 2) domains (Fig 1A). The functional domains are interconnected by flexible linkers, with the one linking UBL and UBA1 being exceptionally long (78 amino acids). This long linker has a protease resistant amino acid sequence (with very few trypsin (K and R) or chymotrypsin (F, W, L and M) cleavage sites). Meanwhile, it has high Pro, Ala, Ser, Thr content, which is the OGT preference sequence for installing an O-GlcNAc moiety. Presence of a Pro/Ser/Thr rich N terminal and Thr/Ser/Ala rich C terminal adjacent to an O-GlcNAcylation site has been commonly found in O-GlcNAcylated peptides identified before, and it is comparable to the linker sequence in the hHR23B(*2*). Thanks to the long flexible linker regions in the hHR23B, it might adopt an open/closed conformation change just as its paralog hHR23A(*16*). As shown in Fig 1B, the UBL and UBA domains might have some intra-molecular interactions, by which the open/close model has been set up.

The ubiquitin proteasome system is responsible for tightly regulated degradation of specific protein substrates, which is critical in protein turnover, cell cycle progression, antigen presentation, and regulation of oncogenes. Proteins are first tagged with polyubiquitin chains by an enzyme cascade consisting of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3)(*17*). Polyubiquitinated proteins are either recognized and degraded by the proteasome, or shuttled to the proteasome for degradation by the assist of ubiquitin receptor proteins like hHR23B. All four domains of hHR23B might contribute to its role in the UPS. The UBL domain resembles ubiquitin structurally, and can bind to Rpn10 (s5a in yeast) and Rpn1 subunits of the 19S regulatory particle of the 26S proteasome, the main protein degradation machinery in the UPS(*15*). The two UBA domains serve to bind to poly-ubiquitin chains on substrate proteins of the proteasome(*17*). The binding of UBL and UBAs to their binding partners ensures a one-directional shuttling of the poly-ubiquitinated protein substrates to the proteasome for degradation. The XPC binding domain is also involved in the UPS. Although its major function is to bind to XPC and thus stabilize it during the lesion recognition phase of GG-

NER, it was found that in yeast the Rad4 (ortholog of XPC in yeast) binding domain of Rad23 also binds to protein glycanase Png1, a protein responsible for the removal of sugar-tagged substrates in the ER-associated degradation (ERAD), and that Rad23 is an essential protein in the ERAD pathway(*18*). Therefore, the O-GlcNAc modification on the hHR23B protein might influence the efficiency of the whole UPS.

In this chapter, a series of chemical biology tools are applied for the visualization, site identification, and unraveling the physiology of O-GlcNAcylation on hHR23B. It proves that, hHR23B is indeed modified by O-GlcNAc. Six O-GlcNAcylation sites were identified on the protein, among which four were only observed after chemical OGA inhibition. While, the cellular O-GlcNAcylation level is increased by an OGA inhibitor treatment, there is almost 50% more free hHR23B molecules existing in the cells. It demonstrates that, the O-GlcNAcylation affects the protein-protein interaction of the hHR23B protein.

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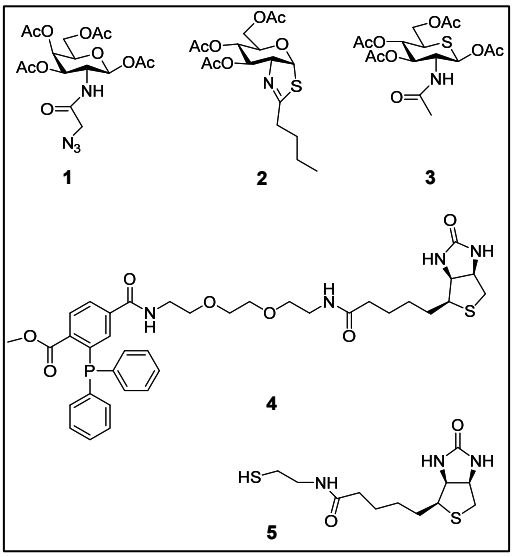

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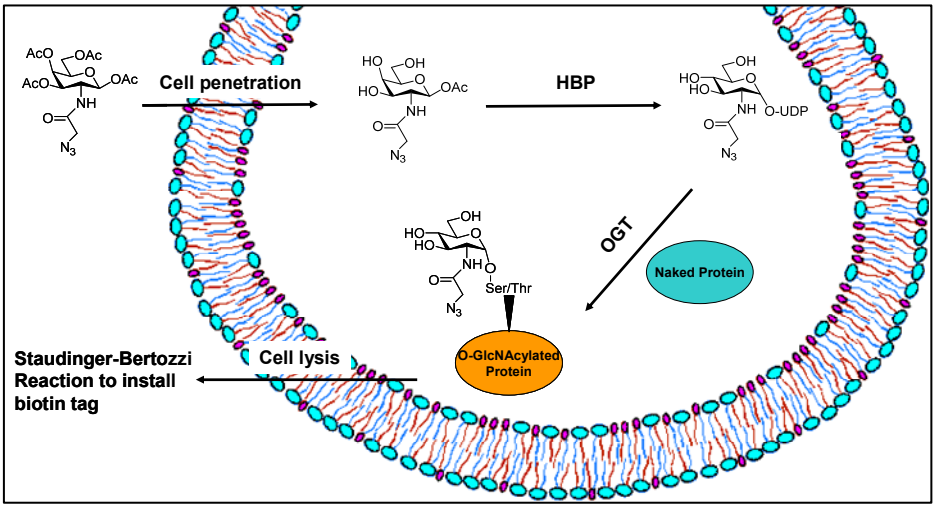

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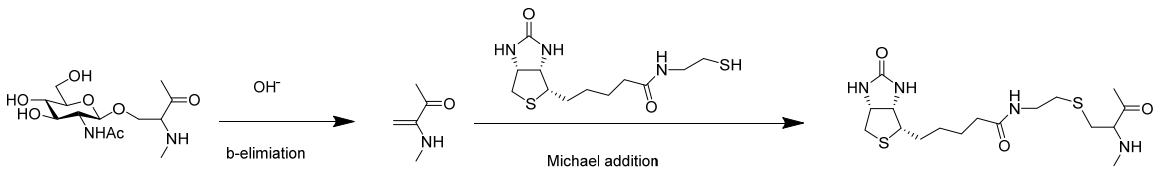

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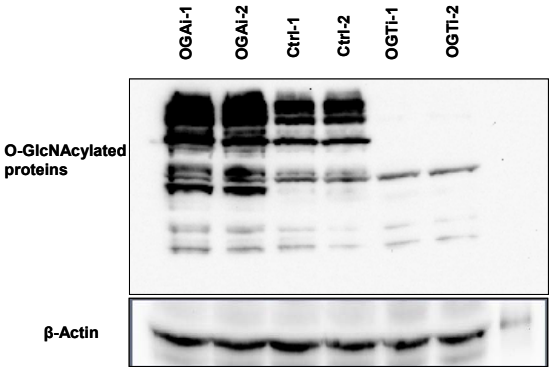

Figure 2: Chemical tools used in this chapter for O-GlcNAcylation study.
(A) Structures of the chemical tools used for the O-GlcNAcylation study.
(B) Schematic presentation of metabolic labeling of O-GlcNAcylated proteins. Cells take up the Ac_4GalNAz in the medium and deacetylate the molecule. GalNAz is converted to UDP-GlcNAz HBP biochemical synthesis enzymes. OGT uses the UDP-GlcNAz as the donor substrate to modify protein substrates. Biotin tag can be installed on the O-GlcNAz modified proteins by bio-orthogonal ligation following lysing the cells.
(C) Schematic presentation of the BEMAB replacement of O-GlcNAc. The labile O-GlcNAc modification on the amino acid residues is removed by a β-elimination under basic conditions, and then biotin-cystamine is added to the amino acid residue by a Michael addition to result in the stable S-linked biotin modification for replacing the O-GlcNAc in LC/MS analysis.
(D) Western blots to test the OGA and OGT inhibitors (**2** and **3**). Cells were treated with or without 50μM **2** or **3** for 24hrs. Full cell lysate were resolved by SDS-PAGE and transferred to PVDF membrane. The O-GlcNAcylated proteins were detected by O-GlcNAc antibody CTD110.6 (upper panel). Anti β-actin blot was performed as loading control (lower panel). Two independent experiments were done and compared on the same blot.

7.2 Results

7.2.1 hHR23B is O-GlcNAcylated

Immunoblot detection is the most straightforward method for proving the existence of O-GlcNAc on the target proteins. To circumvent the disadvantage of sensitivity and specificity of direct visualization by O-GlcNAc antibody, for instance the commonly used O-

GlcNAc antibody CTD110.6 cross reacts with N-GlcNAc$_2$-modified proteins in some cases(*19*), a chemical biological approach was taken. HEK293T cells were transfected with a plasmid (pFLAG-6His-hHR23B) for the expression of Flag tagged hHR23B, followed by treatment with high concentration (250μM) of Ac4-GalNAz (**1** in Fig 2A). The Ac4-GalNAz was deacetylated and converted by the HBP enzymes into UDP-GlcNAz intracellularly, and then OGT used the UDP-GlcNAz as the donor substrate to modify protein substrates. Consequently, proteins got modified by GlcNAz instead of the endogenous GlcNAc(*3*). Then, cells were harvested and lysed. A Staudinger-Bertozzi reaction was performed with biotin-phosphine **4** in the lysate to install a biotin tag on GlcNAz modified proteins and as control for background labeling on cell lysate without GlcNAz exposure (Fig 2B). Afterwards, one immunoprecipitation (IP) against the FLAG tag to purify FLAG-6His-hHR23B and one biotin-streptavidin affinity purification (AP) to enrich for O-GlcNAz modified proteins were done with the lysate. The eluted samples from the IP and AP experiments were detected by both anti-FLAG antibody and streptavidin-HRP, respectively.

As shown in Figure 3A, successful transfection and expression of FLAG-tagged hHR23B was achieved. In all four samples, the bands have sizes of around 57 kD, which is consistent with the molecular weight of FLAG-6His-hHR23B. Lane 1 and lane 2 are the input and elute samples of the anti-FLAG IP without the GalNAz treatment. Lane 3 and lane 4 are the input and elute samples of the IP with GalNAz treatment. The possible presence of O-GlcNAz and biotin on hHR23B does not interfere with antibody binding against FLAG tag (lane 3 and 4). Additionally, the band intensity is slightly stronger in the IP elutes (lane 4), which indicates efficient enrichment. Detection of the same blot with Streptavidin-HRP (Fig 3B) shows clear bands only in the GalNAz treated samples (lane 3 and 4), which indicates that O-GlcNAz was metabolically incorporated as PTM on proteins and that the Staudinger-Bertozzi reaction showed minor background labeling as seen in lanes 1 and 2. The IP sample showed only one intensive band correlating to the same electrophorestic shift of FLAG-6His-hHR23B (lane 4), whereas the input sample showed multiple bands (lane 3). That is because in these two samples, GlcNAz was successfully incorporated as PTM on many cellular proteins, and that, the anti-FLAG IP specifically and efficiently enriched the O-GlcNAcylated hHR23B. Therefore, the anti-biotin immuno blot (IB) detected numerous proteins in lane 3 and only hHR23B in lane 4.

For the affinity purification with streptavidin beads, Figure 3C shows the anti-FLAG detected proteins. Lane 1 and lane 2 are the input and AP elute samples without the GalNAz treatment. Lane 3 and lane 4 are the input and AP samples with GalNAz treatment. In both input samples with or without the GalNAz treatment, the FLAG-6His-hHR23B bands are comparable (lane 1 and 3). The AP elute with GalNAz treatment shows a clear band (lane 4), nonetheless the elution sample without the treatment does not (lane 2),

because no biotin was installed on the proteins. The hHR23B protein shown by the AP is less intensive than the band in the input samples, which might be because the O-GlcNAz modified protein is only a small portion of the whole population or the efficiency of the AP is not 100%. The anti-biotin detection shown in Figure 3D illustrates the enrichment efficiency of the AP experiment, and the efficiency of the metabolic labeling method. The slight bands shown in lane 1 and 2 are just endogenous biotinylated proteins instead of aspecific binding of the biotin-phosphine, considering the results in lane 1 and 2 of Figure 3B. The bands in lane 4 are much more intensive than the ones in lane 3, which is because of the enrichment of biotinylated proteins by the AP.

In summary, the cis-trans pull down experiments show that hHR23B is O-GlcNAcylated. The described strategy of metabolic labeling coupled to bio-orthogonal ligation provided a foundation for target enrichment and allowed straightforward visualization of O-GlcNAcylation on the target protein.

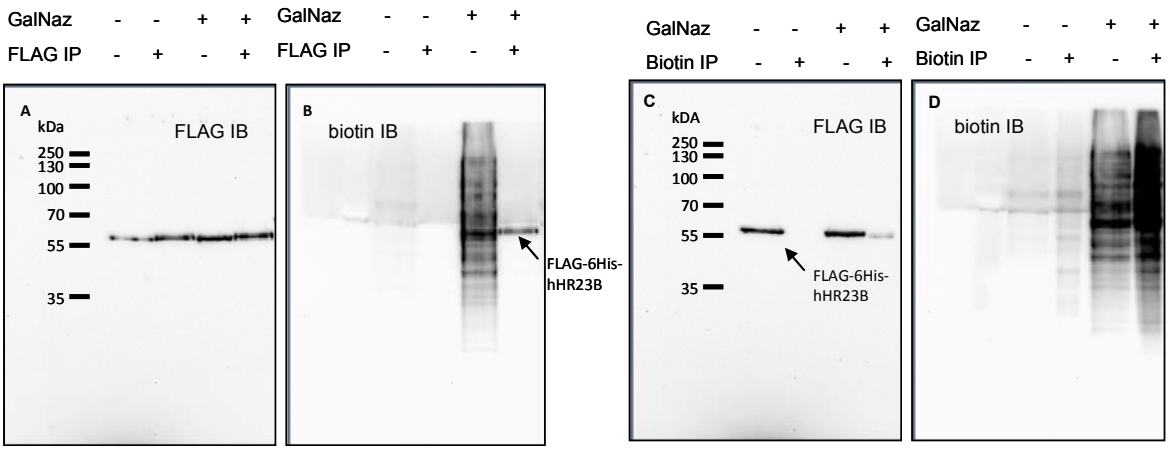

Figure 3: IP and AP to confirm O-GlcNAcylation on hHR23B. Cells tranfected with pFLAG-6His-hHR23B (with or without GalNAz) treatment were lysed. The biotin tag was installed by Staudinger-Bertozzi reaction. The FLAG tagged hHR23B was immunoprecipitated by anti-FLAG beads, and detected by FLAG antibody (A) or Strep-HRP (B). The biotin tagged proteins were affinity purified by Streptavidin beads, and detected by FLAG antibody (C) or Strep-HRP (D). IB demonstrates immuno blot.

7.2.2 Mapping of O-GlcNAcylation sites on hHR23B

To identify the exact O-GlcNAc modification sites on the hHR23B protein, the unstable O-GlcNAc moiety was replaced for a stable biotin-cystamine (**5**, Fig 2A) for the LC/MS analysis. Cells were transfected with plasmid pFLAG-6His-hHR23B, and then grown with or without the OGA inhibitor **2** for 24 hours(*20*) (Fig 2A). As seen in Figure 2D, OGA inhibitor **2** increased and OGT inhibitor **3** decreased the cellular O-GlcNAcylation levels significantly in the living cells. Following harvest and lysis of the cells, an anti-FLAG IP was

performed to enrich the expressed protein. The eluted protein was digested by proteinase K (ProtK) instead of the generally used trypsin, due to the special sequence of the hHR23B protein, which contains long linker parts with very few trypsin cleavage sites(C terminal of Lys and Arg). The digested peptides were dephosphorylated by alkaline phosphatase to prevent β-elimination of the phosphate. The O-GlcNAc groups on the peptides were replaced by biotin-cystamine (**5**) through a β-elimination followed by a Michael addition reaction under basic condition at 52°C(*21*) (Fig 2C). The peptides were then analyzed by nano-LC/MS for identification of the modification sites.

Through this experiment, two O-GlcNAcylation sites were identified in both samples, and four extra modification sites were identified only in the sample with OGA inhibition treatment (Table 1). This suggests the presence of constitutive O-GlcNAc modifications on the hHR23B protein, even under OGA activity in the cell. It is hypothesized that the constitutive O-GlcNAc are for protein functions and the differentially added O-GlcNAc are for regulating the functions by switching between open and closed states. Interestingly, the modification sites identified are mostly located around the linker part between UBL and UBA1, which is indeed the longest linker in the protein. It might play a role in the open-closed mode changes, according to the structural studies on the paralogue protein hHR23A(*16*).

Table 1: O-GlcNAcylation sites identification on hHR23B

samples	peptide identification	mascot score	O-GlcNAc sites
hHR23B-nt	^{29}KIE$_{BiCy}$**S**EKGKDAFPVAGQ^{44}	30	**Ser^{32}**
	^{73}MVTKPKAV$_{BiCy}$**S**TPAPATTQ^{89}	44	**Ser^{81}**
hHR23B-OGAi	^{29}KIE$_{BiCy}$**S**EKGKDAFPVAGQ^{44}	21	**Ser^{32}**
	^{73}MVTKPKAV$_{BiCy}$**S**TPAPATTQ^{89}	45	**Ser^{81}**
	^{74}VTKPKAVSTPAPAT$_{BiCy}$**T**QQ^{90}	23	**Thr^{88}**
	^{79}AVS$_{BiCy}$**T**PAPATTQ^{89}	23	**Thr^{82}**
	^{248}GAPQS$_{BiCy}$**S**AV^{255}	21	**Ser^{253}**
	^{339}GGGGGGG$_{BiCy}$**S**GGIA^{350}	33	**Ser^{346}**

What should also be considered is that a substantial part of the linker sequence Gln^{90}-Lys^{144} was not found by LC/MS analysis. Although the aggressive protease ProtK with little sequence preference was used for the proteolytic degradation of the protein, the linker part of the protein might still be incompletely digested due to the special sequence, and was not analyzed by the mass spectrometer. This could also be the reason that very few reports were published about the O-GlcNAcylation on hHR23B, when trypsin was used for digestion. To solve this problem, studies should be performed with pure hHR23B to find appropriate ProtK digestion condition for increasing the hHR23B coverage and possibly identify additional O-GlcNAcylation sites.

7.2.3 O-GlcNAcylation alters protein-protein interaction of hHR23B

hHR23B functions through binding to other proteins via its four intra-molecular domains. These functions could be influenced by conformational alterations, such as the open/closed model suggested by the hHR23A structural research(*16*). As determined, the O-GlcNAc modification sites are mainly positioned in the linker part between UBL and UBA1, which is the largest linker in the protein and might contribute to the open-closed model. It is to be expected that the O-GlcNAc modifications might influence the changing of the mode from one to the other.

To prove this hypothesis, the first step is to find out whether the interactome of hHR23B is changing depending on the O-GlcNAcylation levels. When hHR23B binds to its interaction partners, the fraction of free hHR23B is expected to be lower. By detecting the free fraction, an estimate of the bound hHR23B can be made, which indirectly reflects on the fraction of hHR23B that is bound to its interaction partners. HEK293T cells were transfected with pFLAG-6His-hHR23B and cultured with or without the OGA and OGT inhibitors (**2** and **3**) for 24 hours(*20, 22*). Subsequently, cellular proteins were cross linked by formaldehyde or not. After quenching the residual free formaldehyde with glycine, the cells were harvested and lysed. The same amount of lysate was loaded on SDS-PAGE from each condition. Samples were run on two gels, one was stained with coomassie blue as loading control correction and visualization of the total protein content, the other one was transferred to a western blot. Free hHR23B protein was detected by anti-hHR23B antibody (Fig 4).

The anti hHR23B western blot shows two bands around 55kD, because there are two populations of the hHR23B protein. The upper band is the transfected FLAG-6His-hHR23B, and the lower one is the endogenous hHR23B. The free hHR23B proteins stays on approximately the same level in the non-cross linked samples with or without inhibitor treatment (lane 1-3, Fig 4A). Because protein analysis by SDS-PAGE is performed under both denaturing and reductive conditions, it is obvious that most non-covalent PPI between hHR23B and its binding partners were lost. The results also demonstrate that the OGA/OGT inhibition did not influence the hHR23B expression level. However, the cross linked samples show some difference between the control and OGA/OGT inhibitions (lane 4-6). The results were then quantified and normalized by the total protein amount quantified from the coomassie stained gel (Fig 4B). The quantification results were shown in Figure 4C. After OGA inhibition, there was around 50% more free hHR23B than in the control cells. Whereas, about 10% less hHR23B monomer was detected in the OGT inhibited samples. These results in total illustrate that, the high O-GlcNAcylation level in cells altered the protein-protein interaction of hHR23B. The evidence indeed correlates to the hypothesis that O-GlcNAc modifications on the hHR23B could change its conformation and further alter its interactome and regulate its functions.

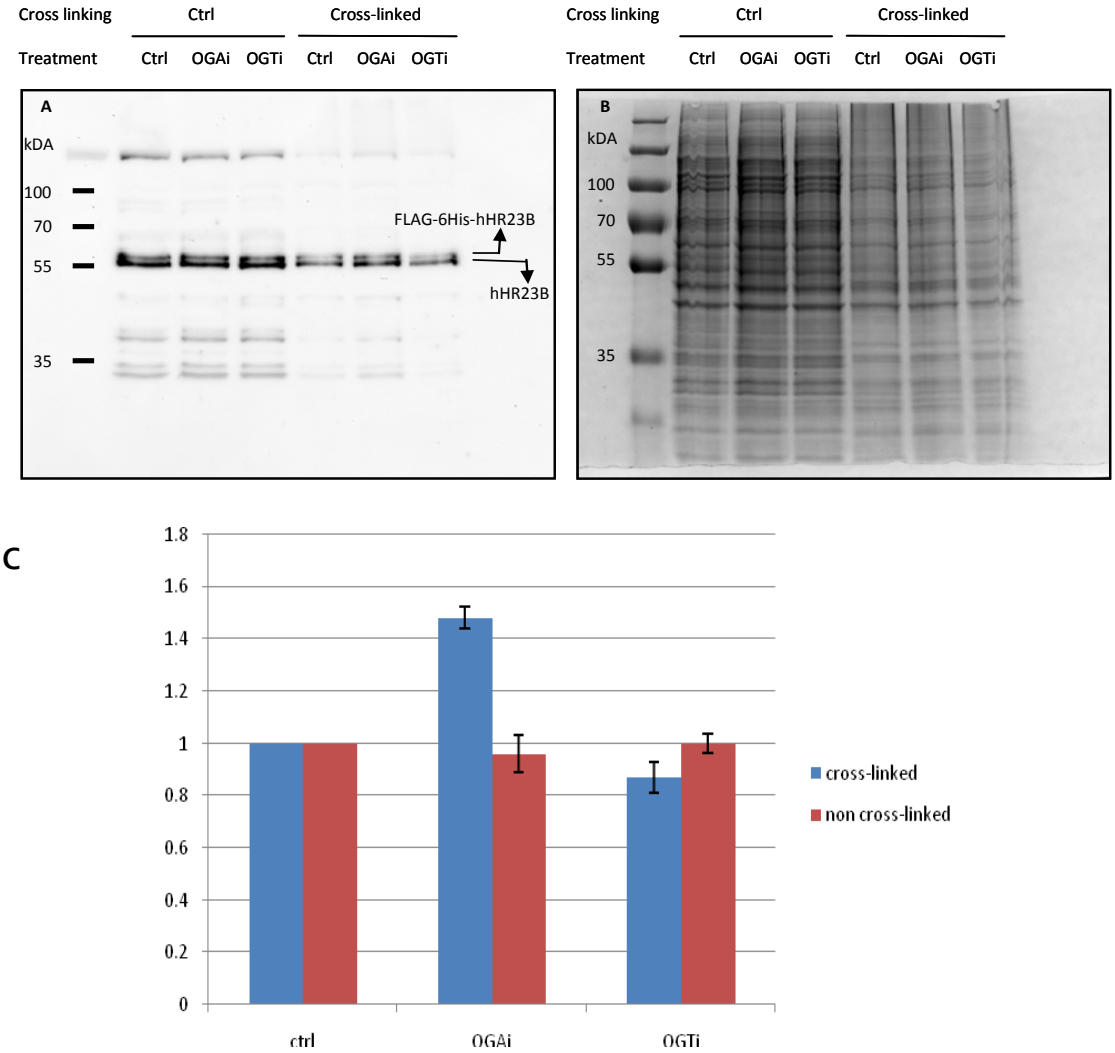

Figure 4: Detection of hHR23B monomer after O-GlcNAcylation regulation. Cellular proteins with or without OGA/OGT inhibitions were cross-linked by a formaldehyde treatment. Control samples and cross-linked samples were separated on SDS-PAGE, and detected by anti-hHR23B immunoblot after transferred to a PVDF membrane (A) or by Coomassie Blue staining (B). The hHR23B level is quantified from the blot (A) and normalized by the loading control (B) to get the quantification graph (C).

7.3 Discussion

O-GlcNAcylation is an essential regulator of many cellular processes, especially by modulation of protein-protein interactions and interplay with other post-translational modifications. The results in this chapter demonstrate that, hHR23B is indeed modified by O-GlcNAc under normal growth condition, which is confirmed by metabolic labeling of GlcNAz followed by the cis-trans pull down experiments. This result was subsequently reconfirmed by LC/MS mapping of the modification sites. Two out of six modification sites were identified on hHR23B in control cells without OGA or OGT inhibition treatment. It

indicates that the modification might be important for the protein functions or that the protein is O-GlcNAcylated under normal condition. Four extra O-GlcNAcylation sites were identified after OGA inhibition, which suggests that the modification is actually highly dynamic on hHR23B and the OGA activity was altered by the chemically synthesized inhibitor. Considering the localization of the O-GlcNAcylation sites identified, the linker between UBL and UBA1 might be the most possible heavily modified region. Moreover, LC/MS coverage of that part of the linker is low due to the difficult digestion, which implies that more O-GlcNAcylation sites might be identified. This also explains why hHR23B has been hardly picked up in previous O-GlcNAcylated protein identification studies, where trypsin was used as the protease for digestion(4).

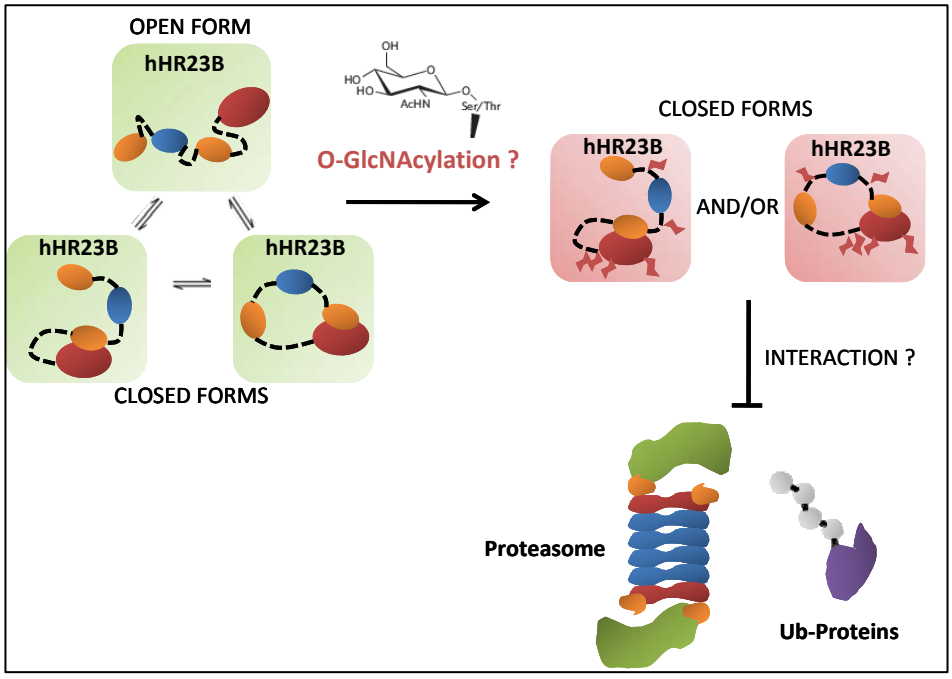

Figure 5: Hypothesis of the O-GlcNAc regulation of hHR23B. The O-GlcNAc moieties on the hHR23B protein might force it to present the closed mode of conformation. Further, the hHR23B interactome is altered by this closed conformation.

hHR23B is known as a key regulator of the global genome nucleotide excision repair pathway (GG-NER) and of the ubiquitin proteasome system (UPS)(15). It functions through binding to the interaction partner XPC that is in charge of the DNA damage recognition and recruitment of other NER factors, and stabilizes it against ubiquitination and proteasomal degradation. In the UPS, hHR23B is one of the major ubiquitin receptor proteins that shuttles polyubquitinated proteins to the proteasome for controlled degradation, by binding to the proteasomal substrates with the UBA domains and interacting with the proteasome using the UBL domain. So, research on the protein-protein interactions of hHR23B is the major focus of characterizing its functions. As an initial experiment, free

hHR23B level in cells with or without OGA/OGT inhibitions was compared. 50% more free hHR23B was observed in the OGA inhibited cells, while 10% less of that was detected in cells with OGT inhibition than in the control cells. This indicates that high O-GlcNAcylation level alters the PPI of hHR23B significantly, which then results in a low free protein ratio in total. Less O-GlcNAcylation does not affect this property of the protein so significantly. That might display the extra sugar moieties on the hHR23B after the OGA inhibition are important regulators for the PPI. These results correlate with previous reports on O-GlcNAcylation research of Sp1 and Tau(*6*, *7*).

The complete molecular mechanism why O-GlcNAcylation level can alter the PPI of hHR23B is still not clear. The hypothesis is that multiple sugars attached to the flexible linker change its property and possibly put the protein conformation into a closed mode, which does not allow the binding partners like proteasome or polyubiquintinated proteins to interact with it anymore (Fig 5). Possibly O-GlcNAcylation is used as a switch to rapidly activate or deactivate a certain pathway, in response to metabolic stimuli. The hypothesis is still to be proven. But recent observation opens new insights into the O-GlcNAcylation functions, that the modification can change the functions of scaffold protein which does not have enzymatic activity by altering its interactome. Furthermore, O-GlcNAcylation can influence the efficiency of the UPS by manipulating the functions of ubiquitin receptor proteins, so that the environmental glucose level and nutrition condition can be linked with the global protein turn over rate.

7.4 Experimental Procedures

7.4.1 Cell culture, transfection and treatment by chemicals

HEK293T cells were purchased from ATCC. The cells were grown in DMEM medium with 10% Fetal Calf Serum and 0.1 mg/ml penicillin and 0.1 mg/ml streptomycin, at 37°C, in a 5% CO_2 humid incubator. The cells were transfected with the PEI transfection reagent to express the target constructs. Ac4-GalNAz (**1**)(*3*), OGA inhibitor (**2**)(*20*) and OGT inhibitor (**3**)(*22*) were dissolved in DMSO before use. 1000x stock solution of the compounds were added to the cell culture for treatment to have the DMSO concentration lower than 1% in the culture medium. All treatments (250μM Ac4-GalNAz, 50 μM OGA inhibitor or 50 μM OGT inhibitor) described were done for 24 hours.

7.4.2 Immuno-precipitation and affinity purification

The cells were lysed in a mild lysis buffer containing 0.1% TX100, 50 mM Tris (pH 7.5), 5 mM EDTA, 250 mM NaCl and 10% Glycerol, supplemented with Protease inhibitor cocktail, phosphostop, and 100 μM OGA inhibitor. 1mg of the lysate was used for Staudinger-Bertozzi reaction with 250 μM biotin-phosphine. After the bioorthogonal ligation, the lysate could directly be used in an anti-FLAG immuno-precipitation

experiment with M2 magnetic agrose beads (Sigma-Aldrich). To do the biotin-streptavidin affinity purification, the protocol described in **Chapter 3** can be used with minor modifications. Reduction and alkylation steps can be skipped, because no LC/MS analysis is done afterwards. The lysis buffer described here can be used for the washing steps.

7.4.3 O-GlcNAcylation sites mapping with BEMAB

The FLAG-6His-hHR23B protein was purified by an anti-FLAG precipitation as described above. After elution, the protein was digested by Proteinase K for 30 min at 37°C. The digested peptides were acidified with Formic Acid to pH<3, cooled on ice. The peptides were desalted by a StageTip. The eluted peptides were evaporated to dryness in a SpeedVac. The dry peptides were dissolved in dephosphorylation buffer (20mM Tris pH7.5 and 10mM $MgCl_2$) and dephosphorylated by TSAP (thermosensitive alkaline phosphatise, Promega) for 3 hours at 37°C. The biotin-cystamine solution (dissolved in 0.4% NaOH and 4% triethylamine) was added at final concentration of 5mM. The reaction mixture was kept at 52°C for 2 hours(*21*). Then, the mixture was acidified and desalted by a StageTip again. The eluted sample was evaporated in a SpeedVac, and reconstituted with LC/MS sample solution (95ml H_2O, 3ml ACN and 0.1ml Fomic Acid). The samples could then be analyzed by nano-LC/MS.

The MS data was searched by Mascot against a self defined database containing only the hHR23B protein sequence to simplify the difficulty of calculations.

7.4.4 Formaldehyde cross linking

Formaldehyde solution was added to the medium of cells with or without OGA/OGT inhibitors treatment to reach a final concentration of 0.75%. The plates were shaken gently for several times to mix well and then incubated at room temperature for 15 minutes. 125mM Glycine was added to quench the cross linking reaction, for 5 minutes at room temperature. The medium was removed and cells were harvested by scraping.

7.4.5 Synthesis of biotin-cystamine

2-(tritylthio)ethan-1-amine A mixture of cysteamine hydrochloride (464mg, 4.1 mmol) and triphenylmethanol (1.06g, 4.1mmol) in 8 mL TFA was stirred for 24h at room temperature. After evaporation of all solvent in vacuum, water (40 mL) was added and pH was adjusted to 9 upon addition of $NaHCO_3$. The product was extracted with chloroform (3×20mL), the combined organic layer was washed by brine, dried over $MgSO_4$, filtered and concentrated in vacuum to product crude product and purification by flash chromatography (2%MeOH in DCM with 0.1% TEA) gave compound (200mg, yield 15.3%).

5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(tritylthio)ethyl) pentanamide Dissolve compound 2-(tritylthio)ethan-1-amine (75.5mg, 0.24mmol) in 5 mL DMF and then

add Biotin 70.8 mg (1.2 equi., 0.29mmol), HCTU 120mg(1.2 equi., 0.29mmol) and DiPEA 0.13mL(3 equi., 0.72mmol) and then stir at room temperature overnight. Remove DMF in vacum and dissolve the residue in EtOAc. The organic layer was washed by 1M HCl(2×20mL), Sat. $NaHCO_3$(3×20mL), and Brine(1×20mL) and dried over $MgSO_4$, giving crude product (126.7mg, yield %). The crude product was used without further purification.

N-(2-mercaptoethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide

Dissolve crude compound 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(2-(tritylthio)ethyl) pentanamide 126.7mg in 6 mL DCM and then add 4 mL TFA and 7 drops of triisopropylsilane. TLC shows complete conversion of the starting material after stirring at room temperature overnight. After evaporation of all solvent in vacuum, product (40mg, yield 56.8%) was obtained by HPLC purification.

^{1}HNMR (400 MHz, CD_3OD): 4.40(dd, 1H, J = 3.2, 4.8Hz), 4.21 (dd, 1H, J = 4.4, 4.4Hz), 3.12 (ddd, 1H, J = 4.4, 3.2, 3.2 Hz), 2.84 (dd, 1H, J = 4.8, 5.2Hz), 2.61 (d, 1H, J= 12.8 Hz), 2.52 (t, 2H, J = 4.8 Hz), 2.12 (t, 2H, J = 7.6 Hz), 1.67-1.28(m, 6H). 13C NMR (CD_3OD): 176.19 (C), 166.13 (C), 63.36 (CH), 61.62 (CH), 57.00 (CH), 43.84 (CH_2), 41.04 (CH_2), 36.72 (CH_2), 29.76 (CH_2), 29.47 (CH_2), 26.33 (CH_2), 24.50 (CH_2). MS $(M+H)^+$: 304.07.

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