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Current Opinion in Chemical Biology

# Molecular mechanisms regulating O-linked *N*-acetylglucosamine (O-GlcNAc)–processing enzymes

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**Abstract**

The post-translational modification of proteins by O-linked *N*-acetylglucosamine (O-GlcNAc) dynamically programmes cellular physiology to maintain homoeostasis and tailor biochemical pathways to meet context-dependent cellular needs. Despite diverse roles of played by O-GlcNAc, only two enzymes act antagonistically to govern its cycling; O-GlcNAc transferase installs the monosaccharide on target proteins, and O-GlcNAc hydrolase removes it. The recent literature has exposed a network of mechanisms regulating these two enzymes to choreograph global, and target-specific, O-GlcNAc cycling in response to cellular stress and nutrient availability. Herein, we amalgamate these emerging mechanisms from a structural and molecular perspective to explore how the cell exerts fine control to regulate O-GlcNAcylation of diverse proteins in a selective fashion.

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**Current Opinion in Chemical Biology** 2019, **53**:131–144

This review comes from a themed issue on **Mechanistic Biology**

Edited by **Hermen S. Overkleeft** and **David J. Vocadlo**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.cbpa.2019.09.001



**Keywords**

Glycosyltransferase, Glycosidase, Glycoprotein, O-GlcNAc, glycosylation, O-GlcNAcylation, Transcription, miRNA, Post-translational modification, Enzyme regulation, GlcNAc, Hexosamine, Subcellular localization, Glycans, Spliceoforms, Post-transcriptional regulation, Retained intron.

## Introduction

The covalent attachment of β-linked *N*-acetylglucosamine to the hydroxyl group of protein serine and threonine residues has been identified on hundreds of nucleocytosolic and mitochondrial proteins and is abundant throughout all multicellular eukaryotes [1]. The donor substrate for O-linked *N*-acetylglucosamine (O-GlcNAc) transferase (OGT)—catalyzed O-GlcNAcylation is uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), the biosynthetic product of the nutrient-responsive hexosamine biosynthetic pathway (HBSP). Protein O-GlcNAc levels are thus tied to nutrient status through HBSP flux, facilitated by the reversible nature of O-GlcNAc that can be installed and removed multiple times during the lifespan of a protein [1]. O-GlcNAc is being found to modulate proteins in an increasing variety of ways including manipulation of protein function, protein–protein interactions, protein localization and protein stability (reviewed by Yang and Qian [1]). In addition, O-GlcNAc has the potential to compete directly and indirectly with phosphorylation sites and may agonize or antagonize other post-translational modifications (PTMs), including ubiquitinylation, sumoylation and acetylation (reviewed by Yang and Qian [1]), although relatively few such interactions have been demonstrated. Nevertheless, taken together, dynamic cycling and interplay with other PTMs make O-GlcNAc ideally suited as a nutrient and stress-responsive molecular switch.

Various noxious stimuli including heat shock, nutrient depletion, endoplasmic reticulum (ER) dysfunction and redox imbalance cause fluctuations in global O-GlcNAc levels to help combat these stressors. Aberrant O-GlcNAc cycling has been linked to chronic human diseases, including neurodegeneration, cancer, diabetes mellitus and X-linked intellectual disability (XLID) [2,3]. The diverse physiological roles of O-GlcNAc and its emerging importance in human health underscore the importance of uncovering the complex molecular mechanisms orchestrating its installation and removal on protein targets, both in normal physiology and pathological states.

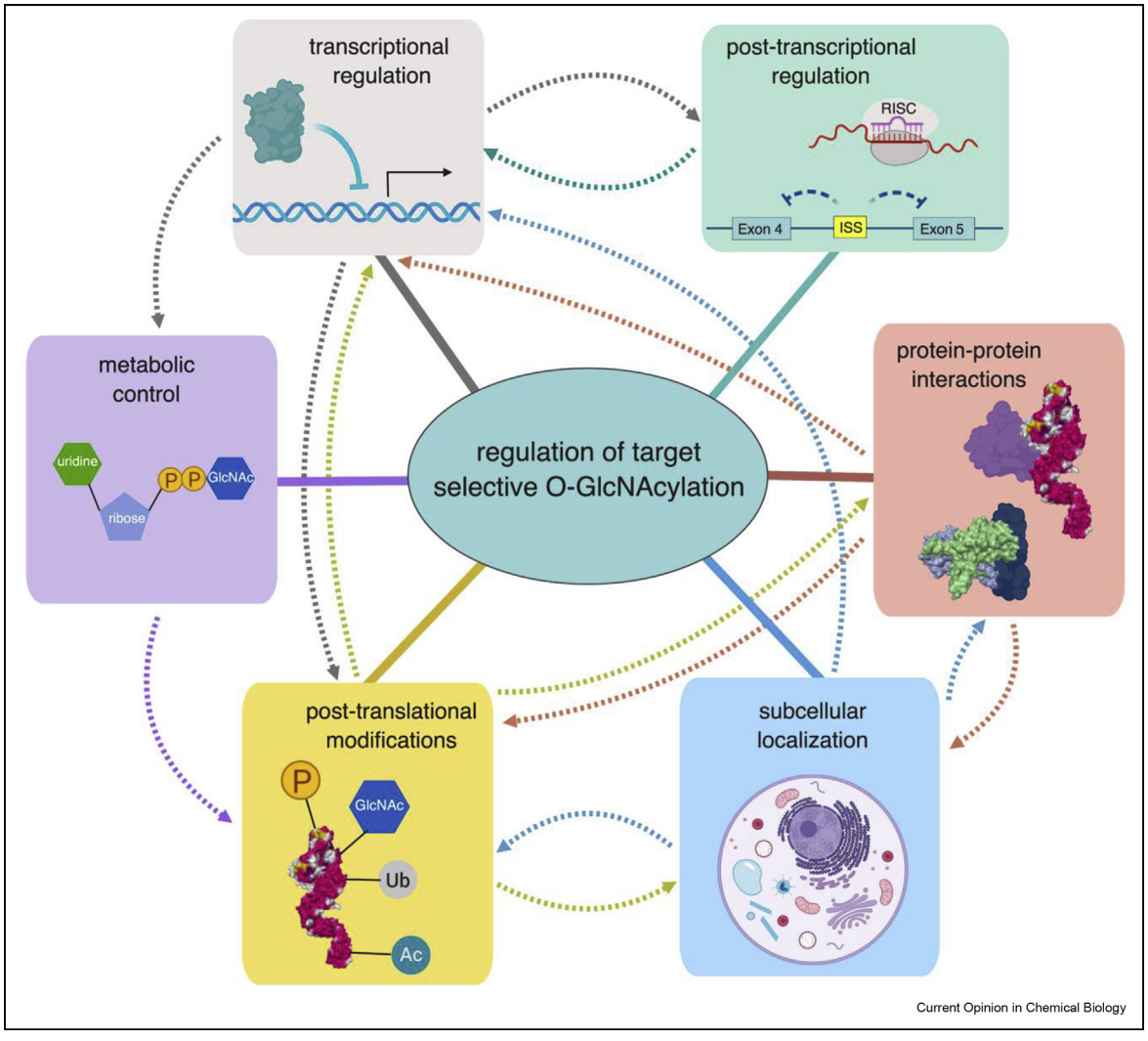
Herein, we summarize the biochemical underpinnings of the O-GlcNAc modification and integrate salient concepts pertinent to its regulation. Notably, the O-GlcNAc enzymes have evolved elaborate multidomain structures that are ideally suited to enable the integrated fine-tuning of their function by diverse regulatory mechanisms. Accordingly, we start with an overview of their structures

and then consolidate select recent literature illuminating key regulatory mechanisms that define the abundance, localization and specificity of the O-GlcNAc cycling enzymes. We focus on emerging interconnected regulatory processes including subcellular localization, PTMs, protein–protein interactions, metabolic control and transcriptional and post-transcriptional regulation (Figure 1). Throughout, we highlight current paradigms and raise key unanswered questions. Owing to space constraints, we regrettably make various omissions to focus on the selected literature most relevant to this topic.

## OGT structure and polypeptide acceptor specificity

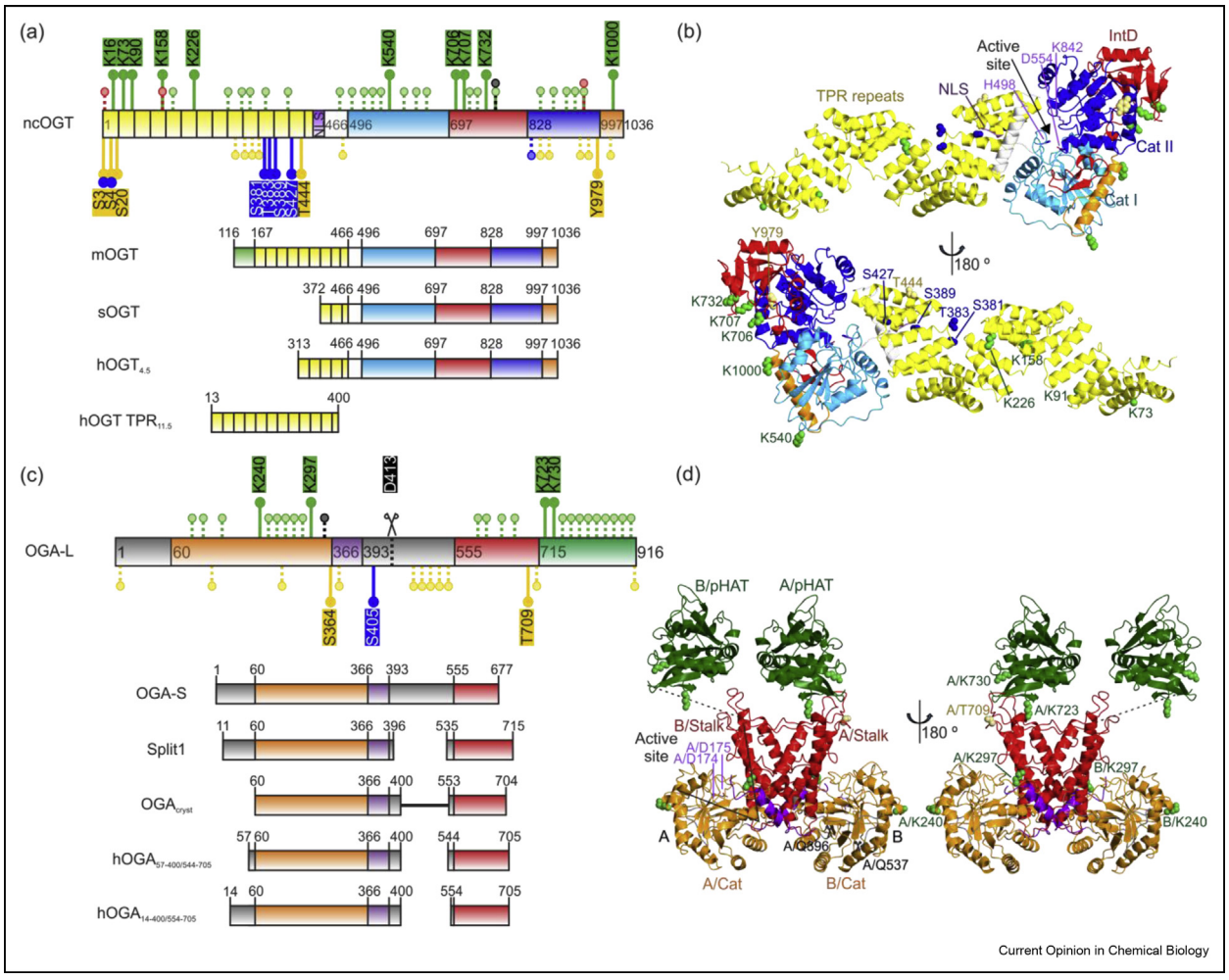
*OGT* encodes a metal-independent CAZY (www.cazy.org) family GT41 β-*N*-acetylglucosaminyltransferase belonging to the GT-B superfamily of glycosyltransferases. This essential gene is located on the X chromosome and encodes a modular protein composed of five distinct regions: (i) an N-terminal region consisting of an isoform-specific number of contiguous helix–turn–helix tetratricopeptide repeats (TPRs), (ii) a transitional (H3) helix that sits between the N- and C-terminal halves, (iii) a C-terminal region containing the GT41 catalytic domain with two catalytic lobes (Cat1 and Cat2) that each have a Rossmann fold, (iv) an intervening domain with a topologically novel fold comprising a seven-stranded β-sheet core stabilized by flanking α-helices (IntD) and (v) a putative C-terminal phosphatidylinositol-binding region (PPO) (Figure 2A). There are three different isoforms of OGT expressed in human cells that vary in the number of N-terminal TPRs. Nucleocytoplasmic OGT (ncOGT) has 13.5 TPRs, mitochondrial OGT (mOGT) has 9

**Figure 1**




**Concept map depicting the interconnected mechanisms regulating target selective O-GlcNAcylation by OGA and OGT.** Each dotted arrow represents higher level crosstalk between the various regulatory mechanisms. OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase; O-GlcNAc, O-linked *N*-acetylglucosamine.

**Figure 2**




**Domain structure and isoforms of OGT and OGA**. **(a)** Schematic of hOGT showing splice variants and constructs used for crystallography. OGT is found in three isoforms distinguished by the number of TPRs. The TPRs are shown in yellow, the NLS (D451-P453) is coloured purple, transitional helix (H3) is white, the glycosyltransferase catalytic domains are illustrated in light blue and dark blue, the Int-D (intervening domain) is red, and the phosphoinositide-binding domain of OGT (PPO) is coloured orange with sequence numbered according to UniProtKB/SwissProt accession (P56558.1). In panels (a) and (c), putative PTMs compiled using PhosphoSitePlus [20] are represented on ncOGT and OGA-L as follows: phosphorylation (yellow), O-GlcNAcylation (blue), ubiquitinylation (green), acetylation (red) and sumoylation (black). High-confidence PTM sites with 5 or more references or experimentally determined by low-throughput mass spectrometry mapping methods are shown with solid lines and bold colours. Lower confidence sites with fewer than 5 references and determined by high-throughput methods are shown with dotted lines and light colours. **(b)** Overall crystallographic model of ncOGT. The model was built by superposing $hOGT_{4.5}$ (PDB ID: 3PE3) and hOGT $TPR_{11.5}$ (1W3B). High-confidence PTMs are labelled, as well as domains and key active site catalytic residues. **(c)** Schematic of human OGA splice variants and constructs used for crystallography. Disordered regions are shown in black, the N-terminal GH84 domain is illustrated in orange, the helical bundle domain is purple, the C-terminal stalk domain is shown in red and the 'pseudo HAT' domain is represented as a green cartoon. The caspase-3 cleavage site is indicated by scissors. **(d)** Overall crystallographic model of hOGA. The hOGA Split1 crystal structure (PDB ID: 6HKI) is shown attached at the C-terminal end (Y715) to a homology model of the OGA pseudo-HAT domain constructed using Phyre2 from the *Oceanicola granulosus* homologue (PDB ID: 3ZJ0, 29% sequence identity). Structures in (b) and (d) are colour coded as in (a) and (c) with high-confidence PTMs labelled on the structures. HAT, histone acetyltransferase; hOGA, human OGA-L; ncOGT, nucleocytoplasmic OGT; OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase; O-GlcNAc, O-linked *N*-acetylglucosamine; PTM, post-translational modification; TPR, tetratricopeptide repeat; NLS, nuclear localization signal.

repeats and short OGT has 2.5 repeats (Figure 2A). These isoforms have distinct subcellular localization patterns and activities (see the following Localization section).

In 2011, the seminal crystallographic structure of human OGT was determined, illuminating its unique atomic architecture. The structure comprises 4.5 N-terminal TPRs and the full C-terminal catalytic region in a ternary complex with UDP and a CKII peptide substrate (hereafter called $hOGT_{4.5}$) [4]. The TPRs in this structure (amino acids (aa) 313–400) overlap with an earlier OGT crystal structure consisting of 11.5 TPRs, and alignment of the two structures provides a putative model of the full-length protein (Figure 2B) [4]. The TPR superhelix consists of two layers of helices, an

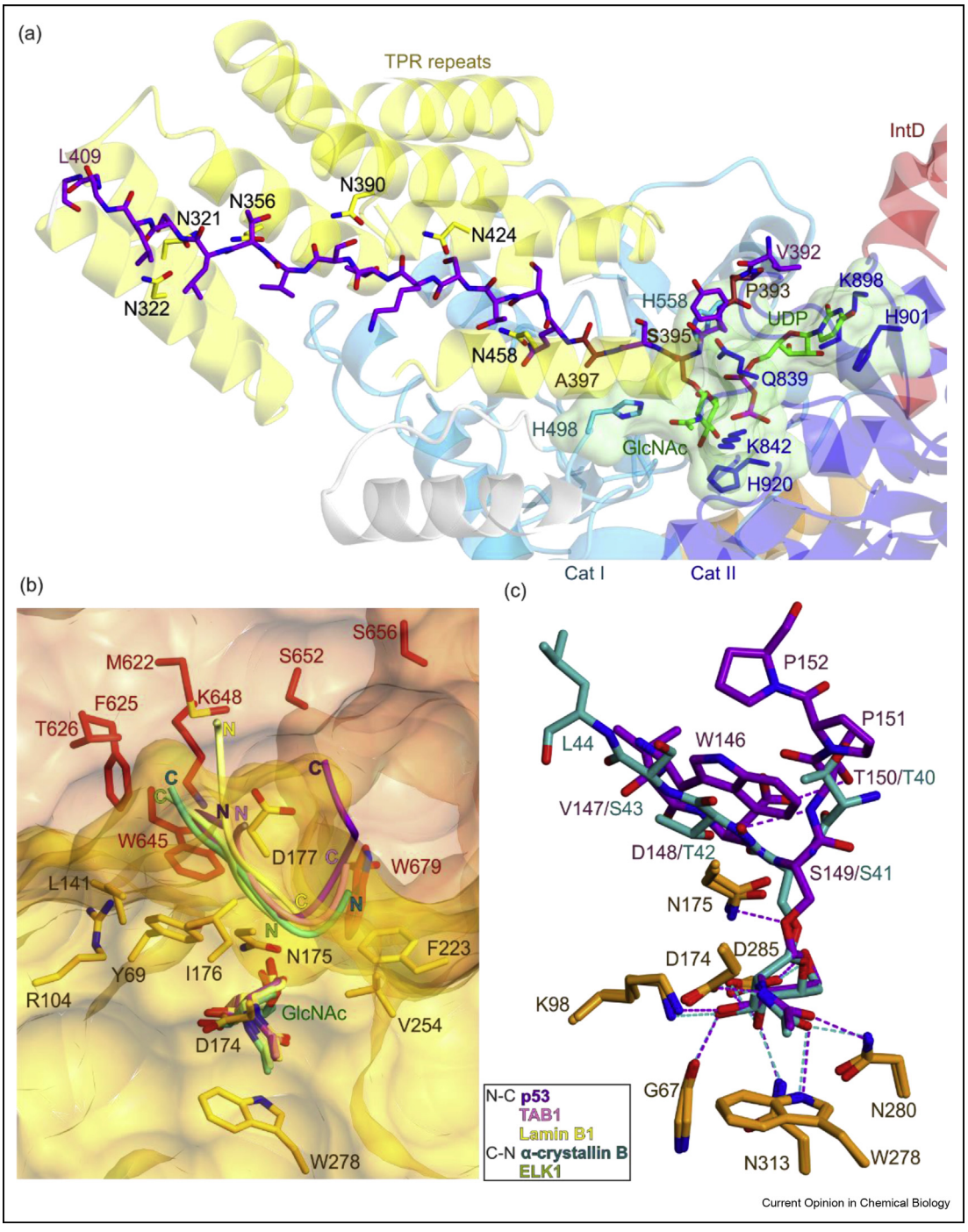
inner concave face formed by helix-A and an outer convex face formed by helix-B. Notably, helix-A has a highly conserved asparagine ladder that runs the length of the TPR domains that is involved in binding to peptide acceptors (see the following section) [4]. The C-terminal end of the TPR superhelix packs against the catalytic domain, carving out a narrow acceptor peptide-binding groove that feeds into the catalytic centre found at the interface of Cat1 and Cat2 [4]. The Int-D domain packs against the outer face of the Cat2 lobe oriented away from the active site core. The function of the Int-D domain remains unknown, but in conjunction with a Cat2 helix and the C-terminal PPO, it forms a large electropositive surface comprising ten lysine residues that may be involved in ligand binding (see the following section) [4].

As an inverting glycosyltransferase, OGT catalyzes the formation of a β-glycosidic linkage between GlcNAc and the acceptor peptide serine. The peptide acceptor binds OGT predominantly through amide backbone hydrogen bonds, with the β-hydroxyl group of its glycosylated serine projecting directly into the nucleotide sugar—binding site. The peptide acceptor covers the solvent-exposed face of the bound UDP-GlcNAc, effectively trapping it within the active site (Figure 3A). This observed binding mode in combination with kinetic data supports the broadly accepted proposal that OGT catalyzes glycosyltransfer via an ordered bi—bi mechanism, whereby UDP-GlcNAc must bind the active site first, followed by the acceptor peptide [4]. Intriguingly, fundamental mechanistic details remain unknown, including the identity of the general base responsible for deprotonating the catalytic serine [4]. Briefly as reviewed [4], three alternative general bases were proposed including His498, Asp554 or the α-phosphate of UDP-GlcNAc. Although mutation of His498 results in inactive enzyme, the side-chain interactions suggest it is protonated at neutral pH, making it poorly suited to serve as a general base. Direct transfer of a proton to the α-phosphate of UDP-GlcNAc appears energetically unfavourable. Structural studies, however, revealed a series of ordered water molecules in the active site acting as a potential proton shuttle adjacent to Asp554, and functional studies reveal defective sugar transfer when this residue was mutated to asparagine, offering the best support for the identity of the general base to date.

OGT lacks a strict consensus sequence governing its activity. However, subtle sequence preferences help govern substrate selectivity. The specificity of OGT was assessed using a saturation library of 3480 unique acceptor peptides displayed in a monolayer. As predicted by earlier work [5], valine and alanine were preferred in the −3 and +2 subsites. In addition, a preference for aromatic residues in the −4 position was uncovered [6]. Notably, proline was previously shown to be preferred at the −2 subsite [5]. In contrast, proline at the +1 position has been found to prevent O-GlcNAcylation within certain peptides [7]. OGT predominantly glycosylates serine and threonine residues located within disordered regions on protein targets but also appears able to glycosylate residues positioned in a variety of different secondary structures [8]. However, these secondary structures may need to be unfolded to be accommodated within the OGT active site. Although not yet characterized, such local unfolding may be triggered in response to various stimuli, or potentially occur during the cotranslational O-GlcNAcylation of some nascent chains [9], before protein folding. The presence of other PTMs can also influence protein targeting as exemplified by the observation that acceptor peptide phosphorylation can reduce OGT-catalyzed O-GlcNAcylation *in vitro* [7]. However, elucidating the physiological significance of this proposed PTM code awaits quantitative analysis of levels on endogenous proteins.

Emerging evidence shows that peptide binding occurs not just in the GT41 active site but also within the TPR domains. A protein microarray in conjunction with an azide-containing analogue of UDP-GlcNAc, UDP-GlcNAz, was used to establish OGT targeting of proteins using downstream click chemistry to incorporate biotin [10]. This method was used to assess the catalytic contribution of a polyasparagine ladder located within the TPR domains, known to engage in numerous bidentate hydrogen bonds with the peptide backbone of Tab1 [11] (Figure 3A). An OGT mutant construct consisting of alanine in place of these 5 asparagine residues (5N5A) retained activity towards short peptides but impaired modification of proteins [10], supporting the importance of these residues in indiscriminate recognition of the polypeptide backbone of proteins distant from the site of glycosylation. et al In a different approach, a GlcNAc electrophilic probe was developed that consists of a UDP-GlcNAc analogue containing an allyl chloride electrophile extending as an N-acyl group from the 2-amino position [12]. This GlcNAc electrophilic probe was used to discern whether mutations in OGT affected sugar binding or protein acceptor binding and glycosylation by examining its relative partitioning to OGT (C917) or the acceptor protein. This method yielded results further supporting an important role for the asparagine ladder in acceptor protein binding [12]. Recently, the XLID OGT mutation, L254F, within the seventh TPR was found to distort the TPR superhelix, resulting in reduced thermal stability and catalytic activity [13]. Furthermore, five additional mutations mapping to the OGT TPR domains have recently been correlated with XLID [13—15]. Very recently, two aspartate residues (D386 and D420) located in the OGT TPR lumen distal from the active site were shown to be important for substrate selection. Interestingly, mutation of these residues to alanine altered substrate

**Figure 3**




**Active-sites of OGT and OGA**. **(a)** OGT in complex with a TAB1 peptide. The bound peptide is fused to the N-terminus of OGT and is shown in purple (PDB ID: 5LVV). Asparagine ladder residues shown in yellow are highlighted against the yellow TPRs. The peptide covers the solvent-exposed face of UDP-GlcNAc, shown in green, with the active site highlighted. **(b)** Overlay of OGA in complex with various peptides. The following peptides are displayed: α-crystallin B (PDB ID: 5VVV, dark green, residues TSTSL), ELK1 (PDB ID: 5VVT, light green, residues TLSP), TAB1 (PDB ID: 5VVU, pink, residues PYSS), Lamin B1 (PDB ID: 5VVX, yellow, residues SSRVT) and p53 (PDB ID: 5UN8, purple, residues WVDSTPP). The peptides are shown as worms with the N-terminus and C-terminus labelled. The catalytic and neighbouring residues are highlighted in the active site in orange. **(c)** Superposition of peptide-bound OGA complexes. The bound peptides α-crystallin B (PDB ID: 5VVV) and p53 are shown in cyan and purple, respectively. Key hydrogen-bond interactions are coloured according to the peptide. OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase; O-GlcNAc, O-linked *N*-acetylglucosamine; TPR, tetratricopeptide repeat; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

specificity and increased rates of protein glycosylation *in vitro*. In particular, a preference was found for substrates with lysine or arginine positioned 7−11 residues away from the site of glycosylation [16]. Taken together, the OGT TPR domains are emerging as essential mediators of substrate binding, enzyme activity and stability.

## OGA structure and target specificity

*OGA* encodes two distinct splice variants referred to as long (OGA-L) and short (OGA-S) isoforms. Human OGA-L (hOGA) consists of three domains connected by disordered regions: (i) an N-terminal *N*-acetyl-β-D-glucosaminidase catalytic domain (CAZy GH84) with a $[(\beta/\alpha)_8]$ triose-phosphate isomerase (TIM) barrel structure, (ii) a helical bundle referred to as the stalk domain composed of four α-helices and (iii) a C-terminal pseudo−histone acetyltransferase (HAT) domain with a GCN5 acetyltransferase−like fold (Figure 2C). The pseudo-HAT domain lacks the conserved P-loop motif required for binding of the cofactor, acetyl-CoA, implying that it likely has a nonenzymatic function. OGA-S lacks the pseudo-HAT domain and part of the stalk domain, leading to greatly reduced enzyme activity.

Recently, three groups independently solved the long-anticipated crystal structure of hOGA. This was achieved by the systematic design of various constructs lacking disordered regions [17−19]. The homodimer is stabilized by a domain-swapped helix (α17: aa 676−694) [18,19]. Notably, a construct lacking this α17 helix (residues 1−662) does not dimerize and displays 100-fold lower catalytic efficiency than wild type (WT) OGA in processing *p*NP-GlcNAc, suggesting that dimerization is required for maximal activity [19].

A V-shaped active site cleft ($\sim 22 \times 25$ Å with an angle of $\sim 70°$) is formed at the interface of the catalytic domain of monomer A and the stalk domain of monomer B [18] (Figure 2D). Multiple hOGA−glycopeptide−bound structures reveal that peptides bind within the cleft in either orientation [18,21,22] (Figure 3B−C). Two conserved aspartate residues flank the glycosidic bond, acting as general acid/base catalysts to facilitate a two-step catalytic mechanism involving a transient oxazoline intermediate [4]. Kinetic analysis of OGA-mediated O-GlcNAc hydrolysis on intact proteins revealed that OGA indiscriminately cleaves diverse O-GlcNAc sites across different targets with roughly equivalent catalytic efficiency [23], an observation that is supported by the enzyme cleaving O-GlcNAc residues incorporated at 'unnatural' sites [24]. This promiscuous substrate selectivity is likely in part due to the predominant interaction of OGA with amide backbone atoms within the bound glycopeptide. As seen elsewhere [23], the rate of OGA-catalyzed hydrolysis of an O-GlcNAcylated serine was shown to be reduced by half in the presence of an adjacent phosphotyrosine on a ZO3-derived model peptide ($RESSYDIY_{(p)}RVPSS_{(g)}QS$) [25]. The ability for PTMs to influence OGA-catalyzed removal merits further investigation. In summary, recent research has provided a vital structural foundation for understanding OGA and OGT. The challenge for the future is to build upon this knowledge to uncover how regulatory factors modulate their structure and function to choreograph target-specific O-GlcNAcylation.

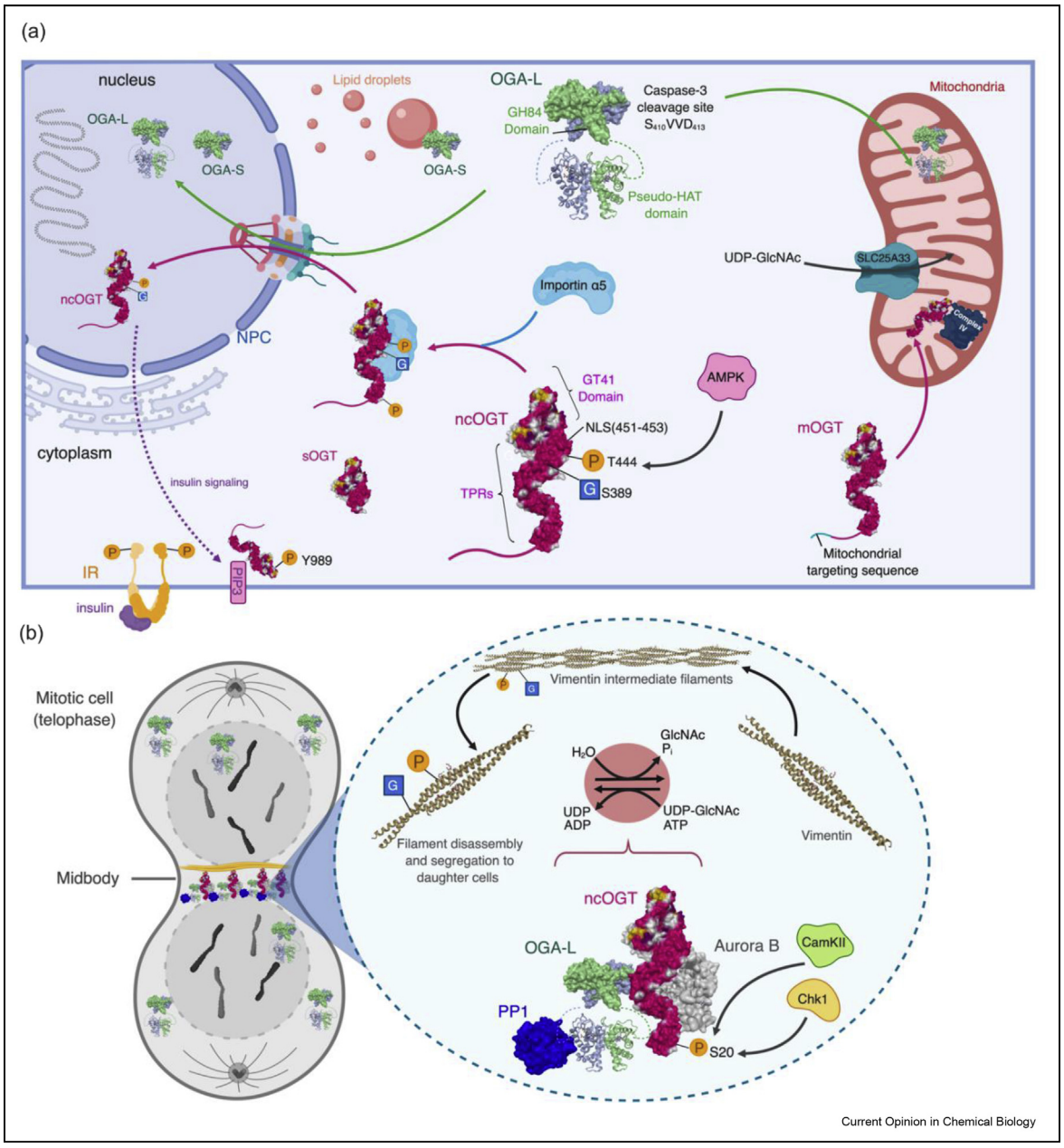
## Localization

The O-GlcNAc cycling enzymes adopt distinct subcellular loci during different phases of the cell cycle, in response to stimuli, and across cell types. The OGT and OGA isoforms (Figure 2) have long been known to display different subcellular localization patterns; ncOGT is found within the nucleus and cytoplasm, short OGT is predominantly localized to the cytosol and mOGT is thought to be largely mitochondrial — although this is a topic of some debate [26,27]. *OGA* has two dominant splice variants, with OGA-L being nucleocytosolic with preferential localization in the cytoplasm and the largely inactive OGA-S residing in the nucleus and on the surface of nascent lipid droplets, where it is thought to regulate proteasome-mediated droplet remodelling (Figure 4A) [1].

Excitingly, the molecular mechanisms regulating these isoform-specific localization patterns are beginning to emerge. Recently, the ncOGT nuclear localization signal (NLS) was characterized (DFP; residues 451-453). Furthermore, the authors found that this NLS is required for interaction with importin a5, and subsequent translocation through the nuclear pore complex (Figure 4A) [28]. mOGT is proposed, based on sequence analysis, to contain a putative N-terminal mitochondrial targeting sequence. Consistent with this hypothesis, OGT appears highly concentrated in the mitochondrial inner membrane where it interacts with electron transport chain complex IV and is suspected of O-GlcNAcylating various mitochondrial proteins [29]. Curiously, the pyrimidine nucleotide carrier 1 transporter protein (SLC25A33) translocates UDP-GlcNAc from the cytosol to the mitochondrial matrix, potentially facilitating mOGT-catalyzed mito-specific glycosyltransfer (Figure 4A) [29]. Isoform-specific siRNA knockdown of mOGT leads to Drp1-dependent mitochondrial fragmentation, reduced membrane potential and loss of mitochondrial content, supporting an important role of OGT in maintaining mitochondrial integrity and function [27].

In addition to these isoform-specific localization patterns, overexpression of tagged O-GlcNAc cycling enzymes shows their partitioning to distinct subcellular locations during cell division and in response to signalling events. During mitosis, OGT is concentrated at the central spindle assembly during anaphase and the midbody

**Figure 4**




**Modulation of OGA and OGT localization by PTMs and protein-binding partners**. **(a)** Subcellular localization of OGA and OGT. Importin α5 facilitates the nuclear import of ncOGT, and this process is dependent on S389 O-GlcNAcylation and AMPK-mediated T444 phosphorylation. The OGT-S isoform is predominantly cytosolic. OGA-L localizes to the cytosol and nucleus, and OGA-S exists in the nucleus and on the surface of lipid droplets. OGA-L and mOGT localize to the mitochondria, and mOGT associates with complex IV. **(b)** OGT and OGA localization and complexation during mitosis. OGT is localized almost exclusively to the midbody during mitosis. In contrast, OGA localizes by diffusion throughout the cell. OGT, OGA, AURKB and PP1 are thought to form a complex at the midbody during mitosis. This complex modulates vimentin intermediate filament formation and disassembly, thereby regulating segregation of daughter cells during cytokinesis. We note that structural details regarding the (OGT, OGA, AURKB, PP1) complex remain unresolved; therefore, our representation of this complex in panel (b) is for illustrative purposes only. AMPK, mOGT, mitochondrial OGT; ncOGT, nucleocytoplasmic OGT; OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase; O-GlcNAc, O-linked *N*-acetylglucosamine; PTM, post-translational modification.

during cytokinesis. This is consistent with the formation of a transient complex between OGT, OGA, mitotic Aurora kinase B (AURKB) and protein phosphatase 1 (PP1), which regulates vimentin filament glycosylation to control intermediate bridge formation and severing during cytokinesis (Figure 4B) [30]. Recently, localization of endogenously expressed O-GlcNAc cycling enzymes and global O-GlcNAcylation was investigated in bovine and human oocytes during meiosis by immunocytochemistry and confocal microscopy [31]. O-

GlcNAcylated proteins were distributed throughout the nucleus and cytoplasm with enrichment at the nuclear envelope during prophase I, whereas during metaphase, O-GlcNAcylated substrates localized throughout the cytoplasm, with slight enrichment at the cortex. OGA was localized at the cortex throughout meiosis, whereas OGT was localized to the meiotic spindles [31]. These results support the idea that localization of the O-GlcNAc cycling enzymes is regulated during cell division. Despite having cell division–dependent localization patterns, the mitotic and meiotic roles of the O-GlcNAc cycling enzymes remain poorly understood, although altering levels of these enzymes clearly affects cell division. On prolonged insulin stimulation of 3T3-L1 adipocytes, a subset of ncOGT is proposed to be recruited to the plasma membrane by phosphatidylinositol-(3,4,5)-triphosphate to modulate the insulin signalling pathway by O-GlcNAcylation (Figure 4A) [32–34]. OGT was initially proposed to have a phosphatidylinositol-(3,4,5)-triphosphate–binding site located in the C-terminal catalytic and Int-D domains; however, this topic remains controversial [4]. We speculate that this type of triggered localization of the O-GlcNAc cycling enzymes may be relatively common to enable temporal control of O-GlcNAc levels at distinct subcellular locations. Yet the molecular bases for these changes need more focused biochemical studies combined with cell-based experiments.

Finally, OGT displays cell-type–specific localization patterns. For example, OGT is enriched in the postsynaptic density of excitatory synapses perhaps to enhance the O-GlcNAcylation of multiple proteins as seen to occur on neuronal stimulation. Postsynaptic knockout (KO) of OGT decreases synaptic expression of ionotropic transmembrane glutamate receptor AMPA subunits (GluA2 and GluA3), reduces presynaptic terminals and stunts the development of mature dendritic spines [35]. Future discovery-based investigations to systematically catalogue OGA and OGT localization patters across diverse cell types and in response to varied stimuli would likely be illuminating, as has been the case for kinases and phosphatases.

## Post-translational modifications

The O-GlcNAc enzymes are decorated with numerous PTMs. In fact, a survey of the PhosphoSitePlus database reveals that proteomic discovery–based mass spectrometry efforts have uncovered a total of 55 and 42 distinct PTMs on human OGT and OGA (Figure 2) [20]. For OGT, a handful of these PTMs have been ascribed to functions including dynamic fine-tuning of its localization, turnover and substrate specificity (Table 1). O-GlcNAcylation of OGT Ser389 within TPR12 enables its nuclear localization [28]. Similarly, AMPK-mediated phosphorylation of Thr444 contributes to OGT nuclear import in myotubes [36]. We note that the T444 and S389 phosphosites are both located within TPR12 in proximity to the recently characterized NLS (451–453) and thus may regulate recruitment of the nuclear import machinery. This NLS is present in all OGT isoforms; however, its contribution to nuclear import has only been investigated in ncOGT. Phosphorylation of OGT T444 changes its substrate selectivity *in vitro* and in proliferating HEK293T kidney cells and alters its binding partners. T444 phosphorylation thus appears to regulate OGT target specificity, localization and protein–protein interactions — phenomena that are likely not mutually exclusive. On glucagon-induced calcium signalling, calcium/calmodulin-dependent kinase II phosphorylates OGT S20, which alters the specificity of OGT to promote O-GlcNAcylation and activation of the Ulk proteins involved in autophagic induction [37]. Similarly, checkpoint kinase 1 also phosphorylates Ser-20, and this event was separately found to stabilize OGT against

**Table 1**

**Key post-translational modifications and processing events on OGT and OGA.**

| Protein (residue) | Modification | Functional role | Enzyme(s) | Reference |
|---|---|---|---|---|
| OGT (S3) | Phosphorylation, O-GlcNAc | Increases activity | GSK3β | [41] |
| OGT (S4) | Phosphorylation, O-GlcNAc | Increases activity | GSK3β | [41] |
| OGT (S20) | Phosphorylation | OGT activity/mitotic localization | Chk1, CamKII | [37,38] |
| OGT (S389) | O-GlcNAc | Required for nuclear localization | OGT | [28] |
| OGT (T444) | Phosphorylation | Nuclear uptake and activity | AMPK | [36] |
| OGT (D451-P453) | Nuclear localization signal | Nuclear uptake | NA | [28] |
| OGT (Y989)[a] | Phosphorylation | Increased activity | IR, Src | [32] |
| OGA (S364) | Phosphorylation | Unknown | Unknown | [20] |
| OGA (S405) | O-GlcNAc | Unknown | Unknown | [20] |
| OGA (S410-D413) | Proteolysis site | Unknown | Caspase-3 | [42] |

CamKII, calcium/calmodulin-dependent kinase II; Chk1, checkpoint kinase 1; GSK3β, glycogen synthase kinase 3β; NA, nonapplicable; OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase; O-GlcNAc, O-linked *N*-acetylglucosamine.
[a] Speculative modification proposed based on blot and sequence analysis on rat OGT Y979 (hOGT Y989).

proteasomal degradation and be required for its previously mentioned localization to the midbody on cytokinesis (Figure 4B) [38]. Understanding the roles of OGT phosphorylation may yield insight into its stimulus-induced spatiotemporal regulation. Even less explored are the multiple putative ubiquitinylation sites discovered on OGT in proteome-wide mass spectrometry experiments (PhosphoSitePlus database [20]). For example, the LSD2 histone demethylase acts as an E3 ligase and targets OGT for polyubiquitinylation at an unknown site, thereby promoting its proteasomal degradation [39], whereas the BAP1 deubiquitinase protects OGT against ubiquitin-proteasome system (UPS)-mediated degradation [40]. However, the identities of the E1 and E2 enzymes regulating these UPS-mediated degradation pathways remain unknown.

PTMs can also directly modulate OGT-catalyzed glycosyltransfer. OGT can be phosphorylated at S3 and S4 by glycogen synthase kinase 3β [41]. Interestingly, GlcNAc has also been identified on OGT S3 and S4, suggesting the possibility that O-GlcNAcylation and phosphorylation may antagonize each other at this site [41]. Although an appealing hypothesis, O-GlcNAc and phosphorylation are both most often substoichiometric, and accordingly, such proposed direct reciprocal crosstalk has been difficult to clearly demonstrate as this would require quantitative assessment of PTMs on endogenous proteins. Taken together, the roles of PTMs in controlling OGT localization, turnover and activity are slowly emerging. The challenge in the future is to confirm these effects, decode the combinatorial influence of these diverse PTMs on OGT function in response to subcellular signalling and delve deeper into the structural and biochemical basis behind these observations.

OGA is also heavily decorated with PTMs; however, very little is understood regarding their functional ramifications. During apoptosis, OGA is cleaved by caspase-3 within a noncanonical $S_{410}VVD_{413}$ recognition site [42]. Coexpression of the two caspase-3 cleavage products recapitulates OGA activity in cells, suggesting the two halves remain associated within cells. However, a functional role for this cleavage has not been defined. Studies have reproducibly identified GlcNAc at S405, yet it is not known whether this modification has a functional role [20]. Bearing in mind that individual sites of modification do not necessarily have physiological roles, identifying those which do and exploring how some of these post-translational events influence OGA function will bridge a key gap in our understanding of the regulation of O-GlcNAc.

## Interaction partners

Interaction partners play an integral role in modulating the activity and localization of the O-GlcNAc cycling enzymes. Here, we focus on select recent examples germane to the regulation of O-GlcNAc. The nuclear receptor REV-ERBα integrates the circadian clock with both glucose and lipid metabolism and has recently been shown to interact with and modulate OGT [43]. REV-ERBα shields cytoplasmic OGT from proteasomal degradation and appears to enhance OGT catalytic activity in the nucleus, thereby increasing O-GlcNAcylation of proteins within both compartments. For example, REV-ERBα is proposed to promote OGT-mediated regulation of insulin signalling by boosting O-GlcNAcylation of AKT in the cytoplasm [43]. Thus, REV-ERBα may exert pleiotropic circadian activities through direct interaction with OGT.

UDP-*N*-acetylglucosamine pyrophosphorylase 1 (UAP1) catalyzes the last step in the synthesis of UDP-GlcNAc. A UAP1 paralog, UAP1-like-1 (UAP1L1), is required for OGT-mediated protein O-GlcNAcylation in HepG2 liver hepatocellular carcinoma cells [44]. Surprisingly, UAP1L1 does not efficiently catalyze UDP-GlcNAc synthesis, but instead interacts directly with the C-terminal catalytic domain of OGT. Knockdown of UAP1L1 markedly reduced global O-GlcNAcylation in HepG2 and TONG cells, yet UAP1L1 alone is not sufficient to bolster OGT activity *in vitro* [44]. Thus, UAP1L1 likely maintains OGT activity within cells through direct binding, although more detailed characterization is necessary.

5-Methylcytosine in DNA plays important roles in transcriptional regulation, genomic imprinting and suppression of transposable elements. The ten-eleven translocation (TET) 1–3 proteins oxidize 5-methylcytosine and thereby facilitate reversal of locus-specific DNA methylation, particularly within promoter regions [45]. Although sometimes contradictory, a range of studies have shown OGT binds to TET proteins and regulates their activity (reviewed by Wu et al [46]). Sadly, aside from the work on REV-ERBα [43], this promising area has largely languished since 2015, and a challenge in the future is to better determine the context-dependent effects of these interactions on the chromatin structure and gene regulation.

To identify putative OGA-binding partners, BioID proximity biotinylation in combination with stable isotope labelling by amino acids in cell culture was used [47]. This analysis revealed 90 proteins displaying stress-dependent interactions with OGA. OGA interactions with fatty acid synthase (FAS), filamin-A, heat shock cognate 70 kDA and OGT were verified by coimmunoprecipitation. *In vitro* analysis of the pool of OGA bound to FAS revealed it had only ~15% of the activity of free enzyme, and FAS overexpression appeared to boost oxidative stress–induced global O-GlcNAc levels in cells [47]. Overall, the structural and biochemical features governing these protein–protein interactions remain unexplored and limit our

understanding of how binding partners functionally regulate OGT and OGA.

## Metabolic regulation

The concentration of the UDP-GlcNAc donor substrate used for OGT-catalyzed glycosyltransfer is responsive to nutrient flux owing to the direct dependency of HBSP on major cellular metabolites. In particular, intracellular concentrations of glucose, glutamine, fatty acids and GlcNAc modulate HBSP flux and UDP-GlcNAc abundance (for an early perspective, see Marshall et al [48]). Notably, HBSP enzyme expression and allosteric modulation are under tight control and respond to various changes in nutrient and metabolite abundance and intracellular signalling (reviewed by Chiaradonna et al [49]). For example, the unfolded protein response signal transducer spliced X-box binding protein 1 acts as a transcriptional activator to induce expression of rate-limiting HBSP enzymes, boosting cellular O-GlcNAcylation in response to ER stress [50]. While nutrient supply clearly alters UDP-GlcNAc levels, nutrient flux may also induce intracellular signalling pathways that ultimately modulate the abundance and function of the O-GlcNAc cycling enzymes. Accordingly, while generally accepted in the field, the effects of nutrients on regulation of O-GlcNAc have surprisingly not been systematically investigated to simultaneously monitor UDP-GlcNAc levels, levels of HBSP enzymes, OGA and OGT and downstream levels of O-GlcNAc. Such studies would help provide a solid foundation to systematically assess the functional and mechanistic basis for the effects of nutrients.

## Transcriptional activation and repression

New findings are emerging to support expression of OGA and OGT being coordinately regulated by control of transcriptional programmes that work to maintain balanced O-GlcNAc levels. For example, increases in OGT lead to concomitant increases in OGA expression, and vice versa, in what appears to be an effort to buffer the cell from rapid changes in O-GlcNAcylation. Furthermore, treatment of cells with the selective OGA inhibitor ThiametG boosts O-GlcNAc levels and induces a compensatory increase in OGA gene expression and a correlated decrease in OGT abundance [51], and the converse is also true for OGT inhibitors [52].

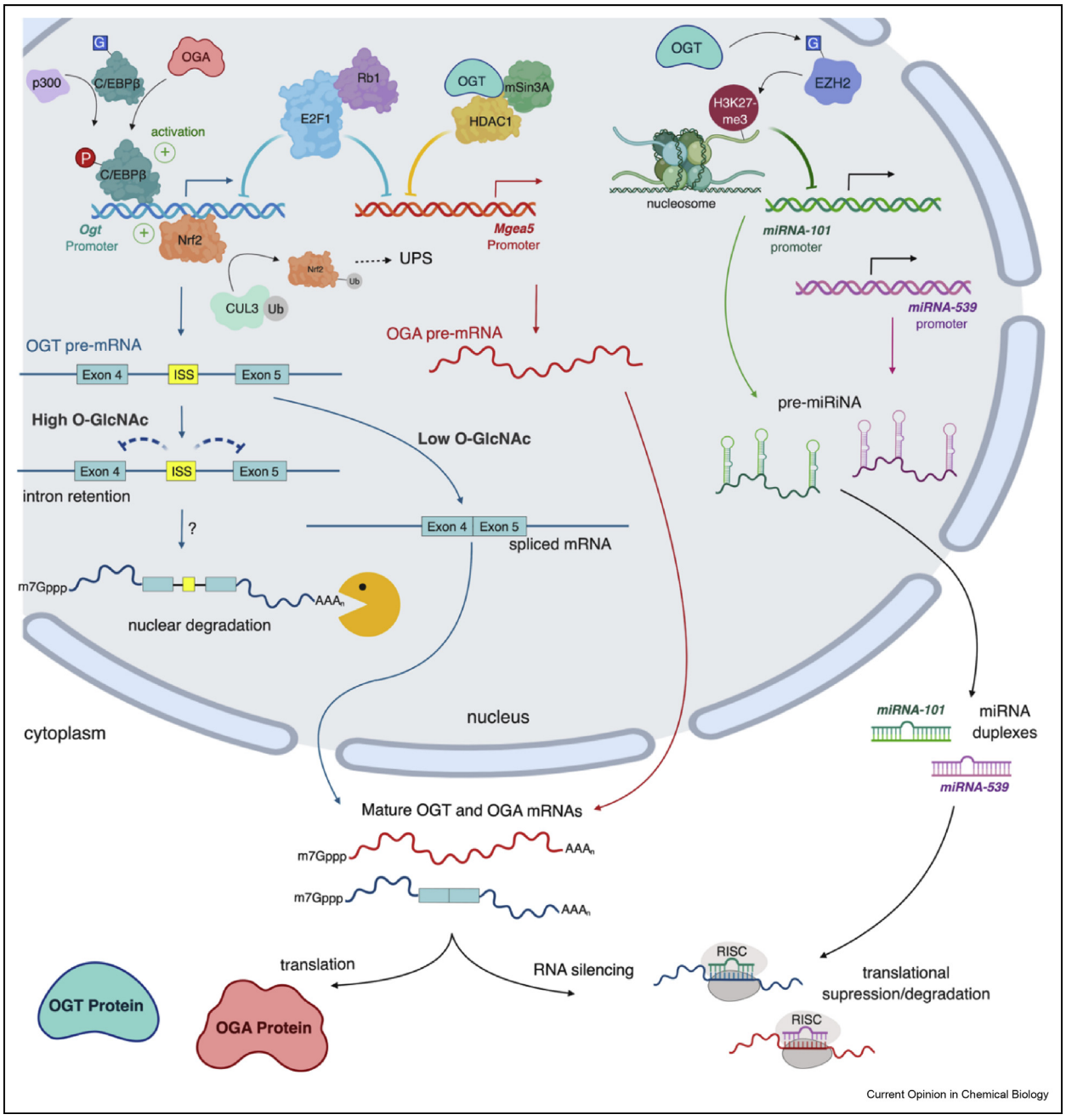
We are just beginning to uncover transcriptional programmes governing these coordinated shifts in expression of *OGT* and *OGA*. For example, OGA serves as a coactivator in conjunction with the HAT p300 to positively regulate the transcription factor CCAAT/enhancer-binding protein beta, among the most common activators occupying the *OGT* promoter (Figure 5) [53]. This transcriptional regulatory mechanism enables OGT expression to be tuned to OGA levels to balance O-GlcNAcylation. In addition, the *OGA* promoter is sensitive to OGT expression, as exemplified by a recent analysis of O-GlcNAcylation in patients with XLID. An XLID variant L254F-OGT results in reduced L254F-OGT protein levels in isolated patient lymphoblastoid cells [3]. However, global O-GlcNAc abundance appeared unaltered, presumably owing to a compensatory decrease in OGA mRNA and downstream OGA levels. OGT appears to be involved in a balancing mechanism that regulates global O-GlcNAc levels in XLID through formation of an OGT–mSin3A–HDAC1 repressive complex at the *OGA* proximal promoter region (Figure 5) [3]. Curiously, E2F transcription factor 1 (E2F1) concurrently represses transcription at both *ogt* and *oga* promoters in a retinoblastoma (pRb)-dependent manner in mice to maintain a balanced mRNA pool (Figure 5) [54]. *OGT* transcription also appears to be regulated by hepatocyte nuclear factor 1 homologue A, which is itself O-GlcNAc modified. Loss of O-GlcNAc appeared to increase transcription of *OGT*, suggesting a mechanism for autoregulation of O-GlcNAc homoeostasis [55]. Overall, a higher order network of multiple transcriptional mechanisms likely acts in concert to maintain O-GlcNAc homoeostasis, but decoding this will require both more detailed biochemical and system-wide studies.

Transcription of O-GlcNAc cycling enzymes is also dysregulated in response to other specific stimuli. For example, OGT levels are markedly increased in human papillomavirus (HPV)–induced cervical neoplasms, and transfection of HPV oncogenes into mouse embryonic fibroblasts induces a pronounced increase in *OGT* mRNA and protein levels [56]. HPV infection alters the expression of several transcription factors with predicted binding sites within the *OGT* promoter. Overexpression of transcription factors AP-1, SP-1, NF-κB, p65 and c-MYC enhanced *OGT* promoter activity on a reporter construct [56]. However, further *in vivo* analysis of the relative contribution of these transcription factors towards enhancement of endogenous *OGT* expression is required. As an additional example, on lipopolysaccharide stimulation, NF-E2-related factor 2 (Nrf2) binds the *ogt* promoter and enhances its transcription in bone marrow–derived mouse macrophages, resulting in amplified O-GlcNAcylation and inhibition of the proinflammatory transcription factor STAT3. Interestingly, Nrf2 is not required for baseline *OGT* expression but rather only boosts its abundance in response to stimuli. Furthermore, the E3 ubiquitin ligase CUL3 negatively regulates *OGT* expression through its targeted degradation of Nrf2 (Figure 5) [57]. Future efforts should aim to uncover additional *cis*- and *trans*-acting factors controlling transcription of *OGT* and *OGA* and illuminate how they are integrated to tailor transcriptional programmes in response to various stimuli.

## Post-transcriptional regulation

MicroRNAs (miRNAs) are short noncoding RNAs that typically hybridize to the 3′ untranslated region (UTR)

**Figure 5**




**Transcriptional and post-transcriptional regulation of *OGA* and *OGT* expression**. OGT is regulated at the transcriptional level by activators (C/EBPβ, Nrf2) that are themselves modulated by secondary regulatory mechanisms. Both OGA and OGT promoters are negatively regulated by E2F1 in an Rb1-pRb dependent fashion. Furthermore, OGA is thought to be transcriptionally repressed by the OGT–mSin3A–HDAC1 complex. The OGT mRNA transcript contains a retained fourth intron with an O-GlcNAc–sensitive internal splicing silencer (ISS). In conditions of high O-GlcNAc, the ISS prevents splicing of the detained intron, leading to nuclear degradation of the transcript, whereas low O-GlcNAc results in splicing of the intron and subsequent nuclear export. The cytoplasmically localized mature OGT and OGA mRNA transcripts are either translated into protein product or can be targeted for degradation via hybridization to miRNAs and subsequent RNA-induced silencing. OGT and EZH2 take part in a negative feedback mechanism to repress the miRNA-101 promoter. C/EBPβ, CCAAT/enhancer-binding protein beta; miRNA, microRNA; Nrf2, ENF-E2-related factor 2; OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase; O-GlcNAc, O-linked *N*-acetylglucosamine.

of targeted transcripts. This binding guides Argonaute proteins to direct targeted mRNAs to the miRNA-induced silencing complex (miRISC), leading to translational repression and/or nucleolytic degradation. An early report showed miRNA-539 destabilizes *OGA* mRNA transcripts [58]. Recently, miRNA-15b has been shown to target *OGT* mRNA in Th17 cells [59], and the oxidative stress–responsive miRNA-200a and miRNA-

200b antagonize the *OGT* mRNA transcript in conditions of high glucose in human aortic endothelial cells [59,60]. Recently, miRNA-101 has been found to target the transcripts of both *OGT* and *EZH2* for degradation. Interestingly, OGT-mediated O-GlcNAcylation has been proposed to stabilize EZH2, thereby promoting EZH2-catalyzed histone H3K27 trimethylation within the miRNA-101 promoter. Because the H3K27me3 mark transcriptionally silences miRNA-101, a negative feedback mechanism that promotes metastatic colorectal cancer may operate (Figure 5) [61]. Finally, in hepatocytes, new data show hepatitis C virus (HCV) infection induces miRNA-501-61p binding to the 3′UTR of OGT transcripts, blocking translation and leading to decreased OGT levels [62]. Moreover, this last observation is recapitulated bidirectionally using OGA and OGT inhibitors. On balance, miRNA-mediated degradation is emerging as a key mechanism regulating context-dependent fine control of expression of O-GlcNAc cycling enzymes.

Another post-transcriptional mechanism controlling OGT expression is the regulated splicing of its fourth intron, through the recently recognized phenomenon of retained introns (RIs) [63]. High cellular O-GlcNAc levels are correlated with increased abundance of the RI spliceoform of the *OGT* transcript (OGT-RI) within the nucleus. Notably, pharmacological inhibition of OGT reduced OGT-RI levels, which correlated with enhanced OGT expression [63]. Furthermore, a 526-nts O-GlcNAc—sensitive intronic splicing silencer (ISS) located within the fourth intron regulates abundance of this nontranslated spliceoform (Figure 5) [63]. Currently, the cellular fate of the OGT-RI and the trans-acting factors that bind and regulate the ISS in response to fluctuating O-GlcNAc levels represent major unknowns of high interest.

## Conclusion

In summary, recent crystallographic investigations of OGT and OGA have provided a structural foundation for understanding the molecular mechanisms regulating O-GlcNAc cycling. On exploring the current state of knowledge about these mechanisms, we emerge with a view that an interconnected network of regulatory factors dynamically tailors the abundance, localization and activity of the O-GlcNAc cycling enzymes. For example, PTMs and protein interaction partners can exert pleiotropic effects on the localization and activity of OGT and OGA (Figure 5). Pretranslational regulatory mechanisms are emerging as major factors orchestrating global O-GlcNAc homoeostasis. In addition, nutrient-based regulation plays defined roles as well, both through modulation of these processes and by directly regulating levels of donor sugar substrates. Although many new regulatory processes are emerging, understanding of events at the atomic level remains largely unknown. A major challenge is to integrate cell- and tissue-based discovery studies with quantitative biochemical analyses to uncover the detailed molecular and atomic basis for regulation of O-GlcNAc. Such findings will illuminate how these events are antagonistic or synergistic in coordinating both global and target-specific O-GlcNAcylation to meet cellular needs. Finally, given that O-GlcNAc is emerging as a druggable pathway, delving deeper to uncover these mysteries will likely enable targeted therapeutic approaches that modulate O-GlcNAc for human benefit.

## Conflict of interest statement

DJV is a cofounder of and holds equity in the company Alectos Therapeutics. DJV serves as CSO and Chair of the Scientific Advisory Board (SAB) of Alectos Therapeutics. GJD serves on the SAB of Alectos Therapeutics. DJV may receive royalties from SFU for commercialization of technology relating to OGA inhibitors.

## Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) (DJV; PJT-148732, PJT-156202) and the Natural Sciences and Engineering Council of Canada (Discovery-RGPIN298406). DJV is a Tier I Canada Research Chair in Chemical Biology. DTK is supported by a postdoctoral fellowship from CIHR and a Trainee Award from the Michael Smith Foundation for Health Research (MSFHR). GJD is a Royal Society Ken Murray Research Professor. AM is supported by the Biotechnology and Biological Sciences Research Council (BBSRC BB/M011151/1).

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