The Impact of the Unfolded Protein Response and the Hexosamine Biosynthetic Pathway on Glycosylation

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ABSTRACT: Glycan changes, in addition to being traditional mediators of protein quality control, cell signaling, and metabolism, are intimately linked to the progression of cancer and can potentially act as novel therapeutic and diagnostic targets. Despite advances in our understanding that glycosylation plays a key role in protein folding, maturation, and function, there is only limited understanding of the interconnected nature of glycan alterations to endoplasmic reticulum stress, the unfolded protein response, and the development of cancer. This review aims to highlight the interdependence of these pathways and the way they feed into each other to maintain protein quality control, regulate key survival mechanisms, and promote cell viability.

KEYWORDS: glycosylation, ER stress, unfolded protein response, cancer, GlcNAc

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Introduction

Tumor cells display altered protein glycosylation patterns, and these changes increase the molecular heterogeneity and functional diversity of the proteins displayed on the cell surface, altering cell-cell and cell-matrix interactions and modulating growth factor signaling. Within the cell, glycosylation of proteins and lipid molecules regulates signal transduction, gene expression, and metabolism in a nutrient-sensitive manner, thereby acting as key modulators of crucial biological functions.[1,2]

One universal characteristic of cancer cells is to increase anaerobic glycolysis under normal oxygen conditions (Warburg effect) producing lactate as a by-product.[3] This altered metabolic state allows cells to take up large quantities of glucose and glutamine to meet the increasing demand for production of essential macromolecules (lipids, amino acids, and nucleotides) that, in addition to sustaining growth and proliferation,[3] feed into biosynthetic pathways that control cellular glycosylation, which affects nutrient utilization by regulating intracellular and extracellular signal transduction.

The increased metabolic capacity accompanied by high nutrient utilization in the cell, sustained by continual growth factor activity, with time, exceeds the capacity of the vasculature supporting the tumor, causing low oxygen tension. This in turn limits nutrient supply and triggers hypoxic stress that leads to the production of reactive oxygen species causing inflammation. Low intracellular cellular adenosine triphosphate (ATP) levels caused by nutrient deprivation can affect correct protein folding and potentially lead to endoplasmic reticulum (ER) stress. During their lifetime, cancer cells inevitably adapt to different types of cellular stressors, caused by both limited and excess nutrients by utilizing and activating nutrient sensing mechanisms that exist in the cell to promote their fitness and survival.

In this review, we will discuss 2 essential pathways that regulate cell fitness: the ER stress response also called the unfolded protein response (UPR) and the hexosamine biosynthetic pathway (HBP) that controls *N*-linked glycosylation and *O*-GlcNAcylation. We will highlight the intertwined nature between the 2 pathways and how they orchestrate together to promote cancer cell survival.

ER Stress and UPR in Cell Fate Decisions

The hallmark of this response is the upregulation of chaperones that bind to the unfolded proteins to prevent their aggregation. A by-product of this process is the transient inhibition of protein synthesis,[4] and this inhibition also conserves energy. Persistent unresolved ER stress leads to activation of a signaling cascade that upregulates intracellular reactive oxygen species that deregulates mitochondrial bioenergetics, triggering the apoptotic cascade leading to cell death.[5] However, during mild ER stress, cells do not undergo apoptosis and they can continue to proliferate.[6]

Three ER-localized proteins (protein kinase RNA-like endoplasmic reticulum kinase [PERK], inositol-requiring enzyme 1 [IRE1], and activating transcription factor 6 [ATF6]) monitor the UPR in the ER lumen and, on activation, regulate downstream responses to this stress. Binding immunoglobulin protein (BiP, also called GRP78) is bound to PERK, IRE1, and ATF6 and in quiescent cells inactivates these pathways. However, when ER stress is sensed, BiP is

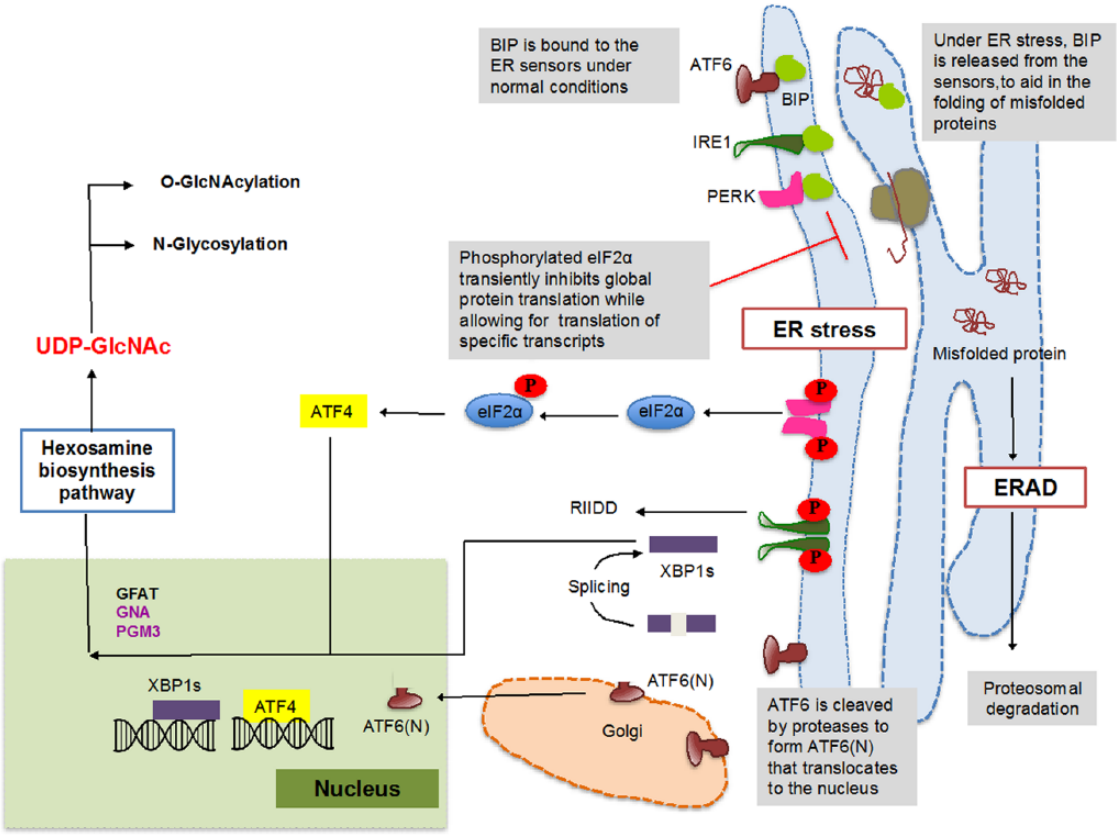

Figure 1. Endoplasmic reticulum stress and the hexosamine biosynthetic pathway (HBP) are integrally regulated. Under normal conditions, BiP is bound to the ER sensors ATF6, IRE1, and PERK. On sensing ER stress, BiP senses the hydrophobic domains in the misfolded proteins and binds to it to facilitate folding. Simultaneously, when BiP is released, the ER sensors IRE1 and PERK undergo homodimerization and autophosphorylation, whereas ATF6 transits to the Golgi. Activated IRE1 exposes its endonuclease domain and cleaves XBP1 mRNA from its unspliced form (XBP1u) to its spliced form XBP1s. XBP1s is an active transcription factor that enters the nucleus and activates the transcription of GFAT1, GNA, and phosphoglucomutase 3 that are involved in the synthesis of UDP-GlcNAc. IRE1 activation also triggers the degradation of mRNAs via regulated IRE1-dependent decay (RIDD) to minimize the substrate for protein translation, thus decreasing ER load. PERK inhibits eiF2α by phosphorylation, attenuating translation, but allows for preferential translation of ATF4. ATF4 then triggers GFAT transcription allowing for increased flux via HBP and increased UDP-GlcNAc synthesis. ATF6 transits to the Golgi, and serine proteases activate ATF6(N) that then mediates increased transcription to restore ER balance. It can increase the abundance of XBP1s, but its direct role in activating genes involved in HBP still remains unknown. ATF4 indicates activating transcription factor 4; ATF6, activating transcription factor 6; BiP, binding immunoglobulin protein; eiF2α, eukaryotic translation initiation factor 2A; ER, endoplasmic reticulum; GFAT1, glucosamine-fructose-6-phosphate aminotransferase; GNA, glucosamine 6 phosphate *N*-acetyl transferase; IRE1, inositol-requiring protein; mRNA, messenger RNA; PERK, protein kinase RNA-like endoplasmic reticulum kinase; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; XBP1, X-box binding protein 1.

released and binds to the unfolded proteins, freeing PERK, IRE1, and ATF6 to regulate ER responses to the stress in an attempt to return it to a normal state. The ER is involved in proper protein folding, and PERK, IRE1, and ATF6 activities are involved in this process and they become activated when ER stress is sensed. Inhibition of *N*-linked glycosylation with tunicamycin induces ER stress, also leading to the activation of PERK, IRE1, and ATF6, whereas, in turn, once these proteins are activated, they are able to regulate glycosylation in an attempt to normalize this process, as discussed below and summarized in detail in Figure 1.

PERK pathway

Protein kinase RNA-like endoplasmic reticulum kinase is a transmembrane protein in the ER and is one of the major transducers of ER stress. It dimerizes and undergoes autophosphorylation upon BiP dissociation and causes the phosphorylation of eukaryotic translation initiation factor 2A (eiF2α) at Ser-51 leading to general translation attenuation, and as a result, induces cell cycle arrest.[7,8] Protein kinase RNA-like endoplasmic reticulum kinase also mediates preferential translation of specific UPR transcripts, such as activating transcription factor 4 (ATF4), and this promotes the transcription of several genes involved in redox response, amino acid synthesis, and lipid biogenesis in an attempt to promote cell survival. Activating transcription factor 4 then activates growth arrest and DNA damage–inducible protein 34, and this dephosphorylates eIF2α which then enables global translation recovery.[9]

Activating transcription factor 4 also activates the transcription of CCAAT-enhancer-binding protein (C/EBP)

homologous protein (CHOP), and this protein is strongly implicated in ER stress–mediated apoptosis.[10] CCAAT-enhancer-binding protein homologous protein activates ER oxidoreductin 1α which promotes oxidative protein folding and ER calcium release via the inositol 1,4,5-trisphosphate receptor that leads to Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) activation of apoptosis.[11] CCAAT-enhancer-binding protein homologous protein can inhibit the expression of antiapoptotic Bcl-2 family proteins and can activate proapoptotic BH3 family proteins: Bcl2-like protein 11, p53 upregulator modulator of apoptosis, and Bcl2-associated X protein.[12] In hypoxic tumors, CHOP activation of autophagy can serve to be a protective tolerance-mediating mechanism, promoting survival.[13]

Protein kinase RNA-like endoplasmic reticulum kinase is able to activate Nrf2 phosphorylation in an attempt to maintain redox balance within the cell.[14] Nrf2 is a transcription factor involved in activating the transcription of antioxidant genes involved in facilitating resistance to reactive oxygen species–mediated cell damage. In addition to PERK-mediated effects on eIF2α, non-PERK kinases can respond to a wide variety of triggers, which include heme and amino acid deprivation and the detection of double-stranded RNA. These kinases along with PERK converge to phosphorylate eIF2α leading to a response called the integrated stress response.[15]

It has been suggested that *N*-glycosylation is regulated during the ER stress response, and it has been found that PERK plays an important role in this process. In addition to transiently inhibiting protein translation, PERK reduces lipid-linked oligosaccharide consumption in the cell, lowering the levels of glycoprotein polypeptide and restoring correct *N*-linked glycosylation.[16]

IRE1-XBP1 pathway

Similar to PERK, IRE1 is also a transmembrane protein located in the ER that undergoes dimerization and requires autophosphorylation to be activated.[17] On activation, IRE1 exposes an RNAse domain that interacts with messenger RNAs (mRNAs) and facilitates their decay. This process is commonly referred as regulated IRE1-dependent decay (RIDD). Induction of RIDD by IRE1 aids in the reduction of ER protein load because nascent mRNAs waiting to be translated are degraded, thus limiting the increase in protein demand.[18] In addition, IRE1 regulates the unconventional splicing of X-box binding protein 1 (XBP1) mRNA. Unspliced XBP1 (XBP1u) contains a nuclear exclusion sequence that is cleaved out of the XBP1 transcript by the release of IRE1 from the ER membrane, forming the spliced version of the protein XBP1s. X-box binding protein 1, being an highly active transcription factor, enters the nucleus and upregulates transcription of several genes involved in the maintenance and expansion of ER function, facilitating the UPR to restore ER homeostasis.[19,20] X-box binding protein 1 regulates differentiation of B cells to highly secretory plasma cells. Overexpression of XBP1 induced activation of genes involved in the secretory pathway and expanded the ER and lysosome content.[21] Indeed, XBP1-based engineering increases the secretory capacity of proteins independent of the protein transcript levels, proving to be a useful tool in biopharmaceutical manufacturing.[22]

In addition to playing a role in the secretory pathway, analysis of XBP1 targets identified 19 genes that were involved in glycosylation and carbohydrate metabolism.[23] In light of this, several recent findings highlight a key role for XBP1 in regulating glycosylation; XBP1 induces the transcription of genes that aid in restoring *N*-glycosylation in the ER[24,25] and *O*-GlcNAcylation in the Golgi[26]; and XBP1-deficient cells demonstrate normal protein folding but have altered protein glycosylation.[27] Supporting and adding to these observations, Dewal et al[28] described a crucial role XBP1 plays in the maturation of *N*-glycans, highlighting an important link between intracellular stress response and *N*-glycan architecture. Elegant studies reported by Termine et al[29] demonstrated that UPR upregulates glycoprotein ER-associated degradation via the downregulation of ER mannosidase I, further supporting the interplay between ER stress and glycosylation.

ATF6 pathway

ATF6, an ER transmembrane protein, when free of BiP, translocates to the Golgi where it is cleaved by serine protease site-1 and metalloprotease site-2 to produce a 50-kDa N-terminal fragment ATF6(N). This fragment is phosphorylated and translocates to the nucleus,[30] where it acts as a transcription factor and upregulates various UPR genes involved in ER-associated degradation and chaperones that enhance protein folding. ATF6 crucially upregulates the expression of XBP1,[31] BiP,[32,33] GRP94,[33] and CHOP.[34] Full-length ATF6 is glycosylated; however, under ER stress, it is underglycosylated causing it to traffic to the Golgi at a faster rate. This suggests that the glycosylation of ATF6 can act as a sensor of ER homeostasis.[35] Therefore, glycosylation can modulate ATF6 function, but whether ATF6 plays a direct role in modulating glycosylation is not yet known.

Hexosamine Biosynthetic Pathway

Hexosamine biosynthetic pathway is a branch of the glucose metabolic pathway that uses 2% to 5% of the total glucose entering the cell to produce the high-energy substrate donor uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) for *N*-linked and *O*-GlcNAcylation reactions. The first step of this pathway is initiated by glucosamine-fructose-6-phosphate aminotransferase (GFAT1), which is rate limiting and catalyzes the conversion of fructose-6-phosphate and glutamine to glucosamine-6-phosphate. Additional steps in this pathway incorporate products from lipid and nucleotide metabolism (acetyl CoA and UTP) to yield UDP-GlcNAc.[36] This pathway is "nutrient responsive" because formation of the

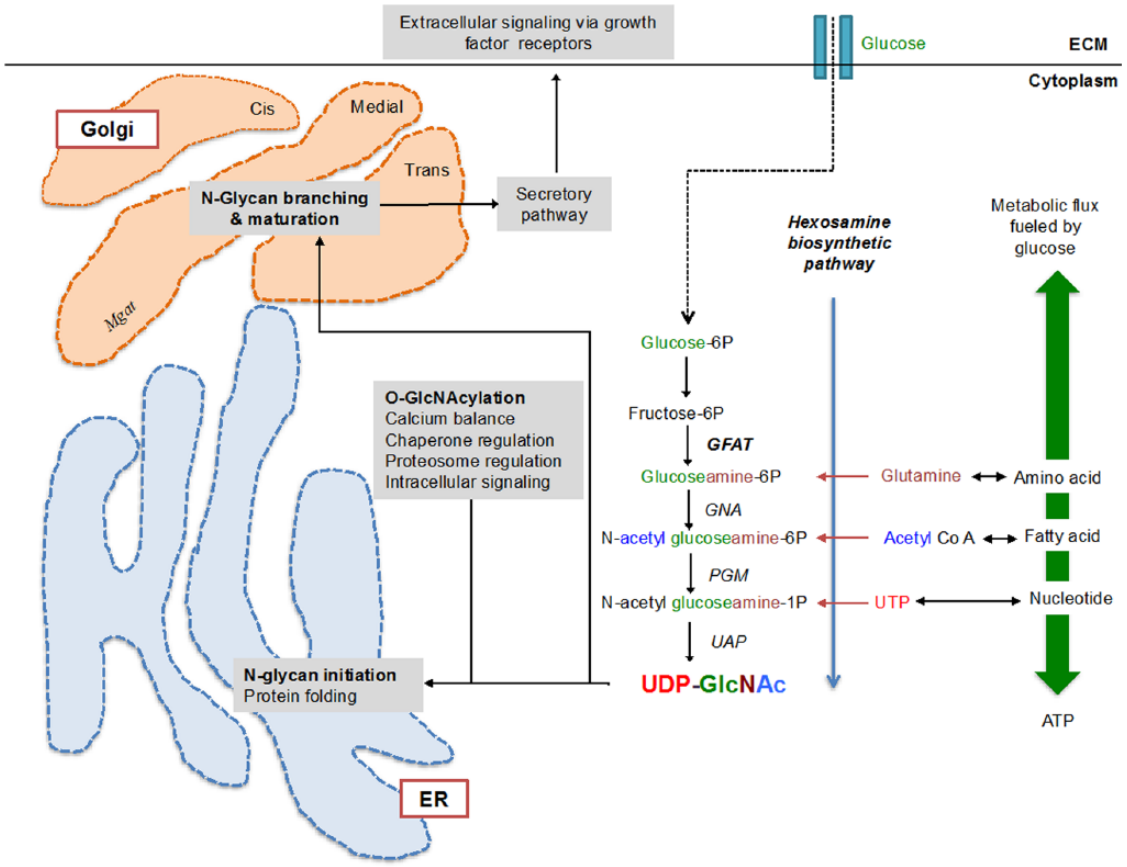

Figure 2. Hexosamine biosynthetic pathway (HBP). HBP integrates all aspects of energy metabolism (amino acid, fatty acid, and nucleotide) to form UDP-GlcNAc. Glucose enters the cell via GLUT transporters, glucose is converted into glucose-6-phosphate (G6P) by hexokinase, and then G6P is isomerized to fructose-6-phosphate (F6P) by glucose-6-phosphate isomerase. Until this step occurs, it is still glycolysis. HBP diverges from glycolysis when the rate-limiting enzyme GFAT (glutamine-6-fructophosphate aminotransferase) converts F6P to glucosamine-6-phosphate. Glucosamine-6-phosphate undergoes acetylation by glucosamine 6 phosphate *N*-acetyltransferase (GNA) to form *N*-acetyl glucoseamine-6-phosphate (GlcNAc-6-P) which is then isomerized by phosphoacetyl-glucosamine mutase (PGM) to *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) and uridinylated by UDP-GlcNAc phosphorylase (UAP, UDP-*N*-acetylglucosamine pyrophosphorylase) to create UDP-GlcNAc. UDP-GlcNAc is essential for the first 2 steps of *N*-glycan initiation in the ER, *N*-glycan branching in Golgi, and cytoplasmic and nuclear protein *O*-GlcNacylation. *N*-glycosylation is involved in protein folding in the ER, and *N*-glycan branching and maturation of growth factor receptors can mediate extracellular signaling. *O*-GlcNAcylation controls intracellular signaling, regulates calcium balance in the cell, and plays an important role in protein folding by regulating chaperones and can also regulate proteosomal degradation and aid in clearing unwanted proteins during an ER stress response. ATP indicates adenosine triphosphate; ECM, extracellular matrix; ER, endoplasmic reticulum; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; UTP, uridine-5′-triphosphate.

UDP-GlcNAc product requires the integration of products derived from glucose, amino acid, lipid, and nucleotide metabolism and hence is sensitive to the intracellular concentrations of each of these individual components. Uridine diphosphate *N*-acetylglucosamine is involved in the initiation of *N*-glycosylation in the ER, *N*-glycan branching and processing occur in the Golgi, and *O*-GlcNAcylation modification of proteins occurs in the cytosol (Figure 2).

N-glycosylation

N-linked glycosylation plays a major role in protein folding and quality control by contributing to the solubility, structure, and stability of the protein. Most secreted proteins, cell surface growth factor receptors, and nutrient transporters are *N*-glycosylated. Initiation of *N*-glycosylation takes place on the ER membrane with the transfer of GlcNac-P from UDP-GlcNAc to dolichol phosphate forming GlcNAc-P-P-dolichol under the action of the enzyme GlcNac-1-phosphotransferase, following which a second GlcNAc and 5 mannose residues are sequentially transferred to generate Man5-GlcNAc$_2$-P-P-dolichol, which is flipped from the cytosol into the ER lumen, where addition of 4 mannose and 3 glucose residues in GlcNAcylation yields the precursor Glc$_3$Man$_9$(GlcNAc)$_2$. Glc$_3$Man$_9$(GlcNAc)$_2$ is then transferred en bloc onto the Asn-X-Ser consensus sequence found on proteins by oligosaccharide transferases. Furthermore, glucosidases in the ER trim off 2 glucose residues to generate GlcMan9-GlcNAc$_2$-Asn-R structure that acts as a ligand for lectin chaperones, calnexin, and calreticulin to aid in the correct folding of the protein. Subsequently, misfolded proteins are identified by the enzyme UDP-glucose glycoprotein transferase 1, which uses UDP-glucose (transported from the cytosol to the ER lumen) as the glucose donor to

reglucosylate and reintegrate the misfolded protein back to the calnexin/calreticulin cycle in an attempt to rescue and refold the protein. If multiple refolding attempts do not result in a correctly folded protein, the ER-associated degradation machinery retrotranslocates the protein back into the cytosol, where it is processed for proteosomal degradation.[37,38] Correctly folded proteins then transit via the *cis*-, medial-, and *trans*-Golgi compartments for additional processing and sequential modifications to the glycan chain. Mannose trimming occurs in the *cis*-Golgi compartment after which the glycoconjugate is branched by *N*-acetyl glucoseaminotransferases (GnTs) in the medial Golgi.

Fang et al[39] identified ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5), an ER UDPase that hydrolyzes UDP to uridine monophosphate (UMP), as a crucial regulatory enzyme in phosphatase and tensin homologue (PTEN) null-PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)/AKT (alpha ser/thr protein kinase)–activated cells where increased cellular translation demands higher ER protein–folding capacity. Increased transcription and expression of ENTPD5 in PTEN null/AKT-activated cells promote UDP-UMP hydrolysis relieving ER protein–folding pressure by decreasing end product inhibition by UDP-glucose glycoprotein glucosyltransferase-1. Uridine monophosphate (UMP) exits the ER via an antiporter system in exchange for another molecule of UDP-glucose from the cytosol, thus promoting *N*-glycosylation and folding. Adenosine triphosphate hydrolysis is required for importing UDP-glucose from the cytosol, and protein folding consumes a large amount of ATP, causing a decrease in the cellular ATP/adenosine monophosphate (AMP) ratio. ENTPD5 participates in an ATP hydrolysis cycle along with cytidine monophosphate kinase 1 and adenylate kinase 1 and increases glucose utilization by enhancing glycolytic rate and promoting ATP consumption. Knockdown of ENTPD5 caused ER stress and decreased cell surface receptor tyrosine kinase (epidermal growth factor receptor, insulin-like growth factor 1, and HER2) expression, indicating that ENTPD5 controls the folding of growth factor receptors and contributes to cancer growth by stabilizing the growth factor receptor expression at the cell surface, supporting a feed-forward mechanism of signaling via the PI3K/AKT axis.

Sensitivity of GnTs to UDP-GlcNAc concentration in the Golgi is variable, with K_m values ranging from lowest to highest (mannosyl-glycoprotein *N*-acetylglucosaminyltransferases [MGAT] 1, 2, 4, 5). The K_m values for UDP-GlcNAc are highly variable among GnTs within the Golgi. For example, GnT-IV and GnT-V are encoded by genes Mgat4 and Mgat5, respectively, and are responsible for the addition of *N*-glycan branches. These proteins have higher affinities for UDP-GlcNAc compared with others and are sensitive to the nutrient flux limitation via HBP.[40,41] GnT-V may also play a role in the regulation of glucose uptake by modifying glycosylation of glucose transporter-1. Decrease in GnT-V expression suppresses intracellular glucose transport, and this contributes to ER stress.[40] Lau et al[41] demonstrated that the degree of branching and the number of *N*-glycans on glycoproteins regulate their cell surface retention and signaling in response to UDP-GlcNAc concentrations, depending on their extent of binding to the galectin (galactose-binding protein) lattice. *Mgat5* is responsible for adding a β-1,6 GlcNAc branch that can be further extended by *N*-acetyllactosamine, and this acts as a high-affinity ligand for galectin-3 to create an extracellular carbohydrate lattice that limits the recycling of the cell surface receptor. As a result, this increases the time the receptor is maintained on the cell surface.[42,43] Oncogenic activation of *Mgat5* and increased levels of tetra-antennary *N*-glycan have been reported in many cancers,[44-48] and this was found to correlate with increased tumor growth, matrix remodeling, and metastases,[49] whereas *Mgat5* knockout mice exhibited delayed tumor growth and decreased growth factor–mediated signaling.[50] GnT-III (coded by *Mgat3*) catalyzes the transfer of a β-1,4 GlcNAc residue to the β mannose of the core glycan to create a bisecting GlcNAc structure. The addition of this branch prevents all further branching and elongation of the glycan structures. Overexpression of GnT-III retards tumor growth and metastases[51] and antagonizes the activity of *Mgat5*.[52] In addition, it is thought that GnT-III is capable of regulating intracellular signaling, but these observations require further investigation.[53,54]

O-GlcNAcylation

UDP-GlcNAc is also a substrate donor for the enzyme *O*-GlcNAc transferase (OGT) that mediates *O*-GlcNAcylation of cytosolic and nuclear proteins. *O*-GlcNAc transferase catalyzes the addition of a GlcNAc residue onto the free hydroxyl group of Ser/Thr residues on target proteins, whereas *O*-GlcNAcase (OGA) catalyzes the hydrolysis of the glycosidic bond and removes the GlcNAc. The enzymatic activity and the substrate specificity of OGT are tightly correlated with the concentration of UDP-GlcNAc in the cell,[55] making *O*-GlcNAcylation a nutrient-sensitive process. The dynamic exchange of *O*-GlcNAc on target proteins regulates the function of multitude of proteins that are involved in cell signaling, including transcription factors, signaling effector molecules, metabolic enzymes, cytoskeletal proteins, ribosomal proteins, and kinases either directly or by interplaying with other post-translational modifications such as phosphorylation[56] and ubiquitination.[57]

O-GlcNacylation modulates chaperone activity. Prompt, global, and dynamic increases in *O*-GlcNAc modifications are observed on nucleocytoplasmic proteins on exposure to different forms of cellular stress (oxidative, thermal, chemical, and biological), and the increase was observed to be protective to the cell contributing to stress tolerance.[58] It has been observed that *O*-GlcNAcylation increases the stability of heat shock

family proteins HSP70 and HSP40. *O*-GlcNAc transferase knockdown reduced HSP70 and HSP40 levels.[58,59] Inhibiting GFAT1 also reduces HSP70 protein levels. Binding immunoglobulin protein belongs to HSP70 family of heat shock proteins and is a crucial factor in regulating UPR (discussed above). HSP70 was also shown to have a lectin-like affinity to *O*-GlcNAc.[60] *O*-GlcNAc may also transcriptionally alter HSP synthesis increasing its abundance.[58] In addition, HSPs such as HSP90, HSP27, and heat shock cognate 70 kDa protein are also regulated by *O*-GlcNAcylation.[61] In addition to modulating chaperone affinity and increasing chaperone abundance, it is interesting to speculate whether a higher preference for *O*-GlcNAcylated proteins to be specifically folded during stress response contributes to stress tolerance.

O-GlcNAcylation modulates intracellular calcium handling. Stress-induced increase in *O*-GlcNAcylation is also dependent on Ca^{2+} levels. Lowering extracellular Ca^{2+} with EGTA, blocking store-operated Ca^{2+} entry with SKF96365 inhibitor, or inhibiting calmodulin-dependent protein kinase II (CaMKII) decreases *O*-GlcNAcylation.[62] Cytosolic Ca^{2+} overload is also known to facilitate ischemia/reperfusion injury, and increased *O*-GlcNAc levels are shown to be cardioprotective in these models.[63] Recovery associated with increased *O*-GlcNAc levels correlates with reduction in Ca^{2+}-induced stress responses, such as calpain-mediated proteolysis of α-fodrin and CaMKII.[64] In addition to being important in stress-induced recovery, *O*-GlcNAcylation may play an important role in maintaining calcium homeostasis. The ER is the principal organelle involved in mediating calcium homeostasis, and chaperone-mediated protein folding and *N*-glycosylation are dependent on ER calcium levels. Cytoplasmic calcium regulation depends on the activity of calcium transporters in the ER membrane. Inositol 1,4,5-trisphosphate receptor and the ryanodine receptor export calcium from the ER into the cytoplasm in response to agonists or intralumen Ca^{2+} levels, whereas the ATPase sarcoplasmic reticulum/ER Ca^{2+} transporting 2 pumps calcium back into the ER lumen.[65] Increasing *O*-GlcNAc levels, either by increasing glucose flux via HBP or by inhibiting OGA activity, regulates intracellular Ca^{2+} homeostasis by inhibiting the inositol 1,4,5-trisphosphate agonist, blocking the release of Ca^{2+} from the ER, and leading to decreased cytosolic Ca^{2+} levels.[66] Also, the inositol 1,4,5-trisphosphate receptor in itself is modified by *O*-GlcNAc, and this modification regulates channel activity[67] in an isoform-specific manner.[68] *O*-GlcNAc transferase overexpression/glucose-induced increase in *O*-GlcNAc is associated with prolonged Ca^{2+} transients and reduced sarcoplasmic reticulum/ER Ca^{2+}-ATPase protein expression in cardiomyocytes. When intracellular calcium stores are depleted, nonvoltage store-operated Ca^{2+} channels in the plasma membrane facilitate Ca^{2+} entry into the cell. This mechanism, termed as capacitative calcium entry, refills intracellular calcium levels and activates a wide variety of calcium-mediated signaling pathways in addition to rapidly replenishing ER calcium stores by interacting with STIM1 (stromal-interacting molecule 1) which is an ER calcium sensor.[69] Depletion of Ca^{2+} levels leads to a conformational change in STIM1, resulting in an STIM1 puncta formation and translocation to ER/plasma membrane junction, where it interacts with store-operated Ca^{2+} entry, initiating capacitative calcium entry. *O*-GlcNAcylation of STIM1 decreases the STIM1 puncta formation and leads to functional decrease in store-operated Ca^{2+} entry where it interacts with store-operated calcium channels, initiating capacitative calcium entry.[70] In addition, by decreasing Ca^{2+} overload in cells, *O*-GlcNAcylation decreases the formation of mitochondrial permeability transition pore and loss of mitochondrial membrane potential, protecting cells from oxidative stress.[71] *O*-GlcNAcylation regulates calcium handling in the cell on various levels and may prove to be important not only for recovery from ischemia reperfusion injury but also potentially for other conditions such as fibrosis, neurodegenerative disorders, and cancer where calcium homeostasis is disturbed.

O-GlcNAcylation modulates proteosomal function. *O*-GlcNAcylation can act as a protective signal against proteosomal degradation by directly inhibiting the proteasome,[72] as it has been found that both the 20S catalytic core and the 19S regulatory subunit of the 26S proteasome are extensively *O*-GlcNAcylated.[73] Zhang et al[74] demonstrated that *O*-GlcNAcylation inhibits the activity of regulatory particle triphosphatase-2, an ATPase in the 19S regulatory subunit, and prevents the ATP-dependent hydrolysis of the transcription factor specificity protein-1 which has been implicated in oncogenesis. *O*-GlcNAcylation can also modify the ubiquitination status of the target substrate by regulating the activity of E1 and E3 ubiquitin ligases either by competing with phosphorylation or by providing a docking site for deubiquitinases that removes ubiquitin from the substrate to stabilize its intracellular levels.[75-77] Increasing cellular *O*-GlcNAcylation via glucosamine supplementation or OGA inhibition augmented ubiquitination, whereas decreasing *O*-GlcNAcylation by OGT knockdown, glucose deprivation, and forskolin treatment activated CAMP-dependent inhibition of GFAT1 and downregulated cellular UDP-GlcNAc levels,[78] indicating that *O*-GlcNAcylation regulates the ubiquitination process.

Glycosylation Regulates and Potentiates the ER Stress Response

It has been observed that the *O*-GlcNAc modification is intimately involved in ER stress responses. The *O*-GlcNAcylation of eIF2α at the Ser-219, Thr-239, and Thr-241 residues prevents the phosphorylation of eIF2α and thus alters the function of the protein.[79] Using the ER stressors tunicamycin and thapsigargin, *O*-GlcNAc modifications are enhanced under ER stress, but the mechanism of this enhancement is currently unclear. However, what is known is that UPR activates HBP, and this is via a mechanism that is dependent on XBP1-induced transcription of HBP-related genes (glucosamine 6 phosphate *N*-acetyl transferase, phosphoglucomutase 3, and UDP-galactose 4 epimerase).[26]

Hexosamine biosynthetic pathway flux to generate UDP-GlcNAc is required for sustaining growth factor receptor signaling and glutamine uptake on glucose depletion.[80] Glucose

starvation is known to activate ER stress and UPR.[81] Recently, 2 independent studies demonstrated a key link between HBP and ER stress, identifying that the rate-limiting enzyme of HBP, glutamine-6-fructoamidotransferase-1 (GFAT1) transcription, is regulated by the key ER stress transcription factor, XBP1, and that this directly mediates UPR to protect cells under stress.[26,82] Overexpression of the spliced form of XBP1 in the absence of ER stress promotes the activation of HBP underscoring the importance of XBP1 in this process.[83] In addition, *N*-glycosylation is important in ER homeostasis as it contributes to the proper folding of proteins, alleviating UPR.

Denzel et al[82] identified that increased GFAT1 activity via IRE1α- and XBP1-dependent pathways was protective against tunicamycin-induced ER stress and that *N*-glycosylation was one of the major protective factors in promoting longevity in *Caenorhabditis elegans*. Furthermore, the increased HBP activity led to an increased capacity of ER-associated degradation by the upregulation of the suppressor of lin-12-like protein-1—a component of the E3 ubiquitin ligase complex—that aids in the retrograde transport of unfolded proteins from ER to cytosol and facilitates their proteasome-mediated degradation. Therefore, increased HBP activity via GFAT1 is directly involved in improving proteostasis by increasing the ER-associated degradation capacity and proteasome-mediated degradation, improving the overall protein quality control in the ER.

In addition to the IRE1-XBP1 pathway being involved in UPR regulation, Chaveroux et al[84] demonstrated a new link between the PERK-eIF2α-ATF4 pathway of the UPR and HBP, that the PERK-eIF2α-ATF4 axis controls the expression of GFAT1 in a time-dependent manner and promotes *O*-GlcNAcylation by enhancing HBP flux in low glucose conditions. These findings highlight the integrated roles between HBP and the UPR and demonstrate that both *N*-linked glycosylation and *O*-GlcNAcylation are modulated by the IRE1α and the PERK axis.

The Reciprocal Roles Between *O*-GlcNAcylation and ER Dynamics

The ER is a highly dynamic structure, and microtubules, microfilaments, and intermediate filaments play a major role in the dynamic remodeling of the ER membrane and maintain it in a constant state of flux. *O*-GlcNAc modification is involved in regulating a variety of cytoskeletal proteins.[85] Microtubule-depolymerizing agents dramatically alter ER shape. Microtubule-associated proteins (MAPs)—MAP1, MAP2, and MAP4—that modulate microtubule dynamics are *O*-GlcNAcylated[86] and microtubule polymerization and elongation of ER are interdependent processes. Microtubule polymerization and elongation of ER are interdependent processes. STIM1 is involved in ER tubule elongation and is identified to be regulated by *O*-GlcNAcylation (discussed above). There is not much evidence to date to indicate that *O*-GlcNAcylation could possibly be involved in modulating ER dynamics, but considering the fact that it regulates Ca^{2+} signaling and modifies several cytoskeletal proteins, it likely contributes to the structural changes in ER and potentially regulates key factors involved in protein folding and the secretory pathway.

Abnormalities in HBP and ER Stress in Cancer

Endoplasmic reticulum stress is a common feature of malignant cells and acts as a critical check point in regulating cell signaling during nutrient excess or shortage.[87] Many tumor cells have posttranslational modifications such as glycosylation that are predominantly linked to serine and threonine residues on the proteins. For example, sphingolipids and many glycoproteins are aberrantly glycosylated, and these aberrations have been directly implicated in the malignancy.[88,89] It is believed that the abnormal glycosylation events are dependent on excessive stimulation of HBP.[90] In light of this, studies have shown that HBP and UPR are functionally linked, where HBP is activated by UPR and HBP provides protection during ER stress.[26,82] Data also strongly suggest that UPR sustains the continued growth of tumors.[91] In breast cancer, it has been shown that there is an augmented flux through HBP-hypoxia-inducible factor 1 via GFAT1. The subsequent inhibition of GFAT1 abrogated this axis and reduced the numbers of cancer stem cells. In breast cancer, it has been shown that there is an augmented flux through HBP-hypoxia-inducible factor 1 via GFAT1. The subsequent inhibition of GFAT1 abrogated this axis and reduced the numbers of cancer stem cells.[92] This suggests that targeting this pathway may be a viable therapeutic option for cancer treatments. However, loss of GFAT1 has been associated with epithelial-mesenchymal transition and a poorer prognosis in gastric cancer.[93] Additional evidence suggests that the role of HBP in cancer is complex. Kaushik et al[94] found that HBP enzyme glucosamine-phosphate *N*-acetyltransferase-1 is decreased in castration-resistant prostate cancer compared with localized prostate cancer and that this increased tumor aggressiveness. However, intriguingly, the addition of UDP-GlcNAc to the castration-resistant prostate cancer cells significantly decreased cell proliferation.[94]

X-box binding protein 1 has been shown to play a crucial role in regulating glycosylation as discussed above. It also transcriptionally upregulates UDP-galactose 4-epimerase to generate substrates for glycosylation, thus allowing the tumor cells to cope with an increase in protein folding.[95] In addition, the splice variant of XBP1 complexes with hypoxia-inducible factor 1-alpha inducing glucose transporter-1 and other glycolytic proteins.[96] X-box binding protein 1 directly controls the expression of the HBP[26] and downregulates the expression of forkhead box O1. The net effect of these changes is increased energy and glucose metabolism.[97]

Although mechanisms are still not clear as to how ER stress can modulate tumorigenesis, it has been reported that the CHOP-p21 axis may play a central role in connecting ER stress and a prosurvival phenotype in cancer,[98,99] as CHOP

Table 1. Fundamental role for ER stress or glycosylation in cancer biology.

TYPE OF CANCER	TYPE OF SAMPLE USED	OBSERVATION RELATING TO ER STRESS AND/OR GLYCOSYLATION	CITATION
Breast	Human breast cancer stem cells (CD44+/CD24– MCF7)	Tunicamycin-induced ER stress decreased invasion, migration, and proliferation of cancer stem cells	Nami et al[100]
	Breast cancer xenograft model (MCF7/HER2 xenografts)	Tunicamycin in combination therapy enhanced antitumor activity	Han et al[101]
	Human breast carcinoma cell lines (MCF-10A-ErbB2, MDA-MB-231, MCF-7)	Reducing *O*-GlcNAcylation induces ER stress–mediated cancer cell apoptosis via CHOP activation	Ferrer et al[102]
	Human breast carcinoma cell lines (MDA-MB-231, MDA-MB-435)	Tunicamycin treatment reduces angiogenesis and growth of triple negative breast cancer tumor, via UPR modulation	Banerjee et al[103]
Lung	Human lung cancer cell line (A549)	Tunicamycin improves anticancer drug efficacy via CHOP suppression of p21	Mihailidou et al[104]
	Non–small cell lung carcinoma cell line	Tunicamycin enhances susceptibility of lung cancer cells to EGFR inhibitor via CHOP activation	Ling et al[105]
	Non–small cell lung carcinoma cell line	Glucosamine treatment represses tumor growth in vivo via modulation of ER stress	Song et al[106]
	Rat lung fibroblast cell line (Rat-1)	Glucose shortage via limitation of hexosamine flux triggers UPR to activate CHOP expression. p58-IPK induced by IRE1 counteracts this to promote tumor progression	Huber et al[107]
Pancreatic	Human pancreatic ductal carcinoma cell lines (MiaPaCa2, Capan-1, Panc-1, HPAFII, PC-3)	Increased HBP flux and hyper-*O*-GlcNAcylation in pancreatic ductal carcinoma	Ma et al[108]
	Human pancreatic ductal carcinoma cell lines (L3.6pl, Panc-1)	Increased GRP78 expression leads to chemoresistance	Gifford et al[109]
	Human pancreatic ductal carcinoma cell lines (MiaPaCa2, Panc0403, SU8686, Panc0327, AsPc1, Panc1005)	Inhibition of IRE1-XBP1 pathway leads to apoptosis of pancreatic cancer cells	Chien et al[110]
Prostate	Human prostate tumors	Last enzyme of HBP1 pathway, UAP, is overexpressed in prostate tumors, and it is protective against tunicamycin-induced stress	Itkonen et al[111]
	Mouse xenograft model, human prostate cancer cell lines (PC-3, DU145, BPH-1, LNCaP)	TUSC3 loss alters the ER stress response and accelerates prostate cancer growth in vivo. TUSC3 is a protein involved in *N*-glycosylation, and inhibiting *N*-glycosylation by tunicamycin increases TUSC3	Horak et al[112]
	Human prostate tumors, human prostate cancer cell lines (LNCaP, PC-3)	Upregulation of GFAT and UAP1 stabilizes C-Myc expression	Itkonen et al[113]
	Human prostate cancer cell lines (LNCaP, C42)	AKT upregulates ER UDPase ENTPD5 that promotes *N*-glycosylation and alleviates ER stress	Shen et al,[114] Fang et al[39]
Ovarian	Human ovarian cancer cell lines (PA-1, MDAH2774, SKOV3)	Resveratrol suppresses HBP by modulating ENTPD5, induces GADD153 expression, and promotes apoptosis	Gwak et al[115]
	Human ovarian cancer cell lines (SKOV, H134, TR170)	TUSC3 prevents EMT by altering ER stress responses	Kratochvilova et al[116]
Liver	Human hepatoma cell line (HepG2)	Tunicamycin promotes efficacy of anticancer treatment via CHOP suppression of p21	Mihailidou et al[104]
	Human hepatocellular carcinoma cells (SMMC-7721)	Downregulation of MGAT5 induces ER stress by modulating Glut1 function	Li et al[117]
	Human hepatocellular carcinoma cells (SMMC-7721)	Increased susceptibility to apoptosis by all *trans*-retinoic acid is mediated by ER stress upon downregulation of *Mgat5*	Xu et al[118]

Table 1. (Continued)

TYPE OF CANCER	TYPE OF SAMPLE USED	OBSERVATION RELATING TO ER STRESS AND/OR GLYCOSYLATION	CITATION
	Hepatoma cell lines (Hep3B, HepG2)	Inhibiting *N*-glycosylation promotes GRP78 and GADD153 and suppresses proliferation via modulating cell cycle regulators	Hsu et al,[119] Chiang et al.[120]
	Hepatoma cell lines (Hep3B)	*O*-GlcNAc transferase activity is increased in response to thermal stress	Guo et al[121]
Osteosarcoma	Osteosarcoma cell line (Saos-2)	XBP1 activation affects *N*-glycan maturation	Dewal et al[28]
Brain	Mouse glioblastoma xenograft model, tumors of patients with human glioblastoma	ENTPD5 overexpression correlates with decreased survival. ENTPD5 modulates GRP78 expression and promotes autophagy	Zadran et al[122]

Abbreviations: AKT, alpha ser/thr protein kinase; CHOP, CCAAT-enhancer-binding protein homologous protein; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; GFAT, glucosamine-fructose-6-phosphate aminotransferase; HBP, hexosamine biosynthetic pathway; MGAT5, mannosyl-glycoprotein *N*-acetylglucosaminyl-transferase 5; UAP, UDP-*N*-acetyl glucosamine pyrophosphorylase; UPR, unfolded protein response; XBP1, X-box binding protein 1.

regulates p21 expression. It is thought that this may be predicated on the level of stress in the cell because CHOP-stimulated p21 expression is pronounced during low/moderate chronic stress. However, during acute stress, this process is blocked and apoptosis ensues. This suggests that in cancer the shift between UPR responses can go from being prosurvival to apoptotic and may be orchestrated by the cross talk in the UPR culminating in CHOP activation leading to p21 upregulation.[98,99]

Targeting ER Stress or Glycosylation as Cancer Therapies

Clearly, ER stress and HBP play a prominent role in tumorigenesis, such that they can drive and maintain the malignancy, and thus, targeting these pathways may prove beneficial in cancer treatments (Table 1). Thus, HBP activation is fundamental in UPR and that HBP can relieve the ER stress response in a feedback loop. In light of this, investigators have proposed to target these pathways as potential sites for therapeutic intervention.

The overexpression of several UPR proteins appears to be prognostic in tumors and was found to be indicative of patient survival.[123,124] Targeting BiP/GRP78 significantly inhibits the tumor burden while not affecting normal cells.[125] Other studies have shown that by specifically inducing ER stress, breast cancer becomes more sensitive to radiation therapy[126] and ovarian cancer is more sensitive to cisplatin.[127] Further studies have demonstrated that ATP kinase inhibitors are antiproliferative, and this was observed across different tumors.[128,129] Targeting GRP78 has shown to be of therapeutic value for the treatment of B-cell acute lymphoblastic leukemia.[130] Targeting PERK seems to be a promising approach for inhibiting tumor cell survival; however, some caution needs to be used because suppressing PERK has led to diabetes in an animal model which could be modestly rescued with interferon inhibition.[131] Recently, we identified an 18-mer endogenous peptide that binds to XBP1 and alters UPR. In a pilot study using the therapeutic homologue of this peptide, Naclynamide, we observed modest tumor regression in canines with various end-stage pathologies and no further treatment options available.[132] Although this study was performed in animals with end-stage disease, further studies will need to be performed to determine the efficacy of Naclynamide in cancers that have been diagnosed early.

Although targeting ER stress could be a possible avenue for cancer treatment, others have considered targeting glycosylation to inhibit tumorigenesis. But targeting this pathway is not without significant repercussions for noncancerous cells, as inhibiting glycosylation will have an effect on every cell and could lead to systemic toxicity. Intriguingly, although studies suggest changes in *N*-glycosylation of tumor cell proteins such as increased branching, it has also been noted that there is decreased fucosylation in some cancers. Currently, there are no inhibitors that target cancer-specific glycosylation patterns. However, there are a number of compounds that inhibit various stages of the glycolytic pathway, and these are currently in development as potential cancer therapeutics.[133,134] Some of these molecules have had a measure of success in preclinical development and some have had modest success in early clinical trials. For example, pyruvate analog 3-bromopyruvate has recently entered into clinical phase I testing. Furthermore, inhibition of GnT-V has shown therapeutic promise in reducing tumor burden in mouse models of breast and lung cancer by facilitating immune cell recruitment at the site of tumor, aiding in clearance. However, further studies evaluating its therapeutic potential as a tumor target are necessary.[135]

Concluding Remarks

Advances in the understanding of cancers have made great progress over the years, especially as they pertain to ER stress and the metabolic regulation of HBP. The discussion above provides support for the role of these crucial pathways in maintaining the tumor. Furthermore, the interaction between HBP and the ER stress response in cancer is integral and tightly intertwined such that targeting or exploiting the signaling of

one or other pathway may have a therapeutic benefit for the treatment of cancer.

Author Contributions

AAR, AM and CMA were all involved in the writing,editing and in the proofreading of this manuscript.

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