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Protein *O*-GlcNAcylation: emerging mechanisms and functions

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Abstract

O-GlcNAcylation—the attachment of *O*-linked N-acetylglucosamine (*O*-GlcNAc) moieties to cytoplasmic, nuclear and mitochondrial proteins—is a post-translational modification that regulates fundamental cellular processes in metazoans. A single pair of enzymes—*O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA)—controls the dynamic cycling of this post-translational modification in a nutrient- and stress-responsive manner. Recent years have seen remarkable advances in our understanding of *O*-GlcNAcylation at levels ranging from structural and molecular biology to cell signalling and gene regulation to physiology and disease. Emerging from these recent developments are new mechanisms and functions of *O*-GlcNAcylation that enable us to begin constructing a unified conceptual framework through which to understand the significance of this modification in cellular and organismal physiology.

Introduction

Post-translational modification (PTM) of proteins enables cells to respond promptly to internal and external cues through direct and dynamic control of protein function. Due to heightened appreciation for their essential roles in regulating diverse cellular processes, PTMs such as phosphorylation, acetylation, and ubiquitylation have garnered considerable interest over the past few decades. However, beyond these most well-studied modifications are many others whose significance in normal and disease physiology is only beginning to be understood. Notable among these emerging PTMs is *O*-GlcNAcylation, a non-canonical glycosylation involving the attachment of single *O*-linked N-acetylglucosamine (*O*-GlcNAc) moieties to serine and threonine residues of cytoplasmic, nuclear, and mitochondrial proteins[1, 2]. *O*-GlcNAcylation is the product of nutrient flux through the hexosamine biosynthetic pathway (HBP), which integrates glucose, amino acid, fatty acid, and nucleotide metabolism to generate the donor substrate for *O*-GlcNAcylation, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (Fig. 1a). In addition to being dependent on nutrient availability, *O*-GlcNAc signalling is highly sensitive to various forms of cellular stress (e.g. heat shock, hypoxia, nutrient deprivation), though the mechanisms mediating this response are only beginning to be described[2]. As a result, *O*-GlcNAcylation has been proposed to function as a nutrient and stress sensor regulating cellular processes ranging

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from transcription and translation to signal transduction and metabolism[1, 2]. Physiologically, disruption of *O*-GlcNAc homeostasis has been implicated in the pathogenesis of a plethora of human diseases, including cancer, diabetes, and neurodegeneration[3–9].

There is a large body of evidence indicating that *O*-GlcNAcylation engages in a complex interplay with other PTMs (Box 1). However, while other PTMs are often regulated by a diverse array of "writers" and "erasers," *O*-GlcNAcylation is controlled by a single pair of enzymes: *O*-GlcNAc transferase (OGT), which catalyzes the transfer of a GlcNAc moiety from the donor substrate UDP-GlcNAc to the hydroxyl groups of target serine and threonine residues; and *O*-GlcNAcase (OGA), which catalyzes the hydrolysis of this sugar modification[1, 2] (Fig. 1a). Through alternative splicing and alternate start codons, multiple isoforms of OGT and OGA can be generated from the *OGT* and *MGEA5* genes, respectively (Fig. 1b). The three isoforms of OGT (nucleocytoplasmic, ncOGT; mitochondrial, mOGT; short, sOGT) share common carboxy-terminal (C-terminal) catalytic and phosphoinositide-binding domains but differ in length due to variable numbers of amino-terminal (N-terminal) tetratricopeptide repeats (TPRs). These isoforms also differ in subcellular localization, with ncOGT and sOGT being found in the cytoplasm and nucleus and mOGT being found in the mitochondria. OGA exists in two isoforms: nucleocytoplasmic (ncOGA), which possesses both an N-terminal *O*-GlcNAc hydrolase and a C-terminal histone acetyltransferase-like (HAT-like) domain; and short (sOGA), which lacks the HAT-like domain and is localized to the endoplasmic reticulum and lipid droplets[6, 10].

Dramatic advances in our understanding of the biochemistry, molecular and cell biology, and physiology of *O*-GlcNAcylation have been made over the past decade. With rapidly accelerating interest in *O*-GlcNAcylation and the development of improved genetic and pharmacological tools to study its function, we expect that our understanding of this modification will continue to expand exponentially over the next several years. Thus, we seek to use this Review as a space to explore major scientific questions in the field and thereby provide a conceptual framework that will guide future studies in this area. Here, we consider potential mechanisms enabling recognition of hundreds of protein substrates by a single OGT and OGA; discuss how *O*-GlcNAcylation regulates cellular processes temporally and spatially in response to nutritional and hormonal cues; and explore how *O*-GlcNAc homeostasis may be maintained in order to achieve optimal cellular function. Since there are already many comprehensive reviews describing the diverse functions of *O*-GlcNAcylation, the focus of this Review is on consolidating this knowledge into unifying concepts, with an emphasis on contextualizing the significant advances the field has seen over the past several years[1, 2, 4, 6, 10–12].

Substrate recognition by OGT and OGA

How a single OGT and OGA are able to recognize hundreds of protein substrates has been a longstanding question in the field. While attempts to identify strict consensus sequences controlling substrate recognition have been unsuccessful, biochemical, biophysical, and computational analyses of the molecular structures of OGT and OGA have revealed potential mechanisms that together may confer substrate specificity to these enzymes.

Structural features and isoforms of OGT

The crystal structure of human OGT in a ternary complex with UDP and a peptide substrate shows that peptides are anchored in the OGT active site primarily by OGT side chain contacts with the peptide amide backbone[13]. The relative importance of active site contacts with the peptide backbone versus those with the peptide side chains reinforces the notion that OGT lacks a strict consensus sequence for substrate recognition. However, OGT has been shown to preferentially modify Ser and Thr residues flanked by amino acids that enforce an extended peptide conformation (e.g. prolines, β-branched amino acids), suggesting that moderate sequence constraints imposed by the active site may have some influence on OGT substrate selection[13, 14].

In the absence of a strict consensus sequence, OGT may achieve some level of substrate specificity through structural motifs outside of the active site. Indeed, the prevailing view in the field is that OGT substrate recognition is mediated by the N-terminal TPR domain, an extended superhelical structure of up to 13.5 TPRs that is thought to function as a scaffold for assembly of protein complexes[15]. Individually or in combination, these TPRs could facilitate substrate recognition by generating unique binding sites that, when occupied, induce a conformational change that permits substrate access to the active site (Fig. 2a). This model is supported by biochemical studies demonstrating that specific TPRs are required for the interaction of OGT with individual substrates such as Sin3a and ten-eleven translocation 2 (TET2)[16–19]. Furthermore, structural analyses and molecular dynamics simulations suggest that the TPR domain can shift from obscuring to fully exposing the active site by pivoting around a “hinge” region between the TPR and catalytic domains[13]. Thus, the TPR domain may confer substrate specificity by acting as a “gatekeeper,” restricting access to the active site until a potential substrate occupies its unique binding site formed by specific TPRs.

The crystal structure of OGT in complex with UDP and an OGT-binding peptide derived from host cell factor C1 (HCF-1) provides insight into how the TPR domain may form binding sites for OGT substrates. Specifically, the Thr-rich HCF-1 peptide occupies a channel generated by the TPR domain where conserved Asn residues engage the peptide backbone and Asp residues form hydrogen bonds with the peptide side chains[20]. This model is consistent with earlier hypotheses derived from structural analysis of the TPR domain alone, which proposed that conserved Asn residues within the TPR motifs define binding pockets that in tandem may facilitate substrate recognition. Intriguingly, the TPR domain of OGT was noted to have structural similarities to the peptide-binding site of importin α, which utilizes an analogous mechanism to bind various nuclear localization signals[21].

Since the three isoforms of OGT differ in TPR domain length and subcellular localization (Fig. 1b), they may target distinct but overlapping subsets of the proteome[22]. Indeed, tau and yes tyrosine kinase have been identified as substrates that are unique to ncOGT and mOGT, respectively, while proteins such as nucleoporin 62 (Nup62) and casein kinase II are *O*-GlcNAcylated by both isoforms[23]. Thus, some evidence suggests that OGT may also achieve some level of substrate specificity through its different isoforms. However, a recent study reported that endogenous expression levels of mOGT are extremely low in various human cell lines and murine tissues and that expression of ncOGT is sufficient to *O*-

GlcNAcylate mitochondrial proteins[24]. These findings highlight the uncertain role of mOGT in *O*-GlcNAc signalling and demonstrate the need for an improved understanding of the alternative OGT isoforms (mOGT and sOGT).

Adaptor protein hypothesis

OGT substrate recognition may be additionally mediated by adaptor proteins that recruit substrates to OGT in a context-dependent manner. For instance, during glucose deprivation, activated p38 mitogen-activated protein kinase (MAPK) interacts with the C-terminus of OGT and recruits OGT to the heavy neurofilament polypeptide NF-H, which leads to increased NF-H *O*-GlcNAcylation and solubility[25]. During fasting, HCF-1 functions as an adaptor protein that targets OGT to peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), enhancing its *O*-GlcNAcylation and stability and thereby upregulating the expression of genes involved in gluconeogenesis[26]. Specifically, OGT, HCF-1, and PGC-1α were shown to form a ternary complex and mutation of the HCF-1 binding motif of PGC-1α abolished PGC-1α *O*-GlcNAcylation. Unexpectedly, OGA has also been implicated as an OGT adaptor protein: OGT and OGA can interact to form an "*O*-GlcNAczyme" complex[27] and under high glucose conditions, acetylation of pyruvate kinase isoform M2 (PKM2) by the HAT-like domain of OGA promotes OGT-PKM2 interaction and PKM2 *O*-GlcNAcylation, leading to increased aerobic glycolysis in cancer cells (unpublished).

Since individual proteins (p38 MAPK, HCF-1, OGA) function as adaptors for OGT primarily in certain conditions (glucose deprivation, fasting, high glucose), together they may facilitate targeting of OGT to the appropriate substrates (NF-H, PGC-1α, PKM2) in these specific cellular contexts. Thus, we hypothesize that interaction with a hierarchy of highly conserved adaptor proteins, each responsible for recognizing and recruiting specific substrates, is an additional mechanism enabling OGT to control *O*-GlcNAcylation of hundreds of proteins in a context-dependent manner (Fig. 2b). The well-characterized ubiquitylation system is known to follow a similar paradigm in which the hundreds of E3 ubiquitin ligases confer substrate specificity to the tens of E2 ubiquitin-conjugating enzymes by acting as adaptor proteins[28].

Non-specific O-GlcNAcylation hypothesis

Since peptides are anchored in the OGT active site by OGT side chain contacts with the peptide backbone, OGT has been proposed to *O*-GlcNAcylate proteins in flexible regions (e.g. loops, termini) that can bind to the active site in an extended conformation to expose the amide backbone[13]. Thus, OGT is thought to preferentially modify substrates that contain flexible elements, which is corroborated by studies showing that proteins with regions of intrinsic disorder (e.g. nuclear pore proteins, Sp1, FOXO1, tau) are heavily *O*-GlcNAcylated in these disordered regions[8, 29–32]. These observations raise the intriguing possibility that OGT can non-specifically modify proteins in unstructured regions without recognizing any specific sequences or structures. In this case, substrate selection would be influenced only by sequence preferences intrinsic to the active site due to size and conformational restrictions[14].

Under normal physiological conditions, non-specific *O*-GlcNAcylation is unlikely to be a major mechanism of OGT substrate selection due to the limited number of flexible elements in most mature proteins. However, during cellular stress, the availability of these elements may increase due to accumulation of unfolded proteins in the cytoplasm or within cellular compartments. Perhaps not coincidentally, cellular *O*-GlcNAcylation levels are also dramatically elevated in response to various stress stimuli[33].

Accumulation of unfolded proteins in the cell activates stress response pathways such as the unfolded protein response (UPR) that can combat proteotoxic stress by blocking protein aggregation, modulating protein degradation, and facilitating protein re-folding[34]. Spliced X-box binding protein 1 (Xbp1s), a conserved UPR signal transducer and highly active transcription factor, has been shown to increase cellular *O*-GlcNAcylation levels through direct upregulation of HBP gene expression[35] (Fig. 2c). Coupling of the HBP to the UPR suggests that *O*-GlcNAcylation may play a critical role in the handling of unfolded proteins during cellular stress. Indeed, accumulating evidence indicates that *O*-GlcNAcylation is a key component of the cellular response to perturbations in protein homeostasis. For instance, *O*-GlcNAcylation has been shown to block proteotoxic aggregation of proteins associated with neurodegenerative disease such as tau (Alzheimer's disease) and α-synuclein (Parkinson's disease) as well as thermal aggregation of unrelated proteins such as TAB1[7, 36]. At the same time, *O*-GlcNAcylation can protect proteins from degradation by inhibiting proteasome function (via direct modification of Rpt2 and inhibition of 26S proteasome ATPase activity) or reducing protein ubiquitylation and may facilitate protein re-folding by recruiting chaperones with *O*-GlcNAc-directed lectin activity[26, 37–42]. Taken together, these observations raise the intriguing possibility that, in response to cellular stress, non-specific *O*-GlcNAcylation occurs in unstructured regions of unfolded proteins in order to block their aggregation and degradation and facilitate their re-folding (Fig. 2c).

This hypothesis is further supported by a recent study, which demonstrates that *O*-GlcNAcylation also occurs co-translationally on nascent polypeptides to protect them from premature ubiquitin-mediated degradation. Specifically, nascent Sp1 and Nup62 polypeptides, which are likely to be highly unstructured, were found to be more heavily *O*-GlcNAcylated than their mature counterparts, reinforcing the notion that unfolded proteins are attractive substrates for OGT. Furthermore, the fact that co-translational *O*-GlcNAcylation regulates protein quality control by reducing nascent polypeptide ubiquitylation is consistent with the notion that *O*-GlcNAcylation of unfolded proteins plays a critical role in maintaining protein homeostasis[30].

Substrate recognition by OGA

Insight into potential mechanisms governing OGA substrate recognition has been limited by the lack of a crystal structure for a eukaryotic OGA[43]. However, multiple studies have elucidated the structures of bacterial glycosidases that are close homologs of human OGA, some of which have been shown to possess *in vitro O*-GlcNAc hydrolase activity toward human proteins[44, 45]. Crystal structures of NagJ from *Clostridium perfringens* (*Cp*OGA) in complex with various synthetic glycopeptides reveal that *Cp*OGA binds different substrates in similar conformations by interacting primarily with the peptide backbone and sugar

moiety and avoiding contact with the peptide side chains[46]. Similar results were obtained from molecular docking and dynamics simulations analyzing models of various *O*-GlcNAcylated peptides in complex with *Bacteroides thetaiotaomicron* GH84 (*Bt*OGA). These findings suggest that OGA substrate recognition generally lacks sequence sensitivity, though variations in sequence near the *O*-GlcNAcylation site are predicted to have some effect on OGA-glycopeptide binding affinity[47]. While structural features and adaptor proteins could also confer substrate specificity to OGA, evidence for these mechanisms is limited at this time. We suspect that intrinsic substrate promiscuity enables OGA to recognize hundreds of *O*-GlcNAcylated proteins, analogous to how promiscuous protein phosphatases control the removal of phosphorylation installed by hundreds of protein kinases.

Functions of *O*-GlcNAcylation

Traditionally, protein glycosylation is thought to be limited to the endoplasmic reticulum, Golgi apparatus, and extracellular matrix. However, protein *O*-GlcNAcylation can be found in the cytoplasmic, nuclear, mitochondrial, and plasma membrane fractions, where it regulates fundamental cellular processes such as transcription, epigenetics, and cell signalling dynamics. By engaging in diverse protein complexes in a context-dependent and cellular compartment-specific manner, OGT and OGA can coordinately regulate complex networks of spatially separated cellular processes.

Regulation of transcription

While OGT and OGA are present in both the nucleus and the cytoplasm, OGT is specifically enriched in the nucleus whereas OGA is predominantly cytosolic[48–50]. Consistent with a significant nuclear role for OGT, early studies demonstrated that transcription factors are modified by *O*-GlcNAc, implicating this modification in transcriptional regulation[51]. As the repertoire of *O*-GlcNAcylated transcription factors and cofactors has steadily expanded, their biological functions have also become increasingly recognized. For instance, in T and B lymphocytes, OGT *O*-GlcNAcylates the crucial transcription factors nuclear factor of activated T cells 1 (NFATc1) and nuclear factor kappa B (NF-κB) and is required for lymphocyte activation[52]. In hepatocytes, *O*-GlcNAcylation of CRTC2, FOXO1, and PGC-1α modulates expression of gluconeogenic genes[26, 53, 54]. Ultimately, regulation of cell type- and tissue-specific genetic programs by *O*-GlcNAcylation gives rise to its many distinct functions in individual cell types and tissues (Table 1).

Mechanistically, *O*-GlcNAcylation can affect the translocation, DNA binding, transactivation, and stability of transcription factors. In the case of NF-κB, *O*-GlcNAcylation of the RelA subunit decreases its binding to NF-κB inhibitor alpha (IκBα) and increases its nuclear translocation and transcriptional activity, whereas *O*-GlcNAcylation of c-Rel is required for its DNA binding and transactivation[52, 55, 56]. However, the effects of OGT on NF-κB are context-dependent: in the presence of the glucocorticoid receptor (GR), OGT mediates the transrepression of NF-κB activity[57]. Sp1 is another prototypical transcription factor that illustrates the role of *O*-GlcNAcylation in transcriptional regulation. Sp1 *O*-

GlcNAcylation has been shown to modulate its nuclear localization, transactivation, and stability[58–60] (Fig. 3a).

It has been known for over two decades that RNA polymerase II (Pol II) is modified by *O*-GlcNAc[61]. The C-terminal domain (CTD) of Pol II is reciprocally *O*-GlcNAcylated and phosphorylated at Ser 2 and 5. *O*-GlcNAcylated Pol II is enriched at transcription start sites and involved in preinitiation complex assembly, whereas removal of *O*-GlcNAcylation from the Pol II CTD allows for its dynamic phosphorylation during transcription initiation and elongation[62, 63] (Fig. 3a). Thus, it is conceivable that, on actively transcribed genes, the sequential action of OGT and OGA is essential for recycling Pol II back to the preinitiation complex after a round of transcription. As a result, perturbation of OGT or OGA function would lead to Pol II stalling and blockage of the transcription cycle.

Regulation of epigenetics

An exciting area of study with regard to the nuclear functions of *O*-GlcNAcylation stems from the discovery that OGT interacts with diverse epigenetic regulators[10, 63–65]. An early study identified an association between ncOGT and the Sin3a/histone deacetylase (HDAC) corepressor complex and proposed that *O*-GlcNAcylation of transcription factors and Pol II acts in parallel with histone deacetylation to promote gene silencing[16]. This model has been greatly expanded by recent findings demonstrating that OGT interacts with various other proteins involved in histone modification and DNA methylation[10, 63–65] (Fig. 3b).

HCF-1 is an auxiliary protein for a battery of histone-modifying enzymes, including histone methyltransferases, demethylases, acetyltransferases, and HDACs[65]. About half of nuclear OGT exists in complex with HCF-1, linking OGT to these various histone modifications[66]. The OGT/HCF-1 complex has been shown to interact with BRCA-associated protein 1 (BAP1), a component of the Polycomb repressive deubiquitylase (PR-DUB) complex that deubiquitylates histone H2A[26, 67]. OGT is also required for the trimethylation of histone H3 at lysine 27 (H3K27me3), likely because it stabilizes the histone methyltransferase enhancer of zeste homolog 2 (EZH2) and maintains the integrity of Polycomb repressive complex 2 (PRC2)[68, 69]. Thus, nuclear OGT may regulate epigenetic programs by modulating histone acetylation, ubiquitylation, and methylation (Fig. 3b). However, the epistatic relationships between OGT and histone-modifying enzymes are largely unclear.

DNA methylation at the 5-carbon position of cytosine (5mC) is a key epigenetic mark. The reversal of DNA methylation is mediated by ten-eleven translocation (TET) proteins that successively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC)[70]. Recent studies have revealed that a large percentage of nuclear OGT exists in complex with TET proteins[63–65]. Some studies suggest that TET proteins facilitate the recruitment of OGT to chromatin to enhance histone *O*-GlcNAcylation, while others suggest that OGT can directly *O*-GlcNAcylate TET proteins and modulate their stability or nuclear localization[18, 19, 71, 72]. Furthermore, OGT and TET proteins may act cooperatively to maintain the integrity and activity of the SET1/COMPASS H3K4 methyltransferase complex[73]. Although the functional relationship between OGT and TET proteins is still controversial, these studies highlight an important link between OGT and DNA methylation in epigenetic regulation.

Temporal regulation of cell signalling dynamics

O-GlcNAcylation is a key regulator of the temporal dynamics of various cell signalling pathways. The most well-studied example of this phenomenon is the temporal regulation of insulin signalling dynamics by *O*-GlcNAcylation[74]. An early study showed that global elevation of *O*-GlcNAcylation levels in cultured adipocytes using the non-selective OGA inhibitor PUGNAc blocks insulin-stimulated phosphorylation of AKT, a Ser/Thr kinase critical for cell survival and metabolism[75]. Conversely, overexpression of OGA in HepG2 hepatoblastoma cells results in decreased AKT *O*-GlcNAcylation and increased AKT phosphorylation and activity[76]. The *O*-GlcNAcylation sites of AKT have been mapped to Thr 308 and Ser 473, which are also crucial phosphorylation sites for AKT activation[77]. These observations suggest that *O*-GlcNAcylation of AKT directly opposes its phosphorylation.

Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is a central mediator of insulin signal transduction. Upon insulin stimulation, phosphoinositide 3-kinase (PI3K) catalyzes the production of PIP_3, which recruits phosphoinositide-dependent kinase 1 (PDK1) and AKT to the plasma membrane, where PDK1 phosphorylates and activates AKT[78]. Interestingly, like PDK1 and AKT, OGT is a PIP_3-binding protein[5]. After prolonged insulin stimulation, PIP_3 recruits OGT from the cytoplasm to the plasma membrane, where it *O*-GlcNAcylates and inhibits multiple components of the insulin signalling pathway, leading to gradual attenuation of insulin signal transduction[5, 79] (Fig. 3c). Although a large body of evidence indicates that *O*-GlcNAcylation negatively regulates insulin signalling, global elevation of cellular *O*-GlcNAcylation levels by selective OGA inhibitors such as NButGT and 6-Ac-Cas fails to induce insulin resistance[80]. These seemingly contradictory results can be reconciled assuming that mutual regulation of OGT and OGA is critical for temporal control of insulin signalling dynamics (see discussion under “Maintenance of *O*-GlcNAc homeostasis”).

Temporal regulation of O-GlcNAc signalling

O-GlcNAcylation is highly dynamic and often occurs transiently in response to diverse environmental and physiological cues, suggesting that *O*-GlcNAc signalling itself is also under tight temporal control. For instance, cellular *O*-GlcNAcylation levels are globally downregulated in the first few hours following glucose deprivation but are markedly upregulated at later time points[25, 81, 82]. In adipocytes, insulin stimulates robust but transient *O*-GlcNAcylation of the insulin signalling pathway within 30 minutes[5]. In T and B lymphocytes, *O*-GlcNAcylation of the transcription factor NFATc1 surges 5–10 minutes after lymphocyte activation[52]. Remarkably, *O*-GlcNAcylation levels in neurons rapidly increase in the first 1–2 minutes following depolarization but return to baseline levels within 5 minutes[83]. Since the transience of *O*-GlcNAcylation cannot simply be explained by changes in the intracellular availability of UDP-GlcNAc, unveiling the molecular mechanisms that dynamically regulate *O*-GlcNAc signalling is one of the most pressing challenges in the field.

To date, little is known about how various stimuli trigger dynamic changes in protein *O*-GlcNAcylation. Reducing UDP-GlcNAc levels via glucose deprivation has been reported to increase OGT expression and decrease OGA expression, pointing to the existence of a

transcriptional feedback loop that helps maintain *O*-GlcNAc homeostasis (see discussion under "Maintenance of *O*-GlcNAc homeostasis")[25, 81, 82]. However, as mentioned above, fluctuations in cellular *O*-GlcNAcylation levels often occur on the time scale of minutes, indicating that modulation of gene expression is unlikely to be the primary mode of regulation for the *O*-GlcNAc signalling pathway. We envision that the PTMs of OGT and OGA are the major regulators of *O*-GlcNAc signalling dynamics; thus, identifying and characterizing the functions of these putative modifications will provide critical insight into the temporal regulation of *O*-GlcNAcylation.

Nutritional and hormonal regulation

At the nexus of glucose, amino acid, fatty acid, and nucleotide metabolism, *O*-GlcNAcylation is especially poised to function as a nutrient sensor, coupling fluctuations in nutrient availability to shifts in downstream signalling pathways. The prevailing view in the field has been that cellular *O*-GlcNAcylation levels positively correlate with the availability of specific nutrients due to corresponding changes in flux through the HBP. However, in recent years, it has become increasingly clear that metabolic regulation of *O*-GlcNAc signalling is far more complex than we originally thought.

Nutritional regulation of O-GlcNAc signalling

Early biochemical analysis of OGT demonstrated that increasing the concentration of UDP-GlcNAc enhances *in vitro O*-GlcNAcylation of various peptide substrates in a dose-dependent manner[84]. Since biosynthesis of UDP-GlcNAc via the HBP requires glucose, glutamine, acetyl-CoA, and UTP (Fig. 1a), cellular *O*-GlcNAcylation levels are thought to be elevated when the availability of these nutrients is increased. To date, numerous groups have observed this trend, particularly in response to modulation of extracellular glucose concentrations *in vitro*[81]. *In vivo*, hyperglycemia has been shown to raise cellular *O*-GlcNAcylation levels in various tissues[85]. Many studies have also demonstrated that even low concentrations of glucosamine, a metabolite that bypasses the rate-limiting step of the HBP (conversion of fructose-6-phosphate to glucosamine-6-phosphate by GFAT) (Fig. 1a), can dramatically enhance *O*-GlcNAcylation, further supporting the notion that HBP flux is a major determinant of cellular *O*-GlcNAcylation levels[81, 82]. However, accumulating evidence indicates that the relationship between nutrient availability and *O*-GlcNAcylation is not a simple positive correlation, suggesting that changes in HBP flux are not the only factor driving the nutrient sensitivity of this modification. For instance, *O*-GlcNAcylation of PGC-1α was shown to peak at 5 mM glucose and decrease gradually as glucose concentrations approached either hypo- or hyperglycemia[26]. Since UDP-GlcNAc levels would be highest under hyperglycemic conditions, this pattern of *O*-GlcNAcylation would be unexpected under the conventional view that *O*-GlcNAcylation levels respond linearly to changes in nutrient availability. Consistent with these observations, glucose production and gluconeogenic gene expression, which are regulated by PGC-1α *O*-GlcNAcylation, were also shown to peak at 5 mM glucose and to be gradually suppressed as glucose availability is increased or decreased[26]. This suggests that *O*-GlcNAcylation levels vary on a substrate-by-substrate basis depending on the overall metabolic needs of the cell and are not simply a direct readout of HBP flux.

How are *O*-GlcNAcylation levels regulated in both a nutrient-sensitive and a substrate-specific manner? In the case of PGC-1α, the interaction between OGT and its adaptor protein HCF-1 displayed the same response to changes in glucose concentration as PGC-1α *O*-GlcNAcylation[26]. This suggests that nutrient availability can also regulate substrate *O*-GlcNAcylation levels by modulating the interactions between OGT and its adaptor proteins (see discussion under “Adaptor protein hypothesis”).

Another unexpected phenomenon that has been consistently observed in numerous studies is a dramatic global increase in cellular *O*-GlcNAcylation levels under conditions of nutrient deprivation[25, 81, 82, 86]. Since UDP-GlcNAc levels are decreased under these conditions, this effect cannot be explained by changes in HBP flux[81, 82]. One potential mechanism mediating this response is upregulation of *OGT* gene expression, which may raise cellular *O*-GlcNAcylation levels despite the reduced intracellular availability of UDP-GlcNAc[25, 81, 82]. Furthermore, accumulation of unfolded proteins due to nutrient stress may increase the abundance of high affinity substrates for OGT, which would enhance the efficiency of protein *O*-GlcNAcylation on a global scale (see discussion under “Non-specific *O*-GlcNAcylation hypothesis”). Thus, we propose that nutrient availability regulates cellular *O*-GlcNAcylation levels not only by determining the abundance of UDP-GlcNAc but also by modulating the levels of OGT, OGA, and their respective adaptor proteins and substrates (Fig. 4a).

Hormonal regulation of O-GlcNAc signalling

Our current understanding of metabolic regulation of *O*-GlcNAc signalling is primarily derived from *in vitro* experiments in which the availability of various nutrients can be dramatically altered by modulating their concentrations in cell culture medium. However, *in vivo* nutrient availability is unlikely to undergo such drastic changes in normal physiological conditions; thus, nutritional regulation of *O*-GlcNAc signalling may function primarily to fine-tune cellular *O*-GlcNAcylation levels in response to local metabolic cues. As a key regulator of cellular pathways that control whole body metabolism, *O*-GlcNAcylation is also sensitive to systemic metabolic changes, which are communicated to the relevant cell types and tissues via hormonal signals. Indeed, several studies have now shown that *O*-GlcNAc signalling is regulated by a variety of hormones that encode information about systemic metabolic status, including insulin, glucagon, and ghrelin.

Since disruption of *O*-GlcNAc homeostasis has been implicated in the pathogenesis of insulin resistance, the effect of insulin stimulation on *O*-GlcNAc signalling has been particularly well studied[5]. Specifically, insulin signalling has been shown to regulate OGT through several distinct mechanisms, including modulation of OGT expression, subcellular localization, and enzymatic activity. In addition to upregulating OGT expression through a PI3K-dependent pathway, insulin stimulation promotes translocation of OGT from the nucleus to the cytoplasm and localization of OGT to lipid rafts in the plasma membrane, where it is activated via tyrosine phosphorylation by the insulin receptor[5, 87, 88] (Fig. 3c). Modulation of *O*-GlcNAc signalling in response to insulin stimulation enables temporal control of insulin signal transduction and its downstream effects on cellular metabolism (see discussion under “Temporal regulation of cell signalling dynamics”).

Recent studies have revealed that other hormones can also regulate *O*-GlcNAc signalling in response to systemic changes in metabolic status. For instance, starvation-induced glucagon signalling in the liver stimulates phosphorylation of OGT by calcium/calmodulin-dependent kinase II (CaMKII), which targets OGT to the autophagy-initiating kinase ULK1 and thereby activates autophagy. Liver autophagy then generates amino acids and fatty acids as substrates for gluconeogenesis and ketogenesis, critical pathways in the metabolic response to starvation (unpublished). Ghrelin, secreted by the empty stomach during fasting, raises cellular *O*-GlcNAcylation levels in the appetite-stimulating Agouti-related peptide (AgRP) neurons, which increases their firing rate via activation of the voltage-dependent potassium channel KCNQ3. Increased firing of AgRP neurons during fasting reduces energy expenditure by suppressing browning of white adipose tissue (WAT)[89]. Thus, modulation of *O*-GlcNAc signalling in specific cell types and tissues (liver, AgRP neurons) by hormones (insulin, glucagon, ghrelin) secreted in response to systemic metabolic changes (feeding, starvation, fasting) leads to regulation of key response pathways (insulin signalling, autophagy, WAT browning) that help maintain metabolic homeostasis (Fig. 4b).

Maintenance of *O*-GlcNAc homeostasis

Since *O*-GlcNAcylation plays a vital role in spatiotemporal regulation of cellular processes in response to nutritional and hormonal cues, maintenance of *O*-GlcNAc homeostasis is essential for optimal cellular function. Indeed, disruption of *O*-GlcNAc homeostasis has been implicated in the pathogenesis of a multitude of human diseases[3–9]. For instance, a single nucleotide polymorphism in the human OGA gene *MGEA5* has been shown to be associated with an increased risk of developing type 2 diabetes mellitus in the Mexican-American population[90]. Consistent with this finding, knockout of the *Caenorhabditis elegans* OGA gene *oga-1* reduces lipid storage, a metabolic perturbation that is associated with insulin resistance in humans[91]. Interestingly, the *C. elegans ogt-1* knockout displayed a phenotype similar to that of the *oga-1* knockout despite the fact that these knockouts had opposite effects on global *O*-GlcNAcylation levels[91, 92]. This intriguing observation points to the existence of an “optimal zone” within which global *O*-GlcNAcylation levels must remain in order to preserve normal cellular function (Fig. 5).

How might cells be able to maintain global *O*-GlcNAcylation levels within the “optimal zone” and under what conditions might these mechanisms become deregulated? We hypothesize that cellular *O*-GlcNAc homeostasis is maintained through mutual regulation of OGT and OGA at the transcriptional and post-translational levels. Specifically, OGT may regulate *MGEA5* transcription as well as OGA enzymatic activity and stability and vice versa. Mutual regulation in this manner would allow cells to maintain a balance between OGT and OGA expression and activity, thereby generating a “buffering” system that enables precise control of global *O*-GlcNAcylation levels (Fig. 5). Indeed, OGA has been shown to upregulate *OGT* gene expression through activation of the transcription factor CCAAT/enhancer-binding protein β (C/EBP-β)[93]. Conversely, pharmacological inhibition of OGA has been shown to increase *MGEA5* gene expression, indicating that elevated cellular *O*-GlcNAcylation levels promote compensatory *MGEA5* transcription, perhaps through enhanced *O*-GlcNAcylation of specific transcription factors and cofactors[94]. Both OGT and OGA are also known to be *O*-GlcNAcylated, suggesting that they are subject to

autoregulation and mutual regulation at the post-translational level[48, 95]. We suspect that *O*-GlcNAcylation of OGT and OGA modulates their activities and stabilities; however, the exact functions of the identified modification sites have yet to be determined.

As with any buffering system, the cellular *O*-GlcNAcylation "buffer" generated by mutual regulation of OGT and OGA can likely tolerate moderate and acute perturbations but may be overcome by severe and chronic insults. Since *O*-GlcNAcylation is a nutrient sensor, physiological variations in nutrient availability will produce mild fluctuations in cellular *O*-GlcNAcylation levels within the limits of the "optimal zone"; however, prolonged nutrient deficiency or excess may drive cellular *O*-GlcNAcylation levels beyond the "optimal zone," resulting in gradual loss of normal cellular function. Similarly, since *O*-GlcNAcylation is protective against cellular stress, global *O*-GlcNAcylation levels will be transiently elevated in response to moderate stress stimuli; however, sustained elevation of global *O*-GlcNAcylation levels beyond the "optimal zone" due to severe and chronic stress may have deleterious effects on overall cellular function despite being protective against the specific cellular insult. We propose that loss of *O*-GlcNAc homeostasis due to disruption of the cellular *O*-GlcNAcylation "buffer" generated by mutual regulation of OGT and OGA is an important factor contributing to the pathogenesis of various human diseases (Fig. 5).

Conclusions and perspectives

Since *O*-GlcNAcylation of intracellular proteins was first described by Torres and Hart in 1984, significant strides have been made in identifying "what" proteins and pathways are regulated by this modification[96]. In this Review, we have focused our discussion on key concepts and hypotheses derived from this extensive body of knowledge: "how" OGT and OGA recognize their numerous substrates; "when" and "where" *O*-GlcNAc signalling regulates the vital processes of the cell; "how" nutrients and hormones control cellular *O*-GlcNAcylation levels; and "how" *O*-GlcNAc homeostasis is maintained in order to achieve optimal cellular function. It is our hope that this framework of ideas, some well established and others more speculative, will guide continued investigation into the "what," "when," "where," and "how" of this blooming field.

Ultimately, however, the question we as a field would like to answer is: "why?" Specifically, "why" did the *O*-GlcNAcylation system first arise and "why" has it been evolutionarily conserved from *C. elegans* and *Drosophila* to mice and humans? One perspective from which to address this question is to consider what cellular and physiological functions of *O*-GlcNAcylation are shared across species. Though this Review has generally focused on studies performed in mammalian systems, valuable insights have also been gained from significant efforts to characterize the role of *O*-GlcNAcylation in other model organisms, including *C. elegans, Drosophila*, and *Arabidopsis*[49, 69, 97–101]. Like those in mammalian systems, studies in these organisms have demonstrated the importance of *O*-GlcNAc signalling in the regulation of diverse cellular processes such as autophagy[102, 103], circadian rhythm[104–106], epigenetics[69, 97, 107], carbohydrate and lipid metabolism[91, 92], hormone signalling[106, 108], protein homeostasis[109, 110], and stress response[111, 112]. Thus, it is likely that the need for a robust biological system to regulate these vital cellular pathways in a

coordinated and integrated fashion contributed to the evolutionary conservation of *O*-GlcNAcylation.

Another perspective from which to address this question is to consider what unique and indispensable molecular role is fulfilled by *O*-GlcNAcylation. We propose that *O*-GlcNAcylation can be viewed as the essential “grease and glue” of the cell: it acts as a “grease” by coating target proteins (folded or unfolded, mature or nascent) and preventing unwanted protein aggregation or modification (see discussion under “Non-specific *O*-GlcNAcylation hypothesis”); it also acts as a “glue” by modulating protein-protein interactions in time and space in response to internal and external cues, thereby affecting the functions of various proteins in the cell (see discussion under “Functions of *O*-GlcNAcylation”).

Though both of these perspectives yield intriguing hypotheses, our knowledge of the “origin story” of *O*-GlcNAcylation is ultimately far from complete. Moving forward, systems biology approaches that enable us to observe how *O*-GlcNAc signalling coordinates and integrates the cell’s diverse molecular networks will become increasingly important as we seek to understand the “why” of this fascinating modification.

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Glossary

Tetratricopeptide repeats (TPRs)
34 amino acid structural motifs that are found in tandem arrays in many proteins; mediate protein-protein interactions and assembly of protein complexes

Molecular dynamics simulation
a computational method that simulates the physical movements of atoms and molecules; can be used to model the internal motions and conformational changes of biological macromolecules to understand the physical basis of their structures and functions

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α)
a transcriptional coactivator and master regulator of gluconeogenesis and mitochondrial biogenesis

Gluconeogenesis
a metabolic pathway that produces glucose from various carbon sources, including glycerol, lactate, and certain amino acids; occurs primarily in the liver and kidney in mammals

Pyruvate kinase isoform M2 (PKM2)
an isozyme of pyruvate kinase, the enzyme that catalyzes the final step of glycolysis; PKM2 has been implicated in the reprogramming of metabolic pathways in cancer

Aerobic glycolysis
the preferential utilization of glycolysis and lactic acid fermentation for ATP production despite the availability of oxygen for oxidative phosphorylation; a hallmark of metabolic reprogramming in cancer

Lectin
a protein that recognizes and binds to specific carbohydrates; these carbohydrates can be mono- or oligosaccharides and soluble or attached to glycolipids and glycoproteins

Molecular docking simulation
a computational method that simulates the process of a ligand binding to an enzyme or receptor; can be used to predict the preferred orientation of a ligand in an enzyme’s active site

Transactivation
the increased expression of a gene that is induced by the expression of a transactivator protein (e.g. transcription factor); transcription factors possess transactivation domains that contain binding sites for transcriptional coregulators

Transrepression
the repression of the activity of one protein (e.g. transcription factor) through its interaction with a second protein

Preinitiation complex
a large protein assembly that performs various functions required for transcription initiation (e.g. recruitment of RNA polymerase II to transcription start sites, unwinding of DNA to allow for RNA polymerase II binding)

Lipid rafts
cholesterol- and sphingolipid-enriched microdomains in the plasma membrane that serve as organizing centres for signal transduction

Ketogenesis
the production of ketone bodies (acetoacetate, acetone, and β-hydroxybutyrate) from the catabolism of fatty acids and certain amino acids; ketone bodies become a major energy source for various organs during fasting and for the brain during long-term starvation

Browning (of white adipose tissue)
the increased white adipose tissue expression of uncoupling protein 1 (UCP1), a protein typically found in the mitochondria of brown adipose tissue; UCP1 uncouples the electron transport chain from ATP production to generate heat

Orexin
a hypothalamic neuropeptide that regulates appetite, arousal, and wakefulness; also known as hypocretin

Biographies

Dr. Xiaoyong Yang is an Associate Professor of Comparative Medicine and of Cellular & Molecular Physiology at the Yale University School of Medicine. He received his M.S. from Peking University and Ph.D. from the University of Alabama at Birmingham. His laboratory currently studies the role of protein post-translational modifications in cell signalling, gene regulation, metabolic physiology, and human disease. Xiaoyong Yang's homepage

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Box 1

O-GlcNAcylation and the post-translational modification code hypothesis

The post-translational modification (PTM) code hypothesis proposes that combinatorial modification of proteins with an array of PTMs generates a dynamic "code" that can be translated into complex biological outcomes[113]. This "code" is edited by "writers" and "erasers" (e.g. kinases and phosphatases in the case of phosphorylation) and translated through direct modulation of protein function or by "readers" (e.g. phosphotyrosine-binding proteins) that subsequently regulate protein complex formation and signal transduction. Since *O*-GlcNAcylation is highly sensitive to changes in the cellular environment and interacts extensively with other PTMs, we hypothesize that *O*-GlcNAcylation plays a unique and essential role in the PTM code. Below, we briefly discuss examples of crosstalk between *O*-GlcNAcylation and other common PTMs.

Phosphorylation

Since phosphorylation can also target serine and threonine residues, the interaction between *O*-GlcNAcylation and phosphorylation has been well studied. *O*-GlcNAcylation has been shown to occur reciprocally or sequentially with phosphorylation on the same or neighboring residues of numerous proteins[1, 2].

Ubiquitylation

Several studies have demonstrated that *O*-GlcNAcylation can block degradation of target proteins by reducing their ubiquitylation. Potential mechanisms include recruitment of deubiquitylases to *O*-GlcNAcylated proteins and indirectly modulating ubiquitylation through crosstalk with phosphorylation[26, 38, 39].

Acetylation

O-GlcNAcylation of nuclear factor kappa B (NF-κB) potentiates its acetylation by p300[114]; conversely, *O*-GlcNAcase (OGA)-mediated acetylation of pyruvate kinase isoform M2 (PKM2) enhances its *O*-GlcNAcylation (unpublished). These modifications also have an intrinsic reciprocal relationship due to the fact that OGA possesses both histone acetyltransferase-like (HAT-like) and *O*-GlcNAc hydrolase domains. Indeed, in cells not expressing orexin, transcription of the *Hcrt* gene encoding prepro-orexin is repressed by *O*-GlcNAc transferase (OGT) and the NAD-dependent deacetylase SIRT1 through histone *O*-GlcNAcylation and deacetylation; however, in orexin neurons, OGA and the histone acetyltransferases p300 and CBP activate *Hcrt* transcription via the reverse reactions[115].

Methylation

Several studies suggest that OGT can regulate transcription in concert with ten-eleven translocation (TET) proteins, which are thought to promote DNA demethylation by hydroxylating 5-methylcytosine (5mC)[18, 19, 71–73]. Thus, crosstalk between *O*-GlcNAcylation and DNA methylation may contribute to regulation of transcription (see discussion under "Regulation of epigenetics").

Key Points

1. *O*-GlcNAcylation is a nutrient- and stress-responsive post-translational modification (PTM) that occurs on serine and threonine residues of cytoplasmic, nuclear, and mitochondrial proteins. A single pair of enzymes—*O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA)—controls the dynamic cycling of this PTM.
2. Potential mechanisms enabling a single OGT to recognize hundreds of protein substrates include substrate-specific interactions with the tetratricopeptide repeat (TPR) domain of OGT and context-dependent recruitment of OGT to its substrates by a hierarchy of conserved adaptor proteins. Furthermore, in response to cellular stress, *O*-GlcNAcylation may occur non-specifically in unstructured regions of unfolded proteins in order to block their aggregation and degradation and facilitate their re-folding.
3. *O*-GlcNAcylation is involved in the spatiotemporal regulation of diverse cellular processes, including transcription, epigenetics, and cell signalling dynamics. *O*-GlcNAcylation is highly dynamic and often transient, but the mechanisms underlying the temporal control of *O*-GlcNAc signalling are largely unknown.
4. Nutrient availability regulates cellular *O*-GlcNAcylation levels not only by determining the abundance of the donor substrate uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) but also by modulating the levels of OGT, OGA, and their respective adaptor proteins and substrates. Hormones such as insulin, glucagon, and ghrelin are secreted in response to systemic metabolic changes and modulate *O*-GlcNAc signalling in specific cell types and tissues to regulate key response pathways that help maintain metabolic homeostasis.
5. Cellular *O*-GlcNAcylation levels may be maintained within an “optimal zone” by a “buffering system” generated by mutual regulation of OGT and OGA at the transcriptional and post-translational levels. Maintenance of *O*-GlcNAc homeostasis is essential for optimal cellular function and disruption of the cellular *O*-GlcNAcylation “buffer” may contribute to the pathogenesis of various human diseases.
6. *O*-GlcNAcylation can be viewed as the essential “grease and glue” of the cell: it acts as a “grease” by coating target proteins (folded or unfolded, mature or nascent) and preventing unwanted protein aggregation or modification; it also acts as a “glue” by modulating protein-protein interactions in time and space in response to internal and external cues, thereby affecting the functions of various proteins in the cell.

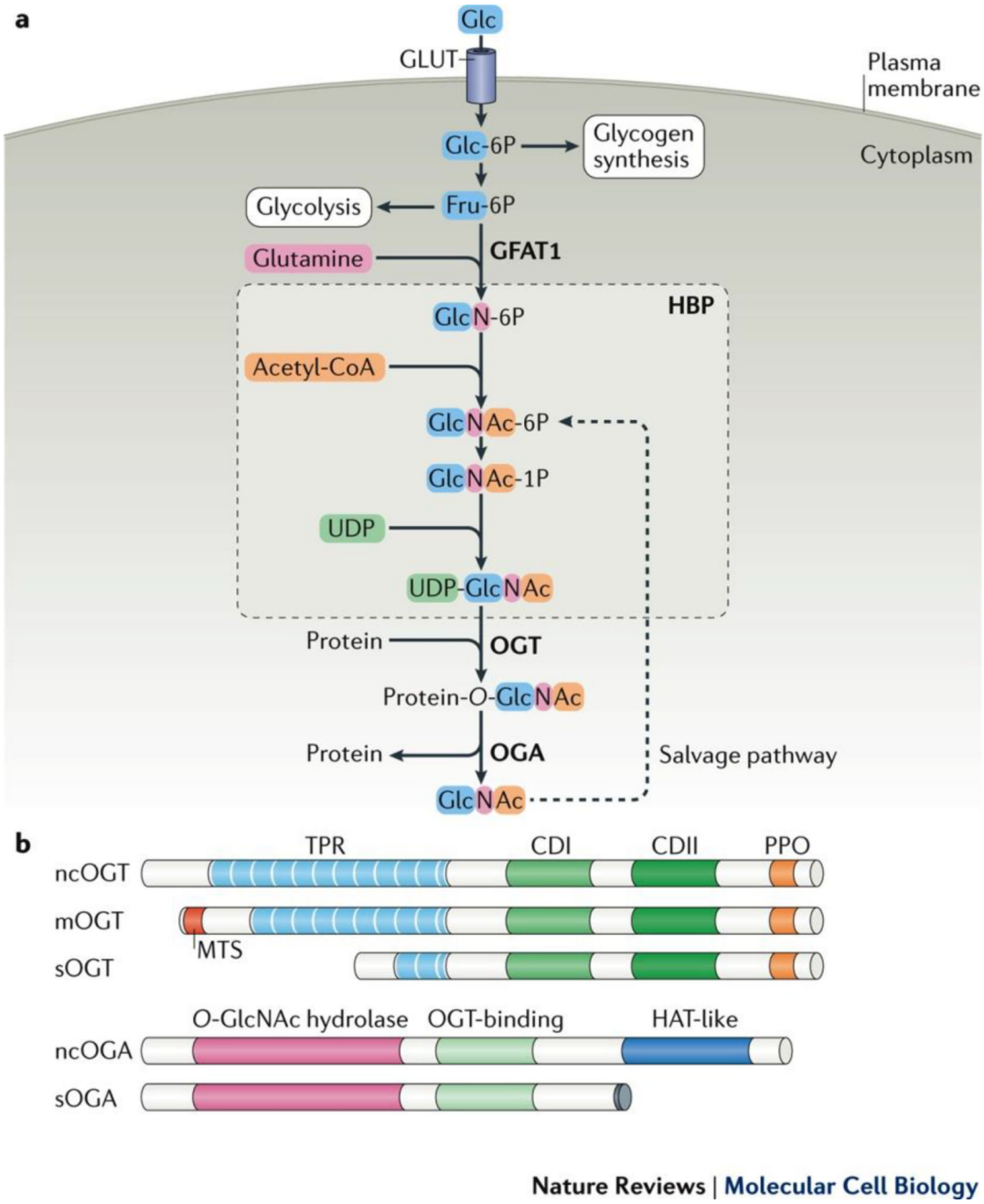

Figure 1. Nutrient flux through the hexosamine biosynthetic pathway regulates protein *O*-GlcNAcylation

a | Glucose (Glc) is taken up from the extracellular milieu by glucose transporter (GLUT) proteins. While a majority of glucose is used for glycolysis and glycogen synthesis, ~2–5% of glucose is channeled into the hexosamine biosynthetic pathway (HBP). Glutamine:fructose-6-phosphate amidotransferase (GFAT) catalyzes the rate-limiting step of the HBP, which converts fructose-6-phosphate (Fru-6P) into glucosamine-6-phosphate (GlcN-6P). Subsequent acetylation and uridylation of GlcN-6P yields the donor substrate for protein *O*-GlcNAcylation, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) catalyze the addition and removal of

O-GlcNAc, respectively. Free GlcNAc can be recycled via the GlcNAc salvage pathway, which converts GlcNAc into GlcNAc-6-phosphate (GlcNAc-6P) that can be utilized by the HBP. **b** | Schematic representation of the OGT and OGA isoforms. The nucleocytoplasmic (ncOGT), mitochondrial (mOGT), and short (sOGT) isoforms of OGT differ in length due to variable numbers of amino-terminal (N-terminal) tetratricopeptide repeats (TPRs) but share common carboxy-terminal (C-terminal) catalytic (CDI and II) and phosphoinositide-binding domains (PPO). mOGT contains a unique N-terminal mitochondrial targeting sequence (MTS). The nucleocytoplasmic (ncOGA) and short (sOGA) isoforms of OGA possess identical N-terminal *O*-GlcNAc hydrolase domains and central OGT-binding regions; however, sOGA lacks the C-terminal histone acetyltransferase-like (HAT-like) domain present in ncOGA.

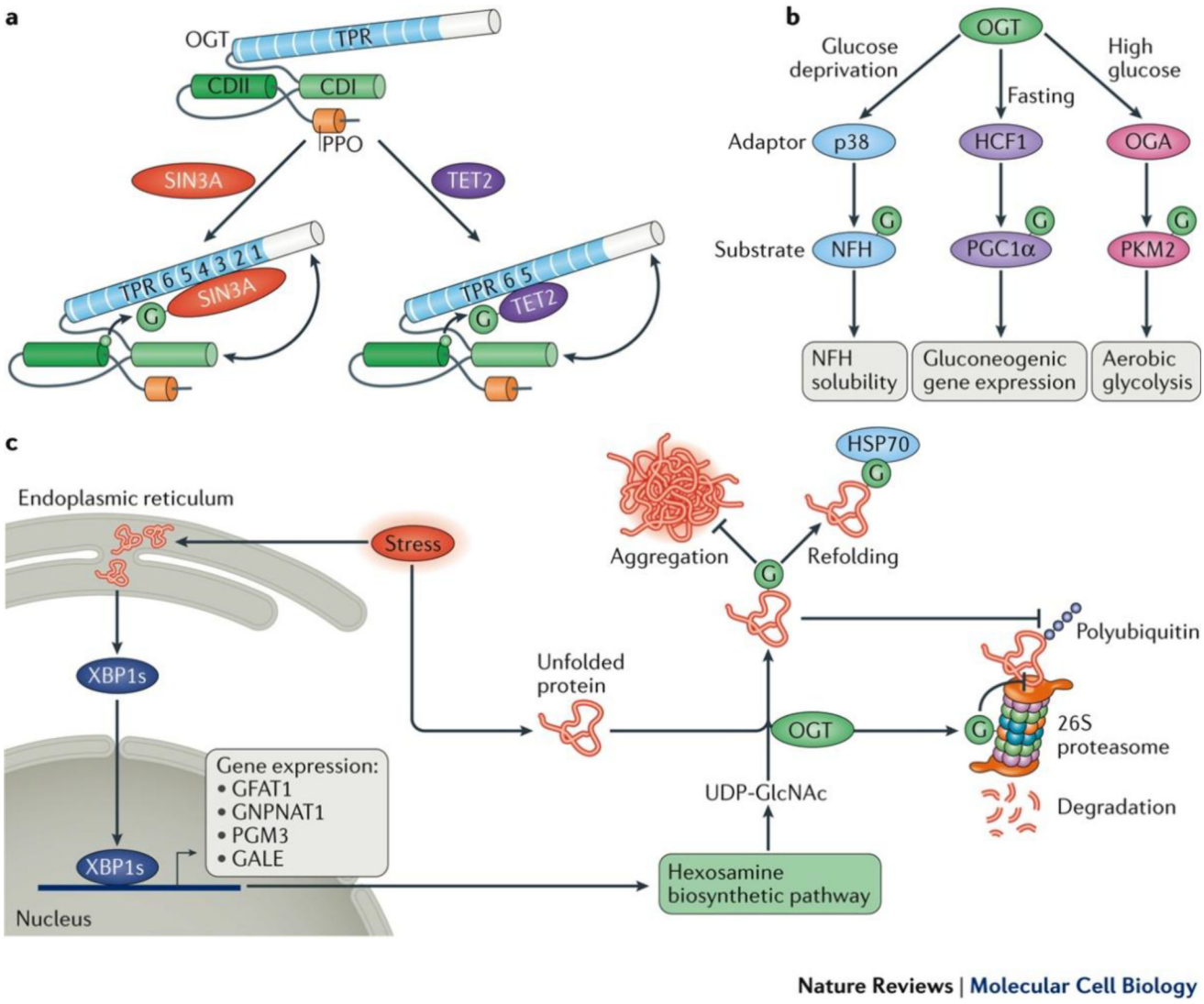

Figure 2. Potential mechanisms of *O*-GlcNAc transferase substrate recognition

a | *O*-GlcNAc transferase (OGT) may achieve some level of substrate specificity through its amino-terminal tetratricopeptide repeat (TPR) domain. Individually or in combination, TPRs could facilitate substrate recognition by generating unique binding sites that, when occupied, induce a conformational change that permits substrate access to the active site. TPRs 1–6 are required for OGT binding to Sin3a and TPRs 5–6 are required for OGT binding to ten-eleven translocation 2 (TET2), though TPRs 9–12 may also be involved. CDI and II, catalytic domains I and II; PPO, phosphoinositide-binding domain; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine. **b** | OGT substrate recognition may be additionally mediated by a hierarchy of highly conserved adaptor proteins, each responsible for recognizing and recruiting specific substrates to OGT in a context-dependent manner. For instance, in each of three different nutrient conditions (glucose deprivation, fasting, high glucose), OGT is recruited by a specific adaptor protein (p38, HCF-1, OGA) to *O*-GlcNAcylate a specific substrate (NF-H, PGC-1α, PKM2), leading to regulation of key downstream cellular pathways (NF-H solubility, gluconeogenic gene expression, aerobic

glycolysis). HCF-1, host cell factor C1; OGA, *O*-GlcNAcase; NF-H, heavy neurofilament polypeptide; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PKM2, pyruvate kinase isoform M2. **c** | Accumulation of unfolded proteins in the endoplasmic reticulum during cellular stress activates the unfolded protein response, which upregulates hexosamine biosynthetic pathway (HBP) gene expression via the transcription factor spliced X-box binding protein 1 (Xbp1s). Increased production of UDP-GlcNAc by the HBP may lead to non-specific *O*-GlcNAcylation of unfolded proteins in the cytoplasm. *O*-GlcNAcylation of unfolded proteins may block their aggregation and proteasomal degradation as well as facilitate their re-folding by chaperones with *O*-GlcNAc-directed lectin activity (e.g. 70 kDa heat shock protein, Hsp70).

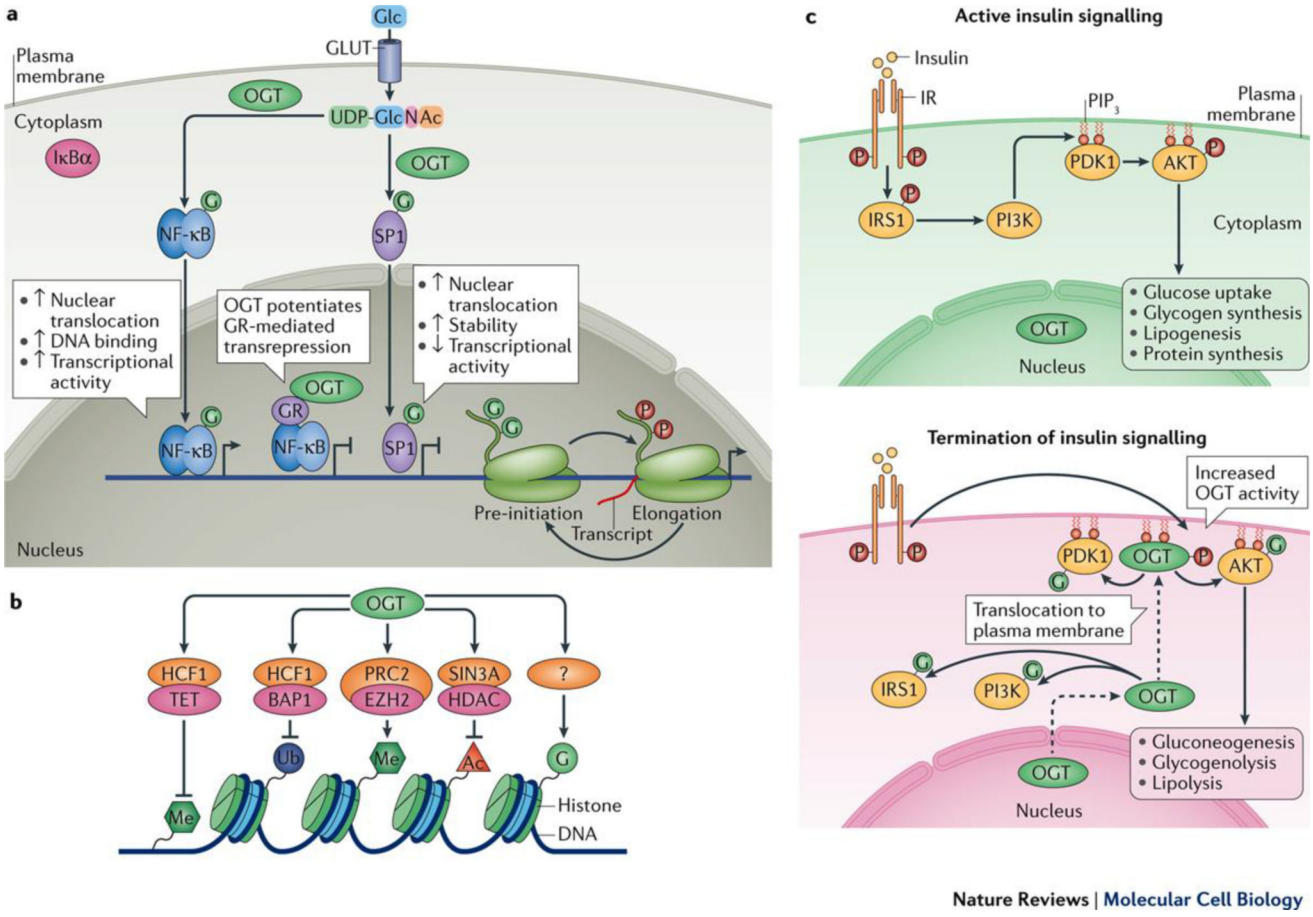

Figure 3. Functions of *O*-GlcNAcylation in time and space

a | *O*-GlcNAcylation of transcription factors and RNA polymerase II (Pol II) regulates transcriptional activation and repression. *O*-GlcNAcylation of nuclear factor kappa B (NF-κB) promotes its nuclear translocation (by blocking its interaction with IκBα) and enhances its DNA binding and transcriptional activity. On the other hand, the glucocorticoid receptor (GR) can directly bind to NF-κB and recruit *O*-GlcNAc transferase (OGT) to repress NF-κB activity. *O*-GlcNAcylation of Sp1 increases its nuclear localization and stability but at the same time inhibits its transactivation. *O*-GlcNAcylation of Pol II on its carboxy-terminal domain (CTD) is important for the assembly of preinitiation complexes at transcription start sites, while removal of *O*-GlcNAcylation from the Pol II CTD allows for its dynamic phosphorylation during transcription initiation and elongation. Thus, reciprocal *O*-GlcNAcylation and phosphorylation of the Pol II CTD is essential for maintenance of an unperturbed transcription cycle. Glc, glucose; GLUT, glucose transporter; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine; IκBα, NF-κB inhibitor alpha. **b** | OGT plays a variety of roles in epigenetic regulation. The OGT/host cell factor C1 (HCF-1) complex binds to ten-eleven translocation (TET) proteins, which catalyze cytosine 5-hydroxymethylation of genomic DNA and thereby promote DNA demethylation. OGT/HCF-1 also binds to BRCA-associated protein 1 (BAP1), which mediates deubiquitylation of histone H2A. OGT also stabilizes enhancer of zeste homolog 2 (EZH2), a component of Polycomb repressive complex 2 (PRC2), to promote histone H3 lysine 27 trimethylation.

Furthermore, OGT acts in concert with the Sin3a/histone deacetylase (HDAC) corepressor complex to silence gene expression by promoting histone deacetylation. OGT may also directly modify histones through unknown mechanisms. **c** | *O*-GlcNAcylation is involved in the temporal regulation of insulin signalling dynamics. Binding of insulin to the insulin receptor (IR) induces IR autophosphorylation and subsequent tyrosine phosphorylation of insulin receptor substrate (IRS). Phosphorylated IRS binds and activates phosphoinositide 3-kinase (PI3K), which catalyzes the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 recruits phosphoinositide-dependent kinase 1 (PDK1) and AKT to the plasma membrane, leading to AKT phosphorylation and activation and increased glucose uptake and anabolic cellular metabolism. In response to prolonged insulin stimulation, OGT translocates from the nucleus to the cytoplasm and localizes to the plasma membrane by binding to PIP_3, which leads to tyrosine phosphorylation and activation of OGT by IR. OGT then *O*-GlcNAcylates and inhibits key insulin signalling mediators such as IRS1, PI3K, PDK1, and AKT, blocking their phosphorylation and/or interactions and thereby facilitating termination of insulin signal transduction.

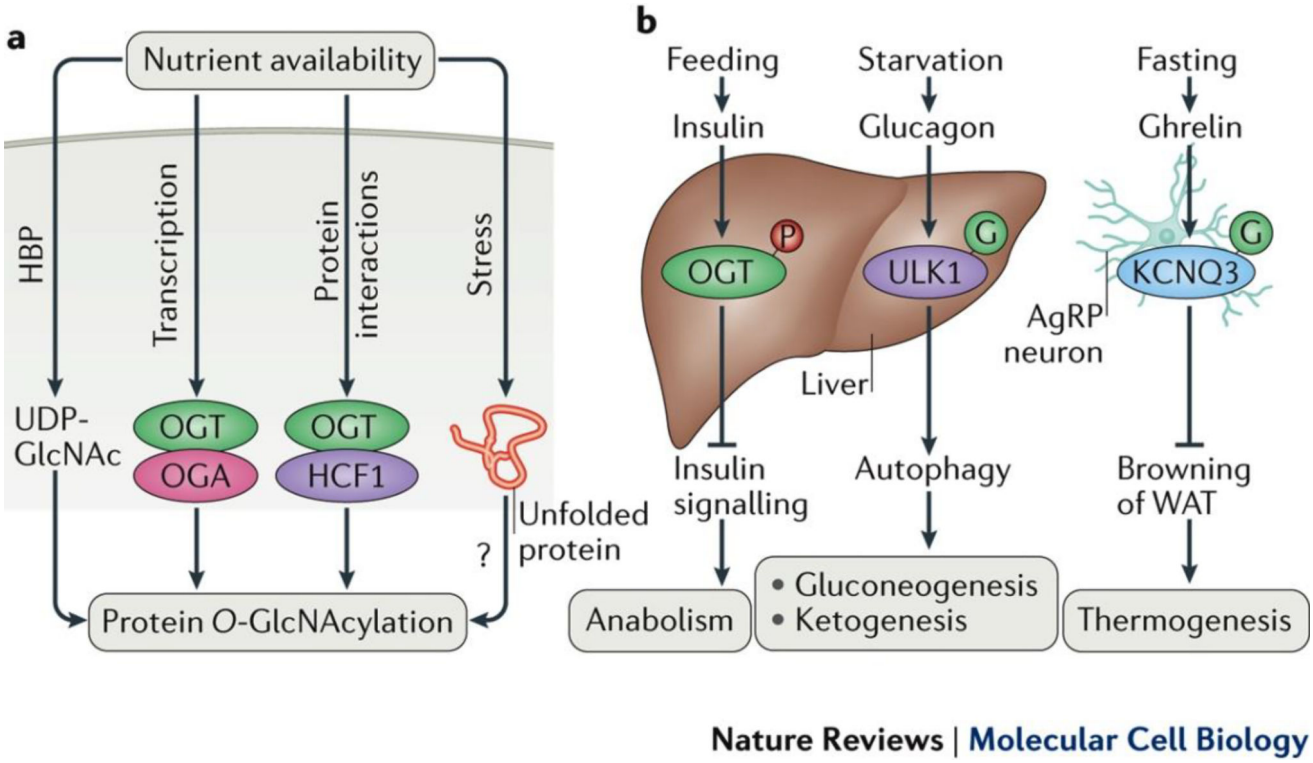

Figure 4. Nutritional and hormonal regulation of *O*-GlcNAc signalling

a | Nutrient availability is thought to regulate cellular *O*-GlcNAcylation levels by controlling flux through the hexosamine biosynthetic pathway (HBP) and thereby determining the abundance of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). We propose that the effect of nutrient availability on *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) expression, OGT-adaptor protein interaction, and high affinity OGT substrate (i.e. unfolded protein) accumulation also drives the nutrient sensitivity of this modification. HCF-1, host cell factor C1. **b** | In response to systemic changes in metabolic status (feeding, starvation, fasting), hormones (insulin, glucagon, ghrelin) modulate *O*-GlcNAc signalling in various cell types and tissues (liver, AgRP neurons), leading to regulation of specific cellular pathways (insulin signalling, autophagy, browning of WAT) that generate an appropriate metabolic response (suppression of anabolism, stimulation of gluconeogenesis and ketogenesis, activation of thermogenesis) to the initial stimulus. AgRP, Agouti-related peptide; WAT, white adipose tissue.

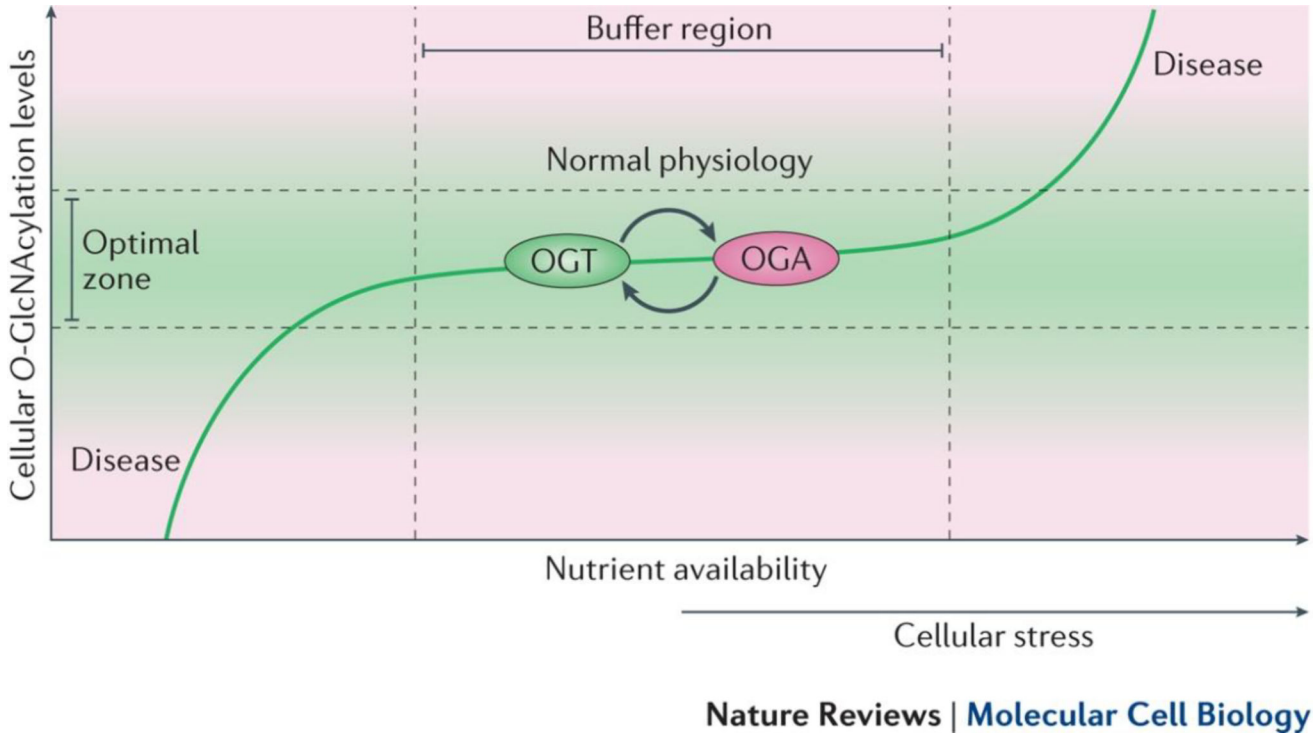

Figure 5. Maintenance of *O*-GlcNAc homeostasis by mutual regulation of *O*-GlcNAc transferase and *O*-GlcNAcase

We propose that there exists an "optimal zone" within which global *O*-GlcNAcylation levels must remain in order to preserve normal cellular function. In response to mild stress stimuli or moderate perturbations in nutrient availability, cellular *O*-GlcNAcylation levels may be maintained within the "optimal zone" by a "buffering" system consisting of mutual regulation of *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) at the transcriptional and post-translational levels. However, severe and chronic insults to the cellular *O*-GlcNAcylation "buffer" may eventually lead to loss of *O*-GlcNAc homeostasis, which is an important factor contributing to the pathogenesis of various human diseases.

Table 1

Cell type- and tissue-specific functions of *O*-GlcNAcylation

Biological system	Cell type or tissue	Targeted pathways	Targeted proteins	References
Circulatory	Heart	Calcium signalling	CaMKII, PLN, STIM1	116–118
		Mitochondrial energy production, dynamics, and membrane potential	Complex I/III/IV, DRP1, VDAC	119–122
		Response to cardiac injury	VDAC	35, 121, 123
Digestive	Liver	Bile acid metabolism	FXR	124
		Circadian rhythm	BMAL1, CLOCK	38
		Gluconeogenesis	CRTC2, FOXO1, PGC-1α	26, 53, 54
		Insulin signalling	IRS1, AKT	5
		Lipogenesis	ChREBP	125
	Pancreas	β-cell survival	-	85, 126
		Insulin secretion	-	127–129
Immune	B cell	B cell activation	NFATc1, NF-κB	52
	T cell	Clonal expansion, self-renewal	-	130
		Cytokine production, T cell activation	NFATc1, NF-κB	52, 56
	Macrophage	Cytokine production, polarization, survival	TAK1	131
	Neutrophil	Chemotaxis	-	132
Integumentary	Adipose tissue	Adipocyte differentiation	C/EBP-β, PPARγ	133, 134
		Browning	-	135
		Insulin signalling	IR-β, IRS1, AKT	5, 75
		Leptin production	-	127
	Keratinocyte	Cell-cell adhesion	Plakoglobin	136
		Keratinocyte differentiation	Sp1	137
		Wound healing	-	138
Musculoskeletal	Bone	Osteoblast differentiation	RUNX2	139, 140
	Cartilage	Chondrocyte differentiation	-	141
	Skeletal muscle	Insulin signalling	-	127, 142, 143
		Mitochondrial function	-	143
		Myogenesis	MEF2D	143–145
Nervous	Neuron (general)	Axonal and dendritic growth	CREB	146, 147
		Mitochondrial motility	Milton	148
		Neurodegeneration	α-synuclein, tau	7, 36, 109, 149

Biological system	Cell type or tissue	Targeted pathways	Targeted proteins	References
		Synapse maturity	-	150
	AgRP neuron	Firing rate, potassium current	KCNQ3	89
	Hippocampal neuron	Long-term depression/potentiation	GluA2	151
	PVN	Excitatory synaptic function	-	152
	Schwann cell	Myelin maintenance	PRX	153
Urinary	Kidney	Mesangial lipogenesis and fibrosis	ChREBP	154
Other	Cancer cell	Invasion and metastasis	c-Myc, FOXM1	155, 156
		Metabolic reprogramming	G6PD, PFK1	3, 157, 158
		Survival	-	158
	Embryonic stem cell	DNA methylation	TET1	71, 72
		Pluripotency, self-renewal	OCT4, SOX2	159, 160

CaMKII, calcium/calmodulin-dependent kinase II; PLN, phospholamban; STIM1, stromal interaction molecule 1; DRP1, dynamin-related protein 1; VDAC, voltage-dependent anion channel; FXR, farnesoid X receptor; CREB, cyclic AMP response element-binding protein; CRTC2, CREB-regulated transcription coactivator 2; FOXO1, forkhead box protein O1; PPARγ, peroxisome proliferator-activated receptor gamma; PGC-1α, PPARγ coactivator 1 alpha; IRS1, insulin receptor substrate 1; ChREBP, carbohydrate response element-binding protein 1; NFATc1, nuclear factor of activated T cells 1; NF-κB, nuclear factor kappa B; TAK1, transforming growth factor beta-activated kinase 1; C/EBP-β, CCAAT/enhancer-binding protein beta; IR-β, insulin receptor beta; RUNX2, runt-related transcription factor 2; MEF2D, myocyte enhancer factor 2D; AgRP, Agouti-related peptide; GluA2, glutamate receptor 2; PVN, paraventricular nucleus; PRX, periaxin; FOXM1, forkhead box protein M1; G6PD, glucose-6-phosphate dehydrogenase; PFK1, phosphofructokinase 1; TET1, ten-eleven translocation 1