30 YEARS OLD: O-GLCNAC REACHES AGE OF REASON – REGULATION OF CELL SIGNALING AND METABOLISM BY O-GLCNACYLATION

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30 YEARS OLD: O-GLCNAC REACHES AGE OF REASON – REGULATION OF CELL SIGNALING AND METABOLISM BY O-GLCNACYLATION

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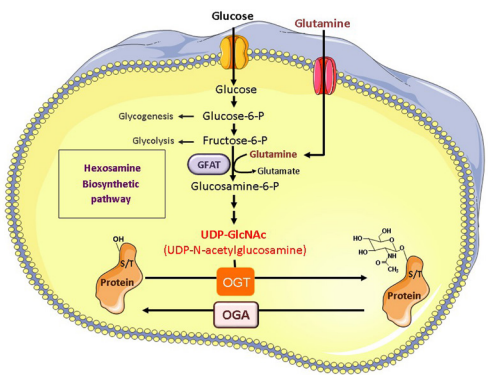

Protein O-GlcNAcylation: a dynamic post-translational modification that regulates cell life according to its nutritional environment.

A small fraction of the glucose entering the cell feeds the hexosamine biosynthetic pathway (HBP) to produce UDP-GlcNAc, the substrate used by the O-GlcNAc transferase (OGT) to add the N-acetyl glucosaminyl group on serine or threonine residues of cytosolic or nuclear proteins. Glucose enters the HBP as fructose-6-phosphate. The latter is converted to glucosamine-6-phosphate by the glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate limiting enzyme of the pathway. After a subset of reactions, UDP-N-acetylglucosamine (UDP-GlcNAc) is generated and used by OGT to add GlcNAc on serine or threonine residues of target proteins. This dynamic and reversible post-translational modification controls the activity, the localization or the stability of proteins according to glucose availability. In addition to glucose, the O-GlcNAc also includes amine and acetyl moieties, and therefore also integrates amino-acids (glutamine) and fatty acid (AcetylCoA) metabolisms, suggesting that availability of other nutrients may also be sensed by this pathway. Thus, OGT regulates cell behavior according to its nutritional environment. The O-GlcNAc moiety is removed from O-GlcNAc-modified proteins by the O-GlcNAcase (OGA).

Adapted from: Baudoin L and Issad T (2015) O-GlcNAcylation and inflammation: a vast territory to explore. Front. Endocrinol. 5:235. doi: 10.3389/fendo.2014.00235

Hundreds post-translational modifications (PTM) were characterized among which a large variety of glycosylations including O-GlcNAcylation. Since its discovery, O-GlcNAcylation has emerged as an unavoidable PTM widespread in the living beings including animal and plant cells, protists, bacteria and viruses. In opposition to N- and O-glycosylations, O-GlcNAcylation only consists in the transfer of a single N-acetylglucosamine moiety through a beta-linkage onto serine and threonine residues of proteins confined within the cytosol, the nucleus and the mitochondria. The O-GlcNAc group is provided by UDP-GlcNAc, the end-product of the hexosamine biosynthetic pathway located at the crossroad of cell metabolisms, making O-GlcNAcylation a PTM which level tightly reflects nutritional status; therefore regulation of cell homeostasis should be intimately correlated to lifestyle and environment. Like phosphorylation, with which it can compete, O-GlcNAcylation is reversible. This versatility is managed by OGT (O-GlcNAc transferase) that transfers the GlcNAc group and OGA (O-GlcNAcase) that removes it. Also, like its unsweetened counterpart, O-GlcNAcylation controls fundamental processes, e.g. protein fate, chromatin topology, DNA demethylation and, as recently revealed, circadian clock. Deregulation of O-GlcNAc dynamism may be involved in the emergence of cancers, neuronal and metabolic disorders such as Alzheimer's or diabetes respectively.

This Research Topic in Frontiers in Endocrinology is the opportunity to celebrate the thirtieth anniversary of the discovery of "O-GlcNAc" by Gerald W. Hart.

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EDITORIAL
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30 years old: *O*-GlcNAc reaches the age of reason – regulation of cell signaling and metabolism by *O*-GlcNAcylation

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Keywords: *O*-GlcNAc, cell signaling, *O*-GlcNAcylation, editorial, proteins

Hundreds of post-translational modifications (PTM) have been characterized on proteins, including a large variety of glycosylations, among which figures *O*-GlcNAcylation. Since its discovery, *O*-GlcNAcylation has emerged as a major PTM that is widespread, being found in viruses, bacteria, and protists through plant and animal cells. In contrast to *N*- and *O*-glycosylations, *O*-GlcNAcylation involves only the transfer of a single *N*-acetylglucosamine moiety through a beta-linkage onto serine and threonine residues of proteins that are localized to the cytosol, nucleus, and mitochondria. The *O*-GlcNAc group is provided by UDP–GlcNAc, the end-product of the hexosamine biosynthetic pathway (HBP), which integrates several metabolic pathways. *O*-GlcNAcylation levels therefore tightly depend on the nutritional status; regulation of functions by this PTM is thus intimately linked to lifestyle and environment (1, 2). As with phosphorylation, with which it can compete, *O*-GlcNAcylation is reversible through opposing actions of *O*-GlcNAc transferase (OGT) that transfers the GlcNAc group, and *O*-GlcNAcase (OGA) that removes it. Also, like its unsweetened counterpart, *O*-GlcNAcylation controls fundamental processes, e.g., protein fate, chromatin topology, DNA demethylation, and the circadian clock. Deregulation of the mechanisms controlling *O*-GlcNAc dynamics may be involved in the development of cancers, neuronal disorders such as Alzheimer's disease, and metabolic conditions such as diabetes (1, 2).

This E-Book, which gathers Original Research papers, Method Articles, and Reviews published as part of a Research Topic in Frontiers in Endocrinology, is the opportunity to celebrate the thirtieth anniversary of the discovery of "*O*-GlcNAc."

Honor to whom honor is due, it is to Gerald W. Hart, the discoverer of *O*-GlcNAc (3), that was assigned the task of writing a historical "Perspective" (4) as an introduction to this "Research Topic."

Protein *O*-GlcNAcylation levels in cells, resulting from the opposing actions of OGT and OGA, are tightly regulated. Most people working in the field have experienced the now commonplace observation that manipulating cellular *O*-GlcNAc levels using drugs, siRNA or cDNA transfection results in counter-regulatory modification in OGT and OGA expression. However, no study had been specifically dedicated to investigate this question. In an original paper by Zhang et al. (5) the effect of a potent and highly selective OGA inhibitor, Thiamet-G, on OGT and OGA mRNA and protein levels, was systematically studied in different cell types. The authors observed that OGA is more sensitive than OGT to *O*-GlcNAc levels. Increases in OGA expression were not due to stabilization of OGA mRNA or protein, suggesting regulation of OGA mRNA via transcription, through as yet unknown mechanisms.

O-GlcNAcylation is generally presented as a glycosylation that occurs only in the cytosol, the nucleus, and to a lesser extent, in mitochondria, in contrast to "classical" and complex *N*- and *O*-glycosylations that take place in the endoplasmic *reticulum* and the Golgi apparatus, and that modify transmembrane, secreted and organelle-confined proteins. However, biology is often made of exceptions to rules, and *O*-GlcNAcylation of protein extracellular domains has been demonstrated in *Drosophila* (6). In this Research Topic, Nagnan-Le Meillour et al. (7) provide original data indicating that olfactory binding proteins (OBPs) secreted in pig nasal mucus are also modified by *O*-GlcNAc. They identified and cloned a conserved eOGT (EGF domain-specific OGT) in *Sus scrofa* and proposed that *O*-GlcNAcylation of OBPs could finely modulate their binding specificities for odors and pheromones.

Increased *O*-GlcNAcylation is involved in insulin resistance associated with diabetes and obesity (2). The adipose cell plays a central role in the regulation of energy homeostasis, in particular, through its capacity to secrete adipokines that modulate insulin sensitivity and pro-inflammatory cytokines. Wollaston-Hayden et al. (8) show that *O*-GlcNAc modulates the transcript levels of multiple secreted proteins in rodent adipocytes, and propose that *O*-GlcNAcylation of transcription factors such as Sp1 plays a role in adipokines gene transcription during insulin resistance.

Whereas OGT or OGA knock down is lethal in higher eukaryotes, *ogt1* and *oga1* null *C. elegans* are viable. Taking advantage of this model organism, Ghosh et al. (9) investigated the consequences of OGT or OGA ablation, and showed that disruption in *O*-GlcNAc cycling alters nucleotide sugar production, overall glycan composition and transcription of genes encoding key members of the HBP pathway.

Although more than 1000 proteins are already known targets for *O*-GlcNAcylation, it is likely that numerous *O*-GlcNAcylated

proteins remain to be identified. In addition, one of the fascinating features of *O*-GlcNAc is its complex interplay with phosphorylation (1), either through regulation of phosphorylation at adjacent sites or by direct competition between *O*-GlcNAcylation and phosphorylation for the same site (the so-called Yin–Yang mechanism). In a Methods article, Cieniewski-Bernard et al. (10) describe the development of a multiplex, fluorescence-based proteomic strategy that permits to detect *O*-GlcNAcylated proteins, phosphoproteins, and the whole proteome on the same bi-dimensional gel.

This Research Topic also includes a number of reviews on some of the important biological and pathophysiological questions linked to *O*-GlcNAcylation. The perturbation of the *O*-GlcNAc cycle recently appeared as a hallmark of cancer cells (11). Jóźwiak et al. (12) review the role of *O*-GlcNAc in metabolic reprograming of cancer cells, through modification of metabolic enzymes, signaling proteins, and transcription factors, and Chaiyawat et al. (13) discuss these alterations specifically in breast and colorectal cancers. Epigenetic alterations also characterize numerous tumors, and recent data reviewed by Dehennaut et al. (14) reveal the involvement of *O*-GlcNAcylation as an epigenetic mark, and its role in chromatin remodeling and DNA methylation.

Numerous studies have provided evidence that *O*-GlcNAc negatively regulates insulin signaling (2), highlighting a link between hyperglycemia, insulin resistance, and glucotoxicity. Zhang et al. (15) review the implication of *O*-GlcNAcylation of signaling components and transcription factors in normal liver metabolism and in liver diseases, including insulin resistance, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis.

In a mini review, Benahmed et al. (16) also discuss the role of transcription factors in the control of energy metabolism, and more specifically the antagonistic relationships between ChREBP, which controls the expression of glycolytic and lipogenic genes, and the nuclear receptor FXR, which controls bile acid metabolism involved in gut–liver homeostasis. Interestingly, both transcription factors are modified by *O*-GlcNAcylation, although the consequences of this modification on ChREBP–FXR interaction remain to be explored.

Finally, as several lines of evidence indicate that *O*-GlcNAcylation regulates immune processes and may participate in hyperglycemia-associated inflammation, Baudoin and Issad (17) review the pro- and anti-inflammatory effects of *O*-GlcNAc, which may appear contradictory depending on cell types and pathophysiological situations. The field reviewed by these authors illustrates the complexity of signaling pathway regulation by *O*-GlcNAcylation. The control of inflammatory processes by *O*-GlcNAcylation is one of the innumerable *Terra incognita* to be explored.

Although not all aspects of *O*-GlcNAc biology could be presented in the Research Topic, we hope that it will excite the curiosity and stimulate the interest of young scientists for this ever-expanding, fascinating field.

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Three decades of research on O-GlcNAcylation – a major nutrient sensor that regulates signaling, transcription and cellular metabolism

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Even though the dynamic modification of polypeptides by the monosaccharide, O-linked N-acetylglucosamine (O-GlcNAcylation) was discovered over 30 years ago, its physiological significance as a major nutrient sensor that regulates myriad cellular processes has only recently been more widely appreciated. O-GlcNAcylation, either on its own or by its interplay with other post-translational modifications, such as phosphorylation, ubiquitination, and others, modulates the activities of signaling proteins, regulates most components of the transcription machinery, affects cell cycle progression and regulates the targeting/turnover or functions of myriad other regulatory proteins, in response to nutrients. Acute increases in O-GlcNAcylation protect cells from stress-induced injury, while chronic deregulation of *O*-GlcNAc cycling contributes to the etiology of major human diseases of aging, such as diabetes, cancer, and neurodegeneration. Recent advances in tools to study O-GlcNAcylation at the individual site level and specific inhibitors of *O*-GlcNAc cycling have allowed more rapid progress toward elucidating the specific functions of O-GlcNAcylation in essential cellular processes.

Keywords: O-GlcNAcylation, O-GlcNAc transferase, O-GlcNAcase, signaling, transcription, diabetes, cancer, Alzheimer's disease

EARLY HISTORY

O-GlcNAcylation was discovered in the early 1980s when bovine milk galactosyltransferase was used as an enzymatic probe of terminal *N*-acetylglucosamine moieties in cells of the murine immune system (1). Later studies established *O*-GlcNAc's surprising nucleocytoplasmic subcellular localization and distribution at a time when dogma stated that protein glycosylation only occurs within the secretory pathway or extracellular compartments (2). O-GlcNAcylation was shown to be highly abundant within the nucleus and particularly enriched at the nuclear envelope and on nuclear pore proteins (3–5). However, O-GlcNAcylation was also found to be abundant on cytoskeletal proteins of human erythrocytes, which lack a nucleus (6). Viruses were also found to contain O-GlcNAcylated proteins, which occur on proteins surrounding their nucleic acid cores, rather than on their capsids, where other forms of "classical" protein glycosylation are found (7). O-GlcNAcylation was subsequently found to be highly enriched on proteins associated with chromatin in *Drosophila* (8), and *O*-GlcNAc was shown to not only be a major modifier of transcription factors (9), but also a major modification of the C-terminal domain (CTD) of RNA polymerase II itself (10). Early studies in lymphocytes showed that cellular activation resulted in rapid changes, suggesting that *O*-GlcNAc cycled like phosphorylation and could be a regulatory modification (11), which was later confirmed by the sugar's rapid cycling on small heat shock proteins, shown by classical pulse-chase analyses (12). An assay for *O*-GlcNAc transferase (OGT), based upon tritiated UDP-GlcNAc as the donor and synthetic peptide acceptors, was developed and OGT activity was identified and characterized (13). OGT was subsequently purified to apparent homogeneity by brute-force biochemical approaches combined with nucleotide affinity chromatography (14). O-GlcNAcase was originally purified from rat spleen cytosol (15) and was found to be similar to hexosaminidase C (16, 17), which was known but had not been purified to homogeneity. Based upon polypeptide sequencing, in conjunction with PCR cloning, the *OGT* cDNA from rat (18), *C. elegans* and human (19) were cloned. OGT was found to be a very highly conserved protein with no homology to other known glycosyltransferases. OGT was also found to have two distinct domains, a catalytic domain and a protein–protein interaction domain consisting of over 11 tetratricopeptide (TPR) repeats separated by a linker region. Likewise, O-GlcNAcase was purified from bovine brain and the protein was sequenced by mass spectrometry, and used to clone the enzyme from a human library (20). The *OGA* gene was found to be identical to *MGEA5* a putative hyaluronidase associated with meningioma (21). Early studies identified *O*-GlcNAc on nuclear receptors, tau protein in the brain, intermediate filament proteins, nuclear oncogenes and tumor suppressors, and many other proteins with a wide-range of functions [reviewed in Ref. (22)].

MORE RECENT FINDINGS

As the tools for the detection and analysis of O-GlcNAcylation improved, it became apparent that this post-translational modification is much more abundant than previously expected [reviewed in Ref. (23, 24)]. In addition, it was soon realized that not only was

the donor for O-GlcNAcylation, UDP-GlcNAc, a major node of metabolism, but also that *O*-GlcNAc has extensive interplay with protein phosphorylation [reviewed in Ref. (23)]. Gene deletion studies have shown that both OGT and O-GlcNAcase are essential genes in mammals and plants (25–27).

Like phosphorylation and ubquitination, O-GlcNAcylation regulates many different cellular processes. O-GlcNAcylation is essential in the process of lymphocyte activation in both B- and T-lymphocytes (28). There are several examples where the glycan regulates protein:protein interactions [e.g., Ref. (29, 30)]. Nutrients fine-tune circadian clocks via O-GlcNAcylation (31–34). *O*-GlcNAc modulates the activity of the proteasome (35–39), and also has interplay with ubquitination (40, 41). Recent studies indicate that O-GlcNAcylation is very important to neuronal and brain functions, including synaptic plasticity, synaptic vesicle trafficking, and axonal branching (42–46). O-GlcNAcylation also regulates growth hormone signaling in plants (27), protects cells from acute stresses (47), and modulates transition through the cell cycle (48). Even though O-GlcNAcylation has not yet been documented to occur in yeast, such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, *O*-GlcNAc does occur in some of oldest known eukaryotes (49), including in some important human parasites (50–52). In certain bacteria, O-GlcNAcylation regulates flagellar motility (53), and in *Streptococcus pneumonia*, O-GlcNAcylation of an adhesion plays a role in infection and pathogenesis (54). However, the bacterial OGT involved in each case is quite different from the eukaryotic enzyme.

As a key nutrient sensor, O-GlcNAcylation is fundamentally important to the regulation of transcription at nearly all levels, including regulation of the functions of RNA polymerase II itself (55, 56), modulating the activities of nearly all transcription factors (30), regulating both histone and DNA methylation (57–61), crosstalking with other epigenetic modifications (62), and serving as an integral part of the histone code (63). Not only does O-GlcNAcylation have extensive crosstalk with protein phosphorylation at the protein site level but also the sugar modifies many kinases and regulates their activities or specificity (64–69).

Given the myriad functions associated with O-GlcNAcylation, it is not surprising that this nutrient sensor plays a fundamental role in the etiology of diabetes and glucose toxicity (70–72). O-GlcNAcylation is elevated in all cancers studied to date and appears to play a role in tumor cell progression (24, 73, 74), and in patient prognosis (75). Given O-GlcNAcylation's abundance and presence on hundreds of proteins in the brain, it is also a major mechanism contributing to neurodegeneration (76–78). After 30-years of research on O-GlcNAcylation, it is now not only more apparent than ever that this post-translation modification plays a central role in the nutrient regulation of cellular physiology but also it is clear that we have a long way to go to fully understand the importance of O-GlcNAcylation in most cellular and disease processes.

FUTURE DIRECTIONS

Many important questions remain with respect to O-GlcNAcylation. (1) How does *O*-GlcNAc cycling achieve substrate specificity with only two known genes in mammals, *OGT* and *OGA (MGEA5)*? Clearly, several different mechanisms are involved. *In vitro*, OGT has remarkable specificity for peptide substrates, which appears to change with UDP-GlcNAc concentrations (79). Most importantly, both enzymes function as part of transient holoenzyme complexes, which number in the hundreds, are cell type specific and serve to target the enzymes to their specific substrates. A key question is how is the formation of these holoenzyme complexes regulated by nutrients and other signals? (2) How are kinases regulated by O-GlcNAcylation? Many kinases are dynamically O-GlcNAcylated, and thus far, those studied are regulated by the glycan. How does this observation alter our view of signaling and system biological studies of cellular physiology? (3) How does O-GlcNAcylation play a role in neuronal functions and in learning and memory? O-GlcNAcylation is incredibly abundant in the mammalian brain, and in neurons, particularly at the synapse and in dendritic spines (42, 45, 80). Elucidation of *O*-GlcNAc's roles in normal neuronal functions and in brain biology will become a huge area of future research. (4) What are the specific roles of O-GlcNAcylation in nutrient regulation of transcription? While it is now clear that O-GlcNAcylation is fundamentally important in nearly every aspect of transcription, we currently know almost nothing with respect to its protein-specific or site-specific roles on individual transcription regulatory proteins. This area will remain an enormous challenge for some time to come.

Finally, while the tools to study O-GlcNAcylation have advanced substantially in the last three decades, there remains an acute need to develop better methods and approaches that can be applied by biologists. These include: (1) The development of many site-specific *O*-GlcNAc antibodies; (2) A molecular biology approach to either mimic O-GlcNAcylation or to generate site-specific O-GlcNAcylation on proteins; (3) Methods are need that can raise or lower O-GlcNAcylation on individual proteins or at individual sites to evaluate functions. Unfortunately, current methods either based upon inhibitors or genetic approaches to alter O-GlcNAcylation, all act globally. (5) There continues to be a need for better methods to both detect and site-map *O*-GlcNAc on proteins. The challenges in this field are large but so is the pay off for our understanding of cellular physiology and chronic disease.

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O-GlcNAcase expression is sensitive to changes in *O*-GlcNAc homeostasis

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O-linked *N*-acetylglucosamine (*O*-GlcNAc) is a post-translational modification involving an attachment of a single β-*N*-acetylglucosamine moiety to serine or threonine residues in nuclear and cytoplasmic proteins. Cellular *O*-GlcNAc levels are regulated by two enzymes: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which add and remove the modification, respectively. The levels of *O*-GlcNAc can rapidly change in response to fluctuations in the extracellular environment; however, *O*-GlcNAcylation returns to a baseline level quickly after stimulus removal. This process termed *O*-GlcNAc homeostasis appears to be critical to the regulation of many cellular functions including cell cycle progress, stress response, and gene transcription. Disruptions in *O*-GlcNAc homeostasis are proposed to lead to the development of diseases, such as cancer, diabetes, and Alzheimer's disease. *O*-GlcNAc homeostasis is correlated with the expression of OGT and OGA. We reason that alterations in *O*-GlcNAc levels affect OGA and OGT transcription. We treated several human cell lines with Thiamet-G (TMG, an OGA inhibitor) to increase overall *O*-GlcNAc levels resulting in decreased OGT protein expression and increased OGA protein expression. OGT transcript levels slightly declined with TMG treatment, but OGA transcript levels were significantly increased. Pretreating cells with protein translation inhibitor cycloheximide did not stabilize OGT or OGA protein expression in the presence of TMG; nor did TMG stabilize OGT and OGA mRNA levels when cells were treated with RNA transcription inhibitor actinomycin D. Finally, we performed RNA Polymerase II chromatin immunoprecipitation at the OGA promoter and found that RNA Pol II occupancy at the transcription start site was lower after prolonged TMG treatment. Together, these data suggest that OGA transcription was sensitive to changes in *O*-GlcNAc homeostasis and was potentially regulated by *O*-GlcNAc.

Keywords: *O*-GlcNAc, *O*-GlcNAc transferase, *O*-GlcNAcase, post-translational modification, transcription

INTRODUCTION

O-linked *N*-acetylglucosamine (*O*-GlcNAc) is a post-translational modification (PTM) first discovered by Gerald W. Hart and Carmen-Rosa Torres in 1984 (1). They initially used bovine milk galactosyltransferase (GalT1) to probe for terminal *N*-acetylglucosamine glycoconjugates on T-cells and unexpectedly discovered the existence of single β-*N*-acetylglucosamine conjugated proteins inside the cell (1). *O*-GlcNAc is a reversible modification that is ubiquitiously expressed in higher eukaryotes. *O*-GlcNAc transferase (OGT) is the enzyme that adds the *O*-GlcNAc modification, whereas *O*-GlcNAcase (OGA) removes it (2, 3). Because uridine diphosphate-*N*-acetyl-glucosamine (UDP-GlcNAc), the end point of the hexosamine biosynthetic pathway, is the high-energy donor substrate for OGT, *O*-GlcNAcylation is sensitive to nutrient availability (4). Furthermore, the removal and addition of *O*-GlcNAc termed *O*-GlcNAc cycling is highly dynamic. Changes in hormones, nutrients, or the environment cause within minutes to several hours changes to the total level of *O*-GlcNAc on proteins (5–7). Importantly, *O*-GlcNAc cycling rates affect transcription regulatory pathways, cell cycle progression, and respiration (8–12).

Since *O*-GlcNAcylation plays a significant role in regulating a wide panel of cellular processes, and aberrant *O*-GlcNAcylation contributes to the development of diseases, understanding the regulation of OGT and OGA is, therefore, important. Several studies report that the expression of OGT and OGA is sensitive to changes in total cellular *O*-GlcNAc levels (13, 14). Elevation of *O*-GlcNAc levels via pharmacological inhibition of OGA causes OGT protein expression to decrease and OGA protein expression to increase (13). A rapid decrease in OGA protein expression occurs in mice embryonic fibroblasts when OGT is knocked out (14). Cells appear to actively keep a specific level of *O*-GlcNAcylation suggesting a certain homeostatic level of *O*-GlcNAc must be maintained for optimal cellular function. Although alterations of cellular *O*-GlcNAc levels affect OGT and OGA expression, the exact mechanism as to explain this phenomenon is still unclear.

An imbalance in the homeostasis of *O*-GlcNAc does contribute to the development of diseases including cancer, diabetes, and Alzheimer's (15–18).

To further address how cells adjust OGT and OGA protein expression in response to alterations in *O*-GlcNAc levels, we measured in different cell lines OGT and OGA protein and mRNA expression and stability after pharmacologically inhibition of OGA by Thiamet-G (TMG, an OGA inhibitor). In these experiments, we were able to show that the OGA mRNA levels were more sensitive compared to OGT to alterations in *O*-GlcNAc, and RNA Pol II occupancy at the OGA transcription start site (TSS) was decreased after prolonged TMG treatments. Altogether, our data show that the protein expression of OGT and OGA is sensitive to changes in *O*-GlcNAc levels, and OGA transcription is sensitive to alterations in *O*-GlcNAc homeostasis.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

All primary and secondary antibodies used for immunoblotting were used at a 1:1,000 and 1:10,000 dilution, respectively. Anti-O-linked *N*-acetylglucosamine antibody [RL2] (ab2739) was purchased from Abcam. Antibodies for OGT (AL-34) and OGA (345) were gracious gifts from the Laboratory of Gerald Hart in the Department of Biological Chemistry at the Johns Hopkins University School of Medicine. Actin (A2066) antibody and anti-chicken IgY HRP (A9046) were purchased from Sigma. Chromatin immunoprecipitation (ChIP) grade mouse (G3A1) mAb IgG1 isotype control (5415) and RNA polymerase II antibody, clone CTD4H8 (05-623) were purchased from Cell Signaling Technologies and Millipore, respectively. Anti-rabbit HRP (170-6515) and anti-mouse HRP (170-6516) were purchased from Bio-Rad.

All reagents were purchased form Sigma unless otherwise noted. Cycloheximide (CHX, C7698, Sigma) was used at 50 μg/ml for HeLa cells and 25 μg/ml for K562 cells (19, 20). Actinomycin D (AMD, A1410, Sigma) was used at 0.5 μg/ml for HeLa cells and 5 μg/ml for K562 cells (20, 21).

CELL CULTURE

HeLa cells and SH-SY5Y neuroblastoma cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gemini) and 1% penicillin/streptomycin (Invitrogen). K562 cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum, 1× MEM non-essential amino acids solution (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2.5 mM HEPES, and 1% penicillin/streptomycin. Cells were treated with 10 μM Thiamet-G (TMG, S.D. Specialty Chemicals) for 6, 8, 24, or 48 h with fresh TMG supplied daily. Cells were also pretreated with CHX for 4 h, followed by TMG treatment for 8 h or AMD for 0.5 h, followed by TMG treatment for 6 h. Cells were infected with OGT, OGA, or green fluorescent protein (GFP) virus at a multiplicity of infection (MOI) of 75 for 24 h. After different treatments, cells were harvested for western blot, quantitative PCR (qPCR), or ChIP assay.

IMMUNOBLOTTING

Cells were lysed on ice for 30 min in Non-idet P-40 (NP-40) Lysis Buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 40 mM GlcNAc, and 1% Non-idet P-40) with 1 mM PMSF, 1 mM sodium fluoride, 1 mM β-glycerol phosphate, and 1× protease inhibitor cocktail I (leupeptin 1 mg/ml, antipain 1 mg/ml, benzamidine 10 mg/ml, and 0.1% aprotinin). Cell lysates were mixed with 4× protein solubility mixture (100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 8% SDS, 50% sucrose, 5% β-mercaptoethanol, 0.08% Pyronin-Y). All electrophoresis was performed with 4–15% gradient polyacrylamide gels (Criterion Gels, Bio-Rad) and separated at 120 V, followed by transfer to PVDF membrane (Immobilon, Millipore) at 0.4 A. Blots were blocked by 3% BSA in TBST (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) at room temperature for 20 min, then incubated with primary antibody at 4°C overnight. After washing with TBST for 5 × 5 min, blots were incubated with HRP-conjugated secondary for 1 h at room temperature, then washed with TBST again and developed using chemiluminescent substrate (HyGlo E2400; Denville Scientific). Blots were stripped in 200 mM glycine, pH 2.5 at room temperature for 1 h and probed with different primary antibodies. All immunoblotting results were repeated in three independent experiments (9). OGA and OGT relative protein levels were measured by analyzing the bands density using ImageJ 1.48 (http://imagej.nih.gov/ij/download.html) then normalized to the density of actin. All experiments were repeated three times, and average relative fold changes were calculated.

TOTAL RNA ISOLATION AND RT-PCR

Total RNA was isolated by TRI reagent solution (AM9738, Ambion) according to manufacture's instruction. Briefly, 2×10^6 cells were resuspended by 1 mL TRI reagent solution. Then, 200 μl of chloroform was added to extract RNA. After spinning down, upper phase containing total RNA was collected and incubated with equal amount isopropanol. RNA pellets were then precipitated by centrifugation, washed once with 70% ethanol, air-dried, and dissolved in nuclease free water (Invitrogen).

RNA concentration was measured by Nanodrop 2000c (Thermo) and 1 μg of total RNA was used for reverse transcription (RT) using iScript Reverse Transcription Supermix (170-8841, Bio-Rad) following manufacturer's instruction. In all, 10 μl of each completed reaction mix was incubated in a thermal cycler (Model 2720, Applied Biosystems) using the following protocol: priming 5 min at 25°C, RT 30 min at 42°C, and RT inactivation 5 min at 85°C. cDNA products were diluted with 90 μl nuclease free water and analyzed by qPCR. All qPCR results were repeated in three independent experiments (22).

ChIP ASSAY

K562 cells were harvested by centrifugation at 1,000 g for 5 min and washed twice with 1× PBS. Cells were then crosslinked by 2 mM EGS (21565, Pierce) in PBS at room temperature for 30 min, followed by 1% formaldehyde (BP531-25, Fisher) for another 10 min. Crosslinking reaction was terminated by 125 mM glycine. Cell pellets were collected and lysed on ice for 30 min by cell lysis buffer (10 mM Tris-HCl, pH 8.1, 10 mM NaCl, 0.5% NP-40) with protease inhibitors. Chromatin was collected by spinning down, and the pellets were resuspended in cold nuclear lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, 25% glycerol) with

protease inhibitors. In total, 300 μl of nuclear lysis buffer was used to resuspend chromatin from 2×10^6 cells.

Chromatin DNA was sheared to the size of 100–300 bp by sonication (Model Q800R, Active Motif) with the following protocol: amplification 75%, pulse on 15 s, pulse off 45 s, temperature 3°C. 200 μl of sheared chromatin was diluted by adding 1 ml of ChIP buffer (20 mM Tris-HCl pH8.1, 1.2% Triton X-100, 1.2 mM EDTA, 20 mM NaCl) with protease inhibitors. 2 μg of control IgG and specific antibody were added to diluted chromatin respectively, followed by end to end rotation at 4°C overnight. At the same time, 12 μl of diluted chromatin was saved as input and processed later. Next day, 30 μl of PBS washed M-280 Sheep Anti-Mouse IgG Dynabeads (11204D, Invitrogen) was added to the chromatin, followed by rotating at 4°C for 4 h. Dynabeads were separated by DynaMag-2 Magnet (12321D, Invitrogen) and subsequently washed with 1 ml of the following buffer for 5 min at 4°C: wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), wash buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 300 mM NaCl), wash buffer 3 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), wash buffer 4 (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0), and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Complexes were eluted from beads with 500 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$, 40 mM Tris-HCl, pH 8.0, 10 mM EDTA) and added with 200 mM NaCl. Eluates and inputs were treated at the same time with RNase A (EN0531, Thermo) at 65°C overnight, followed by proteinase K (25530-031, Invitrogen) treatment for 2 h. DNA was extracted by phenol/chloroform/isoamyl alcohol (AC327111000, Fisher) and precipitated by glycogen (10814-010, Invitrogen) and ethanol (23). DNA pellets were air-dried, dissolved in 50 μl nuclease free water, and analyzed by qPCR.

qPCR ASSAY

cDNA or ChIP DNA samples were analyzed by qPCR using SsoAdvanced Universal SYBR Green Supermix (172-5271, Bio-Rad) according to manufacturer's instruction. Briefly, 2 μl of cDNA or 5 μl of ChIP DNA samples, SYBR green supermix, nuclease free water, and primers (**Table 1**) for the target gene were mixed with a total reaction volume of 20 μl. A 96-well PCR plate (AVRT-LP, Midsci) with the mixture was loaded to CFX96 Touch Real-Time PCR Detection System (185-5195, Bio-Rad) with the following protocol: polymerase activation and DNA denaturation 30 s at 95°C, amplification denaturation 5 s at 95°C and annealing 30 s at 60 or 62°C with 40 cycles, and melt curve 65–95°C with 0.5°C increment 5 s/step.

DATA ANALYSIS

Quantification cycle (Cq) value was recorded by CFX Manager™ software. For cDNA qPCR data, dynamic range of RT and amplification efficiency was evaluated before using ΔΔCq method to calculate relative gene expression change. For ChIP DNA qPCR data, Cq value was normalized to percentage of input. Data generated in three independent experiments was presented as means ± standard error and analyzed using two-tailed Student's *t*-test with $P < 0.05$ as significant difference.

Table 1 | Primer sequences used for qPCR.

Target gene	PrP primer sequence
OGT	Forward: CATCGAGAATATCAGGCAGGAG Reverse: CCTTCGACACTGGAAGTGTATAG
OGA	Forward: TTCACTGAAGGCTAATGGCTCCCG Reverse: ATGTCACAGGCTCCGACCAAGT
HPRT	Forward: ATTGGTGGAGATGATCTCTCAACTTT Reverse: GCCAGTGTCAATTATATCTTCCACAA
−1000 OGA TSS	Forward: TTGGGTCTCCTTGCTGTATG Reverse: ACCTCACAGGTTGAGATAGATTT
OGA TSS	Forward: GGGCTAGCCTATTAAGCTTCTTTA Reverse: AGGGTGGCAAGCAGAAAT
+2700 OGA TSS	Forward: TCCTTTCAGAGTTGCTCCAATA Reverse: CAGTCAACCGAAACCATGAAC

RESULTS

ALTERATION IN *O*-GLcNAc LEVELS CHANGES THE PROTEIN EXPRESSION OF OGT AND OGA

Previous reports demonstrated that different pharmacological inhibitors of OGA, PUGNAc and GlcNAc-thiazoline, rapidly increase the protein expression of OGA (13, 24). We explored this phenomenon using another highly selective inhibitor of OGA Thiamet-G (TMG) (25). We altered the *O*-GlcNAc levels of SH-SY5Y neuroblastoma, HeLa cervical carcinoma, and K562 leukemia cells with TMG and measured *O*-GlcNAc, OGT, and OGA levels at various time points up to 48 h of TMG treatment. The *O*-GlcNAc levels were increased in the TMG treated samples while the pattern of *O*-GlcNAcylation was unique to each of the three cells lines used. OGA protein expression increased while OGT protein expression decreased gradually in the prolonged TMG treatment time points (**Figures 1A–C**). Additionally, in SY5Y cells, we used adenoviral-mediated OGT or OGA infection to alter *O*-GlcNAc levels. GFP was used as a control for the adenoviral infection. Cells overexpressing OGT showed an elevation in *O*-GlcNAc levels and a slight increase in OGA protein expression compared to control, while cells overexpressing OGA showed a decrease in *O*-GlcNAc levels and a slight increase in OGT protein expression compared to control (**Figure 1D**).

TMG DOES NOT STABILIZE OGA PROTEIN EXPRESSION

In order to explore the reason why TMG increases OGA protein level, we asked the question does TMG increase OGA protein stability. We pretreated cells with CHX to inhibit protein translation (26). We treated HeLa (**Figure 2A**) and K562 (**Figure 2B**) cells with TMG and observed a robust increase in OGA protein level (**Figures 2A,B**, Lane 2) compared to control cells without any treatment (**Figures 2A,B**, Lane 1). When HeLa cells were treated with CHX, OGT protein levels dramatically decreased compared to control, and we did not observe much of a decrease in OGA protein expression (**Figure 2A**, Lane 3). However, both OGT and OGA protein levels were dramatically decreased after CHX treatment in K562 cells (**Figure 2B**, Lane 3) compared to control. Combination of CHX and TMG did not change the OGA or OGT protein levels

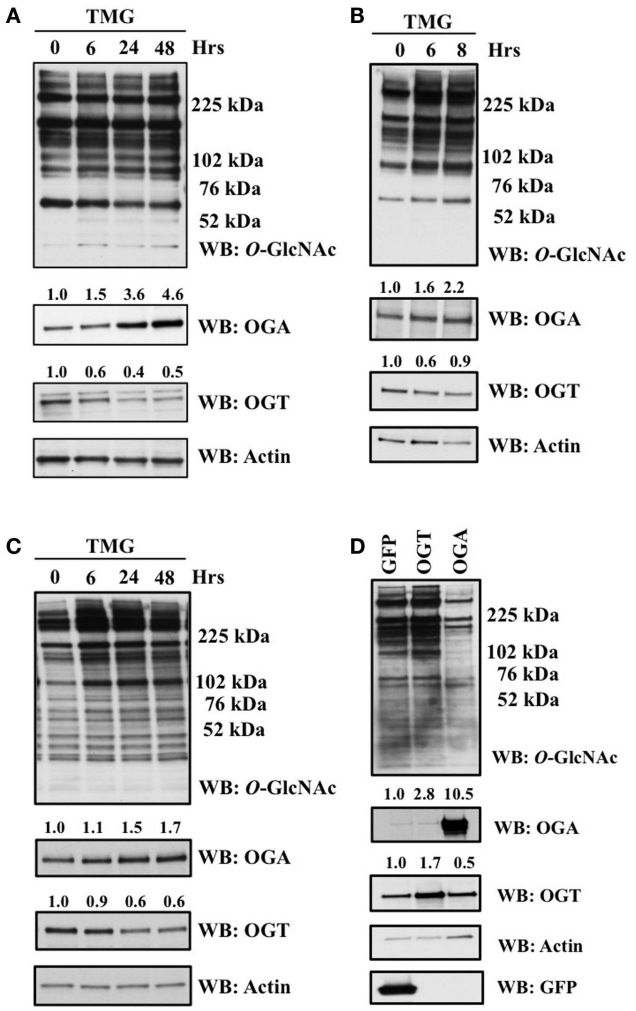

FIGURE 1 | OGA protein level was increased after TMG treatment. **(A)** SH-SY5Y neuroblastoma cells. **(B)** HeLa cervical cells. **(C)** K562 leukemia cells were treated with 10 μM TMG for indicated time. **(D)** SH-SY5Y cells were infected with GFP, OGT, and OGA adenovirus at 75 MOI for 24 h. Cells were lysed, overall *O*-GlcNAc level, OGA and OGT protein level were analyzed by western blot with actin as a loading control. Average fold change for OGT and OGA is listed on the blots.

(**Figures 2A,B**, Lane 4) compared to CHX treatment only suggesting that the TMG mediated increase in OGA protein expression was not due to increased stability of the protein.

OGA TRANSCRIPT LEVEL IS INCREASED AFTER TMG TREATMENT

Next, we investigated if OGT or OGA transcript level was altered after TMG treatment. We analyzed OGA mRNA level in SH-SY5Y (**Figure 3A**), HeLa (**Figure 3B**), and K562 (**Figure 3C**) cells. We found OGA mRNA level increased from 6 h TMG treatment in all three cell lines and was still elevated above control after 48 h TMG treatment (**Figures 3A,C**). The OGA mRNA level corresponded with the increase in protein level in **Figure 1**. However, the OGT mRNA level did not significantly change (**Figures 3D–F**). We also demonstrated that the corresponding OGA and OGT mRNA levels increased slightly but not significantly when OGT or OGA were overexpressed in SH-SY5Y cells (**Figures 3G,H**).

TMG DOES NOT STABILIZE OGA mRNA

Next, we asked the question whether increased OGA mRNA level after TMG treatment was due to stabilized OGA mRNA. AMD, a RNA synthesis inhibitor, was used to test OGA mRNA stability. TMG treated HeLa cells showed an increase of OGA mRNA level compared to control cells without any treatment (**Figure 4A**). When cells were treated with AMD, both OGA and OGT mRNA levels were dramatically decreased compared to control (**Figures 4A,B**). Combination of AMD and TMG did not change the OGA and OGT mRNA levels compared to AMD treatment only (**Figures 4A,B**). The same results were observed when using K562 cells (**Figures 4C,D**).

RNA POL II OCCUPANCY IS DECREASED AT OGA TSS AFTER 48 H TMG TREATMENT

We next investigated RNA Pol II occupancy at the OGA TSS via RNA Pol II ChIP. In control K562 cells, RNA Pol II was bound to OGA TSS with little binding upstream (−1000) or downstream (+2700) of the TSS. However, after 48 h TMG treatment, RNA Pol II occupancy was decreased at the OGA TSS compared to control cells (**Figure 5A**). Normal mouse IgG ChIP was used as a negative control (**Figure 5B**).

DISCUSSION

The production of UDP-GlcNAc, the substrate for OGT, integrates various metabolic substrates allowing the *O*-GlcNAc modification to act as a nutrient sensor (4, 27). Consequently, cells are sensitive to changes in *O*-GlcNAc levels due to nutritional and metabolic flux and will adjust cellular functions accordingly. Prolonged alterations in homeostatic levels of *O*-GlcNAc will cause the protein expression of OGT and OGA to change in an effort to restore *O*-GlcNAc homeostasis (4). Exactly how cells sense alterations to homeostatic levels of *O*-GlcNAc and adjust OGT and OGA expression to compensate for the changes in *O*-GlcNAcylation is unclear. For example, pharmacological inhibition of OGA rapidly increases cellular *O*-GlcNAc levels; however, the protein expression of OGA will also increase in response to the elevation of *O*-GlcNAc (13, 24). We sought to explore the mechanism as to how OGT and OGA protein expression changes in response to alterations in cellular *O*-GlcNAc levels. In agreement with previous reports, we found an increase in OGA protein expression as quickly as 8 h in HeLa cells and 24 h in K562 and SY5Y cells after treatment with TMG. OGT protein expression also decreased in these later time points (**Figure 1**). Due to the fact that increased levels of *O*-GlcNAc can increase the stability of proteins, such as p53 (28) and TET (ten-eleven translocation) (29), we postulated that increased *O*-GlcNAc could stabilize OGA. K562 or HeLa cells exposed to CHX in the presence of TMG showed no difference in the stability of either OGT or OGA (**Figure 2**) suggesting that the increase in OGA protein expression was not due to increased stability and more likely to an increase of OGA transcripts.

Importantly, decreased *O*-GlcNAc levels do not necessarily increase OGT levels in all cell types; for example, blocking GFAT (glutamine fructose-6-phosphate amidotransferase) activity with

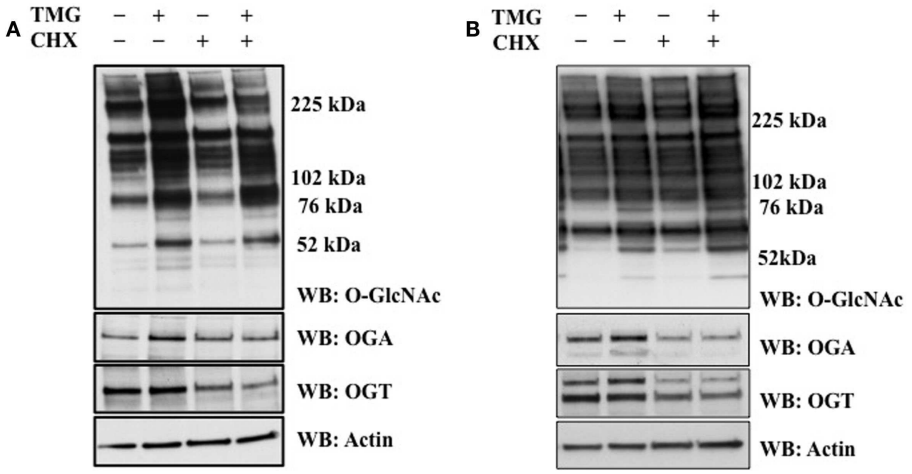

FIGURE 2 | TMG does not stabilize OGA protein. **(A)** HeLa cells and **(B)** K562 cells were treated with TMG, CHX (protein translation inhibitor), and CHX + TMG. Cells were lysed, overall *O*-GlcNAc level, OGA and OGT protein level were analyzed by western blot, with actin as loading control.

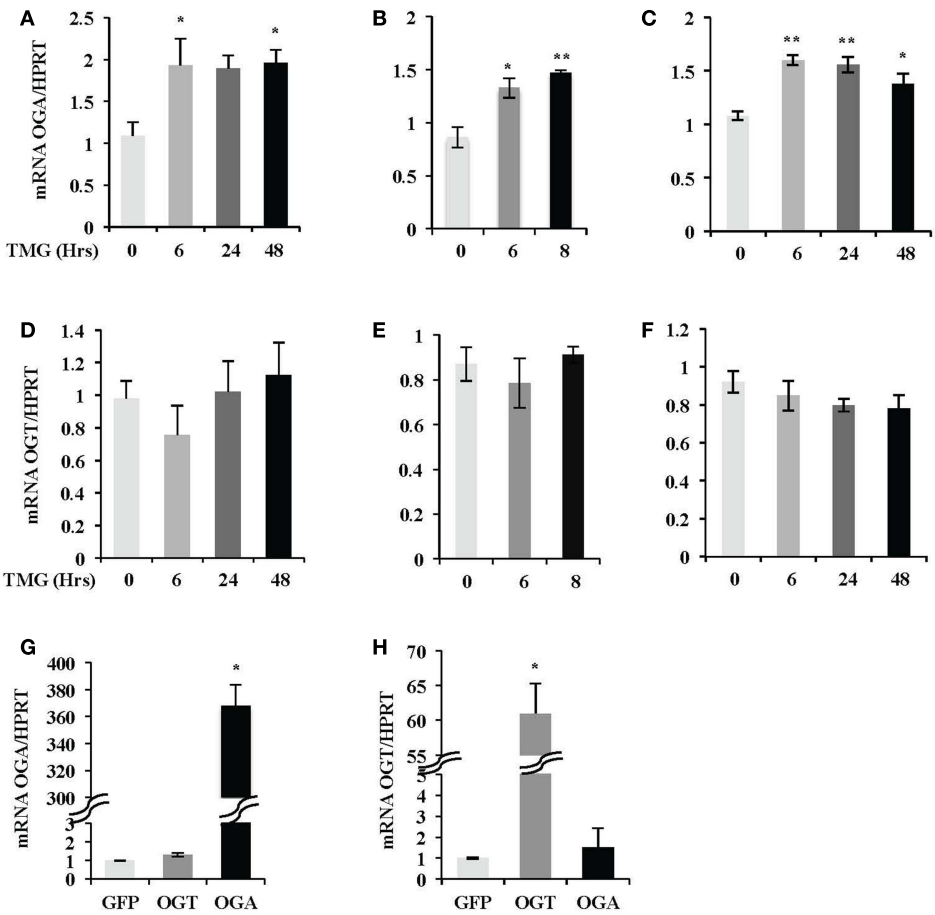

FIGURE 3 | OGA mRNA level was increased after TMG treatment. After TMG treatment, relative OGA mRNA level in **(A)** SH-SY5Y, **(B)** HeLa, and **(C)** K562 cells, as well as OGT mRNA level in **(D)** SH-SY5Y, **(E)** HeLa, and **(F)** K562 cells was analyzed by qPCR. **(G)** OGA mRNA level and **(H)** OGT mRNA level in SH-SY5Y cells infected with GFP, OGT, and OGA adenovirus at 75 MOI for 24 h, respectively, were also analyzed by qPCR. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was served as internal control. $*P < 0.05$. $**P < 0.01$, compared with control (TMG 0 h or GFP), $n = 3$, Student's *t*-test.

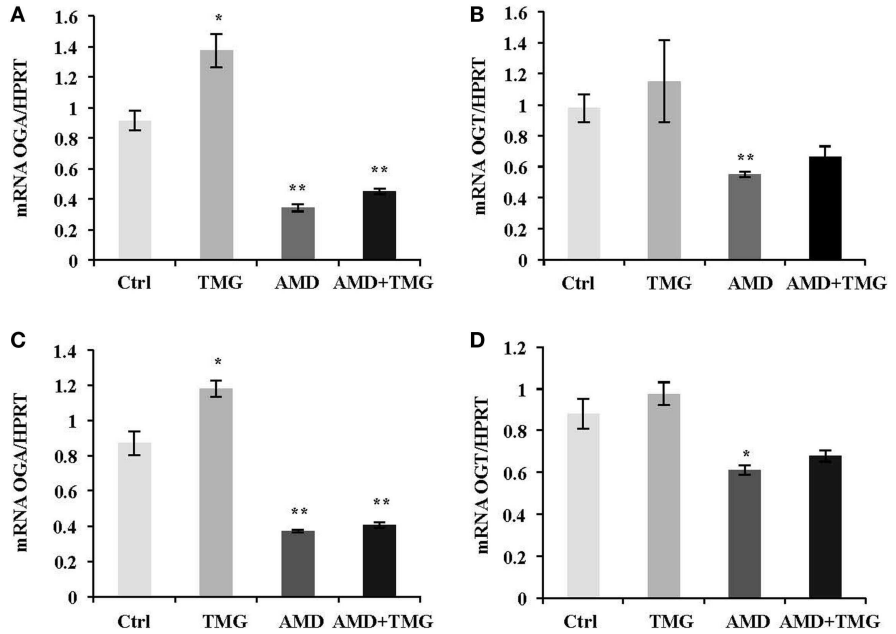

FIGURE 4 | TMG does not stabilize OGA mRNA. HeLa cells **(A,B)** and K562 cells **(C,D)** were treated with TMG, AMD (RNA transcription inhibitor), and AMD + TMG, respectively. **(A)** OGA **(A,C)** and OGT **(B,D)** mRNA level were analyzed by qPCR, with HPRT as internal control. $*P < 0.05$. $**P < 0.01$, compared with control, $n = 3$, Student's *t*-test.

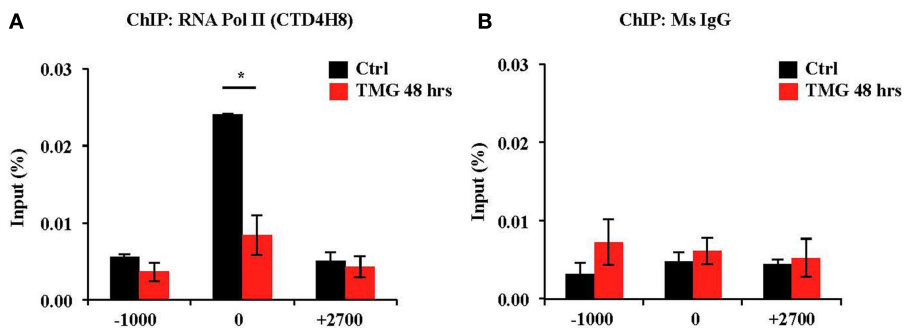

FIGURE 5 | RNA Pol II occupancy at OGA TSS was decreased after 48 h TMG treatment in K562 cells. **(A)** RNA Pol II ChIP assay was performed on control and 48 h TMG treated cells. ChIP DNA was analyzed by qPCR using a set of primer targeting 1000 bp upstream of OGA TSS (−1000), OGA TSS (0), and +700 bp downstream of OGA TSS (+2700). $*P < 0.05$, $n = 3$, Student's *t*-test. **(B)** Normal mouse IgG ChIP served as a negative control.

6-diazo-5-oxo-L-norleucine (DON) in HeLa cells lowered *O*-GlcNAc levels but did not increase OGT protein expression (13). On the other hand, OGA protein levels quickly decreased after *Cre*-mediated knockout of OGT in mouse embryonic fibroblasts (14), but OGA knockdown in colon cancer cells did not significantly decrease OGT protein expression (30). Changes in OGA protein expression appear more sensitive to changes in *O*-GlcNAc than OGT in HeLa cells, while both OGT and OGA expression significantly changed in SY5Y cells (**Figure 1**). Overexpression of OGA did not substantially influence OGT protein expression (**Figure 1**), and OGT overexpression did not change OGA expression (**Figure 1**). Recently, the development of a selective OGT inhibitor allowed for a dramatic reduction in cellular *O*-GlcNAcylation (31), which in turn caused OGA protein expression to rapidly decrease with only a minimal increase in OGT protein expression (31). The dynamic change in OGA protein expression was seen in the development of disease as well. In red blood cells of prediabetic individuals, OGA expression was significantly increased (32), and OGA protein levels correlated with increased blood glucose in these prediabetic patients. These data suggest that higher blood glucose levels promote increased flux through the hexosamine biosynthetic pathway leading to elevated OGT activity, followed by OGA protein levels increasing to restore cellular *O*-GlcNAc homeostasis in erythrocyte precursor cells. Together, these data support the proposed hypothesis that if OGT acts as a nutrient sensor allowing for rapid changes in

O-GlcNAcylation due to alterations, the cellular concentration of UDP-GlcNAc (33), then OGA should be less sensitive to nutrient changes and more sensitive to changes in *O*-GlcNAcylation.

In order to respond to changes in *O*-GlcNAc levels, cells rapidly and dramatically alter the expression of OGA mRNA (**Figure 3**). In the case of OGT, we did not detect a significant change in OGT mRNA levels after TMG treatment. The rapid increase in OGA mRNA levels after TMG treatment would argue that either OGA transcripts were more stable or transcriptional activity at the OGA promoter was increasing. We tested transcript stability by inhibiting RNA polymerase II with AMD (21). Interestingly, OGA and OGT transcript levels were not more stable after TMG treatment in the presence of AMD (**Figure 4**) suggesting that the increase in OGA mRNA levels with TMG was due to an increase in OGA gene transcription.

Next, we performed ChIP at the OGA promoter with an antibody that recognized all forms of RNA Pol II (phosphorylated and non-phosphorylated forms). After 48 h of prolonged TMG treatment in K562 cells, total RNA Pol II at the promoter was decreased compared to the control samples (**Figure 5**). Potentially, an antibody directed against the actively transcribing phosphorylated C-terminal domain (CTD) of RNA Pol II might have demonstrated an increase in enrichment of the phosphorylated forms of RNA Pol II at the promoter while non-phosphorylated forms of RNA Pol II would be less associated with the promoter. Interestingly, RNA Pol II is *O*-GlcNAcylated on the CTD at the fourth position of the CTD repeat, which is between the two activating phosphorylations at serine two and serine five on the CTD, which is needed for transcriptional elongation (34). Both *O*-GlcNAcylation and phosphorylation appeared to be mutually exclusive suggesting a cycle of *O*-GlcNAcylation and phosphorylation on the CTD repeats (35). Several groups have suggested that OGT and OGA work together to promote gene transcription by organizing the RNA Pol II preinitiation complex (PIC) (11, 36). *O*-GlcNAcylation was shown to promote the formation of the PIC in an *in vitro* transcription assay system; however, OGA activity was required for full transcriptional activation suggesting that OGT modified RNA Pol II, which initiated the formation of the PIC, while OGA was then required to remove the *O*-GlcNAc on the stalled RNA Pol II allowing for phosphorylation and transcription elongation (11). We have yet to explore RNA Pol II occupancy at the OGA promoter after a short TMG treatment (for example 6 h), which might yield a different result and needs to be studied further. The mRNA levels of OGA in K562 cells did begin to decrease at the 48 h TMG treatment suggesting that the OGA promoter might become inactive after prolonged TMG treatment. Reciprocal binding of OGT and OGA at active gene promoters provides several interesting future questions into the nature of transcriptional regulation, and the control of both the OGT and OGA promoter might be regulated in this manner.

Many transcription factors are modified by *O*-GlcNAc (15) and likely alteration of the *O*-GlcNAcylation level of a transcription factor could mediate the change in OGA transcription. We used the predictive software TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) to identify potential transcription factor-binding sites in the first 1000 base pairs upstream of the OGA TSS (37). Among the transcription factor-binding sites in this sequence, GATA and MZF were the most predicted transcription factors. Due to the essential and ubiquitous expression of OGA (4), we anticipated that several housekeeping transcription factors might bind to this region, but we found only few of these. Interestingly, both GATA and MZF are important transcription factors regulating hemopoietic development (22, 38). Perhaps the increased in OGA expression in prediabetic red blood cells (32) was partially due to changes in either of these two proteins. *O*-GlcNAcylation changes might lead to alteration of GATA or MZF occupancy at the OGA promoter. Some GATA family members are modified by *O*-GlcNAc (39); thus, this presents an interesting avenue to explore in more detail.

Together, our data demonstrate that OGA protein and mRNA expression is sensitive to cellular levels of *O*-GlcNAc. Some disease states have OGA expression uncoupled from *O*-GlcNAc levels (40). In many different cancers, *O*-GlcNAc homeostasis appears to be disrupted with increased OGT protein expression and *O*-GlcNAc levels (41). Several pancreatic cancer cell lines have increased *O*-GlcNAc levels when compared to an immortalized control cell line; importantly, OGT protein expression was increased while OGA protein expression was decreased (40). The uncoupling of OGA expression to *O*-GlcNAc homeostasis could be an indicator of cancer progression and suggest that an increase of OGA protein expression would be beneficial therapeutically. Determining how *O*-GlcNAc regulates OGA expression and transcription will be crucial for understanding the biology of *O*-GlcNAcylation and how *O*-GlcNAc homeostasis is disrupted in disease.

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Proteomic analysis of pig (*Sus scrofa*) olfactory soluble proteome reveals *O*-linked-N-acetylglucosaminylation of secreted odorant-binding proteins

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The diversity of olfactory binding proteins (OBPs) is a key point to understand their role in molecular olfaction. Since only few different sequences were characterized in each mammalian species, they have been considered as passive carriers of odors and pheromones. We have explored the soluble proteome of pig nasal mucus, taking benefit of the powerful tools of proteomics. Combining two-dimensional electrophoresis, mass spectrometry, and western-blot with specific antibodies, our analyses revealed for the first time that the pig nasal mucus is mainly composed of secreted OBP isoforms, some of them being potentially modified by *O*-GlcNAcylation. An ortholog gene of the glycosyltransferase responsible of the *O*-GlcNAc linking on extracellular proteins in *Drosophila* and Mouse (EOGT) was amplified from tissues of pigs of different ages and sex. The sequence was used in a phylogenetic analysis, which evidenced conservation of EOGT in insect and mammalian models studied in molecular olfaction. Extracellular *O*-GlcNAcylation of secreted OBPs could finely modulate their binding specificities to odors and pheromones. This constitutes a new mechanism for extracellular signaling by OBPs, suggesting that they act as the first step of odor discrimination.

Keywords: olfaction, odorant-binding protein, extracellular *O*-linked-N-acetylglucosaminylation, olfactory secretome, *O*-GlcNAc transferase

INTRODUCTION

Olfaction is generally considered as a minor, primitive sense for human beings, and the study of this sense in mammals has been neglected. However, many odor-guided behaviors are involved in the establishment of biological functions: reproduction *via* the choice of mate partner, establishment of the mother–young bond, maintenance of the social hierarchy, and to a less extent choice of food. This dialog between partners of the same species is driven by pheromones. The sex pheromone of pig is one of the few characterized in mammals: a mixture of androstenol and androstenone (1) secreted in testis is transported by lipocalins in blood to the saliva. During sex behavior, the male produces high quantity of saliva that, when perceived by the female, evokes a typical posture called lordosis, meaning the male acceptation by the female (2). Besides the identification of pheromones, studies have focused for the two past decades on the molecular and cellular mechanisms involved in pheromone reception, starting with the discovery of a gene family encoding odorant receptors (3). A general scheme of olfactory coding hypothesized that pheromones are detected by sensory neurons of the vomeronasal organ (VNO), while other odors are detected by the main olfactory epithelium (MOE) sensory neurons [reviewed in Ref. (4, 5)]. There is a large body of evidence that the coding of olfactory signals is more complex. Some pheromone-mediated behaviors are still effective after VNO lesions (6–8). Conversely, mouse VNO neurons can be stimulated by odorants emitted by other species, such as floral and woody smelling compounds (9).

The reception of olfactory signals takes place in the nasal mucus. The biochemical players are olfactory receptors (ORs), olfactory binding proteins (OBPs), and odorant degrading enzymes, whose kinetic interactions are not fully understood. Among them, OBPs are the best characterized. They are small water-soluble proteins secreted in high quantity in the nasal mucus by Bowman's gland of the olfactory epithelium (10, 11). One major unresolved question in mammalian olfaction is the nature of the ligand of ORs. Two hypotheses have been proposed: (1) the ligand is the odorant molecule itself solubilized and transported to the receptor by OBPs. In this scheme, the binding between odorant molecules and OBPs is unspecific, which is supported by the small number of OBP genes in each animal species [reviewed in Ref. (12)]. OBPs are also assumed to concentrate odors and/or to scavenge them from receptors in a deactivation process (13). (2) The ligand is the complex formed by the specific binding between a given odorant molecule and a specific OBP. This hypothesis

involves a conformational change of the protein upon ligand binding, which confers an "activated" form to the complex, able to interact with a specific OR. Recently, it was shown that the complexes are internalized by the olfactory epithelium after activation of the receptors (14), supporting the hypothesis that OBP/odor complexes are the ligand of OR. Contrary to insects, where c. a. 30 OBP genes were identified in olfactory tissues (15, 16), no more than 3–4 OBP genes have been characterized in pig, rat, and human (17–19). As the few number of OBPs limits the possibility of a key-role in the coding of pheromones and odors, they have been considered as passive carriers in mammals (20).

However, the possibility of OBP diversity at the protein level has been evoked since the time of their discovery (17, 21–23). Recently, Stopkova et al. (24) identified eight OBP genes in mouse genome, suggesting a larger OBP diversity than previously described. In pig, we have demonstrated that post-translational modifications (PTM) generate OBP isoforms with specific binding properties, reinforcing the possibility of an active role of mammalian OBPs in pheromone and odor coding. Thus, we have demonstrated that two OBPs in pig, the OBP (*stricto sensu*) and the VEG (Von Ebner's Gland protein), can undergo phosphorylation, which generates several isoforms for each corresponding primary sequence (25). Moreover, we have shown that the binding specificity for pheromones is driven by phosphorylation (26) and/or *O*-GlcNAcylation for two VEG isoforms (27). This diversity of isoforms with different binding abilities rekindles the debate on the OBPs role in pheromone coding in the pig, and in mammals in general. Previous studies were performed in one-dimensional electrophoresis and did not allow a precise characterization of the pig OBP diversity. To go further in the characterization of both OBP isoforms and their PTM, in particular the *O*-GlcNAcylation unexpected for secreted proteins, we have explored the soluble proteome of pig nasal mucus, taking benefit of the powerful tools of proteomics. Combining two-dimensional electrophoresis (2-DE), mass spectrometry, and western-blot with specific antibodies, our analyses revealed for the first time that the pig nasal mucus is mainly composed of OBP isoforms, some of them being potentially modified by *O*-GlcNAcylation. As a glycosyltransferase (GT) responsible for *O*-GlcNAcylation of extracellular domains has been reported in *Drosophila* (28), we have searched for such a GT in the pig olfactory tissues. The encoding cDNA was cloned and the obtained sequence was used in a phylogenetic analysis to determine whether this modification could eventually occur in other model species used for the study of olfaction mechanisms.

MATERIALS AND METHODS

ANIMALS AND TISSUES

Animals (Large White *Sus scrofa*) were maintained at the Experimental Farm of INRA (UEPAO, Nouzilly, France). Individuals coming from the same offspring (brothers and sisters) were slaughtered in agreement with EU directives about animal welfare. Pigs were sacrificed by a licensed butcher in an official slaughterhouse (authorization No. A37801 E37-175-2 agreement UEPAO). Four animals of different physiological status were used in this study: a pre-pubertal male, a pubertal male, a pre-pubertal female, and a pubertal female. Respiratory mucosa (RM) and VNO were dissected immediately after death from each anesthetized animal and stored half in tubes at −80°C for protein extraction, and half in RNA*later* RNA Stabilization Reagent for RNA extraction (Qiagen).

PROTEIN EXTRACTION

The proteins were extracted from pig frozen tissues by phase partition using chloroform/methanol (v:v, 2:1) on ice. The resulting samples were centrifuged (15,000 *g* for 15 min at 4°C) and the methanol phase was collected then evaporated in a Speed-vac concentrator. Aliquots were tested by native-polyacrylamide gel electrophoresis as already described (29) in order to obtain a standard quantity of proteins for each tissue (1X corresponds to 1 microg/band). The relative quantification of protein bands was calculated by using Image J software. Dried samples were stored at −20°C.

TWO-DIMENSIONAL ELECTROPHORESIS

All chemicals and reagents were from Sigma-Aldrich. Dried proteins (100 μg) were solubilized in 150 μl of rehydration buffer [8 M Urea, 2 M Thiourea, 2% (w/v) CHAPS, 10 mM dithiothreitol (DTT), 1.2% (v/v) Immobilized pH Gradient (IPG) buffer (pH 3–5.6) (GE Healthcare), and bromophenol blue]. After vigorous shaking, proteins were loaded onto 7-cm IPG strip (pH 3–5.6, GE Healthcare) by overnight passive rehydration at room temperature. The first-dimensional isoelectric focusing (IEF) was carried out on a Protean IEF Cell (Bio-Rad) using the following program: 300 V for 30 min (rapid voltage ramping), 1000 V for 1 h (rapid voltage ramping), 5000 V for 2 h (rapid voltage ramping), and 500 V for 3 h (rapid voltage ramping), with a current limit at 50 μA/gel. When IEF was complete (10,000 VH final), strips were incubated twice for 15 min in the equilibration buffer [375 mM Tris-HCL pH 8.8, 6 M urea, 2% (w/v) SDS, and 30% (v/v) glycerol] containing 1.5% (w/v) DTT then 2% (w/v) iodoacetamide. The second dimension separation was performed using 16.8% SDS–PAGE in Mini PROTEAN Tetra Cell (Bio-Rad).

STAINING AND WESTERN-BLOT AFTER 2-DE

After 2-D electrophoresis, gels were either stained with colloidal Coomassie blue R solution (12% trichloroacetic acid, 5% ethanolic solution of 0.035% Serva blue R 250), or transferred onto nitrocellulose (Hybond C-Extra, GE Healthcare) or PVDF (Immobilon P, Millipore) membranes. For immunodetection, membranes were blocked in 5% (w/v) non-fat dry milk in Tris-Buffered Saline-0.05% Tween-20 (TBS-T) for probing with polyclonal antibodies (anti-OBP, anti-SAL, and anti-VEG) and 3% Bovine Serum Albumin (BSA, Sigma-Aldrich) in TBS-T for probing with monoclonal anti-*O*-GlcNAc antibodies. Membranes were then incubated with antibodies in TBS-T 1 h at room temperature [RL2 (Thermofisher) 1:2,000; CTD 110.6 (Thermofisher), 1:2,000; anti-OBP, 1:30,000; anti-VEG, 1:5,000; and anti-SAL, 1:10,000]. After three washes in TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG-HRP linked, 1:30,000, Thermofisher, for RL2; anti-mouse IgM-HRP linked, 1:30,000, Thermofisher, for CTD 110.6; and anti-rabbit IgG-HRP linked, 1:30,000, Thermofisher for polyclonal antibodies) for 1 h at room temperature. After three washes in TBS-T, blots were developed using enhanced chemiluminescence (ECL Plus and ECL Prime Reagents, Hyperfilm™ MP, GE

Table 1 | Primers used in the RACE-PCR experiments for amplification of putative OBP and VEG splice variants.

	5′ RACE		3′ RACE	
	5′ Primer	**3′ Primer**	**5′ Primer**	**3′ Primer**
OBP	GeneRacer™ 5′ primer	3′ OBP primer: TCACTTGGCAGGACAGTCATCTCT	5′OBP primer: CACCCAGGAACCTCAACCTGAACA	GeneRacer™ 3′ primer
VEG	GeneRacer™ 5′ primer	3′ VEG primer: CTAGTTCCCTCCTG-GAGAGCAGGTTTCGCTTT	5′ VEG primer: CAGGAGTTCCCGGCCGTGGGGCA	GeneRacer™ 3′ primer

The sequence of the GeneRacer primers is available at www.invitrogen.com. Gene specific 5′ primers correspond to the first codons coding for the N-terminal of protein sequence (without signal peptide) and gene specific 3′ primers to the last codons of the C-terminal of the protein sequence.

Healthcare). Coomassie blue stained gels and membranes were scanned on GS800 calibrated imaging Densitometer including the Quantity One® software for image acquisition and analysis (Bio-Rad, Marnes-La-Coquette). Images were merged using Image J® software.

MASS SPECTROMETRY ANALYSIS

The spots of interest were excised from the gels, destained and digested by Trypsin Gold Mass Spectrometry grade (Promega) overnight at 37°C as previously described (26). Protein identification was performed either by MALDI-TOF MS or by Nano-LC-ESI-MS/MS. MALDI-TOF MS analysis was performed on an Applied Biosystem, Voyager DE Pro MALDI-TOF. The instrument was used in reflector mode, measuring peptide masses on a range of 500–4,000 Da. Calibration points were based on the masses of the matrix cluster or trypsin autolysis products (m/z 842, m/z 2211). Protein identification was performed by comparison of measured peptide masses with the theoretical mass fingerprint of porcine OBP (GenBank accession number NP_998961), VEG (S77587), and SAL (P81608)[1] obtained with the following parameters: trypsin digestion with two missed cleavages allowed, cysteines in reduced form, acrylamide adducts, methionine oxidized (MSO), and monoisotopic peptide masses as $[M+H]^+$. Nano-LC-ESI-MS/MS was carried on a hybrid quadrupole time-of-flight mass spectrometer (Q-Star, Applied Biosystems) equipped with a nano-electrospray ion source coupled with a nano HPLC system (LC Packings Dionex). Database searching was performed using Mascot software (MS/MS ion search module) in the NCBInr database. Search parameters were as follow: other mammalians as taxonomy, 50 ppm tolerance for the parent ion mass and 50 amu for the MS/MS fragment ions, one missed cleavage allowed, carbamidomethylation of cysteine and methionine oxidation as possible modifications. Only candidate proteins with a significant Mascot score were taken into consideration (significance threshold for candidate <0.05 using MudPIT scoring method and an expectation value for ion peptides <0.05).

SEARCH FOR PUTATIVE OBP AND VEG SPLICE VARIANTS BY RAPID AMPLICATION OF cDNA ENDS-POLYMERASE CHAIN REACTION AND *IN SILICO* ANALYSIS

Total RNA was extracted from 30 mg of each tissue conserved in RNA*later* with RNeasy Fibrous tissue Mini Kit according to manufacturer's instructions (Qiagen, Courtaboeuf). Rapid amplication of cDNA ends-polymerase chain reaction (RACE-PCR) was performed with the GeneRacer™ kit according to manufacturer's instructions (Invitrogen). Capped mRNAs (for 5′ RACE) and native mRNAs (for 3′ RACE) were reverse transcribed into cDNA with the Sensiscript III RT kit (Invitrogen) and oligo dT primer. Amplification of cDNA coding for *OBP* and *VEG* putative variants was performed with 1 µl of each RT product, Platinum® Taq DNA polymerase High Fidelity (Invitrogen), and the set of primers described in **Table 1**, by touchdown PCR, as follows: 94°C for 2 min, followed by 5 cycles of 30 s at 94°C, 2 min at 72°C, 5 cycles of 30 s at 94°C, 2 min at 70°C, 25 cycles of 30 s at 94°C, 30 s at 65°C, 2 min at 68°C, and a final elongation of 10 min at 68°C. The PCR products were cloned into *pCR4®-TOPO* vector (Invitrogen). The recombinant plasmids were amplified into *Escherichia coli* One Shot Top 10 Chemically Competent cells (Invitrogen), then purified with the QIAprep Spin Miniprep kit (Qiagen, Courtaboeuf), and sequenced in both senses (GATC Biotech). In parallel, splicing variants were searched in the pig genome (Ensembl *Sus scrofa*) by performing Blastn analysis with mRNA *OBP*, *VEG*, and *SAL* sequences (NCBI Ref Seq: NM_213796, NM_213856, NM_213814, respectively).

AMPLIFICATION AND MOLECULAR CLONING OF *SUS SCROFA EOGT*

Messenger RNA obtained by total RNA extraction (see above) were reverse transcribed into cDNA with the Sensiscript III RT kit (Invitrogen) using a 3′-end gene specific primer (5′-TCACTGGGATCCCCGAGCTCATCATGTTTGTTCTT-3′) designed from the transcript ID ENSSSCT00000012589 (Ensembl database, *Sus scrofa* genome). PCR amplification of *EOGT* in each tissue was carried out on a thermal cycler (Bio-Rad) with 1 µl of RT product as template with Platinum® Taq DNA polymerase High Fidelity (Invitrogen), with primers (Sense 5′-ATGTTAATGTTGCTTGTCTTTGGAGCATTGCTT-3′ and antisense 5′-TTAGAGCTCATCATGTTTGTTCTTAAAAGGCCACTT-3′), according to manufacturer's instructions. Touchdown PCR was performed as described above. The single product obtained by amplification from adult male RM was cloned into *pCR® 4-TOPO* vector (Invitrogen). The recombinant plasmid was amplified and sequenced as described above.

SEQUENCE ANALYSIS AND PHYLOGENETIC RECONSTRUCTION

Sequences were retrieved by BLASTp searches of the NCBI non-redundant database using *S. scrofa* EOGT protein sequence

[1]http://expasy.org/tools/peptide-mass.html

(ENSSSCP00000012260) and *S. scrofa* OGT protein sequence (ENSSSCP00000013198) as a query (Data 1 in Supplementary Material). Only sequences that aligned the entire length with an *e*-value not higher than 10^{-6} were kept for multiple sequence alignment in order to keep as much informative sites as possible for phylogenetic reconstruction. Sequences of a same species that were 100% identical to one another or entirely included in a longer one were eliminated to remove redundancy. We performed phylogenetic analyses using two different approaches, Bayesian estimation and bootstrapped maximum likelihood (ML). The phylogenic reconstruction was performed with 48 sequences from model insect species (*Drosophila melanogaster*, *Bombyx mori*, *Anopheles gambiae*, *Aedes aegypti*, and *Apis mellifera*) as well as from other models or agronomical species (*Mus musculus*, *Rattus norvegicus*, *Danio rerio*, *Tetraodon nigroviridis*, *Takifugu rubripes*, *Xenopus laevis*, *Xenopus tropicalis*, *Bos taurus*, *Equus caballus*, *Sus scrofa*, *Gallus gallus*, and *Homo sapiens)*. Sequences were aligned using MUSCLE (30) with default parameters and the multiple sequence alignment was manually examined using JALVIEW (31, 32) (Data 2 in Supplementary Material). We performed phylogenetic analyses using ML estimation with the RAxML software (33). We systematically ran 100 bootstrap replicates followed by a ML search for the best-scoring tree. We chose WAG model of amino acids evolution because it returned the best posterior probability score in the corresponding Bayesian phylogenetic analysis (data not shown) done using MRBAYES software (34). We used a model with four categories of estimated gamma rates of evolution as well as an estimate of the proportion of invariable sites. The tree was generated using MEGA 5 (35) and is presented with bootstrap values. Each protein was also assessed for targeting to the extracellular space using two different prediction methods: WoLF PSORT (36) and SignalP 4.0 (37).

RESULTS

ANALYSIS OF THE PROFILE OF NASAL MUCUS SOLUBLE PROTEINS BY ONE-DIMENSIONAL NATIVE-PAGE

Soluble proteins were extracted from each tissue (RM or VNO) of each animal (pre-pubertal male, pre-pubertal female, pubertal male, and pubertal female). Proteins were separated by one-dimensional native-PAGE, in order to confirm the range of pH to be used further in 2-DE. The most abundant proteins are localized in the lower part of the gel (**Figure 1**) indicating an acidic pI, consistent with the theoretical values calculated from porcine OBPs primary sequences: 4.23 for OBP, 4.89 for VEG, and 5.07 for SAL. Strips in the pI range of 3–5.6 were thus used in 2-DE analyses. The quantity of each sample corresponds to 1/250 of the proteins extracted from 30 mg of tissue. This analysis allowed standardization of the protein amount for 2-DE, the quantity 1X corresponding to 1 μg of the faster migrating band (**Figure 1**, arrow). Mainly quantitative changes in the protein composition of samples were observed, with higher quantities in pubertal male and female RM and VNO than in pre-pubertal samples (**Figure 1**).

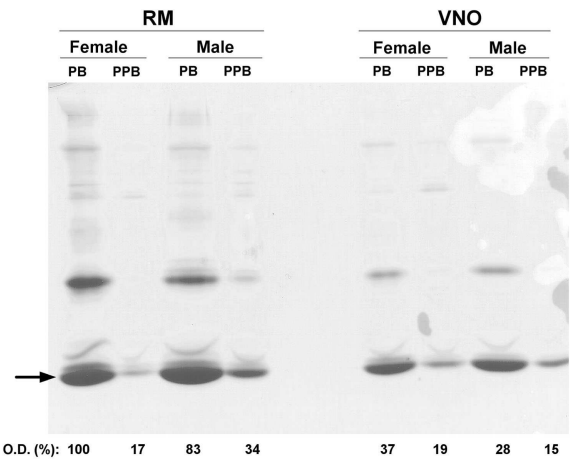

FIGURE 1 | One-dimensional native-PAGE of soluble proteins extracted from respiratory mucosa (RM) and vomeronasal mucosa (VNO) of pigs. The gel was stained with colloidal Coomassie blue. Wells were loaded for each tissue as follows: pubertal female (PB), pre-pubertal female (PPB), pubertal male (PB), and pre-pubertal male (PPB). Arrow: faster migrating band used as a standard for quantification and normalization of the samples. Relative optical density (in %) was calculated by using ImageJ software.

ANALYSIS OF OLFACTORY PROTEOME FROM MALE RM BY 2-DE

We proceeded with 2-DE, as it provides a more detailed pattern of soluble proteome diversity. Image analysis of 2-D gels of an adult male sample (**Figure 2A**) revealed a majority of protein spots in the predicted region of pI (3–5.6) and in the MW range of 10–50 kDa. After trypsin digestion, 39 protein spots (numbering in **Figure 2A**) were subjected to Nano-ESI-LC-MS/MS (protein spots of minor intensity, **Table 2**) or to MALDI-TOF MS (protein spots of major intensity, **Table 3**) analyses. The 39 spots were analyzed successfully, resulting in the identification of 10 different proteins in the gel. Among them, seven proteins dispatched in eight spots did not belong to the OBP family: selenium-binding protein (SELENBP1), alpha-1-acid glycoprotein (AGP), vitamin D-binding protein (DBP), haptoglobin precursor (HG), hemopexin precursor (HPX), superoxide dismutase (SOD), and lactoylgluthathione lyase (LGL).

The most abundant proteins (in number and quantity) identified in male RM proteome are isoforms of the three proteins already described in the nasal mucus of pig (29, 38): 15 isoforms of OBP (spots 8, 22–23, 28–36, 37–39), 11 isoforms of SAL (spots 11–21), and 13 isoforms of VEG (spots 9, 24–33, 37–39). The protein spots 28–31, 33, and 37–39 contained OBP and VEG in mixture, spot 32 contained OBP and SAL in mixture. OBP isoforms were found in the acidic part of the gel, except spot 8 (**Figure 2A**). The main difference between OBP isoforms lies in molecular weight. Isoforms at around 20 kDa correspond to the theoretical MW of 17835 Da, while those at around 50 kDa correspond to dimers, which are ever observed, even in denaturing conditions. This tendency to strong aggregation is a typical feature of porcine OBP, and dimers are difficult to dissociate without 8 M urea treatment and overnight heating (39). More surprising is the observation of OBP isoforms at lower molecular weight, between 13 and 15 kDa. In MALDI-TOF MS, we have analyzed the spectra to find out what part of the sequence is missing (**Table 3**). In shorter forms, the N-terminal part of the protein is missing [peptide (1–15)], never the C-terminal ones.

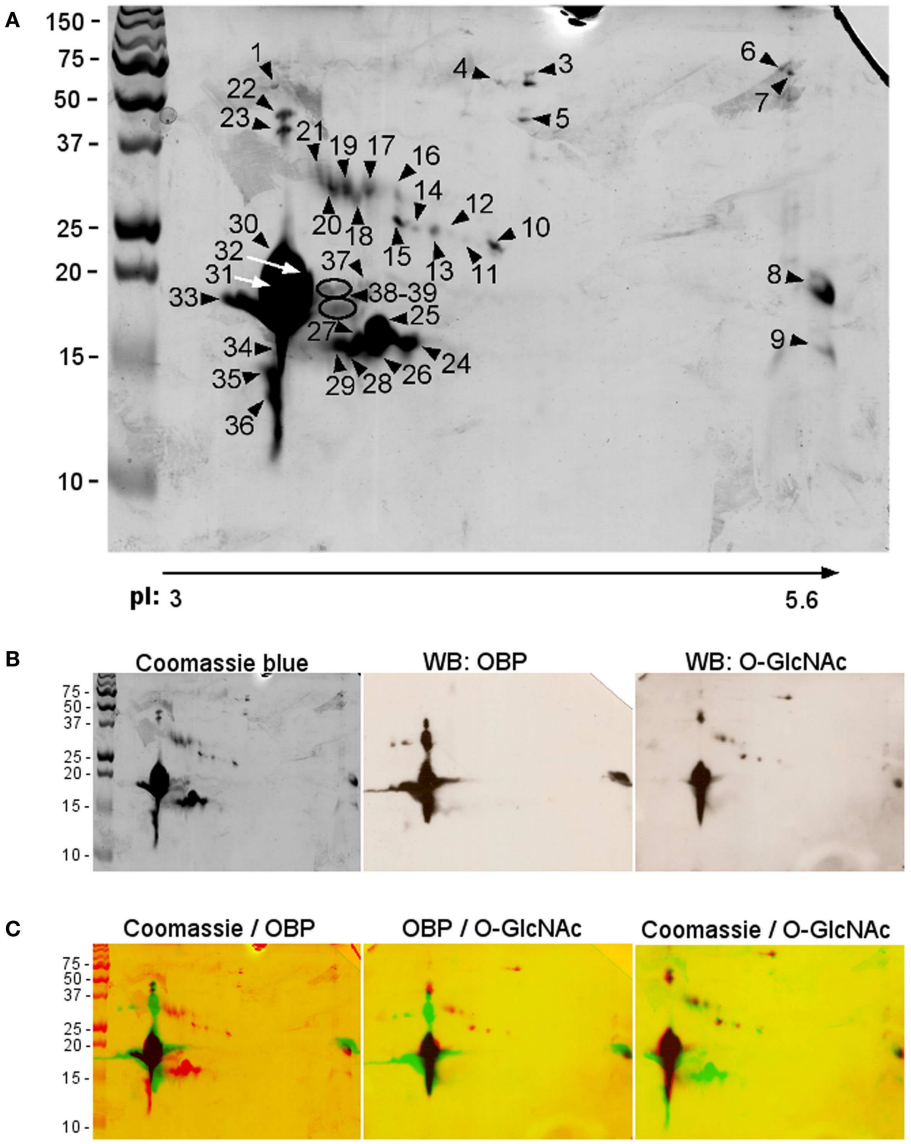

FIGURE 2 | Two-dimensional electrophoresis of soluble proteins extracted from pubertal male respiratory mucosa. **(A)** Coomassie blue staining. Protein spots were identified by mass spectrometry (numbering corresponds with data in **Tables 2** and **3**). **(B)** Raw images used in the merges, Coomassie blue staining, western-blot with anti-OBP, and RL2 antibodies. **(C)** Merges of the three images above.

Thirteen isoforms of VEG were separated by 2-DE. Isoforms in spots 30, 31, 33, 37, 38, and 39 were in mixture with OBP isoforms and have an expected apparent molecular mass of 17–18 kDa. Isoforms of spots 24, 26, 27, 28, and 29 have a lower apparent molecular mass of 15 kDa, corresponding to the loss of the first N-terminal 16 amino acids. VEG isoform of spot 25 has a higher MW and an N-terminus, but has a lower apparent MW than the forms contained in spots 37–39. Isoform of spot 9 has a MW of 15 kDa and an apparent "basic" pI of 6, as well as spot 8 containing OBP. The spot 33, containing both OBP and VEG, is the most acidic ones of the profile. Only one primary sequence was observed, corresponding to the expected form described in RM (Leu141), and not to the VNO form (Pro141) (38). It is interesting to notice that VEG dimers were not observed, as spots 22 and 23 contained only OBP isoforms.

The pattern of the 11 SAL isoforms is in accordance with the theoretical MW of 19916 Da (**Figure 2A**). Two nucleotide sequences were reported for porcine SAL (38). The mass spectra obtained by MALDI-TOF MS after trypsin digestion of spots 13, 15, 17, 19–21 indicated that the primary sequence corresponds to the product of gene SAL1 referenced as NM_213814. The masses corresponding to amino acids replacements Val45→Ala, Ile48→Val, and Ala73→Val described in Scaloni et al. (38) were not found. The increase of molecular weight from spot 11 to spot 21 can easily been explained by the fact that SAL is *N*-glycosylated on Asn53 (38, 40). The polymorphism of glycan

Table 2 | Identification by Nano-ESI-LC-MS/MS of proteins from pubertal male respiratory mucosa.

Spot No.	Protein identification	NCBI accession number	Score	Percentage sequence covered (peptide matched)	*O*-GlcNAc signal
1	Selenium-binding protein (SELENBP1)	gi|194036227	113	19 (2)	
	Alpha-1 acid glycoprotein (AGP)	gi|164302	40	5 (1)	
3	Vitamin D-binding protein (DBP)	gi|335293644	137	12 (9)	+
4	Vitamin D-binding protein (DBP)	gi|335293644	149	10 (8)	
5	Haptoglobin precursor (HG)	gi|47522826	139	15 (6)	
6	Hemopexin precursor (HPX)	gi|47522736	47	4 (3)	
7	Hemopexin precursor (HPX)	gi|47522736	53	23 (25)	
9	VEG	gi|27657971	405	28 (20)	
	Superoxide dismutase (SOD)	gi|15082144	51	6 (3)	
11	SAL	gi|21465464	110	28 (5)	
12	SAL		132	20 (6)	
14	SAL		477	52 (20)	
16	SAL		298	41 (16)	+
18	SAL		622	37 (28)	
37	OBP	gi|3122574	560	51 (27)	
	VEG	gi|27657971	85	12 (3)	
38	OBP	gi|3122574	512	32 (23)	
	VEG	gi|27657971	50	4 (1)	
39	OBP	gi|3122574	418	44 (18)	+
	VEG	gi|27657971	63	12 (4)	

Spots labeled by RL2 antibody are indicated in the last column.

chains, the structure of which is still unknown, could generate SAL sub-populations of different MW, and, if processed as other mammalian *N*-glycans, of different pI due to sialic acids in terminal position. It could be noticed that SAL isoforms are of expected MW or higher, contrary to OBP and VEG that display shorter isoforms. Shorter forms lacking the N-terminus could come from protease degradation, but there are no obvious reason to explain why SAL would be protected against enzymatic action, and not OBP and VEG. In addition, action of proteases would generate much more random forms and not specifically forms lacking the N-terminal peptide.

SEARCH FOR OBP AND VEG SPLICING VARIANTS BY RACE-PCR AND "*IN SILICO*" ANALYSIS

Interestingly, the sequence of the identified OBP and VEG isoforms with apparent MW of around 15 kDa always lacks the N-terminus (**Table 3**). VEG isoforms of lower MW were already observed in human tears [four isoforms of 14–16 kDa in Ref. (41, 42)]. The origin of N-terminal heterogeneity of VEG has been debated and led authors to speculate involvement of genetic polymorphism. The structure of porcine VEG encoding gene (called *LCN1*, GenBank #U96150) is known (43), but not its regulation. So, we have first hypothesized that this short OBP and VEG isoforms could come from alternative splicing of their coding gene. Amplification of potential transcripts of different sizes was undertaken several times and on different animals by RACE-PCR. We obtained each time a single transcript of full-length sequence for VEG (data not shown). This is consistent with expression studies of human *VEG/LCN1* gene, that showed a single size of RT-PCR products obtained with specific primers in not only olfactory tissue, but also in lachrymal gland, placenta, and mammary gland (19). The search for short variants of OBP by RACE-PCR was also unsuccessful and we obtained a single-size PCR product around 500 bp (data not shown). Since these results, the pig genome has been sequenced and made available at Ensembl database. We have thus performed blast analysis to search for putative splicing variants for OBP, VEG, and SAL. The *SAL1* gene (Ensembl: ENSSSCG00000005474) has only one transcript (ENSSSCT00000006020) corresponding to the coding of the full-length (mature) protein of 175 amino acids. The gene encoding porcine VEG, *LCN1* (ENSSSCG00000024779) has two transcripts corresponding to a mutation leading to the VNO form (AAB34720.1, Pro141) and the RM form (AAO18367.1, Leu141), both already described (38). The sequences of the two variants coming from the alternative splicing of exon 3 of the OBP gene (ENSSSCG00000012095) were obtained from Ensembl: transcript ID ENSSSCT00000013229 of 522 bp coding for the mature protein of 158 amino acids already described (GenBank RefSeq NM_213796, OBP1), and transcript ID ENSSSCT00000033772 of 605 bp encoding a mature protein of 156 aminoacids (OBP2, Ensembl ENSSSCP00000028674). Both proteins have the same theoretical pI of 4.23 (calculated with expasy.org/protparam/). The proteins differ from the amino acid 82 (underlined): ^{81}N<u>Y</u>AGNNKFV^{89} for OBP1, ^{81}N<u>C</u>NNKFV^{87} for OBP2. On MALDI-TOF spectra, the two proteins could be distinguished by the peptide at m/z 1498.6227, only found for OBP2 ([73–85] peptide) but this mass is closed to that of peptide at m/z 1498.7424 obtained only for OBP1 digestion ([16–28] peptide). The two OBP transcripts encode a C-terminal Lysine as already

Table 3 | Identification by MALDI-TOF MS of proteins from pubertal male respiratory mucosa.

Spot No.	Protein identification	Percentage sequence covered (peptides matched)	Observed MW (kDa)	Comments	*O*-GlcNAc signal
8	OBP	72 (9)	18	With N-ter & C-ter	+
10	Lactoylgluthathione lyase (LGL)	15 (9)	23		+
13	SAL	32 (7)	25		+
15	SAL	35 (6)	26		+
17	SAL	60 (8)	32		+
19	SAL	25 (5)	32		
20	SAL	38 (6)	32		+
21	SAL	21 (4)	34		+
22	OBP	62 (7)	48	With N-ter & C-ter	+
23	OBP	33 (6)	40	With C-ter	+
24	VEG	50 (6)	15	No N-ter	
25	VEG	82 (9)	17	With N-ter & C-ter	
26	VEG	66 (9)	15	No N-ter	
27	VEG	62 (8)	15	No N-ter	
28	VEG	60 (6)	15	No N-ter	
	OBP	32 (6)		No N-ter	
29	VEG	57 (6)	15	No N-ter	
	OBP	15 (3)		No N-ter	
30	OBP	27 (5)	21	With N-ter & C-ter	+
	VEG	10 (1)		With N-ter & C-ter	
31	OBP	45 (5)	20	With N-ter & C-ter	+
	VEG	10 (1)		With N-ter & C-ter	
32	OBP	41 (7)	20		+
	SAL	18 (5)			
33	OBP	57 (9)	18	With N-ter & C-ter	
	VEG	10 (1)		With N-ter	
34	OBP	50 (6)	15	No N-ter	+
35	OBP	31 (6)	14	No N-ter	+
36	OBP	40 (7)	13	No N-ter	+

Spots labeled by RL2 antibody are indicated in the last column.

described (29). However, OBP isoforms of male RM all miss the Lysine in C-terminal, indicated by the peak at m/z 2296.0714 Da corresponding to the peptide [138–157] with the Alanine as terminus. This could come from proteolysis degradation or by alternative expression of two alleles of the OBP gene as it was already suggested (29).

IMMUNODETECTION OF *O*-GLcNAcylation

In a previous work, we have shown that a VEG isoform is modified by an *O*-GlcNAc moiety (27). We have performed western-blot on an aliquot of male RM soluble proteome separated by 2-DE in the same conditions as above. The specific antibody RL2 (**Figure 2B**, WB: *O*-GlcNAc) labeled mostly OBP isoforms (**Figure 2B**, WB: OBP), including the "acidic" full-length (spot 8) or short-length (spots 34–36) isoforms, and the "dimers" in spots 22–23. This labeling is highly specific because short-length VEG isoforms in spots 24–29 (and VEG in spot 25 of MW 17 kDa) are not labeled at all, despite their quantity (**Figures 2B,C**: Coomassie/*O*-GlcNAc). This constitutes a negative control of the RL2 specificity for these proteins. The "basic" spot (9) contains also a short-length VEG isoform and is not labeled by RL2 (**Figure 2C**: Coomassie/*O*-GlcNAc). Contrary to OBP isoforms of short size, VEG isoforms of short size are never labeled by RL2, suggesting that the modification occurs on the N-terminal part that is missing in these forms (peptide [1–16]). This also suggests that the VEG isoform identified previously (27) as *O*-GlcNAc modified is of full-length sequence and probably contained in spots where OBP and VEG are in mixture, and labeled by RL2 antibodies (spots 30–32, 39). Meanwhile, VEG isoform of spot 25, with N-terminal and C-terminal has a smaller apparent molecular weight than those of isoform contained in spots 39. This difference could be attributed to the modification of VEG 39 by *O*-GlcNAc, immunoreactive to RL2, whilst VEG 25 is not labeled by RL2. Eight spots containing SAL isoforms were labeled by RL2: 13, 15–17, 20–21, and spot 32 containing OBP and SAL in mixture (**Figure 2C**). Concerning proteins unrelated to OBPs, spot 3 containing DBP and spot 10 containing LGL were labeled by RL2.

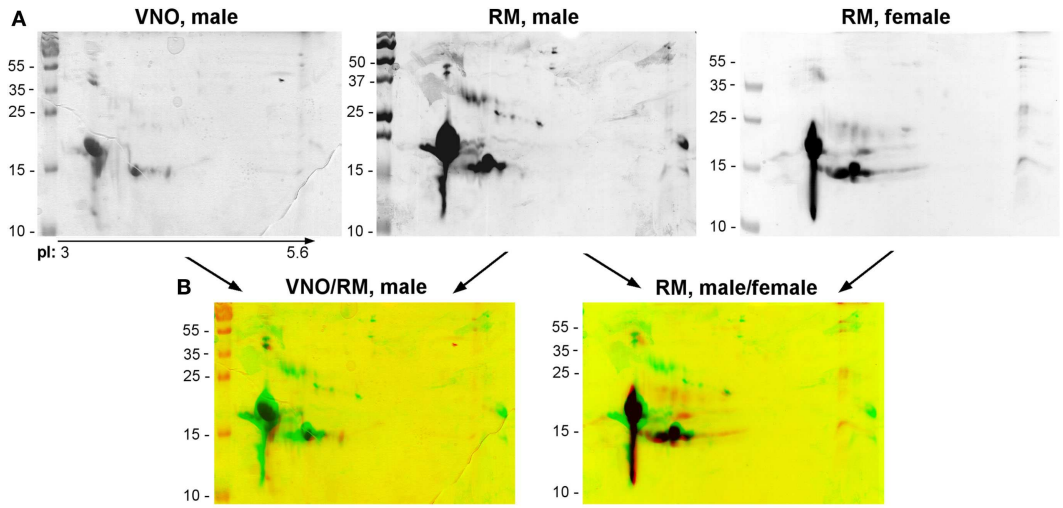

FIGURE 3 | Comparison of soluble proteins profiles between tissues (RM and VNO) of adult male, and between RM of male and female. **(A)** Coomassie blue staining. **(B)** Merges of Coomassie blue stained gels.

COMPARISON OF OLFACTORY PROTEOME BETWEEN TISSUES OF THE SAME ANIMAL (MALE RM AND VNO) AND BETWEEN MALE AND FEMALE RM

For ethical reasons, it is difficult to obtain littermates to constitute replicates. So, we have in a first approach compared the soluble proteome: (1) between VNO and RM of pubertal male and (2) between male and female RM. Soluble proteins from these tissues were separated in the same conditions than the RM sample analyzed above (**Figure 3A**). The three profiles obtained after 2-DE are similar, but slight differences were observed. The quantity and number of isoforms is higher in the male RM soluble proteome, since the samples correspond each to the same weight of tissue (30 mg) before protein extraction. In addition, the merge of images of the two tissues stained by Coomassie blue (**Figure 3B**: VNO/RM, male) indicates a different pattern of OBP, VEG, and SAL isoforms. In the VNO, six isoforms of OBP and three to four isoforms of VEG are visible. The comparison between male and female RM showed less differences (**Figure 3B**: RM, male/female). In particular, the pattern of OBP and VEG isoforms in female sample is similar to those of male, except that SAL and the basic OBP are not visible in the female. Interestingly, a spot of the VEG pattern is similar to spot 25 of male RM. At first glance, no spot can be observed at the position of SAL isoforms migration on the gel stained by Coomassie blue, either in VNO nor in female RM. However, western-blot with anti-SAL antibodies revealed 13 spots in male VNO (**Figure 4**, WB: SAL) in a similar distribution to isoforms of RM extract (**Figure 3A**). An aliquot of male VNO was treated by western-blot with CTD110.6 antibodies, and only OBP isoforms were labeled (**Figure 4**: WB: CTD110.6). This result is surprising because SAL isoforms are labeled by RL2 in male RM (**Figure 2C**: OBP/*O*-GlcNAc).

MOLECULAR CLONING OF *SUS SCROFA EOGT* cDNA AND PHYLOGENETIC ANALYSIS

The report of a new OGT in *Drosophila*, linking a single *O*-GlcNAc moiety on Ser and Thr residues of secreted proteins (28) supported our findings on OBP *O*-GlcNAcylation. The availability of pig genome sequence gave us the opportunity to identify and clone the *EOGT* cDNA from pig olfactory tissues. We have blasted the pig genome (Ensembl database) with *D. melanogaster EOGT* sequence and designed primers from the 3′ and 5′ ends of the transcript ID ENSSSCT00000012589. A single PCR product was obtained in all tissues of RM and VNO from pre-pubertal and pubertal males and females (**Figure 5**), but not in the controls (PCR mix without DNA template or without DNA polymerase). The PCR product from male RM was cloned and sequenced. The cDNA sequence is 1696 bp long, and starts with the ATG codon for initiating the translation and a predicted signal peptide of 18 amino acids [SignalP, (37)], leading after cleavage to a mature protein of 509 amino acids. The *EOGT* sequence obtained from pig tissue was 100% identical to the unique transcript ID ENSSSCT00000012589 reported in Ensembl database (pig genome). The full-length cDNA sequence of *S. scrofa EOGT* was deposited in GenBank database under accession number JX546149. *S. scrofa* EOGT is 48.9% identical to *D. melanogaster* EOGT.

To confirm the cloning of an *EOGT* in *S. scrofa* we performed a phylogenetic analysis. Glycosyl transferases (EC 2.4.x.y) are classified according to their enzymatic activities as well as their structure in the Carbohydrate-Active enzyme (CAZy) web site [(44)][2]. *S. scrofa* OGT is a GT from the characterized family GT41 with UDP-GlcNAc (EC 2.4.1.94) and UDP-Glc (EC 2.4.1.-) known activities, whereas *S. scrofa* EOGT and *S. scrofa* AGO61 belong to the characterized family GT61 with β-1,2-xylosyltransferase (EC 2.4.2.38) and *O*-β-N-acetylglucosaminyltransferase (EC 2.4.1.94). OGT, EOGT, and AGO61 proteins clustered in two different branches of our phylogenic tree (**Figure 6**) with EOGT and AGO61 proteins belonging to two different branches of the same sub-tree. OGT and EOGT groups sequences from deuterostomes and protostomes (in gray) phyla whereas AGO61 branch only groups deuterostome

[2]http://www.cazy.org

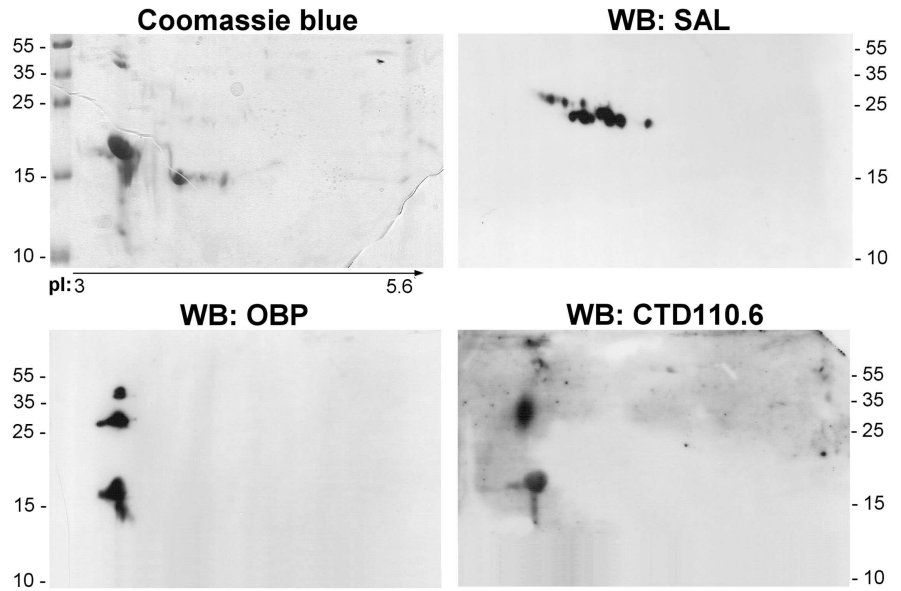

FIGURE 4 | Analysis of male VNO soluble proteome. Aliquots of the same sample were used for Coomassie blue staining, and western-blot with anti-OBP, anti-SAL, and CTD110.6 antibodies.

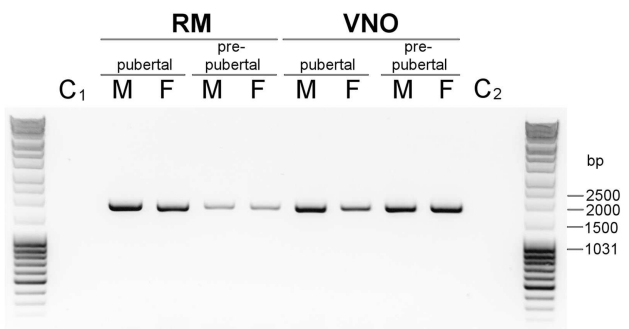

FIGURE 5 | Expression of *Sus scrofa* EOGT in RM and VNO of males and females. Agarose gel (1% in Tris Acetate EDTA buffer) electrophoresis of PCR products amplified with specific primers designed from 5′ and 3′ ends of transcript ID ENSSSCT00000012589 (Ensembl database, *Sus scrofa* genome). C_1, and C_2, controls: PCR mix without cDNA.

sequences. Most of the sequences clustered in the EOGT branch are predicted as targeted to the extracellular space, which is in agreement with the function of the EOGT described by Sakaidani et al. (28).

DISCUSSION

The proteomic analysis of pig nasal mucus described above brought important novelty at several aspects:

SOLUBLE PROTEOME OF PIG NASAL MUCUS IS MAINLY COMPOSED OF OBP, VEG, AND SAL ISOFORMS

The composition of the pig nasal mucus has never been investigated by 2-DE. However, body fluids such as tears, saliva, and nasal mucus could provide important information on health conditions of animals and humans, as alterations in their profile could be associated with specific diseases (45). Until now, such studies concerned proteomic analyses of body fluids in which VEG and SAL are also secreted, tears (41) and saliva (46, 47), but not OBP, as it is specific to the nasal area. Three VEG and five SAL isoforms were identified in porcine saliva (47), and four VEG isoforms in human saliva (46). The human saliva did not contain any SAL isoform, as the encoding gene *SAL1* is a pseudogene in this species (48). But in both cases, these isoforms are not the major proteins (<1% of the total spots). On the contrary, porcine nasal mucus is mainly composed of OBP, VEG, and SAL isoforms, whatever the animal (male or female) or tissue (male RM or VNO). Indeed, only seven proteins identified in eight spots were not OBPs: SELENBP1, AGP, DBP, HG, HPX, SOD, and LGL. Most of these proteins have antioxidant properties and are involved in the protection of cells against oxidative stress. They function probably to protect the cells of the olfactory epithelium exposed to oxygen. Their expression level could be a marker of oxidative stress in nasal mucus. The 32 other spots contained isoforms of OBP, VEG, and SAL, alone or in mixture. The comparison between tissues and animals of the two sexes showed similar profiles, which gives a good confidence in the reproducibility of our analyses. The differences observed in isoforms number and distribution could either be due to inter individual variability, frequently observed in mammals, or to differential expression under hormonal control. This later hypothesis is supported by numerous works on the olfactory system, which displays a high plasticity at different moments in the individual life, determined by hormonal switch, such as puberty [e.g., Ref. (49, 50)]. In pig, the perception of pheromones is differently interpreted according to age and sex of the animals. For example, the sex steroid androstenone is abundant in the saliva of sexually

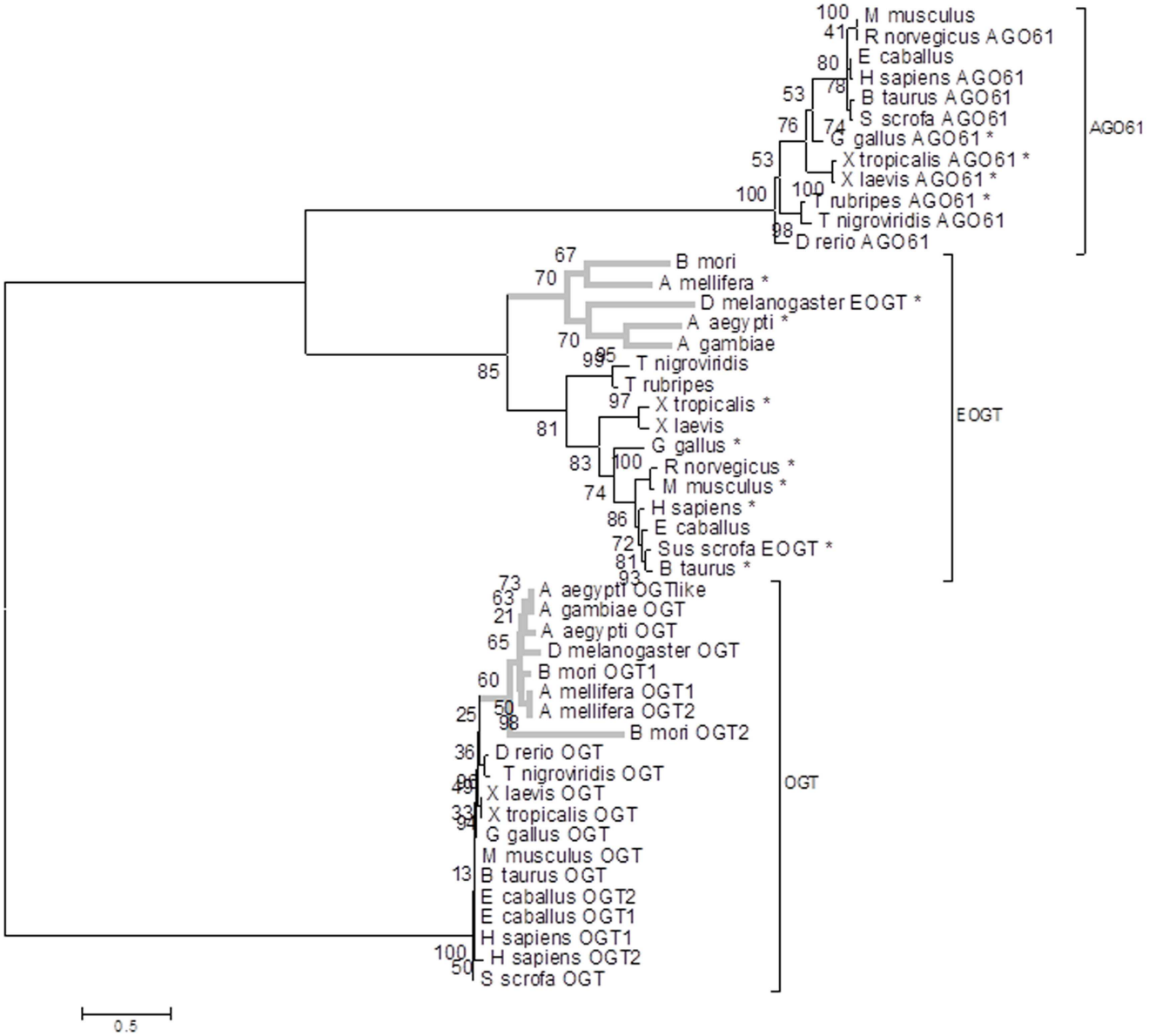

FIGURE 6 | Maximum likelihood phylogenetic tree of genes encoding OGT, EOGT, and AGO61. RAxML bootstrap support values are shown at the nodes of the tree. *Outlined secreted proteins detected by WoLF PSORT or SignalP 4.0. Protostomes are indicated in gray lines.

active males and induces acceptation of the male in estrus females (heat period), but not in di-estrus females (2). When perceived by pre-pubertal animals, it has appeasing effects and is a signal of submission for piglets (51). This multiple role in social relationships is due to the endocrine status of the receiver animal, in particular to the level of testosterone, the hormone of puberty in males that is very low in pre-pubertal individuals of both sexes, and in adult females. Considering the high and rapid turnover of OBPs (14) at the peripheral level, these proteins are good targets for olfactory plasticity and we could hypothesize that their post-translational regulation is under control of hormones determining the endocrinal status of animals.

OBP ISOFORMS ARE POTENTIALLY MODIFIED BY ATYPICAL *O*-GLcNACYLATION

In a previous work, we have identified an *O*-GlcNAc moiety on a VEG isoform that seems to determine its specific binding to testosterone (27). This PTM affects nuclear and cytoplasmic proteins (52, 53), *via* the action of a cytosolic GT, the *O*-linked *N*-acetylglucosaminyltransferase [OGT, (54)]. This dynamic modification of Ser/Thr residues was not supposed to affect proteins of the secretion pathway that are processed in Endoplasmic Reticulum, Golgi, and secretion vesicles. Meanwhile, since 2008, some authors reported the presence of *O*-GlcNAc on extracellular domains of receptors, which are synthesized through the secretion

pathway. Atypical *O*-GlcNAcylation was initially reported as specific to the 20th EGF domain of recombinant Notch expressed in insect cells (55). Thus, the enzyme that catalyzes the *O*-GlcNAcylation of extracellular domains of Notch or Dumpy in *Drosophila*, EOGT, was proposed to modify EGF repeats of these proteins (28, 56). Our results clearly show that RL2 and CTD110.6 antibodies labeled some OBP isoforms in a specific manner, as VEG isoforms were never labeled. OBP do not display any EGF repeat in its sequence (nor VEG or SAL), but is potentially modified by *O*-GlcNAcylation. Interestingly, ORs and VNO receptors, which do not have EGF repeats in their sequence, have been characterized by Click-chemistry as *O*-GlcNAc modified (57). Extensive data have now highlighted the importance of *O*-GlcNAcylation in intracellular signaling (58), and it is not so surprising to consider its involvement in extracellular signaling, especially crucial in pheromone communication. Indeed, at an evolutionary point of view, the accurate perception of pheromone determines the fitness of an individual, and more largely, is one of the strongest prezygotic reproductive barriers involved in interspecific isolation (59). OBPs and ORs are parts of the first step of odors and pheromone reception, and so, it is reasonable to suggest that their binding properties need to be regulated by fine molecular mechanisms, such as *O*-GlcNAcylation.

IS *SUS SCROFA* EOGT RESPONSIBLE FOR *O*-GLCNACYLATION OF OBP ISOFORMS?

We have cloned and sequenced the cDNA encoding EOGT in *Sus scrofa* from tissues coming from RM and VNO in animals of different age and sex. Thus, it seems that EOGT is expressed whatever the physiological status of pigs. In addition, the phylogenetic analysis confirmed that our corresponding *Sus scrofa* protein clustered with the characterized DmEOGT (28) as well as with mammalian EOGT orthologs. They belong to a specific EOGT phylogenic subgroup where the majority of proteins are predicted as targeted to the extracellular space. This EOGT sub-group clearly segregates from the two other sub-groups AGO61 and OGT. AGO61 is a putative GT from the same family as EOGT in the CAZy database (60). One *Sus scrofa* gene is found in each of these sub-groups but the *Sus scrofa* gene described in this paper is the ortholog of *DmEOGT*. This reinforces the claim that extracellular *O*-GlcNAcylation is a fundamental biochemical mechanism conserved during evolution. In particular, this process is theoretically conserved in model species where the role of OBPs is extensively studied: *B. mori* (moth), *A. mellifera* (bee), *D. melanogaster*, and *A. aegypti/gambiae* (mosquitoes) for insects, and *S. scrofa*, *R. norvegicus*, and *M. musculus* for mammals. It would be of interest to search for OBP *O*-GlcNAcylation in these species, as a potential mechanism for odor and pheromone discrimination by OBPs.

The fact that an ortholog *EOGT* gene exists in the pig genome does not mean that the enzyme is responsible for *O*-GlcNAc modification of secreted OBPs. The EOGT-like AGO61 could be a candidate for *O*-GlcNAcylation of secreted proteins, but this hypothesis was eliminated after demonstration that mouse EOGT was the sole enzyme to catalyze the linking of *O*-GlcNAc on Notch1 (61). More experiments are required to demonstrate the role of *Sus scrofa* EOGT. The EOGT could be expressed in the yeast, since no similar sequence to those of EOGT has been found yet in the databases. Thus, the EOGT purified from the yeast would come only from overexpression of the porcine form. We have already overexpressed the porcine OBP in *Pichia pastoris* with very good yields (26) and the linking of *O*-GlcNAc on recombinant OBP by recombinant EOGT could easily be monitored with UDP-GlcNAc as substrate. Alternatively, expression of OBP in mammalian cell lines deficient in EOGT would be useful.

BINDING SPECIFICITIES OF OBP ISOFORMS COULD BE DRIVEN BY PTM PATTERN

Previously, we have shown that OBP and VEG are modified by phosphorylation (25), which is also an unusual PTM for secreted proteins, although several reports of extracellular phosphorylation were published (62, 63). Moreover, binding experiments indicated that recombinant OBP isoforms display different binding affinities for the pheromone components in the pig (26). None of these isoforms binds testosterone, the natural and specific ligand of *O*-GlcNAc modified VEG (27). The results obtained here indicate that porcine OBP is modified by *O*-GlcNAc. Implications of PTM in OBP binding properties need to be investigated. First of all, purification of these isoforms has to be performed as it was done for recombinant OBP isoforms (26). Then, localization of PTM sites (phosphorylation and/or glycosylation) by high-resolution mass spectrometry would permit to rely their PTM patterns to specific binding properties. Our results indicate that *O*-GlcNAcylation could be a fine mechanism to control OBP binding specificity. These data draw a new scheme of interactions between OBPs and their odorant ligands. OBP should no more be considered as passive carriers, but more likely as players of the first step of odor and pheromone coding by the olfactory system.

AUTHOR CONTRIBUTIONS

Patricia Nagnan-Le Meillour, Anne-Sophie Vercoutter-Edouart, Frédérique Hilliou, Chrystelle Le Danvic, and Frédéric Lévy conceived and designed the experiments. All authors performed experiments and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fendo.2014.00202/abstract

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Global O-GlcNAc levels modulate transcription of the adipocyte secretome during chronic insulin resistance

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Increased flux through the hexosamine biosynthetic pathway and the corresponding increase in intracellular glycosylation of proteins via O-linked β-*N*-acetylglucosamine (O-GlcNAc) is sufficient to induce insulin resistance (IR) in multiple systems. Previously, our group used shotgun proteomics to identify multiple rodent adipocytokines and secreted proteins whose levels are modulated upon the induction of IR by indirectly and directly modulating O-GlcNAc levels. We have validated the relative levels of several of these factors using immunoblotting. Since adipocytokines levels are regulated primarily at the level of transcription and O-GlcNAc alters the function of many transcription factors, we hypothesized that elevated O-GlcNAc levels on key transcription factors are modulating secreted protein expression. Here, we show that upon the elevation of O-GlcNAc levels and the induction of IR in mature 3T3-F442a adipocytes, the transcript levels of multiple secreted proteins reflect the modulation observed at the protein level. We validate the transcript levels in male mouse models of diabetes. Using inguinal fat pads from the severely IR *db/db* mouse model and the mildly IR diet-induced mouse model, we have confirmed that the secreted proteins regulated by O-GlcNAc modulation in cell culture are likewise modulated in the whole animal upon a shift to IR. By comparing the promoters of similarly regulated genes, we determine that Sp1 is a common *cis*-acting element. Furthermore, we show that the LPL and SPARC promoters are enriched for Sp1 and O-GlcNAc modified proteins during insulin resistance in adipocytes. Thus, the O-GlcNAc modification of proteins bound to promoters, including Sp1, is linked to adipocytokine transcription during insulin resistance.

Keywords: O-GlcNAc, insulin resistance, adipose tissue, adipocytokines, transcription

INTRODUCTION

It is estimated that diabetes affects 8.3% of the United States population (1). Type 2 diabetes mellitus (T2DM) is characterized by both hyperinsulemia and hyperglycemia, which result from a combination of whole-body insulin resistance and pancreatic beta-cell dysfunction that leads to insulin insufficiency (2). T2DM can lead to a wide-range of severe and costly complications, such as blindness, kidney failure, stroke, and cardiovascular disease (3). The abundance of associated complications reflects the number of interrelated systems involved in T2DM pathogenesis (4).

White adipose tissue is an important mediator of energy homeostasis. In addition to its role as an energy storage depot, it acts as an endocrine organ by secreting adipocytokines, such as leptin and adiponectin. Adipocytokines can affect both local and distant tissue insulin sensitivity and energy homeostasis (5, 6). Obesity alters the ability of adipose tissue to properly express and secrete adipocytokines. Obesity, which affects more than 10% of adults world-wide, is the leading environmental risk factor for the development insulin resistance and T2DM (7–9). Importantly, several adipocytokines have been implicated in the development of insulin resistance and the pathogenesis of T2DM (10). The mechanism by which adipocytes respond to the excess nutrient-flux during obesity and insulin resistance and alter the secretion of adipocytokines is not completely understood.

One way for cells to sense nutrient abundance and thereby alter their metabolism and gene expression is through the hexosamine biosynthetic pathway (HBP). In 1991, Marshall et al. first implicated the HBP in the development of insulin resistance (11). The HBP has been proposed to be a nutrient-flux sensor, since it utilizes 2–5% of intracellular glucose, and acts to limit the amount of glucose uptake by inducing insulin resistance (11). The end product of the HBP, uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), is the sugar donor for the enzyme O-GlcNAc transferase (OGT), which transfers the O-GlcNAc post-translational modification onto serine or threonine residues of nuclear and cytoplasmic proteins (12–15). It has been demonstrated by many groups, including our own, that in multiple systems the elevation of O-GlcNAc levels is sufficient to induce insulin resistance (16–21).

The expression of several adipocytokines has been shown to be regulated at the level of transcription (22–26). Additionally, the transcription and secretion of several adipocytokines is modulated

by altered HBP flux (22, 27, 28). Transgenic mice overexpressing OGT in peripheral tissues have both glucose disposal defects and hyperleptinemia, suggesting that the O-GlcNAc modification is intricately tied to the development of insulin resistance and the regulation of adipocytokines (16).

We have recently used shotgun proteomics to identify multiple murine secreted proteins from adipocytes (adipocytokines) whose levels are modulated upon the induction of insulin resistance by indirectly and directly modulating O-GlcNAc levels (29). In this study, we investigate the transcriptional regulation of several of the secreted proteins identified by proteomics. We explore whether O-GlcNAc modified transcription factors are regulating these proteins, since several adipocytokines are known to be regulated at the level of transcription and O-GlcNAc has been demonstrated to modify and alter the function of many transcription factors (30, 31). Here, we show that these secreted factors are co-regulated in a mouse adipocyte cell line and two mouse models of insulin resistance. We demonstrate that the promoters of these genes contain a common *cis*-acting motif for Sp1. We determine that Sp1 is more heavily O-GlcNAc modified during insulin resistance. Finally, we determine that Sp1 and O-GlcNAc modified proteins are enriched on the LPL and SPARC promoters. Our findings suggest that the O-GlcNAc modification of proteins regulates adipocytokine transcription during chronic insulin resistance.

MATERIALS AND METHODS

MATERIALS AND REAGENTS

Tissue culture media, serum, and antibiotics were purchased from Gibco (Grand Island, NY, USA). 3-isobutyl-1-methyxanthine and dexamethasone were from Sigma (St. Louis, MO, USA). Recombinant insulin, human, was from Roche Diagnostics (Indianapolis, IN, USA). O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenyl carbamate (PUGNAc) was from Toronto Research Chemicals Inc. (North York, ON, USA). GlcNAcstatin was a kind gift from Dr. Daan van Aalten (University of Dundee, Dundee, Scotland). Anti-Sp1 (PEP 2), anti-LPL (H-53), anti-Angiotensin I/II (N-10), anti-ERK-2 (C-14), normal sera, and agarose conjugated beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PEBP1 was from Novus Biologicals (Littleton, CO, USA). Anti-SPARC was from Abcam (Cambridge, MA, USA). Anti-O-GlcNAc (RL2) was from Enzo Life Sciences (Farmingdale, NY, USA). Anti-O-GlcNAC (CTD110.6) was previously generated in Dr. Gerald W. Hart's Laboratory (Johns Hopkins University, Baltimore, MD, USA). Dynabead Protein G was from Life Technologies (Carlsbad, CA, USA).

CELL CULTURE AND TREATMENTS

3T3-F442a preadipocytes were maintained and differentiated as previously described (29, 32). On day 6 after the induction of differentiation, the adipocytes were maintained in the appropriate low (1.0 g/L) or high glucose (4.5g/L) DMEM media containing 10% FBS, antibiotics, and vitamins with or without 100 μM PUGNAc, 20 nM GlcNAcstatin, or 100 nM insulin. After 24 h incubation, cells were washed either three times or five times (for media immunoblotting) with low or high glucose serum free media without antibiotics and vitamins. Following the rinses, cells were incubated for 16 h in the appropriate low or high glucose media without serum, antibiotics, and vitamins and with or without 100 μM PUGNAc, 20 nM GlcNAcstatin, or 1 nM insulin. After the incubation, the conditioned media was carefully collected, filtered, and buffer exchanged as previously described (29). The remaining cells were washed two times with ice cold PBS and then harvested by scraping and stored at −80°C until further analysis.

ANIMALS

Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Animals were group housed with a 12-h light, 12-h dark cycle. Inguinal and retroperitoneal fat tissues from 12-week-old-male C57BL/6J wt (wt), C57BL/6J *db/db* (6J), and C57BL/3J *db/db* (3J) mice were isolated along with serum. Mice were fed *ad libitum* normal rodent chow. After sacrifice by decapitation, the inguinal fat was weighed, snap frozen, and stored at −80°C until transcript analysis. Both the 3J and 6J mice had both inguinal and retroperitoneal fat masses three times greater than the wildtype littermates as well as elevated serum glucose levels (>1.5×) and insulin levels (>5×) with the 3J mice having higher levels than 6J mice. For the diet-induced insulin resistance experiment, young (~9 weeks) C57BL/6 male mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Both treatment groups were fed *ad libitum* normal rodent chow and water. The mice in the high fat high sucrose (HFHS) treatment group were given free access to a 30% sucrose solution and lard in addition to their normal chow and water. After week 1, 2, and 3 of treatment, an insulin sensitivity test (ITT) was performed on a pair of mice closest to the average weight of each treatment group. The insulin sensitivity test was performed as previously described (33). Weights were recorded every week. After 3 weeks of treatment, six mice from each treatment group were sacrificed by decapitation. Trunk blood was collected for the measurement of serum insulin using a LINCO rat insulin RIA kit (EMD Millipore Corporation, Billerica, MA, USA). The liver and four fat pads (inguinal, epididymal, mesenteric, and retroperitoneal) were weighed, snap frozen, and stored at −80°C until transcript analysis.

CELL LYSATES, WESTERN BLOTTING, AND IMMUNOPRECIPITATION

For immunoprecipitations and anti-O-GlcNAc Western blots, 3T3-F442a cell pellets were lysed in 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1:100 protease inhibitor cocktail set V, EDTA-free (Calbiochem), and 1 μM PUGNAc. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The CTD110.6 Western blots were performed essentially as described (34). Immunoprecipitations were carried out at 4°C overnight using anti-Sp1 or normal rabbit IgG with 750 μg of precleared protein lysate. Immunocomplexes were collected using Protein A/G-PLUS agarose beads for 2 h. Beads were washed four times with a modified RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS) and one time with a high salt modified RIPA buffer (same as above except 500 mM NaCl). Proteins were eluted by boiling beads in 1× laemmli buffer and then transferred to a fresh tube for Western blotting. For the concentrated media Western blots, the protein concentration of the concentrated 3T3-F442a

media was determined using the Bradford method and verified by Coomassie staining. Equal amounts of protein were separated by SDS-PAGE with Tris–HCl precast minigels (Bio-Rad, Hercules, CA, USA) and transferred to poly(vinylidene difluoride) (PVDF) membranes (for concentrated media) or nitrocellulose membranes (for immunoprecipitations) for Western blot analysis. After blocking for at least 1 h, membranes were incubated with the appropriate primary antibody overnight at 4°C. Membranes were incubated with the appropriate horseradish peroxidase-coupled secondary antibodies for 1 h, followed by extensive washing and Pierce ECL detection. ImageJ was used for densitometry (35).

CHROMATIN IMMUNOPRECIPITATION

Chromatin Immunoprecipitations were performed as in the Millipore EZ-ChIP kit with some modifications. Day 8 adipocytes were washed once with room temperature PBS and then crosslinked by adding 1% formaldehyde in PBS and incubating for 10 min. The adipocytes were washed three times with cold PBS and then harvested by scraping. The adipocytes were resuspended in hypotonic lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1:100 Calbiochem protease inhibitor) and incubated on ice then dounce homogenized. The nuclei were collected by centrifugation and then resuspended in SDS lysis buffer. DNA was sheared to between 200 and 1000 base pair fragments using a Misonix S-4000 sonicator. Protein concentration was quantified using the Pierce BCA Protein Assay Kit. One hundred micrograms of chromatin was used per immunoprecipitation. Sonicated chromatin was diluted 1:10 with dilution buffer and precleared using Protein A/G-PLUS agarose, normal goat IgG, and sheared salmon sperm DNA (ssDNA) (Ambion). Three percent of the sample was saved as Input. One microgram of anti-Sp1, anti-O-GlcNAc (RL2), or normal IgG was used for the immunoprecipitation. Immunocomplexes were collected for 1 h using Protein G Dynabeads that were blocked with ssDNA and BSA (New England Biolabs). The Dynabeads were washed five times and then eluted with 1% SDS and 0.1 M $NaHCO_3$. The elutions were decrosslinked at 65°C overnight with NaCl and RNase A (Ambion). After Proteinase K treatment (New England Biolabs), samples were purified by a Phenol–Chloroform extraction followed by ethanol precipitation overnight at −20°C using glycogen as a carrier. Precipitated DNA was resuspended in 3 mM Tris–HCl pH 8.0, 0.1 mM EDTA. qPCR was performed using primers for the proximal mouse SPARC and LPL promoter Sp1-binding sites. Sequences of primers were SPARC primers 5'-AGGCAAGTTCACTCGCTGGCT-3' (forward) and 5'-AGACACCCTGGCCCCACCTG-3' (reverse) and LPL primers 5'-CCTTCTTCTCGCTGGCACCGTT-3' (forward) and 5'-GGGCAGAACAGTTACAAGGGGCA-3' (reverse). The fold enrichment was calculated for each primer/antibody/treatment combination. First the normalized ChIP C_t values were calculated: $\Delta C_{t(\text{normalized ChIP})} = \{C_{t(\text{ChIP})} - [C_{t(\text{Input})} - \text{Log}_2$ (Input Dilution Factor)]}. The % Input was calculated: % Input $= 2^{(-\Delta\text{Ct [normalized ChIP]})}$. Lastly, fold enrichment was calculated: fold Enrichment = (% Input of antibody/% Input of IgG).

GENE EXPRESSION ANALYSIS

RNA was isolated from 3T3-F442a cell pellets and inguinal fat pads using the Invitrogen PureLink Micro-to-Midi RNA Total RNA Purification System with Trizol reagent and on column DNase I treatment. The Invitrogen Superscript III First-Strand Synthesis System for RT-PCR was used to synthesize cDNA (Life Technologies, Carlsbad, CA, USA). All RT-qPCR primers were obtained from Qiagen QuantiTect Primer Assays and used with Qiagen QuantiTect SYBR Green PCR Kits (Qiagen, Valencia, CA, USA). ChIP-qPCR primers were used with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Amplifications were performed in a Bio-Rad 96-well iCycler or myIQ real-time detection system using the appropriate QuantiTect or iQ SYBR Green cycling protocol. Changes in target gene expression were normalized to TATA box binding protein (Tbp) and ribosomal protein L4 expression (Rpl4). Relative transcript levels were calculated using the ΔΔCt method (36). Normoglycemic transcript levels were set to 100.

MOTIF ANALYSIS

Promoter sequences containing 500 bp upstream of the transcriptional start site were collected for human, mouse, and rat using the UCSC Genome Browser (37). No rat ortholog was found for Quiescin Q6. The human set was used as the main set and was supported by the mouse and rat ortholog sets. Three genes that were identified in the rodent adipocyte secretome but did not change in expression during insulin resistance were used as the negative set for human, mouse, and rat (29). Seven motif finding tools were used for primary motif finding: AlignACE (38), Bioprospecter (39), CONSENSUS(40), CUBIC (41), MDscan (42), MEME (43), and BOBRO (44). For each candidate, a position weight matrix and scoring matrix were generated (Table S1 in Supplementary Material). Corresponding transcription factor binding motifs were determined by analyzing the position weight matrix with TOMTOM (45). Conserved transcription factor binding motifs were confirmed using human and mouse sequences in rVISTA 2.0 (46).

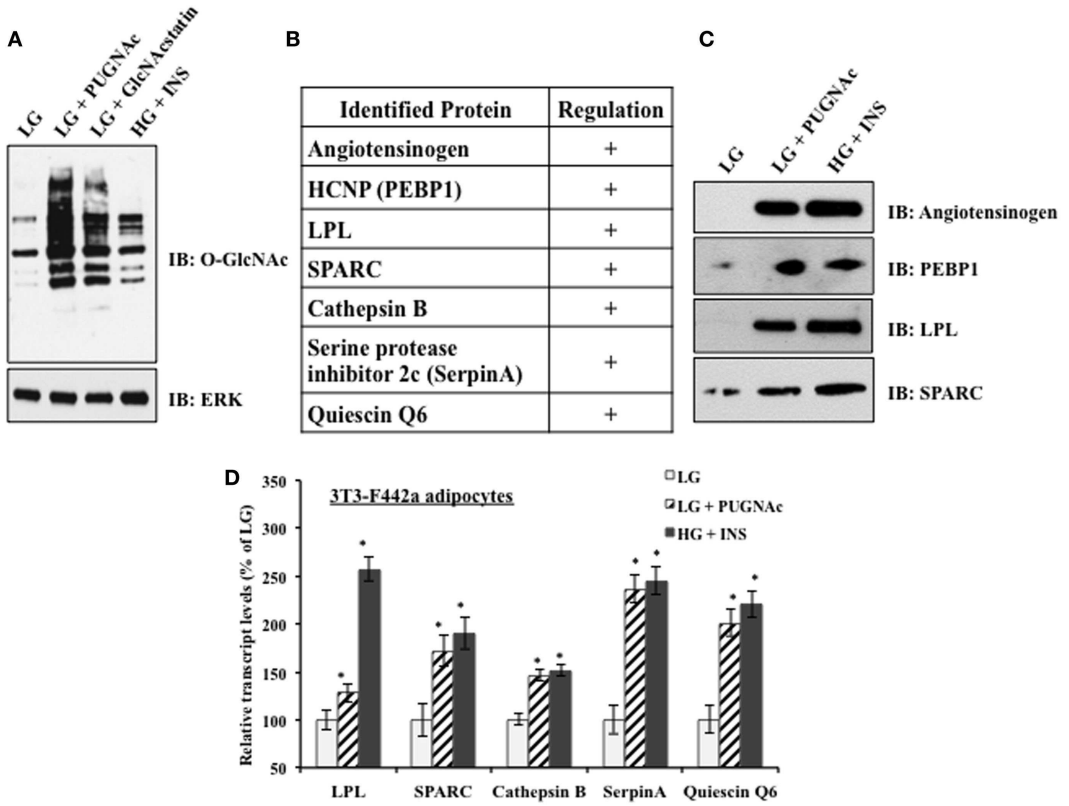
STATISTICAL ANALYSIS

All statistics were performed using the General Linear Model Analysis of Variance [GLM AOV, Statistix (Statistix 10.0, 2010, Tallahassee, FL, USA)]. Error bars represent the SEM of independent experiments. *P*-values under 0.05 were considered significant and represented using an * in all figures. All experiments shown were replicated three to five times.

RESULTS

THE INDUCTION OF INSULIN RESISTANCE IN 3T3-F442a ADIPOCYTES MODULATES SECRETED STEADY-STATE PROTEIN LEVELS AND TRANSCRIPT LEVELS IN THE SAME MANNER

3T3-F442a preadipocytes were differentiated into mature adipocytes before experimental treatments. Mature adipocytes were either maintained in insulin sensitive conditions [low glucose (LG)] or shifted to insulin resistant conditions by the classical treatment of high glucose and chronic insulin (HG + INS) to generate hyperglycemia and hyperinsulemia or by treatment with low glucose and the OGA inhibitors PUGNAc (LG + PUGNAc) or GlcNAcstatin (LG + GlcNAcstatin) to more specifically elevate global O-GlcNAc levels. **Figure 1A** shows that all insulin resistant conditions generated elevated global O-GlcNAc levels as evaluated by immunoblotting with an O-GlcNAc specific antibody. Previously, our group used shotgun proteomics to characterize the secreted proteome of rodent adipocytes and to identify



Identified Protein	Regulation
Angiotensinogen	+
HCNP (PEBP1)	+
LPL	+
SPARC	+
Cathepsin B	+
Serine protease inhibitor 2c (SerpinA)	+
Quiescin Q6	+

FIGURE 1 | Insulin resistant 3T3-F442a adipocytes display altered adipocytokine expression. **(A)** 3T3-F442a adipocytes were grown under insulin responsive (LG) or insulin resistant (LG with PUGNAc, LG with GlcNAcstatin, or HG with insulin) conditions as described in Section "Materials and Methods." Equal amounts of protein from whole cell lysates were separated by SDS-PAGE and Western blotting was performed using anti-O-GlcNAc (CTD110.6). Equal loading was confirmed by Western blotting with ERK2. **(B)** A partial list of rodent adipocytokines found to be regulated by insulin resistance based on proteomic quantification. $^{+}$Indicates that the protein expression is upregulated upon the induction of insulin resistance. **(C)** Proteomic quantification of protein expression was confirmed by Western blotting in 3T3-F442a adipocytes. Equal amounts of concentrated media were separated by SDS-PAGE and Western blotting was performed with the designated antibodies. **(D)** The steady-state transcript levels were evaluated using qPCR in 3T3-F442a adipocytes. Data are presented so that 100% represents the transcript level in the insulin responsive condition (LG). $^{*}P < 0.05$.

multiple proteins whose levels are modulated upon the induction of insulin resistance by indirectly and directly modulating O-GlcNAc levels in rodent adipocytes as described above (29). **Figure 1B** shows a shortened list of adipocytokines whose protein expression was found to be positively regulated by the induction of insulin resistance using quantitative proteomics in our previous studies. Here, we validated the relative levels of several of these secreted proteins using immunoblotting as an orthogonal method. 3T3-F442a adipocyte conditioned media from each treatment group was concentrated and buffer exchanged before immunoblotting with selected antibodies. **Figure 1C** shows that the regulation observed by quantitative proteomics is recapitulated by immunoblotting as an independent method. Since adipocyte insulin resistance was induced by either indirectly (HG + INS) or directly (LG + PUGNAc) altering O-GlcNAc levels, it is likely that O-GlcNAc is modulating the secretion of these adipocytokines. Since the secretion of many of the adipocytokines studied thus far is regulated at the level of transcription (22–26) and O-GlcNAc has been shown to modify and alter the function of many transcription factors (47), we hypothesized that the elevation of O-GlcNAc levels was regulating many of the identified adipocytokines at the level of transcription. **Figure 1D** shows that upon the elevation of O-GlcNAc levels and the induction of insulin resistance in 3T3-F442a adipocytes, the steady-state transcript levels of many of the identified secreted proteins, as measured by qPCR, reflect the modulation observed at the protein level.

THE INDUCTION OF INSULIN RESISTANCE MODULATES SECRETED PROTEIN STEADY-STATE TRANSCRIPT LEVELS IN A GENETIC INSULIN RESISTANT MOUSE MODEL

The mouse preadipocyte cell lines are a very useful system for studying adipocyte biology; however, their ability to secrete proteins at the high levels measured *in vivo* is impaired in many cases (48). Additionally, the complex paracrine interactions between adipocytes and the stromal-vascular cell fraction that comprises adipose tissue as well as the signaling between tissues in a whole animal are lost in adipocyte cell lines *in vitro* (49). Therefore, the regulation of adipocytokine transcript levels upon the induction of

insulin resistance was examined in a biologically relevant mouse model. The inguinal fat pads from severely insulin resistant 12-week-old male leptin receptor mutant (*db/db*) mice were used for transcript analysis. *db/db* mice produce leptin but fail to respond to it. The C57BL/6J *db/db* (6J) mice produce only the short-form leptin receptors (Ob-Ra, Ob-Rc, Ob-Rd) and the circulating form leptin receptor (Ob-Re) but not the long signaling form of the receptor (Ob-Rb). The C57BL/3J *db/db* (3J) mice produce only the circulating form leptin receptor (Ob-Re) (50). **Figure 2** shows the inguinal fat pad transcript levels in the *db/db* mouse models vary significantly from the *wt* mice and reflect the modulation shown at the transcript and protein levels in the 3T3-F442a adipocytes. All of the transcripts were elevated with the exception of the control gene, adipsin. Adipsin transcript levels have been shown to be downregulated in many models of rodent obesity (51).

THE INDUCTION OF INSULIN RESISTANCE MODULATES STEADY-STATE TRANSCRIPT LEVELS IN A DIET-INDUCED IR MOUSE MODEL

Evidence suggests that T2DM develops from a combination of genetic and environmental factors but the relative contribution of each is unclear (52). A monogenic genetic mouse model (*db/db*) does not represent the true genetic heterogeneity that is present in most cases of human T2DM (53). In addition, the genetic defect is in an adipocytokine pathway, which could lead to potentially confounding effects for this experiment (54). To address these concerns, a diet-induced insulin resistant mouse model was developed by feeding *ad libitum* sucrose and lard (HFHS) to approximately 9-week-old C57BL/6 mice as described in Section "Materials and Methods." After 3 weeks of treatment, the live weight as well as the wet weight of the inguinal, epididymal, mesenteric, and retroperitoneal fat pads was significantly increased in the HFHS mice compared to the mice on the normal chow diet (Figure S1A in Supplementary Material). The mice on the HFHS diet had elevated glucose levels and an attenuated response to insulin (Figure S1B in Supplementary Material). In addition, the mice displayed significantly elevated insulin levels (Figure S1C in Supplementary Material). After 3 weeks on the HFHS diet, the mice displayed mild insulin resistance and obesity so the inguinal fat pads were used for transcript analysis. Since the diet-induced insulin resistant mice were mildly obese and insulin resistant, we would expect the adipsin levels to only change slightly in contrast to the *db/db* mice, which were extremely obese and insulin resistant. **Figure 3** shows the transcript levels were significantly elevated in the HFHS mice inguinal fat pads for all genes excluding adipsin. The diet-induced insulin resistant mice transcript levels reflect the modulation shown at the transcript level in the *db/db* mouse fat pads and at the transcript and protein levels in the 3T3-F442a adipocytes. **Table 1** shows the relative increase in transcript levels in the insulin resistant mice (HFHS and 6J) compared to the transcript levels of the insulin sensitive mice (N and wt), which are set to 100%. Given that transcript level regulation is consistent for both insulin resistant mouse models, the transcript regulation observed in cell culture during insulin resistance is validated.

SP1 IS A COMMON CIS-ACTING ELEMENT FOR THE ADIPOCYTOKINE PROMOTERS AND THE O-GLCNAC MODIFICATION OF SP1 IS ALTERED DURING INSULIN RESISTANCE

We hypothesized that a common transcription factor or cofactor was responding to the elevation of O-GlcNAc levels and altering the transcription of the observed secreted proteins. Multiple complementary motif finding programs were used to analyze the same set of orthologous proximal promoters in order to find a more accurate set of regulatory motifs. Human, mouse, and rat promoters were used to identify conserved motifs, with the hope

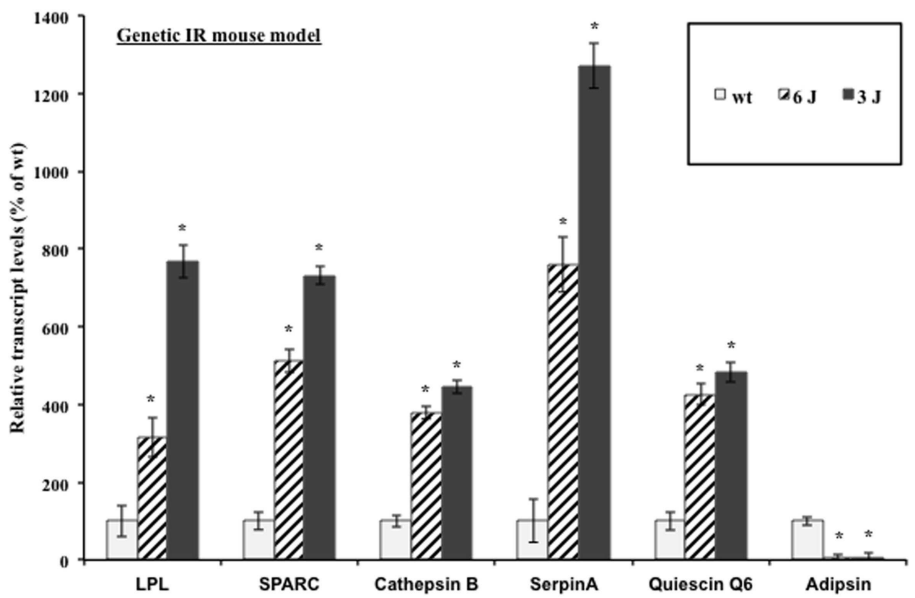


FIGURE 2 | Genetic insulin resistant mice display altered adipocytokine steady-state transcript levels. Inguinal fat pads from 12-week-old male *wt, 6J,* and *3J* ($n = 6$) mice were used for transcript analysis by qPCR. Data are presented so that 100% represents the transcript level in the insulin responsive condition (*wt*). $^*P < 0.03$.

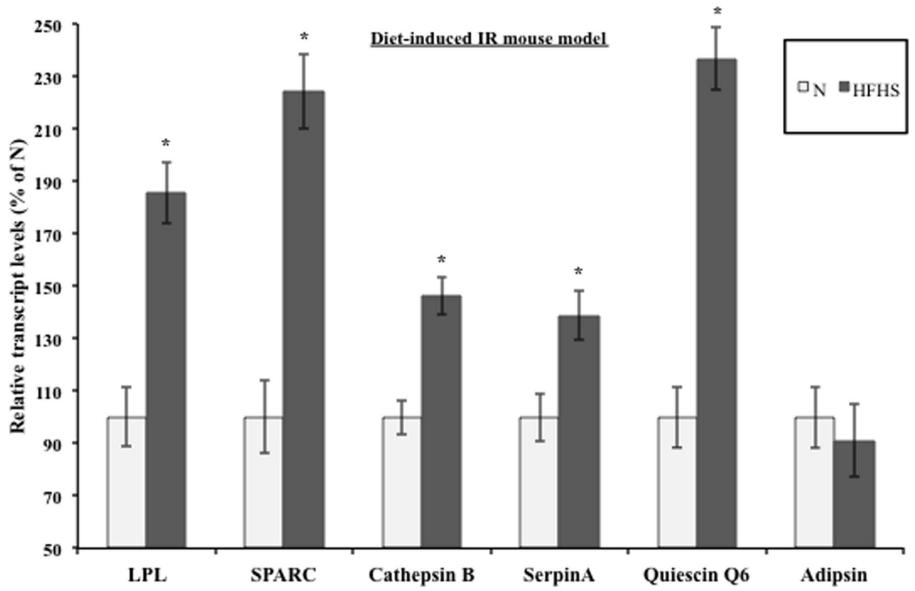


FIGURE 3 | Diet-induced insulin resistant mice display altered adipocytokine steady-state transcript levels. Inguinal fat pads from C57BL/6 mice fed normal chow (N) ($n = 4$) or a high fat, high sucrose (HFHS) ($n = 3$) diet for 3 weeks were used for transcript analysis by qPCR. Data are presented so that 100% represents the transcript level in the insulin responsive condition (N). $^*P < 0.05$.

Table 1 | Secreted protein transcript levels elevated in mouse models of insulin resistance.

Mouse model	LPL	SPARC	Cathepsin B	Serpin A	Quiescin Q6
Insulin sensitive	100	100	100	100	100
Diet-induced	185	224	146	139	237
Genetic	316	511	379	760	425

that the most important regulatory motifs would be under stronger evolutionary pressure (**Figure 4A**) (55). Twenty-four common putative regulatory motifs were identified using motif analysis programs as described in Section "Materials and Methods" (Table S1 in Supplementary Material). The putative regulatory motifs were compared to known transcription factor binding motifs. The Sp1-binding motif was found to match putative regulatory motif 3 (**Figure 4B**). The conservation of the Sp1 sites between human and mouse promoters was verified using rVista 2.0. Sp1 is relevant to adipocytokine transcription since it is a target of the insulin signaling cascade and many promoters of genes regulated during insulin resistance have Sp1 motifs (56–61). In addition, Sp1 is known to be dynamically modified by O-GlcNAc (47). Sp1 O-glycosylation is reported to be elevated in the liver, kidney, and adipose tissue of *db/db* mice (62). Many studies have associated the altered O-GlcNAc modification of Sp1 with altered transcriptional activation of target genes (63–68). **Figure 4C** shows that immunoprecipitated Sp1 has greater O-GlcNAc modification during insulin resistance in 3T3-F442a adipocytes. Both direct (LG + GlcNAcstatin) and indirect (HG + INS) modulation of O-GlcNAc levels trended toward elevated Sp1 O-GlcNAc modification although only the GlcNAcstatin reached statistical signifcance. The more modest O-GlcNAc modification seen in the HG + INS condition was most likely due to the more modest increase in global O-GlcNAc levels (**Figure 1A**).

SP1 AND O-GLcNAc MODIFIED PROTEINS ARE ENRICHED ON THE PROXIMAL SPARC AND LPL PROMOTERS DURING INSULIN RESISTANCE

We noticed that two of the identified motif three positions on the promoters corresponded with known biologically relevant Sp1-binding sites for LPL and SPARC. Since these sites are reported to be important for transcriptional activation, we wanted to determine whether Sp1 and O-GlcNAc modified proteins were enriched at these sites during insulin resistance in 3T3-F442a adipocytes. ChIP was performed with Sp1 and O-GlcNAc specific antibodies. Enrichment on the promoters was determined by analyzing purified DNA using qPCR with primers designed to amplify the region containing the Sp1-binding motif on either the LPL or SPARC promoter. **Figure 5** shows both of the promoter regions showed significant enrichment of both Sp1 and O-GlcNAc modified proteins during insulin resistant conditions. These results suggest that the elevation of global O-GlcNAc levels, either directly or indirectly, leads to increased O-GlcNAc modification of Sp1 and increased Sp1 enrichment on the SPARC and LPL proximal promoters. Since the O-GlcNAc antibody will bind any protein modified with O-GlcNAc, the enrichment of O-GlcNAc on the LPL and SPARC promoters could be due to O-GlcNAc modified Sp1 or potentially other O-GlcNAc modified proteins.

DISCUSSION

White adipose tissue plays an important role in maintaining energy homeostatis by mediating lipid flux and altering the secretion of adipocytokines. Adipocytokines can act in an autocrine, paracrine,

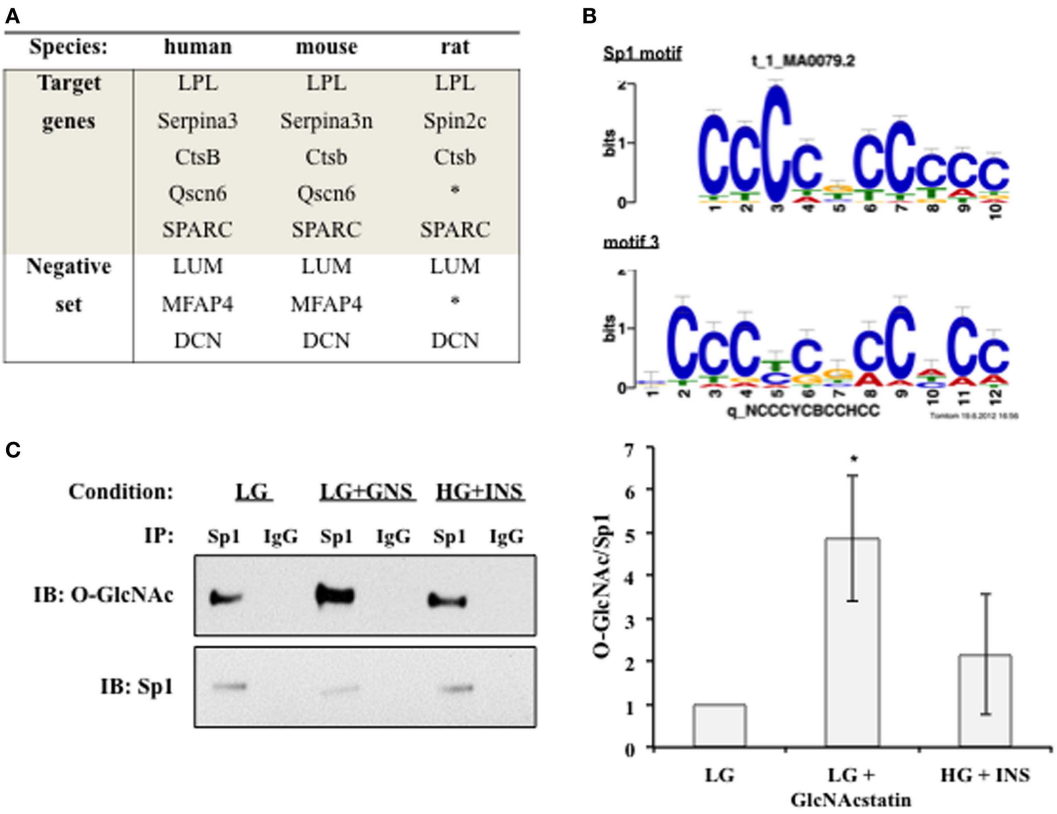


FIGURE 4 | An O-GlcNAc modified protein is identified as a common regulatory element. **(A)** The promoters of target co-regulated genes for human, mouse, and rat were analyzed for common regulatory motifs as described in Section "Materials and Methods." Three genes that were not co-regulated were used as a negative set to avoid identifying non-regulatory motifs. *Denotes no orthologous gene in rat. **(B)** TOMTOM was used to assign identified regulatory motifs to known transcription factor binding motifs. Regulatory motif 3 matches the Sp1 DNA binding motif with a *p*-value of 1.3×10^{-6}. **(C)** Whole cell lysates from insulin responsive and insulin resistant 3T3-F442a adipocytes were subjected to immunoprecipitation with anti-Sp1 or normal rabbit IgG followed by immunoblotting with anti-Sp1 or anti-O-GlcNAc (RL2). A representative immunoblot is shown (*right panel*). The ratio of O-GlcNAc modified Sp1 to total Sp1 was quantified using densitometry of independent experiments ($N = 4$).

or endocrine manner to regulate a variety of processes, including energy homeostasis (5). Genetic mouse models showing that the induction of insulin resistance in white adipose tissue induces whole-body insulin resistance have highlighted the importance of adipocytokines during insulin resistance (69–71). In addition, adipocytokines are implicated in many of the complications leading to and resulting from T2DM, especially the tissue remodeling during nephropathy, cardiovascular disease, and obesity (72).

Many of the secreted proteins we studied are extracellular matrix (ECM) modulators and associated with inflammatory states. SPARC is a modulator of cell – ECM interactions and has diverse roles in osteogenesis, angiogenesis, fibrosis, tumorigenesis, and adipogenesis (73). Cathepsin B is associated with ECM degradation, apoptosis, and inflammation (74). SerpinA is an acute phase response protein that is involved in inflammation (75). Quiescin Q6 is upregulated in pancreatic cancer and may promote tumor cell invasion by upregulating matrix metalloproteinases (76, 77). Involvement in tumorigenesis is another common theme for these adipocytokines. During obesity, extensive remodeling is required for the expansion of fat pads (78). These ECM modulators may play an important role in local tissue remodeling. Obese adipose tissue is associated with an inflammatory response, which may also be mediated in part by these adipocytokines (78–83).

In this study, we have attempted to better define the relationship between O-GlcNAc modification and adipocyte-secreted protein transcription during insulin resistance. Several studies have suggested that leptin and adiponectin are regulated primarily at the level of transcription in adipocytes (22–25, 84). We investigated whether the secreted factors that we identified by quantitative proteomics were similarly regulated after confirming the elevation via an orthogonal method, Western blotting, for several of these secreted proteins. We have also recently evaluated many of these secreted factors in human adipose tissue with similar findings (85). We found that the induction of insulin resistance in mouse adipocytes elevated transcript levels in the same manner as protein levels for several of the secreted proteins identified by proteomics (**Figure 1**). Although a role for the transcriptional regulation of adipocytokine secretion has been established for SPARC (86), there are conflicting reports for LPL (87–90), and it was not known whether Cathepsin B, Quiescin Q6, and SerpinA were transcriptionally regulated in adipocytes. In addition, the biological relevance of the transcriptional upregulation of the proteins

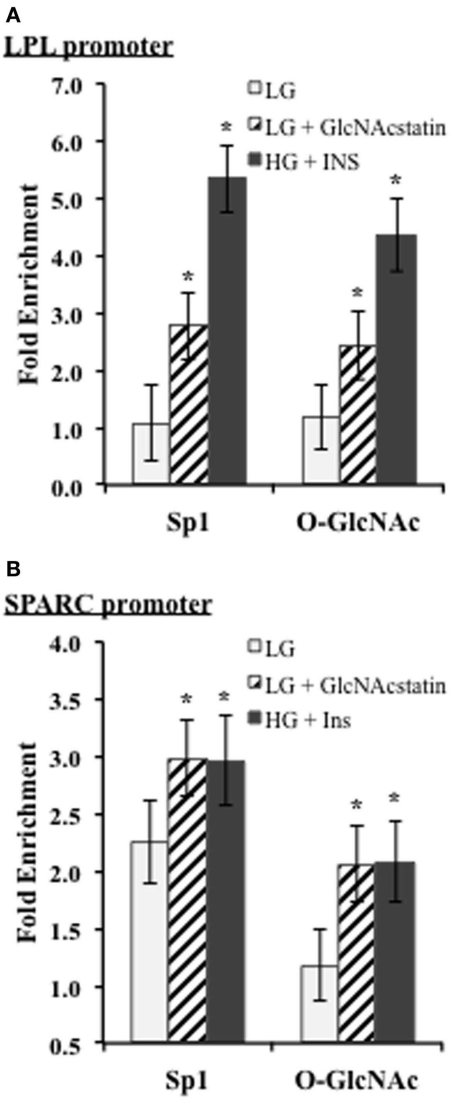


FIGURE 5 | ChIP analysis of conserved Sp1 sites on the SPARC and LPL promoters. Insulin responsive and insulin resistant 3T3-F442a adipocytes were subjected to chromatin immunoprecipitation with anti-Sp1, anti-O-GlcNAc (RL2), or normal IgG. Quantitative PCR was performed with primers designed to amplify the conserved Sp1-binding site motif on the LPL **(A)** or SPARC **(B)** promoter. Fold enrichment was calculated using% Input as described in Section "Materials and Methods." $*P < 0.05$.

during insulin resistance was verified using both a genetic and diet-induced mouse model of insulin resistance (**Figures 2** and **3**).

Several studies have suggested that adipocytokine expression is regulated by the HBP and O-GlcNAc. Infusions of metabolites that increased HBP flux into rats increased leptin expression (22, 91). Both GFAT and OGT transgenic mice displayed hyperleptinemia (16, 27, 28). GFAT transgenic mice also displayed decreased adiponectin levels (28). In primary human adipocytes and 3T3-L1 mouse adipocytes, HBP flux was shown to correlate with leptin expression (27, 92). Although many studies have manipulated the HBP, studies that manipulate O-GlcNAc levels more directly and examine adipocytokine expression have been lacking. We found that both the direct modulation of O-GlcNAc levels by the addition of OGA inhibitors and the indirect modulation of O-GlcNAc levels by hyperglycemia and chronic hyperinsulinemia in mouse adipocytes elevated transcript levels in the same manner as protein levels (**Figure 1**). It was not known whether inducing insulin resistance solely by raising global O-GlcNAc levels would regulate these secreted proteins at the level of transcription.

It is reasonable to assume that co-regulated genes have a similar upstream regulator. Since we found that the expression of these proteins was similarly regulated by both classical insulin resistance and by solely raising global O-GlcNAc levels, we hypothesized that O-GlcNAc was a regulator. O-GlcNAc has been proposed to be a "nutrient sensor" because the levels of the end product of the HBP, UDP-GlcNAc, are regulated by the flux of glucose, uridine, glutamine, and FFA's (93, 94). OGT is responsive to physiological levels of UDP-GlcNAc, so increased HBP flux results in globally elevated levels of O-GlcNAc modification (95). The regulation of OGT is complex and still being elucidated but it is clear that it has a preference for certain proteins and sites and does not universally add O-GlcNAc to all proteins (96–98). A large body of literature has shown that the O-GlcNAc modification plays an important role in transcriptional regulation. O-GlcNAc modifies transcription factors and cofactors, RNA Pol II, chromatin remodelers, and has even been identified as part of the histone code. O-GlcNAc modification of proteins can affect protein stability, protein–protein interactions, chromatin remodeling, transcriptional initiation and elongation, DNA binding, and localization (47, 99).

The secreted factors were regulated at the level of transcription, so we looked for common transcription factor binding motifs. After determining that Sp1 was a common *cis*-acting motif for these genes, we found that the O-GlcNAc modification of Sp1 trended toward an increased level during insulin resistance in mouse adipocytes (**Figure 4**). Sp1 has been implicated in the transcriptional regulation of LPL, SPARC, Cathepsin B, and SerpinA.

A role for Sp1 as a regulator of SPARC transcription has been established in transformed cells. The proximal promoter of SPARC contains several modified GC-boxes that are binding sites for Sp1 and/or Sp3. Sp1 and/or Sp3 are required for SPARC transcriptional activation in chickens, mice, and human beings (100–102). In chick embryonic fibroblasts, v-Jun represses SPARC promoter activation and initiates cell transformation by targeting the minimal promoter region. It was shown that v-Jun does not bind this DNA region directly but binds Sp1 and/or Sp3 to target promoter activation (101). c-Jun activates SPARC transcription in human MCF7 cells through the activation of Sp1 (100). In mammary carcinoma, Brg-1, a SWI/SNF chromatin remodeling complex ATPase, was shown to interact with Sp1 to activate SPARC transcription (102). Sp1's involvement in SPARC transcription in adipocytes has not previously been described.

Several studies have associated Sp1 and/or Sp3 with LPL transcriptional regulation. Interferon-γ (IFNγ) decreases macrophage LPL transcription by decreasing Sp3 protein levels and Sp1 DNA binding to sites in the 5′ UTR, which is mediate by casein kinase 2 (CK2) and Akt (103, 104). Transforming growth factor-β (TGF-β) represses macrophage LPL transcription through Sp1 and/or Sp3 sites in the 5′-UTR (105). Sp1 and/or Sp3 also bind an

evolutionarily conserved CT element (−91 to −83), also known as a GA box, in the proximal promoter. Sterols regulate LPL through a SRE site that is close to the CT element (89, 106). A T(−93)G SNP that is close to the CT element has been associated with a predisposition to obesity and familial combined hyperlipidemia in some studies in human beings. The minor allelic frequency is highly variable for difference ethnic populations and the SNP effect may be influenced by the synergistic effects of a Asp9Asn and T(-93)G haplotype that is present in some populations (107–110). People with both the Asp9Asn and T(-93)G mutations have been shown to have an increased risk of cardiac disease and decreased LPL activity in some studies (111–113). In the South African black population, the SNP was associated with mildly lower triglyceride levels and was associated with higher promoter activation in smooth muscle cells (108, 114). This is in contrast to other studies, which show that the mutation decreases Sp1 and/or Sp3 DNA binding leading to lowered transcriptional activation (106, 107, 109, 110). Sterol regulatory element-binding protein (SREBP) was found to act synergistically with Sp1 to activate the promoter in macrophages. Mutation of the CT element is also reported to decrease promoter reporter activity in 3T3-F442a pre-adipocytes (115). The importance of these Sp1/Sp3 binding sites has not been previously explored in mature adipocytes.

Both Sp1 and O-GlcNAc modified proteins were found to be significantly enriched in the region of the conserved Sp1 site on both the LPL and SPARC promoters (**Figure 5**). In our experiments in mature mouse adipocytes, Sp1 is most likely facilitating transcriptional activation. The studies described above have begun to shed light on the role of O-GlcNAc in modulating adipocytokine transcription through the modification of Sp1. Although Sp1 in ubiquitously expressed and often thought of as a housekeeping transcription factor, the diversity of Sp1 post-translational modifications and the wide-range of interaction partners can fine tune Sp1 activity in a context specific manner (116). Sp1 is subject to many forms of post-translational modification including phosphorylation, acetylation, sumoylation, ubiquitylation, and glycosylation. The sites of phosphorylation on Sp1 can either increase or decrease Sp1 DNA binding and transcriptional activation (117). Glycosylation can affect Sp1 stability, protein–protein interactions, DNA binding, degree of phosphorylation, and localization (47). These other modifications as well as the recruitment of other proteins to the promoters may explain the differences between HG + INS and LG + GNS. Sp1 has at least eight sites of O-GlcNAc modification, but the specific roles of each site is still being elucidated (118). Five sites of modification have been mapped to the DNA binding domain, and the mutation of these sites can disrupt Sp1 transcriptional activation in hepatocytes (62, 68). O-GlcNAc modification of the Sp1 activation domain inhibits Sp1 transactivation (64, 119). Since O-GlcNAc acts as a nutrient-flux sensor, many studies manipulate the glycosylation of Sp1 by manipulating nutrient flux. Studies using glycosylation site-specific Sp1 mutants would help to clarify the specific role of O-GlcNAc modification; however, determining the action of a specific glycosylation site could be complicated by the complex interplay between phosphorylation and O-GlcNAc modification as well as the presence of several other O-GlcNAc sites. In addition, site-specific Sp1 studies in adipocytes would be challenging since adipocytes are notoriously difficult to transfect. Other O-GlcNAc modified proteins could also be modulating the adipocytokine transcription since the O-GlcNAc enrichment on the promoters could be due to proteins other than Sp1. ChIP-reChIP would help to determine, which proteins are in complex on the promoters.

In conclusion, these experiments serve to identify a possible mechanism by which adipocytes respond to insulin resistance and regulate the expression of adipocytokines. Future work is aimed at identifying the specific function of the O-GlcNAc modification on Sp1 during insulin resistance in adipocytes. In addition, the mechanism of adipocytokine transcriptional upregulation in animal models should be investigated. Understanding the transcriptional regulation of adipocytokines by O-GlcNAc may provide therapeutic targets for normalizing the expression of adipocytokines during obesity and T2DM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fendo.2014.00223/abstract

Figure S1 | Characterization of the diet-induced IR mouse model. (A) After 3 weeks on either the HFHS diet ($n = 6$) or normal diet ($n = 6$), mice were weighed, sacrificed, and the liver and four fat pads were dissected and weighed. Data are presented so that 100% represents the weight of the normal diet mice. **(B)** Insulin sensitivity test was performed as in Section "Materials and Methods." **(C)** Trunk blood was used for an Insulin RIA. $^*P < 0.05$.

Table S1 | Common motifs for adipocytokine promoters.

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Disruption of O-GlcNAc cycling in *C. elegans* perturbs nucleotide sugar pools and complex glycans

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The carbohydrate modification of serine and threonine residues with O-linked beta-*N*-acetylglucosamine (O-GlcNAc) is ubiquitous and governs cellular processes ranging from cell signaling to apoptosis. The O-GlcNAc modification along with other carbohydrate modifications, including N-linked and O-linked glycans, glycolipids, and sugar polymers, all require the use of the nucleotide sugar UDP-GlcNAc, the end product of the hexosamine biosynthetic pathway (HBP). In this paper, we describe the biochemical consequences resulting from perturbation of the O-GlcNAc pathway in *C. elegans* lacking O-GlcNAc transferase and O-GlcNAcase activities. In *ogt-1* null animals, steady-state levels of UDP-GlcNAc/UDP-GalNAc and UDP-glucose were substantially elevated. Transcripts of genes encoding for key members in the HBP (*gfat-2, gna-2, C36A4.4*) and trehalose metabolism (*tre-1*, *tre-2*, *tps-2*) were elevated in *ogt-1* null animals. While there is no evidence to suggest changes in the profile of N-linked glycans in the *ogt-1* and *oga-1* mutants, glycans insensitive to PNGase digestion (including O-linked glycans, glycolipids, and glycopolymers) were altered in these strains. Our data support that changes in O-GlcNAcylation alters nucleotide sugar production, overall glycan composition, and transcription of genes encoding glycan processing enzymes. These data along with our previous findings that disruption in O-GlcNAc cycling alters macronutrient storage underscores the noteworthy influence this posttranslational modification plays in nutrient sensing.

Keywords: O-GlcNAcylation, nucleotide sugars, hexosamines, *C. elegans*/nematode, glycogen, trehalose

INTRODUCTION

Posttranslational modifications ranging from glycosylation to phosphorylation play critical roles in biological processes including protein localization, transcription, and cellular signaling [see Ref. (1) and references therein]. The posttranslational modification O-linked beta-*N*-acetyl glucosamine (O-GlcNAc) is ubiquitous throughout the nucleus and cytoplasm modifying over 4000 protein substrates including nuclear pore and transcription complexes, proteasomes, and kinases (2). The addition and removal of O-GlcNAc to serine and threonine residues is governed by two enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively (3). This dynamic cycling occurs faster than protein turnover poising O-GlcNAc to act as a signaling molecule (4).

UDP-GlcNAc, the activated nucleotide sugar utilized by OGT, is the end product of the hexosamine biosynthetic pathway (HBP). A metabolite responsible for sensing the nutrient status of the cell, UDP-GlcNAc is synthesized by a series of enzymes utilizing key metabolites including glucose, L-glutamine, acetyl-CoA, and UTP (5). Perturbation of the HBP is linked with modulation in insulin signaling and glucose toxicity [see Ref. (6) and references therein]. Indeed, globally decreased levels of UDP-GlcNAc can be profoundly damaging to mammals (7). The way in which the HBP plays a role in the modulation of insulin signaling and glucose toxicity is currently being defined with implications that O-GlcNAcylation plays an important mechanistic role [see Ref. (8) and references therein].

We previously demonstrated that *C. elegans* loss-of-function *ogt-1* and *oga-1* animals have altered insulin signaling and carbohydrate–fat metabolism (9). Importantly, *ogt-1* and *oga-1* null *C. elegans* are viable while loss of OGT and OGA activity in higher eukaryotes yields embryonic lethality (10, 11). *C. elegans* lacking OGT-1 activity exhibit no addition of O-GlcNAc to protein serine and threonine residues and animals without OGA-1 activity lack the capacity to remove the modification. In order to better understand the repercussions of impaired O-GlcNAc cycling in a whole organism, we chose to define the way in which these perturbations altered UDP-GlcNAc concentrations, glycan composition, and transcription of important metabolic enzymes. Among the most striking differences, we found that animals lacking OGT-1 or OGA-1 activities exhibit increases in UDP-HexNAc pools and differences in their overall glycan compositions compared to wild type (N2). Indeed, along with these changes, *ogt-1* null animals also show increased transcription of metabolic and HBP genes suggesting that the animals are modulating or attempting to compensate for the altered carbohydrate profiles. Although O-GlcNAc cycling has been linked to nutrient sensing, we provide the first evidence in a whole organism that O-GlcNAc plays an important role in modulating nucleotide sugar utilization and the steady-state

levels of transcripts encoding key HBP enzymes. We suggest that O-GlcNAc acts as a rheostat to fine-tune evolutionarily conserved components of *C. elegans* metabolism giving a defined model to study how changes in O-GlcNAc may impact human health.

MATERIALS AND METHODS

C. ELEGANS AND BACTERIAL STRAINS AND MAINTENANCE

The following *C. elegans* strains were used in this study: N2 Bristol (WT), *ogt-1 (ok430)*, *ogt-1 (tm1046)*, and *oga-1 (ok1207)*. The *ogt-1 (ok430)* and *oga-1 (ok1207)* strains were provided by the *C. elegans* Gene Knockout Consortium (CGC, Oklahoma Medical Research Foundation, Oklahoma City) and the *ogt-1 (tm1046)* was provided by the National Bioresource Project of Japan. All strains were backcrossed four times to N2 prior to use in experiments. The presence of the deletion alleles was confirmed by nested PCR primers as described by the CGC. The OP50 *E. coli* was cultured without antibiotic at room temperature in Luria–Bertani (LB) broth and plated on nematode growth media (NGM) plates or 2% agarose-topped LB plates. NGM plates were made with tryptone rather than peptone. *C. elegans* were maintained on NGM plates supplemented with OP50 *E. coli* and animals were manipulated using standard techniques (12, 13). Two percent agarose-topped LB plates were used for RNA isolation experiments only.

ISOLATION OF NUCLEOTIDE SUGAR

C. elegans strains were grown in large quantities starting from synchronous L1 stage and collected when animals reached the gravid adult stage (after approximately 72 h incubation at room temperature, 22°C). Animals were washed from NGM plates with water, washed twice with water, counted, and purged by rocking animals for 30 min in water at room temperature as previously described (9). Animals were isolated by centrifugation and the supernatant removed first by pipette and then by lyophilization. To 8 mg lyophilized worms, 0.75 ml cold 0.5 N perchloric acid was added. The suspension was vortexed vigorously for 20 s in an ice bath followed by centrifugation at 15,000 × *g* for 10 min at 4°C and the supernatant was collected. The remaining pellet was extracted second time and the two supernatants was pooled. Two hundred microliters of charcoal suspension (30 mg of Mallinckrodt charcoal/ml of 1 N perchloric acid) was added to the cold supernatant and stirred vigorously in an ice bath. The activated charcoal in acidic solution binds the nucleotide sugar and the sugars are then released in the alkaline solution in the following steps. After centrifugation at 15,000 × *g* for 10 min at 4°C, the supernatant was discarded. The charcoal pellet was eluted three times with 750 μL of a solution containing 50% ethanol + 1% NH_4OH. The EtOH solutions were isolated, pooled, frozen, and lyophilized. This experiment was performed in triplicate. Isolated sugars were analyzed by high performance anion exchange chromatography (HPAEC) with pulse amperometric detector (PAD) using a PA10 anion exchange column as described by Suriano et al. (14). Error bars represent SD of an experiment done in triplicate and *P* values were calculated by an unpaired Student's *t*-test. ns: $P > 0.05$; $^{*}P < 0.05$ compared to N2.

ISOLATION OF PNGase-SENSITIVE GLYCANS

Glycoprotein rich fractions were isolated from *C. elegans* at synchronous L4 larval stage by procedures described by Cipollo et al. (15, 16). Further processing was analogous to preparation described in the aforementioned papers with minor modifications. Isolated glycoproteins were treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin for 4 h at 37°C in 50 mM ammonium bicarbonate, pH 8.5 buffer. The reaction was stopped by boiling the samples twice for 10 min. The resulting peptides were isolated by acetone (80%) precipitation. N-glycans were released from these glycopeptides by incubating with PNGase A (Roche) and F (Prozyme) enzymes according to the manufacturers' protocols. PNGase-sensitive glycans were pooled and purified by passing through LudgerClean™ EB10 Glycan Cleanup Cartridge following the manufacturer's protocol. Resulting glycans were hydrolyzed with 2 M TFA for 4 h at 100°C to yield monosaccharides. Samples were evaporated to dryness and resuspended in 100 μL of deionized water. This evaporation and resuspension was repeated twice with the last 100 μL water suspension passed through a cation exchange resin to remove amino acids and peptides. A portion of this resulting monosaccharide-containing solution was profiled by HPAEC-PAD as described elsewhere (14, 17). To measure GalNAc and GlcNAc, the concentrations of monosaccharides galactosamaine (GalN) and glucosamine (GlcN) were measured by HPAEC as, immediately following their release, the *N*-acetyl sugars undergo quantitative deacetylation. Error bars represent SD of an experiment done in triplicate, and *P* values were calculated by an unpaired Student's *t*-test. ns: $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ compared to N2.

ISOLATION OF RNA FROM L4 STAGE *C. ELEGANS*

OP50 grown on agarose coated LB plates were seeded with synchronized L1 N2, *ogt-1 (ok430)*, and *oga-1 (ok1207)* strains. Animals incubated at room temperature (22°C) were collected after roughly 48 h when N2 animals reached a synchronous L4 stage. Although animals were synchronous at L1, we noticed that *ogt-1 (ok430)* animals had a difference in growth rate and we accommodated by plating them on OP50 4 h prior to the other strains. Collected L4 populations contained 97, 95, 80, and 60% L4 animals in N2, *oga-1*, *ogt-1 (tm1046)*, and *ogt-1 (ok430)* strains, respectively. The remaining animals ranged from stages L1 to L3. *C. elegans* were isolated from plates, thoroughly washed with water to remove bacteria, and worm pellets were stored −80°C for further use. RNA was isolated from each strain using a Qiagen RNAeasy Mini kit and quantified spectrophotometrically as described in Ref. (4, 18).

qRT-PCR ANALYSIS

RNA was treated with DNAse from Invitrogen to destroy contaminating genomic DNA in RNA sample (4, 18). SuperScript III (Invitrogen) was used to produce cDNA according to manufacturer's protocols with random primer from Promega. Following cDNA synthesis, quantitative real-time PCR (qRT-PCR) was performed using SYBR Green to quantitatively determine gene expression. RNA treated without reverse transcriptase served as the negative control. The experiment was performed in biological triplicate and samples were normalized to the control gene *act-4*. qRT-PCR data were analyzed by the comparative C_t method, error bars represent SD, and *P* values were calculated by

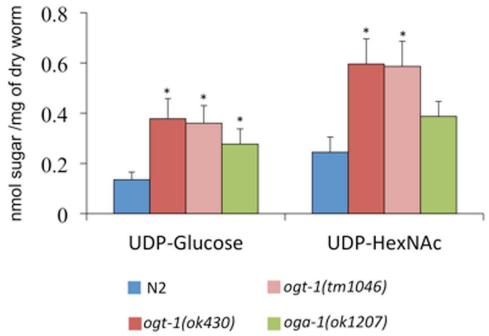

FIGURE 1 | O-GlcNAc cycling mutants exhibit increased concentrations of nucleotide sugar. UDP-nucleotide sugars were assessed by HPAEC-PAD detection. Compared to N2 animals, *ogt-1 (ok430)*, *ogt-1 (tm1046)*, and *oga-1 (ok1207)* animals have increased levels of UDP-nucleotide sugar. Error bars represent SD of an experiment done in triplicate. ns: $P > 0.05$; $^{*}P < 0.05$ compared to N2.

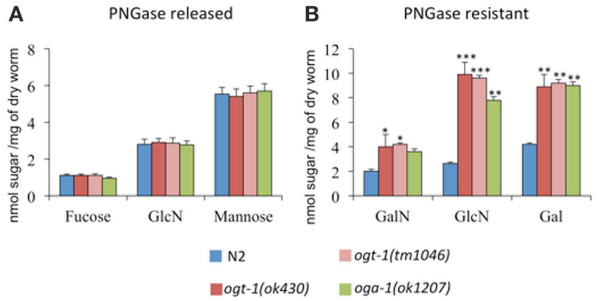

FIGURE 2 | *C. elegans* lacking OGT-1 and OGA-1 activity show increased GalN, GlcN, and Gal monosaccharides compared to N2 animals (B). Concentrations of monosaccharides galactosamaine (GalN) and glucosamine (GlcN) were measured by HPAEC as, immediately following their release, the *N*-acetyl sugars GalNAc and GlcNAc, respectively, undergo quantitative deacetylation. No statistically different changes were observed for the composition of PNGase A and F sensitive glycans for O-GlcNAc cycling mutants when compared to N2 animals **(A)**. Error bars represent SD of an experiment done in triplicate. ns: $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ compared to N2.

an unpaired Student's *t*-test. ns: $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ compared to N2.

GENE ACCESSION INFORMATION

Gene public name, Gene WormBase ID: *ogt-1*, K04G7.3, WBGene 00003858; *oga-1*, T20B5.3, WBGene00020596; *gfat-2*, F22B3.4, WBGene00009035; *gna-1*, B0024.12, WBGene00001646; *gna-2*, T23G11.2, WBGene00001647; C36A4.4, WBGene00007965; *tre-1*, F57B10.7, WBGene00006607; *tps-2*, F19H8.1, WBGene00006603; *gsy-1*, Y46G5A.31, WBGene00001793; Y73B6BL.4, WBGene00022 233; *act-4*, M03F4.2, WBGene00000066.

RESULTS

NUCLEOTIDE SUGAR LEVELS ARE INCREASED IN *C. ELEGANS* ogt-1 AND oga-1 MUTANTS

The *C. elegans* nucleocytoplasmic enzyme OGT-1 relies on a portion of the UDP-GlcNAc pool to glycosylate serine and threonine residues with O-GlcNAc. We sought to determine the upstream consequences when either OGT-1 activity, or the activity of its counterpart OGA-1, were absent in *C. elegans*. We initially profiled activated nucleotide sugar concentrations by isolating the nucleotide sugar pools, treating them with mild acid hydrolysis, and profiling the freed monosaccharides by HPAEC-PAD (**Figure 1**). We were intrigued to find that animals lacking either OGT-1 or OGA-1 activity demonstrated increased pools of UDP-glucose and *ogt-1* animals had increased UDP-GlcNAc/UDP-GalNAc (represented as UDP-HexNAc) pools. Loss of OGT-1 yielded animals with over a twofold increase in these nucleotide sugars compared to N2 while *oga-1* animals demonstrated a more modest increase. Importantly, the increased nucleotide sugar pool was similarly elevated in both *C. elegans ogt-1* null alleles that we used throughout the paper to further support our conclusions (**Figure 1**). Additional nucleotide sugars profiled (GDP-mannose, GDP-fucose, and UDP-galactose) were present in minimal amounts and their concentrations remained unchanged in *ogt-1 (ok430)*, *ogt-1 (tm1046)*, and *oga-1 (ok1207)* animals compared to N2 (data not shown).

PNGase RESISTANT MONOSACCHARIDES ARE INCREASED IN *ogt-1* NULL ANIMALS

UDP-GlcNAc is required for the production of a myriad of glycans, which suggests that changes in its concentration may affect glycan synthesis. Indeed, increased pools of UDP-GlcNAc have been correlated with the production of tri- and tetra-antennary N-glycans in mammary carcinoma cells (19). To determine whether complex glycan synthesis was affected by deletion of the *C. elegans* O-GlcNAc cycling enzymes, we assessed whole animal glycan composition. Briefly, isolated N-glycans were released by PNGase A and F, pooled, purified, and hydrolyzed with TFA. The resulting monosaccharide concentrations were assessed by HPAEC-PAD. Although *ogt-1* animals exhibit over a twofold increase in UDP-HexNAc levels compared to N2 (**Figure 1**), the released monosaccharides from N2, *ogt-1 (ok430)*, *ogt-1 (tm1046)*, and *oga-1 (ok1207)* animals' N-glycans remain largely similar (**Figure 2A**). Specifically, the ratio of mannose to GlcNAc suggests N-glycans form normally in these mutants.

Glycan structures not cleaved by PNGase A and F include O-linked glycans, glycolipids, and glycopolymers. To determine whether the PNGase-insensitive glycans were altered in *ogt-1* and *oga-1* null animals, the residual glycoprotein pellets were treated with 2 M TFA and the freed sugar pools were assessed. The *ogt-1* and *oga-1* null animals exhibited up to 3.7-fold higher GalNAc, GlcNAc, and galactose levels compared to N2 worms suggesting structural changes in glycan composition for PNGase-insensitive glycans when O-GlcNAc cycling is perturbed (**Figure 2B**).

GENES ENCODING ENZYMES INVOLVED IN UDP-GlcNAc SYNTHESIS AND METABOLISM ARE PERTURBED IN O-GlcNAc CYCLING MUTANTS

With nucleotide sugar pools and PNGase-insensitive glycan structures affected by the loss of *ogt-1* and *oga-1*, we hypothesized that the transcription of genes encoding enzymes involved in nucleotide sugar production and metabolism would be affected.

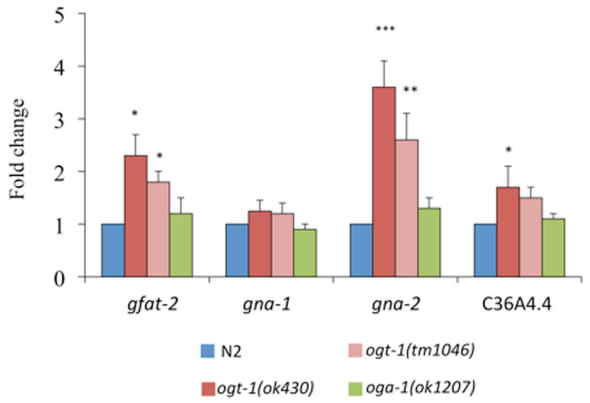

FIGURE 3 | Transcripts of genes encoding key HBP enzymes are elevated in *ogt-1* null animals. Animals lacking OGT-1 activity show increased transcripts of *gfat-2*, *gna-2*, and C36A4.4 genes while animals lacking OGA-1 activity are not statistically different from N2. Error bars represent SD of an experiment done in triplicate. ns: $P > 0.05$; $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$ compared to N2.

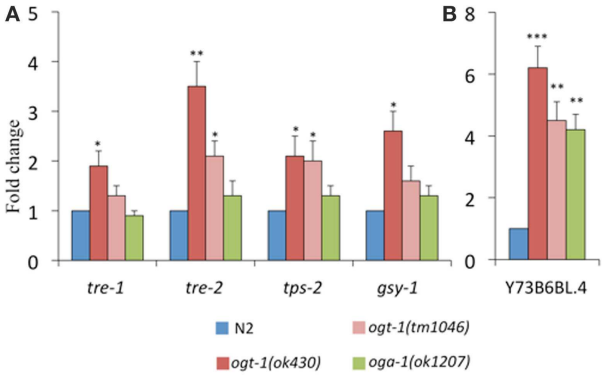

FIGURE 4 | Transcripts of genes encoding enzymes critical for metabolism are altered in *C. elegans* O-GlcNAc cycling mutants. *ogt-1 (ok430)* animals exhibit an increases in **(A)** *tre-1, tre-2, tps-2, gsy-1*, and **(B)** Y73B6BL.4 while *oga-1 (ok1207)* animals show an increase in only Y73B6BL.4. Error bars represent SD of an experiment done in triplicate. ns: $P > 0.05$; $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$ compared to N2.

To address this question, we examined the expression of key components of the HBP as well as trehalose and glycogen metabolism modules by qRT-PCR.

The following genes encode key enzymes in the HBP: *gfat-2* (F22B3.4 encodes the HBP's rate-liming glucosamine:fructose-6-phosphate aminotransferase – GFAT-2), *gna-2* (a glucosamine-6-phosphate *N*-acetyltransferase), and C36A4.4 (the putative UDP-GlcNAc pyrophosphorylase orthologous to human UAP1) (20). Transcripts for *gfat-2*, *gna-2*, and C36A4.4 are all elevated in *ogt-1 (ok430)* mutants while the transcript levels in *oga-1 (ok1207)* mutants remain unchanged (**Figure 3**). Elimination of OGT-1, an enzyme that utilizes a portion of the UDP-GlcNAc pool, affects the transcription of genes required for the synthesis of the same nucleotide sugar. This is not surprising as the HBP is exquisitely sensitive to nucleotide sugar concentrations and we suggest that gene transcription changes may be due to changes in feedback inhibition within the HBP. It is possible that there are no changes in HBP gene transcription for animals lacking OGA-1 activity as OGT-1 can still actively use UDP-GlcNAc.

Our previous work identified that total amounts of glycogen and trehalose, two important forms of energy storage, which can be enzymatically broken down to glucose, were increased in *ogt-1* and *oga-1* null animals (9). Here, we find that in comparison to N2 animals, *ogt-1* null animals exhibit increased transcription for enzymes involved in trehalose and glycogen metabolism (*tre-1*, *tre-2*, *tps-2*, and *gsy-1*) (**Figure 4A**). Interestingly, while *ogt-1 (ok430)* animals exhibit nearly twofold changes for all four genes, the *oga-1 (ok1207)* animals exhibited no statistical changes from N2 suggesting that changes in the ability of the animal to add and remove the O-GlcNAc modification have different biological consequences.

Triglyceride levels are significantly altered when OGT activity is perturbed in mice and *C. elegans* (9, 10, 21). To better understand our data showing 40–70% decreases in triglyceride levels in O-GlcNAc cycling mutants (9, 10), we assessed the level of transcription of Y73B6BL.4, a gene encoding a phospholipase (22). Indeed, this phospholipase increased over fourfold for both *ogt-1* alleles and the *oga-1 (ok1207)* animals (**Figure 4B**).

DISCUSSION

Cellular signaling depends on a series of protein posttranslational modifications working in concert to define protein localization, enzyme activity, and recognition events. Modifications ranging from glycosylation to phosphorylation are among the most widely recognized and defined posttranslational modifications with protein glycosylation being the most heterogeneous. The HBP produces a nutrient-sensitive nucleotide sugar, UDP-GlcNAc, that is utilized by glycosyltransferases in endo-membrane organelles (Golgi and endoplasmic reticulum) (23) as well as enzymes in the nucleus and cytoplasm (**Figure 5**). Optimal levels of UDP-GlcNAc are required for maintenance of cellular homeostasis in mammals with profound consequences including embryonic lethality resulting from loss of the nucleotide sugar synthesis (7, 24). The importance of this nucleotide sugar is evolutionarily conserved as loss or knockdown of enzymes required for synthesis of UDP-GlcNAc yields phenotypes including lethality in *C. elegans* (25–29).

Endo-membrane enzymes utilize UDP-GlcNAc to initiate N-glycosylation and further glycan branching (30). A portion of the UDP-GlcNAc pool is also used by OGT to glycosylate a myriad of nucleocytoplasmic targets ultimately influencing proteins' localization, activity, and/or folding [see Ref. (8, 31) and references therein]. Perturbation of O-GlcNAc cycling yields a wide range of biological consequences; indeed, OGT and OGA are essential for viability in mice and other higher eukaryotes (11, 32). In viable *C. elegans* animals with loss-of-function of *ogt-1* and *oga-1* genes, we note altered carbohydrate and lipid metabolism as well as severely deregulated insulin signaling (9, 10, 33). Furthermore, we found that *C. elegans* animals with altered O-GlcNAc cycling have striking changes in fertility and reproductive timing during glucose stress suggesting that O-GlcNAc acts as a buffer to sense glucose

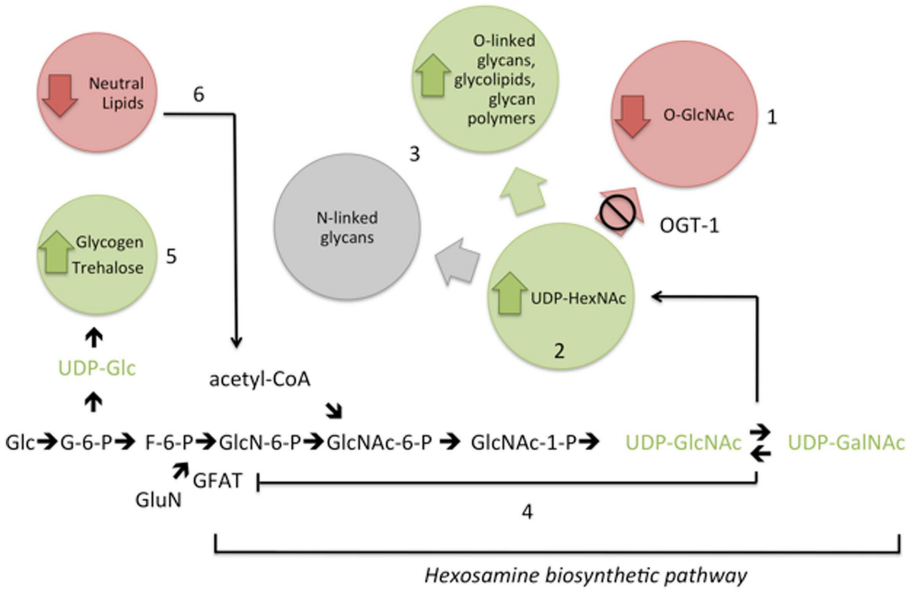

FIGURE 5 | Interference with the addition of O-GlcNAc (1) results in elevation of UDP-GlcNAc and UDP-GalNAc (UDP-HexNAc) levels (2). Compensatory metabolic changes include increased flux into some complex glycans (3). Feedback inhibition of GFAT (4) is insufficient to fully normalize UDP-HexNAc levels in the absence of OGT-1. In addition, with increased nucleotide sugar pools, there are changes in upstream complex sugar levels (5) and decreased macronutrient storage (6). These changes in cellular nucleotide sugar pools are associated with altered transcriptional regulation of genes encoding hexosamine biosynthetic pathway and macronutrient storage enzymes (see text). Metabolite levels are increased (green), decreased (red), remain the same (gray), or were untested (black). Glc, glucose; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; GluN, glutamine; GlcN-6-P, glucosamine-6-phosphate; GlcNAc-6-P, *N*-acetylglucosamine-6-P; GlcNAc-1-P, *N*-acetylglucosamine-1-phosphate; acetyl-CoA, acetyl coenzyme A.

availability (34). The work herein details the way in which perturbations in O-GlcNAc cycling influences changes in overall *C. elegans* carbohydrate composition as well as the transcription of key metabolic enzymes.

Our first efforts focused on identifying the ways in which changes to O-GlcNAc cycling affected nucleotide sugar concentrations and overall cellular carbohydrate structure. We noted that UDP-Glc and UDP-HexNAc (a combination of UDP-GlcNAc and UDP-GalNAc) were elevated 1.5- to 3-fold in the *ogt-1* and *oga-1* animals (**Figure 1**). Importantly, other nucleotide sugars such as GDP-mannose were not altered suggesting specific changes to the pool of activated sugars directly downstream of glucose metabolism. The elevation of UDP-Glc and UDP-HexNAc in both O-GlcNAc cycling mutants intrigued us. It is clear that in *ogt-1* loss-of-function animals, the UDP-GlcNAc pools would be elevated: the animals no longer utilize the activated nucleotide sugar to produce the O-GlcNAc PTM. Conversely, in animals that lack OGA-1 activity, while OGT-1 remains capable of utilizing UDP-GlcNAc to glycosylate protein substrates, the cycling of the PTM is likely altered and may influence the turnover of the nucleotide sugar pool. We speculate that this may explain the modest changes in nucleotide sugar concentrations and alterations in PNGAse-insensitive glycans but yield milder effects on the transcription of HBP members.

We hypothesized that among the consequences for even modest changes in activated nucleotide sugar pools, animals lacking *ogt-1* and *oga-1* would exhibit changes in N-glycan structures. Indeed, N-glycan structures have been shown to be ultrasensitive to UDP-GlcNAc concentration (19). We were, thus, surprised to find that N-glycan monosaccharide composition in both *ogt-1* and *oga-1* animals was indistinguishable from N2 animals (**Figure 2A**). These data suggest that the *C. elegans* mutants lacking O-GlcNAc cycling to have no global defect in constructing glycans sensitive to PNGase F and A. Interestingly, we found that glycans resistant to PNGase A or F (heterogeneous O-linked glycans, glycolipids, and glycan polymers) exhibited marked increases in galactose, GalNAc, and GlcNAc in both *ogt-1* and *oga-1* animals (**Figure 2B**). Recent work catalogs 14 types of mucin type O-linked glycans in the *C. elegans* WT strain with changes to O-linked glycans in another glycosyltransferase mutant (35). Future work will define the structural changes found in *ogt-1* and *oga-1* null animals' PNGase resistant glycans. The compiled data suggest that the inability to add and remove O-GlcNAc on nuclear and cytoplasmic targets has far-reaching cellular affects affecting global glycosylation. These effects are likely to be pleiotropic and result from changes in transcription, metabolic flux, signaling, or direct enzyme activation. Among the reasons for these changes could be both direct and indirect perturbation of enzymes required for nucleotide sugar synthesis or the speed at which glycosylated proteins traffic through the secretory pathway (36).

With evidence suggesting that O-GlcNAc cycling is a key player in sensing cell nutrient status from our work and the work of others [see Ref. (31, 37) and references therein], we next hypothesized that the transcription of critical players in the HBP would be altered in the *ogt-1* and *oga-1* animals. The first, and rate limiting, enzyme of the HBP is GFAT, which is responsible for the conversion of

fructose-6-phosphate to glucosamine-6-phosphate (**Figure 5**). To note, the activity of GFAT is modulated by UDP-GlcNAc itself through a feedback mechanism to reduce HBP flux (38). Two glucosamine-6-phosphate *N*-acetyltransferases (*gna-1* and *gna-2*) are required for an intermediary step in the HBP, and C36A4.4 is the presumptive pyrophosphorylase required for the last step in the synthesis of UDP-GlcNAc. We examined the expression levels for *gfat-2*, *gna-1*, *gna-2*, and C36A4.4 and noted that only in *ogt-1* mutants were there statistically significant changes in transcription for three of the four transcripts (**Figure 3**). Although feedback inhibition should occur in *ogt-1* animals due to increased UDP-HexNAc levels, it is insufficient to normalize the nucleotide sugar pool with increased *gfat-2* transcription. Moreover, these findings were surprising as PNGase-insensitive glycans were altered in both mutants suggesting a complex interplay between nutrient flux and appropriate substrate O-GlcNAc modification. (37).

Given that the O-GlcNAc cycling mutants exhibit variations in activated nucleotide sugar pools, changes in glycosylation (**Figures 1** and **2**), and increased glycogen and trehalose storage (9), we next assessed the levels of transcription for genes encoding enzymes involved in trehalose and glycogen metabolism. *C. elegans* encodes four putative glycoside hydrolases – enzymes responsible for catalyzing the conversion of trehalose to glucose – including *tre-1* and *tre-2*. *tps-2* encodes for one of two enzymes responsible for trehalose-6-phosphate synthesis and *gsy-1* is ortholog to the human glycogen synthase 1. All four of these transcripts were found to be elevated in *ogt-1* mutants while the levels remain unchanged in *oga-1* animals (**Figure 4**). These findings are consistent with our previous reports suggesting major changes in trehalose and glycogen metabolism upon genetic interference with O-GlcNAc cycling. The present findings suggest that these changes are associated with increased metabolic flux to produce UDP-Glc and with transcriptional changes in the transcripts encoding the relevant enzymes mediating interconversion.

Triglyceride levels have been shown to correlate with perturbations in O-GlcNAc cycling in both mice and *C. elegans* (9, 10, 21) and free fatty acids, usually derived from triglycerides or phospholipids, are known to be potent HBP modulators (39). Our previous work demonstrated that triglyceride levels are decreased by 70% in *ogt-1* and 40% in *oga-1* compared to N2 (9). These data suggest that either the production of triglycerides was hampered or their hydrolysis was increased. To test whether triglycerides are catabolized more rapidly in the O-GlcNAc cycling mutants, we assessed the transcriptional expression of Y73B6BL.4, a gene encoding for a phospholipase. We noted a significant increase in transcription for Y73B6BL.4 in both O-GlcNAc cycling mutants supporting that the decrease in triglyceride levels for both *ogt-1* and *oga-1* animals is likely associated with increased hydrolysis.

Perturbations of OGA activity in cell culture (40–42), loss of OGA-1 activity in *C. elegans* (9, 10), and OGT overexpression in mouse liver or fat promotes insulin resistance (43). Furthermore, loss of OGT-1 yields insulin sensitivity in *C. elegans* and altered lipid and carbohydrate metabolism (9, 10). Together, these data support a strong role for O-GlcNAc in insulin signaling metabolism maintenance. Our results reveal that loss of *ogt-1* and *oga-1* changes the nucleotide sugar pools and the production of PNGase-insensitive glycans. These changes along with altered transcriptional expression of genes encoding key HBP and metabolic enzymes in *ogt-1* null animals suggest that the addition of O-GlcNAc to appropriate target proteins is critical for appropriate HBP flux. Additional roles of OGT, including its non-catalytic role in protein–protein interactions (44), could also influence this signaling paradigm. We propose that with O-GlcNAc cycling profoundly affecting the HBP, *C. elegans* is an excellent model to studying metabolic changes associated with insulin signaling in viable *ogt-1* and *oga-1* null alleles (**Figure 5**). Using *C. elegans ogt-1* and *oga-1* animals, we will be able to further define the molecular details of the HBP's role in insulin resistance.

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Multiplexed detection of *O*-GlcNAcome, phosphoproteome, and whole proteome within the same gel

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The cellular diversity of proteins results in part from their post-translational modifications. Among all of them, the O-GlcNAcylation is an atypical glycosylation, more similar to phosphorylation than classical glycosylations. Highly dynamic, reversible, and exclusively localized on cytosolic, nuclear, and mitochondrial proteins, O-GlcNAcylation is known to regulate almost all if not all cellular processes. Fundamental for the cell life, O-GlcNAcylation abnormalities are involved in the etiology of several inherited diseases. Assessing to O-GlcNAcylation pattern will permit to get relevant data about the role of O-GlcNAcylation in cell physiology. To get understanding about the role of O-GlcNAcylation, as also considering its interplay with phosphorylation, the *O*-GlcNAc profiling remains a real challenge for the community of proteomists/glycoproteomists. The development of multiplexed proteomics based on fluorescent detection of proteins permits to go further in the understanding of the proteome complexity. We propose herein a multiplexed proteomic strategy to detect *O*-GlcNAcylated proteins, phosphoproteins, and the whole proteome within the same bidimensional gel. In particular, we investigated the phosphoproteome through the ProQ Diamond staining, while the whole proteome was visualized through Sypro Ruby staining, or after the labeling of proteins with a T-Dye fluorophore. The *O*-GlcNAcome was revealed by the way of the Click chemistry and the azide–alkyne cycloaddition of a fluorophore on GlcNAc moieties. This method permits, after sequential image acquisition, the direct in-gel detection of *O*-GlcNAcome, phosphoproteome, and whole proteome.

Keywords: O-GlcNAcylation, phosphorylation, proteomic analysis, 2D-electrophoresis, click-chemistry, multiplexed proteomics technology, fluorescent dyes

BACKGROUND

O-GlcNAcylation got 30 years old, the "age of reason." Since its discovery in 1984 by Gerald W. Hart (1, 2), O-GlcNAcylation was demonstrated by turn to be involved in numerous cellular processes, in particular, transcription, translation, signal transduction, proteasomal degradation, cellular stress, and so on (3–5). Nowadays, no one could refuse that O-GlcNAcylation is a key modulator in almost all if not all cellular processes. Furthermore, a dysregulation of O-GlcNAcylation cycling is associated to the physiopathology of several acquired diseases, such as cancers, type 2 diabetes, neurodegeneration, or cardiovascular disorders (6–9). The *O*-GlcNAc profiling, assessing to O-GlcNAcylation pattern and the quantification of variation of O-GlcNAcylation on proteins, remains an important challenge for the understanding of the role of this atypical glycosylation on the regulation of cellular processes or on the physiopathology of several inherited diseases.

Several antibodies directed against *O*-GlcNAc moieties are currently available [detailed in Ref. (5, 10)], enhancing greatly the probe and/or the enrichment of *O*-GlcNAcylated proteins. Classical methods of quantification of O-GlcNAcylation variation on given proteins are based on immunoprecipitation coupled to western blot analysis. Thus, upstream enrichment of *O*-GlcNAc bearing-proteins (through immunoprecipitation or lectin enrichment) followed by antibody-based detection of a protein of interest through western blot analysis remains a common practice to quantify relative changes of *O*-GlcNAc level on a target protein. This method remains a suitable tool for "oriented" investigation about the role of O-GlcNAcylation in a given cellular pathway. However, while largely and routinely used in laboratories, this classical approach suffers from an important limitation due to the selection of the proteins of interest by the researchers: only a slight number of proteins could be considered.

Furthermore, it is well-admitted that O-GlcNAcylation could not be considered alone, because of its dynamic interplay with phosphorylation (4, 11–13). This interplay could be investigated using the approach described above with minor changes, the antibody used in western blot being directed against the phospho-epitope of the protein. However, while many phospho-specific antibodies are currently available (for example, those directed against proteins from key intracellular processes), several proteins known to be phosphorylated suffer from the lack of a specific antibody directed against their phosphorylated epitope. Thus, we have recently coupled this immunoprecipitation/western blot methodology with the Phos-Tag electrophoresis to quantify the variation of O-GlcNAcylation on proteins separated according to their phosphorylation status (14). Interestingly, using a Phos-Tag

acrylamide incorporated directly into the monodimensional gel, different states of a given protein could be separated according to the number of phosphate moieties and the variation of O-GlcNAcylation could be determined on each phosphorylated form of the protein.

Indeed, to gain in understanding of O-GlcNAcylation dynamics occurring during cell or tissue status changes, proteomic analysis remains a method of choice to undergo changes in glycosylation level of proteins. Despite the fact of intrinsic limitations of 2D-electrophoresis (analysis of membrane proteins, divergent proteins expression in cells or tissues, high-chemical diversity of proteins . . .) (15), bidimensional electrophoresis enables to get relevant information through the cartography of the proteome at a given time and under particular physiological conditions, and is a powerful strategy to characterize multiple modified proteins (16). The consideration of *O*-GlcNAcome map was recently successfully investigated using a gel-based strategy. Proteins were separated on 2D-gels and were transferred on membrane for detection of *O*-GlcNAc moieties using CTD110.6 or RL-2 antibodies or lectins, leading to the identification of *O*-GlcNAcylated proteins, and/or those presenting a modulation in their O-GlcNAc level (17–20). Based on western blot analysis, this kind of approach could be coupled to detection of phosphoproteome using antibodies directed against the phosphoamino acids (21). One of the major difficulties in this kind of approach is the alignment between the 2D-western blot and Coomassie- or silver-stained 2D-gels using images software. To avoid this difficulty, fluorescent detection of proteins in gels is gaining popularity and large-scale use since it gains in reproducibility and its linear dynamic range of detection. In this way, the Van Eyk group's assessed the N-linked and O-GlcNAcylation in human and simian immunodeficiency viruses using a 2D-gel approach, and a detection of glycosylated proteins using the ProQ Emerald staining (22). In a previous study, the use of this fluorescent dye was coupled to ProQ Diamond staining in order to detect glycosylated and phosphorylated forms of proteins, the whole proteins pattern being detected using Sypro Ruby staining (23).

We propose herein a multiplexed proteomic strategy to detect *O*-GlcNAcylated proteins, phosphoproteins, and the whole proteome within the same gel. Detection of *O*-GlcNAcylated proteins was done after labeling of sugar moieties by a fluorophore (TAMRA or Alexa Fluor® 488), the (3 + 2) azide–alkyne cycloaddition of the fluorophore required the preliminary incorporation of an azide function on the *O*-GlcNAcylated moieties. We compared the metabolic incorporation of GlcNAz (azido-modified *N*-acetylglucosamine) moieties and the labeling of *O*-GlcNAcylated proteins with GalNAz (azido-modified *N*-acetylgalactosamine) through the engineered β-1,4-galactosyltransferase (Y289L GalT). While the detection of proteins phosphorylated on serine, threonine, and tyrosine residues was previously performed with success, in particular, in view of a bottom-up proteomic strategy (24–26), we detected the phosphoproteome using the ProQ Diamond dye. Finally, the global proteome was detected through the fluorescent dye Sypro Ruby, as it was described in classical multiplexed approaches, or after labeling of proteins using the T-Dye. The sequential image acquisitions permitted, from only one gel, a direct visualization of *O*-GlcNAcylated proteins, phosphorylated proteins, and the whole proteins pattern.

DETAILED EXPERIMENTAL PROCEDURES

C2C12 CELL CULTURE

Proliferation and differentiation of C2C12 cells

The C2C12 mouse myoblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). C2C12 myoblasts were grown to 80–90% confluence in Dubelcco's Modified Eagle Medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and 1% antibiotics/antimycotics, at 37°C in a 5% CO_2-humidified atmosphere. They were then induced to differentiate into myotubes by switching to DMEM containing 2% heat-inactivated horse serum (differentiation medium, DM). The shifting time to DM was assigned to day 0 of differentiation. Media were changed every 48 h, and myotubes formation was monitored daily. All experiments were performed on 5-day differentiated myotubes, this state of differentiation, corresponded to mature myotubes, was chosen according to preliminary experiments.

Metabolic labeling

In certain experiments, 50 μM of Ac_4-GlcNAz (tetraacetylated *N*-azidoacetylglucosamine, Molecular Probes), diluted in DMSO, was added in cell medium for 48 h. Control condition corresponded to cells incubated with DMSO alone (vehicle condition). It is noteworthy that we evaluated the efficiency of the metabolic incorporation of Ac_4-GlcNAz after coupling the GlcNAz moieties to Alexa Fluor® 488 fluorophore, as well as cell viability. Several concentrations of Ac_4-GlcNAz and treatment durations were tested.

Cell viability test

C2C12 myoblasts (20,000 cells/well) were grown in 96-well plates and then differentiated in myotubes as described above. Cell viability was assessed by methylthiazoletetrazolium (MTT) assay. Briefly, myotubes were rinsed with PBS to remove the interfering phenol red providing from DMEM media. Fifty micrograms of MTT in 100 μl of PBS was added to each well for 4 h at 37°C. MTT was then removed, and replaced by 100 μl of DMSO to dissolve the resulting formazan. Absorbance was quantified at 570 nm on micro-plate reader.

PROTEIN EXTRACTIONS

Whole cellular extract

C2C12 myotubes were rinsed three-times in cold PBS. They were then scrapped in cold RIPA lysis buffer (10 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% TritonX-100; 0.5% sodium deoxycholate; 0.1% SDS) or in NP-40 buffer (20 mM Tris-base, pH 8.0; 150 mM NaCl; 1% NP-40), both containing anti-proteases (Complete EDTA-free, Roche Diagnostic), anti-phosphatases (Phos-Stop, Roche Diagnostic), and 50 μM PUGNAc [*O*-(2-acetamido-2-deoxy-D-glucopyranosilidene)amino-*N*-phenyl-carbamate, Sigma]. Proteins extracts were rapidly sonicated using ultra-sonic cell disruptor, and then homogenized with gentle agitation for 1 h at 4°C. Protein estimation of these whole cellular extracts was done using Bradford assay (Biorad).

Protein subfractionation

In some cases, proteins extracts were subfractionated as previously described (27). Briefly, myotubes were scrapped in cold cytosol lysis buffer (50 mM Tris/HCl, pH 7.5; 5 mM EGTA; 2 mM EDTA; 5 mM DTT; 0.05% digitonin) containing the inhibitors as described just above. Cell lysates were then centrifuged at 9,500 rpm for 30 min at 4°C, and supernatants (corresponding to the cytosol-enriched fraction) were carefully removed while the pellets (included the membrane-enriched fraction and the myofilament fraction) were discarded. The protein content of the cytosol-enriched fraction was assayed using the Bradford's method.

PROTEIN DESALTING

Chloroform/methanol precipitation

Briefly, the sample volume was adjust to 200 µl. Six hundred microliters of methanol were added and sample was vortexed. After addition of chloroform (150 µl volume) and a brief vortex, 400 µl of water were finally added, and sample was briefly vortexed. After centrifugation at 13,000 rpm for 5 min, the upper aqueous phase was carefully discarded while the interface layer (corresponded to proteins precipitate) was leaved intact. A volume of 450 µl of methanol was added, then the sample vortexed before being centrifuged at 13,000 rpm for 5 min. The supernatant was removed and the pellet dried. To ensure the later resolubilization of the pellet, the use of speed-vacuum was avoided, and the sample was air-dried for few minutes.

Zeba spin column

In some cases, desalting was performed using the Zeba Spin Desalting Column (Thermo Scientific) with a cut-off of 7 kDa. Column was prepared by a simple centrifugation at 1500 g for 1 min, followed by the deposit of proteins sample to the top of the resin. A stacker of ultrapure water was applied to ensure maximal protein recovery.

ENZYMATIC AND CHEMICAL DEGLYCOSYLATION

Before deglycosylation reaction being performed, samples were desalted using the Zeba Spin Column. Each reaction was performed on 200 µg of proteins from whole cellular extract as well as on proteins from cytosol-enriched fraction. Anti-proteases were added to reaction mixtures. Quantity and incubation time were determined for each deglycosylation protocol to ensure a total deglycosylation. Once reaction was achieved, proteins were precipitated using the chloroform/methanol protocol as described above for each deglycosylation protocol.

Peptide: N-glycosidase F (PNGase F) deglycosylation

Proteins were denatured by boiling 10 min in SDS-containing denaturing buffer (10× Glycoprotein Denaturing Buffer, New England Biolabs, NEB). SDS, which could lead to an inhibition of glycosidase activity, was then neutralized by adding NP-40 (10% NP40 buffer, NEB). The 10× G7 reaction buffer (NEB) was finally diluted to a final concentration of 1×. Five hundred units of PNGase F (NEB) were added, and the reaction mix was incubated for 4 h at 37°C.

Beta-elimination

The GlycoProfile™β-Elimination kit (Sigma-Aldrich) was used to release *O*-glycans from proteins. It removes efficiently and specifically O-linked carbohydrates from glycoproteins without protein degradation (28), permitting consequently downstream proteomics analyses. The β-elimination reagent mixture was prepared as described by the manufacturer, and added equal to 20% of the sample volume. Incubation was performed at 4°C for 8 h. This incubation time was chosen since shorter reaction time was insufficient to be efficient on deglycosylation, whereas longer reaction time (or incubation at room temperature) leads to a slight protein degradation observed by SDS-PAGE profile (data not shown), which is incompatible with the downstream proteins analysis. Once reaction achieved, 1 M HCl was added to bring pH to 6–8.

β-*N-acetyl-hexosaminidase*

The removal of β-linked *N*-acetyl-hexosamine was performed enzymatically using the β-*N*-acetyl-hexosaminidase (NEB). This enzyme was chosen since it did not lead to protein degradation as we observed for enzymes from other manufacturers. The G2 buffer (NEB) was added to proteins sample, as well as 50 U of enzyme. Reaction was performed overnight at 37°C.

PROTEINS LABELING

Labeling and/or coupling of O-GlcNAcylated proteins

Galactosyltransferase labeling. Two hundred micrograms of proteins were chloroform/methanol precipitated. Resulting pellet was resuspended in 20 mM HEPES, pH 7.9 added with 1% SDS, and heated at 90°C for 10 min. Sample was then homogenized at room temperature to ensure the solubilization of proteins. To label *O*-GlcNAcylated proteins with GalNAz, the Click-iT™*O*-GlcNAc Enzymatic Labeling System was used (Molecular Probes). Briefly, Gal-T1 (Y289L) was incubated with proteins in labeling buffer (containing 20 mM HEPES, pH 7.9; 50 mM NaCl; 2% NP-40; 5.5 mM $MnCl_2$; 25 µM UDP-GalNAz), according to manufacturer's recommendations. Reaction was performed at 4°C under gentle agitation for 20 h. All reagents were provided in the kit. Once labeling achieved, proteins were chloroform/methanol precipitated.

Note that the volume of each reagent was adjusted for higher proteins quantity, i.e., when 500 µg of proteins were labeled. This quantity of proteins was suitable to detect with a good sensitivity the cytosolic *O*-GlcNAcome on 2D-gel, according to preliminary experiments.

Fluorophore coupling. All coupling reactions were performed using commercially available kits on proteins metabolically labeled with GlcNAz, or on proteins enzymatically labeled with GalNAz. Briefly, proteins pellet was resuspended in 50 mM Tris/HCl, pH 8.0, 1% SDS. The Click-It Reaction Buffer containing the fluorophore was added, followed by $CuSO_4$ (2 mM final concentration) and Click-iT™ Reaction Buffer Additives 1 then 2. Incubation was performed under rotation end-over-end for 20 min in dark and at room temperature. Once coupling performed, sample was chloroform/methanol precipitated. A second step of methanol wash was added to remove the residual reaction components. The pellet was heated at 70°C in Laemmli buffer for monodimensional

gel electrophoresis, or diluted in solubilization buffer, heated at 37°C for 10 min for bidimensional gel electrophoresis.

When the coupling was performed with TAMRA, the Click-iT™ Protein Analysis Detection Kit was used (Molecular Probes). When coupling was done with Alexa Fluor® 488, the Click-iT® Protein Reaction Buffer Kit, and the Alexa Fluor® 488 alkyne (Molecular Probes) were used. Note that Alexa Fluor® 488 alkyne was diluted in DMSO at a concentration of 4 mM, for a final concentration of 40 μM in the Click-It Reaction Buffer.

Labeling of whole proteins

The whole proteins pattern was labeled using the T-Red 310 fluorescent chromophore (T-Dye Series, NH DyeAGNOSTICS). Briefly, 100 μg of proteins were desalting using Zeba Spin Column. The T-Dye, diluted in T-Dye solvent, was added to proteins sample, and then incubated for 30 min on ice. The proteins label with T-Red 310 were then mixed with 400 μg of non-labeled proteins from the same biological sample, and submitted to chloroform/methanol precipitation.

ELECTROPHORESIS

Monodimensional gel electrophoresis

Proteins extracted from myotubes were boiled for 5 min in Laemmli buffer (62.5 mM Tris/HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol; 0.02% bromophenol blue) and resolved by SDS-PAGE. Proteins were separated by 7.5% acrylamide:bisacrylamide [(37.5:1), Biorad] SDS-PAGE. Image acquisitions were done with the ChemiDoc MP Imager, a CCD imager, using the Image Lab 4.0.1 software (Biorad).

Bidimensional gel electrophoresis

Isoelectrofocalisation. Five hundred micrograms of proteins from cytosolic-enriched fraction were precipitated using the chloroform/methanol protocol. The pellet were resolubilized in rehydration buffer (7 M urea, 2 M thiourea, 100 mM dithiothreitol, 4% CHAPS, 4% ASB-14, 1% IPG buffer pH 3–10 non-linear, 0.002% bromophenol blue). To ensure a total resolubilization of the proteins pellet, sample was incubated at 37°C for 5–10 min, following by homogenization under vigorous agitation at room temperature for 1 h. The sample was applied on a pre-cast immobilized pH gradient (IPG) strips (18 cm, pH 3–10, non-linear, GE Healthcare Life Science). Complete sample uptake was carried out overnight for a passive rehydration at room temperature. Focusing was carried out at 20°C under a current limit of 50 μA per strip on PROTEAN® i12 isoelectrofocalisation (IEF) cell (Biorad), and performed at 50 V for 5 h (active rehydration step), 250 V for 1 h (fast progression), followed by a ramping to 10,000 V for 4 h (gradual progression), and was completed at 10,000 V (fast progression) for a total of 60,000 V/h.

Second dimension. After isoelectric focusing, the IPG strips were equilibrated for 20 min at room temperature under gentle agitation in reducing solution [6 M urea; 0.375 M Tris/HCl, pH 8.8; 30% glycerol (v/v); 2% SDS (w/v); 2% DTT (w/v)]. They were then equilibrated for a further 20 min in an alkylating solution, which was identical to the reducing solution except that the DTT was replaced by 2.5% (w/v) iodoacetamide. The equilibrated IPG gels were applied to the top of a 8.5% StrenghtAcryl™(Proteomic Solutions) gel and sealed with concentrating acrylamide gel. Electrophoresis was carried out at 10°C with the Protean II XL Cell (Biorad) in running buffer (0.02 M Tris-base, 0.2 M training ion, 0.1% SDS) at 60 V for 22 h. Training ion was glycine for lower buffer, and tricine for upper buffer.

PROTEINS VISUALIZATION

Fluorescence detection

Once electrophoretic separation achieved, gels were rinsed in ultrapure water to remove the excess of SDS. Gels were immediately scanned using the Chemidoc MP imager (Biorad) for monodimensional gels or with Typhoon 9400 (GE Healthcare) for 2D-gels.

Chemidoc MP acquisitions. Image capture of monodimensional gels images was performed using Chemidoc MP imager under Image Lab™ software. Detection of fluorophores was done with epi-illumination blue, green, or red, in combination with emission filter of 530 BP (band pass) 30, 605 BP 50, and 695 BP 55 nm, for detection of Alexa Fluor® 488, ProQ Diamond, and Sypro Ruby, respectively. The exposure times were chosen to obtain the higher signal/background ratio without a saturation of signals.

Typhoon 9400 acquisitions. Images acquisition on Typhoon 9400 was performed with the Typhoon Control Software. The detection of fluorophores was done with blue, green, or red excitation lasers (wavelength of 488, 532, or 633 nm), in combination with emission filters of 520 BP 40, 580 BP 30, 670 BP 30, or 610 BP 30 for detection of Alexa Fluor® 488, ProQ Diamond, T-red 310, or Sypro Ruby. To set PMT (Photo Multiplier Tube) gain, acquisition was performed with low resolution (1,000 μm for pixel size) under normal sensitivity. The PMT was progressively increased until the signals were saturated. The pixel values were determined with ImageQuant Software, if they were comprised between 1 and 100,000, signals were not saturated. When optimal PMT determined (between 600 and 850 V according to fluorophore), acquisition was done under high sensitivity with 200 μm resolution.

Proteins staining

After visualization of fluorescent proteins, gels were fixed in methanol (50%, v/v)/ATCA (10%, w/v) at least for 1 h 30 min. Gels were then rinsed with ultrapure water, 6 min × 10 min.

ProQ diamond staining. Gels were stained using ProQ Diamond (Molecular Probes, Invitrogen) to detect the phosphoproteome. Incubation was performed overnight under gentle agitation. To avoid background, gels were extensively destained in 50 mM sodium acetate, pH 4.0, containing 15% 1,2-propanediol (29), at least 6 h × 1 h or beyond when background is high. After several washes with ultrapure water, gels were scanned on Typhoon 9400 or on Chemidoc MP. All steps were performed in dark, and gels were immediately scanned after the end of the protocol. To control the specificity of staining, negative control experiments were done on proteins dephosphorylated prior to their electrophoretic separation. All staining and destaining steps were optimized according to the detection of Peppermint Stick markers (Molecular Probes).

Sypro ruby staining. Total levels of proteins were revealed on the same gels using Sypro Ruby (Molecular Probes, Invitrogen) staining. Gels were incubated in staining solution overnight at room temperature under gentle agitation. Gels were successively washed once with ultrapure water, twice in destaining solution [10% methanol (v/v), 7% acetic acid (v/v)], and finally twice with ultrapure water, each bath with a duration of 10 min. All steps were performed in dark, and gels were immediately scanned after the end of the protocol.

RESULTS

COMPARISON OF THE LABELING WITH TAMRA OR ALEXA FLUOR® 488

We have firstly compared the signals obtained after labeling of *O*-GlcNAcylated proteins with two different fluorophores: the TAMRA or the Alexa Fluor® 488. Data were presented on **Figure 1**. Briefly, 25 or 100 μg of proteins (lanes 1 and 2, respectively) corresponding to whole proteins extract were labeled using galactosyltransferase in order to add a residue of azido-modified *N*-acetylgalactosamine on GlcNAc moieties. Through the Click chemistry, the azide group was coupled to an alkyne-modified fluorophore. Proteins were separated by SDS-PAGE, and fluorescent proteins were detected immediately after electrophoresis. As shown on **Figure 1A**, blue epi-illumination lead to the detection of Alexa Fluor® 488-labeled proteins, the intensity of signals being proportional to the amount of proteins per lane. As expected, TAMRA-labeled proteins, as well as non-labeled proteins, were not detected using blue epi-illumination. A green epi-illumination was used to detect the TAMRA-labeled proteins (**Figure 1B**). Similarly to the Alexa Fluor®488-labeled proteins, the intensity of signals was proportional to the quantity of proteins. Nevertheless, the signals were a little more intense for Alexa Fluor®488 labeling than TAMRA-labeling; in addition, the TAMRA signals were a little less defined than those obtained after Alexa Fluor®488 labeling. In both cases, when galactosyltransferase was omitted from reaction buffer, we did not observe any signal in blue nor green epi-illumination, demonstrating the specificity of fluorophore coupling (lanes 3). Note that the proteins patterns were identical in both cases (lanes 1 or 2).

After the detection of *O*-GlcNAcylated proteins, gels were fixed, then rinsed, and scanned again with blue and green epi-illuminations. We observed a slight decrease in the intensity of signals (data not shown), so that the detection of the fluorescent *O*-GlcNAcylated proteins should be done immediately after electrophoresis. The phosphoproteome was then detected after the ProQ Diamond staining as shown on **Figure 1C**. The proteins patterns were identical for Alexa Fluor® 488- or TAMRA-labeled proteins as well as for the non-labeled proteins, suggesting that the fluorophores did not affect the ProQ Diamond staining. The TAMRA fluorophore and the ProQ Diamond staining were detected with green epi-illumination, thus, as expected in view of excitation and emission wavelengths, the TAMRA labeling was not compatible with the downstream detection of phosphorylated proteins with ProQ Diamond staining. In contrast, the Alexa Fluor® 488-labeled proteins were detected in the same extent than the post-fixation detection of proteins (data not shown).

The proteins were finally stained with Sypro Ruby, leading so to the detection of whole proteome (**Figure 1D**). The proteins profiles were identical for labeled proteins (with both

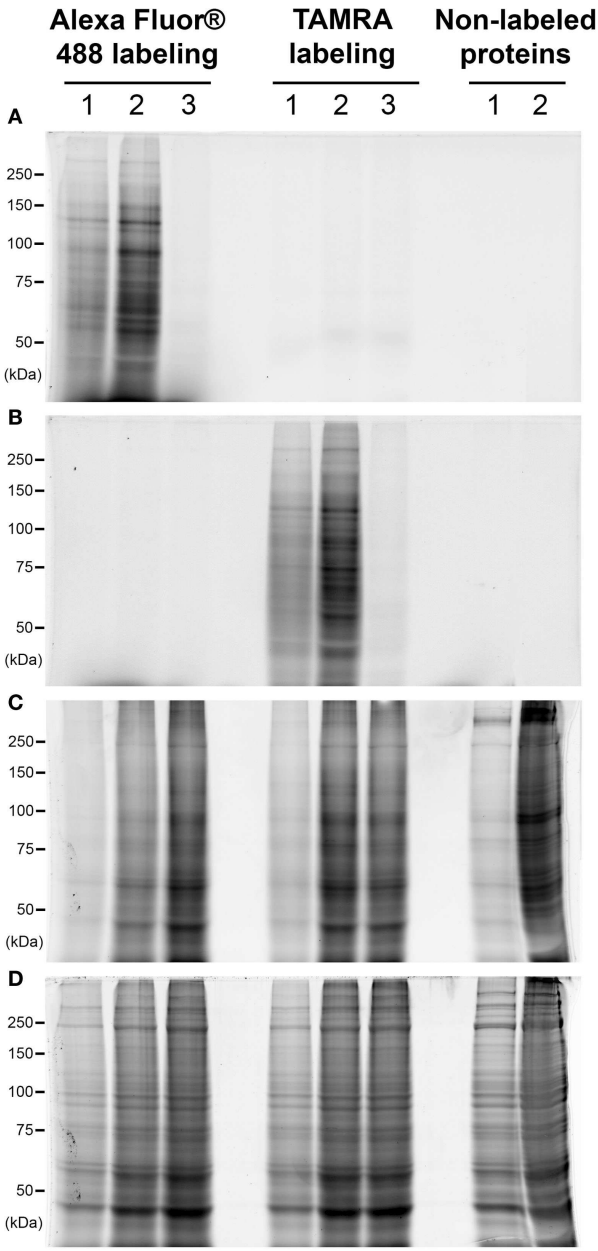

FIGURE 1 | Comparison of the labeling with TAMRA or Alexa Fluor® 488. Proteins corresponded to 25 or 100 μg of whole cellular extract (lanes 1 and 2, respectively) were labeled with galactosyltransferase and coupled with two different fluorophores: Alexa Fluor® 488 or TAMRA. In each case, the same protocol was applied on 100 μg of proteins but omitting the galactosyltransferase, corresponding so to non-labeled proteins (lane 3). Proteins were then separated on a 7.5% SDS-PAGE, and fluorophores detection was done at the end of the electrophoresis. Gel was then fixed and successively stained with the ProQ Diamond and Sypro Ruby. Images acquisition was performed sequentially after each staining. All acquisitions were done using the Chemidoc MP imager. **(A)** Blue epi-illumination was applied on the gel to detect the Alexa Fluor® 488-labeled proteins. **(B)** Green epi-illumination was applied on the gel to detect the TAMRA-labeled proteins. **(C)** Green epi-illumination was applied on the gel to detect the phosphorylated proteins after the ProQ Diamond staining. **(D)** Red epi-illumination was applied on the gel to detect the whole proteins extract after the Sypro Ruby staining.

fluorophores) as well as non-labeled proteins. In view of these data, the Alexa Fluor® 488 labeling was preferred to TAMRA labeling in all the following experiments.

COMPARISON OF GlcNAz METABOLIC INCORPORATION VERSUS GALACTOSYLTRANSFERASE LABELING AFTER N- OR O-DEGLYCOSYLATION

We compared the metabolic and the enzymatic incorporation of azide function on whole cellular extract. All results were presented on **Figure 2**, those concerning the cytosol-enriched fraction were presented only for GalNAz enzymatic labeling. Indeed, the buffer we used for subfractionation contained dithiothreitol, which could reduce the azide function and therefore interfere with the downstream coupling of alkyne-modified fluorophore in the case of the metabolic incorporation of GlcNAz. We also compared the pattern of labeled proteins after N- or O-deglycosylation.

All results obtained for Ac_4-GlcNAz metabolic incorporation were presented on **Figure 2A**. Lane 1 corresponded to non-deglycosylated proteins pattern. When proteins were *N*-deglycosylated with PNGase F prior to the coupling of Alexa Fluor® 488 coupling, we observed a drastic loss of signals (**Figure 2A**, lane 2). The loss of signals was also observed after chemical O-deglycosylation through β-elimination (**Figure 2A**, lane 3), but in a lesser extent. In contrast, when deglycosylation was done by β-*N*-acetyl-hexosaminidase, proteins profile was quite similar to non-deglycosylated proteins profile (**Figure 2A**, lane 4). All together, these data suggested that the Ac_4-GlcNAz was preferentially incorporated in complex *N*- and *O*-glycans rather than in *O*-GlcNAcylated proteins. It is noteworthy that no signal was observed when C2C12 myotubes were cultured with vehicle, i.e., DMSO, whereas proteins profiles corresponding to Sypro Ruby staining were identical in vehicle or Ac_4-GlcNAz culture conditions.

Results corresponding to the GalNAz enzymatic labeling were presented on **Figure 2B**, for whole proteins extract and for cytosol-enriched fraction. As previously, lane 1 corresponded to non-deglycosylated proteins. These profiles were totally different to that obtained after metabolic incorporation of GlcNAz. In contrast, the two profiles (non-deglycosylated whole proteins and cytosolic proteins) were quite similar. The proteins profiles of *N*-deglycosylated proteins were identical to the non-deglycosylated profile (lane 2 compared with lane 1), for whole cellular extract as well as for cytosolic fraction. In contrast, we observed the total loss of signal after chemical and enzymatic O-deglycosylation (lanes 3 and 4, respectively). All together, these data strongly suggested that proteins labeled with the use of galactosyltransferase corresponded exclusively to *O*-GlcNAcylated proteins. This strategy was chosen for the following experiments.

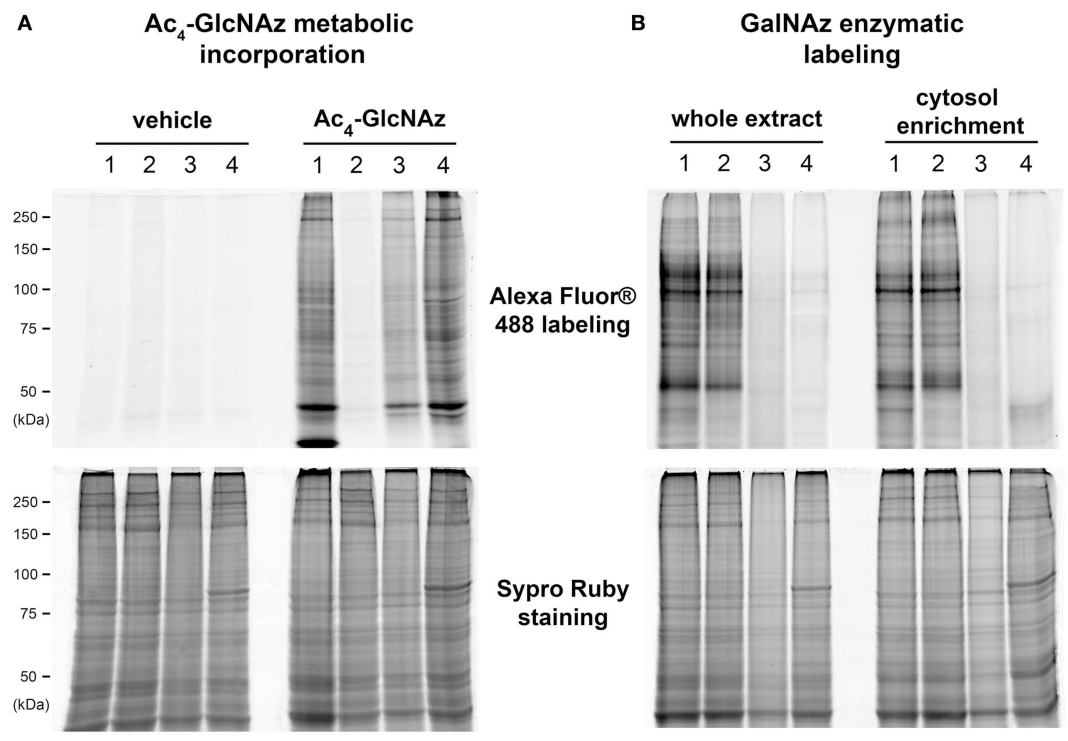

FIGURE 2 | Comparison of GlcNAz metabolic incorporation versus the galactosyltransferase labeling after N- or O-deglycosylation. A 100 μg of proteins corresponding to whole proteins extract or to cytosol-enriched fraction were labeled with Alexa Fluor® 488 after metabolic incorporation of Ac_4-GlcNAz **(A)** or enzymatic labeling with galactosyltransferase **(B)**. After electrophoresis, blue epi-illumination was applied on gels to detect the Alexa Fluor® 488-labeled proteins. Gels were fixed and stained with Sypro Ruby; red epi-illumination permitted the detection of the whole pattern of proteins or cytosol-enriched proteome. Proteins were deglycosylated prior to the labeling with fluorophore. (1) Corresponded to control conditions, i.e., the non-deglycosylated proteins; (2) to *N*-deglycosylated proteins using peptide:N-glycosidase F; (3) to chemically *O*-deglycosylated proteins after β-elimination; and (4) to enzymatically deglycosylated proteins by β-*N*-acetyl-hexosaminidase.

2D-gel OF *O*-GlcNAcome, PHOSPHOPROTEOME, AND WHOLE PROTEOME

To be in adequacy with the multiplexed proteomic strategy, we compared two workflows permitting the sequential visualization of the *O*-GlcNAcome, the phosphoproteome, and the whole proteome, as presented in **Figure 3**. Briefly, in the first strategy, the cytosolic *O*-GlcNAcome was detected through the Click chemistry (labeling of *O*-GlcNAcylated proteins with galactosyltransferase and coupling with Alexa Fluor® 488), followed by the detection of phosphoproteome and the whole cytosolic proteome. The corresponding sequential image acquisition of this strategy was presented on **Figure 4A**. As shown on this figure, different proteins profiles were obtained from the same gel, corresponding to *O*-GlcNAcome, phosphoproteome, and whole proteome, respectively. It is noteworthy that the image acquisitions were performed on a 3-day period, since the ProQ Diamond and the Sypro Ruby required overnight staining.

The second workflow, described on **Figure 3**, was done as followed. A double labeling was performed. The *O*-GlcNAcylated proteins were labeled as previously described, while the whole proteins were labeled with the T-Red 310 fluorescent chromophore, a dye having excitation and emission wavelengths compatible with the use of Alexa Fluor® 488 and the ProQ Diamond. We have tested different combination of labeling, i.e., Alexa Fluor® 488 firstly followed by T-Dye labeling, and reciprocally. When the sequence of labeling was Alexa Fluor®488 labeling, and then T-dye labeling, we were neither able to obtain a well-resolved image of the *O*-GlcNAcome nor the whole proteome, suggesting that this combination of labeling was problematic and need to be discarded (data not shown).

The second combination of labeling was T-Dye labeling followed by the labeling of *O*-GlcNAcylated proteins as described in the Section "Detailed Experimental Procedures." After both labeling, proteins were resolved on bidimensional gel electrophoresis, and images were sequentially acquired according to excitation and emission wavelengths of both dyes. After images acquisition, the ProQ Diamond staining was done to imaging the phosphoproteome. All these results were presented on **Figure 4B**, in the same order than the sequential image acquisition. It is noteworthy that this strategy required only 2 days for all the workflow. On this figure were indicated three squared zones (one squared zone per gel), each being zoomed on **Figure 4C**. On this panel, plain arrows and blank arrows indicated spots detected on whole proteome pattern, but detected or not on *O*-GlcNAcome and phosphoproteome images. Thus, proteins spots could be detected on phosphoproteome pattern but not on *O*-GlcNAcome pattern, or vice-versa. We could hypothesized that these spots could correspond to phosphorylated but non-glycosylated proteins, and reciprocally. Of course, we can not exclude that the signals obtained after ProQ Diamond staining or after *O*-GlcNAcylated proteins labeling were under the detection threshold. In some case, spots were detected in all images, suggesting that the corresponding proteins could bear simultaneously *O*-GlcNAc and phosphate moieties. It should be mentioned that the same data were obtained from each zone of interest in the first strategy, but only results corresponding to the second strategy were included in **Figure 4**.

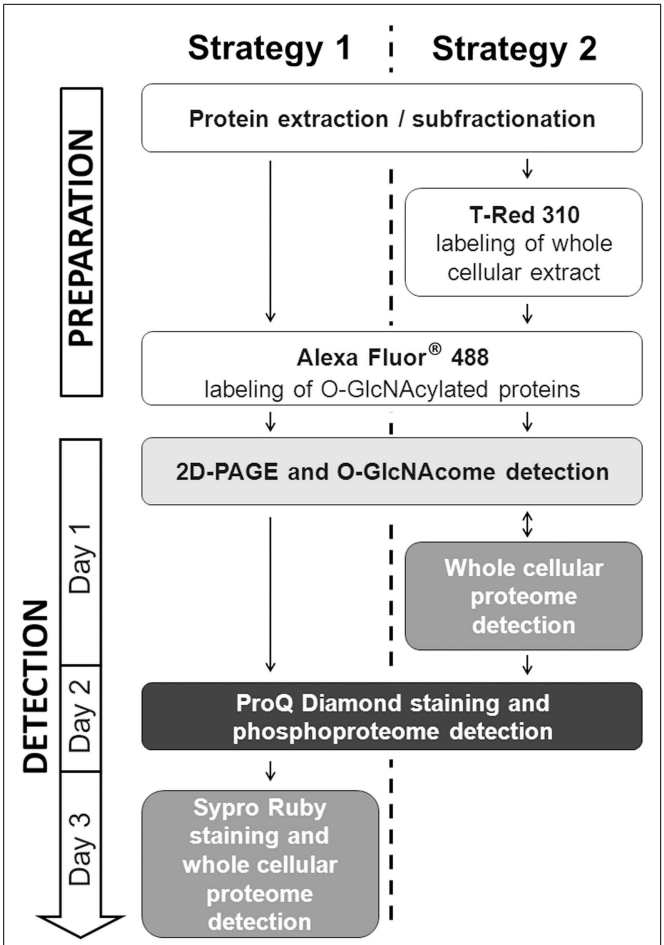

FIGURE 3 | Schematic representation of the two alternatives for in-gel detection of *O*-GlcNAcome, phosphoproteome, and whole proteome. The two strategies were indicated in parallel. Note that several experimental steps are common between the two strategies.

DISCUSSION

Proteomics community celebrates the 20 years old of the term proteomic, proposed to the Sienna conference in 1994 (30). Historically, bidimensional gel electrophoresis was largely used in proteomic approaches. However, 15 years ago, the benefits/advantages of bidimensional gel electrophoresis approach were questioned. In fact, as pointed out by Fey and Larsen (31), 2D gel requires manual dexterity and precision to reproduce precisely and is thus not well-suited as a high-throughput technology. However, despite these drawbacks, 2D-electrophoresis offers a resolution and sensitivity, which are exquisite and unsurpassed if one wants a global view of "cellular activity" (31). Nevertheless, though sometimes criticized, bidimensional gel electrophoresis remains one of the most widespread techniques in the field of functional proteomics. Through proteomes comparison between cells, tissues, or organs, providing from different physiological or pathological conditions, 2D-electrophoresis allowed a proteome map at a given time, and offered a large-scale analysis about the alterations occurring in protein expression levels and modifications. Moreover,

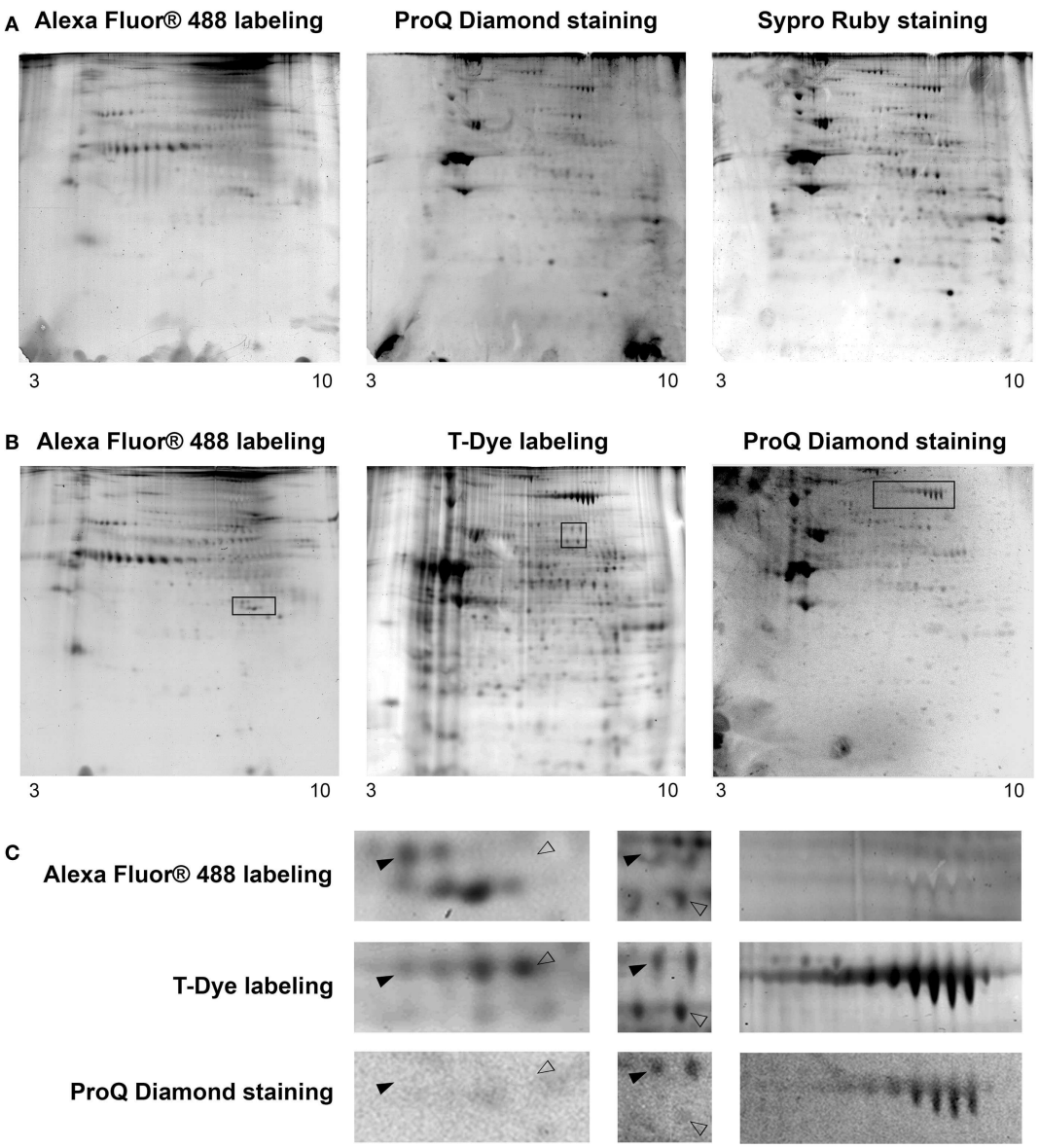

FIGURE 4 | Cartographies of the *O*-GlcNAcome, the phosphoproteome, and the whole proteome within the same gel. Five hundred micrograms of cytosol-enriched extract were resolved on bidimensional gel electrophoresis; isoelectrofocalisation was done on non-linear 3–10 IPG dry strip, while second dimension was performed on 8.5% resolving gel. The *O*-GlcNAcome was detected through Click chemistry and Alexa Fluor® 488. The phosphoproteome was imaged after ProQ Diamond staining. The whole proteome was visualized through two approaches: the Sypro Ruby staining, or the T-Dye labeling. **(A)** This workflow corresponded to *O*-GlcNAcome imaging, followed by ProQ Diamond and then Sypro Ruby staining. **(B)** This workflow corresponded to *O*-GlcNAcome imaging, in parallel of whole proteome imaging through T-Dye labeling; the phosphoproteome was then investigated using the ProQ Diamond staining. **(C)** Zoom of zones of interest squared on gels from previous panel. Plain or blank arrows indicated proteins differentially detected on *O*-GlcNAcome, phosphoproteome, or whole proteome images, corresponding so to proteins bearing or not *O*-GlcNAc and/or phosphate moieties.

consecutively to the numerous approaches used by the proteomic community, the advantages and the drawbacks of gel-based proteomic methods, in particular, 2D-electrophoresis, are well-known nowadays, and should be considered when proteomic approach is initiated (32, 33). Since this kind of approach could be done without the need of high-sophisticated equipment (and so accessible to a large panel of laboratories), we proposed in this paper, a method based on 2D-gel electrophoresis, to detect in a same gel the *O*-GlcNAcome, the phosphoproteome (and therefore the interplay between both post-translational modifications), and the whole cellular proteome.

The use of fluorescence detection in 2D-differential gel electrophoresis had substantially upgraded the potential and the power of bidimensional electrophoresis for the analysis of

protein expression differences, and for the detection of post-translational modifications. Through multiplexed technologies, reproducibility, robustness, and technical confidence greatly increased for several years (33, 34). This methodology, based on the use of non-overlapped fluorescent dyes such as ProQ Diamond, ProQ Emerald, and Sypro Ruby, allowed the parallel determination of phosphorylation, glycosylation, and whole proteins patterns through the comparison of different images acquired from the same gel (22, 23, 35). However, the ProQ Emerald, while more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye, also detected the *N*-glycosylated proteins. Thus, the ProQ Emerald is not the exclusive method for the detection of O-GlcNAcylation. Few years ago has emerged the use of chemical reporters of glycosylation, originally developed by the Bertozzi's laboratory [Ref. (36); reviewed in Ref. (37)], and azide- or alkyne-bearing analogs of monosaccharide were currently available. In this way, the azido-modified monosaccharide GlcNAz could be used for metabolic incorporation of azide group on *O*-GlcNAcylated proteins. Alternative method of post-lysis was also developed in Hsieh–Wilson's group, based on the transfer of azido-modified *N*-acetylgalactosamine on *O*-GlcNAc moieties through a mutant galactosyltransferase (38, 39), this advanced chemoenzymatic strategy for proteomic analysis lead to the development of commercially available reagents for fluorescent labeling of *O*-GlcNAcylated proteins (38). These bioorthogonally functionalized proteins extract were then labeled with a probe, permitting downstream the purification or the detection of *O*-GlcNAcylated proteins after Staudinger ligation or copper-catalyzed azide–alkyne cycloaddition (37).

This Click chemistry is nowadays a promising method for detection and/or purification of *O*-GlcNAcylated proteins. This approach is characterized by a relative simple and improved methodology and allowed a good sensitivity, as well as reproducibility. In this paper, we compared both methods (metabolic and enzymatic) of incorporation of azide group on *O*-GlcNAcylated proteins. According to our data, the chemoenzymatic labeling should be preferred for labeling of GlcNAc moieties since this strategy offers a serious specificity of GlcNAc moieties detection after incorporation of the azido-modified *N*-acetylgalactosamine compared with GlcNAz incorporation (in particular, in complex glycans). In addition, the subcellular fractionation remains a helpful device to reduce the contamination of proteins with the glycoproteins bearing complex *N*- or *O*-glycans and enhanced the specificity as well as the sensitivity for *O*-GlcNAcylated proteome analysis.

The major finding in this paper was the detection of *O*-GlcNAcome, phosphoproteome, and whole proteome in only one gel. Briefly, the chemical labeling of *O*-GlcNAc moieties with Alexa Fluor® 488 lead to the detection of the *O*-GlcNAcome, and downstream detection of phosphoproteome was done after ProQ Diamond staining. The global proteome could be detected either Sypro Ruby staining or after covalent labeling of proteins using the T-Dye fluorophore. In both cases, the proteins profiles were similar. In our finding, the double labeling was preferred since the *O*-GlcNAcome and the whole proteome images could be acquired simultaneously. Whatever the approach used, we were able to discriminate unmodified proteins from proteins which were *O*-GlcNAcylated, or phosphorylated or both. It remains important to keep in mind the limitation of this approach, inherent to 2D-gel approach as well as sensitivity of dyes, such as ProQ Diamond staining or the labeling of *O*-GlcNAc proteins, in particular, in view of the detection threshold. In this way, a relative important amount of proteins are necessary to the detection of *O*-GlcNAcome and the phosphoproteome, leading so to a slight decrease in spot resolution of 2D-gel. It should also be mentioned that this method should be optimized according to the studied tissues or the cell lines in terms of the amount of labeled proteins and the electrophoretic conditions. In addition, the effective phosphorylated and/or *O*-GlcNAcylated state of a protein should be attempt in the validation steps.

Indeed, the final purpose of this method was to propose a simple methodology to determine the variation of O-GlcNAcylation, and/or phosphorylation, and/or protein expression. This semi-quantification could be thereafter determined through images analysis using specific proteomic softwares, by comparison of proteins extract, resulting from different physiological or cellular conditions, for example, healthy versus pathological conditions, or untreated versus treated cells, and so on. Differential spots could be then excised from gel to be submitted to proteolytic digestion and mass spectrometry analysis through a bottom-up proteomic approach. All data providing from this kind of approach need to be validated, and suffer from the lack of a precise quantification. In this way, recent developments in mass spectrometry, more particularly with the breakthrough of stable isotope labeling with amino acids in cell culture (SILAC) or the label-free quantification, are nowadays powerful and adaptable tools for quantitative proteomic (5).

To conclude, we propose herein a method for the profiling of *O*-GlcNAcome, phosphoproteome, and whole proteome in a completely blind and global approach. The recent developments render 2D-electrophoresis to be still considered seriously for proteome analysis and to be again one of the preferred methods in many laboratories. This method remains fast, simple, and easy to use, without the need of high-sophisticated equipment, and so accessible to a large panel of laboratories. The major finding was the proof-of-concept of a 2D-gel-based multiplexed strategy, in which three important informations were gained within only one gel. The use and large choice of fluorescent probes enhanced the sensitivity and powerful of this technique and allowed multiplexed proteomic technology to detect O-GlcNAcylation and phosphorylation two key post-translational modifications in the regulation of many cellular processes.

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O-GlcNAcylation and metabolic reprograming in cancer

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Although cancer metabolism has received considerable attention over the past decade, our knowledge on its specifics is still fragmentary. Altered cellular metabolism is one of the most important hallmarks of cancer. Cancer cells exhibit aberrant glucose metabolism characterized by aerobic glycolysis, a phenomenon known as Warburg effect. Accelerated glucose uptake and glycolysis are main characteristics of cancer cells that allow them for intensive growth and proliferation. Accumulating evidence suggests that *O*-GlcNAc transferase (OGT), an enzyme responsible for modification of proteins with *N*-acetylglucosamine, may act as a nutrient sensor that links hexosamine biosynthesis pathway to oncogenic signaling and regulation of factors involved in glucose and lipid metabolism. Recent studies suggest that metabolic reprograming in cancer is connected to changes at the epigenetic level. *O*-GlcNAcylation seems to play an important role in the regulation of the epigenome in response to cellular metabolic status. Through histone modifications and assembly of gene transcription complexes, OGT can impact on expression of genes important for cellular metabolism. This paper reviews recent findings related to *O*-GlcNAc-dependent regulation of signaling pathways, transcription factors, enzymes, and epigenetic changes involved in metabolic reprograming of cancer.

Keywords: *O*-GlcNAcylation, cancer, metabolism, PI3K/Akt pathway, transcription factors, glycolytic enzymes, epigenetics

CANCER CELL METABOLISM

Most early studies concerning cancer biology focused only on molecular alterations in signaling pathways that led to uncontrolled proliferation, while changes in cancer metabolism were treated as a secondary effect. However, in recent years, a growing body of evidence has demonstrated that metabolic reprograming can be a key process during tumorigenesis and many oncogenes and tumor suppressors are, in fact, regulators of metabolism. Changes in metabolism are necessary for the shift from normal to malignant growth (1).

Cancer cell metabolism is characterized by an enhanced uptake and utilization of glucose (2–6). In normal cells, glucose is catabolized to pyruvate. Pyruvate is further converted to acetylo-CoA and oxidized to carbon dioxide through the mitochondrial tricarboxylic acid (TCA) cycle, which generates NADH and $FADH_2$. The transfer of electrons from NADH and $FADH_2$ to oxygen through respiratory chain is an energy-efficient process. Together, glycolysis, TCA cycle, and electrons transfer phosphorylation produce 36 ATP molecules per glucose molecule. In cancer cells, oxidative phosphorylation is inhibited and cells use glycolysis to provide

Abbreviations: 4EBP1, factor 4E binding protein 1; α-KG, alpha ketoglutarate; ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; Akt, v-akt murine thymoma viral oncogene; ALDA, aldolase A; AMPK, 5′ adenosine monophosphate-activated protein kinase; BADGP, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; CAD, carbamoyl phosphate synthetase aspartate transcarbamylase and dihydroorotase; CBP, CREB binding protein; ChRE, carbohydrate response element; ChREBP, carbohydrate responsive element-binding protein; eIF4E, eukaryotic translation initiation factor 4E; ENO1, enolase; ERK, extracellular signal-regulated kinase; ERα, estrogen receptor α; EZH2, enhancer of zeste homolog 2; F6P, fructose-6-phospate; $FADH_2$, reduced flavin adenine dinucleotide; FAS, fatty acid synthase; FBP1, fructose-1,6-bisphosphatase-1; FoxO1, forkhead box protein O1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAT, glutamine fructose-6-phosphate amidotransferase; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GLS, glutaminase; GLUT, glucose transporter; GSK3β, glycogen synthase kinase 3b; H2Bub, monoubiquitinated H2B; HBP, hexosamine biosynthetic pathway; HDAC1/2, histone deacetylases 1/2; HIF-1, hypoxia-induced factor 1; HK, hexokinase; HR6A/B, human orthologs of the *S.cerevisiae* ubiquitin conjugated enzyme Rad6; IκB, inhibitor of kappa B; IKK, I kappa B kinase; LDHA, lactate dehydrogenase A; LKB1, liver kinase B1; L-PK, liver pyruvate kinase; mTOR, mammalian target of rapamycin; mTORC1/2, mammalian target of rapamycin complex 1/2; NADH, nicotinamide adenine dinucleotide; NButGT, 1,2-dideoxy-2′-propyl-alpha-D-glucopyranoso-[2,1-D]-delta 2′-thiazoline; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ODC, ornithine decarboxylase; OGA, *O*-GlcNAcase, *O*-GlcNAc hydrolase; OGT, *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase; OXPHOS, oxidative phosphorylation; PDK, pyruvate dehydrogenase kinase; PDK1, phosphoinositide-dependent kinase-1; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PGK, phosphoglycerate kinase-1; PGM, phosphoglycerate mutase; PHD2, prolyl hydroxylase domain protein 2; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; *PIK3CA*, gene encoding the p110alpha catalytic subunit of PI3K; PKA, protein kinase A; PKM2, pyruvate kinase M2; PP2A, protein phosphatase 2A; PPP, pentose phosphate pathway; PTEN, phosphatase and tensin homolog; PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate; Rheb, Ras homolog enriched in brain; RNF20, ring finger protein 20; S6K, ribosomal protein S6 kinase; SAGA, Spt-Ada-Gcn5 histone acetyltransferase complex; SCD1, stearoyl-CoA desaturase; SHMT, serine hydroxymethyl transferase; *SLC2A1*, solute carrier family 2 member 1, gene encoding GLUT1; *SLC2A3*, solute carrier family 2 member 3, gene encoding GLUT3; SOC2, cytochrome *c* oxidase 2; TCA, tricarboxylic acid cycle; TET, ten–eleven translocation methylcytosine dioxygenase; TNF, tumor necrosis factor; TPI, triose phosphate isomerase; TSC1/2, tuberous sclerosis complex 1/2; Ubp8, ubiquitin-specific peptidase 8; UDP-GlcNAc, Uridine diphosphate *N*-acetylglucosamine; USP22, ubiquitin-specific protease 22; VHL, von Hippel–Lindau tumor suppressor protein; Xyl-5-P, xylulose-5-phosphate.

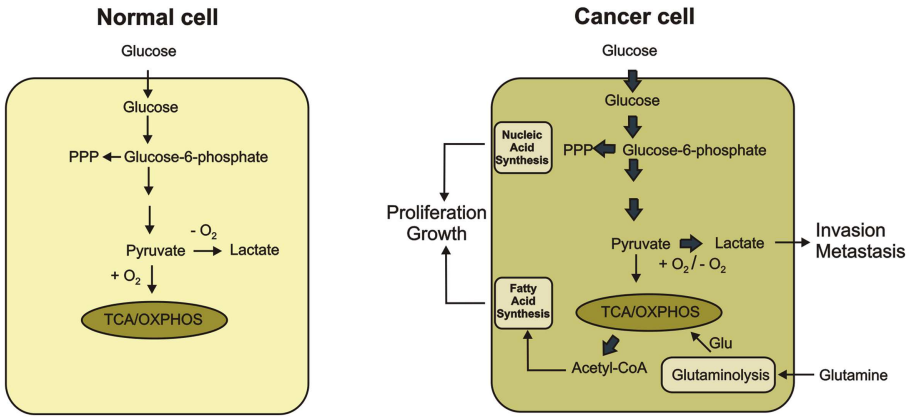

FIGURE 1 | Metabolic differences between normal and cancer cells are shown. In normal cells, glucose is metabolized to pyruvate, which is completely oxidized to CO_2 through the TCA cycle and the oxidative phosphorylation process in the mitochondria. Only if O_2 is limited, pyruvate is metabolized to lactate. Cancer cells convert most glucose to lactate regardless of the availability of O_2 (the Warburg effect). Secretion of lactate favors tumor progression. The increased glucose utilization through glycolytic pathway generates metabolic intermediates such as glucose-6-phosphate, which is used for the synthesis of nucleic acids through the pentose phosphate pathway. Glutamate produced during glutaminolysis serves as the major substrate to refuel the TCA cycle. Citrate-derived acetyl CoA is used for lipid production. The increased synthesis of nucleic acid and lipids promote proliferation and growth of cancer cells.

them with the necessary energy. Glycolysis can only provide 2 ATP molecules per glucose molecule producing lactic acid as the end product. Cancer cells preferentially use glycolysis even in the abundance of oxygen whereas normal cells use only when oxygen supply is limited (4–6). The increased glucose uptake with concomitant lactate production, even under aerobic conditions, is known as the Warburg effect or aerobic effect (2, 3) (**Figure 1**).

It was originally hypothesized that these metabolic changes in cancer cells reflected damage to mitochondrial oxidative phosphorylation, suggesting that cancer cells are forced to use glycolysis instead of oxidative phosphorylation (1–3). However, it has been revealed that many cancer cells are capable of synthesizing ATP through mitochondrial respiration (7, 8). There is also no strong evidence that respiration is less active in cancer cells than in normal cells. Additionally, mitochondria play important role in cancer because they are involved in biosynthesis of molecules necessary for growth and proliferation. Impairment of mitochondrial function has been shown to suppress tumor growth (9). Therefore, increased glycolysis is not just a consequence of impaired mitochondria but rather constitutes a primary change of cancer metabolism.

In fact, increased glycolytic flux is very beneficial to cancer cells because the glycolytic intermediates fuel several biosynthetic pathways that produce *de novo* nucleotides, lipids, amino acids, and NADPH. Reprograming of cellular metabolism toward synthesis of precursors for macromolecules allows for the accumulation of biomass during cell growth and proliferation (10, 11). Moreover, cancer cells are more resistant to hypoxia condition associated with tumor growth by switching their metabolism from oxidative phosphorylation to oxygen-independent glycolysis (12). By producing an increased amount of lactic acid, cancer cells can lower the pH of extracellular microenvironment, which induces the activity of metalloproteases and facilitates degradation of extracellular matrix components. Thus, lactate can be an inducer of cancer invasion and metastasis (13–15) (**Figure 1**).

The molecular mechanisms that control metabolic reprograming in cancer cells are complex. Tumors conduct aerobic glycolysis and upregulate glutaminolysis, lipid metabolism, and pentose phosphate pathway (PPP), partly through the activation of oncogenes or loss of tumor suppressor activity. Oncogenes such as Akt or c-Myc are promoters of cancer metabolic changes. In contrast, tumor suppressors such as p53 or AMP-activated protein kinase (AMPK) prevent those alterations (6, 16, 17). It is also suggested that epigenetic changes may contribute to the Warburg effect (18).

O-GlcNAcylation

O-GlcNAcylation is a post-translational modification of cellular proteins that is suggested to play a role in the nutrient sensing mechanism (19, 20). This modification results from the enzymatic addition of the *N*-acetylglucosamine (GlcNAc) moiety to the hydroxyl groups of serines or threonines. *O*-GlcNAcylation is dynamically regulated by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which are respectively responsible for *O*-GlcNAc addition and removal (21, 22). *O*-GlcNAc modification level of proteins is dependent on the concentration of UDP-GlcNAc, which is a donor substrate for OGT. UDP-GlcNAc is the end product of the hexosamine biosynthetic pathway (HBP), which directly uses cell glucose input. Consequently, *O*-GlcNAcylation is modulated by nutrients availability. Therefore, *O*-GlcNAcylation is proposed as a nutrient sensor and metabolic regulator (19, 20, 23). Glucose and glutamine are the two most abundant extracellular nutrients and cancer cells are highly dependent on availability of these compounds. Glutamine is the donor substrate in the conversion of fructose-6-phosphate to glucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT) in the HBP. Thus, an excess in both glutamine and glucose uptake in cancer cells contributes to an

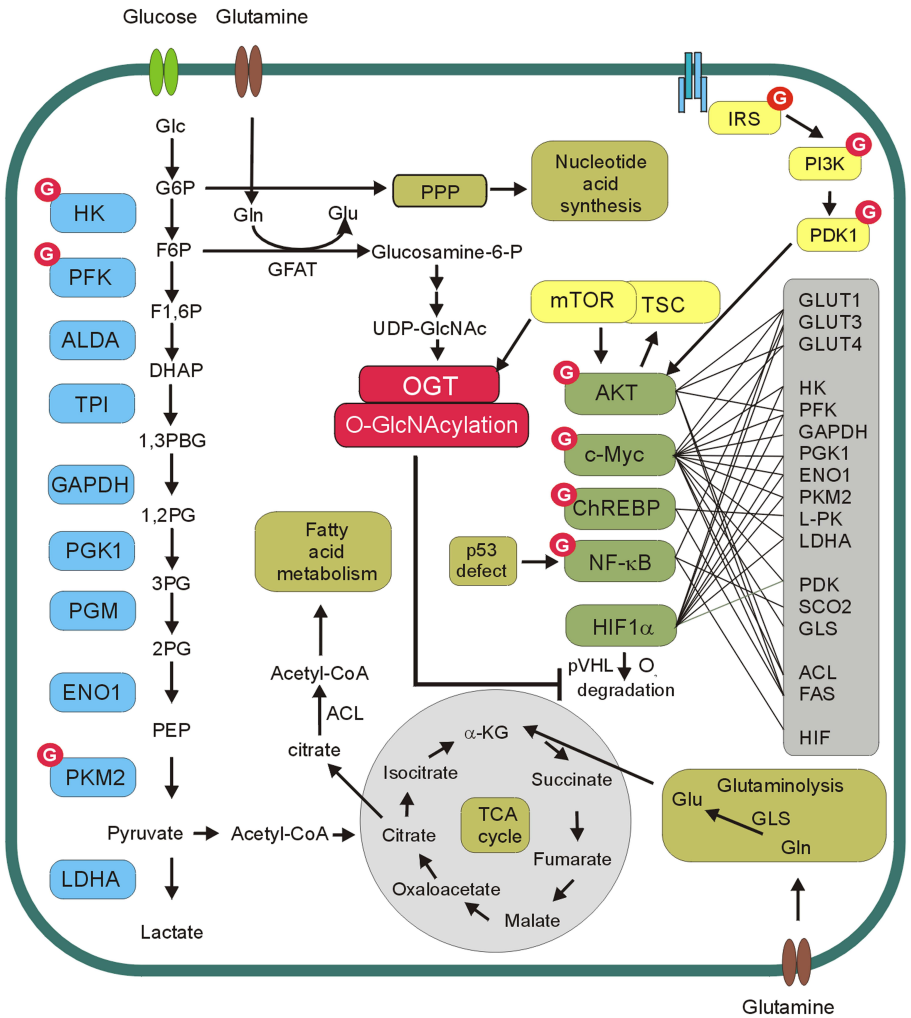

FIGURE 2 | *O*-GlcNAc and cancer metabolism. *O*-GlcNAcylation level of proteins is dependent on concentration of UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is a donor substrate for *O*-GlcNAc transferase (OGT). UDP-GlcNAc is derived from glucose through hexosamine biosynthetic pathway. In this pathway, fructose-6-phospate (F6P) is converted to glucosamine-6-phosphate by the glutamine:fructose-6-phosphate amidotransferase (GFAT) and after the subset of reactions UDP-GlcNAc is generated. OGT modifies and regulates several glycolytic enzymes, transcription factors as well as components of PI3K/Akt/mTOR pathway. Akt, c-Myc, ChREBP, NF-kB, and HIF-1α reprogram cellular metabolism by direct or indirect regulation of expression of glucose transporters (GLUT1, GLUT3, GLUT4), glycolytic enzymes (HK, PFK, GAPDH, PGK1, ENO1, PKM2, L-PK, LDHA), pyruvate dehydrogenase kinase (PDK), glutaminase (GLS), cytochrome *c* oxidase 2 (SCO2), fatty acid synthase (FAS), ATP citrate lyase (ACL).

increased flux through the HBP. This in turn contributes to an increased level of the HBP end product, i.e., UDP-GlcNAc and increased *O*-GlcNAcylation (19, 20, 23) (**Figure 2**).

O-GlcNAcylation occurs on serine or threonine residues of proteins at sites that may also be phosphorylated. Therefore, extensive crosstalk exists between phosphorylation and *O*-GlcNAcylation. At first, it was suggested that *O*-GlcNAcylation is a reciprocal to phosphorylation and these modifications are mutually exclusive. However, recent studies have shown that some cellular stimuli are able to increase both modifications on the same proteins. Thus, the interplay between *O*-GlcNAcylation and phosphorylation is more complex than previously assumed (24).

The results of many studies suggest that increased expression of OGT and hyper-*O*-GlcNAcylation are the universal features of cancers [for review see Ref. (25–27)]. Aberrant *O*-GlcNAcylation seems to be involved both in tumorigenesis and cancer progression. *O*-GlcNAcylation of oncogenes, tumor suppressors, and other proteins involved in cell signaling pathways may significantly impact tumor growth, cell proliferation, angiogenesis, invasion, and metastasis. A growing body of evidence suggests that hyper-*O*-GlcNAcylation may also be an important factor in reprograming of cancer cell metabolism (**Figure 2**).

IMPACT OF *O*-GlcNAcylation ON KEY FACTORS IN CANCER METABOLISM

PI3K/Akt/mTOR PATHWAY

Phosphatidylinositol 3-kinase/Akt/mTOR signaling pathway is a key mechanism involved in both growth and glucose metabolism

control in cells. Constitutively activated PI3K/Akt/mTOR signaling as a consequence of *PIK3CA* mutations or *PTEN* loss is one of the most common lesion in human cancers (28–30).

The activation of phosphatidylinositol 3-kinase (PI3K) leads to the phosphorylation of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate and subsequent recruitment of Akt to the plasma membrane where this kinase is activated (31). Akt is partially activated through an initial phosphorylation at Thr308 by phosphoinositide-dependent kinase-1 (PDK1) and then fully activated by the phosphorylation at Ser473 by a mammalian target of rapamycin complex 2 (mTORC2) (32–36). Akt can directly or indirectly affect the activity of many transcription factors and enzymes mediating multiple effects (35, 36). One of the major downstream effectors of Akt is the serine/threonine kinase mTOR. mTOR constitutes catalytic subunit of the functionally distinct mTORC1 and mTORC2 complexes. Akt can activate mTORC1 indirectly through phosphorylation and inhibition of tuberous sclerosis complex 2 (TSC2) (37) (**Figure 3**).

Phosphatidylinositol 3-kinase/Akt/mTOR signaling pathway plays a central role in cancer cell metabolism reprogramming (30). PI3K/Akt pathway regulates glucose uptake and utilization (5). Activation of PI3K/Akt causes increased glucose transporters expression on the cell surface, activation of hexokinase (HK) that phosphorylates glucose to keep it in cell and phosphofructokinase-2-dependent allosteric activation of phosphofructokinase-1 (PFK1), which catalyzes the committed step of glycolysis (1). Moreover, activation of PI3K/Akt/mTOR pathway enhances the biosynthesis of macromolecules. PI3K and Akt stimulate expression of lipogenic genes and lipid synthesis in many cell types, while mTOR regulates protein translation (38–40).

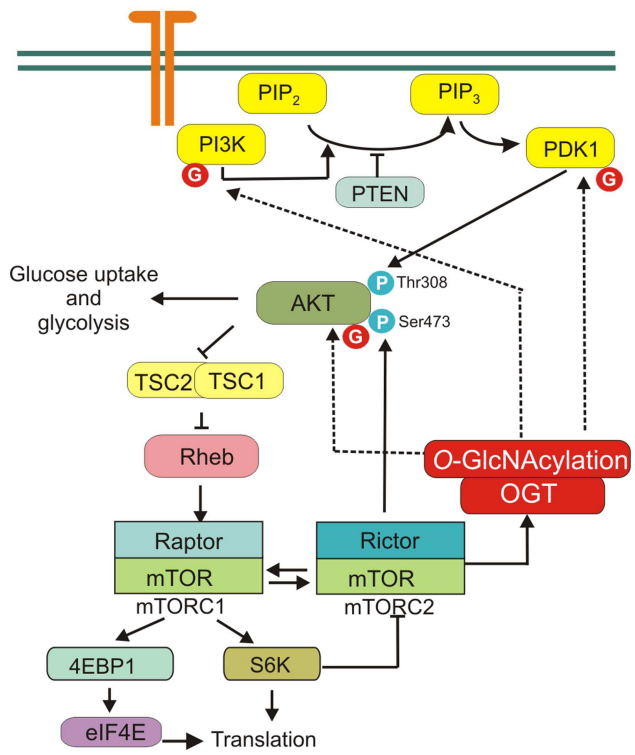

FIGURE 3 | PI3K/Akt/mTOR signaling pathway. mTORC1 is activated by receptor signaling through the PI3K/Akt pathway. mTORC2 is crucial factor in PI3K/Akt signaling, phosphorylating Akt on Ser473 to promote its maximal activation. mTOR regulates protein *O*-GlcNAcylation through affecting OGT stability. Several proteins of PI3K/Akt/mTOR pathway are modified by OGT, i.e., PI3K, PDK1, and Akt.

The role of *O*-GlcNAcylation in regulation of PI3K/Akt signaling pathway was extensively studied especially in adipocytes and muscle cells (41–45). It was shown that overexpression of OGT and increased *O*-GlcNAcylation in muscle, adipocytes, or liver cells inhibited insulin signaling (23, 43, 46, 47). However, studies using OGA inhibitors gave contradictory results. Inhibition of OGA by PUGNAc [*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate] increased global *O*-GlcNAc levels and caused insulin resistance in 3T3-L1 adipocytes and skeletal muscle (40, 43). But the other studies showed that more selective than PUGNAc inhibitor NButGT (1,2-dideoxy-2′-propyl-alpha-D-glucopyranoso-[2,1-D]-Delta 2′-thiazoline) did not induce insulin resistance in 3T3-L1 adipocytes (48, 49).

Akt is one of the most frequently investigated *O*-GlcNAcylated proteins. In murine pancreatic β-cells, Akt1 Ser473 may undergo both phosphorylation and *O*-GlcNAcylation and the balance between these modifications may regulate cell apoptosis (50). However, the relationship between *O*-GlcNAcylation and phosphorylation of Akt in cancer cells is not fully elucidated. Wang et al. showed that *O*-GlcNAcylations at Thr305 and Thr312 inhibited Akt phosphorylation at Thr308 via disrupting the interaction between Akt and PDK1 in MCF-7 cells (51). The impaired Akt activation affected functions of Akt, as evidenced by suppressed cell proliferation and migration capabilities. On the other hand, Kanwal et al. showed that in MCF-7 cells treated with PUGNAc and glucosamine the phosphorylation of Akt Ser473 was higher (52). Similarly, in thyroid anaplastic cancer cells, down-regulation of OGA and increased *O*-GlcNAcylation caused increased Akt1 Ser473 phosphorylation and enhanced proliferation (53). Onodera et al. found that OGT inhibition by BADGP (benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside) or down-regulation by siRNA led to suppression of Akt signaling in 3D cultures of breast cancer cells (54).

Additionally, PI3K/Akt pathway is sensitive to extracellular glucose. Jones et al. have shown that short-term glucose deprivation significantly restricts insulin-stimulated Akt activation and inhibits growth of U2OS cancer cells (55). The authors found that insulin signaling can be rescued by extracellular glucosamine and increased flux through the HBP and *O*-GlcNAcylation (55). Together, these data seem to support the concept that in cancer cell metabolism, reprograming increased HBP flux and *O*-GlcNAcylation may play an important role.

Recent studies have also shown that mTOR regulates protein *O*-GlcNAc modification through affecting OGT stability. Inhibition of mTOR causes a decrease in global *O*-GlcNAcylation due to decreased OGT protein level (56).

Thus, many studies have pointed to *O*-GlcNAcylation as a key regulatory modification of PI3K/Akt/mTOR pathway. But further

studies are necessary to provide direct evidence for the role of *O*-GlcNAcylation in PI3K/Akt/mTOR pathway in cancer metabolism regulation.

HYPOXIA-INDUCED FACTOR

Hypoxia is an important characteristic of the tumor microenvironment (57–59). Decreased oxygen availability stimulates cells to consume more glucose and produce lactate (59). This adaptive response to reduced O_2 availability is mediated by hypoxia-induced factors 1 and 2 (HIF-1 and HIF-2). These factors are composed of the constitutively expressed HIF-1β subunit and either the HIF-1α or HIF-2α subunit, which are stable only in hypoxia conditions (17, 58). HIF-1α is ubiquitously expressed whereas HIF-2α expression is restricted to several cell types. Under normoxic conditions, the HIF-1α subunit undergoes hydroxylation on Pro402 and/or Pro564 by prolyl hydroxylase domain protein 2 (PHD2), which uses O_2 and α-ketoglutarate (α-KG) as substrates (58). Hydroxylated HIF-1α is recognized by von Hippel–Lindau (VHL) tumor suppressor protein, which recruits an E3-ubiquitin ligase that targets HIF-1α for proteasomal degradation. Under hypoxic conditions, the prolyl hydroxylation reactions are inhibited by O_2 deprivation and HIF-1α accumulates and dimerizes with constitutively expressed HIF-1β. HIF-1 dimer binds to the hypoxia response element of target genes and causes their transcriptional activation. HIF-1's targets include *SLC2A1* and *SLC2A3* genes encoding for glucose transporters (GLUT1 and 3, respectively) as well as genes encoding for most of glycolytic enzymes (58) (**Figure 4**).

Recent studies revealed that *O*-GlcNAcylation may affect cancer metabolism reprograming by regulation of HIF-1 pathway (60). In human breast cancer cells, high level of HIF-α is associated with elevated OGT level. Ferrer et al. showed that reduction of *O*-GlcNAcylation in cells increased HIF-1α hydroxylation and interaction with VHL resulting in HIF-1α degradation and reduction of GLUT1 expression (60).

c-Myc

c-Myc is a helix–loop–helix leucine zipper transcription factor, which participates in many cellular processes including cell proliferation, apoptosis, and differentiation (61, 62). This transcription factor is also a key regulator of cancer cell metabolism. In transformed cells, c-Myc is often expressed at constitutively high levels and promotes energy production and biomolecule synthesis independent of growth factor stimulation (16). Activated c-Myc induces the expression of almost all glycolytic enzymes, particularly hexokinase 2 (HK2), phosphofructokinase-1 (PFK1), phosphoglycerate kinase-1 (PGK1), lactate dehydrogenase A (LDHA), and pyruvate kinase M2 (PKM2) (17, 63). c-Myc not only promotes energy production by enhancing glycolysis but also increases biomolecule synthesis by targeting genes of anabolic enzymes such as carbamoyl phosphate synthetase aspartate transcarbamylase and dihydroorotase (CAD), serine hydroxymethyl transferase (SHMT), fatty acid synthase (FAS), and ornithine decarboxylase (ODC) (17). Moreover, multiple studies have demonstrated that c-Myc stimulates glutamine uptake and metabolism. c-Myc directly stimulates expression of glutamine

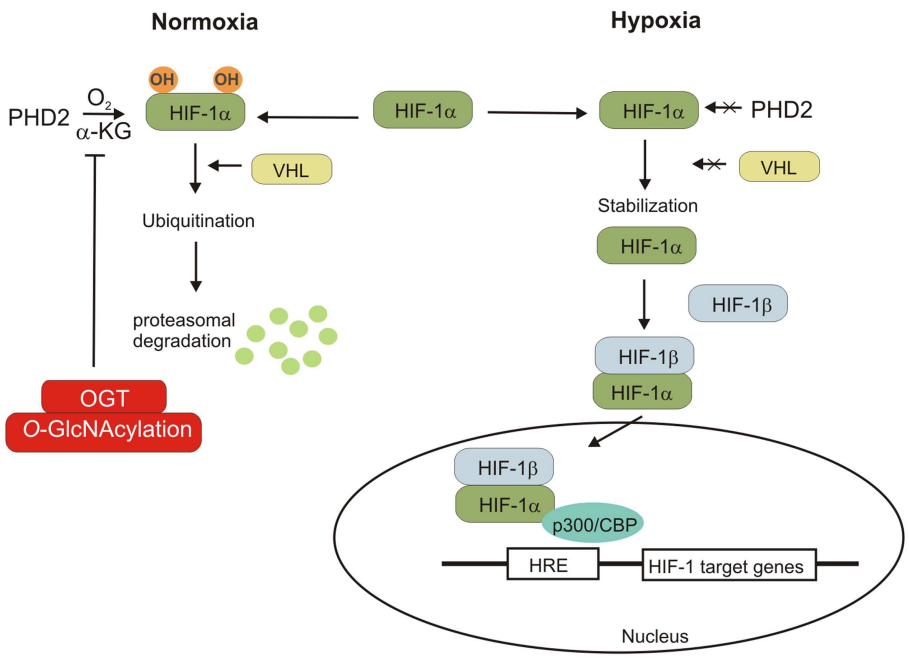

FIGURE 4 | Hypoxia-inducible factor regulation is shown. Under normal condition, HIF-1α subunit hydroxylated by PDH2 can bind to VHL protein, which promotes the polyubiquitination of HIF-1α and its degradation. The lack of oxygen prevents the hydroxylation of HIF-1α, leading to its stabilization. HIF-1α can associate with HIF-1β and the cofactor p300/CBP. The HIF-1 complex induces the transcription of genes containing hypoxia-responsive elements (HRE). OGT regulates stability of HIF-1α via regulation of α-ketoglutarate levels and inhibiting HIF-1α hydroxylation.

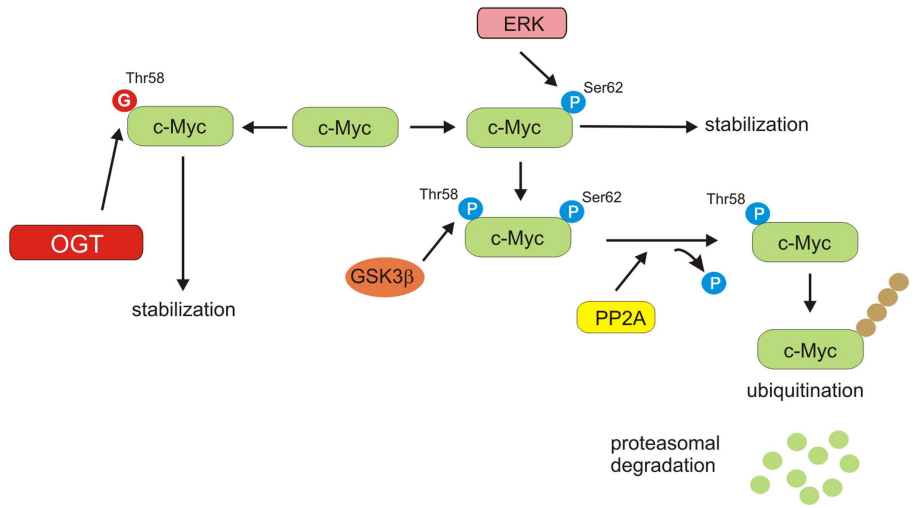

FIGURE 5 | c-Myc stability regulation. ERK phosphorylates c-Myc on Ser62, resulting in stabilization of protein. Phosphorylated on Ser62 c-Myc can be modified by GSK3β on Ser58. PP2A can dephosphorylate Ser62. Thr58 mono-phosphorylated c-Myc is a target for ubiquitin ligase complex, leading to proteasomal degradation. *O*-GlcNAcylation of Thr58 can compete with phosphorylation and potentially increase stability of c-Myc.

transporters and indirectly promotes glutaminase (GLS) activity by repressing expression of miR-23a/b, which targets *GLS1* transcript (64–66). High level of c-Myc in cancer cells causes glutamine addiction, and cells undergo apoptosis when deprived of glutamine (64).

Stability of c-Myc is controlled by phosphorylation of specific sites (67, 68). Activated extracellular receptor kinase (ERK) stabilizes c-Myc by phosphorylation at Ser62. Once c-Myc phosphorylated at Ser62, it is recognized by GSK3β, which phosphorylates it at Thr58. At that time, dephosphorylation of Ser62 is mediated by protein phosphatase 2A (PP2A) (69). c-Myc phosphorylated at Thr58, but not at Ser62 is recognized by the E3 ligase, which ubiquitinates c-Myc at the N-terminus and targets it for proteasome-dependent degradation (69, 70). Thus, phosphorylation of Thr58 is a key event in c-Myc regulation (**Figure 5**). Mutation of Thr58 has been observed in Burkitt's lymphomas and is associated with increased c-Myc protein stability. It was shown that c-Myc could be also *O*-GlcNAcylated at Thr58 (71–73). Increased Thr58 *O*-GlcNAcylation could compete with phosphorylation and potentially stabilize c-Myc. Moreover, PP2A has been found to be *O*-GlcNAcylated in oocytes of *Xenopus laevis* (74). However, the significance of its *O*-GlcNAcylation in cancer cells has not been established. Recently, Itkonen et al. have shown that OGT is, in fact, a central regulator of c-Myc stability in prostate cancer cells (75). OGT inhibition elicited a dose-dependent decrease in the levels of c-Myc protein but not c-Myc mRNA in prostate cell lines (75). Collectively, these data suggest that OGT by modification of c-Myc and PP2A could potentially regulate c-Myc stability and affect its function in cancer cell metabolism.

NF-κB

NF-κB is a glucose-responsive transcription factor that is involved in many biological processes such as inflammation and immune response, cell survival, growth, and development (76). Five members of NF-κB transcription factors family have been identified: p65 (RelA), RelB, c-Rel, p105/p50, and p100/p52. Activation of NF-κB proteins is tightly regulated and altered activation of the NF-κB signaling pathways has been linked to autoimmunity, chronic inflammation, and various cancers. In basal state, NF-κB is sequestered by inhibitor of κB (IκB) in the cytosol. Upon stimulation, IκB is phosphorylated by the IκB kinase (IKK) complex and is then degraded by the ubiquitin–proteasome system. The freed NF-κB translocates into the nucleus and induces gene transcription (76, 77) (**Figure 6**).

It has been suggested that NF-κB may be an important factor promoting the switch of cellular glucose metabolism from oxidative phosphorylation to oxygen-independent glycolysis in tumor cells (18). Kawauchi et al. showed the link between p53, NF-κB, and glycolysis (78). In p53-deficient cells, the activity of NF-κB was found to be enhanced and that caused an increase in the rate of aerobic glycolysis via upregulation of glucose transporter GLUT3 (78). On the other hand, it was found that NF-κB as a regulator of mitochondrial respiration, suppressed reprograming to aerobic glycolysis and prevented necrosis in cells upon nutrient starvation. But this function of NF-κB was p53-dependent and involved upregulation of mitochondrial synthesis of cytochrome *c* oxidase 2, which increased oxidative phosphorylation and reduced glycolytic flux in cells (79, 80). NF-κB is also involved in metabolism via p53-independent mechanisms. Kumar et al. have found that transglutaminase-2 regulates metabolic reprograming in mammary epithelial cells by constitutively activating nuclear factor NF-κB, which binds to hypoxia-inducible factor promoter and induces its transcription even under normoxic conditions (81).

Activation of NF-κB requires post-translational modifications such as phosphorylation and acetylation. Growing evidence also suggests a pivotal role for *O*-GlcNAcylation in the activation of NF-κB (82–86) (**Figure 6**). The *O*-GlcNAc modification sites within NF-κB p65 have been identified as Thr322 and Thr352.

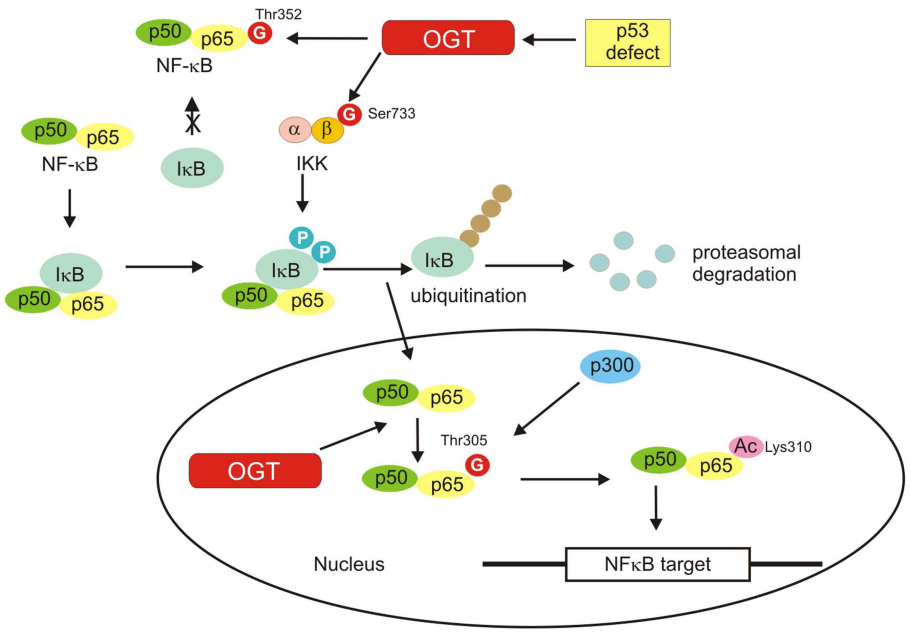

FIGURE 6 | NF-κB activation is shown. Inactive NF-κB is located in the cytosol complexed with the inhibitory protein IκB. Activated by extracellular signals IκB kinase (IKK) phosphorylates the IκB protein, which results in dissociation of IκB from NF-κB, ubiquitination, and degradation of IκB by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA. *O*-GlcNAcylation favors activation of NF-κB by regulation of IKKβ activity, inhibition of the interaction of NF-κB with IκB, and promotion of NF-κB acetylation.

O-GlcNAc modification of NF-κB p65 at Thr352 in response to high glucose has been shown to inhibit the interaction of NF-κB with IκB, causing the nuclear translocation of NF-κB and activation of its target genes (83). Recently, Allison et al. have demonstrated that OGT localizes to chromatin and drives p300-mediated acetylation of p65 at Lys310 in response to tumor necrosis factor (TNF) (84). The studies revealed that Thr305 was an important residue required for an attachment of the *O*-GlcNAc moiety on p65. The attachment of the *O*-GlcNAc moiety to p65 at Thr305 is a precondition for Lys310 acetylation, which is necessary for full NF-κB-dependent transcription (84).

IκB kinase is also *O*-GlcNAcylated. Kawauchi et al. showed that loss of p53 enhanced catalytic activity of IKKβ through *O*-GlcNAcylation in mouse embryonic fibroblasts (MEFs) and transformed human fibroblasts (87). *O*-GlcNAcylation of IKKβ occurred at Ser733 in the C-terminal domain, which was identified as an inactivating phosphorylation site. Thus, *O*-GlcNAcylation of IKKβ regulates its catalytic activity (87) (**Figure 6**).

The direct link between HBP, OGT, and NF-κB was shown in human pancreatic ductal adenocarcinoma cells (PDAC) (85). Ma et al. have observed increased HBP flux and hyper-*O*-GlcNAcylation in PDAC cells, which was associated with increased OGT and decreased OGA levels (85). In these cells, the NF-κB p65 subunit and upstream kinases IKKα/IKKβ were *O*-GlcNAcylated. Reducing p65 *O*-GlcNAcylation specifically by mutating two p65 *O*-GlcNAc sites caused the reduction of PDAC cells anchorage-independent growth (85).

p65 is not the only *O*-GlcNAcylated NF-κB family member. Ramakrishnan et al. examined the *O*-GlcNAcylation status of all of the NF-κβ family proteins in lymphocytes under hyperglycemic conditions (86). They have shown that c-Rel is the major *O*-GlcNAcylated NF-κβ subunit in lymphocytes, and that enhancement of its *O*-GlcNAcylation increases its transcriptional activity. They have identified Ser350 as the site of *O*-GlcNAcylation. Mutation of Ser350 blocked the *O*-GlcNAcylation of c-Rel and greatly reduced DNA-binding ability and transactivation potential in cells in response to stimulation of the T cell receptor (86).

CARBOHYDRATE RESPONSIVE ELEMENT-BINDING PROTEIN

Carbohydrate responsive element-binding protein (ChREBP) is helix–loop–helix leucine zipper transcription factor, which mediates glucose-dependent induction of glycolytic and lipogenic enzyme genes (88–94). ChREBP is involved in the induction of liver pyruvate kinase (L-PK) and acting synergistically with sterol regulatory element-binding protein 1c (SERBP-1c) activates genes encoding lipogenic enzymes: acetyl-CoA carboxylase (ACC) and FAS (88–94). ChREBP is expressed in most tissues but the highest level of this protein is observed in liver and adipocytes (94). The function of ChREBP in hepatocytes has been extensively studied but its role in cancer cell metabolism has not been fully elucidated. However, the studies of Tong et al. suggest that ChREBP plays a key role in regulation of proliferating cells metabolism (95). This study demonstrated that induction of ChREBP in response to mitogenic stimulation was required for proliferation of HCT116 colorectal cancer cells and HepG2 hepatoblastoma cells. Suppression of ChREBP causes a reduction of aerobic glycolysis, *de novo* lipogenesis, and nucleotide biosynthesis but stimulated

mitochondrial respiration (95). Thus, ChREBP seems to contribute to the glycolytic phenotype exhibited by cancer cells. It plays a key role in directing glucose metabolism into anabolic pathways, i.e., lipid and nucleotide biosynthesis during cell growth (95).

Carbohydrate responsive element-binding protein contains several phosphorylation sites recognized by protein kinase A (PKA) such as Ser196, Ser626, and Thr666 that are involved in negative regulation of its nuclear import and DNA-binding activity (94). However, mutations of Ser196, Ser626, and Thr666 did not significantly affect the glucose-responsiveness of ChREBP. It appears that PKA-mediated phosphorylation and glucose activation are independent regulatory mechanisms (94).

Carbohydrate responsive element-binding protein is modified by *O*-GlcNAcylation and this modification increases its protein level and transcriptional activity (96, 97) (**Figure 7**). *O*-GlcNAcylation affects ChREBP protein stability and protects it against proteasomal degradation. *O*-GlcNAcylated ChREBP under hyperglycemic conditions shows increased activity toward its target glycolytic (*LPK*) and lipogenic (*ACC*, *FAS*, *SCD1*) genes (97).

Ido-Kitamura et al. have shown that FoxO1 is a negative regulator of ChREBP activity (98) (**Figure 7**). FoxO1 decreases glucose utilization and lipid synthesis by reducing ChREBP activity. Overexpression of FoxO1 in hepatocytes attenuated ChREBP activity by suppressing *O*-GlcNAcylation and reducing the protein stability. FoxO1 inhibits high glucose- or OGT-induced L-PK promoter activity by decreasing ChREBP recruitment to the L-PK promoter (98). However, the exact mechanism by which FoxO1 inhibits ChREBP *O*-GlcNAcylation is not known.

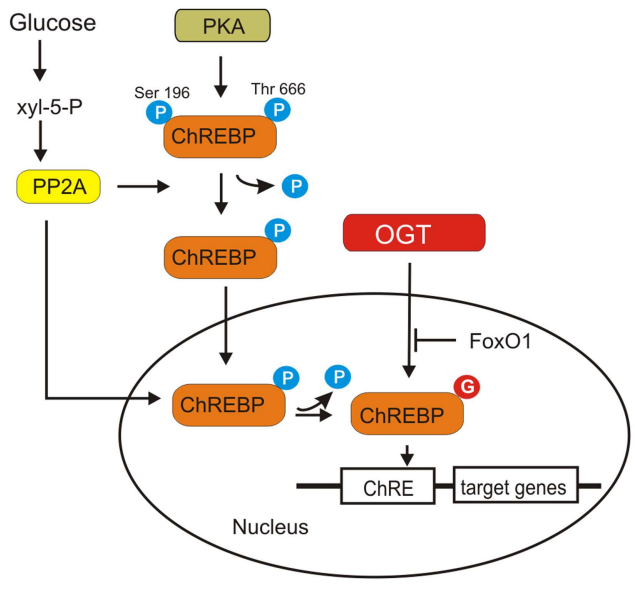

FIGURE 7 | ChREBP activation is shown. ChREBP is phosphorylated by PKA. Dephosphorylation of Ser196 and Thr666 ChREBP is required for its translocation into the nucleus and DNA binding. A particular isoform of protein phosphatase 2A (PP2A), selectively activated by Xyl-5-P, an intermediate of pentose phosphate pathway, is responsible for both cytosolic and nuclear dephosphorylation of ChREBP. *O*-GlcNAcylation of ChREBP increases its protein level and transcriptional activity. Overexpression of FoxO1 attenuates ChREBP activity.

GLYCOLYTIC ENZYMES

Several glycolytic enzymes related to Warburg effect are *O*-GlcNAcylated (99–101). One of the most important enzymes involved in cancer cell metabolism reprograming is pyruvate kinase (PK) (17). This enzyme catalyzes a reaction generating pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP (102). There are four isozymes of PK (L, R, M1, and M2) and these vary in tissue distribution, kinetic characteristics, and regulatory mechanism. PKL and PKR are products of PKL gene, transcribed with different promoters (103). PKM1 and PKM2 are encoded by the PKM gene and are the products of two alternatively spliced exons (exon 9 and exon 10, respectively) (104). Isozyme M1 is expressed in most adult differentiated tissues, whereas M2 is expressed in embryonic cells, adult stem cells, and cancer cells (105). PKM2 possesses unique properties important in the reprograming of cell metabolism. Active PKs consist of four subunits, and PKL, PKR, and PKM1 form stable tetramers. PKM2 can exist as an active tetramers and much less active dimers (102). When PKM2 is in dimeric form, glycolytic intermediates above PK accumulate and may be directed toward anabolic pathways for synthesis of amino acids, nucleic acids, and phospholipids (17).

O-GlcNAcylation may be involved in regulation of PKM2 activity. The site of *O*-GlcNAcylation on PKM2 has not been established. However, the increased *O*-GlcNAcylation in cells is associated with a decrease in general PK activity. It is suggested that hyper-*O*-GlcNAcylation in cancer cells would likely decrease PKM2 activity contributing to directing glycolytic intermediates toward biosynthetic pathways (25). Interestingly, Champattanachai et al. showed that PKM2 is *O*-GlcNAc modified only in breast cancer tissues but not in normal samples (101).

To form the active tetramer, PKM2 requires fructose-1,6-bisphosphate, which is produced in reaction catalyzed by phosphofructokinase-1 (17). Yi et al. have demonstrated that PFK1 is *O*-GlcNAcylated at Ser529 in response to hypoxia in cancer cells (100). Glycosylation inhibits PFK1 activity and redirect the flux of glucose from glycolysis through the PPP (100). Yi et al. have also examined the impact of OGT overexpression on HK, PGK, and PK activities (100). Direct *O*-GlcNAcylation status of these proteins has not been studied but in cancer cells with increased OGT activity, HK activity was increased while PGK and PK activities were decreased (100).

EPIGENETICS, *O*-GlcNAcylation, AND CANCER METABOLISM

The connection between cancer metabolism reprograming and epigenetics may be considered in two aspects. Changes in cancer cell metabolism may impact epigenetic gene regulation since the enzymes involved in modification of histones or chromatin remodeling utilize substrates generated by metabolic pathways (106, 107). On the other hand, through modification and remodeling of chromatin, extracellular signals from tumor microenvironment or nutrition compounds can regulate the expression of genes involved in cellular proliferation as well as cellular metabolism (18).

The studies of Gao et al. have revealed that high glucose is an inducer of monoubiquitination of histone H2B at Lys120 in cultured glioma cells (108). Nutrient deprivation causes decrease of H2B ubiquitination (109). Compared to the other histone modifications, ubiquitination is less well studied and its specific roles in tumors remain to be clarified. However, de-regulation of H2Bub has been suggested as an etiology factor of cancer development (110, 111). The enzymes responsible for H2B monoubiquitination were first identified in *Saccharomyces cerevisiae* as Rad6 (E2) and Bre1 (E3). In humans, there are two homologs of Rad6 (HR6A and HR6B) and Bre1 (RNF20 and RNF40) (110). The latest seem to play main role in ubiquitination of H2B in humans (112, 113). RNF20 physically interacts with the tumor suppressor protein p53, functioning as a transcriptional co-activator of p53 (112). RNF20 is also required for p53 expression and RNF20 depletion leads to more than 10-fold decrease in expression of p53 (114). Monoubiquitination of histone H2B can be reversed by Ubp8, a component of the transcriptional activator SAGA in yeast. USP22 is the human homolog of this protein (110). The results showed also that USP22 is a positive regulator of c-Myc-dependent transcription and induction of c-Myc targeted genes is impaired in USP22-depleted cells (115). Although most data indicate that H2Bub and its ubiquitin ligases act as tumor suppressors, a few studies suggest that their activity may promote tumorigenesis (116–118). The discrepancies found may be due to different role of H2B ubiquitination in tumorigenesis and tumor progression. H2B ubiqiutination may be involved in arising of tumors and proliferation of cancer cells but may suppress cancer stem cell phenotypes. In fact, it has been found that RNF20 and RNF40 knockdown decrease cell proliferation but increase cell migration (111).

Recent studies have also shown that *O*-GlcNAcylation plays an important role in H2B ubiquitination. H2B is *O*-GlcNAcylated by OGT at Ser112 (119–122). H2B Ser112 *O*-GlcNAcylation changes in response to extracellular glucose (120). It is suggested that H2B Ser112 *O*-GlcNAcylation promotes Lys120 monoubiquitination because GlcNAc moiety can serve as an anchor for a histone H2B ubiquitin ligase (122). *O*-GlcNAcylation of H2B is probably important for transcriptional activation since modified by *O*-GlcNAc H2B is frequently located near transcribed genes (120). H2B Ser112 *O*-GlcNAcylation depends on TET2/3 (ten-eleven translocation), which is an enzyme that catalyzes the conversion of 5-methylcytosine to 5-hydoxymethylcytosine (121). TET2 and 3 directly interact with OGT (121, 122). TET2 promotes OGT activity and facilitates OGT-dependent histone modification (121). Xu et al. have found that AMPK could suppress histone H2B *O*-GlcNAcylation (123). AMPK directly phosphorylates OGT and this modification inhibits OGT–chromatin association, histones *O*-GlcNAcylation, and gene transcription. The authors have suggested that there is a crosstalk between the LKB1-AMPK and the hexosamine biosynthesis (HBP)-OGT pathways, which coordinate together for the sensing of nutrient state and regulation of gene transcription (123).

Additionally, it has been recently found that methyltransferase EZH2, which is a component of Polycomb repressive complex 2, is *O*-GlcNAcylated at Ser75 in breast cancer cells (124). This modification stabilizes EZH2 and facilitates the trimethylation of histone H3 at Lys27. Thus, the study of Chu et al. uncovered a unique epigenetic role of OGT in regulating histone methylation (124). It is also possible that OGT by regulation of EZH2 may be involved in metabolic reprograming. Polycomb group protein EZH2 is a direct upstream regulator of c-Myc oncogene (125). c-Myc is one of the main regulators of cancer cell reprograming process. EZH2 was found to activate c-Myc in breast cancer cells through the ERα and the Wnt pathways (126).

O-GlcNAcylation plays an important role in activation of NF-κB and this factor seems to be also involved in epigenetic regulation of Warburg effect. Liu et al. have shown that fructose-1,6-bisphosphatase-1 (FBP1), which is gluconeogenesis regulatory enzyme and functions to antagonize glycolysis has been down-regulated through NF-κB pathway in Ras-transformed NIH3T3 cells (127). The authors have found that inhibition of NF-κB restored FBP1 expression, partially through demethylation of FBP1 promoter. NF-kB can be involved in negative regulation of gene expression through interaction with transcription co-repressors such as histone deacetylase HDAC1 and HDAC2 (128, 129). Interestingly, HDAC1 has been found to be *O*-GlcNAcylated in HepG2 liver carcinoma cells (130). It is suggested that OGT can contribute along with HDAC to the repression of genes. Moreover, histone deacetylases can interact with DNA methyltransferases that by methylation of promoters can cause stable silencing of gene expression (18).

CONCLUSION

There is no doubt that metabolic reprograming is one of the main hallmarks of cancer cells. The most important changes in cancer metabolism include elevation of glucose uptake and glycolysis, enhanced glutaminolysis, induction of PPP, and upregulation of macromolecule synthesis. These changes are beneficial for cancer proliferation, growth, metastasis, and angiogenesis. Many studies have shown that *O*-GlcNAcylation, which acts as a nutrient sensor, is elevated in different cancers and seems to be responsible for coupling cell metabolic status to signal transduction and transcription. It is strongly suggested that increased glucose flux through HBP and elevated UDP-GlcNAc is a general feature of cancer cells that contributes to hyper-*O*-GlcNAcylation. High activity of OGT as a result of both high substrate level and gene overexpression favors modification of several key factors involved in cancer metabolism reprograming. *O*-GlcNAcylation impacts their stability, activity, localization, interaction with other proteins, and in consequence, enhances their effect on reprograming of cell metabolism. Akt, c-Myc, ChREBP, NF-κB, and HIF-1 affect metabolism by direct or indirect regulation of expression of glucose transporters as well as glutaminolytic, glycolytic, and lipogenic enzymes. OGT and *O*-GlcNAcylation may also constitute a link between nutrient status and epigenetic regulation of gene expression. In response to nutrient availability, OGT may directly affect histone code by attachment of *N*-acetylglucosamine residues. Moreover, OGT can indirectly influence gene expression by interactions with histone modifying enzymes and modulation of their stability and activity. However, although a large body of evidence has demonstrated the significance of *O*-GlcNAcylation in metabolism regulation, there is still much to learn about its role in cancer metabolism reprograming. Elucidating the relationship

between O-GlcNAc cycling controlling mechanism and cellular metabolic activity of cancer cells is an exciting challenge for future research.

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Aberrant *O*-GlcNAcylated proteins: new perspectives in breast and colorectal cancer

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Increasing glucose consumption is thought to provide an evolutionary advantage to cancer cells. Alteration of glucose metabolism in cancer influences various important metabolic pathways including the hexosamine biosynthesis pathway (HBP), a relatively minor branch of glycolysis. Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), an end product of HBP, is a sugar substrate used for classical glycosylation and *O*-GlcNAcylation, a post-translational protein modification implicated in a wide range of effects on cellular functions. Emerging evidence reveals that certain cellular proteins are abnormally *O*-GlcNAc modified in many kinds of cancers, indicating *O*-GlcNAcylation is associated with malignancy. Since *O*-GlcNAc rapidly on and off modifies in a similar time scale as in phosphorylation and these modifications may occur on proteins at either on the same or adjacent sites, it suggests that both modifications can work to regulate the cellular signaling pathways. This review describes the metabolic shifts related to the HBP, which are commonly found in most cancers. It also describes *O*-GlcNAc modified proteins identified in primary breast and colorectal cancer, as well as in the related cancer cell lines. Moreover, we also discuss the potential use of aberrant *O*-GlcNAcylated proteins as novel biomarkers of cancer.

Keywords: breast cancer, cancer biomarker, colorectal cancer, hexosamine biosynthesis pathway, *O*-GlcNAcylation, phosphorylation

INTRODUCTION

Glucose consumption is required by living cells. Through glycolysis, glucose is mainly broken down into pyruvate, which enters into the tricarboxylic acid (TCA) cycle for maximum energy production. Cancer cells, however, uptake glucose at a higher rate and produce lactic acid rather than metabolizing pyruvate through the TCA cycle. This adaptive metabolic shift is termed the Warburg effect (1), leading to anaerobic glycolysis, and is thought to provide an evolutionary advantage to cancer cells by providing both increase bioenergetics and biosynthesis (2). Many proto-oncogenes (e.g., Ras and Myc) and tumor suppressors (e.g., p53) influence metabolism, and mutations in these genes can upregulate glucose uptake in cancer cells and promote a metabolic phenotype supporting tumor cell growth and proliferation (3). Elevated glucose uptake in cancer cells can be applied to monitor the location of primary and metastatic tumor sites; for an example, using F-18 fluorodeoxyglucose (FDG), a glucose analog, with a combination of positron emission tomography/computed tomography (PET/CT) (4). In general, most glucose enters into the glycolytic pathway, but a small fraction of glucose goes to the hexosamine biosynthesis pathway (HBP). This pathway generates a nucleotide sugar, uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), used for many reactions including a sugar donor, and in multiple glycosylation reactions such as proteoglycan synthesis, *N*-linked glycosylation, and the formation of *O*-linked glycoproteins or *O*-GlcNAcylation (5).

The *O*-GlcNAc is dynamically regulated by two key enzymes: *O*-GlcNAc transferase (OGT) (6) and *O*-GlcNAcase (OGA) (7), for the addition and removal of a single GlcNAc residue from proteins, respectively. Unlike classical glycosylation present in endoplasmic reticulum (ER) and golgi apparatus, *O*-GlcNAcylation takes place in the cytoplasm, nucleus, and mitochondria, and is implicated in a wide range of effects on cellular function and signaling in metabolic diseases and cancer (8). Almost three decades since its discovery, more than 1,000 *O*-GlcNAcylated proteins have now been identified (9). Growing evidence reveals that *O*-GlcNAcylation has extensive crosstalk with phosphorylation either on the same or adjacent sites of various proteins (10). The interplay of these two post-translational protein modifications (PTMs) can work to fine-tune the regulation of target protein functions, stabilization, translocation, complex formation, and enzyme activity, which subsequently affects cellular signaling pathways. Glucose flux into the HBP resulting in increased *O*-GlcNAcylation is an emerging paradigm of the integration of metabolic and signaling networks. This review emphasizes the recent connection between the HBP and metabolic shifts, *O*-GlcNAc cycling enzymes, and *O*-GlcNAcylation and phosphorylation found in most cancers. In addition, *O*-GlcNAcylated proteins have been identified in primary breast and colorectal cancer (CRC) as well as in their cancer cell lines. Moreover, *O*-GlcNAcylated proteins are discussed as potential candidates for novel biomarkers of cancer.

HBP, METABOLIC SHIFTS, AND CANCER

The final end product of the HBP is UDP-GlcNAc (**Figure 1**), which is built up from glucose, glutamine, fatty acid (acetyl-CoA), and uridine. *O*-GlcNAc level may, therefore, be considered as a

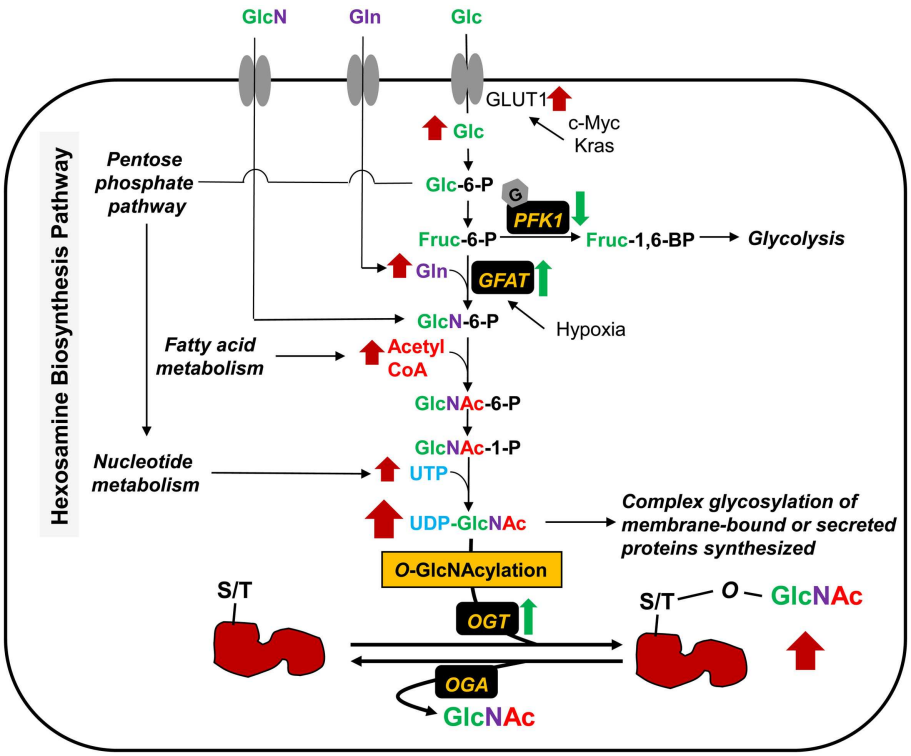

FIGURE 1 | Metabolic shifts through the hexosamine biosynthesis pathway (HBP) and protein *O*-GlcNAcylation in cancer. The HBP produces UDP-GlcNAc from its parts, glucose (Glc), glutamine (Gln), acetyl-CoA, and UTP. The levels of these various metabolic inputs are all increased in cancer cells. Glucose is transported into cells by glucose transporters (e.g., GLUT1). Overexpression or mutation of *c-Myc* and *Kras* leads to an increase of glucose uptake through GLUT1 activation. *O*-GlcNAcylation of PFK1 suppresses the enzyme activity, resulting in redirection of glucose metabolism. GFAT is the rate-limiting enzyme for glucose entry into the HBP, which converts Fruc-6-P and glutamine (Gln) into GlcN-6-P. Hypoxia induces the GFAT transcription and expression. Glucosamine (GlcN) enters into cells via the glucose transporters and is phosphorylated to GlcN-6-P by hexokinase, bypassing GFAT. UDP-GlcNAc serves as a sugar donor of classical glycosylation and *O*-GlcNAcylation. The later glycosylation, taking place in cytoplasm, nucleus, and mitochondria, is controlled by *O*-GlcNAc cycling enzymes; OGT and OGA for the addition and removal of sugar in and out of proteins, respectively. OGT level is also upregulated, and consequently results in an increase of *O*-GlcNAcylation in several kinds of cancers.

nutrient sensor for both normal physiology and disease pathophysiology, such as diabetes and cancer. Approximately 2–4% of glucose uptake into the cells enters into the HBP (11). Oncogenic genes (e.g., *c-Myc*, *Kras*) and hypoxia contribute in glucose metabolism. Osthus et al. showed that overexpression of c-Myc directly transactivates genes encoding glucose transporter GLUT1 and increases glucose uptake (12). Ying et al. also reported that activation of oncogenic Kirsten rat sarcoma viral oncogene homolog (Kras) is required in stimulating glucose uptake in an *in vivo* model of pancreatic ductal adenocarcinoma (PDAC) (13). Loss of Kras functions led to the downregulation of glucose uptake. Moreover, the levels of metabolites of the pentose phosphate pathway (PPP) and the HBP, as well as *O*-GlcNAcylation were decreased upon Kras inactivation. The rate-limiting enzyme in the HBP, glutamine:fructose-6-phosphate amidotransferase (GFAT) catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate. Hypoxia has been reported to induce the transcription of GFAT gene through the hypoxia responsive element (HRE) (14). Guillaumond et al. also showed that hypoxia increases the levels of GFAT mRNA expression and *O*-GlcNAcylation in pancreatic cancer cells (15). Blocking of GFAT activity by azaserine led to a decrease in hypoxic cell number, suggesting that activation of the HBP is required for survival of hypoxic pancreatic cancer cells. Glucosamine, although normally present at low levels in bodily fluids, enters into cells via the glucose transporters (16) and is phosphorylated to glucosamine-6-phosphate by hexokinase, thereby bypassing GFAT and elevating UDP-GlcNAc levels. Recently, Yang et al. demonstrated that radiolabeled glucosamine analogs can be introduced as novel agents to complement FDG imaging to increase specificity and improve the accuracy of lesion size in oncology applications (17). Glucosamine analogs become UDP-GlcNAc analogs and the newly modified *O*-GlcNAc analog proteins catalyzed by OGT are found in the cytoplasm and nucleus, whereas FDG, a glucose analog, is not metabolized and remains in the cytoplasm of cells. This method can be used to tag transcription factors known to be modified by *O*-GlcNAc (e.g., Sp1 and NF-κB), which are moved from the cytoplasm into the nucleus upon stimulation or activation in cancer cells. Glucosamine analogs can, therefore, be used in nuclear imaging to observe bio-activity in tumors.

In cancer cells, hyperglycemia is thought to feed the HBP and promote abnormally elevated *O*-GlcNAcylation of key signaling

proteins (18, 19). Phosphofructokinase 1 (PFK1), one of the key enzymes in the glycolysis pathway, is modified by *O*-GlcNAc at Ser-529 in response to hypoxia in the lung cancer cell line, H1299 (20). This glycosylation suppressed PFK1 activity and redirected glucose flux through the PPP, thus increasing nucleotide metabolism and providing a growth advantage for cancer cells. Blocking of *O*-GlcNAcylated PFK1 led to a reduction of cancer cell proliferation *in vitro* and impaired tumor formation *in vivo*. Other glycolytic enzymes also reported to be modified by *O*-GlcNAc include triose phosphate isomerase (TPI) (21), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (22–25), enolase 2 (Eno2) (22, 25–27), and pyruvate kinase M2 (PKM2) (25). Although the glycosylation sites of these glycolytic enzymes have not been identified, their modifications could potentially modulate tumor cell metabolism promoting proliferation.

Glutamine is a major source of energy for rapidly dividing cells and is also an amino donor substrate for the conversion of fructose-6-phosphate to glucosamine-6-phosphate by GFAT in the HBP. Glutamine enters into cells via the glutamine transporter, which is found to be overexpressed in various cancers (28, 29). If GFAT is active, glutamine uptake in cancer cells can increase the HBP flux, as well as UDP-GlcNAc, and *O*-GlcNAcylation. This augmentation, for example, is found in human PDAC (30). In a related context, inhibition of glutaminase, an amidohydrolase enzyme, which generates glutamate from glutamine, led to a lower proliferation rate in human breast cancer cells (31). This inhibition also caused a reduction of GFAT activity and changes in *O*-GlcNAc targets, such as OGT and transcription factor Sp1. Moreover, removal of glutamine from the culture medium promotes tumor cell differentiation and decreased proliferation; conversely, addition of glutamine protects cells from apoptosis and induces proliferation (32). Thus, metabolic shifts through the HBP flux, hyperglycemia, glutamine consumption, elevation of UDP-GlcNAc, and *O*-GlcNAcylation contribute in regulating signaling cascades and cell proliferation in cancer.

REGULATION OF *O*-GLcNAc CYCLING ENZYMES AND *O*-GLcNAcylation

O-GlcNAcylation is tightly regulated by *O*-GlcNAc cycling enzymes, OGT and OGA for the addition and removal of sugar in and out of proteins, respectively. In human beings, there is only a single *OGT* gene localized at chromosome Xq13.1 (33). The alternative splicing of the *OGT* gene translates into at least three different isoforms of OGT enzyme, including the 110-kDa nucleocytoplasmic isoform (ncOGT), the 103-kDa mitochondrial isoform (mOGT), and the short 78-kDa isoform (sOGT) (34). The different isoforms of OGT differ in the number of tetratricopeptide repeats (TRP) located at the N-terminal domain, which is involved in protein interaction and is important for protein substrate recognition (33). OGT is inhibited by various inhibitors such as ST045849 (35). A single OGA encoding gene, identified as meningioma-expressed antigen 5 (*MGEA5*), is localized on chromosome 10q24.1–q24.3 (33). The two isoforms of OGA from alternative splicing have been identified (7). The 130-kDa isoform localizes predominantly in the cytoplasm, while the 75-kDa isoform, lacking one-third of the C-terminal domain, resides in the nucleus (33). The N-terminal domain of OGA is the catalytic domain, while the C-terminal domain contains a histone acetyltransferase (HATs) sequence (33). This enzyme can be inhibited with inhibitors such as PUGNAc, Thiamet G (36), and GlcNAcstatin (37). A number of studies have revealed complex formation between OGA and OGT. In a yeast-two hybrid screening study, OGA has been reported as a binding partner of OGT (38). The OGA-OGT complex has also been identified in combination with mSin3A, and histone deacetylase-1 (HDAC1) when the estrogen and progesterone signaling are stimulated in CHO cells (39). Therefore, it is possible that the binding between OGA and OGT may affect the regulation of each other under specific conditions.

O-GlcNAcylation in cancer has been increasingly studied for the past decade. Accumulating evidence reveals that the levels of *O*-GlcNAcylation and its cycling enzymes in malignant tissues are altered in various cancers. Recently, we showed that *O*-GlcNAcylation and OGT expression levels are increased in both breast and CRC (25, 40). As mentioned above, the *O*-GlcNAc cycling enzymes OGT and OGA tightly regulate the level of *O*-GlcNAcylation. Enhanced *O*-GlcNAcylation level corresponding to increased OGT and decreased OGA expression is commonly observed in various cancers including bone (41), bladder (42), breast (26, 43–45), bile duct (46), colon (47–49), leukemia (50), liver (51), lung (47), ovary (36), pancreas (30), and prostate (52, 53). Alteration of this glycosylation in thyroid cancer, however, occurs in the opposite way (54). Interestingly, no mutations in OGT and OGA genes have been reported in human cancers, suggesting that these enzymes are tightly conserved. Therefore, besides increased flux through the HBP, the altered expression of OGT and OGA also contributes to enhancement of *O*-GlcNAcylation in most cancers.

Manipulation and regulation of *O*-GlcNAc cycling enzymes in cancer may be a way of stopping cancer growth. We have shown that OGT silencing led to a reduction of anchorage-independent growth of a breast cancer cell line, MDA-MB-231 (25). Caldwell et al. also reported that reduction of OGT in breast cancer cells caused inhibition of tumor growth, both *in vitro* and *in vivo* (43). OGT knockdown did not block cell growth in a non-transformed breast cell line, MCF-10A (43). Consistent with this finding, reduction of *O*-GlcNAcylation had no effect on non-transformed pancreatic epithelial cell growth, but inhibited human PDAC cell proliferation and anchorage-independent growth, and triggered apoptosis (30). Because cancer cells appear to overexpress OGT, strategies to reduce OGT activity or expression level are attractive as an anti-cancer approach. Recently, the crystal structure of human OGT was solved by Lazarus et al. (55). As mentioned above, there is only one OGT gene in mammals with three alternative splicing isoforms, but more than one thousand *O*-GlcNAc proteins have been identified so far (dbOGAP: Database of *O*-GlcNAcylated Proteins and Sites). How does one OGT specifically modify a wide range of target proteins? OGT actually has protein-binding partners, which can form a transient complex under specific stimulation such as by nutrient, stress, and hormone. These partners include p38, OIP106, and OGA (38). The binding between OGT and its adaptor specifically targets the catalytic site of OGT to *O*-GlcNAcylate its target proteins (55). This knowledge will accelerate the rational design of OGT inhibitors for anti-cancer drug in the future.

O-GLcNAc MODIFICATION AND PHOSPHORYLATION IN CANCER

O-GlcNAcylation has been studied widely for 30 years. Novel methodologies for enrichment of *O*-GlcNAc modified proteins are now available, as well as mass spectrometric methods for their characterization. Increasing study of *O*-GlcNAc proteins suggest extensive crosstalk between *O*-GlcNAcylation and phosphorylation. Crosstalk between these two modifications occurs not only by sharing their protein substrates but also by regulating each other's cycling enzymes. Both post-translational modifications share many characteristics including the cycling of their substrates at a similar time scale, the site of modification, and cellular state (33). This becomes more complicated when several studies show *O*-GlcNAcylation of many kinases (56), as well as phosphorylation of OGT and OGA (10).

The *O*-GlcNAc attachment sites can be predicted using online software (57) such as Yin-Yang and dbOGAP (Database of *O*-GlcNAcylated Proteins and Sites). These programs can be used to determine the interplay between *O*-GlcNAcylation and phosphorylation. The crosstalk between *O*-GlcNAcylation and phosphorylation in cancer has been observed in various biological signaling regulators, including c-Myc (58, 59), p53 (57, 60), Snail1 (61), and NF-κB p65 subunit (30).

c-Myc is a transcription factor regulating transcription of many genes involved in cell proliferation, cell differentiation, and programed cell death, and displays a reciprocal interplay between both modifications. c-Myc at Thr-58 can be both a target for phosphorylation by GSK3 and *O*-GlcNAcylation (58, 59). Crosstalk between these two modifications is competitive depending on certain conditions. For example, *O*-GlcNAc modification at Thr-58 of c-Myc was higher than phosphorylation at the same site when cells are starved of serum. Serum stimulated cells showed the opposite result, since Thr-58 shows enhanced phosphorylation and decreased *O*-GlcNAcylation. Moreover, point mutation at Thr-58 in the coding region of c-*myc* is frequently found in human Burkitt lymphomas (59, 62). Therefore, modifications of this site might be crucial for tumor progression.

Yang et al. reported *O*-GlcNAc modification of p53 at Ser-149 in a breast cancer cell line, MCF-7 and a lung cancer cell line, H1299 (57, 60). *O*-GlcNAcylation at Ser-149 reduced phosphorylation at Thr-155, leading to disruption of binding between Mdm2 and p53, which consequently reduced p53 ubiquitin-proteasome degradation (57). Another study by Park et al. showed the important role of *O*-GlcNAcylation of Snail1, a transcriptional repressor of E-cadherin (61). Snail1 is phosphorylated by GSK-3β, promoting its ubiquitination and degradation (63). Elevated *O*-GlcNAc level caused by OGA inhibitors inhibited the phosphorylation-mediated proteasomal degradation of Snail1 and consequently increased Snail1 half-life in a similar manner to p53.

Nuclear factor-kappa B (NF-κB) is a well-known transcription factor regulating cytokine production, lymphocyte activation, and proliferation. NF-κB activation was found in lymphoma and many solid tumors (64). NF-κB is a dimer of p65 (RelA) and p50 subunits. Ma et al. reported *O*-GlcNAc modification of NF-κB p65 subunit and IKKα/IKKβ in human PDAC (30). Phosphorylation of the p65 subunit was increased when global *O*-GlcNAcylation was reduced.

Crosstalk between *O*-GlcNAcylation and phosphorylation in cancer are not always reciprocal. Examples include vimentin and heat shock protein 27 (HSP27), as well as keratin 8 and 18. *O*-GlcNAc sites of vimentin are on Ser-7, Thr-33, Ser-34, and Ser-54 (65). Alteration of *O*-GlcNAc level by OGA overexpression in HeLa cells led to decreased pSer-82 level and increased pSer-71 level in vimentin (66). Guo et al. also showed that nuclear translocation of HSP27 observed in liver cancer cells is regulated by both *O*-GlcNAc and phosphate groups (67). Another study from Srikanth et al. displayed the synergistic effects of *O*-GlcNAcylation and phosphorylation on keratin 8 and 18 (68). The more *O*-GlcNAc modification occurs, the more phosphorylation was observed in soluble keratins compared to filamentous form. Moreover, increased *O*-GlcNAcylation and phosphorylation of keratin 8 and 18 were observed in heat stress-induced HepG2 cells.

COLORECTAL CANCER AND *O*-GLcNAcylation

THE EXPRESSION LEVELS OF *O*-GLcNAc, OGT, AND OGA IN CRC

Colorectal cancer is one of the most common cancers worldwide. It ranks the third in men and second in women according to World Health Organization GLOBOCAN database, in 2012 (69). Even though CRC is a curable cancer, its mortality rate is remarkably high, accounting for 8% of all cancer deaths. Many studies are focused on molecular targets of CRC for finding biomarkers and improving treatments, but there is a little research on the regulation of *O*-GlcNAc, OGT, and OGA in CRC.

Significantly elevated levels of OGT and *O*-GlcNAcylation were observed in CRC tissues compared to adjacent normal tissues (47). However, OGA level was not significantly enhanced in such cancer tissue samples (47). Consistent with this finding, Phueaouan et al. also reported that, in primary CRC patients (grade II), the upregulation of *O*-GlcNAcylation and OGT enzyme was found in CRC tissues, but the expression of OGA did not differ in CRC tissue extracts compared to normal samples (40).

Two studies reported changes in global *O*-GlcNAcylation of CRC cell lines in association with biological effects. A colorectal adenocarcinoma cell line, HT29 was transfected with the sh*OGT* expressing lentiviral vector in order to knockdown the *OGT* gene (47). The level of *O*-GlcNAcylation was decreased, but this did not reduce invasion in HT29, but did diminish anchorage-independent growth. Increasing *O*-GlcNAc levels in Thiamet-G treated HT29 cells markedly enhanced colony formation in soft agar. Another study on the association of *O*-GlcNAcylation and CRC recently indicated that the metastatic SW620 clone showed higher *O*-GlcNAcylation level than the primary SW480 clone (48). Enhanced *O*-GlcNAcylation by si*OGA* knockdown in SW620 resulted in the alteration of morphology to a fibroblast-like morphology, associated with the epithelial metastatic progression, and growth retardation. In addition, transcriptomics by microarray analysis revealed that silencing of *OGA* can affect the expression of many genes involved in cell movement and growth, as well as in lipid and carbohydrate metabolism (48).

O-GLcNAcylated PROTEINS AND O-GLcNAc TARGETING IN CRC

A multistage carcinogenesis model of CRC progression was proposed by Vogelstein et al. (70). The initial step, which changes normal epithelium cells to early adenoma, includes mutation of

APC (adenomatous polyposis coli) gene resulting in nuclear accumulation of protooncogene β-catenin (49). Nuclear β-catenin activates the transcription of c-Myc and cyclin D1, which are important for cell proliferation. β-Catenin is negatively regulated by phosphorylation leading to proteasomal degradation. Mutation of β-catenin at specific amino acids stabilizes β-catenin and subsequent nuclear localization (71). Little information has been reported on *O*-GlcNAc modified proteins in CRC (**Figure 2**). *O*-GlcNAcylated proteins were identified in the CRC molecular signaling pathway, including β-catenin (49) and Snail1 (61). Olivier-Van Stichelen et al. found that β-catenin of the normal colon cell line, CCD841CoN, showed less *O*-GlcNAcylation compared to two other CRC cells, HT29 and HCT116 (72). Other work from the same group demonstrated that β-catenin and global *O*-GlcNAc levels were increased in proteins extracted from colons of mice fed with high carbohydrate diet and Thiamet G (49). Four *O*-GlcNAcylation sites on β-catenin including Ser-23, Thr-40, Thr-41, and Thr-112 were mapped by ETD-MS/MS. Increased global *O*-GlcNAcylation of CRC cells reduced phosphorylation of β-catenin at Thr-41, located in the D box of β-catenin, which is important for proteasomal degradation.

Snail1, a transcriptional suppressor of E-cadherin, is upregulated in CRC tissues (73, 74). E-cadherin functions in cell adhesion, which is required for cell differentiation and homeostasis of epithelium. Suppression of E-cadherin accelerates invasion and is associated with a more malignant phenotype and poor differentiation in CRC (75). Snail1 was pulled down using sWGA affinity from SW480 CRC cell (61), and although *O*-GlcNAcylated Snail1 function was not studied in this cell line, its role was proposed according to data from HEK293 and A549 cells. *O*-GlcNAcylation at Ser-112 of Snail1 has been mapped in HEK293 cell (61). As mentioned earlier, Ser-112 is crucial for phosphorylation-mediated proteasomal degradation of Snail1. *O*-GlcNAc modification can stabilize Snail1 and subsequently inhibit mRNA expression levels of E-cadherin. Hyperglycemia also induced *O*-GlcNAcylation of Snail1 and suppressed E-cadherin expression level, resulting in stimulation of epithelial–mesenchymal transition (EMT).

Sp1, a specificity protein 1 transcription factor, regulates various genes encoding for growth factors, receptors, and proteins involved in cell growth, apoptosis, differentiation, and immune responses (76). *O*-GlcNAcylation of immunoprecipitated Sp1 in HT29 cell was reported by Haltiwanger et al. (77). Increased *O*-GlcNAcylation by treatment with PUGNAc showed a reciprocal effect to phosphorylation on Sp1. However, the consequence of *O*-GlcNAcylation of Sp1 in HT29 cell was not reported. Interestingly, other works showed the function of *O*-GlcNAcylation in protecting Sp1 from the ubiquitin-proteasome pathway (76, 78).

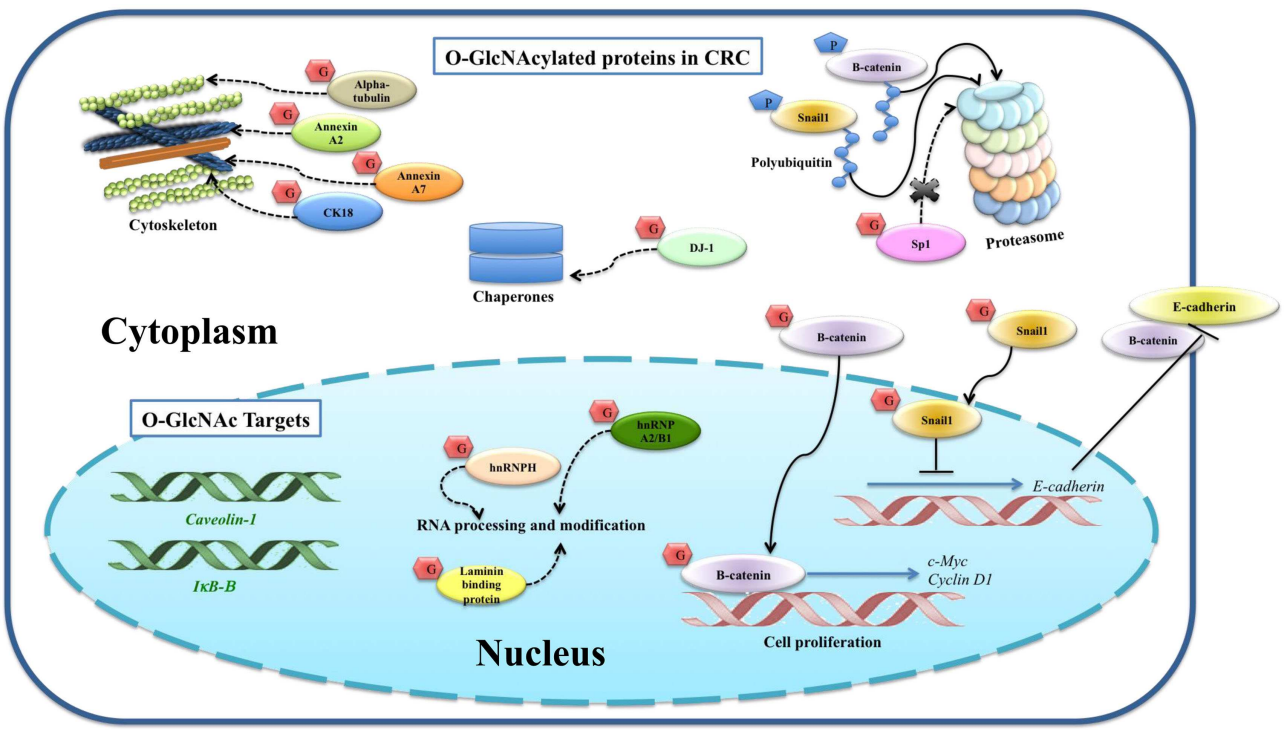

FIGURE 2 | *O*-GlcNAcylated proteins and their targets identified in colorectal cancer. *O*-GlcNAcylation stabilizes β-catenin and subsequently translocates into the nucleus for its gene activation. *O*-GlcNAcylation stabilizes Snail1, which subsequently represses E-cadherin expression level observed in other cancer cells, suggesting a proposed mechanism for colorectal cancer. Phosphorylation of β-catenin and Snail1 is proposed to activate proteasomal degradation. The proposed mechanisms for other *O*-GlcNAcylated proteins, including SP1, CK18, α-tubulin, hnRNPA2/B1, hnRNPH, annexin A2, annexin A7, laminin-binding protein, and protein DJ-1, are also indicated. Expression levels of *E-cadherin*, β-*catenin*, *caveolin-1*, and *IκB*-β are altered corresponding to increased global *O*-GlcNAcylation, so they are categorized as *O*-GlcNAc downstream targets. Solid lines indicate known mechanisms, whereas dashed lines are proposed mechanisms.

Gel based proteomics was used by our group to study the *O*-GlcNAc profile in CRC tissues (40). Eight *O*-GlcNAc modified proteins showed an increase in *O*-GlcNAcylation including cytokeratin 18 (CK18), α-tubulin, heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1 (hnRNPA2/B1), hnRNPH, annexin A2, annexin A7, laminin-binding protein, and protein DJ-1 (40). CK18 was reported to be modified by *O*-GlcNAc in at least three sites (Ser-30/Ser-31/Ser-49) (79). Increased *O*-GlcNAc-CK18 was associated with increased solubility and decreased cellular levels, while absence of *O*-GlcNAc on CK18 increased stability (68). α-tubulin was also identified as an *O*-GlcNAc modified protein by Walgren et al. (80). Increased *O*-GlcNAc α-tubulin resulted in a reduced hetero-dimerization into microtubules (81). Annexin A2 and A7 play important roles in cytoskeletal formation and cell matrix interaction. *O*-GlcNAcylation of annexin A2 was found to be overexpressed in all cancer samples (7/7) (40). HnRNPA2/B1 and H are a group of RNA-binding proteins involved in various processes in RNA metabolism including pre-mRNA splicing, mRNA transport, and translation. Protein DJ-1 plays a role as antioxidant and/or a molecular chaperone. Laminin-binding protein is involved in the assembly and/or stability of the ribosome in the nucleus. Although the sites of *O*-GlcNAcylation of such proteins have not been mapped in CRC tissues, the results presented here showed detectable *O*-GlcNAc modified proteins in clinical samples, which are promising as novel potential CRC biomarkers.

Furthermore, *O*-GlcNAcylation affects expression levels of a number of genes in the SW620 cell (48). Silencing of *OGA* affected the expression of about 1300 genes associated with cell movement and growth, as well as metabolic pathways involving lipids and carbohydrates. Among these, E-cadherin, β-catenin, and caveolin-1 proteins were upregulated, while IκB-β was downregulated when the cell was transfected by *siOGA* or Thiamet-G treatment, respectively. This also suggests that alteration of *O*-GlcNAcylation plays a vital role in the regulation of gene expression in CRC.

BREAST CANCER AND *O*-GLcNAcylation

THE EXPRESSION LEVELS OF *O*-GLcNAc, OGT, AND OGA IN BREAST CANCER

Breast cancer is the most frequently observed cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). Moreover, breast cancer ranks as the fifth cause of death from cancer overall (69). Many women who develop breast cancer have no obvious risk factors for breast cancer. Since intervention cannot always guarantee prevention of breast cancer, more research is required for identification and development of early stage biomarkers and molecular targets for effective drug treatment.

Several studies of *O*-GlcNAc and its cycling enzyme expression have been studied in breast cancer. In 2001, Slawson et al. found that *O*-GlcNAcylation was decreased and this is due to an increase in hexosaminidase and OGA activity in primary breast tumors, compared to matched normal adjacent breast tissues (82). However, Dahl et al. reported a gene named *MGEA5* coding for OGA, which showed about 56% reduction in expression in breast cancer tissues (83). Several groups, including Gu et al. (44), Caldwell et al. (43), Krzeslak et al. (84), and our group (25), showed that the expression of *O*-GlcNAcylation and OGT are upregulated, while the OGA expression is downregulated in breast cancer. Manipulation of OGT and OGA activities in breast cancer cells showed that overexpression of OGT enhanced the migration/invasion of breast cancer cells *in vitro* and lung metastasis *in vivo*, but did not affect cell proliferation (44). Conversely, reduction of *O*-GlcNAcylation through RNA interference of OGT in breast cancer cells led to inhibition of tumor growth both *in vitro* and *in vivo* (43). Similarly, OGT silencing resulted in a reduction of anchorage-independent growth of a breast cancer cell line, MDA-MB-231 (25). Recently, increased *O*-GlcNAcylation level was found to protect the breast cancer cell line, MCF-7 from tamoxifen induced cell death, whereas siRNA mediated OGT knockdown had opposite effects (85). These data suggest that OGT may represent a novel therapeutic target of cancer, especially in overcoming tamoxifen resistance in breast cancer.

O-GLcNAcylated PROTEINS AND *O*-GLcNAc TARGETING IN BREAST CANCER

Many groups of researchers have reported *O*-GlcNAc targets in breast cancer (**Figure 3**).Growing evidence suggests that *O*-GlcNAc plays vital roles in the regulation of cellular adhesion and cytoskeletal formation. Gu et al. showed that *O*-GlcNAcylation of p120 and β-catenin played roles in decreasing the level of E-cadherin at the cell surface (44). E-cadherin was also reported to be modified by *O*-GlcNAc at its cytoplasmic domain in breast cancer (86). Later, Geng et al. showed that *O*-GlcNAcylated E-cadherin interferes with the binding of Type I gamma phosphatidylinositol phosphate kinase (PIPKIγ), a protein required for recruitment of E-cadherin to adhesion sites, leading to reduced E-cadherin trafficking to the plasma membrane and accelerated apoptosis (87). Other *O*-GlcNAcylated proteins found in breast cancer and described earlier in terms of their function are p53 (60) and Snail1 (61).

In a related context, cofilin, a family of actin-binding proteins, which disassembles actin filaments, has also been reported to be modified by *O*-GlcNAc at Ser-108 and this glycosylation is essential for invadopodia formation, a process involving extracellular matrix (ECM) degradation during cancer invasion and metastasis (88). Decrease in its *O*-GlcNAcylation leads to the destabilization of invadopodia and impairs the invasion of breast cancer cells. Komura et al. reported that GlcNAc polymers and *O*-GlcNAc proteins induce the expression of vimentin and cell migration in MCF-7 (89). Regulation of vimentin expression by GlcNAc may play a crucial role for the EMT. We also showed that vimentin, cytokeratin 18 (CK18), β-actin, and keratin 7 appeared to show increased *O*-GlcNAcylation in breast cancer tissues, but their glycosylation sites were not yet mapped (25).

Glycolytic enzymes are also targets of *O*-GlcNAcylation. Several glycolytic enzymes were hyper-*O*-GlcNAcylated in breast cancer tissues. Using *O*-GlcNAc gel based proteomics, four glycolytic enzymes including enolase 2 (ENO2), TPI, pyruvate kinase M2 (PKM2), and GAPDH were identified (25). *O*-GlcNAcylation of enolase 1 (ENO1) was also reported in a breast cancer cell line, T47D (26), but the glycosylation sites have not yet been identified. Interestingly, as described earlier, *O*-GlcNAc at Ser-529 of phosphofructokinase 1 (PFK1) inhibited its activity and redirected glucose flux through the PPP (20). Recently, Ferrer et al. demonstrated

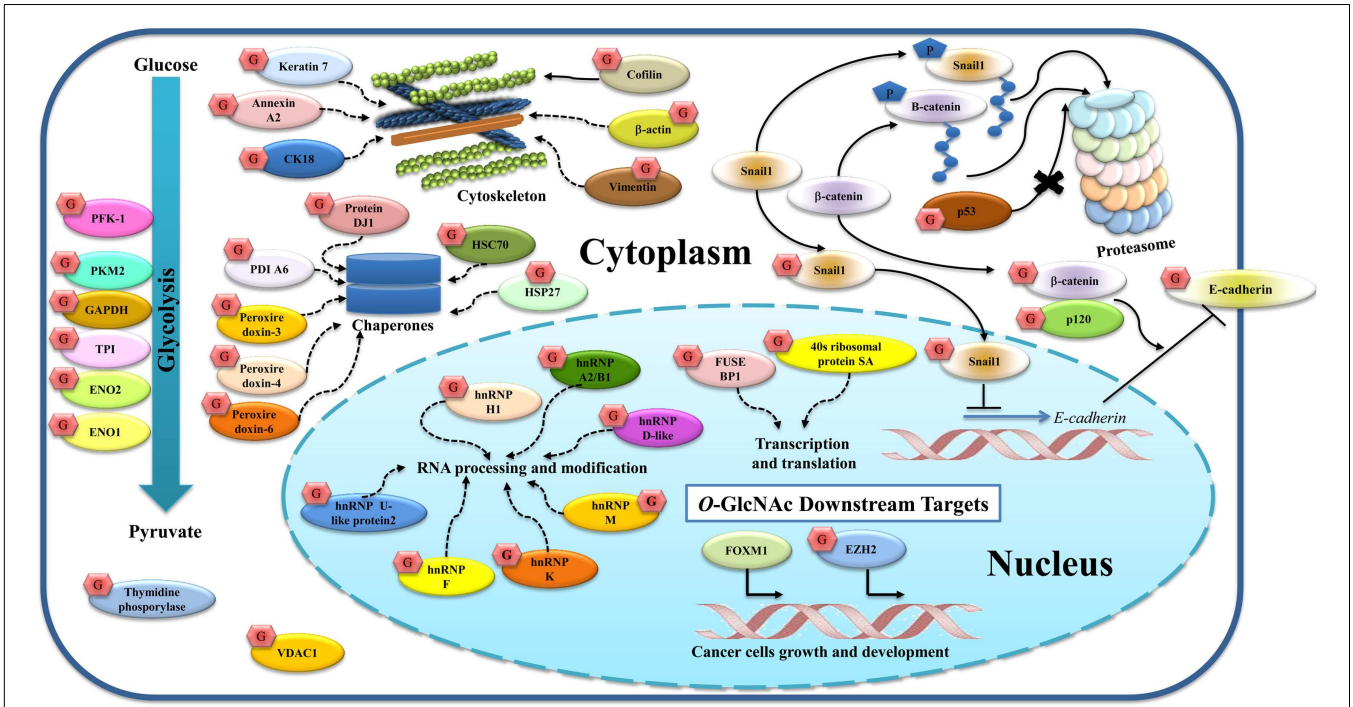

FIGURE 3 | *O*-GlcNAcylated proteins and their targets identified in breast cancer. *O*-GlcNAcylation modifies many proteins in both the cytoplasm and nucleus. p120, β-catenin, and E-cadherin are glycosylated, and this modification regulates E-cadherin localization and stability. *O*-GlcNAc Snail1 suppresses E-cadherin expression. Phosphorylation of β-catenin and Snail1 can activate proteasomal degradation while *O*-GlcNAc p53 protects this event. Other cytoskeletal proteins (cofilin, β-actin, vimentin, keratin 7, annexin A2, and CK18), glycolytic enzymes (PKF1, PKM2, GAPDH, TPI, ENO2, and ENO1), chaperones (protein DJ1, HSC70, HSP27, PDIA6, peroxiredoxin 3, 4, and 6), thymidine phosphorylase, and VDAC1 are also *O*-GlcNAcylated. Nuclear proteins including hnRNPs and proteins related to transcription and translation are *O*-GlcNAcylated as indicated. Alteration of this modification also regulates gene and protein expressions in breast cancer including FoxM1 and EZH2. Solid lines indicate known mechanisms, whereas dashed lines are proposed mechanisms.

that *O*-GlcNAcylation regulates glycolysis in cancer cells via hypoxia-inducible factor 1 (HIF-1α) and glucose transporter type 1, GLUT1 (90). Reducing *O*-GlcNAcylation led to HIF-1α degradation and activation of ER stress and apoptosis. In addition, human breast cancers with high HIF-1α and OGT levels, and low OGA levels are correlated with poor patient outcome. This suggests that the combined detection of HIF-1α, OGT, and OGA in clinical samples may be useful as potential breast cancer biomarkers.

Heterogeneous nuclear ribonucleoproteins are also a major group of proteins modified by *O*-GlcNAc in breast cancer. The hnRNPs are complexes of RNA and proteins that are involved in multiple aspects of RNA processing and modifications. Five members of hnRNPs including hnRNP U-like protein 2, hnRNPK, hnRNPF, hnRNPM, and hnRNPA2/B1 showed increased *O*-GlcNAcylation in breast cancer tissues (25), while other work by Rambaruth et al. showed that hnRNP H1, hnRNP D-like, hnRNP A2/B1 were hyper-*O*-GlcNAcylated in breast cancer cells (26). Although their glycosylation sites were not identified, increased *O*-GlcNAcylation in the hnRNP family may act as a novel regulation of alternative mRNA processing and gene expression, and that promotes a beneficial phenotype for cancer. In addition, two proteins in the nucleus involved in transcription and translation, ribosomal protein SA (RSSA) and far upstream element-binding protein 1 (FUSE-BP1), showed increased *O*-GlcNAcylation in cancer (25).

Chaperones and stress response proteins also show *O*-GlcNAc modification. We reported that heat shock cognate proteins (HSC70), protein disulfide isomerase (PDI) A6, peroxiredoxin 3, 4, and 6, and protein DJ-1 are *O*-GlcNAcylated in breast cancer tissues (25), while Rambaruth et al. showed *O*-GlcNAcyation of HSP27 (26). However, the effect of this modification on chaperones and stress response function is still not clear.

Two other proteins may be hyper *O*-GlcNAcylated: thymidine phosphorylase (TP), an enzyme involved in nucleic acid metabolism, and voltage dependent anion selective channel protein 1 (VDAC1), a mitochondrial protein that may contribute in triggering apoptosis (25).

O-GlcNAcylation also regulates gene and protein expression in breast cancer. Caldwell et al. showed that decreasing *O*-GlcNAcylation using RNA interference against OGT led to decreased cell invasion, tumor growth, and angiogenesis, both *in vitro* and *in vivo*, and this reduction is associated with decreased expression and activity of the oncogenic transcription factor FoxM1 (43). Recently, Chu et al. showed that the enhancer of zeste homolog 2 (EZH2), an enzyme, which acts as a gene silencer by histone methylation, is *O*-GlcNAcylated at Ser-75 (91). OGT knockdown reduced the EZH2 expression and H3 trimethylation at K-27 in MCF-7, indicating that *O*-GlcNAcylation of EZH2 is required for EZH2 stability.

CONCLUSION AND PERSPECTIVES

Current information strongly suggests that alteration of cellular metabolism from mitochondrial oxidative phosphorylation to aerobic glycolysis provides both bioenergetics and biosynthesis capability for cancer cells. Increasing glucose uptake and the redirection of glucose to the HBP flux can lead to an increase of UDP-GlcNAc and *O*-GlcNAcylation levels in cancer. Mutation of genes involving glucose uptake also contributes to this. In addition, *O*-GlcNAc cycling enzymes are altered in most cancers. Changes in *O*-GlcNAc and OGT levels clearly show positive relationship with the histological grade of breast and colorectal tumors. Greater increases in *O*-GlcNAc levels correlated with higher grades of tumor development. Importantly, this modification has extensive crosstalk with phosphorylation, which consequently affects cellular signaling. Thus, metabolic shifts through the HBP flux and *O*-GlcNAcylation contribute to regulation of signaling cascades and cell proliferation in cancer.

Study of *O*-GlcNAcylation in cancer is rapidly growing in terms of both detection and functional studies using *in vitro* and *in vivo* models. Several *O*-GlcNAc modified proteins have been discovered in breast and CRC. However, more *O*-GlcNAcylated proteins have been identified in breast cancer in comparison with CRC. This may result from differences in protein expression in each specific organ, as well as the tumor grade examined in the study. In addition, the levels of *O*-GlcNAc cycling enzymes differ. In breast cancer, major identified proteins are glycolytic enzymes and proteins, which function in biosynthesis (nucleic acid metabolism). The modified protein groups shared in breast and CRC are (1) proteins, which function in the stress responses; (2) hnRNPs and proteins involved in transcription and translation; (3) proteins related to the cytoskeleton and their regulation; and (4) transcription factors (e.g., Snail1 and β-catenin), which are difficult to observe and need to be enriched before detection. In addition, a number of *O*-GlcNAc targets in breast and CRC are not shown to be modified directly but rather regulate gene and/or protein expression such as FoxM1, Caveolin-1, and IκB-β. Moreover, more *O*-GlcNAc modified proteins were identified from breast cancer tissues than from breast cancer cell lines, indicating the complexity of *O*-GlcNAc regulation *in vivo* (25). *O*-GlcNAc modified proteins identified from clinical samples are thus more realistic as potential novel cancer biomarkers. Examples are PKM2 in breast cancer (25) and annexin A2 in CRC (40). However, larger scale studies need to be performed to obtain information with greater accuracy and reliability for possible use in clinical detection. On another hand, *O*-GlcNAc research on cell lines is also needed in order to test mechanisms and functions. A good example is that of *O*-GlcNAcylation of PFK1, which leads to decrease in activity and redirection of glucose metabolism in cancer cell lines (20). Alteration of OGT expression both in breast cancer cells (*in vitro*) and in animal model (*in vivo*) also suggests a promising approach for anticancer therapy (43). Research from both specimen samples and cell lines is, therefore, needed to provide a better understanding of *O*-GlcNAc biology in cancer. Taken together, this review shows the current findings on *O*-GlcNAcylation in breast and CRC. Many *O*-GlcNAc modified proteins are promising as potential novel cancer biomarkers or may be used in combination with standard detection (e.g., serum biomarkers) to enhance specificity and accuracy. Ongoing research will aim to detect these modified proteins in large-scale samples specifically and rapidly. Many detection techniques such as advanced mass analyzers with new fragmentation techniques including electron transfer dissociation (ETD) are being developed. Specific *O*-GlcNAc modified proteins in cancer specimens, therefore, are challenging to discover as potential candidates for cancer diagnosis, especially in breast and CRC.

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O-GlcNAcylation, an epigenetic mark. Focus on the histone code, TET family proteins, and polycomb group proteins

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There are increasing evidences that dietary components and metabolic disorders affect gene expression through epigenetic mechanisms. These observations support the notion that epigenetic reprograming-linked nutrition is connected to the etiology of metabolic diseases and cancer. During the last 5 years, accumulating data revealed that the nutrient-sensing *O*-GlcNAc glycosylation (*O*-GlcNAcylation) may be pivotal in the modulation of chromatin remodeling and in the regulation of gene expression by being part of the "histone code," and by identifying OGT (*O*-GlcNAc transferase) as an interacting partner of the TET family proteins of DNA hydroxylases and as a member of the polycomb group proteins. Thus, it is suggested that *O*-GlcNAcylation is a post-translational modification that links nutrition to epigenetic. This review summarizes recent findings about the interplay between *O*-GlcNAcylation and the epigenome and enlightens the contribution of the glycosylation to epigenetic reprograming.

Keywords: *O*-GlcNAcylation, OGT, histones, TET family proteins, polycomb, epigenetic, cancer

INTRODUCTION

It is widely accepted that cancer is a group of genetic diseases initiated by a sequential acquisition of mutations leading to the constitutive activation of oncogenes and/or the loss of function of tumor suppressor genes. However, numerous studies demonstrated that tumoral development also implies epigenetic modifications, i.e., an alteration of gene expression through mechanisms that do not affect the primary sequence of DNA (1). These epigenetic modifications include perturbations of DNA methylation patterns (repression of tumor suppressor genes and activation of oncogenes by hypermethylation and hypomethylation of their promoter region, respectively) and post-translational modification (PTM) of histone tails that drive chromatin compaction and relaxation that is chromatin dynamics.

An increasing number of studies tend to demonstrate that the "epigenome" is capable of integrating and transmitting nutrient information across generations. For example, the dietary intake of the methyl group donor folate and vitamin B12 to pregnant mice influences the expression of the *Agouti* gene, whose methylation rate defines the color of the coat of the offspring (2). It has also been demonstrated that young mice arisen from mothers undernourished during the pregnancy had defects in the methylation and in the expression of the *leptin* gene encoding a factor controlling satiety and that this phenotype was maintained in adults (3). Therefore, it is obvious that nutrient intake or metabolic disorders could influence the emergence of cancers by modifying the epigenome (4). In this way, a recent study highlighted that, when compared with individuals placed on a normal diet, individuals fed a high lipid diet on a very short period exhibit a modification of the methylome of muscular cells affecting principally genes implicated in the inflammatory response, the reproductive system, and cancer (5). Another recent study also showed that a deprivation in folate led to an increase in the invasive character of human colic cancer cells through hypomethylation of the promoter region of the *Sonic-hedgehog* oncogene and activation of the NF-κB signaling pathway (6). Whereas these two studies among many others lend weight to the hypothesis of a close relationship between nutritional disorders, epigenetic reprograming, and cancer, the underlying mechanisms are still poorly understood. Therefore, the nutrient sensor and chromatin modifier *O*-GlcNAc should be specifically considered as a candidate connecting nutrition to epigenetic and cancer.

O-GLcNAcylation: A NUTRIENT SENSOR IMPLICATED IN CANCER EMERGENCE

O-GlcNAcylation or *O*-linked β-D-*N*-acetylglucosaminylation is a reversible PTM of cytosolic, nuclear, and mitochondrial proteins that consists in the covalent linkage of a unique residue of *N*-acetylglucosamine (GlcNAc) to serines and threonines of target proteins. *O*-GlcNAcylation levels are regulated by a unique couple of enzymes: OGT (*O*-GlcNAc transferase) that catalyzes the transfer of GlcNAc from UDP-GlcNAc onto the protein and OGA (*O*-GlcNAcase) that hydrolyzes the residue (**Figure 1**). *O*-GlcNAcylation levels are closely dependent upon the concentration of UDP-GlcNAc, the second most abundant nucleotide structure in the organism, ATP being the first (40 and 100 nmol/g of tissue, respectively). The glucose, glutamine, fatty acids, uridine, and ATP metabolisms converge on the hexosamine biosynthetic pathway (HBP) to produce the nucleotide-sugar. Thus, UDP-GlcNAc and *O*-GlcNAcylation are considered as sensors of the nutritional state of the organism (7, 8), which can relay the effects of an excessive food supply, malnutrition, obesity,

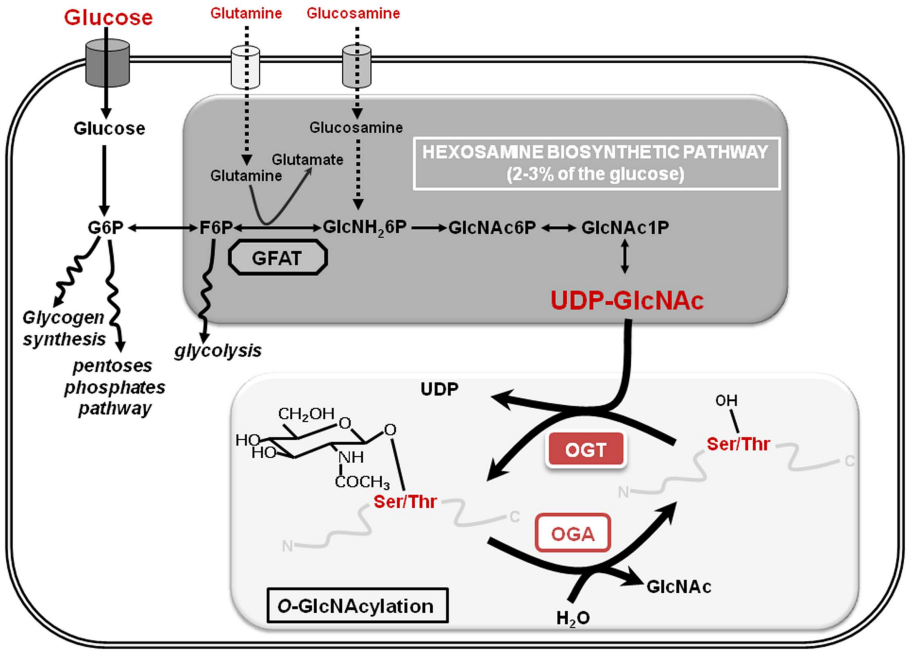

FIGURE 1 | The hexosamine biosynthetic pathway and *O*-GlcNAcylation are shown. The hexosamine biosynthetic pathway (HBP) whose key limiting enzyme is GFAT (glutamine:fructose-6-phosphate amido transferase) uses 2–3% of the extracellular glucose to produce UDP-GlcNAc (uridine-di-phospho-*N*-acetyl-glucosamine), the substrate that provides the GlcNAc residue for the *O*-GlcNAcylation processes. This dynamic and reversible post-translational modification of nuclear and cytosolic proteins controls the target proteins fate according to glucose and nutrients availability: it is therefore considered as a nutritional sensor. A single residue of GlcNAc is transferred to a serine or a threonine residue of the protein by the unique *O*-GlcNAc Transferase (OGT) and *O*-GlcNAcase (OGA) hydrolyzes the residue. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; $GlcNH_2$6P, glucosamine-6-phosphate; GlcNAc6P: *N*-acetyl-glucosamine-6-phosphate; GlcNAc1P: *N*-acetyl-glucosamine-1-phosphate.

and other metabolic problems that represent high risk factors of cancerization processes (9–11), e.g., overweight and obesity account for more than two-thirds of new cases of type-2 diabetes that in turn doubles colorectal cancer emergence (12). In this way, numerous studies clearly show that *O*-GlcNAcylation plays a significant role in the etiology of cancers at different levels: (i) increased contents of *O*-GlcNAcylation and OGT were characterized in different types of cancer (breast, prostate, colon . . .); (ii) a modulation of the expression or of the activity of OGT influence the proliferation and/or the invasiveness of cancer cells; (iii) several oncogenic and anti-oncogenic proteins are modified and regulated by *O*-GlcNAc (p53, HIC1, c-myc, FOXM1, NF-κB, β-catenin . . .); (iv) *O*-GlcNAcylation also participates in the metabolic reprograming of cancer cells. The different aspects of the role of *O*-GlcNAcylation in cancer emergence have been extensively reviewed (13, 14). In recent years, *O*-GlcNAcylation has also emerged as an important regulator of chromatin dynamics since this PTM contributes both to the extensively described chemical modifications of histones (acetylation, methylation, ubiquitination, phosphorylation . . .) and to DNA methylation patterns that affect chromatin structure. The goal of this review is to summarize recent data showing how *O*-GlcNAcylation is involved in the regulation of the epigenome (**Figure 2**) and consequently how it could contribute to epigenetic reprograming.

O-GLcNAcylation: A NUTRIENT SENSOR REGULATING CHROMATIN DYNAMICS

O-GLcNAcylation IS PART OF THE "HISTONE CODE"

The chromatin compaction status governs the accessibility of the transcriptional machinery to DNA and so it has a crucial role in the establishment, the maintenance, and the propagation of gene expression patterns. The nucleosome is the basic unit of chromatin. This is an octamer made of a tetramer of histones H3 and H4 and of two dimers of histones H2A–H2B around which DNA rolls up on 147 bp and is locked by the linker histone H1. The chromatin organization is managed in part by a complex network of PTMs of histones called the "histone code" (15). Histones are methylated, acetylated, phosphorylated, ubiquitinated, ADP-ribosylated, and SUMOylated (**Figure 2**). These PTMs regulate the interaction of histones with DNA and the ability to recruit chromatin remodeling complexes necessary for transcription, replication, recombination, repair, and mitosis. Several recent studies revealed that the core histones H3–H4–H2A–H2B are also modified with *O*-GlcNAc, adding a supplementary level of complexity to the histone code that is far from being entirely deciphered (16–20). Sakabe and Hart (21) showed that the overexpression of OGT in HeLa cells synchronized in M-phase prevented the increase in histone H3 Ser10 phosphorylation, whereas it attenuated the decrease in H3K9 acetylation and H3K27 trimethylation observed during mitosis (22); these observations point out the

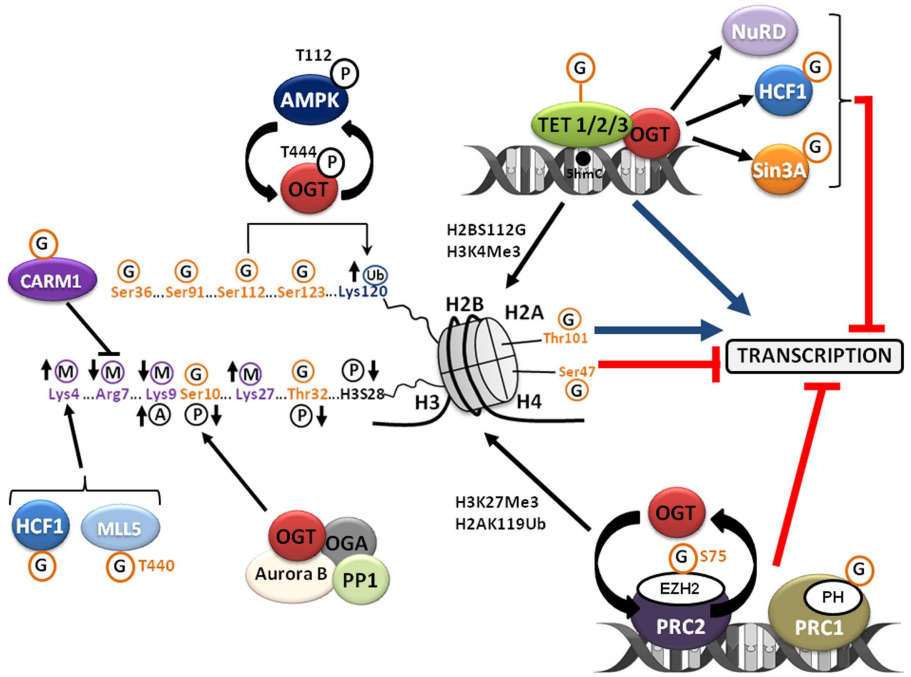

FIGURE 2 | *O*-GlcNAcylation regulates the chromatin dynamics. *O*-GlcNAcylation regulates chromatin compaction and accordingly gene transcription by interfering with the other post-translational modifications of histones that define the "histone code" and indirectly by its complex relationship with the PcG proteins and the TET family proteins. See the text for details. A, acetylation; G, *O*-GlcNAcylation; M, methylation; P, phosphorylation; Ub, ubiquitination.

crucial role of OGT in modifying histone H3 during mitosis. The same laboratory was the first to report *O*-GlcNAcylation of the four core histones in HeLa cells. *O*-GlcNAcylation levels of histones, especially those of histone H3, decrease during mitosis whereas glycosylation status of histones increases upon heat-shock concomitantly with DNA condensation (16). Three sites of *O*-GlcNAcylation were mapped by MS/MS on histone H2A Thr101, histone H2B Ser36, and histone H4 Ser47, respectively. The *O*-GlcNAcylation of core histones was also reported by Zhang et al. in HEK 293 cells (18). These authors showed that the glycosylation of histones fluctuates all along the cell cycle with a lower level in S-phase. More particularly, histone H3 Ser10 was identified as a site of *O*-GlcNAcylation and, in accordance with Sakabe and Hart findings (21), it was observed that increasing *O*-GlcNAcylation by treating cells with glucosamine was associated with a decreased phosphorylation of histone H3 Ser10. These data demonstrate a direct competition between phosphorylation and *O*-GlcNAcylation at histone H3 Ser10 (18). It was also demonstrated that glucosamine induced an increase of the active epigenetic mark H3K4Me3 concomitantly with a decrease of the repressive mark H3K9Me3. This suggests that *O*-GlcNAcylation influences indirectly the occurrence of other PTMs of histones. The precise function of H3 Ser10 phosphorylation is still currently not fully understood but is clearly associated with regulation of the condensation and/or segregation of chromosomes during mitosis (23). Intriguingly, histone H3 Ser10 phosphorylation is catalyzed by the kinase Aurora B (24) whereas it is dephosphorylated by the phosphatase PP1 (25). These two enzymes physically interact with OGT and OGA in a complex located at the midbody to regulate cytokinesis and exit from mitosis (26). In HeLa cells, Thr32 of histone H3 was highlighted as another site of *O*-GlcNAcylation with a higher level in interphase than in mitosis, which inversely correlated with mitosis-specific phosphorylations on histone H3 (19). The mitosis-specific phosphorylations of histone H3 at Thr32, Ser10 (18), and Ser28 (also phosphorylated by Aurora B) (27) are reduced by treating M-phase synchronized-cells with the two inhibitors of OGA, PUGNAc, or thiamet G, or by overexpressing OGT. Fujiki et al. identified three sites of *O*-GlcNAcylation of histone H2B at Ser91, Ser112, and Ser123 (17). *O*-GlcNAcylation of histone H2B at Ser112 fluctuates in response to extracellular glucose and promotes its monoubiquitination on Lys120 by favoring the interaction of histone H2B with the E3 ubiquitin ligase complex BRE1A/B. In this study, genome-wide analysis revealed that H2B Ser112 *O*-GlcNAcylation was frequently located near transcribed genes, suggesting that histone H2B *O*-GlcNAcylation facilitates gene transcription. A very recent study identified the AMP-activated protein kinase (AMPK) as a regulator of H2B Ser112 *O*-GlcNAcylation (20). AMPK is a sensor of energy status which activity is controlled by the ATP/AMP ratio. AMPK controls cell metabolism and cell growth in response to changes in nutrient availability. AMPK dysfunctions are associated with diseases including diabetes and cancers (28, 29). In their study, Xu et al. demonstrated that activating AMPK with AICAR in mouse embryonic fibroblasts (MEFs) resulted in a decrease in H2B

Ser112 *O*-GlcNAcylation and H2B K120 ubiquitination. AMPK directly phosphorylates OGT on Thr444; this phosphorylation does not interfere with OGT activity *per se* on H2B Ser112, but it prevents its loading on chromatin as demonstrated by ChIP experiments. The authors also demonstrated that the catalytic subunit of AMPK, AMPKα1, is *O*-GlcNAcylated and that the knock-down of OGT by RNAi in MEFs led to a decrease in the activating phosphorylation of AMPK on Thr112. As a whole, these results highlight the occurrence of a feedback regulatory loop between OGT and AMPK.

It was also reported that OGT and *O*-GlcNAcylation control the activity of histones methyltransferases (**Figure 2**). OGT associates with and modifies CARM1 (co-activator associated arginine methyltransferase 1) (21, 30). The overexpression of OGT in HeLa cells decreased the phosphorylation of CARM1 and its methyltransferase activity at Arg7 of histone H3 (H3R7) (21). MLL5, an H3K4 histone methyltransferase and co-activator of RARα, interacts with OGT in a multimeric complex (31). *O*-GlcNAcylation of MLL5 Thr440 potentiates its H3K4 methyltransferase activity and increases granulopoiesis of HL60 promyelocytes in response to retinoic acid (31). Host cell factor 1 (HCF1) is a component of the H3K4 methyltransferase complex SET1/COMPASS (32, 33). *O*-GlcNAcylation of HCF1 enhances the stability of the SET1/COMPASS complex. OGT is necessary for the binding of SETD1A, the component of the complex that bears the methyltransferase activity, to chromatin (33).

In addition to the glucosaminidase activity, OGA exhibits a histone acetyl transferase (HAT) property in its C-terminal region and is sometimes called NCOAT for nuclear and cytoplasmic *O*-GlcNAcase and acetyl transferase (34). Therefore, *O*-GlcNAcylation would be able to regulate the acetylation of the histones tails, but this function remains controversial (35). Whisenhunt et al. demonstrated that OGA/NCOAT, OGT, the co-repressor Sin3A (Switch-independent 3A), and HDAC1 (Histone Deacetylase 1) co-exist in a complex that was named *O*-GlcNAczyme (36). ChIP experiments performed in MCF7 cells revealed a specific enrichment of the *O*-GlcNAczyme on promoters of repressed genes (36). Nevertheless, the existence of this *O*-GlcNAczyme complex has not yet been confirmed by other studies.

O-GLcNAcylation MODIFIES MEMBERS OF THE TET FAMILY PROTEINS

In 2013, several studies, albeit sometimes contradictory, provided compelling evidences of a close relationship among OGT, *O*-GlcNAcylation, and the DNA hydroxylase properties of the TET (Ten–Eleven Transcription) family proteins involved in the DNA demethylation on CpG islands (**Figure 2**). TET1, TET2, and TET3 convert 5-methyl-cytosine (5mC) to 5-hydroxy-methyl-cytosine (5hmC) and are necessary for gene transcription, pre-mRNA splicing, and zygotic genetic reprograming (37). Chen et al. demonstrated that OGT interacts but does not *O*-GlcNAcylate or influence the function of TET2 and TET3 (38). However, these authors showed that TET2 and TET3 promote the recruitment of OGT to the chromatin in order to modify histones (38). Such a role for TET3 in the loading of OGT to chromatin has been also reported by Ito et al. at the same time (39). In their study, Chen et al. performed ChIP-Seq experiments that revealed the presence of OGT and H2B Ser112-*O*-GlcNAc on a large number of TET2 target genes and more especially around transcription start sites (38). Contrary to the observations of Chen et al., *O*-GlcNAcylation of transiently transfected TET3 was reported in two other studies, but *O*-GlcNAcylation of the endogenous protein was not observed (39, 40). The *O*-GlcNAcylation of TET1 and TET2 were also independently reported (40–42). OGT promotes the cytoplasmic relocation of a myc-TET3 construction according to a mechanism that remains to be deciphered whereas *O*-GlcNAcylation has no effect either on TET1 or on TET2 subcellular localization (40). However, *O*-GlcNAcylation of TET1 regulates its expression level in mouse embryonic stem (ES) cells (42) and stabilizes the hydroxylase on the promoters of target genes (41). The occurrence that OGT and TET proteins form a complex with co-repressors reinforces the importance of *O*-GlcNAcylation processes in the regulation of gene transcription. By proteomic analyses performed in mouse ES cells, Shi et al. demonstrated that TET1 and OGT interact with the chromatin regulator Sin3A and with several members of the NuRD (nucleosome remodeling and deacetylase) complex (**Figure 2**) (40). The authors also found that OGT is required for maintaining ES cells pluripotency since depletion of this enzyme induces a derepression of several markers of differentiation (40). The interaction between TET1, TET2, Sin3A, HCF1, and OGT was also reported (41). In this study, using genome-wide ChIP-seq experiments, 11552 binding sites for OGT among which 62% are located within promoter regions were identified. A co-localization of OGT, TET1, and H3K4Me3 was also observed near the transcription start sites; this demonstrates that TET1 is necessary to recruit OGT to the chromatin.

OGT BELONGS TO THE POLYCOMB GROUP PROTEINS

In 2009, two independent studies surprisingly revealed that the fly gene *Sxc* (*Supersexcomb*) initially characterized as a gene belonging to the polycomb group (PcG) proteins (43) is the gene that encodes OGT (44, 45). PcG proteins represent a family of transcriptional repressors discovered in *Drosophila melanogaster*. These proteins are necessary for the maintenance of the repression of the homeotic genes (*Hox*) whose expression patterns govern the establishment of the antero-posterior axis of the embryo. In mammals, PcG proteins also repressed *Hox* genes and numerous other genes controlling the programing of adult and ES cells, cell proliferation, and differentiation. In *Drosophila* and mammals, PcG proteins are found in two main large complexes, PRC1 and PRC2 (Polycomb Repressive Complexes 1 and 2), whose members have been conserved during evolution (46). These two complexes act in a sequential manner: first PRC2 is recruited to the promoter region of its target genes where the histone methyl transferase EZH2 (Enhancer of Zest Homolog 2) is responsible for H3K27 di- and trimethylation (H3K27Me2 and H3K27Me3), a repressive epigenetic mark. H3K27Me3 is subsequently recognized by the chromodomain of the PC (Polycomb) protein, a core component of PRC1. The catalytic activity of PRC1 is driven by the E3 ubiquitin ligase RING that catalyzes the monoubiquitination of histone H2A Lys119 (H2AK119Ub). The precise mechanisms by which Polycomb complexes repress transcription are not fully understood

while it involves both inhibition of the transcriptional machinery and chromatin compaction and recruitment of DNA methyl transferases (DNMTs). Polycomb responsive elements (PRE) were characterized in *Drosophila*, but such nucleotide sequences are not found in mammals. Therefore, in mammals, the mechanisms of PRC2 recruitment to its target genes remain to be elucidated. One proposed targeting mechanisms together with sub-stoichiometric components of PRC2 (e.g., JRID2) or the implication of specific long non-coding RNAs in the interaction of PRC2 components with sequence-specific transcription factors (47). For example, the transcriptional repressor HIC1 (Hypermethylated in cancer 1) could recruit PRC2 on a subset of target genes through its interaction with human polycomb-like (hPCL) proteins (48). For note, we previously demonstrated that HIC1 is *O*-GlcNAcylated, but the role of its glycosylation is still not deciphered (49). In their original study performed in *Drosophila*, Gambetta and collaborators identified by ChIP-seq experiments 1138 sites occupied by *O*-GlcNAcylated proteins among which 490 colocalized with PREs (44). The authors demonstrated a decrease of the fixation of the polyhomeotic (PH) protein, a core component of PRC1, on the majority of the PREs in *sxc/ogt* mutants in comparison with wild-type *Drosophila*. The same authors also showed that PH is itself *O*-GlcNAcylated (**Figure 2**). The catalytic core component of PRC2 EZH2 interacts with OGT inside PRC2 and the knockdown of OGT in MCF7 or MDA-MB231 cells led to a 50% decrease in H3K27Me3 due to EZH2 and PRC2 destabilization (50). *O*-GlcNAcylation of EZH2 has been mapped at Ser75, and the S75A mutant is less stable than the wild-type protein (**Figure 2**). By a combination of microarrays and ChIP experiments, Chu et al. (50) identified 16 genes co-regulated by OGT and EZH2. Knockdown of OGT affects the fixation of EZH2 and the deposit of the repressive mark H3K27Me3 on these genes, some of which like *UNC5A* or *IL1R1* have been reported as tumor suppressor genes (51, 52). A study conducted in mouse highlighted a decrease in OGT and in nuclear *O*-GlcNAcylation in $eed^{-/-}$ and $suz12^{-/-}$ ES cells, two genes encoding core components of PRC2 (53). This set of data suggests a complex feedback relationship between *O*-GlcNAcylation and PcG proteins, notably with core components of PRC2, which remains to be fully understood.

CONCLUSION AND FUTURE DIRECTIONS

O-GlcNAcylation has recently emerged as a novel epigenetic mark affecting chromatin remodeling and gene expression according to several mechanisms (**Figure 2**). First, *O*-GlcNAcylation modifies histone tails and depending on the residue, *O*-GlcNAcylation either favors chromatin relaxation and gene transcription or chromatin compaction and thus it prevents transcription. *O*-GlcNAcylation also regulates the occurrence of other PTMs defining the histone code and more particularly methylation by modulating the activity of several methyltransferases like CARM1, MLL5, and HCF1. OGT and *O*-GlcNAcylation regulates the activity of different co-repressors among which NuRD and mSin3A; but, especially, *O*-GlcNAcylation displays a complex relationship with the PcG proteins to prevent gene transcription. At last, *O*-GlcNAcylation may promote DNA demethylation by interacting with members of the TET family proteins thus favoring gene transcription.

At this time, the crucial role played by *O*-GlcNAcylation in metabolic disorders and neuronal diseases etiology is indisputable. Regarding cancer, *O*-GlcNAcylation interfered with cell biology through a large panel of mechanisms among cell proliferation, adhesion, migration, and metabolic reprograming. In this review, we summarized recent evidences suggesting that *O*-GlcNAcylation also highly coordinates chromatin dynamics adding a further level of regulation of cancer emergence through *O*-GlcNAcylation of the epigenome. However, the role of *O*-GlcNAcylation in cancer-associated epigenetic reprograming is far from being fully deciphered and further studies are required to understand the impact of aberrant *O*-GlcNAcylation in tumorigenesis and to identify new targets that could be used for prevention, diagnosis, or treatment of cancers.

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O-GlcNAc: a bittersweet switch in liver

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The liver is a vital organ responsible for maintaining nutrient homeostasis. After a meal, insulin stimulates glycogen and lipid synthesis in the liver; in the fasted state, glucagon induces gluconeogenesis and ketogenesis, which produce glucose and ketone bodies for other tissues to use as energy sources. These metabolic changes involve spatiotemporally co-ordinated signaling cascades. *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification has been recognized as a nutrient sensor and regulatory molecular switch. This review highlights mechanistic insights into spatiotemporal regulation of liver metabolism by *O*-GlcNAc modification and discusses its pathophysiological implications in insulin resistance, non-alcoholic fatty liver disease, and fibrosis.

Keywords: *O*-GlcNAc, insulin, glucagon, liver metabolism, insulin resistance, NAFLD, liver fibrosis

INTRODUCTION

The liver is the second largest organ and accounts for about 2% of the body mass of an adult human being. It is a major metabolic organ responsible for maintaining whole-body homeostasis in a changing nutritional environment. Dysregulation of liver metabolism is associated with a wide range of chronic liver disorders.

LIVER METABOLISM DURING THE FEEDING/FASTING CYCLE

The liver plays a key role in maintaining normal glucose levels between meals. When blood glucose is in excess (e.g., after a meal), the liver rapidly takes up glucose to produce glycogen (glycogenesis). When blood glucose levels fall below a normal range (72–85 mg/dL for healthy individuals), glycogen is broken down into glucose (glycogenolysis), which is then exported to the bloodstream. If the glycogen reserve is exhausted, the liver will generate glucose from non-carbohydrate carbon substrates, such as lactate, pyruvate, glycerol, and glucogenic amino acids (gluconeogenesis).

The liver also oxidizes triglycerides to produce energy during fasting. When carbohydrates and proteins are in excess, they are converted into fatty acids and triglycerides in the liver, and these are then exported and stored in adipose tissue. The liver is also responsible for producing lipoproteins, cholesterol, and phospholipids.

The metabolic function of the liver during the feeding/fasting cycle is tightly regulated by several endocrine hormones, particularly insulin and glucagon. Insulin enhances glucose uptake in muscle and adipose tissue and inhibits glucose production in the liver, thereby regulating blood glucose concentration. Insulin also stimulates the synthesis of fatty acids, glycogen, and proteins. The opposing effects are largely mediated by glucagon – it raises blood glucose concentration by promoting glycogenolysis and gluconeogenesis. These signaling and biochemical pathways can be regulated by insulin and glucagon at the transcriptional, translational, and post-translational levels.

INSULIN RESISTANCE AND NAFLD

Since the liver is a vital organ to sustain metabolic homeostasis, chronic liver disorders are among the most devastating human diseases. Liver dysfunction can be a consequence of infection, immune disorders, alcohol- or drug-induced liver damage. It can contribute to a constellation of common metabolic disorders, including insulin resistance, non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH).

Hepatic insulin resistance causes impaired glycogen synthesis and failure to suppress glucose production, eventually resulting in hyperglycemia. Insulin resistance is often associated with increased lipogenesis and hepatic steatosis (fatty liver).

Non-alcoholic fatty liver disease is now the most common liver disorder in the US, where 30% of the general adult population suffers from the disease (1). NAFLD can be characterized by excessive lipid accumulation in hepatocytes. In addition, a strong correlation between NAFLD and type 2 diabetes (T2D) has been reported: 70–80% of T2D and obesity patients have NAFLD and most patients with NAFLD have hepatic insulin resistance (2).

The majority of patients with NAFLD starts with simple steatosis and is often asymptomatic. However, a subset of NAFLD patients with simple steatosis can progress to NASH with manifestations of inflammation, hepatocellular injury, and fibrosis (3). Patients with fibrosis tend to have poor prognosis, often progressing to cirrhosis and even hepatocellular cancer (4). However, the molecular basis for the development of NAFLD and NASH is not clearly understood.

O-GlcNAc MODIFICATION

O-GlcNAcylation has emerged as an important regulatory mechanism underlying normal liver physiology and liver diseases. This post-translational modification uses UDP-GlcNAc as the substrate for the attachment of the acetylglucosamine (GlcNAc) moiety to

the hydroxyl groups of serine and threonine residues of proteins (5, 6). Since UDP-GlcNAc is synthesized via the hexosamine biosynthesis pathway (HBP), which involves nutrients such as glucose, free fatty acids, uridine, and glutamine, *O*-GlcNAcylation can serve as a nutrient sensor, tuning various cellular processes in response to systemic metabolic status (7–9). The cycling of *O*-GlcNAcylation is controlled by two highly conserved enzymes: *O*-GlcNAc transferase (OGT) catalyzes the addition of *O*-GlcNAc to proteins and *O*-GlcNAcase (OGA) catalyzes the removal of the monosaccharide (10–12). This dynamic modification is prevalent on signaling proteins, transcription factors, metabolic enzymes, and histones and has emerged as a key regulator of diverse cellular processes, including signal transduction, gene expression, and protein degradation (7, 13–17).

This review focuses on the spatiotemporal regulation of key signaling pathways in glucose and lipid metabolism by *O*-GlcNAcylation. Understanding the regulatory role of this modification provides significant insight into normal liver physiology and liver disease processes.

O-GlcNAc IN NORMAL LIVER METABOLISM

O-GlcNAc REGULATION OF FEEDING RESPONSE

Insulin plays a central role in glucose and lipid metabolism. After a meal, a sizable amount of insulin is rapidly released from the pancreas and is circulated to critical organs such as skeletal muscle, adipose tissue, and liver. In the liver, insulin induces acute activation of the insulin signaling cascade (acute postprandial response) followed by the attenuation of this pathway (prolonged postprandial response) (18). The temporal patterns of signal transduction are largely dictated by dynamic protein phosphorylation (19, 20). Similar to protein phosphorylation, *O*-GlcNAc modification can influence protein function by regulating protein–protein interaction, protein stability, nuclear-cytoplasmic shuttling, and intrinsic protein activity (21). The interplay between phosphorylation and *O*-GlcNAcylation has been implicated in the regulation of critical cellular processes. Here, we review the known effects of *O*-GlcNAc modification on insulin signal transduction in acute and prolonged postprandial responses, focusing on the role of *O*-GlcNAc in attenuating insulin signaling (**Figure 1**).

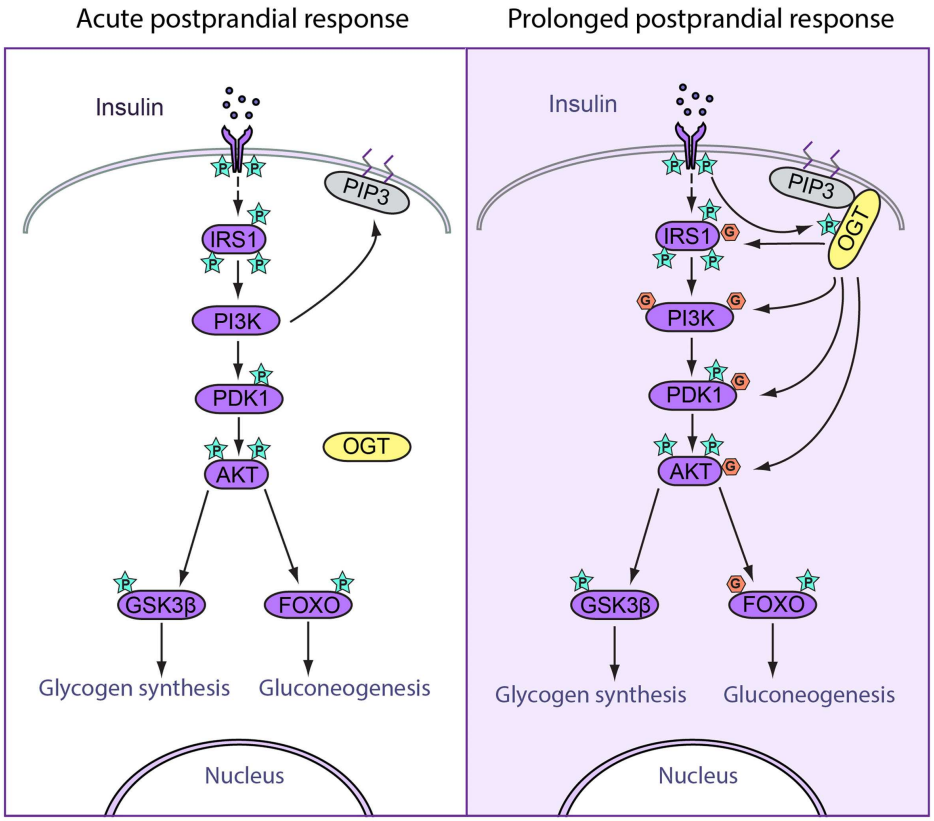

FIGURE 1 | Spatiotemporal regulation of feeding response by *O*-GlcNAcylation. (Left) Acute postprandial response. During early insulin signaling, OGT remains in the cytosol. Insulin binds to insulin receptor (IR) and triggers its autophosphorylation. Phosphorylation of IR recruits IRS1 to be phosphorylated, after which IRS1 binds to PI3K. PI3K catalyzes the production of PIP3, which recruits PDK1 to be phosphorylated and activated. Activated PDK1 phosphorylates and activates AKT, which further phosphorylates and activated downstream targets, including GSK3β and FOXO, which enhances glycogen synthesis and suppresses gluconeogenesis. (Right) Prolonged postprandial response. The insulin signaling pathway needs to be attenuated after a period of stimulation in order to maintain homeostasis. *O*-GlcNAcylation of insulin signal proteins contributes to the attenuation of the pathway. During prolonged insulin signaling, OGT translocates to the plasma membrane and binds with PIP3 through the PIP3-binding domain. OGT is then phosphorylated and activated by IR. Activated OGT *O*-GlcNAcylates key insulin signaling proteins including IRS1, PI3K, PDK1, and AKT, antagonizing the activation by phosphorylation on these proteins. These events lead to decreased glycogen synthesis and increased gluconeogenesis.

Acute postprandial response

In acute postprandial response, insulin binds to the insulin receptor (IR) and triggers the autophosphorylation of various tyrosine residues within the intracellular tyrosine kinase domain of the IR. This leads to the recruitment and phosphorylation of downstream proteins, including insulin receptor substrate (IRS). Subsequently, IRS binds and phosphorylates phosphatidylinositol-3-kinase (PI3K), which mediates a variety of critical signaling events. PI3K catalyzes the formation of membrane phosphatidylinositol 3,4,5-bisphosphate (PIP3), which recruits AKT to be activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) through phosphorylation at threonine 308. AKT then phosphorylates several target proteins, including glycogen synthase kinase (GSK3), AS160, and forkhead box protein O (FOXO). In glycogen synthesis, AKT phosphorylates and deactivates GSK3 (22), the enzyme responsible for phosphorylating and deactivating glycogen synthase. This leads to increased glycogen synthesis. In gluconeogenesis, phosphorylation of FOXO by AKT triggers FOXO export from the nucleus, thereby preventing FOXO from promoting gluconeogenic gene transcription (23). Thus, in acute insulin response, sequential phosphorylation events lead to increased glycogen synthesis and decreased gluconeogenic gene expression (**Figure 1**).

Prolonged postprandial response

The precise control of the duration of signal transduction is critical for maintaining physiological homeostasis. For instance, at some point after acute activation, insulin signal transduction is dampened through several feedback mechanisms (18). First, protein tyrosine phosphatases, such as PTP1B, have been shown to act as negative regulators of insulin signaling through dephosphorylation of IR (24). Lipid phosphatases, specifically PTEN and SHIP2, can dampen the PI3K pathway both *in vitro* and *in vivo* (25). Second, phosphorylation of specific Ser/Thr sites on IRS by protein kinases such as ribosomal protein S6 kinase beta-1 (S6K1) terminates insulin signaling (26, 27). Third, recent studies have indicated that *O*-GlcNAcylation plays a profound role in attenuating insulin signaling (28, 29).

In response to prolonged insulin stimulation, OGT translocates from the cytoplasm to the plasma membrane through the C-terminal PIP3-binding domain (28), leading to phosphorylation and activation of OGT by IR (30). Active OGT is known to *O*-GlcNAcylate and deactivate key insulin signaling proteins, including IRS-1, PI3K, PDK1, and AKT, thereby facilitating insulin signal attenuation (29, 30) (**Figure 1**).

Insulin receptor substrate deactivation is an important mechanism for terminating insulin signaling. IRS-1 is a direct substrate of OGT (29). Increased *O*-GlcNAcylation of IRS-1 in 3T3-L1 adipocytes reduces IRS-1 interaction with PI3K p85 and Tyr phosphorylation of IRS-1 at the Tyr 608 and increases IRS-1 phosphorylation at Ser 307 and Ser 632/635 (29). PI3K and PDK1 are also direct substrates of OGT and are implicated in insulin signaling attenuation (28).

Decreased AKT activity is essential for insulin signal termination. Increased *O*-GlcNAcylation of AKT at Thr 305/312 decreases AKT activity by reducing Thr 308 phosphorylation, which disrupts AKT/PDK1 interaction. In contrast, Ser 473 phosphorylation is unaffected (31).

Decreased AKT activity also reduces glycogen synthesis by decreasing phosphorylation of GSK3β. GSK3β is known to be modified by *O*-GlcNAcylation, and the inhibition of GSK3β by lithium alters global *O*-GlcNAc levels (32). However, the function of GSK3β *O*-GlcNAcylation has not yet been elucidated. Furthermore, *O*-GlcNAcylation of glycogen synthase itself is responsive to high glucose or glucosamine treatment and reduces the activity of the enzyme (33).

Lipogenesis in feeding response

Hepatic *de novo* lipogenesis allows for the conversion of glucose into fatty acids during feeding. Recent evidence indicates that glucose flux promotes lipogenesis through *O*-GlcNAcylation. The role of *O*-GlcNAcylation in both activating lipogenesis and attenuating insulin signaling raises an interesting question regarding the temporal regulation of insulin signaling. However, the studies on *O*-GlcNAcylation of lipogenic proteins have not addressed the dynamics of this modification in relation to the temporal regulation of lipogenesis.

The liver X receptors (LXRs) have long been viewed as nutrient sensors for lipid metabolism, glucose homeostasis, and inflammation. LXRs have been found to be *O*-GlcNAcylated in human Huh7 cells (34). High glucose increases LXR *O*-GlcNAcylation and transcriptional activity on the promoter of the sterol regulatory element-binding protein 1c (SREBP-1c), the master transcriptional regulator of hepatic lipogenesis (34). *In vivo* studies have shown that increased hepatic LXR *O*-GlcNAcylation can be observed in refed mice and in streptozotocin-induced diabetic mice (34).

The carbohydrate-responsive element-binding protein (ChRE BP) plays a significant role in glycolysis and lipogenesis. In HEK293T cells and hepatocytes, *O*-GlcNAcylation of ChREBP has been shown to stabilize the protein and to increase its transcriptional activity on lipogenic genes (35).

O-GlcNAc REGULATION OF FASTING RESPONSE

During fasting, energy metabolism shifts from glucose utilization to fat burning. In the liver, fasting induces glycogenolysis and gluconeogenesis in order to fuel glycolytic tissues, such as the brain and red blood cells. In short-term fasting, gluconeogenesis is mainly induced by glucagon through the cyclic AMP-CREB pathway. During a period of prolonged fasting, hepatic gluconeogenesis has shown to be sustained through the peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α)-dependent mechanisms (36).

Short-term fasting

During short-term fasting, glucagon stimulates gluconeogenesis by enhancing the activity of the cyclic AMP-responsive element-binding protein (CREB). CREB is phosphorylated at Ser 133 by cAMP-dependent Ser/Thr kinase protein kinase A (PKA) (37). Phosphorylation of CREB increases its interaction with CBP/p300 (38–40), which has been shown to promote gluconeogenic gene expression by acetylating nucleosomal histones (41–44). CREB directly enhances the expression of pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase 1 (PEPCK1), and glucose-6-phosphatase (G6PC) genes upon its binding to cAMP response

elements (CREs). Phosphorylated CREB also promotes the expression of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α), which is a critical co-activator for prolonged stimulation of gluconeogenic gene transcription (45).

O-GlcNAcylation of many gluconeogenic transcription factors and cofactors has been reported to promote glucose production in the liver. OGT can induce hepatic gluconeogenesis by *O*-GlcNAcylation of CRTC2, the co-activator of CREB. At basal levels, CRTCs are phosphorylated at Ser 70 and Ser 171 by salt-inducible kinases (SIKs) and other members of the AMP-activated protein kinase (AMPK) family and are sequestered in the cytoplasm by 14-3-3 proteins (46). In response to cAMP and calcium signals, CRTC2 is dephosphorylated and *O*-GlcNAcylated at the same site. This promotes CRTC2 translocation into the nucleus and binding to CREB, which induce gluconeogenesis (47) (**Figure 2**).

Prolonged fasting

During prolonged fasting, OGT primarily affects PGC1α-mediated expression of gluconeogenic genes. PGC1α acts as a co-activator for the glucocorticoid receptor, the hepatocyte nuclear factor 4 (HNF4), and FOXO1, which further stimulates the expression of gluconeogenic genes (45). OGT can target PGC-1α via host cell factor C1 (HCF-1) (8). *O*-GlcNAcylation stabilizes PGC-1α by recruiting BAP1 for de-ubiquitination (8). PGC-1α helps recruit OGT to *O*-GlcNAcylate and activate FOXO1 (48), which further promotes hepatic glucose production (**Figure 2**). Our previous work also demonstrates that OGT can physically and functionally interact with the glucocorticoid receptor. It is, therefore, plausible that OGT is also involved in glucocorticoid induction of gluconeogenesis (49).

In the above sections, we have provided a snapshot view of the molecular events regulated by *O*-GlcNAcylation in the different phases of the feeding/fasting cycle. It should be noted that these phases are not strictly divided but exist on the continuum of time. The feeding and fasting responses are directed by precise spatiotemporal regulation of insulin signaling cascades. Despite remarkable advances in our understanding of the role of *O*-GlcNAcylation in insulin signaling, how *O*-GlcNAcylation crosstalks with phosphorylation is not well known. Exploring the mechanistic and kinetic features of *O*-GlcNAcylation on key signaling proteins holds great promise for a better understanding of normal liver metabolism.

O-GlcNAc IN LIVER DISEASES

Non-alcoholic fatty liver disease is now the leading cause of liver disease in the US. NAFLD refers to a wide spectrum of liver disorders from simple steatosis (fatty liver) to NASH. One of the earliest features of NAFLD is accumulation of lipids in hepatocytes. A proportion of patients progress to NASH, which is characterized by ballooned hepatocytes, inflammatory infiltrate and fibrosis in the liver. Fatty liver disease is reported to be strongly associated with insulin resistance. Hepatic insulin resistance has a major impact on whole-body energy metabolism. Recent studies on *O*-GlcNAcylation shed light on the etiology of hepatic insulin resistance, fatty liver, and associated fibrosis.

O-GlcNAc AND HEPATIC INSULIN RESISTANCE

The liver is an insulin-sensitive organ critical for the maintenance of nutrient homeostasis. Hepatic insulin resistance produces derangements in liver metabolism such as uncontrolled glucose production, impaired glycogen synthesis, and enhanced

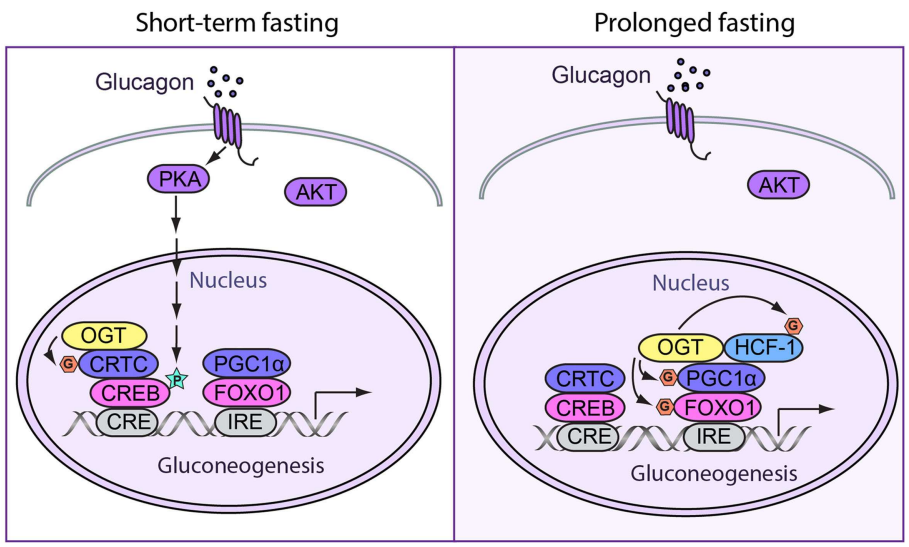

FIGURE 2 | Spatiotemporal regulation of fasting response by *O*-GlcNAcylation. (Left) During short-term fasting, glucagon stimulates gluconeogenesis by enhancing the activity of CREB. Phosphorylation of CREB by PKA directly promotes gluconeogenic gene expression. Additionally, OGT can induce gluconeogenesis by *O*-GlcNAcylating CRTC2, the co-activator of CREB. When CRTC2 is dephosphorylated and then *O*-GlcNAcylated at the same site, it translocates into the nucleus and binds to CREB to induce gluconeogenesis. (Right) In prolonged fasting, OGT targets PGC-1α via a complex with HCF-1. Both PGC-1α and HCF-1 can be *O*-GlcNAcylated. *O*-GlcNAcylated PGC-1α helps recruit OGT to glycosylate and activate FOXO1, which further promotes hepatic glucose production.

lipogenesis. In the development of hepatic insulin resistance, *O*-GlcNAcylation is associated with various changes in gluconeogenesis, glycogenesis, and glycolysis. As discussed above, *O*-GlcNAcylation has been identified as a negative regulator of insulin signal transduction. Hepatic overexpression of OGT in mice impairs the expression of insulin-responsive genes and causes insulin resistance and dyslipidaemia (28, 50) (**Figure 3**).

Uncontrolled gluconeogenesis is one of the hallmarks of diabetic liver and contributes to hyperglycemia. *O*-GlcNAcylation has been found on many gluconeogenic transcription factors and cofactors, including CRTC2, HCF-1, PGC-1α, and FOXO1. Global *O*-GlcNAcylation levels have been shown to be elevated in the liver of high fat diet-fed and *db*/*db* mice. Hepatic overexpression of OGA in these mice decreases *O*-GlcNAcylation of CRTC2, downregulates gluconeogenic gene expression, and attenuates hyperglycemia (47). OGT *O*-GlcNAcylates and activates FOXO1 during prolonged fasting to stimulate gluconeogenesis. The levels of HCF-1 are elevated in the liver of high fat diet-fed and *db*/*db* mice, which is causally linked with uncontrolled gluconeogenesis and hyperglycemia. Consistently, knockdown of OGT and HCF-1 restores glucose homeostasis in *db*/*db* mice (8).

The liver undergoes glycogenesis to absorb excessive blood glucose. Glycogen synthase is activated when insulin signaling turns on and inhibits GSK3β. Activation of glycogen synthase is often suppressed in insulin resistance. High glucose has been shown to enhance *O*-GlcNAcylation of glycogen synthase, which is associated with reduced enzymatic activity in a cell culture model (33). This finding suggests that *O*-GlcNAcylation of glycogen synthase impairs glycogenesis and exacerbates hyperglycemia. This might produce a vicious cycle between hepatic insulin resistance and *O*-GlcNAcylation.

Recent studies also indicate that *O*-GlcNAcylation modulates glycolysis by inhibiting phosphofructokinase 1 (PFK1) activity and redirecting glucose flux into the pentose phosphate pathway (PPP) (17). Overexpression of OGT or pharmacological inhibition of OGA in many cell lines leads to increased global *O*-GlcNAcylation, decreased glycolysis, and decreased ATP concentration. Further studies should clarify whether *O*-GlcNAcylation inhibits glycolysis in the liver and whether this contributes to the pathogenesis of insulin resistance.

O-GlcNAc AND NAFLD

Non-alcoholic fatty liver disease is characterized by triglyceride accumulation in the cytoplasm of hepatocytes, arising from an imbalance between lipid acquisition and removal. Insulin insensitivity presumably leads to suppressed lipogenesis, which may alleviate NAFLD symptoms. However, diabetic animals often have "selective insulin resistance," where insulin fails to suppress gluconeogenesis but retains its ability to activate lipogenesis (51). The conundrum of selective insulin resistance has not been resolved. It is also under debate whether selective insulin resistance and NAFLD are inherently related to each other.

The role of *O*-GlcNAcylation in regulating lipogenesis might hold the key to this paradox because *O*-GlcNAcylation suppresses insulin signaling but activates lipogenic pathways. *O*-GlcNAcylation increases ChREBP protein level and transcriptional activity on lipogenic genes (35). Importantly, ChREBP is hyper-*O*-GlcNAcylated in the liver of *db*/*db* mice. OGA overexpression reduces ChREBP glycosylation and protects these mice from hepatic steatosis. Another paradox involves farnesoid X receptor (FXR), a nuclear receptor that inhibits expression of SREBP1c and LXRα (52). Patients with NAFLD have lower levels of FXR mRNA and protein (53). An independent study has demonstrated that high glucose concentrations, which are believed to be a common pathophysiological condition in NAFLD patients, increases FXR *O*-GlcNAcylation and enhances FXR gene expression and protein stability (54). More direct evidence is required to reveal how *O*-GlcNAcylation affects NAFLD progression through FXR (**Figure 3**).

O-GlcNAc AND LIVER FIBROSIS

As NAFLD progresses to NASH, fibrosis becomes one of the common features among NASH patients and often correlates with poor prognosis. Fibrosis is characterized by excessive deposition of the extracellular matrix and can be regarded as a scarring process of the liver in response to repeated injury. NASH patients with liver fibrosis are more susceptible to cirrhosis (4), which is believed to be more irreversible than fibrosis. Therefore, fibrosis serves as a valuable therapeutic target to retard the progression of NASH. To date, the evidence that links *O*-GlcNAcylation to fibrosis is still limited but does shed some light on the topic.

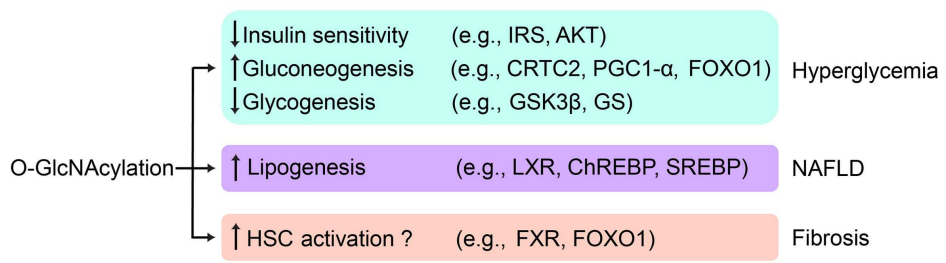

FIGURE 3 | *O*-GlcNAc regulation of liver disease. Aberrant hepatic *O*-GlcNAcylation leads to hyperglycemia by attenuating insulin signaling, activating gluconeogenesis, and suppressing glycogen synthesis. *O*-GlcNAcylation may contribute to NAFLD by stimulating *de novo* lipogenesis. *O*-GlcNAcylation may also play a role in the initiation and the progress of fibrosis by activating HSCs. Key targets of *O*-GlcNAcylation involved in related pathways are listed.

Activated hepatic stellate cells (HSCs) are the major source of the extracellular matrix in the liver (55). It was reported that elevated levels of *O*-GlcNAcylation are essential for HSC activation and upregulation of collagen expression *in vitro* (56). Both *in vitro* and *in vivo* studies confirm that FXR activation limits the transdifferentiation of HSCs from a resting, fat-storing phenotype toward a myofibroblast-like phenotype (57, 58). Given their effects on lowering inflammatory and fibrogenic processes, a number of synthetic FXR agonists are being used to treat different hepatic and metabolic disorders (59, 60). Nevertheless, the role of FXR *O*-GlcNAcylation in the context of fibrosis has not been elucidated. FOXO, which can be *O*-GlcNAcylated, also has a potential role in fibrosis. A study showed that HSC transdifferentiation was suppressed by FOXO1 (61). Paradoxically, enhanced FOXO1 expression and nuclear localization were reported in NASH patients (62). Further study is required to address whether *O*-GlcNAcylation of FOXO1 plays a role in liver fibrosis (**Figure 3**).

CONCLUSION

It is becoming clear that protein *O*-GlcNAcylation is critical for metabolic control in time and space. In hepatocytes, cytosolic *O*-GlcNAc is crucially involved in resetting insulin signaling, whereas nuclear *O*-GlcNAc has a key role in transcriptional regulation of gluconeogenesis and lipogenesis. This regulatory mechanism may serve as a "rheostat" that ensures the fluctuation of circulating nutrients within a limited range during the feeding/fasting cycle. Under pathophysiological conditions such as overnutrition or stress, aberrant cellular *O*-GlcNAcylation leads to excessive glucose production and lipid accumulation in the liver. As such, *O*-GlcNAc disturbance is likely a unifying cause of hyperglycemia, fatty liver, and fibrosis. Small molecules that target *O*-GlcNAc signaling should be explored to treat these medical conditions.

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O-GlcNAcylation links ChREBP and FXR to glucose-sensing

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Accumulating evidence suggests that O-GlcNAc transferase, an enzyme responsible for O-GlcNAc post-translational modification acts as a nutrient sensor that links glucose and the hexosamine biosynthetic pathway to the regulation of transcriptional factors involved in energy homeostasis. In liver, glucose signaling is mediated by carbohydrate response element-binding protein (ChREBP), which stimulates glycolytic and lipogenic gene expression through its binding on a specific ChoRE DNA sequence. Modulation of ChREBP by O-GlcNAcylation increases its DNA binding affinity and its activity. ChREBP transcriptional activity also depends on the presence of several other co-factors and transcriptional factors. Among them, the nuclear Farnesoid X Receptor (FXR), a key transcription factor of bile acid metabolism involved in the gut–liver axis homeostasis was recently shown to directly interact with ChREBP, acting as a repressor on the ChoRE of glycolytic genes. Interestingly, similarly to ChREBP, FXR is O-GlcNAcylated in response to glucose. This review discusses the importance of ChREBP and FXR modifications through O-GlcNAcylation in liver and how glucose can modify their mutual affinity and transcriptional activity.

Keywords: ChREBP, FXR, glucose-sensing, O-GlcNAcylation, liver metabolism

INTRODUCTION

The liver plays a central role in the control of energy homeostasis. In the liver, glucose does not only serve as an energy source but also acts as a signaling molecule to control the expression of key genes of glucose, fatty acid, and bile acid metabolism. Once in the hepatocyte, glucose is converted into glucose 6-phosphate (G6P) by the glucokinase enzyme (GK) leading, in turn, to the activation of glycolytic and lipogenic enzymes including L-pyruvate kinase (L-PK), acetyl-CoA carboxylase (ACC), and fatty-acid synthase (FAS). The positive effects of glucose on gene expression are mediated by the transcription factor carbohydrate-responsive element-binding protein (ChREBP). ChREBP, which belongs to the Mondo family of bHLH/Zip transcription factors, is a large protein (864 a.a) that contains several regulatory domains including a nuclear localization signal (NLS, amino acids 158–173) near the N-terminus, polyproline domains, a bHLH/LZ domain (amino acids 660–737), and a leucine zipper-like (Zip-like) domain (amino acids 807–847) (1). A conserved consensus sequence, named carbohydrate response element (ChoRE), the ChREBP-binding site, is required for glucose responsiveness. Modulation of ChREBP expression and/or activity by glucose occurs at multiple levels. In the presence of high glucose concentrations, ChREBP mRNA levels are increased (2, 3). ChREBP is also regulated at the post-translational level: in response to high glucose concentrations, ChREBP translocates into the nucleus (4) where the protein undergoes several post-translational modifications (PTMs), including acetylation and O-GlcNAcylation, which stimulates ChREBP activity and affinity for ChoRE sequences (5–7). The O-GlcNAc modification requires the hexosamine biosynthetic pathway (HBP): in response to high glucose concentrations, HBP synthesizes *N*-acetyl-glucosamine (UDP-GlcNAc), an obligatory substrate for β-*N*-acetylglucosaminyltransferase (OGT), a key enzyme allowing O-GlcNAcylation of proteins (8). This modification is reversible and is able to alter several protein properties such as stability, degradation, and/or modulation of transcriptional activity. Recently, key transcription factors involved in energy homeostasis, including ChREBP, have been reported to be modified by O-GlcNAc in liver. Among them, the nuclear receptor Farnesoid X receptor (FXR) (9) is a regulator of gene expression involved in bile acid synthesis and transport in the liver and the intestine (10). Interestingly, FXR

Abbreviations: ACC, acetyl-CoA carboxylase; AF-1, activation function-1; ChIP, chromatin immunoprecipitation; CBP, CREB-binding protein; ChoRE, carbohydrate response element; ChREBP, carbohydrate-responsive element-binding protein; FAS, fatty-acid synthase; FXR, farnesoid X receptor; FXRE, FXR response element; G6P, glucose-6-phosphate; GSM, glucose-sensing module; GK, glucokinase; G6PDH, glucose 6-phosphate dehydrogenase; GRACE, glucose-response activated conserved element; HBP, hexosamine biosynthetic pathway; LID, low glucose inhibitory domain; L-PK, L-pyruvate kinase; Mlx, max-like protein X; NAFLD, non-alcoholic fatty liver disease; NRB, nuclear receptor box; OGT, β-*N*-acetylglucosaminyltransferase; p300, histone acetyl transferase p300; PPP, pentose phosphate pathway; PEPCK, phosphoenolpyruvate carboxykinase; PTM, post-translational modification; UDP-GlcNAc, *N*-acetyl-glucosamine; NLS, nuclear localization signal; RXR, retinoid X receptor; X5P; xylulose-5-phosphate.

was also recently reported to be involved in the control of glucose homeostasis via its direct interaction with ChREBP (9, 11). This review will discuss how ChREBP and FXR, both regulated by O-GlcNAcylation, modulate the signaling pathway that controls glucose homeostasis.

ChREBP: A KEY REGULATOR OF GLUCOSE HOMEOSTASIS

The discovery of ChREBP as key regulator of glycolysis and lipogenesis has shed light on the mechanism by which glucose transcriptionally regulates gene expression. ChREBP stimulates the expression of several genes involved in glucose and lipid metabolism such as L-PK, FAS, and ACC not only in the liver (3, 12) and also in adipose tissue (13) and in pancreatic β cells (14). ChREBP directly binds a conserved consensus sequence, ChoRE, present on the promoter region of its target genes (15, 16). The ChoRE sequence is composed of a tandem E box element separated by 5 nucleotides (5'-CACGTGnnnnnCACGTG-3'). ChREBP interacts with Max-like protein X (Mlx), its functional partner to form a heterodimer. The association of two heterodimers is necessary to bind the ChoRE motif and to provide a transcriptional complex regulated by glucose (17).

SEVERAL KEY GLUCOSE METABOLITES ACTIVATE ChREBP IN RESPONSE TO GLUCOSE

The regulation of ChREBP activity by glucose is complex and brings in different steps [see Ref. (18) for review]. The laboratory of K. Uyeda was the first to describe a mechanism of activation dependent on a glucose metabolite. Kabashima et al. (19) demonstrated that xylulose-5-phosphate (X5P), a metabolite of the pentose phosphate pathway (PPP), is central for ChREBP translocation and DNA binding activity in response to glucose. Under high glucose concentrations, X5P activates the protein phosphatase PP2A, which dephosphorylates ChREBP on the serine residue 196 (Ser196), allowing its translocation to the nucleus. In a second step also occurring in a X5P and PP2A-dependent manner, ChREBP is dephosphorylated on the threonine residue 666 (Thr666) leading to its binding to DNA and to transactivation (19). However, this mechanism is controversial as several distinct hypotheses were proposed to explain the glucose-mediated activation of ChREBP [see Ref. (18) for review]. For instance, a structure–function analysis of the ChREBP protein identified an N-terminal domain, named the glucose-sensing module (GSM), a highly conserved sequence through evolution (20). The GSM contains two domains, the low glucose inhibitory domain (LID, residues 1–197) and the glucose-response activated conserved element, residues 197–298 (GRACE), both implicated in the regulation of ChREBP in response to glucose (21). Under low glucose concentrations, the GRACE domain is inhibited by the LID domain, leading to a lack of induction of ChREBP activity. Under high glucose concentrations, the inhibitory effect of the LID is relieved, thereby allowing the GRACE domain to stimulate ChREBP activity. In agreement with this hypothesis, deletion of the 196 first amino acids encompassing most of the LID yields a constitutive active form of ChREBP, independent of glucose concentrations (21). Interestingly, McFerrin and Atshley (22) identified a G6P binding pocket using structure prediction of the ChREBP protein. G6P, produced by the GK enzyme, after binding onto the GSM could induce a conformation change, dissociating the LID from the GRACE domain and therefore supporting ChREBP transactivation. More importantly, G6P could "open/derepress" the ChREBP protein structure allowing interaction with co-activators such as CBP and p300 (22). Arguments in favor of a role for G6P in activating ChREBP in hepatocytes and other cell types were reported. Overexpression of glucose-6-phosphate dehydrogenase (G6PDH), a rate limiting enzyme of the PPP in the pancreatic β cell line INS1 deprives cells from G6P and inhibits ChREBP transcriptional activity. In contrast, G6P accumulation driven by the specific inhibition of G6PDH activity increases ChREBP transcriptional activity in these cells (23). Using G6PDH overexpression and silencing approaches in hepatocytes, our laboratory showed that G6P, but not X5P, is required for ChREBP translocation to the nucleus and transactivation, suggesting that G6P is necessary and sufficient to induce ChREBP activity (24). The glucose-mediated activation of ChREBP remains complex and additional studies will be required to elucidate the exact contribution of the proposed metabolites. A step forward concerning ChREBP regulation was recently made when Herman and colleagues identified a novel variant of ChREBP named ChREBP-β (13). This variant arises from an alternative promoter located in exon1b of the ChREBP gene. This new transcript, which results from the splicing of exon 1b to exon 2, is translated at the next start-site located in exon 4 and produces a shorter protein of 687 amino acids (ChREBP-β) compared to the full-length protein ChREBP, re-named ChREBP-α (13). According to the hypothesis raised by Herman and co-workers, glucose metabolism (potentially G6P) would first induce the transcription of ChREBP-α. ChREBP-α would in turn bind the ChoRE identified in exon1b to enhance ChREBP-β transcription. In adipose tissue, ChREBP-β was described as a much more potent transcriptional regulator than ChREBP-α. In the liver, ChREBP-β expression seems to be less sensitive than that of ChREBP-α to nutritional regulations (fasting versus refeeding). However, the physiological contribution of ChREBP-β to the glucose-induced transcriptional response in the liver remains to be determined.

A CENTRAL ROLE FOR ChREBP IN REGULATING HEPATIC GLYCOLYSIS AND LIPOGENESIS

Convincing *in vitro* and *in vivo* evidences revealed that ChREBP is required for the induction of glycolytic and lipogenic genes in response to glucose (3, 12). Stimulation of primary cultured hepatocytes with high glucose concentrations (25 mM) leads to the induction of ChREBP expression and activity allowing stimulation of its target genes (25). In contrast, inhibition of ChREBP expression by a siRNA approach prevents this induction and blunts the accumulation of lipids in response to glucose (3). Importantly, global inactivation or liver-specific inhibition of ChREBP leads to a decrease in glycolytic and lipogenic gene expression associated with a significant decrease in triglyceride synthesis under both physiological and pathophysiological conditions (12, 26). The mirror experiment in which the ChREBP protein was overexpressed through an adenoviral approach in liver of mice led to an exacerbation of the glycolysis and lipogenesis pathways associated with the development of hepatic steatosis (27).

FXR: A NEW MODULATOR OF GLUCOSE HOMEOSTASIS

Once activated by its ligands such as natural bile acids, FXR binds, alone or with its partner Retinoid X receptor (RXR), onto its response elements (FXRE) to regulate its target genes. While largely implicated in the transcriptional control of genes controlling bile acid metabolism, FXR also recently emerged as a novel modulator of glucose homeostasis (28, 29). FXR is necessary for the control of blood glucose concentrations in response to starvation in mice. FXR knockout mice ($FXR^{-/-}$, whole body inactivation) were reported to be hypoglycemic in response to a short time (6 h) fasting. This phenotype can be, in part, explained by an alteration of the expression of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme of gluconeogenesis. Interestingly, the response to longer fasting (24–48 h) was not affected in the absence of FXR, suggesting a defective adaptative response in $FXR^{-/-}$ mice. Surprisingly, activation of FXR by GW4064, a specific synthetic ligand, did not increase PEPCK expression in primary mouse hepatocytes (30). However, primary human and rat hepatocytes stimulated with GW4064 displayed an increase in PEPCK expression that was correlated with enhanced glucose output (31) suggesting either species differences or dependence on changes such as nutritional or environmental stimuli.

Interestingly, $FXR^{-/-}$ mice respond more rapidly to high carbohydrate feeding with an accelerated induction of glycolytic and lipogenic genes without, however, any difference in ChREBP mRNA levels (29). In addition, nuclear translocation of ChREBP protein was not affected by FXR activation. FXR was shown to directly interact with the ChREBP protein in different cell lines. *In vitro* GST pulldown experiments showed that FXR interacts with ChREBP, irrespective of its ligation to GW4064. Analysis of FXR deletion mutants revealed that FXR interacts with ChREBP via its N-terminal activation function-1 (AF-1) domain (amino acids 1–127) and via the N-terminal part of its ligand-binding domain (amino acids 215–300) (11).

At the functional level, treatment of primary hepatocytes with the FXR agonist GW4064 decreased the glucose-induced expression of L-PK, ACC, and FAS. This inhibition was prevented in $FXR^{-/-}$ hepatocytes. Importantly, using a ChoRE luciferase promoter construct, the authors reported that FXR transfection and/or activation prevented the stimulation of ChoRE-driven target genes. Gel shift analysis revealed that FXR was indeed able to bind to the L3 site (contained within the ChoRE) but not to the L4 site of L-PK promoter (29). These results were confirmed in the immortalized human hepatocyte (IHH) cell line: when activated by either its most potent natural ligand (CDCA: chenodeoxycholic acid) or synthetic agonists (GW4064; INT747; and WAY362450), FXR was able to bind the L-PK promoter. Finally, using chromatin immunoprecipitation (ChIP) assays, Caron et al. (11) were able to demonstrate the concomitant recruitment of ChREBP, HNF4α, p300, CBP, and FXR on the genomic L4/L3 region of the L-PK promoter in the presence of high glucose concentrations. According to this model, agonist-mediated activation of FXR leads to the release of CBP and p300, while allowing the recruitment of the co-repressor SMRT (**Figure 1**). This study reveals that FXR acts as a transrepressor and provides a novel mechanism by which FXR directly controls ChREBP-dependent genes, such as the L-PK gene.

The structural base of the repressive activity of FXR on ChREBP activity may rely on the existence of the LxQLLT motif, called the nuclear receptor box (NRB) within the ChREBP protein (22). This NRB matches the consensus LXXLL motif primarily found in co-activators of nuclear receptors that confers agonist-induced binding to nuclear receptors suggesting a potential ligand-dependent interaction between ChREBP and nuclear receptors such as FXR (22) and recruitment of FXR on ChoRE-bound ChREBP. SMRT tethering to the FXR–ChREBP complex could then occur through the second co-activator binding motif specifically found in FXR (32), although this awaits formal investigation. It would be of interest to mutate this NRB motif and study the modification of interaction between ChREBP and FXR as well as the consequences on the transcriptional regulation of the L-PK gene. One can also speculate that under appropriate conditions, ChREBP might serve as a FXR co-regulator, hence conferring glucose responsiveness to FXR. Such a possibility could be investigated by high resolutive genomic binding studies such as ChIP-Exo assays. Another hypothesis is that glucose metabolism could act as a signal that activates FXR independently of ChREBP via PTMs such as O-GlcNAcylation, as discussed below.

ChREBP AND FXR ARE O-GlcNAcylated

O-GLcNAcylation STABILIZES THE ChREBP PROTEIN AND STIMULATES ITS TRANSCRIPTIONAL ACTIVITY

Approximately 2–5% of total glucose in the cell is used through the HBP. O-GlcNAcylation is a dynamic reaction catalyzed by two enzymes: (i) O-GlcNAc transferase (OGT), which adds a monosaccharide to serine/threonine residues of target proteins; (ii) the *O-GlcNAc hydrolase* (OGA), which hydrolyzes the monosaccharide. Sakiyama and co-workers (33) first showed that when Hepa1–6 hepatoma cells are treated with PUGNAC, a drug that increases O-GlcNAc, the transcriptional activity of ChREBP is exacerbated under high glucose concentrations, without any change in protein levels. In contrast, in cells treated with DON (6-diazo-5-oxo-L-norleucine), a drug that decreases O-GlcNAc, the stimulatory effect of high glucose on ChREBP activity is prevented (33). Our laboratory showed that ChREBP directly interacts with OGT in HEK293 cells and hepatocytes (6). ChREBP is O-GlcNAcylated in hepatocytes treated with high concentrations of glucose or glucosamine and in the liver of refed mice, demonstrating a nutritional regulation of ChREBP O-GlcNAcylation ($ChREBP^{OG}$). In mouse hepatocytes, overexpression of OGA led to an inhibition of ChREBP-target genes associated with a decrease of lipid droplets under high glucose concentrations. *In vivo*, OGT overexpression in mouse liver was associated with an increase of $ChREBP^{OG}$, correlated with the induction of L-PK expression and with ChREBP recruitment to the L-PK promoter. OGT overexpression also increased ChREBP protein content without modifying ChREBP mRNA levels suggesting that the protein may be stabilized by O-GlcNAcylation (6). Interestingly, in a follow-up study, Ido-Kitamura and co-workers suggested that ChREBP poly-ubiquitination ($ChREBP^{ub}$) was reduced when ChREBP is O-GlcNAcylated (7) suggesting that these two PTMs may interfere to regulate ChREBP stability.

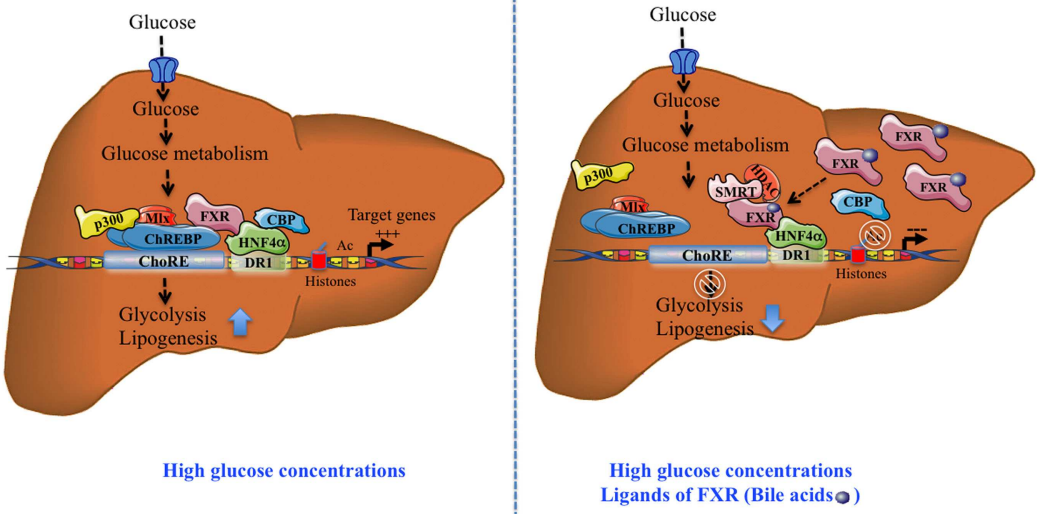

FIGURE 1 | Activation and transrepression of ChREBP-target genes by ChREBP and FXR. After a meal, in the presence of high glucose concentrations, without FXR activation, ChREBP binds together with HNF4α, to ChoRE region of the L-PK promoter and transactivates gene expression, in part due to the recruitment of co-activators p300 and CBP. Due to its direct interaction with ChREBP and HNF4α, FXR interacts with this complex. The complex formation leads to the stimulation of the glycolytic and lipogenic pathways. The synergistic presence of high glucose concentrations and FXR ligands (bile acids, CDCA), activated FXR recruits the co-repressor SMRT. This recruitment leads to the release of ChREBP, CBP, and p300 leading to the inhibition of ChREBP-target gene expression. Tethered to the promoter through its interaction with HNF4α, FXR recruits the transcriptional co-inhibitor SMRT and represses transcription through the recruitment of HDACs and deacetylation of H3 histones. This effect leads to inhibition of the glycolytic and lipogenic pathways.

FXR IS REGULATED BY O-GlcNAcylation

While the impact of nutrients and glucose on bile acid homeostasis is not fully understood (10), it was recently shown that FXR can be modified through O-GlcNAcylation in response to high glucose concentration level (9). *Berrabah* et al. revealed that FXR is modified by O-GlcNAcylation through its interaction with OGT, which catalyzed this reaction in response to high glucose level. O-GlcNAcylation leads to an increase of FXR protein stability, transcriptional activity, and chromatin binding through SMRT inactivation. O-GlcNAcylation of FXR occurs on serine 62 (Ser62) within the AF-1 domain. In agreement, mutation of Ser62 decreased FXR O-GlcNAcylation that correlated with an inhibition of its transcriptional activity. *In vivo*, nutritional experiments reveals that FXR is O-GlcNAcylated under fed conditions, which correlates with an induction of its target genes (Shp, Cyp7A1) and a decrease in hepatic bile acid content. Interestingly, a recent study reported that FXR can also be modified by SUMOylation (34). Ligand-dependent SUMOylation of FXR leads to a decrease of FXR transcriptional activity and consequently to a down regulation of its target genes. Interestingly, O-GlcNAcylation (Ser62) and sumoylation (Lys122) of FXR occur within the same domain, the A/B-domain known to play gene-specific role in transactivation and cofactor recruitment (11). It would be of interest to determine whether O-GlcNAcylation of FXR prevents and/or interferes with its SUMOylation and vice versa.

RELEVANCE OF ChREBP AND/OR FXR O-GlcNAcylation TO PHYSIOPATHOLOGY

Hyperglycemia and diabetes result in an increased flux through the HBP, which, in turn, increases PTM of Ser/Thr residues of proteins by O-GlcNAcylation. Altered O-GlcNAc signaling has been implicated in the pathogenesis of diabetes and may play an important role in its complications including non-alcoholic fatty liver disease (NAFLD), diabetic nephropathy, and/or retinopathy (35). Indeed, we have reported that the hepatic content of ChREBP^{OG} is increased in liver of diabetic db/db mice, and correlated to the pathophysiology of hepatic steatosis in this mouse model. OGA overexpression in the liver of db/db mice reduced ChREBP^{OG} concentrations leading to an inhibition of its target genes involved in *de novo* lipogenesis. Consequently, hepatic steatosis was prevented and correlated to an improvement of several physiological parameters (improved glucose tolerance and insulin sensitivity). The improved phenotype in OGA-treated db/db mice was also associated with a significant decrease in O-GlcNAcylation of the transcriptional co-activator CRTC2 (6) involved in the control

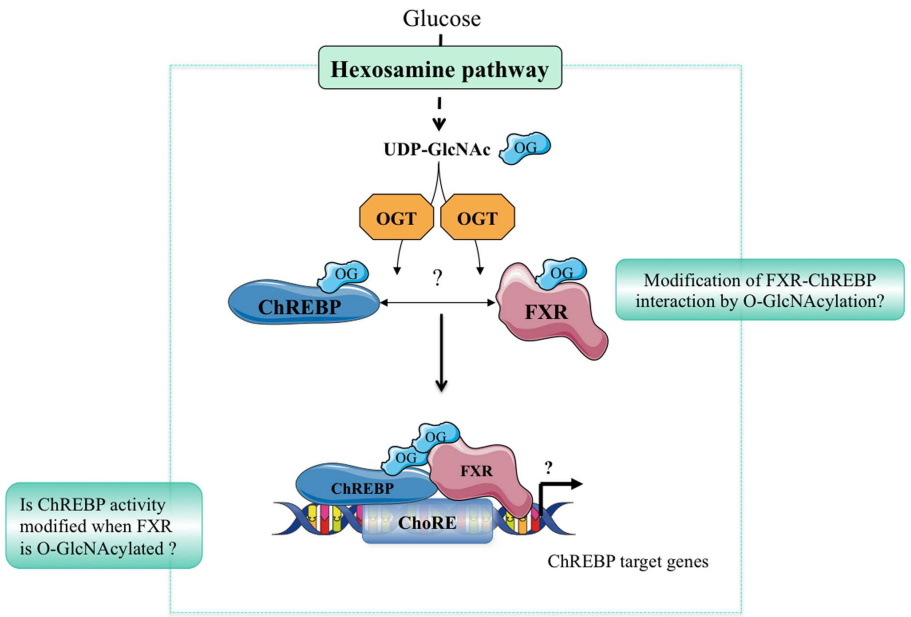

FIGURE 2 | Hypothetical model of ChREBP and FXR interaction. In response to high glucose concentrations, the hexosamine biosynthesis (HBP) pathway is activated leading to UDP-GlcNAc production. ChREBP and FXR are in turn O-GlcNAcylated through direct binding with the OGT enzyme. O-GlcNAcylation of ChREBP and FXR may represent an important feature of their interaction. The physiological or pathophysiological consequences of such a modification remains, however, unknown.

of gluconeogenic genes (36). Recently, the role of ChREBP^{OG} in diabetic nephropathy was also investigated (37). Treatment with high glucose concentrations increased cellular O-GlcNAc and ChREBP^{OG} levels in mesangial cells compared with low glucose concentrations. PUGNAc treatment increased ChREBP-target expression in mesangial cells, whereas DON blunted the stimulatory effect of high glucose. Mechanistically, O-GlcNAc augmented protein stability, transcriptional activity, and nuclear translocation of ChREBP in these cells, leading to an exacerbated lipid accumulation. Importantly, in a pathophysiological context, ChREBP^{OG} was elevated in mesangial cells from streptozotocin-induced diabetic rats. Altogether, this study suggests that the hyperglycemia-mediated induction of ChREBP O-GlcNAcylation in mesangial cells may drive excess lipid accumulation and fibrosis, characteristic features of diabetic nephropathy (37). The potential contribution of FXR O-GlcNAcylation to the pathophysiology of liver and/or of other organs has not yet been addressed. Interestingly, FXR deficiency was previously reported to improve several of the metabolic abnormalities observed in ob/ob mice. Indeed, FXR$^{-/-}$ mice crossed on ob/ob background are less obese, more tolerant to glucose and more sensitive to insulin than controls (38). The finding that FXR is modified by O-GlcNAcylation (9) further links bile acid metabolism to nutrient availability as observed in human in physiology (39), but also in a context of metabolic dysfunctions such as type 2 diabetes (40). In fasting–refeeding experiments, an FXR-dependent correlation between hepatic bile acid content, FXR transcriptional activity, and plasma glucose concentration has been established, suggesting that O-GlcNAcylation of FXR might regulate bile acid production (9). However, this awaits a formal demonstration using O-GlcNAcylation-deficient FXR *in vivo*. Importantly, the concomitant regulation of ChREBP and FXR by O-GlcNAcylation in liver cells in response to hyperglycemia may trigger and/or enhance their physical interaction, modulating in turn the transcriptional regulation of their common target genes involved in glycolysis, lipogenesis, and/or bile acid metabolism (**Figure 2**). Interestingly, FXR was reported to interact with the ChREBP protein through its AF-1 domain (11), a domain also shown to be the site of FXR O-GlcNAcylation (9). Further analysis of this interaction in response to high glucose concentrations, as well as the identification of O-GlcNAc residues within the ChREBP protein should provide a better understanding of the relevance of the coordinated O-GlcNAcylation of ChREBP and FXR under physiological and pathophysiological conditions (**Figure 2**).

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O-GlcNAcylation and inflammation: a vast territory to explore

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O-GlcNAcylation is a reversible post-translational modification that regulates the activities of cytosolic and nuclear proteins according to glucose availability. This modification appears to participate in several hyperglycemia-associated complications. An important feature of metabolic diseases such as diabetes and obesity is the presence of a low-grade chronic inflammation that causes numerous complications. Hyperglycemia associated with the metabolic syndrome is known to promote inflammatory processes through different mechanisms including oxidative stress and abnormally elevated protein O-GlcNAcylation. However, the role of O-GlcNAcylation on inflammation remains contradictory. O-GlcNAcylation associated with hyperglycemia has been shown to increase nuclear factor κB (NFκB) transcriptional activity through different mechanisms. This could contribute in inflammation-associated diabetic complications. However, in other conditions such as acute vascular injury, O-linked *N*-acetyl glucosamine (*O*-GlcNAc) also exerts anti-inflammatory effects via inhibition of the NFκB pathway, suggesting a complex regulation of inflammation by *O*-GlcNAc. Moreover, whereas macrophages and monocytes exposed to high glucose for a long-term period developed a pro-inflammatory phenotype, the impact of O-GlcNAcylation in these cells remains unclear. A future challenge will be to clearly establish the role of O-GlcNAcylation in pro- and anti-inflammatory functions in macrophages.

Keywords: *O*-GlcNAc glycosylation, diabetes, metabolic syndrome, inflammation, cytokines, macrophages, nitric oxide, NFκB

INTRODUCTION

In the last decades, changes in lifestyle, including excessive energy intake and consumption of food enriched in saturated fat, combined with the lack of physical activity, have led to a dramatic increased prevalence of pathologies such as diabetes, obesity, and atherosclerosis. These pathologies are part of the metabolic syndrome, which constitutes one of the major threats to global health.

It is now well established that these metabolic diseases are associated with a low-grade chronic inflammation (1) that causes complications such as nephropathy, neuropathy, retinopathy, and atherosclerosis, and contributes to morbidity and mortality associated with the metabolic syndrome. This low-grade inflammation is characterized by an abnormal cytokine production. Thus, it has been demonstrated that the adipose tissue of obese individuals produce higher levels of the pro-inflammatory cytokine tumor-necrosis factor α (TNFα) and other pro-inflammatory factors such as interleukin (IL) 6 (1). The excessive amount of nutritional lipids might have a role not only in the pathogenesis of obesity-associated insulin resistance but also in the chronic inflammation associated with this condition. Indeed, free fatty acids can activate the lipopolysaccharide (LPS) receptor toll-like receptor (TLR) 4 and induce the production of pro-inflammatory cytokines by macrophages (2). Not only lipids but also high-glucose concentrations are involved in inflammatory processes (3, 4). High glycemic index diets appeared to play a key role in the establishment and persistence of inflammation (5–7). In contrast, a 4 weeks food restriction in obese patients was sufficient to significantly reduce oxidative stress (8).

It is well documented that hyperglycemia associated with the metabolic syndrome promotes abnormally elevated protein O-GlcNAcylation, which participates in the glucotoxicity phenomenon (9). O-GlcNAcylation is a reversible post-translational modification consisting in the addition of *N*-acetylglucosamine to serine or threonine on cytosolic and nuclear proteins (**Figure 1**). Only two enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), control the level of O-linked *N*-acetyl glucosamine (*O*-GlcNAc) on proteins. OGT uses UDP-GlcNAc, produced through the hexosamine biosynthetic pathway (HBP) to *O*-GlcNAcylate proteins, whereas OGA removes *O*-GlcNAc from proteins. Thus, according to glucose availability and its flux through the HBP, O-GlcNAcylation modulates protein functions by regulating their sub-cellular localization, stability, interaction with protein partners, or activity. More than 1000 proteins have now been identified as target of this modification, including transcription factors (10–17) and signaling molecules (9, 18–22) involved in glucose and lipid metabolism, insulin resistance, and inflammation. In addition to glucose, the *O*-GlcNAc also includes amine and acetyl moieties, and therefore also integrates amino-acids (glutamine) and fatty acid (AcetylCoA) metabolisms, suggesting that the availability of other nutrients may also be sensed by this pathway. Thus, infusion of a lipid emulsion in rats induced a twofold increase in

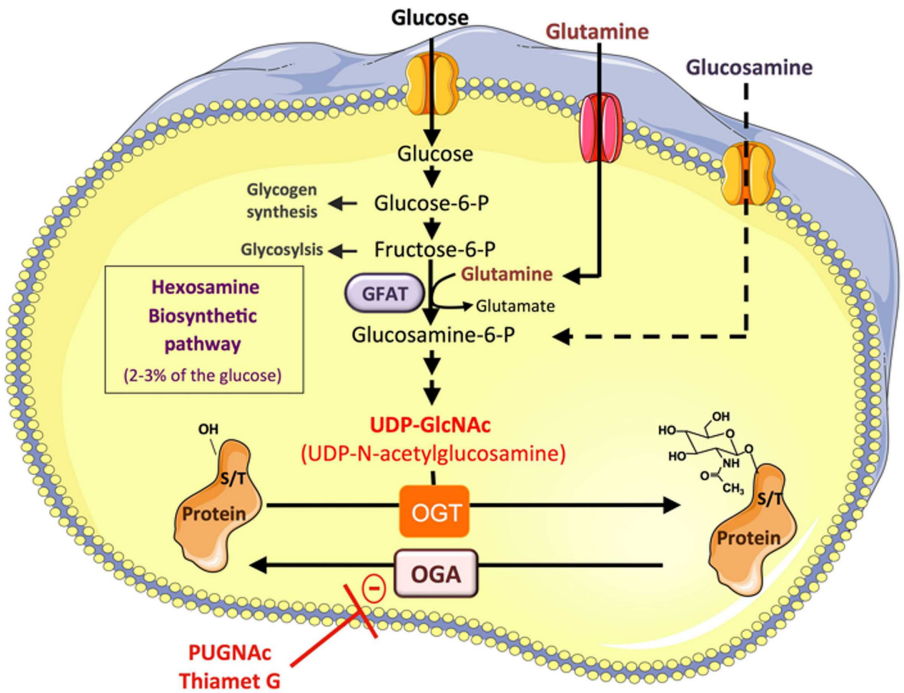

FIGURE 1 | Protein O-GlcNAcylation depends on the flux of glucose through the hexosamine biosynthesis pathway. A small fraction of the glucose entering the cell feeds the hexosamine biosynthetic pathway (HBP) to produce UDP-GlcNAc, the substrate used by *O*-GlcNAc-transferase (OGT) to add *N*-acetyl glucosamine on serine or threonine residues of cytosolic or nuclear proteins. This dynamic and reversible post-translational modification controls the activity, the localization, or the stability of proteins according to glucose availability. Glucose enters the HBP as fructose-6-phosphate. The latter is converted to glucosamine-6-phosphate by the glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate limiting enzyme of the pathway. After a subset of reactions, UDP-*N*-acetylglucosamine (UDP-GlcNAc) is generated and used by OGT to add GlcNAc on serine or threonine residues of target proteins. The *O*-GlcNAc moiety is removed from *O*-GlcNAc-modified proteins by the *O*-GlcNAcase (OGA). Experimentally, O-GlcNAcylation of proteins can be increased by incubating the cells with high concentrations of glucose, or with glucosamine, which bypass the rate limiting step catalyzed by GFAT. Inhibitors of OGA such as O-[2-acetamido-2-deoxy-D-glucopyranosylidene] amino-*N*-phenylcarbamate (PUGNAc) or (3aR,5R,6S,7R,7aR)-2-(ethylamino)-3a,6,7,7a-tetrahydro-5-(hydroxymethyl)-5H-Pyrano[3,2-d]thiazole-6,7-diol (Thiamet-G) can also be used to increase the *O*-GlcNAc level on proteins.

UDP-GlcNAc content in skeletal muscle, associated with insulin resistance. Moreover, fatty acids can directly regulate the expression of glutamine:fructose-6-phosphate amidotransferase (GFAT) (23) and other enzymes of the HBP pathway (24) in muscle and pancreatic β-cell. Therefore, increased nutrients, and particularly increased blood glucose and fatty acids levels associated with excess food intake, obesity, and/or diabetes, are likely to impact numerous cellular processes, including those involved in inflammation, through protein O-GlcNAcylation.

O-GlcNAcylation, DIABETIC COMPLICATIONS, AND INFLAMMATORY PROCESSES

A number of experimental data have suggested the involvement of the HBP in pathological manifestations of the metabolic syndrome, such as diabetic associated-kidney disease. Indeed, one-third of diabetic patients will develop diabetic nephropathy, a chronic microvascular complication leading to a progressive decline in renal function, decreased glomerular filtration rate and proteinuria. Clinical trials have demonstrated that high glucose is central to the pathogenesis of diabetic nephropathy (25), and the beneficial effect of glycemia correction on renal complications has been demonstrated (26). Mesangial cells are smooth muscle-like pericytes that surround the filtration capillaries within glomerulus (27). In these cells, glucose flux, through the HBP pathway, regulates the expression of pro-fibrotic factors such as transforming growth factor β1 (TGFβ1) and plasminogen activator inhibitor 1 (PAI-1), and extracellular matrix components (28, 29), at least in part via the O-GlcNAcylation of transcription factors such as Sp1 (11, 30). In mesangial cells, the HBP pathway also regulates the expression of pro-inflammatory factors such as vascular cell adhesion molecule-1 (VCAM-1), IL6, and TNFα, through the nuclear factor κB (NFκB) pathway (31). Abnormal activation of the NFκB pathway is certainly a major contributor in inflammation-associated diabetic complications. In vascular smooth muscle cells, high-glucose conditions resulted in NFκB activation (32). Peripheral blood mononuclear cells isolated from patients with diabetic nephropathy showed an increased activation of NFκB that could be corrected by anti-oxidant treatment (33, 34). Glucose oxidative stress is obviously central to glucotoxicity in diabetic conditions (35), and a link between hyperglycemia, oxidative stress, and O-GlcNAcylation has been proposed, reinforcing the potential involvement of O-GlcNAcylation in inflammation

(36, 37). Therefore, exploring the potential regulation of NFκB activity by O-GlcNAcylation in different settings is of paramount importance.

O-GlcNAcylation AND THE NFκB PATHWAY

The transcription factor NFκB is involved in a large number of cell functions including apoptosis, cell survival, and differentiation, and is critical to immune response and inflammation. NFκB family comprises five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NFκB1), and p100/52 (NFκB2) that associate to form distinct homo and hetero-dimeric complexes (38–40). In non-stimulated cells, NFκB is inactive and is retained in the cytoplasm by the inhibitor of κB (IκB) (**Figure 2**). Upon stimulation by pro-inflammatory cytokines, LPS, or growth factors, IκB is phosphorylated by the IκB kinase (IKK). This phosphorylation leads to IκB ubiquitination and proteosomal degradation. Free NFκB can then translocate into the nucleus to activate its target genes (38–40).

Nuclear factor κB activation has been implicated in the metabolic syndrome and in diabetes pathogenesis (43–46). Because NFκB is mainly regulated by post-transcriptional modifications (with an important role of phosphorylation and acetylation), and because high glucose is known to activate NFκB and stimulate its target genes, different studies focused on the potential role of *O*-GlcNAc on NFκB activation.

O-GlcNAcylation AS A POSITIVE REGULATOR OF NFκB ACTIVITY

In the first study addressing this question, mesanglial cells treated with glucosamine or high-glucose exhibited an increased nuclear protein binding to NFκB consensus sequences in an electromobilty shift assay, correlated with O-GlcNAcylation of p65 (31). This observation suggested that NFκB O-GlcNAcylation could play a part in inflammatory processes. However, in that first study, the *O*-GlcNAc modification sites on NFκB had not been identified and the mechanism by which *O*-GlcNAc modification led to NFκB activation remained unclear (31).

It now clearly appears that different mechanisms, acting at various cellular levels, are involved in the effects of O-GlcNAcylation on activation of NFκB signaling. First, O-GlcNAcylation can regulate the interaction between NFκB and its inhibitor IκB. In porcine vascular smooth muscle cells, it has been demonstrated that down-regulation of O-GlcNAcylation mediated by OGA over-expression inhibits hyperglycemia-induced NFκB activation. In contrast, an increase in O-GlcNAcylation mediated by OGT over-expression increases NFκB activity (41). These effects were due to an increase in O-GlcNAcylation of RelA on T352 that decreases its affinity for IκB, leading to an increased nuclear translocation of RelA [**Figure 2A** (I)]. This could contribute to the sustained activation of NFκB that is associated with diabetes (41). Another study indicated that O-GlcNAcylation increases NFκB transcriptional activity by promoting its acetylation (42). Indeed, chromatin immunoprecipitation assays demonstrated that, upon induction with TNFα , OGT localizes to NFκB-regulated promoters. OGT siRNA experiments showed that OGT protein was required for NFκB-dependent transcription. The mechanism involved was the attachment of *O*-GlcNAc moiety to T305 on RelA that promoted NFκB transcription by potentiating p300-dependent acetylation on K310 [**Figure 2A** (II)] (42).

The O-GlcNAcylation of NFκB also appears to play an important role in the immunity and the production of pro-inflammatory cytokines by T lymphocytes. Golks et al. first showed that OGT was necessary for activation of T lymphocytes by the T-cell receptor (TCR), inducing O-GlcNAcylation of p65 and stimulation of NFκB-dependent transcription (47). More recently, it was reported that in these cells, the c-Rel subunit of NFκB was modified by O-GlcNAcylation on Ser 350 [**Figure 2A** (III)]. This modification increased c-Rel transcriptional activity and was necessary for c-Rel mediated expression of *IL2*, *IFNG*, and *CSF2* in response to TCR activation (48). Importantly c-Rel O-GlcNAcylation was not required for TNFα- or TCR-induced expression of other NFκB target genes, such as *NFKBIA* (which encodes IκBα) and *TNFAIP3* (which encodes A20), indicating a gene specific requirement of c-Rel O-GlcNAcylation (48). These results suggest that during chronic hyperglycemia, an increase in c-Rel O-GlcNAcylation could contribute to type-1 diabetes progression by enhancing the production of Th1 pro-inflammatory cytokines, leading to pancreatic β cells destruction (48, 49). Finally, O-GlcNAcylation of IKK [**Figure 2A** (IV)] has also been demonstrated, resulting in an increase in its kinase activity, leading to subsequent increase in phosphorylation, and degradation of IκB and stimulation of NFκB activity in cancer cells (50). Whether this mechanism is also operative in the context of hyperglycemia-induced inflammation remains to be evaluated.

O-GlcNAcylation AS A NEGATIVE REGULATOR OF NFκB ACTIVITY

Whereas O-GlcNAcylation is generally found associated with an increased in NFκB activity in diabetic conditions, in some situations, *O*-GlcNAc appears, however, to reduce its pro-inflammatory activity (51–53). Thus, in a rat model of trauma-hemorrhage followed by fluid resuscitation, increased O-GlcNAcylation induced by glucosamine or PUGNAc significantly improved cardiac function and peripheral organ perfusion, and decreased the circulating levels of pro-inflammatory cytokines TNFα and IL6 (51, 52). These authors observed that increased O-GlcNAcylation reduces IκB phosphorylation and NFκB signaling in cardiac tissue from trauma-hemorrhage treated rats. Moreover, O-GlcNAcylation-inducing treatments appear to have anti-inflammatory and vasoprotective effects during acute vascular injury (54, 55). Indeed, Xing et al. showed that in rat aortic smooth muscle cells, O-GlcNAcylation of p65 NFκB upon PUGNAc or glucosamine treatment was accompanied by a reduction in TNFα-induced phosphorylation on serine 536, resulting in increased association of NFκB with IκB, decreased NFκB activity and inhibition of the production of pro-inflammatory mediators (**Figure 2B**) (53).

It therefore appears that, depending on the cellular context and type of insult (chronic hyperglycemia versus acute vascular injury), O-GlcNAcylation may have different effects on the NFκB pathway, resulting in either pro- or anti-inflammatory outcomes.

O-GlcNAcylation AND MACROPHAGE ACTIVITY

Monocytes and macrophages play central roles in acute and chronic inflammatory processes. As mentioned previously, insulin resistance, obesity, and diabetes are associated with recruitment of pro-inflammatory monocytes/macrophages in different organs, including adipose tissue, liver, pancreas, as well as

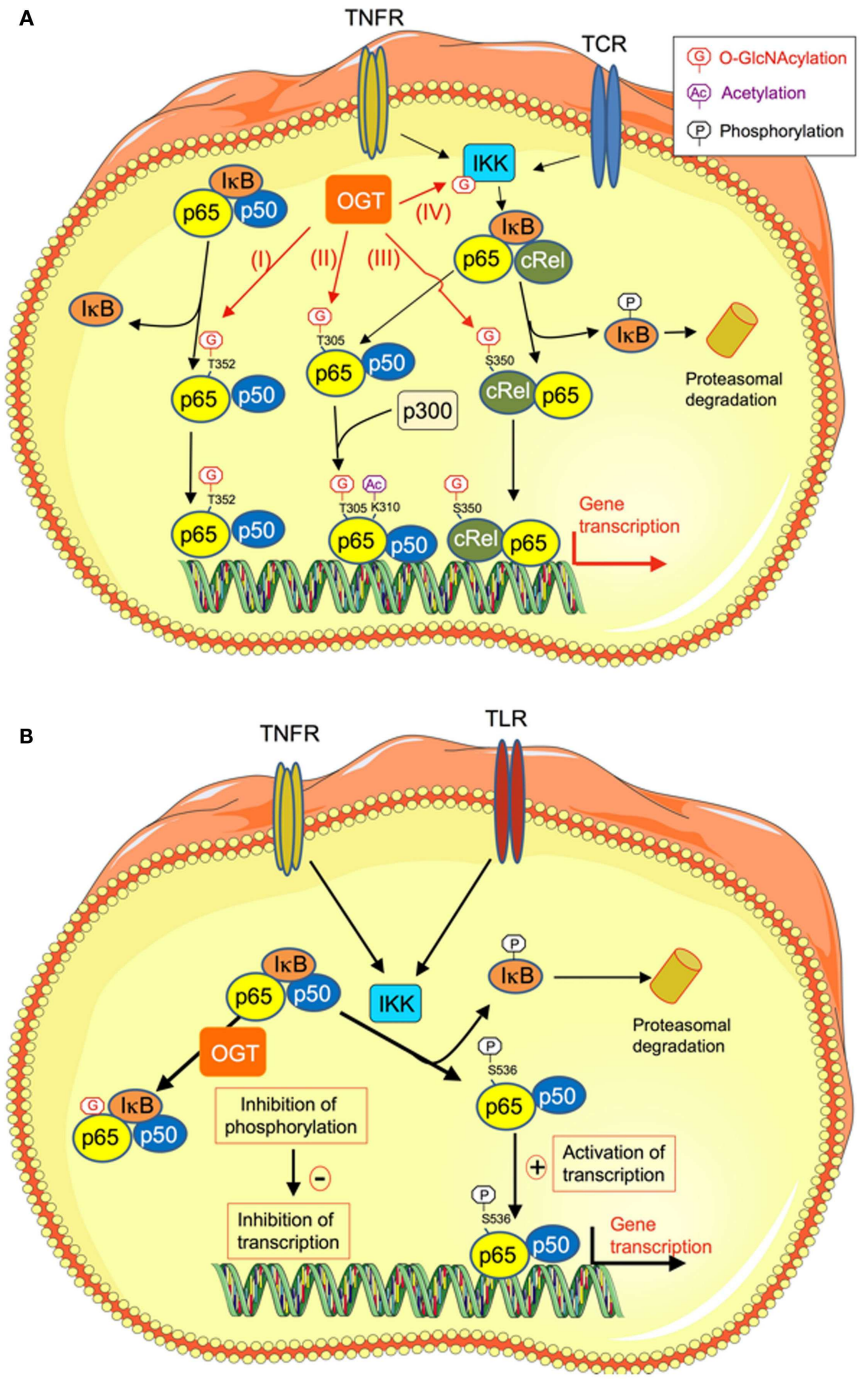

FIGURE 2 | O-GlcNAcylation regulates NFκB transcriptional activity through different mechanisms. **(A)** O-GlcNAcylation stimulates NFκB transcriptional activity. High-glucose conditions are known to promote inflammatory processes through different mechanisms, including increased O-GlcNAcylation of NFκB. Several mechanisms have been described that could account for increased transcriptional activity of this factor upon O-GlcNAcylation. (I) O-GlcNAcylation of p65/RelA on T352 decreases its affinity for IκB, resulting in increased in its nuclear localization and transcription of its target genes (41). (II) O-GlcNAcylation of T305 on RelA promotes NFκB transcriptional activity by potentiating its p300-dependent acetylation on K310 (42). (III) O-GlcNAcylation of c-Rel on S350. This modification increases c-Rel DNA binding and transcriptional activity. (IV) O-GlcNAcylation of the β-subunit of IKK on Ser733 stimulates its activity, resulting in increased phosphorylation and degradation of IκB, and thereby increased NFκB activity.
(B) O-GlcNAcylation inhibits NFκB transcriptional activity. O-GlcNAcylation-inducing treatments appear to have anti-inflammatory and vaso-protective effects during acute vascular injury. In rat aortic smooth muscle cells, O-GlcNAcylation of NFκB specifically inhibits its phosphorylation on Ser 536, while leaving other phosphorylation sites unaffected. This results in increased NFκB binding to IkB, inhibition of TNFα-induced NFκB DNA binding, and reduction of expression of genes coding for inflammatory mediators (TNFR, TNFα receptor; TCR, T-cell receptor; IKK, Iκ kinase).

blood vessels wall (56–62). Numerous studies have shown that macrophages/monocytes submitted to long-term exposure to high-glucose concentrations developed a pro-inflammatory phenotype. Indeed, in human monocytic cells THP1, high glucose (15 mmol/L) for 72 h increased gene expression of the pro-inflammatory factors monocyte chemotactic protein 1 (MCP1), IL1β, and TNFα. Of interest, in this study, the NFκB activation played an important role in the high glucose-induced MCP1 transcription (63). In THP1 cells, exposure to high glucose also increased the RNA and protein levels of TLR2 and TLR4, which play key roles in innate immune response and inflammation. TLR2 and TLR4 activate MyD88 dependant signaling and induce NFκB transactivation, leading to the production of pro-inflammatory cytokines. These up-regulations of TLR2 and TLR4 under high-glucose condition seemed at least in part mediated by protein kinase C (PKC) (64). In RAW 264.7, a murine macrophages cell line, high-glucose alone did not induce inflammatory mediator expression but increased inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in response to LPS. This effect appeared to be mediated by NFκB activation (65). High-glucose also increased IL1β secretion from LPS activated macrophages, a risk factor in diabetes that contributes to pancreatic β-cell damage (66). This effect appeared to involve activation of ERK1/2, JNK1/2, and PKCα and δ in macrophages cultured in high-glucose conditions (65).

In vivo hyperglycemia also affects the inflammatory profile of macrophages. An increased pro-inflammatory profile was observed in peritoneal macrophages from mice two weeks after diabetes induction with alloxan or streptozocin (67, 68). However, peritoneal macrophages from mice with 4 months streptozotocin-induced diabetes displayed complex modification of the pro-inflammatory profile, with increased NO production but decreased TNFα and IL6 in response to LPS stimulation (69). Another study showed impaired inflammatory response to multiple TLR ligands in alveolar macrophages from 2 weeks streptozotocin-induced diabetic mice (70). Therefore, *in vivo* hyperglycemia may have complex effects on macrophages functions, depending on their tissue of origin and on the duration of the diabetes.

High-glucose concentrations may affect macrophages functions through numerous mechanisms, including oxidative stress, activation of PKC, and/or MAP kinases, advanced glycation end products, as well as protein O-GlcNAcylation. Only a few studies evaluated the role of O-GlcNAcylation in macrophages functions, and contradictory results were obtained.

In the human monocyte THP1 cell line, high-glucose concentrations and PUGNAc increased the expression and the secretion of macrophage inflammatory protein MIP1α and β through OGT dependent epigenetic mechanisms (71).

On the other hand, glucosamine exerted neuroprotective effects via suppression of post-ischemic microglia inflammation in rat brain after ischemia/reperfusion injury (72). Accordingly, in cultured mouse BV2 microglial cells and RAW264.7 macrophages, Hwang et al. observed that glucosamine suppressed LPS-induced up regulation of pro-inflammatory molecules by inhibiting NFκB activation by LPS. Glucosamine, which bypass the rate limiting step of the HBP, is often used to increase O-GlcNAcylation in cells. Unexpectedly, in this study, glucosamine induced a decrease in NFκB O-GlcNAcylation. This counter-intuitive result was explained by an inhibitory effect of glucosamine on an LPS-induced interaction between OGT and NFκB (72). More recently, the same group obtained similar results with cRel in BV2 microglial cells, showing glucosamine inhibition of LPS-induced cRel-OGT interaction, associated with decreased O-GlcNAcylation of c-Rel and subsequent inhibition of its transcriptional activity (73). However, the mechanism by which glucosamine may interfere with the LPS pathway and affect OGT-NFκB interaction was not elucidated. For instance, the specific effect of increasing O-GlcNAcylation levels using PUGNAc or Thiamet-G was not evaluated in theses studies. Glucosamine, by increasing UDP-GlcNAc in the cell, may also affect complex glycosylations of proteins. Thus, it is possible that glucosamine effects were mediated by modification of N-linked glycosylation of receptors and/or secreted proteins, as suggested previously in a study using macrophage cell lines (74). Moreover, depending on the experimental setting, glucosamine may also induce ATP depletion (75) or promote oxidative stress (76). Therefore, glycosylation-independent effects might also play a role in the paradoxical effect of glucosamine on NFκB O-GlcNAcylation state. Further confusion was provided by an additional study by Hwang et al. (77), which showed that over-expression of OGT unexpectedly reduced the transcriptional activity of NFκB both in the absence and presence of glucosamine, resulting in inhibition of LPS-mediated expression of the NFκB target gene iNOS.

Innate immune signaling initiated by interaction of pathogen ligands with TLRs induces iNOS expression, and, subsequently, the production of NO, which not only plays a role as a bactericidal agent but also act as an intracellular mediator. Indeed, S-nitrosylation of cysteine thiols regulates protein activities in NO-generating cells. Complex interactions between NO signaling and O-GlcNAcylation pathway have been suggested. Thus, in RAW264.7 cells and in mice peritoneal macrophages, Ryu et al. observed that LPS treatment induces increased global S-Nitrosylation of proteins, concomitant with a paradoxical denitrosylation of *S*-nitrosylated OGT (78). Denitrosylation of OGT was associated with an increase in its catalytic activity, suggesting a potential mechanism for LPS-induced O-GlcNAcylation of p65 and subsequent production of pro-inflammatory cytokines (78). On the other hand, in N9 microglia cells, Zheng et al. observed that LPS induced a (modest) reduction in global O-GlcNAcylation of proteins, associated with a reduction in OGT protein level (79). Clearly, additional work will be needed in order to untangle the complex relationships between OGT and p65 and their potential regulation by LPS, glucosamine, and S-nitrosylation signaling pathways, and to firmly establish their relative role in pro- and anti-inflammatory functions in macrophages.

CONCLUSION

Whereas the implication of hyperglycemia in metabolic syndrome-associated inflammation is now well established, the involvement of O-GlcNAcylation appears complex, with both pro- and anti-inflammatory effects associated with this modification, depending on the type and duration (acute versus chronic) of the insult (80). In agreement with a dual effect of *O*-GlcNAc on inflammation,

O-GlcNAcylation of NFκB, through an array of different mechanisms, can have both positive and negative effects on its activity depending on pathophysiological models and cell types (31, 41, 42, 47, 48, 51, 52, 81).

Recent data suggested that O-GlcNAcylation in the immune system may participate in the pathogenesis of both type-1 and type-2 diabetes (48, 49). Interestingly, O-GlcNAcylation was discovered 30 years ago in immune cells (82), and dynamic changes in *O*-GlcNAc levels upon lymphocyte activation were detected as early as the beginning of the nineties (83). However, only a limited amount of studies have investigated the function and regulation of this modification in immune cells, and very few works concern macrophages biology. This is indeed an emerging field, with many deficiencies in the existing knowledge. Several important points should be addressed in the future. Thus, the role of OGT and O-GlcNAcylation on macrophage functions (phagocytosis, ROS production in the phagosome, cytokine expression and secretion, M1 versus M2 polarization, etc.) should be thoroughly investigated. Ideally, these studies should be performed using primary cultured macrophages rather than in cell lines. In addition, the consequences of *in vivo* chronic hyperglycemia on protein O-GlcNAcylation in macrophages should also be evaluated. In this context, the development of macrophages specific OGT or OGA knock-out mice should provide important clues on the role of this modification in hyperglycemia-induced inflammation. Therefore, a large continent in the *O*-GlcNAc world remains to be explored.

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