O-GlcNAc transferase – an auxiliary factor or a full-blown oncogene?

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Abstract: The β-linked N-acetyl-D-glucosamine (GlcNAc) is a post-translational modification of serine and threonine residues catalyzed by the enzyme O-GlcNAc transferase (OGT). Increased OGT expression is a feature of most human cancers and inhibition of OGT decreases cancer cell proliferation. Anti-proliferative effects are attributed to post-translational modifications of known regulators of cancer cell proliferation, such as MYC, FOXM1 and EZH2. In general, OGT amplifies cell-specific phenotype, for example, OGT overexpression enhances reprogramming efficiency of mouse embryonic fibroblasts into stem cells. Genome-wide screens suggest that certain cancers are particularly dependent on OGT, and understanding these addictions is important when considering OGT as a target for cancer therapy. The O-GlcNAc modification is involved in most cellular processes, which raises concerns of on-target undesirable effects of OGT targeting therapy. Yet, emerging evidence suggest that, much like proteasome inhibitors, specific compounds targeting OGT elicit selective anti-proliferative effects in cancer cells, and can prime malignant cells to other treatments. It is therefore essential to gain mechanistic insights on substrate specificity for OGT, develop reagents to more specifically enrich for O-GlcNAc modified proteins, identify O-GlcNAc 'readers' and develop OGT small molecule inhibitors. Here, we review the relevance of OGT in cancer progression and the potential targeting of this metabolic enzyme as a putative oncogene. Contrasting the functions of any candidate oncogene between normal and cancer cells is rarely done, but only by understanding the normal functions of a given factor, it is possible to understand these functions gone awry. Here we review oncogenic functions of OGT.

1. Introduction: what is OGT?

The β-linked N-acetyl-D-glucosamine (GlcNAc) modification was discovered over 30 years ago (1) and O-GlcNAc transferase (OGT), the sole enzyme responsible for this modification, has since been linked to most biochemical processes in mammalian cells (2-4) (**Fig. 1**). In the early studies, OGT and O-GlcNAc were found to be enriched in the nucleus, in particular in the nuclear envelope (5,6), and subsequently OGT has been established to function also in the cytoplasm and mitochondria (7,8). In these subcellular compartments, O-GlcNAcylation encounters a vast array of other post-translational modifications, most notably phosphorylation. In many instances, O-GlcNAcylation and phosphorylation occur on the same residues, and OGT acts reciprocally with kinases.

Human OGT gene was sequenced in 1997 and it was found to be highly conserved within eukaryotes (9,10). OGT is essential for dividing mammalian cells (11-13), while lower eukaryotes survive without a functional copy (14,15). Evolutionary conservation and OGT being essential in only higher eukaryotes, imply that OGT has acquired additional roles during the evolution, but currently these roles remain obscure. Despite conservation of the OGT gene, the genetic architecture of the OGT locus is drastically different between vertebrates and lower eukaryotes, and only the mammalian OGT gene contains hyper-conserved intron, intron 4, which has been identified as the key means to regulate O-GlcNAc homeostasis (16,17). By comparing common and unique features of OGT in lower and higher eukaryotes, it may be possible to describe the essential functions OGT has in human cells.

Crystal structure of human OGT established the major domains of the protein and proposed regions that are important for substrate recognition. In contrast to kinases, which have a consensus phosphorylation site that is defined by the few flanking residues mediating interactions with the enzyme (18), the active site of OGT is relatively large (19) and its role in substrate selectivity has not been established. Instead, OGT substrate specificity is defined in significant part through interactions between the substrate peptide and the corkscrew-shaped N-terminal tetratricopeptide-domain that is far from the active site (20,21). The framework of how OGT selects its substrates has been described; however, the functional importance of this modification, and in particular proteins that recognize the O-GlcNAc mark on specific proteins, the ‘readers’, have not been described.

OGT activity is sensitive to a number of different inputs, most of which culminate to the hexosamine biosynthetic pathway (HBP) that produces OGT's co-substrate, UDP-GlcNAc (**Fig. 1**). HBP enzymes are frequently overexpressed in cancer, which confers growth-advantage to these cells (22-24). In addition, increased glucose uptake and many other inputs affect OGT activity (25-29).

OGT is overexpressed in cancer cells and the protein is essential only in proliferating cells, the two key features that position this enzyme as a drug-target (11-13,30-32). OGT acts reciprocally with kinases and, similar to phosphorylation, also O-GlcNAcylation is reversible through the action of an enzyme termed O-GlcNAcase (OGA, MGEA5, **Fig. 1**). Due to the large repertoire of OGT substrates and the large number of cell types in which OGT is active, there is no clearly defined single pathway that is impacted by OGT activity to drive tumorigenesis. This makes it very difficult to classify OGT as an oncogene in the classical sense. It is clear that high OGT activity confers growth-advantage to cancer cells, and while OGT may be important for adapting to stress induced during transformation and other cancer-specific features, the adaptive mechanisms have not been established in most instances. In this review, we describe current understanding of OGT biology with a particular focus on cancer.

2. OGT functions in normal cells

2.1. Evolutionarily conserved functions of OGT

Classically oncogenes and also proto-oncogenes are regulators of cell proliferation. OGT is not necessary for cell cycle progression in *Caenorhabtidis elegans*. Instead, OGT functions in metabolic processes and knockout phenotypes in the worm relate to insulin signaling and altered innate immunity (33,34). Worms without the functional copy of OGT also show decreased life-span (34,35), reduced glycogen and lipid storages (14), and increased autophagy (36). Notably, all these effects are regulated by OGT also in mammalian cells but the molecular details and contextual specificities are largely unknown (2,37).

In *Drosophila melanogaster*, OGT is required for development and contributes to DNA damage response, but the protein appears not to be necessary for cell division (38,39). Deletion of the OGT gene in the fly results in developmental defects in body segmentation, and these effects have been attributed to the role OGT plays in transcriptional repression. In more detail, OGT glycosylates the transcriptional repressor Polyhomeotic, the core subunit of the Polycomb

Repressive Complex 1 (PRC1) in *D. melanogaster*, and this glycosylation promotes stabilization of the ordered PRC1 oligomers (40,41). Similarly, OGT glycosylates the key components of the PRC1 (BMI1 and RING1B) and PRC2 (EZH2) in mammalian cells (42). PRC-complexes are important during development, and their altered activity in cancer cells is important for cancer cell plasticity and treatment resistance (43). OGT-dependent epigenetic regulation emerged early in the evolution, and it remains to be determined if OGT activity is important for the cancer cell plasticity.

2.2. Why is OGT essential for cell division in higher eukaryotes?

OGT is required in actively dividing mammalian cells and attempts to make OGT knockout animals have failed (11-13). In contrast, OGA knockout mice survive past birth, which implies that O-GlcNAc cycling itself is not necessary for cell division (44). It is particularly puzzling that OGT is essential for cell division in higher eukaryotes when the gene is not necessary for this in lower eukaryotes (38).

OGT is a well-established regulator of cell cycle progression and both decreased and increased activity impair mitosis. OGT overexpression results in defective mitosis due to inhibition of CDK1 (45), while inhibition of OGT activity results in decreased CDK1 expression with overall decrease in cell cycle progression (46,47). Notably, CDK1 is the only necessary cell cycle CDK in mammalian cells (48). Another important function for OGT is the proteolytic cleavage of a transcription co-factor, host cell factor 1 (HCF-1), a protein that has to be cleaved to perform specific activities during cell cycle (49-51). OGT and HCF-1 form a stable complex that is present in most cell types. Loss of HCF-1 causes defects in cytokinesis and accumulation of binucleated cells (50). In contrast, loss of OGT causes senescence and cell death (11,13). In addition to controlling unperturbed cell cycle, OGT also dampens DNA damage-signaling (52,53), and inhibition of OGT activity sensitizes cancer cells to DNA damage-inducing agents (54-56). Overall, OGT reduces DNA repair efficiency, and it is possible that the loss of OGT impairs progression through cell cycle through combinatorial effects on CDK1, HCF-1, DNA damage-signaling, and yet-to-be-identified factors, which would explain the distinct phenotypes seen for the knockout of OGT and HCF-1. Utilization of fast-acting means to deplete OGT (inhibitor or degron-based approaches) in specific cell cycle phases will help to clarify these data.

As described in the introduction, the OGT locus is drastically different between vertebrate and insect cells. OGT mRNA contains a detained intron (DI) only in vertebrates, and this DI is one of the most abundant DI-containing mRNAs (16). DIs are present in the poly-adenylated transcripts and these mRNAs are not subject to nonsense-mediated decay. DI-containing mRNAs can be matured by splicing of one intron and exist as an mRNA-population that can rapidly respond to external cues independent of transcriptional activation. For example, OGT intron 4, which is the OGT DI, is spliced rapidly in response to OGT inhibition (17). It is possible that an increase in DI-mRNAs confers stress-resistance as these mRNAs, at least theoretically, represent a pool of mRNAs that can be rapidly processed for translation independent of transcription. Remarkably, when OGT activity is inhibited, all DI-containing mRNAs are spliced more efficiently (57), but it has not been established if this is important for cell survival.

Overall, OGT performs important functions during cell cycle progression. It may be that OGT acquired some of these functions during evolution, which would explain lack of effects on cell cycle in *C. elegans* and *D. melanogaster*. OGT is integrated to almost all aspects of cellular physiology, and it may not be possible to pinpoint a single activity that explains the essentiality of this enzyme to higher eukaryotes.

2.3. OGT functions in terminally differentiated cells

Mammalian cells that have exited the cell cycle can survive in the absence of OGT. An important finding from OGT knockout studies in adult neurons is that there is no overt loss of cell number or activation of the cell death response (30,58). However, loss of OGT results in altered physiological functions, in particular in the regulation of metabolic homeostasis, as could be expected from the evolutionarily conserved functions of OGT. More specifically, OGT regulates satiety and nutrient-uptake in a cell-type specific manner: deletion of OGT from specific neurons (αCaMKII-positive) increases food uptake (58), while deletion from others (AgRP neurons) protects mice from diet-induced obesity (30), and if OGT is deleted from sensory neurons (Nav1.8), mice lose weight (59). These studies underline that OGT has clear context-dependent functions.

Deletion of OGT even from terminally differentiated cells can have severe physiological effects. For example, OGT deletion from the cells of the target tissue results in progressive cardiomyopathy (31), apoptosis of pancreatic β-cells (60) and necroptosis of hepatocytes (61). In

addition, deletion of OGT from pre-B cells decreases the number of mature B cells, an effect that can in part be attributed to the essential functions OGT has during the cell cycle (62). Most of these studies describe developmental knockouts of OGT in specific tissues, and in the future, it is important to assess what happens to terminally differentiated cells when OGT is depleted. Curiously, developmental deletion of OGT from the insulin-secreting cells (β cells) results in a significant decrease of these cells, while deletion of OGT from the terminally differentiated β cells does not affect cell number (60). It is possible that OGT is essential only during the development and has limited functions in the terminally differentiated cells; if this is correct, OGT-targeting therapies would likely be safe.

In all these studies, OGT was deleted from cells of a specific organ, and the effects may be more severe than using pharmacological strategies. It is not possible to predict what the systemic effects would be in response to a small molecule inhibitor targeting OGT, but there is a clear risk for severe on-target toxicities.

3. OGT expression and mutations in human cancer

3.1. OGT expression and activity are increased in cancer

We asked how OGT activity is altered in human cancers (**Suppl. Table 1**). Differences in OGT activity can arise from altered expression of OGT or OGA, the enzyme removing O-GlcNAc mark from target proteins. In addition, OGT activity may be affected via substrate-availability, which is challenging to accurately measure, but a good compromise as a read-out of OGT activity is to use O-GlcNAcylation, the catalytic product of OGT.

Increased expression of OGT was reported in 49 out of 59 studies that assessed this, and patients whose tumors had high levels of OGT, had poor prognosis. Overall, OGT expression is increased during carcinogenesis. OGA antagonizes OGT activity; however, also OGA is upregulated in over half of all the studies assessing this (**Suppl. Table 1**). OGT and OGA expression are regulated in a coordinated fashion in cell line models and the expression of the two is correlated in most cancers (63,64). The mechanistic basis for the coordinated regulation has not been established, but is likely to involve O-GlcNAcylated component(s) as an activity-dependent switch(es).

OGT catalyzes the addition of GlcNAc modification on target proteins' serine and threonine residues, and O-GlcNAcylation is used as a measure of OGT activity. O-GlcNAcylation is

increased almost universally in all cancers, and increased levels are associated with poor survival in the two studies evaluating this (**Suppl. Table 1**).

The levels of OGT, OGA and O-GlcNAc have been evaluated in most human cancers, but there is lack of data for myelomas and cancers of the central nervous system. Curiously, out of all major cancer types, OGT expression is the highest in cancer cells of the blood lineage, in particular models of myeloma (**Fig. 2**). Another limitation of the current studies is that the expression of OGT, OGA, and the catalytic product, O-GlcNAcylation, are rarely assessed in the same tissue sample, and it is therefore not possible to conclude if increased O-GlcNAc levels are a result of dysregulation of OGT/OGA enzyme pair, or largely independent of them. In addition, while it has been established that the expression of OGT and OGA are coordinately regulated, it has not been established if increased expression of both leads to an increase in the steady-state O-GlcNAcylation and / or whether this increases the dynamic range of O-GlcNAcylation.

3.2. OGT mutations in patient samples

Mutations are a well-established means to alter enzyme function and confer growth-advantage to cancer cells. Data available through the cBioPortal shows that the cancer-associated OGT mutations are enriched in the tetratricopeptide repeat (TPR) region and in the catalytic domain / near the active cite of the enzyme (**Fig. 3**). Most frequent mutations are conversions of an arginine residue to either cysteine or histidine (A>C/H). A>C mutations confer a pH-dependent gain-of-function to EGFR and p53 proteins that alter functions of these proteins in cancer cells that typically have lower pH (65). It is not known if the cancer-associated OGT mutations can promote or "drive" tumorigenesis. To test this, cancer-associated OGT mutations can be modeled to existing crystal structures to select the most interesting once for functional studies using *in vitro* enzyme assays. The ultimate experiment to test if OGT mutations promote tumorigenesis requires replacement of the endogenous OGT with a mutant version, which could be achieved using the degron-tagged OGT cell lines recently described by Levine *& al.* (2020) (66).

Systematic analysis of protein-protein interaction surfaces revealed that the interface between OGT and HCF-1 is a mutation hotspot (67). It has been proposed that mutations in the OGT-HCF-1 binding interface affect proteolytic processing of HCF-1, but this has not been assessed.

Functional relevance of the cancer-associated mutations in the *OGT* gene may be better understood based on hereditary conditions. OGT expression and activity are high in the central

nervous system and a number of studies have reported X-linked intellectual disability with mutations in the OGT locus. These mutations are typically found far from the active site and are located in the tetratricopeptide (TPR) domain of OGT (68-70), the domain that functions as the substrate recognition module of OGT (20,21). In addition, two studies reported mutations in the catalytic domain of OGT (71,72). The mutations in the catalytic domain decrease OGT activity, and one of these mutations was shown to delay neuronal differentiation, implying causality (72).

Data obtained through biochemical studies and from patient samples propose that OGT selects its substrate in a significant part through the TPR region. It would be interesting to assess the functional relevance of these mutations in isogenic cell line pairs. Overall, OGT mutations are rare, but as more and more tumors are sequenced, certain regions of the OGT gene show accumulation of mutations, similar to consensus point mutations in classical oncogenes.

3.3. Overexpression of OGT promotes malignant phenotype of cancer cells

OGT overexpression (OE) is advantageous to cancer cells. In particular, OGT OE promotes proliferation of colon (73), gastric (74,75), thyroid (76), breast (77) and liver cancer cells (78). In addition, OGT OE has been shown to enhance invasion / motility of cancer cells of breast (79,80), cervix (81) and thyroid (82). In all these studies, forced OGT overexpression promotes the malignant phenotype of cancer cells.

Resistance to targeted therapies has been attributed in large part to cancer-cell plasticity and reprogramming of the cells to a more primitive, stem cell-like state. Interestingly, OGT OE promotes reprogramming efficiency of mouse embryonic fibroblasts via glycosylation of the Yamanaka factors (c-Myc, Oct4, Sox2 and Klf4) (83). It is conceivable that increased OGT activity, as observed in tumors, may promote establishment of the stem cell-like state through Yamanaka factors, but this has not been formally tested.

Overall, forced overexpression of OGT promotes the malignant phenotype of cancer cells but in the molecular level, it is not known what the key mediators of this response are. In many instances, OGT behaves similar to metabolic genes: it is critical for tumor growth but may also be essential for the functions of the normal metabolically active cells for example by affecting mitochondrial respiration (66,84,85). When evaluating these results, it is important to note possible context-dependency.

3.4. OGT-dependent control of transcription in cancer cells

OGT and O-GlcNAc modification can directly regulate the transcriptional program of the cell. RNA polymerase II transcribes majority of the protein-encoding part of the human genome, and its activity is regulated by the kinases that phosphorylate the carboxy-terminal domain (CTD) of the polymerase. OGT-dependent glycosylation of the CTD was described over 30 years ago, and we have shown that the chromatin O-GlcNAc overlaps with active promoters; however, decreasing OGT activity has only minimal effects on transcription (47,86,87). Recently, OGT inhibition was shown to result in acute depletion of detained intron-containing mRNAs (57). OGT-dependent control of the splicing machinery may enable cells to couple nutrient-availability to the transcriptional program of the cell, and it becomes important to assess how OGT affects splicing in cancer cells. Interestingly, alternative splicing, particularly accumulation of the detained intron-containing mRNAs, was recently identified as a hallmark of aggressive prostate cancer, the most common cancer in men (88). It is not known if accumulation of the detained intron containing mRNAs is a result of defective splicing machinery or whether it is an adaptation to maintain transcriptional program that is insensitive to perturbations in transcription. By depleting OGT activity from prostate cancer cells, it is possible to test if depletion of detained intron containing mRNAs can sensitize these cells to other therapies, for example, to anti-androgens.

3.4. Inhibition of OGT activity suppresses cancer cell proliferation

Reducing OGT activity has been shown to suppress proliferation of all cancer cells tested. In xenograft models, knockdown of OGT decreases the growth of cancer models of breast (89,90), prostate (91), pancreatic (92-94), liver (95), colon (96), and bladder (97,98). Based on these studies, and others, no canonical ‘OGT-circuit’ has been established. Instead, OGT is expressed in all cells, and appears to act on proteins available. In cancer cells, OGT glycosylates known oncogenes such as c-MYC (63), NF-κB (92), YAP (99), and EZH2 (100) to promote their activity. It is therefore not at all clear what defines cancer cells’ dependency on OGT, or if this is dictated by the tissue of origin.

Decrease in OGT activity is tolerated by certain cancer cells, while others are more dependent on OGT (**Fig. 4**). For example, MYCN-amplified neuroblastoma cells are particularly dependent on OGT. It is notable that the neuroblastomas and prostate cancers harboring MYCN

amplification have short survival and currently no curative treatment strategy (101). As with all possible therapies, identification of biomarkers that can predict the response is extremely important, and exploring the role of OGT in MYCN-amplified tumors is an excellent starting point to identify cancer cells addicted to OGT.

Existing data proposes that the relative importance of high OGT activity is defined by the cell-specific proteome. This proposes that targeting cancer-specific features will sensitize these cells to OGT inhibition. Indeed, depletion of OGT activity greatly potentiates the efficacy of clinically tested cancer therapies targeting estrogen receptor (tamoxifen) (54,102,103), phosphoinositide 3-kinase (GDC-0941) (104), AMPK-activator (metformin) (46), glycolysis (2-deoxy-D-glucose) (105), cyclin-dependent kinase 9 (106) and proteasome (Bortezomib (107). These reported sensitization effects are based on known cancer cell addictions; however, the advent of genome wide CRISPR inhibition and activation screens enables unbiased discovery of synthetic lethal interactions with lowered OGT activity.

To summarize, decrease in OGT activity suppresses proliferation of all cancer models tested, and enhances the effects of clinically approved therapies. OGT is clearly an exciting candidate drug target, and particular focus of research should be on cancers that do not have effective therapy, in particular pancreatic cancer and treatment-resistant prostate cancer (castration-resistant and neuroendocrine, the latter being driven frequently by MYCN).

4.1. The OGT toolkit

Phosphorylation has been established as one of the major mechanisms in cell signaling, and it may be possible to use similar tools to better understand glycosylation. However, glycosylation and phosphorylation have fundamental differences, in particular, OGT is the sole enzyme catalyzing all nucleocytoplasmic glycosylation, and it is not at all clear if OGT can mediate signal amplification, as occurs in the kinase-world. In addition, phosphorylation induces conformational changes in the protein, which can generate binding motif for an interactor or otherwise alter protein function, but examples of OGT-dependent glycosylation exerting similar effects are limited. Finally, it has been challenging to develop glycoform-specific antibodies, and the reasons for these challenges likely include relatively small size / charge-change introduced by O-GlcNAcylation and the fact that glycosylated proteins are found in high abundance in the extracellular milieu. Scientific community has developed tools to study phosphorylation, and

development of similar tools is needed for glycosylation. In particular, there is a need for tools to detect glycosylation on specific proteins, identify reader proteins for O-GlcNAc, develop better OGT-targeting strategies and evaluate these in animal models.

4.2. Reagents to detect O-GlcNAcylation

There are three major approaches to detect O-GlcNAc in cells: lectins, antibodies and chemoenzymatic labeling. Lectins are specific carbohydrate-binding proteins (108). Succinylated wheat germ agglutinin conjugated to either fluorophore / horseradish peroxidase and different matrices can be used to image and enrich for O-GlcNAcylated proteins, respectively. In addition to lectins, also antibodies can be used to detect O-GlcNAcylation, and the most widely used antibodies are RL2 and CTD110.6, two mouse monoclonal antibodies that were generated against rat liver nuclear envelope (109) and a synthetic glycopeptide (110), respectively. There are two major limitations associated with these, and in practice, all existing anti-O-GlcNAc antibodies: 1) they do not detect all glycoproteins, and 2) they do not detect a specific protein that is O-GlcNAcylated in a defined residue. Therefore, tools to detect all O-GlcNAc and also site-specific O-GlcNAcylation are needed, and it should be possible to generate antibodies for the latter. It has been challenging to develop these antibodies due to numerous reasons, including: 1) O-GlcNAc modified epitopes are self-antigens / tolerated by the immune system, 2) the O-GlcNAc on protein is small relative to the peptide backbone, and 3) the interactions between carbohydrates and antibody appear to be weak. However, there are a few examples of antibodies that are specific for a single O-GlcNAc-modified protein, such as the antibody against TAU-protein O-GlcNAcylated on Ser-400 (111) and an antibody against SIRT1 glycosylated on Ser-549 (112). In addition, there are few commercially available antibodies with claimed specificity for O-GlcNAc; however, these antibodies have not always been properly characterized, which is needed to draw conclusions on their utility as tools for researchers. With the advent of new antibody-generation techniques, including phage-display system that allows repeated rounds of antigen-guided selection (113), it should be possible to generate specific antibodies against O-GlcNAc-modified proteins. In the future, it is important for academic groups to pair with industrial partners to generate and validate specific antibodies against O-GlcNAcylated proteins.

Chemoenzymatic labeling methodology is based on a mutant galactosyltransferase enzyme (GalT), which enables modification of all O-GlcNAc residues using azido-modified sugar-analogs and chemoselective ligation between an azide and an alkyne. Coupling this technique with different analysis methods has enabled detection of O-GlcNAc on proteins of interest, monitoring changes in the total levels of O-GlcNAcylation and also in mapping the stoichiometry of O-GlcNAc to specific amino acid residues (114).

There are a number of tools that enable enriching for O-GlcNAcylated proteins. Important next steps include generation of tools for enrichment of O-GlcNAc on specific proteins, and characterization of how protein function changes when it is O-GlcNAc-modified, something that generation of specific antibodies will enable researchers to do.

4.3. Identification of O-GlcNAc reader proteins

In general, it is not known how O-GlcNAcylation affects protein function and if O-GlcNAcylation is recognized, or read, by specific protein(s). There has been one report that used consensus OGT substrate peptide to show that the 14-3-3 proteins bind the O-GlcNAcylated peptide but not to the naked peptide (115). Since there are thousands of proteins that can be O-GlcNAc modified, there are thousands of potential O-GlcNAc modified sites, and utilization of specific proteins as baits is required to understand the true complexity of reader proteins.

A myriad of post-translational modifications (PTM) has encouraged researchers to develop tools to identify proteins that recognize specific PTMs, and the O-GlcNAc-field should make use of the existing tools. Immobilization of histone peptides has enabled discovery of reader proteins from nuclear extracts (116). Similar, but more sensitive method is based on SILAC-technology, in which proteins from heavy and light amino acid labeled cells are incubated with immobilized biotinylated peptides, and the enriched proteins are pooled prior to analysis with mass spectrometry, allowing quantitative identification (117).

When moving to identification of the reader proteins for O-GlcNAc modification, it is important to select bait-proteins carefully as the interaction between the PTM reading domain and the modified protein may be weak, especially as the peptide baits will unlikely adopt completely similar conformation than the full protein does (118). Utilization of proteins with multiple adjacent O-GlcNAc-modified residues should be prioritized. Overall, identification of the O-GlcNAc-recognition modules and the possible common motif-features is an important

milestone in the O-GlcNAcylation field, and this will open new research avenues on O-GlcNAc-dependent signaling.

4.4. Development of small molecule inhibitors targeting OGT

There is a relatively large number of compounds claimed to inhibit OGT, however, many of these lack specificity and even the best of them have not been vigorously tested in animal models. Common features of most of these compounds is that they are analogs of OGT co-substrate, UDP-GlcNAc. Alloxan (uracil mimic) (119) and BAGDP (N-acetylgalactosamine mimic) (120) are early probe compounds that act in millimolar doses. BZX2 is a di-phosphate mimic and the first covalent OGT inhibitor (121), while Ac4-5SGlcNAc is converted into its active form UDP-5SGlcNAc in cells (122); the limitation of these two interesting compounds is that they target other carbohydrate processing enzymes as well. ES1 compound is a UDP-GlcNAc analog carrying an electrophile that reacts with a cysteine-residue in the OGT active site, and the compound has promising specificity against OGT (123). Walker laboratory has long-standing experience in developing OGT inhibitors, starting from development of suitable screening platform that initially discovered ST045849-compound (124), through a quinolinone-6-sulfonamide (Q6S) class of compounds / analog synthesis that yielded OSMI-1 (125) and with the most recent compound, OSMI-4, which was a result of solving co-crystal structures with probe compounds and iterative improvement of the molecules (19). Finally, L01 compound was identified through a structure-based virtual screening approach, and presents a molecule that was not discovered through a rational design (126).

Overall, there is a large repertoire of OGT targeting compounds. The use of multiple, structurally unrelated compounds, represents an excellent strategy to probe OGT biology *in vitro*. An important next step is to take the best OGT inhibitors and test them in animal models. There are few studies that have explored this. 5SGlcNHex is a metabolic inhibitor that is converted into its active form inside the cell and the compound decreases O-GlcNAcylation in multiple tissues (127). OSMI-1 was shown to cause a delay in tumor progression of pancreatic cancer but no delivery route and PK/PD data was reported (128). One option to test the anti-cancer efficacy of the compound of interest, is to pre-treat cells *in vitro* prior to inoculation as has been done to test OSMI-1 efficacy in suppressing survival of lung and colon cancer cell line-derived tumors in

mice (129). An obvious limitation of these studies is that they do not assess on-target systemic toxicity. Finally, ES1 compound was tested in a zebrafish model, and while no gross toxicity was observed after 24 hours, authors did not evaluate if OGT was inhibited (126).

First studies using OGT small molecule inhibitors in animal models have shown promising anti-tumor effects. However, significant concerns in interpreting these results exist, in particular, evaluation of PK/PD parameters, on-target effect on OGT in target tissue and other tissues of concern (in particular brain, which has high OGT activity) are currently missing. It is also noteworthy that the most recently developed OGT inhibitors, in particular OSMI-4 and L01, are yet to be tested in animal models. Finally, an excellent tool to acutely deplete OGT activity from a model-system of interest, would be to use degron-based approaches that enable acute and selective control of protein abundance (130). Scientific community should see new developments in these fields in the near future.

5. Concluding remarks

OGT is overexpressed in most cancers, enzyme is essential to dividing cells, and overexpression confers significant growth-advantage to cancer cells. As such, OGT is a prototypical target for cancer therapy. The fact that OGT is expressed in most cells, and particularly high levels have been measured in the central nervous system, raises concerns about toxicity. However, recent development of potent and specific OGT targeting compounds show limited or no toxicity to normal cells, which implies that these cells can adapt to decreased O-GlcNAcylation and are therefore not acutely dependent on high level of unperturbed activity. It is vital to develop specific OGT inhibitors suitable for animal experiments, generate *in vivo* PK and PD data, and evaluate the systemic effects these compounds have on both OGT-activity and physiological effects. The cancer models of special interest are diseases with no curative treatment options, in particular pancreatic cancer, myeloma and castration-resistant / neuroendocrine prostate cancer.

OGT has a remarkably wide range of potential targets, and the scientific community has only started to elucidate the mechanisms how OGT selects its substrates. It is important to use different versions of OGT (mutant / differently modified / include co-factors) to describe substrate repertoire. In addition, the sequence motif recognized by OGT can be refined using the microarray strategy.

Phosphorylation status of a protein can be mimicked with phosphomimetic amino acids, but there are no amino acids that would mimic the O-GlcNAcylated residue. In part due to these obstacles, it has been challenging to generate antibodies that recognize the O-GlcNAc epitope. However, novel approaches, such as the phage-display technology, will improve the quality of antibodies.

An important task is to describe the natural motifs that recognize O-GlcNAc-mark on proteins. This could be approached using similar tools that were used to identify motifs that recognize differently phosphorylated peptides; for example by utilizing glycosylated peptide as a bait. By using multiple O-GlcNAcylated (and naked control) baits, it is possible to describe either one, predominant reader protein, or multiple readers.

OGT is important for cancer progression in part due to its functional diversity. In many instances, OGT appears to amplify cell-specific features, rather than conferring an 'OGT-unique' phenotype. Some of the tools and technologies to study OGT have been developed, and in the next years we will likely see progression of OGT-targeting therapies to animal studies, and, in the long run, also to clinical trials. Addition of new drug targets to treat cancer and metabolic disorders has tremendous value, and in this process, scientific community will describe new functions for this intriguing enzyme.

OGT-dependent glycosylation was discovered 30 years ago, but we still have a lot to learn. With the current evidence, OGT cannot be classified as an oncogene; however, no study has overexpressed OGT in normal cells and assessed if this is sufficient to drive tumorigenesis – an experiment required to test if OGT actually is an oncogene.

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Figure legends

Figure 1. OGT transfers N-Acetylglucosamine (GlcNAc) from uridine diphosphate-GlcNAc to target proteins' serine and threonine residues. OGT directly glycosylates proteins involved in transcription (RNA polymerase II, transcription factors) and also components of the spliceosome, ribosome and proteasome. As such, OGT is positioned to integrate information from multiple cues, and to act on downstream targets to maintain cellular homeostasis.

Figure 2. OGT expression in different cancers. Data was downloaded from the DepMap-portal (131,132). Each dot represents a cell line and the red bar graphs are mean of expression in that tissue type along with standard error of mean. Engineered samples were excluded from the figure.

Figure 3. OGT mutations detected in human cancer. Data was accessed through the cBioPortal (133,134). Mutated arginine residues are highlighted. Below the OGT gene are depicted known modifications as reported in dbPTM (135,136).

Figure 4. Low levels of OGT expression is sufficient in most cells. Value of less than 0.5 represents a noteworthy depletion and is highlighted with color red. MYCN amplification was identified as one of the dependencies based on the DepMap portal, and is highlighted in the y-axis (MYCN copy number log2(relative to ploydy+1). Most of the cell lines screened are cancer cell lines. **A)** OGT is defined as a common essential gene in every cell line assessed based on CRISPR-mediated knockout (710 cell lines). Dependency is plotted on the x-axis (lower the value, more important the gene is in a given cell line). Figure was generated using data available through the DepMap-portal (137,138). **B)** OGT knockdown using RNAi-strategy shows that most cell lines can survive even if OGT expression is lowered. Dependency is plotted on the x-axis (lower the value, more important the gene is in a given cell line). Figure was generated using data available through the DepMap-portal (138,139).

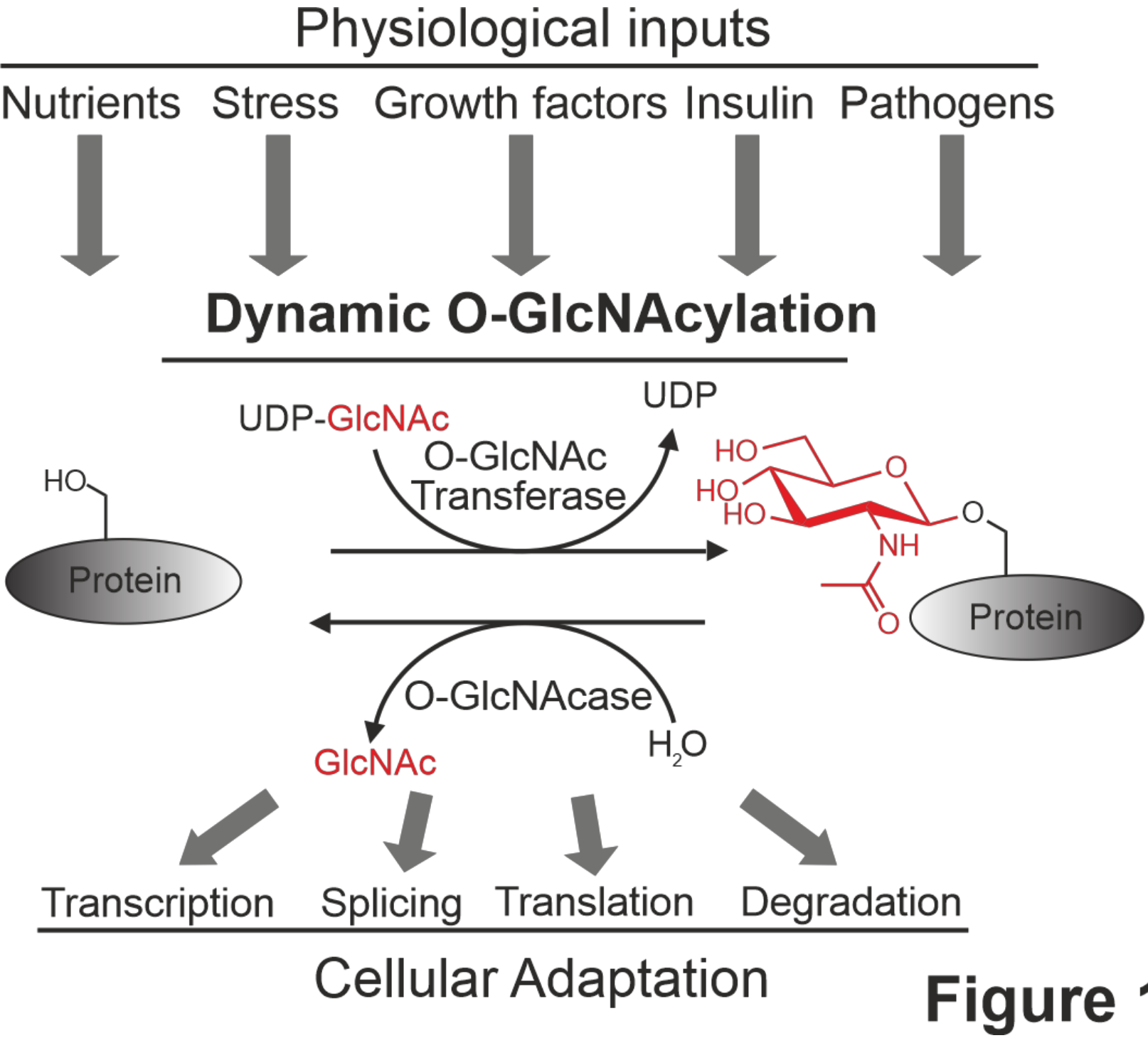

Physiological inputs
Nutrients
Stress
Growth factors
Insulin
Pathogens
Dynamic O-GlcNAcylation
UDP-GlcNAc
UDP
O-GlcNAc Transferase
HO
Protein
HO
HO
HO
O
O
NH
O
Protein
O-GlcNAcase
H_2O
GlcNAc
Transcription
Splicing
Translation
Degradation
Cellular Adaptation

Figure 1

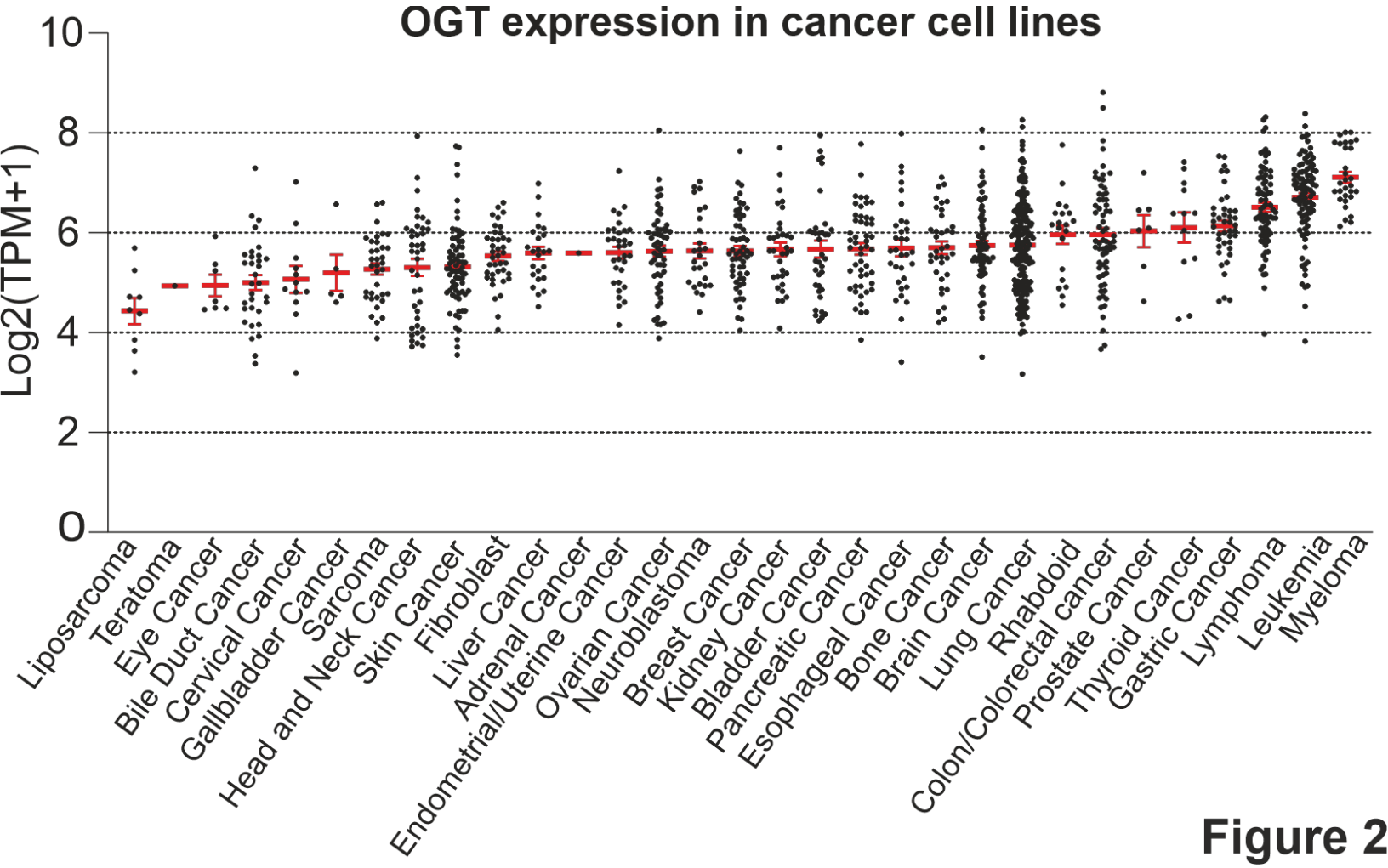

OGT expression in cancer cell lines
Log2(TPM+1)
10
8
6
4
2
0
Liposarcoma
Teratoma
Eye Cancer
Bile Duct Cancer
Cervical Cancer
Gallbladder Cancer
Sarcoma
Head and Neck Cancer
Skin Cancer
Fibroblast
Liver Cancer
Adrenal Cancer
Endometrial/Uterine Cancer
Ovarian Cancer
Neuroblastoma
Breast Cancer
Kidney Cancer
Bladder Cancer
Pancreatic Cancer
Esophageal Cancer
Bone Cancer
Brain Cancer
Lung Cancer
Rhabdoid
Colon/Colorectal cancer
Prostate Cancer
Thyroid Cancer
Gastric Cancer
Lymphoma
Leukemia
Myeloma

Figure 2

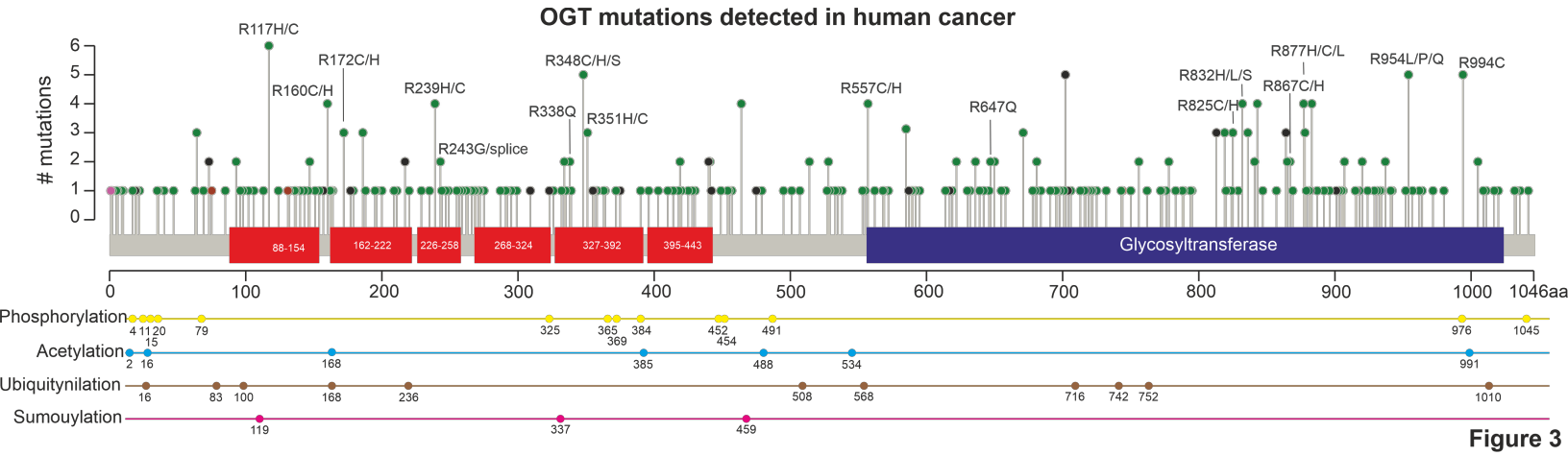
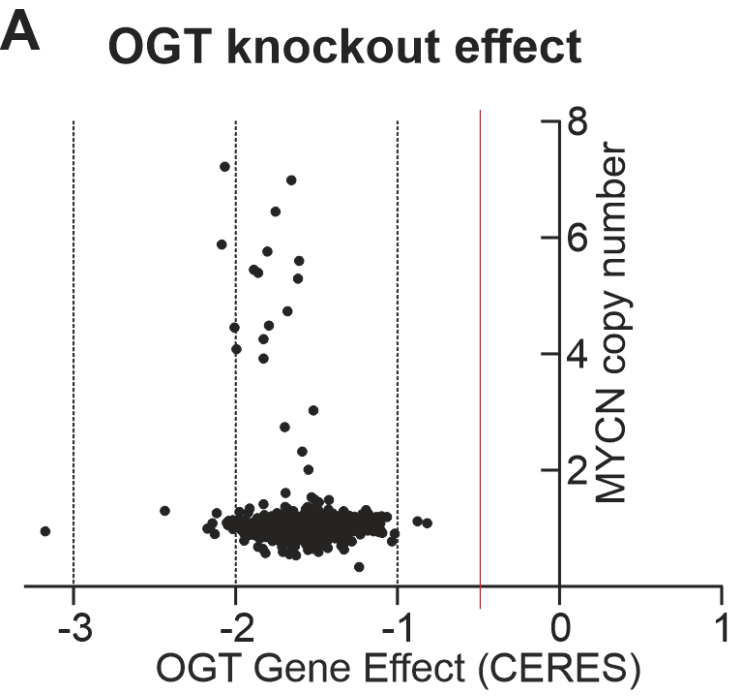

OGT mutations detected in human cancer
mutations
R117H/C
R172C/H
R160C/H
R239H/C
R243G/splice
R348C/H/S
R338Q
R351H/C
R557C/H
R647Q
R832H/L/S
R825C/H
R877H/C/L
R867C/H
R954L/P/Q
R994C
88-154
162-222
226-256
268-324
327-392
395-443
Glycosyltransferase
1046aa
Phosphorylation
Acetylation
Ubiquitynilation
Sumouylation

Figure 3


A
OGT knockout effect
MYCN copy number
8
6
4
2
-3
-2
-1
0
1
OGT Gene Effect (CERES)

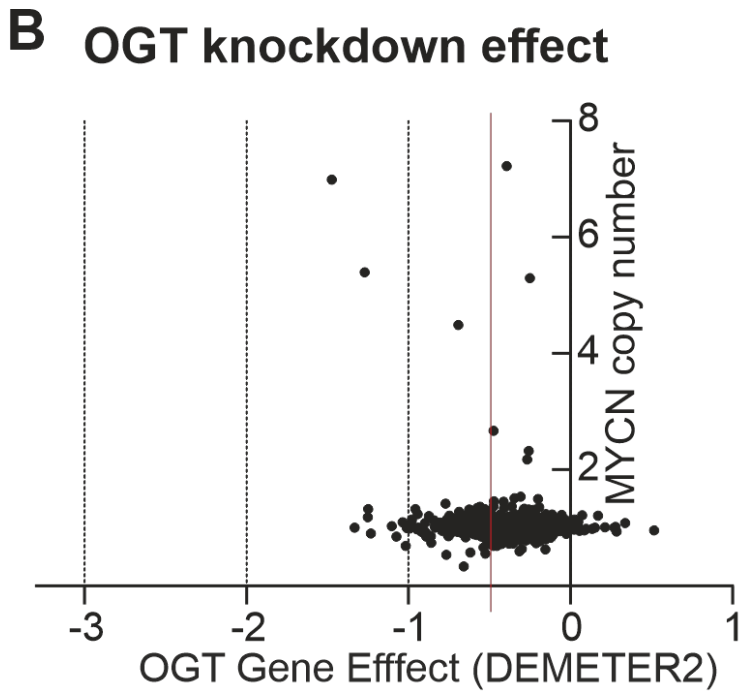

B
OGT knockdown effect
MYCN copy number
8
6
4
2
-3
-2
-1
0
1
OGT Gene Efffect (DEMETER2)

Figure 4