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Review

Histone *O*-GlcNAcylation and Potential Biological Functions

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Abstract:
Histone modifications play an important role in the control of DNA-based processes by altering the structure and function of chromatin. *O*-linked N-acetylglucosamine (*O*-GlcNAc) modification is a form of post-translational modification of proteins that affects the serine (Ser)/threonine (Thr) residues. This process is controlled by a single pair of enzymes, i.e. *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). Recent evidence indicates the existence of *O*-GlcNAc modification of histones, with 16 histone *O*-GlcNAc sites reported to date. *O*-GlcNAc modification is a nutrient-sensitive modification; therefore, it is likely to serve as a molecular mechanism linking nutrient conditions and epigenetic status. Recently, functional analyses have been advanced by the acquisition of antibodies for the specific detection of *O*-GlcNAcylation of histone residues. Here, we discuss the current knowledge of histone *O*-GlcNAc modification, with a view to elucidating its comprehensive biological functions.

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Keywords
Histone modification; *O*-GlcNAcylation; chromatin

1. Introduction

Chromatin, which is a complex consisting mainly of histone proteins and DNA, is indispensable for packaging of the entire genome [1-3]. The compaction status of chromatin governs the accessibility of the transcriptional machinery to DNA; thus, it has plays a crucial role in establishing gene expression patterns [4, 5]. The nucleosome is the building block of chromatin, containing approximately ~147 base pairs of DNA wrapped around a histone octamer consisting of 2 two copies each of the histones H2A, H2B, H3, and H4 [6, 7]. Post-translational modifications (PTMs) of histones may regulate histone-histone and/or histone-DNA interactions related to genome activity, including transcription. Histones are subject to numerous types of PTMs with acetylation, methylation, and phosphorylation being the most abundant modifications forms [8.9]. Recently, accumulating evidence has revealed that histones are also modified by a the addition of a monosaccharide, GlcNAc (Table 1). In this review, we provide an overview of the current understanding of histone *O*-GlcNAcylation and discuss its potential roles in biological functions.

2. Regulation of *O*-GlcNAcylation

The nucleotide sugar UDP-GlcNAc serves as a donor for *O*-GlcNAcylation of Ser/Thr residues of nuclear and cytosolic proteins, which was first reported over 30 years ago [18]. Unlike complex *N*- and *O*-linked glyco-chains, the GlcNAc moiety is not further glycocylated following *O*-GlcNAcylation [19, 20]. Compared to other PTMs, the on/off cycling of *O*-GlcNAcylation of various types of proteins is uniquely regulated by a single pair of enzymes, i.e. *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) [21-25] (Figure 1)

The mechanism by which a single OGT enzyme processes the *O*-GlcNAcylation of thousands of substrates is believed to be dependent on the protein-protein interaction domain of OGT, the N-terminal tetratricopeptide-repeat (TPR) [23, 26]. Although the large diversity of OGT substrates is thought to be achieved by interactions between the TPR of OGT and so-called adaptor proteins, this mechanism is not yet fully understood [22, 32-37].

In humans and mice, two isoforms of OGA are produced by the alternative splicing of *MGEA5*/*Mgea5* mRNA [27]. The longer isoform, full-length OGA, is composed of an N-terminal GlcNAc hydrolytic cleavage domain for the removal of *O*-GlcNAc from target proteins [28]. Although there is a C-terminal acetyltransferase-like domain that shares sequence homology with GCN5 histone acetyltransferase (HAT) [29, 30], there are discrepancies among several reports on the HAT activity of OGA [38, 39]. Moreover, since structural analysis indicates that the C-terminal acetyltransferase-like domain lacks essential residues for the binding of acetyl-CoA as a substrate [40], it is likely that the acetyltransferase-like domain has no HAT activity. However, overexpression studies have confirmed the dual function of Mgea5/OGA as HAT and *O*-GlcNAcase in neuronal differentiated mouse ES cells [41]. Given the lack of an acetyl-CoA-binding site on OGA itself, it can be speculated that OGA interacts with other proteins with HAT activity. Furthermore,

the dual function of Mgea5/OGA is of great interest as an example of crosstalk between histone modifications.

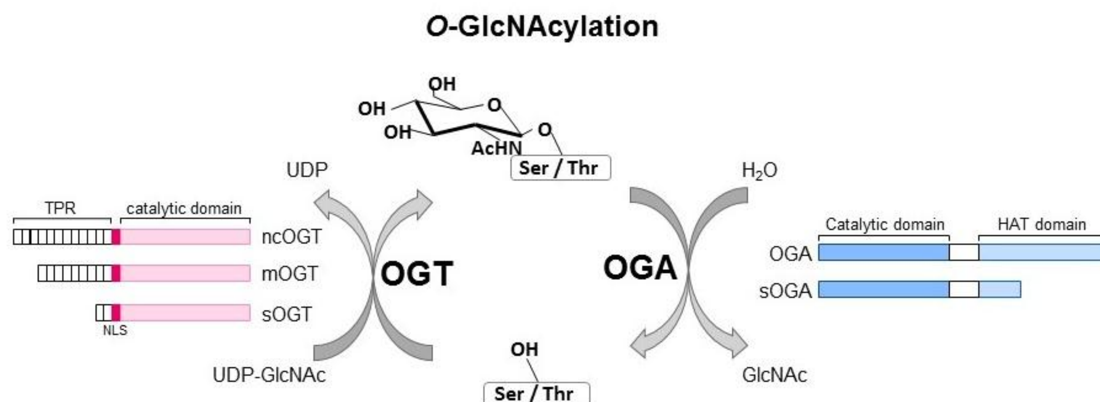

Figure 1 *O*-GlcNAcylation enzymes. The on/off cycling of *O*-GlcNAc on Ser/Thr residues is catalyzed by a writer (OGT) and an eraser (OGA) enzyme. Alternative splicing produces three isoforms of OGT with different lengths of tetratricopeptide-repeats (TPRs). Nucleocytoplasmic OGT (ncOGT) contains 13 TPRs, whereas mitochondrial OGT (mOGT) and short OGT (sOGT) contain nine and two TPRs, respectively [23, 26]. mOGT and sOGT have been implicated in apoptosis. Two isoforms of OGA are produced by alternative splicing [27]. The full-length OGA consists of catalytic and HAT-like domains. The short OGA isoform (sOGA) has a catalytic domain but no HAT-like domain [28-30]. Instead, the C-terminal domain of sOGA functions in targeting sOGA to intracellular lipid droplets for regulation of lipid storage [31].

As *O*-GlcNAcylation occurs on the Ser/Thr residues that are phosphorylated, crosstalk between these two PTMs is suggested to occur through reciprocal competitive block of sites involved in functional switching [42-44]. However, when >800 phosphorylation sites were monitored following inhibition of OGA, elevated *O*-GlcNAcylation resulted in lower phosphorylation at approximately 30% of the monitored sites, and unexpectedly caused increased phosphorylation at approximately 20% of the sites [45]. Therefore, crosstalk between *O*-GlcNAcylation and phosphorylation cannot be explained only by competitive substitution of both modifications with the same amino acid residue.

3. *O*-GlcNAcylation is a Nutrient-Sensitive PTM

The interactions between nutrient-sensing mechanisms, such as hexosamine biosynthesis pathway (HBP), and cellular pathways are key to energy homeostasis [46, 47]. Less than 5% of total cellular glucose flows into the HBP, leading to the production of UDP-GlcNAc, which is a donor for *O*-GlcNAcylation [48].

As the HBP depends on the availability of glucose, increasing extracellular glucose elevates the flux through the HBP and results in increased UDP-GlcNAc production. A dynamic change in the *O*-GlcNAcylated protein pattern is observed even when the concentration of UDP-GlcNAc is only slightly increased due to nutrient excess [49-52]. Therefore, since cellular levels of UDP-GlcNAc

and protein *O*-GlcNAc fluctuate with the availability of glucose, *O*-GlcNAcylation is recognized as a nutrient sensor.

As shown in Figure 2, UDP-GlcNAc biosynthesis by the HBP integrates flux not only from carbohydrate metabolism (glucose), but also from other metabolic pathways linked to nutrient intake. Fluctuation in the availability of these nutrients also affects the production of UDP-GlcNAc, causing a dynamic change in GlcNAcylation levels. Thus, *O*-GlcNAc modification can be considered as a broad range nutrient-sensing PTM [48, 53, 54].

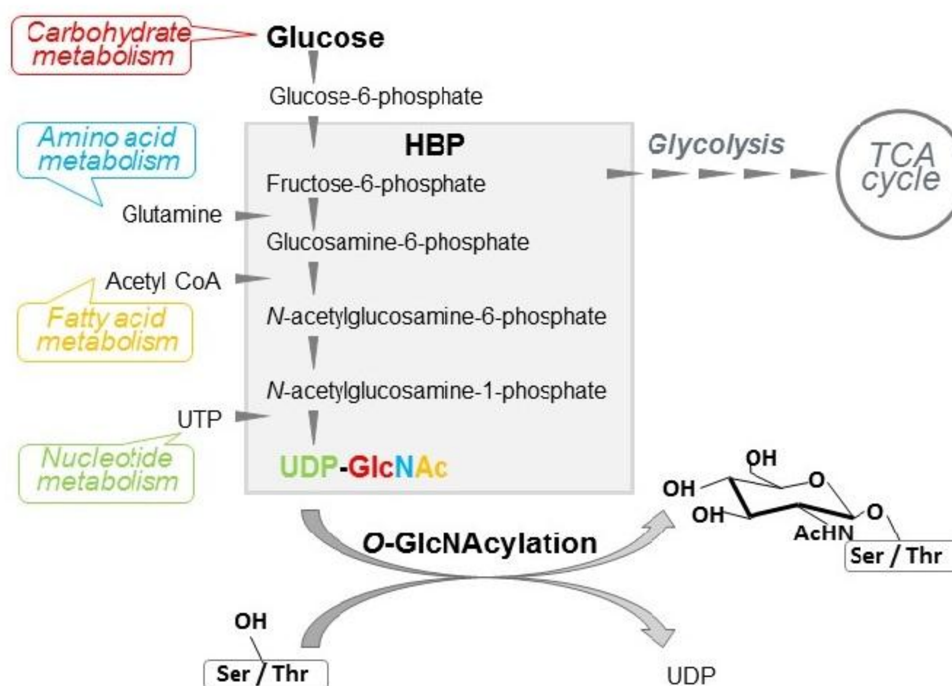

Figure 2 The hexosamine biosynthetic pathway (HBP). The donor for *O*-GlcNAc modification, UDP-GlcNAc, is the final product of HBP. The HBP integrates metabolites of carbohydrates (glucose), amino acids (glutamine), fatty acids (acetyl-CoA), and nucleotides (UTP) into the synthesis of UDP-GlcNAc, suggesting that the HBP may function as a nutrient-sensing pathway [48, 53, 54].

4. *O*-GlcNAcylation is one of a Variety of Histone Modifications

The first study of histone *O*-GlcNAcylation was reported by Sakabe *et al.* in 2010 [11]. Since then, 16 histone *O*-GlcNAcylation sites have been reported (Table 1) [10-17, 55-58]. Among these, all but two sites were identified by indirect techniques, such as immunoblotting, selective enzymatic labeling, chemoenzymatic detection, and lectin staining, used in combinations with mutation experiments, leaving to skepticism about the true existence of histone *O*-GlcNAcylation [11-16, 59]. However, the presence of *O*-GlcNAc at the two sites, H2AS40 and H3T32, was confirmed based on the detection of endogenous *O*-GlcNAc by mass spectrometry (MS) analysis of histones isolated from mammalian cells [10, 17], even though it is generally acknowledged that direct identification of peptidyl *O*-GlcNAcylated Ser/Thr by MS is challenging due to its unstable nature. Although the existence of some reported histone *O*-GlcNAcylations remains controversial [59, 60], evidence suggests that *O*-GlcNAcylation is a form of histone PTM.

Table 1 Overview of published strategies for detection of *O*-GlcNAc sites on histones.

Histone	Sites	Sample	Enrichment	Detection	References
H2A	S40	mES cells	RP-HPLC and mAb	*O*-GlcNAc site by HCD tandem MS	10
	T101*	HeLa cells	GalNAz labeling and DTT tagging	DTT tag by CID tandem MS	11
		Recombinant histone	*In vitro* reaction with OGT	*O*-GlcNAc site by ETD tandem MS	12
H2AX	T101	HeLa cells		Abolished *O*-GlcNAc signal by CTD110.6 Ab for FLAG-tagged H2AXT101A mutant	13
	S139	HeLa cells		Abolished *O*-GlcNAc signal by CTD110.6 Ab for FLAG-tagged H2AXS139A mutant	13
H2B	S36	HeLa cells	GalNAz labeling and DTT tagging	DTT tag by CID tandem MS	11
	T52, S55, S56, S64*	Various cell lines		Large scale CID tandem MS using the Oscore software, which assesses presence of *O*-GlcNAcylation	14
	S64*	Calf thymus	Lectin-pulldown and butylamine tagging	Butylamine tagging by CID tandem MS	15
	S91, S112, S123	Recombinant histone	*In vitro* reaction with OGT	*O*-GlcNAc site by ETD tandem MS	12
H3	S10	HEK239 cells	Overexpression and IP by tag Ab	Abolished *O*-GlcNAc signal by lectin staining of FLAG-tagged H3S10A mutant	16
	T32	HeLa cells	IP by anti-H3 Ab	*O*-GlcNAc site by ETD tandem MS	17
H3.3	T80	Calf thymus	Lectin-pulldown and butylamine tagging	Butylamine tag by CID tandem MS	15
H4	S47	HeLa cells	GalNAz labeling and DTT tagging	DTT tag by CID tandem MS	11

**O*-GlcNAcylated sites identified by independent research. S, Serine; T, Threonine; mES cells, mouse Embryonic Stem cells; RP-HPLC, reversed-phase high performance liquid chromatography; Ab, Antibody; mAb, monoclonal Antibody; GalNAz, azide-modified galactose; DTT, dithiothreitol; IP, immunoprecipitation; MS, mass spectrometry analysis; HCD, higher-energy collisional dissociation; CID, collision-induced dissociation; ETD, electron-transfer dissociation.

5. Biological Functions of *O*-GlcNAcylated Histones

The histone PTM *O*-GlcNAcylated H2BS112 was presumed by incubating unmodified histones with OGT and UDP-GlcNAc *in vitro*. A monoclonal antibody for the specific detection of this modification was originally raised against synthetic *O*-GlcNAcylated oligopeptides corresponding to a region flanking H2BS112. At present, only polyclonal anti-*O*-GlcNAcylated H2BS112 antibodies are commercially available. In HeLa cells, H2BS112 *O*-GlcNAcylation has been shown to promote H2BK120 mono-ubiquitination by providing GlcNAc as an anchor for ubiquitin ligase, leading to transcriptional activation via H3K4me3 [12]. In contrast, *O*-GlcNAcylation of H2BS112 preserves stable chromatin in the early stages of cell differentiation and may repress gene transcription in adipocytes [56]. Thus, the cellular functions associated with *O*-GlcNAcylation of H2BS112 appear to be quite diverse.

Mitosis is another cellular event in which histone *O*-GlcNAcylation is reported to play a role [61, 62]. Phosphorylation may occur on the same residues as *O*-GlcNAcylation; therefore, histone *O*-GlcNAcylation sites have been functionally analyzed in relation to phosphorylation. H3S10 and H3T32 are known to be phosphorylated when cells enter mitosis [61, 62]. The increase in *O*-GlcNAcylation of H3T32 causes a decrease in mitosis-specific phosphorylation of S10, S28, and T32 [16, 17, 61, 62], indicating that *O*-GlcNAcylation of H3T32 regulates mitosis by modulating mitosis-related histone phosphorylation. In addition, mitosis-specific phosphorylation of H3S10 can be competitively reduced by the level of *O*-GlcNAcylation at this site. Therefore, it can be speculated that *O*-GlcNAcylation of H3S10 regulates the pathways involving H3S10 phosphorylation, such as the G2/M checkpoint [9, 16, 17].

Functional analysis of *O*-GlcNAcylation at H2AT101 (H2AT101Gc) has been advanced by nucleosome reconstitution methods using synthesized GlcNAcylated histones. H2AT101Gc has been shown to influence nucleosome structure through destabilization of H2A/H2B dimers, causing the promotion of relaxed chromatin [55].

6. Newly Discovered *O*-GlcNAc Modification of H2AS40 in Placental Mammals

As part of the search for novel histone *O*-GlcNAcylation sites, a series of monoclonal antibodies were generated using an *O*-GlcNAcylated oligopeptide library containing several putative *O*-GlcNAcylation sites designed based on preliminary MS analyses of purified histones derived from mouse ES cells. One of the obtained antibodies, 20B2, was found to specifically recognize *O*-GlcNAcylated H2AS40 (H2AS40Gc) [10], which functions to maintain genome integrity through the DNA repair mechanism in coordination with γH2AX and AcH2AZ [58]. As *O*-GlcNAcylation of H2AXS139 [13] and H2BS112 [57] has also been implicated in DNA repair, we propose a DNA repair response mechanism centered on histone *O*-GlcNAc modifications.

The majority of histone modifications are highly conserved within the animal kingdom [63]. In contrast, a genomic database survey revealed that H2AS40Gc, with Ser at position 40, instead of Ala in the “universal” H2A, appears to be a modification that is unique to placental mammals. Chromatin immunoprecipitation sequencing (ChIP-seq) for genome-wide localization of H2AS40Gc in mouse trophoblast stem cells showed a dynamic change in the distribution pattern with differentiation [10]. Oxidative stress occurs in a normal placenta with the establishment of

maternal circulation [64-66]; therefore, placental mammal-specific H2AS40Gc might play a role in genome protection against DNA damage by reactive oxygen species (ROS) produced under oxidative stress in the placental environment. However, further investigations are required to verify this hypothesis**.**

7. Perspectives on *O*-GlcNAcylated Histones

As mentioned previously, *O*-GlcNAcylation-specific antibodies are a very powerful tool for functional analyses [10, 12, 56, 58]. However, historically, it has been difficult to obtain specific antibodies against sugar moieties. Even when such antibodies are successfully produced, they are usually IgM antibodies, which have limited use [67]. IgG antibodies specifically directed against *O*-GlcNAc histone modifications that are suitable for use in ChIP-seq analysis are required to progress our understanding of *O*-GlcNAc modification. We have attempted to produce such antibodies; the characterization of these antibodies and the validation of their targets is currently underway.

In hyperglycemia (insulin resistance), a rich UDP-GlcNAc pool produced by HBP flux results in an abnormally high level of protein *O*-GlcNAcylation. In the case of some key transcription factors or coactivators, increased levels of *O*-GlcNAcylation are known to stimulate gluconeogenesis/lipogenesis transcription, which further diminishes insulin sensitivity [20, 68, 69]. Histone *O*-GlcNAcylation could be regarded as a new factor in insulin resistance through epigenetic regulation. Therefore, investigations of the responses of histone *O*-GlcNAc modification to hyper-or hypoglycemic status are of great interest, suggensting that O-GlcNAcylated histone might have the potential in the diagnosis and/or prevention of chronic metabolic diseases such as diabetes.

Recent studies have shown that unbalanced *O*-GlcNAc modification leads not only to metabolic disease [46, 70, 71], but also to various types of other conditions, including neurological disorders [72, 73], cardiovascular disease [74, 75], and cancer [76-78]. In particular, high levels of *O*-GlcNAc modifications have been observed in breast [79-81], prostate [82, 83], lung, and colon cancers [84, 85], as well as in hepatocellular carcinoma [86, 87]. Although *O*-GlcNAc modification levels have been typically analyzed using only a pan-*O*-GlcNAcylation antibody, however, we speculate that histones are the key targets of *O*-GlcNAcylation and that as epigenetic mechanisms underlie the onset of chronic diseases [88-91].

The combination of the wide variety of histone modifications allows for increased complexity of epigenetic regulation [8]. With advances such as ChIP-seq, it is now possible to map the genome-wide distribution or colocalization of histone modifications at high resolutions, revealing the many combinations of histone modification-crosstalk, such as mutual exclusion, precondition, or coexistence [92]. The identification of the colocalization of H2BS112Gc and H2BK120 mono-ubiquitination [12], and that of H2S40Gc and γH2AX [58], has contributed to the elucidation of the biological functions of these histone *O*-GlcNAcylations. Thus, it is worth focusing on the crosstalk between *O*-GlcNAcylation and other histone modifications with the aim of determining the function of histone *O*-GlcNAc modifications.

The interplay between phosphorylation and *O*-GlcNAcylation may be one example of such crosstalk. Competition between phosphorylation and *O*-GlcNAcylation has been reported only for the H3S10, H3T32, and H2AXS139 sites [13, 16, 17]. Future studies should, therefore, investigate

phosphorylation of other residues for which *O*-GlcNAc modification has been reported. In addition, considering the presence of a HAT-like domain in OGA, the crosstalk between *O*-GlcNAcylation and acetylation should be validated, despite the controversy regarding the true HAT enzymatic activity of OGA [18, 30, 39-41, 93].

Studies of histone PTMs have focused mainly on the flexible N/C-terminal tails as the targets of numerous functional modifications that act by recruiting effector proteins [94,95]. Whereas modifications in the histone tails might have a limited structural impact on the nucleosome itself, PTMs in the globular domain of histones have a direct structural effect on the nucleosome through their influence on histone-histone or histone-DNA interactions [96, 97]. Of the 16 *O*-GlcNAcylation sites of histones reported, 13 sites are not present in the tail, but are located on the surface of the histone octamer (5 sites) or even on the inside of the nucleosome (8 sites) [10-17, 55-58, 98] (Figure 3). Therefore, it can be hypothesized that most *O*-GlcNAc modifications of histones function at the level of chromatin dynamics based on the structural changes of the nucleosome. This raises the new question as to whether such internal Ser/Thr residues are *O*-GlcNAcylated before or after nucleosome assembly. It is possible that *O*-GlcNAcylation site-specific adapter proteins facilitate the access of OGT to the inside of nucleosomes. Reconstitution experiments using a synthetic pure *O*-GlcNAcylated histone performed by Lercher *et al.* for H2AT101 [55], could help to clarify this issue.

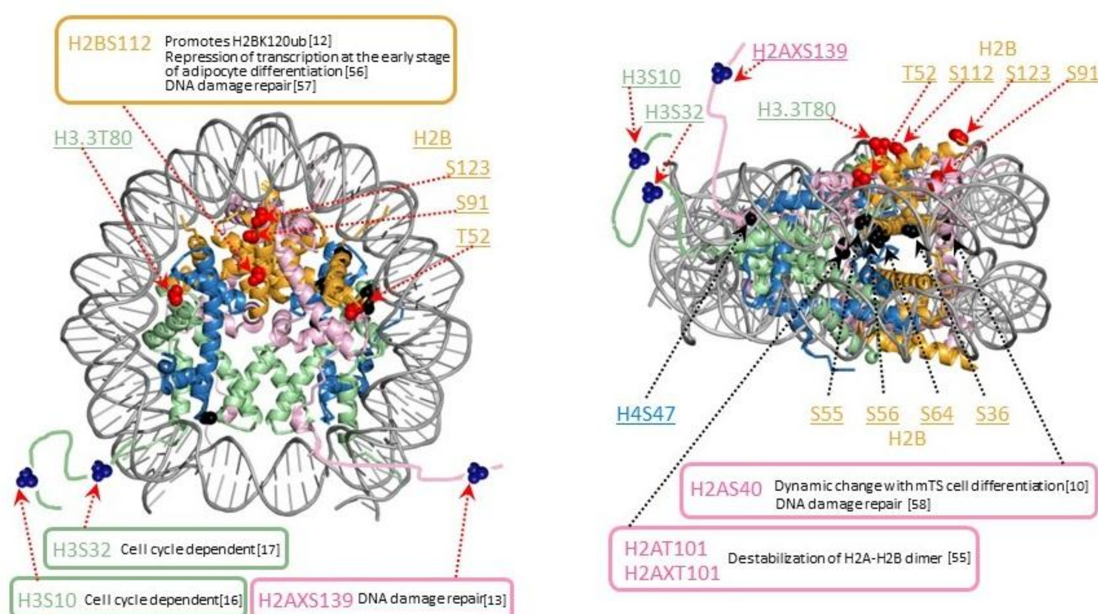

Figure 3 *O*-GlcNAcylated histone residues mapped onto the nucleosome structure. Top (left) and lateral (right) view of nucleosome structure (PDB ID: 3AFA) [98]. H2A: pink, H2B: orange, H3: green, H4: blue. The *O*-GlcNAcylated Ser or Thr side chains are indicated by spheres. *O*-GlcNAcylated residues in the histone tail: blue spheres, *O*-GlcNAcylated residues at the nucleosome surface: red spheres, *O*-GlcNAcylated residues inside the nucleosome: black spheres, reported characteristics.

8. Conclusions

Although the biological functions of histone *O*-GlcNAcylation have been gradually unveiled since its discovery nearly a decade ago, our knowledge of this protein modification is still limited. On the basis that *O*-GlcNAcylation functions as a nutrient sensor, histone *O*-GlcNAcylation can be regarded as a molecular mechanism linking metabolism and epigenetics, thus establishing a new paradigm of the epigenetic basis of chronic metabolic diseases.

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Author Contributions

M.H. wrote the manuscript with input from all authors.

Competing Interests

The authors declare no competing interests.

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