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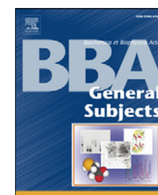

# Short O-GalNAc glycans: regulation and role in tumor development and clinical perspectives☆

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ABSTRACT

*Background:* While the underlying causes of cancer are genetic modifications, changes in cellular states mediate cancer development. Tumor cells display markedly changed glycosylation states, of which the O-GalNAc glycans called the Tn and TF antigens are particularly common. How these antigens get over-expressed is not clear. The expression levels of glycosylation enzymes fail to explain it.
*Scope of Review:* We describe the regulation of O-GalNAc glycosylation initiation and extension with emphasis on the initiating enzymes ppGalNAcTs (GALNTs), and introduce the GALA pathway — a change in GALNTs compartmentation within the secretory pathway that regulates Tn levels. We discuss the roles of O-GalNAc glycans and GALNTs in tumorigenic processes and finally consider diagnostic and therapeutic perspectives.
*Major conclusions:* Contrary to a common hypothesis, short O-glycans in tumors are not the result of an incomplete glycosylation process but rather reveal the activation of regulatory pathways. Surprisingly, high Tn levels reveal a major shift in the O-glycoproteome rather than a shortening of O-glycans. These changes are driven by membrane trafficking events.
*General Significance:* Many attempts to use O-glycans for biomarker, antibody and therapeutic vaccine development have been made, but suffer limitations including poor sensitivity and/or specificity that may in part derive from lack of a mechanistic understanding. Deciphering how short O-GalNAc glycans are regulated would open new perspectives to exploit this biology for therapeutic usage. This article is part of a Special Issue entitled "Glycans in personalised medicine" Guest Editor: Professor Gordan Lauc.
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## 1. Introduction

### 1.1. Glycosylation in health and cancer

Cancer remains one of the leading causes of mortality and a major loss of years of potential life, with an estimated 8.2 million deaths worldwide in 2012 [1]. Cancer continues to pose tremendous challenges for treatment and diagnosis. This is due to the complicated pathology of the disease that involves a whole panel of dysregulated cellular processes, which are interconnected and often vital for proper cell functioning. Identifying the key alterations specific to the neoplastic tissues and understanding the underlying mechanisms would allow precise targeting and detection of the disease. Current detection and treatment methods still suffer insufficient selectivity between tumor and normal tissues. This could be due in part to a primary focus on genetic aspects of the disease. In tumor formation, genetic changes in tumor cells and interaction with normal cells lead to emerging cellular states that often cannot easily be traced to specific changes in DNA. These cellular states ultimately define the behavior and evolution of a tumor and their understanding could be exploited for better specificities in diagnosis and treatment.

Among the processes defining cellular states, the most frequently occurring and also the most complicated post-translational modification (PTM) is glycosylation. Glycosylation is the enzymatic process that adds carbohydrate chains or glycans on protein and lipids. Glycosylation occurs in a complex and concerted series of steps taking place in the endoplasmic reticulum (ER) and more predominantly, in the Golgi apparatus [2,3]. Unlike other biopolymers, glycan synthesis is not template-driven, not directly encoded in the genome and as a consequence it is not well understood how this synthesis is controlled. Yet, the result is a vast diversity of glycan structures with many important functions.

*Abbreviations:* GalNAc, N-acetylgalactosamine; Tn antigen, T antigen nouvelle; S-Tn, sialylated Tn; TF antigen, Thomsen-Friedenreich antigen; S-TF, sialylated TF; ppGalNAcT (GALNT), polypeptide N-acetylgalactosaminyltransferase; GALA pathway, GALNT Activation pathway; C1GALT1, core 1 β3-galactosyltransferase; EMT, epithelial-mesenchymal transition; sLe$^x$, sialyl Lewis x.

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In mammals, glycan structures are assembled from ten monosaccharides: fucose (Fuc), galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), N-acetyl-galactosamine (GalNAc), glucuronic acid (GlcA), iduronic acid (IdoA), sialic acid (Sia), mannose (Man) and xylose (Xyl) [4–6]. Combinations of these monosaccharides coupled with differences in linkages (1–3 versus 1–4, etc.), anomeric states (α versus β), branching, length and substituted components (phosphate, sulfate, etc.) creates further diversity.

Glycan addition to substrate proteins and lipids also further generates different subsets of glycoconjugates. In proteins, glycans can be attached to at least nine out of 20 types of amino acids and the two prevailing processes involve amide linkages to asparagine residues (N-glycosylation) and glycosidic linkages to serine and threonine (Ser/Thr) side chains (O-glycosylation) [7,8]. Even within O-glycosylation, there are subtypes. Several sugars can be added to the Ser/Thr to yield different classes of O-linked glycans, such as α-linked O-GalNAc, α- or β-linked O-galactose, α- or β-linked O-glucose, α-linked O-fucose, α-linked O-mannose, β-linked O-GlcNAc and β-linked O-xylose. O-GalNAc glycans represent the most abundant O-glycans type. In this review, we will sometimes refer to O-GalNAc glycosylation as the process of sugar addition and O-GalNAc glycans as the various products of this process. We will sometimes use simply O-glycosylation or O-glycans when there is no risk of confusion.

Hence, glycosylation exponentially expands and diversifies the encoded genomic information [9,10], increasing the biochemical complexity of eukaryotes [11]. However, it should not be perceived that glycosylation occurs randomly with huge variations even within a specific protein. Normally, only specific sites of a given protein are glycosylated, and with a limited number of glycan structures at each site (microheterogeneity) [12]. This suggests precise regulatory mechanisms in place to control glycan biosynthesis, particularly within the Golgi, where most of the glycosylation machinery resides [13].

Glycans contribute a wide range of biological functions in the organism, particularly as part of glycoproteins. This is emphasized by the fact that some glycan functions are well conserved in the evolution of multicellular vertebrates [14]. Glycosylation by GlcNAc can occur in the cytosol. By contrast, most glycosylation pathways occur within the secretory pathway and generate the complex glycans.

In the early secretory pathway, glycans are essential for protein stability and secretion by regulating the folding of newly synthesized proteins, for quality control in the ER and for protein targeting in the secretory pathway [15,16]. At the cell surface, glycans contribute to multiple processes such as cell-cell communication, cell adhesion and migration, signal transduction, immune surveillance and host-pathogen interactions [14,17]. In fact, nearly all cell surface and secreted proteins are glycosylated [5,18–20].

Recent studies have found that O-GalNAc addition occurs on at least one Ser/Thr residue in more than 85% of secreted proteins [19]. Cell surface glycans can modulate their carrier protein conformation as well as provide ligands for glycan-binding proteins such as selectins, galectins and siglecs. Glycans are often required for physiological processes such as cell-matrix adhesion and cell–cell interactions [21–24]. Altogether, this stresses the importance of glycosylation for coordinating multi-cellular life. Interestingly, many glycoproteins have been implicated in tumor pathology [2,25,26]. It therefore makes sense that glycosylation would be significantly perturbed in cancer.

Altered glycosylation was first described more than 60 years ago and has since been recognized as a hallmark in oncogenic transformation [2]. Various glycan changes including under- or over-expression of specific glycan structures, expression of unprecedented or incomplete/truncated glycan structures or increased levels of precursor structures have been observed on tumor cells compared to their normal counterparts [27]. It is apparent that different glycan structures affect the cellular processes, as well as the tumor microenvironment, that play a pivotal role in cancer progression, angiogenesis, metastasis, cell-cell contact and epithelial-mesenchymal transition (EMT) in cancer cells. A few glycans are markedly associated with malignant transformation and progression. Given that a key aspect in tumor progression is clonal selection of the fittest cells from a genetically heterogeneous population, it suggests that these cancer-specific glycans are selected for and are likely to promote tumor cell survival [27].

### *1.2. Cancer-specific O-GalNAc glycans: the Thomsen-Friedenreich related antigens*

Among the cancer-specific glycans are the Thomsen-Friedenreich (TF)-related antigens, which comprise several short O-GalNAc glycans: T antigen nouvelle (Tn antigen), TF (aka T antigen or core 1) and their downstream sialylated counterparts (S-Tn and S-TF). The Tn antigen consists of a monosaccharide GalNAc alpha-O-linked to Ser/Thr in the polypeptide chain (GalNAc-α1-Ser/Thr) and the TF antigen is formed from the subsequent addition of Gal to GalNAc (Gal-β1–3GalNAc-α1-Ser/Thr). Sialylation of Tn and TF antigens involves the addition of terminal sialic acid to carbon 6 on GalNAc (S-Tn) and to carbon 3 on galactose (S-TF) respectively which prevents further elongation of the structure (Fig. 1). Because they tend to be observed also during development, they were initially named oncofetal antigens [28].

The initial discovery of these antigens came from the observation of occasional agglutination of stored blood cells in 1930. The agglutination was due to contaminating bacterial neuraminidases that exposed the TF antigen on blood cells and the TF antigen was recognized by anti-TF antibodies in the sera, contributing to hemagglutination [29]. Tn was subsequently discovered in 1957 to be expressed in the subpopulations of blood cells of lineages in patients with Tn syndrome, a rare hematological disorder [30]. Tn on the cell surface leads to increased polyagglutinability of erythrocytes and consequent hemolytic anemia, possibly due to the anti-Tn IgM [31].

The link between Tn and cancer was first observed in 1969 based on the binding of tumor cells to the snail *Helix pomatia* lectin (HPL) [32], and subsequent work by Springer and colleagues showing that Tn was highly expressed in around 90% of breast tumors [33]. Subsequent studies in the 1970–1980s established Tn to be a pan-carcinoma antigen as it is frequently expressed in cancers. Tn is expressed in 70–90% of most human solid tumor tissues, such as breast, colon, lung, bladder, cervix, ovary, stomach, and prostate while there is very low expression in the corresponding normal tissues [34–36]. Only the embryonic brain has been reported to express high levels of Tn [37]. Tn appears to be expressed mainly in epithelial carcinomas and less in blood cancers [38,39]. Expression of Tn appears in early tumor stages [40–42] and correlates with cancer progression, tumor metastasis and poor patient prognosis [43–46].

The mechanistic understanding of Tn expression has long been unclear. As high levels of Tn suppose large amounts of unmodified GalNAc residues, a long-standing hypothesis is loss of activity of the downstream core 1 β3-galactosyltransferase (C1GALT1) and/or the Core 3 synthase Core 3 β1–3 N-acetylglucosaminyltransferase (B3GNT6) [47–49]. As most tissues display Core1 glycans, loss of C1GALT1 activity was the driving hypothesis and proposed to be due to defects in Cosmc, a dedicated molecular chaperone required for proper folding of C1GALT1 [47,50]. As Cosmc is X-linked, its loss in cancers could arise more easily through gene mutations, chromosomal deletions and epigenetic silencing. These changes were indeed observed in a few specimens of cervical, colon, pancreatic tumor samples and cell lines derived from leukemia and melanoma [47,51,52]. However, the proposed mechanism is unlikely in most cancer types for various reasons described below. The main argument is that the TF antigen is also highly prevalent in many of the same cancers [53–57]. Yet, loss of Cosmc/C1GALT1 would abolish its expression.

In this review, we examine the regulation of O-GalNAc glycosylation, focusing on the initiating enzymes GALNTs. We describe current knowledge on the roles of O-GalNAc glycans and GALNTs in cancer

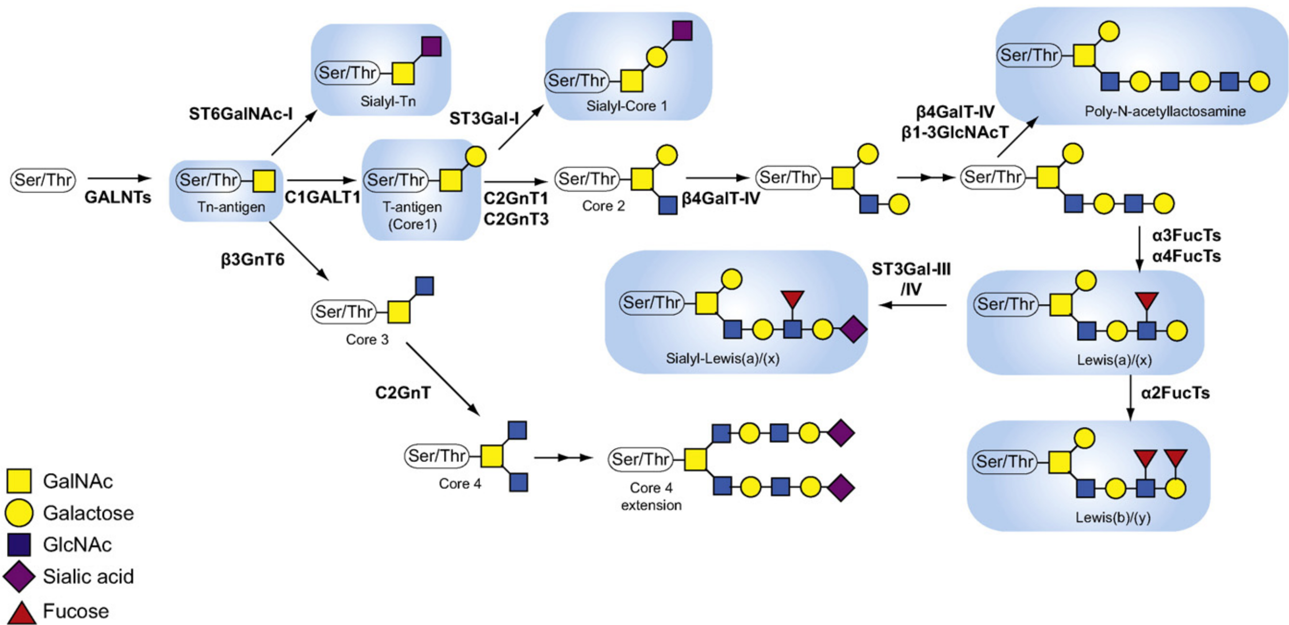


**Fig. 1.** The O-GalNAc glycosylation biosynthetic pathway. Cancer-associated structures are highlighted with blue boxes.

progression. Finally, we illustrate the clinical perspectives of the use of cancer-specific O-GalNAc glycans for cancer diagnosis and therapeutics.

## 2. The diversity and functions of O-GalNAc glycosylation

### 2.1. The GALNTs: a huge and diverse family of enzymes

The Tn antigen is synthesized by a large family of enzymes, the polypeptide N-acetylgalactosaminyltransferases (GALNTs), that are localized at the Golgi apparatus. GALNTs catalyze the transfer of GalNAc sugar from UDP-GalNAc to the hydroxyl group of Ser/Thr residues α-linked with an O-glycosidic bond (Fig. 1). This forms the first step of O-GalNAc glycosylation cascade. In normal cells, this GalNAc sugar is extended to form various core O-glycans, branching out the glycosylation pipeline. The core O-glycans are further extended and capped by histo-blood group-related structures or sialic acid [58] (Fig. 1).

There are about 20 different GALNT isoforms in humans [59–61]. The family is evolutionarily conserved with numbers increasing across evolution: *C. elegans*, *Drosophila* and humans have 9, 12 and 20 GALNTs isoforms respectively [58,62,63]. This is in contrast to other glycosylation initiation processes which usually involve only one or two isoenzymes or complexes. Interestingly, GALNTs are present in all metazoans, but not in yeast or plants [64], suggesting an expanding role of GALNTs during the evolution of metazoans [60].

GALNTs are type II transmembrane proteins with a short N-terminal cytoplasmic tail and a C-terminal domain comprising a stem region and a globular catalytic domain that extend in the Golgi lumen. GALNTs are about 600–800 amino acids and are generally larger than most other glycosyltransferases due to an additional C-terminal ricin-like-type lectin domain [65]. Both domains are connected by a short flexible linker region that varies in length among isoforms [65–67].

GALNT isozymes have distinct, yet partially overlapping substrate specificities with no clear global consensus motifs or isoform-specific motifs [60]. All GALNTs (except for GALNT10) appear to show a preference for protein regions with a Pro residue at the +3 position of Ser/Thr, which possibly increases exposure of Ser/Thr residues in a β-turn conformation, and varying degrees of preference when the Pro is at -3, -1 and +1 positions depending on the GALNT isozyme [68–70]. The lectin domain also acts in concert with the catalytic domain to facilitate further addition of GalNAc on existing substrates as the enzyme slides along the protein [71].

Phylogenetic analysis has allowed the classification of GALNTs into two major families (I and II) based on their peptide or glycopeptide substrate preferences respectively. While “peptide-preferring” isoforms readily glycosylate peptide substrates and some glycopeptides [72], “glycopeptide-preferring” members mostly act on Ser/Thr-O-GalNAc glycosylated peptides solely, although some isoforms (GALNT4 & -T12) can also act on unglycosylated peptides [73,74]. Within each family, they are divided into subfamilies of similar functions (Ia–g and IIa–b) based on their amino acid similarities [60,62]. Subfamily members tend to have similar substrate selectivity but generally show tissue specific expression, resulting in partial functional redundancy [75].

The catalytic domain is thought to be the main driver for substrate selectivity; it is poorly conserved between isoforms, yet highly conserved among orthologous isoforms among different species [59]. This suggests that during evolution, orthologous GALNTs maintain similar substrate repertoires while newly evolved GALNTs acquired new substrate preferences, allowing a diverse range of substrates to be O-glycosylated.

Another level of complexity derives from the lectin domains. While the lectin domain was thought to primarily promote further GalNAc addition on neighboring sites of existing GalNAc residues [71,73,76,77], lectin domains of different isoforms also show differential substrate selectivity [78]. Furthermore, the orientation of a remote Thr-O-GalNAc (N- or C-terminal to glycosite), recognized by the lectin domain, also influence the catalytic activity of isoforms in subfamilies Ia–Id and the preferences for this orientation also varies among isoforms [72].

More recently, depending on the isoform, glycosylation is observed at -3, -1 and +1 residues relative to the neighboring Thr-O-GalNAc and both catalytic and remote lectin interactions have to work in concert to control O-glycosylation [79]. Consequently, the addition of a GalNAc also alters the binding of and competition for the substrate to other GALNT isozymes, given that GALNTs have overlapping substrate specificities [80]. This, hence, adds additional levels of regulation and complexity in the process.

Variations in patterns and density have been thought to mostly depend on the differential control of GALNT expression. However, the localization of GALNTs in the Golgi may also be important for regulating O-glycan density as the lectin domain of GALNTs might compete with elongation enzymes for access to the GalNAc residue. Various GALNT

isoforms have been found to localize in different Golgi cisternae of HeLa cells [81]. More recently, we reported that the trafficking of GALNTs between the Golgi and the ER is regulated and can influence O-glycan patterns [82].

In sum, while all other types of glycosylation initiation occur by one or two enzymes or an oligomeric enzyme complex (in N-glycosylation), the huge number of isoenzymes in the GALNT family allows much more room for differential regulation of O-GalNAc glycosylation initiation, allowing an incomparable cell- and protein-specific regulation of O-GalNAc glycan attachment sites. This eventually leads to a diverse array of O-GalNAc glycoproteomes existing in nature [60].

### 2.2. The O-GalNAc glycoproteome and functional roles of O-GalNAc glycosylation

O-GalNAc glycoproteins were traditionally defined as mucins and mucin-like proteins. Mucins carry dense clusters of O-GalNAc glycans on PTS (Pro, Thr and Ser) repeats and are expressed on the mucous membranes of various organs, such as ocular, gastrointestinal, respiratory and genitourinary tracts. The hydrophilic and negatively charged O-glycans form highly hydrated dense arrays that provide tissue lubrication and as a protective barrier against pathogens and physical or chemical damage [21]. Hence, O-GalNAc glycosylation is often termed mucin-type O-glycosylation [83,84].

More recently, it is increasingly evident that O-GalNAc glycans are widely distributed on numerous proteins that do not have mucin-like features [83–86]. Many glycosites tend to be GALNT isoform-specific as verified by in vitro glycosylation assays [58,87,88]. These isoform-specific O-glycans have specialized functions in the cell, including hormonal regulation, lipid metabolism and growth factor signaling [10] as verified by animal and disease association studies. The high incidence of embryonic lethality or severe phenotypes in Drosophila GALNT knockouts also illustrates the importance of site-specific O-glycosylation during development [89,90].

For instance, O-GalNAc glycosylation of extracellular (ECM) proteins are important for their secretion and, in turn, influencing ECM composition and affecting cell adhesion, growth and overall organogenesis. Loss of drosophila *pgant3* disrupts wing development due to defective secretion of the ECM protein and integrin ligand Tiggrin [91]. Similarly, mice deficient in *Galnt1* suffered growth defects in submandibular glands (SMGs) [92] and heart [93] due to defective secretion of ECM proteins and altered ECM composition. O-glycosylation of tissue-specific membrane proteins also impacts organ development. For instance, specific O-glycan changes on podocalyxin and podoplanin affect kidney formation [94] and endothelial development [95] respectively. O-glycosylation also controls secretion directly by affecting regulators of the secretory pathway. Tango1, a player in secretory cargo formation, requires O-glycosylation to be protected from furin-mediated proteolysis [96]. This protective effect of O-glycosylation against proteolytic cleavage appears to be a recurrent theme.

Perhaps one of the well-established regulatory mechanisms of site-specific O-glycosylation is the protection from proprotein convertase (PC) processing [10], an essential PTM for protein maturation. As many as 700 proteins could be regulated by both site-specific O-glycosylation and PC processing [10]. However, the regulation mechanisms of only a few of these proteins have been well elucidated.

A clear example involves the processing of fibroblast growth factor-23 (FGF23) whose defects have been linked to the metabolic disorder familial tumoral calcinosis (FTC; OMIM 211900). FGF23 is a circulating peptide hormone that decreases phosphate reabsorption in the kidney [97,98] and is processed in the cell by subtilisin-like proprotein convertases (SPC) at the furin processing site RHTR↓SA$^{181}$ [99–101]. Based on genetic-linkage studies, GALNT3 was found to glycosylate FGF23 at Thr$^{178}$ that blocks PC processing and enhances intact FGF23 secretion [102]. Loss of GALNT3 led to increased secretion of the cleaved C-terminal region FGF23 that inhibits signaling and renal phosphate reabsorption [103]. Similarly, in lipid metabolism, high serum triglyceride and high-density lipoprotein cholesterol (HDL-C) levels is associated with loss of GALNT2 based on genome-wide associations and mouse model studies [104–106]. GALNT2 glycosylates Thr$^{226}$ next to the furin processing site of Angiopoeitin like-3 protein (ANGPTL3) (RAPR↓TT$^{226}$), inhibiting processing and allowing secretion of full length ANGPTL3 [107]. Cleaved N-terminal ANGPTL3 inhibits endothelial lipase, lipoprotein lipase and hepatic triacylglycerol lipase [107]. Premature growth factor pro-IGF-II has to undergo sequential cleavage to generate a mature 67 amino acid IGF-II. Interestingly, both endogenous and recombinant pro-IGF-II are O-glycosylated at various sites (Ser$^{71}$, Thr$^{72}$ and Thr$^{139}$) [108]. Aberrant O-glycosylation of IGF-II has been described in non-islet cell tumor hypoglycemia. Non-glycosylated recombinant pro-IGF-II E-domain showed greater growth stimulatory effects compared to the glycosylated variant [109,110], indicating the importance of O-glycosylation in modulating its processing and activity.

PC processing occurs differentially in different tissue types and this could be regulated by differential GALNT expression. For instance, pro-opiomelanocortin (POMC), the precursor of peptide hormones adeno corticotropic hormone (ACTH), α-, β- and γ-MSH (melanocyte stimulating hormone) and β-endorphin, is differentially processed in different tissues, resulting in various peptide hormones in distinct lobes of the pituitary gland [111]. The longest peptide, N-POMC$^{1-77}$ is O-glycosylated at Thr$^{45}$, suggesting inhibition of PC processing of N-terminal POMC [112]. In the heart, PC processing of the pro-brain natriuretic peptide (pro-BNP) releases a C-terminal peptide hormone during cardiomyocyte stress, leading to vasodilation and natriuresis. Pro-BNP is O-glycosylated at Thr$^{71}$ close to the furin cleavage site (LYTLRAPR↓SP$^{78}$), protecting against pro-BNP cleavage [113,114]. Indeed, plasma concentration of pro-BNP is increased in heart failure [115].

The mechanism by which the site-specific O-glycan modulates PC processing has recently been explored. *In silico* modeling revealed that a single hydrophilic O-GalNAc sugar ±3 residues to the furin cleavage site, is sufficient to block processing [115] and this appears to be more specific for furin proteases. This has been validated in in vitro cleavage studies of the substrate glycoproteins [116]. These studies hence demonstrate that the biological function and activity of the O-glycoprotein depends on the delicate balance between the two processes.

Apart from intracellular PC processing, O-GalNAc glycans also protect membrane and secreted proteins such as the low-density lipoprotein receptor (LDLR) from extracellular proteolytic cleavage, affecting their stability. O-glycans added on the stem region of LDLR and several of its family members such as VLDLR, LRP1, and ApoER2, protect them from proteolytic cleavage by secretase and ectodomain shedding [117–119]. Lack of O-glycosylation on LDLR consequently alters cellular LDL uptake [117]. O-glycans on the extracellular tails of G-protein coupled receptors (GPCRs) also protect from disintegrin and metalloprotease (ADAM) protease processing and has implications in heart function with the ®1 adrenergic receptor. Similarly, O-glycosylation on Thr$^{27}$ on the extracellular region of copper transporter CTR1 protects it from proteolytic cleavage, altering its activity [120]. Secreted proteins such as TNFα mature and are secreted via cleavage by ADAM-17 on membranes (QA↓VR$^{78}$) [121,122]. Interestingly, O-glycosylation at Ser$^{80}$ blocks cleavage, resulting in various truncated peptides (QA↓V↓R↓SSSR$^{82}$) observed in lymphoblastic leukemia B-cells, explaining the disease [123]. Secretion of amyloid precursor protein (APP) is dependent on its site-specific O-glycosylation on various residues, particularly at Tyr$^{10}$ close to the β-secretase cleavage site, which has implications with Alzheimer's disease [124].

In addition to the above-mentioned proteins, the repertoire of identified O-GalNAc glycosylated proteins has exponentially expanded in recent years, mostly due to the development of the 'Simple cell' technology developed by H. Clausen's lab. 'Simple cells' are knockouts of the Cosmc gene and hence, have an inactive C1GalT1, resulting in all O-

GalNAc glycans being Tn or S-Tn. This greatly reduces O-glycan heterogeneity, allowing lectin enrichment and facilitating mass spectrometry identification of glycosites on O-glycoproteins. A screen of cell lines and their secretomes from various tissues identified about 3000 glycosites in >600 proteins [19]. In addition to the general role in blocking protein proteolysis [10,113], the data suggests that there is still a vast number of biological functions modulated by O-glycans that remains undiscovered. It is notable that many O-GalNAc glycosylated proteins are ECM (fibronectin, collagen, etc) and cell surface proteins (such as receptors GPCRs LDLR, VLDLR, LRP1, LRP1B, LRP2, LRP8, adhesion proteins CD44, cadherins, integrins, etc). Given that many of these proteins are linked to cancer processes, it would not be too surprising if O-GalNAc glycosylation is directly involved in the molecular mechanisms of cancer progression.

## 3. Regulation of O-GalNAc glycosylation initiation and extension

The regulation of O-GalNAc glycosylation occurs on two different levels: first, which proteins and which sites are being glycosylated and second, how is GalNAc further modified into the final O-glycan. In other words, there is regulation at the initiation as well as elongation level. This review is mostly focused on regulation of initiation and we will discuss only briefly elongation. Initiation is controlled by GALNTs, which can be regulated at the expression level as well as through compartmentation through the GALA pathway [125].

### 3.1. Evidence of transcriptional regulation of GALNTs

Given that different GALNT isoforms have partially overlapping but distinct substrate specificities, increasing the number of family members directly increases the overall repertoire of potential substrates. The diversity of GALNT isoforms increases with the complexity of organisms, suggesting that this evolutionary expansion allowed for a better modulation of the O-glycoproteome. Congruently, the human GALNTs exhibit differential expressions in cells and tissues during development and differentiation [60]. Some members are more ubiquitously expressed in organs, such as GALNTs 1 and 2, whose broad expression patterns have been demonstrated by Northern studies [126,127], RT-PCR expression profiling [128] as well as immunohistochemistry. However, some cell type-specific expression patterns were also revealed within tissues [129]. GALNTs 4 and 6 in salivary glands are strongly expressed in mucous cells but only weakly in serous cells [60,130], and GALNTs 11 and 14 in the kidney are found only in tubules but not glomeruli [62].

Other family members show more organ-restricted expression. For example, Northern blot analysis of GALNT3 located it mainly in the pancreas and testis, and only weakly in a few other organs [131]; GALNT5 showed abundant expression in rat sublingual gland and lower expression in stomach, small intestine and colon [132]; while human GALNT9 and rat GALNT19 appeared largely brain-specific [133,134]. Developmentally, in a study of spatial expression patterns of seven GALNTs in mouse embryo, *Galnt3*, *5* and *7* were found to have more restricted expression than *Galnt1*, *2*, *4* and *9*. Furthermore, during organ differentiation there were instances of GALNT expression being restricted to either mesenchymal or epithelial tissue; concurrent expression of a specific GALNT in both tissue types was never observed [135]. In a *Drosophila* development study of 12 GALNT isoforms, four displayed unique expression patterns, two of which (*pgant2* and CG30463) were specifically related to eye development [136].

The variable expression of the members of this large family suggests precise individual regulation. However, little is known yet about how this regulation is achieved and integrated in regulatory networks. These expression patterns also point to specialized roles for each isoform. Indeed, although no overt phenotypes have been reported in knockout mice for *Galnt* 4,5,8,10, or 14 [137–139], other *Galnt* knockouts were found to have various deficiencies. For example, *Galnt1* deficient mice displayed disorders in bleeding and B-cell maturation [140]. Mutations in Drosophila pgant35a, ortholog of human GALNT11, were recessive lethal due to defects in tracheal epithelia morphogenesis [141], and pgant3 deficient Drosophila show defects in wing development due to inappropriate secretion of ECM components which affect cell adhesion events [91], while another four Drosophila pgant genes (CG31956, CG31651, CG6394 and CG30463) have been identified to be essential for viability [90]. Xenopus xgalnt11, ortholog of human GALNT16, has been suggested to have specialized and necessary roles in normal neural and mesodermal differentiation [142].

In humans, GALNT3 has been shown to be required for proper FGF23 activation, and gene inactivation results in familial tumoral calcinosis, as mentioned above [102,143]. Genome-wide association and other studies have linked other GALNTs with specific functions and diseases, including GALNT2 in plasma lipid level regulation and cardiovascular disease [104,105], GALNT4 in acute coronary disease [144], GALNT14 in death receptor-mediated apoptosis resistance [145], and GALNT11 in left–right body patterning and congenital heart disease [146].

These examples illustrate the importance of O-GalNAc glycosylation in diverse fundamental biological pathways, and specifically of differential regulation of the initiating event in organismal development and survival. However, an earlier study linking a GALNT1 single nucleotide polymorphism with decreased epithelial ovarian cancer risk [154] was later found by the same authors to be non-replicable in a larger study [147], underscoring the importance of providing a mechanistic understanding to explain genetic association studies.

The expression of some GALNTs has been proposed to be regulated by microRNAs. In a bioinformatics analysis of miRNA regulation of glycosylation related genes, three GALNTs (1, 7 and 3) were among the top 10 most "highly regulated" glycogenes identified [148].

In cervical cancer, miR-214 was found to be frequently downregulated; its expression in cervical cancer cells reduces proliferation, migration and invasion, which can be countered by restoring expression of its target GALNT7 [149]. miR-34a/c was also found to be downregulated in laryngeal squamous cell carcinoma, with enhancement of cell proliferation and migration potentially mediated by its direct target GALNT7 [150]. On the other hand, upregulation of miR-17 in hepatocellular carcinoma (HCC) appeared to enhance tumor growth and vascularization via silencing of three proteins including GALNT7 [151]. In melanoma cells, GALNT7 was found to be downregulated by increased expression of miR-30b/30d, correlating with poorer prognosis; its downregulation possibly lead to immunosuppression by increasing IL-10 synthesis and secretion, which reduced immune cell activation and recruitment [152]. These examples illustrate how GALNTs can be regulated by miRNAs. However, the apparently opposite trends observed with GALNT7 depending on context invite prudence and suggest that a better understanding of GALNT substrate repertoire in cancer is required.

The involvement of miRNAs provide a probable way in which context specificity can be rendered, via co-regulation of GALNTs with other proteins. Indeed, a recent systems-based study on a panel of 60-cancer cell lines integrated lectin array data and miRNA expression data to generate miRNA/glycogenes/glycans regulatory networks. They identified a cluster linked to Tn and TF expression involving 15 miRNAs with a large number of predicted GALNT targets [153]. Focusing on targets of the miR-200 family, regulators of EMT, the same group identified "promesenchymal glycosylation enzymes" and proposed their likely involvement in TGFβ-induced EMT, indicating that miRNA networks can be used to identify important glycosylation enzymes critical in disease development [154].

### 3.2. Regulation of GALNT activity through subcellular localization: the GALA pathway

An additional layer of GALNT regulation is happening at the level of spatial localization. As mentioned above, we recently reported that activation of the proto-oncogene Src activates Golgi-to-ER trafficking of

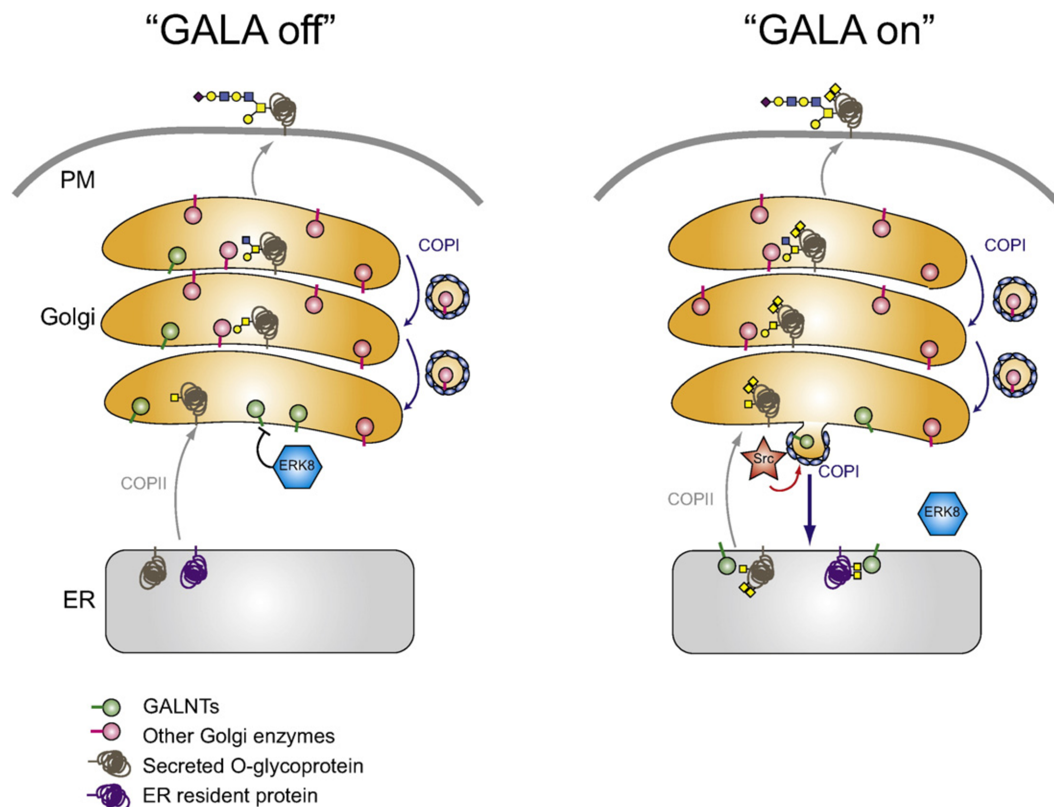


**Fig. 2.** The molecular regulation of the GALA pathway. Under normal conditions without GALA activation, most O-GalNAc glycosylation initiation of secreted/membrane glycoproteins occurs in the Golgi. The GalNAc residues are rapidly modified by downstream galactosyltransferase C1GALT1, in turn, preventing lectin domain binding and secondary GalNAc addition by the same or different GALNTs. Upon GALA activation with Src activation/loss of ERK8, ppGalNAcTs are trafficked to the ER and can glycosylate ER-resident proteins. ER localization of GALNTs may facilitate lectin-dependent GalNAc addition on secondary glycosites and also in unexposed regions of the proteins that are normally folded when present in the Golgi. For reasons still unclear, some GalNAc added after GALA remain unextended and appear at the cell surface, where it typically concentrates in focal adhesions.

GALNTs, resulting in a strong increase in intracellular Tn levels [82] (Fig. 2). Src is a tyrosine kinase that associates with multiple cellular membranes [155], and can be activated by cell surface receptors such as EGFR. A fraction of Src in the cell is localized at the Golgi [156], and has been shown to have consequences on Golgi organization [157, 158]. The Src-dependent enzyme relocation appears to be specific to the GALNTs and not other glycosylation enzymes (hence we have termed it "GALA", for GALNT Activation), and requires the COPI trafficking machinery [82,159]. COPI vesicles are required for transport within the Golgi and Golgi-to-ER trafficking and the latter is known to transport a number of proteins including KDELR [160,161]. Interestingly, GALNT containing COPI vesicles appears to be differentially regulated to the KDELR containing carriers [159]. However, what drives GALNTs recruitment in COPI vesicles is not known at this stage. It is therefore not clear why GALNTs and not other Golgi enzymes are relocated to the ER upon Src activation.

Evidences indicate that Src is able to activate the formation of COPI vesicles, however it is not known how Src is connected with the COPI machinery. The basic elements of this machinery comprise a heptameric COPI coatomer protein complex, the Arf small GTPases and guanine nucleotide exchange factors (GEFs) among other elements [161]. The vesicles that transport from the Golgi to ER are controlled mostly by Arf1, 3 and 5 GTPases and their GEF GBF1 [162]. Upon reaching the ER, the transport carriers fuse with ER membranes and discharge their content.

GALNTs are enzymatically active in the ER and seem to have access to a host of protein substrates, including ER resident proteins [59,159] (Fig. 2). C1GALT1 does not seem to be relocated by the GALA pathway, and would probably be inactive in the ER anyway due to binding by its ER-resident chaperone Cosmc [50]. Thus Tn generated by GALNTs in the ER cannot be extended in the same compartment. This lack of extension allows for further GALNT activity due to their lectin domain, and indeed Tn levels after GALA activation highly depend on a functional GALNT lectin domain [82], suggesting that lectin-dependent glycosylation are the key events promoted by ER relocalization [59]. Altogether, these various elements probably explain why relocation leads to high intracellular Tn levels.

Semi-quantitative analysis revealed that most Tn is localized in the ER with a small fraction at the cell surface [82]. This suggests that most GalNAc residues added in the ER are extended when their cargo proteins are trafficking through the Golgi. Why some residues are able to reach the cell surface without being modified is not clear at present.

Activation of GALA and loss of C1GALT1 activity lead to very different predictions for O-GalNAc glycans intracellular patterns and nature in tumors. With GALA, most Tn is expected to be in the ER with a small pool at the Golgi and cell surface, while loss of T-synthase leads to Tn being mostly at the Golgi and cell surface. GALA will affect the pattern of glycosites (the glycoproteome) and maybe to some extent the distribution of extended glycans. By contrast, loss of C1GALT1 activity should not affect glycosites but induce a drastic reduction in extended glycans. In human solid tumors, extended O-glycans are often observed, being the TF antigen or the Lewis type antigens. The intracellular pattern strongly supports an ER accumulation of Tn. Therefore, GALA is the more likely mechanism driving high Tn in human solid tumors.

GALA is an analog rather than binary phenomenon, displaying varying levels of Tn elevation depending on the method of stimulation. GALNTs relocation irreversible upon release of activation, with Tn levels reverting to baseline within hours and GALNTs returning to the Golgi apparatus [159]. This suggests that GALNTs are constantly cycling between the Golgi and ER, their steady-state distribution being determined by the balance between retrograde export from the Golgi by COPI carriers and anterograde transport from the ER by COPII. This

pathway thus affords a dynamic way to acutely regulate Tn levels independent of enzyme expression level, and may account partly for some of the differences, as described above, in GALNT carcinogenicity in different tissues.

A systematic RNAi screen of ~1000 signaling genes with stringent selection criteria found 12 regulators of GALA, with most of the proteins being previously described as Golgi-localized or interacting with Golgi-related proteins, suggesting a complex signaling network regulating GALNT export from the Golgi [159]. A key node in this network is the serine/threonine kinase ERK8, which has high basal activity independent of growth factor stimulation [163,164], and in fact displaces from the Golgi upon growth factor stimulation [159] (Fig. 2). Its depletion increases Golgi-associated phosphotyrosines, suggesting that it actively suppresses a Golgi-localized tyrosine kinase, possibly Src. These observations indicate that GALA seems to be continuously inhibited by a network of regulators, the perturbation of which allows GALA activation.

Expression of a chimeric construct expressing ER-localized GALNT1 or −2 is sufficient for strong Tn elevation, suggesting that GALNT Golgi-to-ER relocation is the only trafficking event necessary for GALA and that UDP-GalNAc is present at sufficient levels in the ER. Furthermore, ER-localized GALNT2 induces morphological changes in epithelial cells similar to those caused by Src activation or ERK8 depletion [159], and increases their adhesion to various ECM substrates [165] as well as stimulates cell migration and invasiveness. Thus GALA seems to play a role in activating a migration process involving enhanced adhesion, possibly mesenchymal-type migration [166]. When an ER-targeted soluble GALNT lectin domain is expressed in cells with activated GALA, Tn levels are reduced, suggesting that it functions as a competitive inhibitor of full-length enzymes [165]. Expression of this lectin domain also reduces cell adhesion and motility.

### 3.3. GALA impact on TF expression and O-GalNAc glycans extension

O-GalNAc glycans can be elongated in various ways (Fig. 1). The resulting O-glycans can have various physiological effects, for example by forming potential binding sites for endogenous lectins. It is not clear at this stage how initiation and extension are influencing each other. It is likely that activation of the GALA pathway influences extension. Indeed a higher density of O-glycans might hinder extension reactions, favoring shorter O-glycans.

However, extension is probably controlled by various other mechanisms. The TF antigen, alias Core-1, is only one biosynthetic step from the initiation reaction (Fig. 1). TF is over-expressed in many tumors, yet levels of TF are not correlated with Tn levels [167]. In addition, in vitro, activation of the GALA pathway yields only very moderate levels of TF [167]. Similarly, in an RNAi screen for signaling regulators of Golgi organization, TF and Tn levels did not correlate [168]. Interestingly, multiple signaling molecules were found regulate TF levels, suggesting that TF regulation might occur at the Golgi level. It also suggests that high TF expression is not restricted to cancer but a physiological phenomenon. Accordingly, high TF levels have been known to occur in situ in maturing T- and B-cells [169,170], activated T cells [171,172], as well as other developing tissues such as colon [173] and neurons [174] for many years.

### 3.4. GALA and competition with other O-glycosylation pathways

An interesting potential consequence of GALNT relocation to the ER is competition between O-GalNAc glycosylation and other O-glycosylation events. Besides O-xylose proteoglycan biosynthesis which is initiated in the Golgi and O-GlcNAc glycosylation which occurs in the cytoplasm and nucleus, all other types of protein O-glycosylation (including O-mannose, O-fucose, O-glucose and O-galactose) are initiated in the ER. The GALA pathway may enable GALNTs to compete in the ER for O-glycosylation sites that are normally modified by the time they reach the Golgi, thus tipping the balance towards more O-GalNAc glycosylation versus other types. A recent study of the O-mannose glycoproteome revealed sites in target proteins which were also known to be GalNAc-modified, including PDIA3 [230]. The discovery of the GALA pathway opens up the possibility for direct competition between O-Mannose and O-GalNAc pathways and their associated biological functions.

## 4. Role of O-GalNAc glycosylation in tumorigenesis

### 4.1. O-GalNAc glycans in tumor progression: general considerations

There are probably multiple ways by which O-GalNAc glycans can regulate tumor formation, cell invasion and cancer progression (Fig. 3). The O-glycans on a receptor protein tend to affect its properties such as binding properties, activity, expression level, and/or stability. In fact, O-GalNAc glycans have been implicated in numerous molecular mechanisms that could play key roles in tumorigenesis (Fig. 3). While many evidences point to such roles, what is missing in most cases is a clear definition of target proteins and especially an understanding of the mechanisms regulating glycosylation itself. Evidences also suggest that both the presence or not of an O-glycan and the type of O-glycan can be regulated and have biological effects on the target protein.

### 4.2. GALNTs, Tn and GALA in cancer

GALNTs have been associated with a multitude of human cancers. Mutations in glycosyltransferases have not generally been commonly found in tumor cells, but one study did find that GALNT12 is a mutational target in colon cancers, with two somatic and six germline inactivating mutations identified [175].

Misregulation of GALNT expression has been described more frequently and in many cancers, with differing trend depending on both tissue context and GALNT isoform. In HCC, GALNT1 expression was found to be frequently upregulated and associated with poor patient survival, possibly by decreasing EGFR degradation, enhancing its activation and consequently cell migration and invasion [176]. On the other hand, GALNT2 was found to be frequently downregulated especially in cases with vascular invasion and recurrence, via inhibition of ligand-induced endocytosis and downstream signaling of EGFR [177]. Similarly, different isoforms can have opposing effects in gastric cancer. GALNT5 expression was found to be markedly reduced in gastric cancer tissues compared to non-malignant gastric mucosa [178], and other studies associated expression of GALNT3 [179] and GALNT10 [180] in gastric cancer with good prognosis. GALNT2 expression was described to be significantly higher in a gastric cancer cell line compared to other poorly differentiated human cancer cell lines [181], and GALNT6 expression was associated with venous invasion in gastric carcinoma [182]. GALNT6 was also detected to be overexpressed in breast cancer with consequences on MUC1 stabilization and carcinogenesis [183]. GALNT3, the family member most similar to GALNT6 in amino acid sequence, has also been observed to be overexpressed in multiple cancers, including renal cell carcinoma in which it predicts high tumor grade and poor prognosis [184], high-grade serous epithelial ovarian tumors in which it correlates with shorter progression-free survival [185], and early-stage oral squamous cell carcinoma in which it correlates with poor differentiation, vascular invasion, and recurrence [186].

On the other side of the coin, strong GALNT3 expression was correlated with good prognosis in human colorectal carcinomas [187] and non-small cell lung cancer [188]; in a mouse colon cancer model, loss of expression was correlated with higher metastatic potential [189]. Similarly, loss of GALNT6 expression in pancreatic tumors was correlated with poor clinical outcome [190]. These examples illustrate the complexity of GALNT glycosylation and are consistent with their diversity and distinct substrate specificities enabling specific roles in specific cellular contexts.

While GALNTs expression modulation may affect the repertoire of glycosites, upregulation of a specific GALNT is not likely to explain the

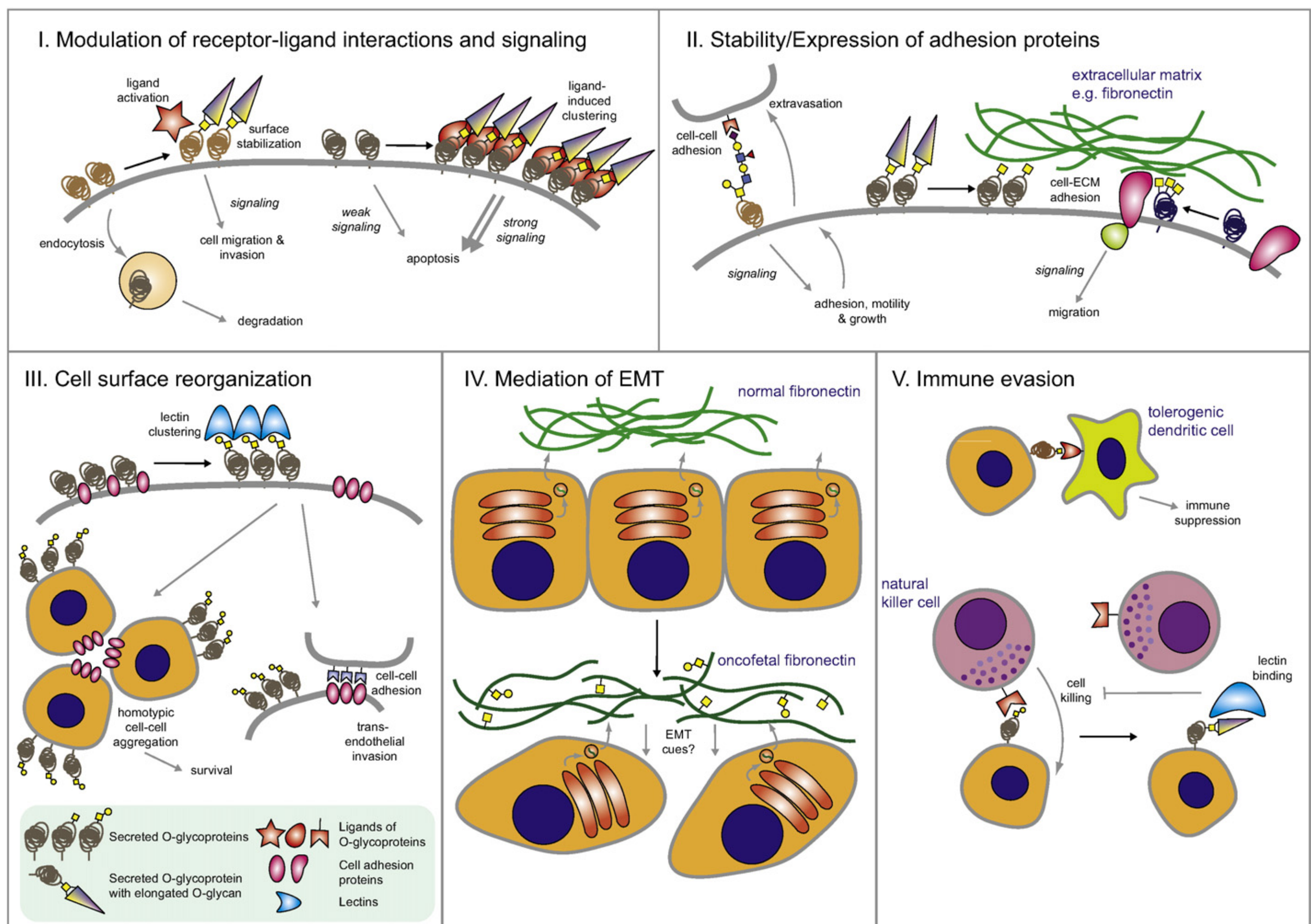


**Fig. 3.** The roles of O-GalNAc glycans in cancer cell invasion. Black arrows indicate a change in O-glycosylation. Gray arrows indicate signaling pathways or other cellular processes. (I) O-glycans can stabilize receptor expression on the cell surface by reducing endocytosis, or promote ligand-induced clustering, both of which allow stronger signaling. (II) Specific O-glycans on cell adhesion or ECM proteins can regulate cell-cell or cell-ECM adhesion by directly influencing O-glycoprotein binding interactions, as well as modulating related signaling pathways. (III) O-glycans can allow cell surface reorganization of O-glycoproteins to expose other proteins essential for cell-cell adhesion. (IV) O-glycans can trigger immune suppression by binding to tolerogenic dendritic cells, or modulate cell killing by natural killer cells via competitive lectin binding. (V) O-glycans can modulate EMT by modifying ECM protein glycosylation. Changes in O-glycosylation initiation induced by GALA activation could modulate these processes to affect tumor cell invasiveness.

high Tn levels observed in cancer. As discussed above, this probably involves GALA activation [167]. The effect of GALA appears to be a significant change in the O-glycoproteome of tumor cells, with for instance the increased glycosylation of ER resident proteins. How this induced glycoproteome affects cancer cells remains to be determined.

GALA activation does not entail that Tn glycans themselves play active roles. Indeed, after increased synthesis in the ER, many of the GalNac residues generated will probably be elongated as their carrier protein traffics through the Golgi apparatus. These GALA-dependent elongated O-glycans may correspond to some of the O-glycans described below and have unique effects on their carrier proteins. Still, our recent work in breast cancer cells showed that Tn could be observed at the lamellipodia and was associated with increased cell adhesion, motility and invasiveness [167]. In addition, O-glycans on ER resident proteins will probably remain mainly in the Tn form. Modification of ER protein dynamics could play a role in tumor physiology as processes such as the Unfolded Protein Response have been linked to tumor cell adaptation.

The effects on cell behavior suggest that GALA is affecting cell surface proteins, presumably by glycosylating important sites in these proteins. Additionally, GALA could modulate the activity of ER-resident proteins such as chaperones to affect the folding or trafficking of cell surface proteins. Interestingly, although most Tn detected in tumor cells is in the ER, GALA induces some cell surface Tn expression, which tends to localize to adhesion structures. These Tn-modified proteins appear to be essential for GALA's effects since Tn binding lectins can inhibit cell migration [167]. At present, it is not clear why this small fraction of Tn is not modified as it traffics through the Golgi apparatus.

### *4.3. The TF antigen in cancer*

Similar to Tn, the TF antigen is highly expressed in >90% of all human cancers including colon, breast, bladder, prostate, liver, ovary and gastric tissues [53–57]. TF seems to correlate less than Tn with cancer progression and patient prognosis [36]. However, this could in part be due by the greater variability of the detecting reagents for TF antigen which could result in cross-reactivity with normal tissues [191].

Upregulated TF expression was also reported to be correlated with metastasis [53,192]. Shortening of O-glycans into TF was shown to affect mammary cancer development in mice [193]. Targeting the TF antigen with an anti-TF monoclonal antibody (mAb) showed improved prognosis of mice with mammary tumors [194]. Vaccination with purified TF antigen-conjugated immunogenic carriers led to prolonged survival of breast and ovarian cancer patients, likely through complement-mediated tumor cell killing, in a few clinical trials [195–197].

At the moment, it is unclear how tumor cells upregulate the expression of the TF antigen. The GALA pathway only drives a modest increase

of TF cell culture in vitro and is an unlikely mechanism. However evidences suggest that like Tn, TF expression in cancer may not result from an aberrant, cancer specific truncation of O-glycans but rather from activation of an endogenous pathway (See above 3.3).

### 4.4. Extended O-GalNAc glycans and tumor biology

Various extended O-GalNAc glycans have been associated with tumor progression. The core structure on the O-glycan seems to be important. For example, core 2 O-glycans are upregulated in various malignant tumors including colorectal, lung, prostate and bladder [191,194, 198–200], while core 3 and core 4 O-glycans appear to be generally downregulated in cancer [48,201]. However, core 2 O-glycan expression is reduced in breast cancers [202–204], illustrating that there are no general roles for branching, and the functions of specific glycan branches are likely tissue- and context-specific.

Sialylation of O-GalNAc glycans, probably by providing a negative charged residue on glycans also seems to play critical roles in governing the properties of proteins on the cell surface. For instance, Core 2 O-glycans are extended into poly-N-acetyllactosamine (poly-LacNAc) chains (Fig. 1), which in their sialylated and/or fucosylated forms participate in various steps of tumor formation [27,205]. Core 2-based structures are the main carriers of the sialyl Lewis$^x$ (sLe$^x$) glycan motif (Fig. 1), which is expressed on tumor cells. Control of the core 2/core 1 ratio by change of C2GnT1 expression seems to regulate the expression of sLe$^x$ in maturing dendritic cells [192], T cells [206,207], and pre-B lymphocytic cells [208]. sLe$^x$ is predominantly formed on Type 2 poly-LacNAc chains, and the expression of sLe$^x$ in colon and pancreatic cancer has been attributed to the relative levels of β3Gal-T and β4Gal-T, which synthesize Type 1 and Type 2 poly-LacNAc chains respectively [209, 210].

### 4.5. O-GalNAc glycans in the regulation of cell-cell and cell-ECM adhesion

Various O-GalNAc glycans on different proteins have been shown to have marked effects on cell-cell and cell-ECM adhesion. For instance, sLe$^x$ binds to E-selectins on endothelial cells and P-selectins on platelets, and these interactions are critical for the extravasation of the tumor cells from circulation into tissue [211,212]. Expression of S-Tn decreases cell adhesion and increases cell metastatic potential in T-47D breast cancer cells [213]. Blocking O-glycan extension enhances E-cadherin-dependent cell-cell adhesion by decreasing the O-glycan dependent inhibitory action of dysadherin [214].

O-GalNAc glycans also impact the protein stability and expression levels of cell surface adhesive and ECM proteins such as integrins and MMPs or fibronectin and collagen respectively. These effects probably modulate invasiveness via affecting cell interactions with their microenvironment. Treatment of lymphoma cell lines with an inhibitor of O-GalNAc extension, benzyl-α-GalNAc, seemed to enhance their adhesion to fibronectin mediated by integrin very late antigen (VLA)-4 [215].

An inverse correlation between sialidase NEU1 expression and metastatic potential has been found in a number of cell types [216–218]. Expression of NEU1 in colon cancer cells was found to reduce their metastatic potential by decreasing cell migration, invasion and adhesion both in vitro and in vivo. This was mediated by decreasing sialylation of O-GalNAc glycans on integrin beta-4, decreasing its phosphorylation and attenuating the focal adhesion kinase and Erk1/2 signaling pathway, which is possibly linked to the subsequent downregulation of ECM metallopeptidase MMP7 [219]. Sialylation of integrins regulate their adhesion to the ECM protein fibronectin [220], and increased sialylation inhibits adhesion [221,222], which can facilitate cancer spread and metastasis [223].

Sialic acids on core 2-derived sLe$^x$ on mucins are also important for colon cancer cell adhesion and migration. In colon cancer, sialidase NEU4 is also downregulated. NEU4 was the only sialidase found to act effectively on mucins, preferentially cleaving the sLe$^x$ motif [224]. E-selectin binding to sLe$^x$ stimulates colon cancer cell adhesion, motility and growth via the p38-Hsp27-actin reorganization pathway, thus downregulation of NEU4, which inhibits this pathway, enhances colon cancer cell invasiveness.

The high metastasis of some lung cancer sublines has been attributed to trimeric Tn (tTn) on syndecan 1 (Sdc1), generated by upregulation of GALNT13. tTn-glycosylated Sdc1 promotes integrin-dependent cell adhesion to fibronectin which induces phosphorylation of FAK and paxillin, events known to facilitate cell migration. tTn-Sdc1 also forms a molecular complex with integrin α5β1 and MMP9 in glycolipid-enriched microdomains/rafts [225], which was previously shown to increase metastatic potential [226].

O-GalNAc glycans can be ligands for members of the β-galactoside-binding family galectins, which can bind to cell surface glycoproteins via their carbohydrate recognition domains. This binding can clusters and polarizes molecules like the cell surface MUC1, allowing exposure of smaller adhesion proteins such as CD44 and E-selectin ligands, thus promoting cell adhesion and transendothelial invasion [227]. The galectin-3-induced polarization of MUC1 also exposes E-cadherin, enhancing anchorage-independent homotypic cell-cell aggregation which prevents anoikis, thus promoting survival of circulating tumor cells and facilitating their metastatic spread [228]. Thus O-GalNAc glycans can affect cell physiology through enhanced binding to galectins [229]. Recently, O-GalNAc glycosylation has been proposed to be important in the process of epithelial-mesenchymal transition (EMT). EMT involves a decrease in expression of epithelial markers and increase in that of mesenchymal markers, which lead to changes in cell morphology and increased cell motility. It was found that treatment of prostate epithelial cell lines with TGF-β, a known inducer of EMT, induced upregulation of oncofetal fibronectin which bears a specific O-GalNAc glycosylated threonine in the IIICS domain, along with typical EMT characteristics. TGF-β-induced oncofetal fibronectin upregulation and the EMT process were both inhibited by knockdown of GALNT6 and GALNT3 [230]. In a different study, GALNT6 overexpression in a nontumorigenic mammary epithelial cell line was also shown to stabilize fibronectin by increased O-glycosylation, leading to EMT-like phenotypes, including morphological changes which disrupted acinar morphogenesis, and cadherin switching (a shift from E-cadherin expression to N-cadherin expression) [231].

### 4.6. O-GalNAc glycans in regulating cell proliferation and cell death

As mentioned above, GALNT expression can affect EGFR endocytosis and subsequently its downstream signaling as well as degradation, having consequences on cell migration and invasion. Site-specific O-GalNAc glycosylation on secreted ligands such as IGF-II affect their processing by proprotein convertase and thus their secretion, thereby modulating their ability to effect growth stimulation. O-GalNAc glycans can also influence cell number via modulation of death receptors. O-GalNAc glycosylation of pro-apoptotic death receptors DR4 and DR5 on cancer cells is critical for promoting their TRAIL-induced clustering, which leads to activation of the apoptosis-initiating caspase-8 [145].

### 4.7. O-GalNAc glycans in regulating interaction with the immune system

O-GalNAc glycan-lectin interactions can also play important roles in tumor cell evasion of immune surveillance. Tn-modified MUC1 on colon carcinoma cells, but not MUC1 on normal epithelial cells, were found to bind to macrophage galactose-type lectin (MGL) on myeloid antigen presenting cells such as macrophages and dendritic cells (DCs) [232]. Binding triggers internalization of both proteins and subsequent presentation of the Tn-MUC1 ligand on cell surface major histocompatibility complex class II (MHC II), which can trigger either immune response and cell killing, or immune tolerance via inhibition of immune response. Interestingly, MGL is preferentially expressed on tolerogenic DCs [233], and MGL-positive DCs were detected at colon carcinoma tumor sites

[232] whose tendency to express Tn correlates with poor prognosis, suggesting that Tn binding to MGL on these DCs promotes tumor progression via immune suppression.

Another case of immune evasion is tumor cells expressing major histocompatibility complex class I-related chain A (MICA), where core 2-derived poly-LacNAc on MICA promotes binding to galectin-3 instead of NKG2D on natural killer (NK) cells. Binding to NKG2D would activate release of apoptotic factors by NK cells to kill the tumor cells, and C2GnT-expressing bladder tumor cells appear to better evade NK cell killing and have improved survival in the circulation, thus the O-glycan-galectin-3 interaction confers protective effects for tumor cells [200].

## 5. Clinical perspectives

### 5.1. Glycosylation in cancer diagnostics and prognostics

Cancer biomarkers are useful for diagnosis, prognosis, staging and monitoring therapeutic response, as well as to aid therapy selection and follow-up. Ideally, a cancer biomarker should show high sensitivity and specificity and be detectable in the early stages of the disease. Its levels should also correlate with cancer progression to allow better indications for staging, measuring responses to therapy and checking for relapses.

There are only about 20 FDA approved and clinically used biomarkers, among which only five are validated for diagnostic use [234, 235]. Many of these biomarkers still suffer from insufficient specificity and sensitivity [235]. As a result, clinical decisions can often only be obtained with the combinatorial use of biomarkers and other tests such as imaging and tissue biopsy. For instance, HCC diagnosis requires both alpha fetoprotein (AFP) levels readout and image-based liver ultrasonography [236–238].

One potential reason for the insufficient specificity and sensitivity of current biomarkers may be that readout is based solely on protein levels. Protein levels may not vary enough to correlate sufficiently with disease state, they can also vary between individuals and fluctuate with other diseases, particularly of inflammatory origin [239]. Interestingly, most biomarkers in the clinic are glycoproteins, such as the prostate-specific antigen (PSA) for prostate cancer, carcinoma antigen (MUC16, aka CA125) for ovarian cancer, human chorionic gonadotrophin β subunit (hCG-b) for non-seminomatous testicular carcinoma, and carcinoembryonic antigen (CEA) for colon cancer. These proteins display distinct changes in glycosylation in malignant tissues [114, 240–243], particularly in terms of O-GalNAc glycan patterns [114, 243–245]. However, glycoforms of these biomarkers have yet to be exploited.

An example of glycoform-based biomarker is the core fucosylated AFP (AFP-L3) in HCC diagnosis [246,247]. AFP-L3 shows higher specificity than AFP in differentiating HCC from cirrhosis and hepatitis, and is used in the clinics [248,249]. A drawback that remains is its low sensitivity (60%) which hampers its full potential as a HCC biomarker [250]. However, glycoforms of other proteins may present better sensitivity. Given the very frequent changes in O-glycosylation, it is possible that targeting O-GalNAc glycoforms may provide interesting biomarkers.

O-GalNAc glycans themselves, such as Tn, S-Tn, TF, $SLe^x$ and $SLe^a$, have been proposed as cancer biomarkers, but robust antibodies are difficult to raise. These glycans have mostly been targeted by lectins, which remain the mainstay of tools for glycan detection. Various lectin-based methods, such as immunohistochemistry, blots, liquid chromatography and microarrays, have been tested and employed. Some antibodies have also been raised to detect tumor-associated O-glycans or protein glycoforms [251–258].

The use of tumor-associated antigens has been applied in three main areas: (1) detecting biomarkers in the serum and tissue biopsies, (2) tumor imaging and (3) assessing immune responses or autoantibodies that are triggered against tumor O-glycans, which are discussed in the following sections.

### 5.2. Detection of serum glycoprotein biomarkers

Serum biomarkers provide a non-invasive avenue for cancer detection. Their pathological presence in the serum may stem from the loss of epithelial polarity during cancer transformation [259]. Loss of epithelial polarity results in aberrant secretion of various glycoproteins such as mucins into the serum. Currently, several tumor-associated O-GalNAc glycoproteins are being used in serological assays in clinics. These include MUC1 (CA15-3) and MUC16 (CA125), as well as, O-glycan $SLe^a$ (CA19-9 are used in breast, ovarian and pancreatic cancer monitoring respectively. S-Tn on TAG-72 (CA72-4) serves as a pan-carcinoma marker and is used concurrently with other tests in cancer screening.

All these biomarkers, however, suffer from lack of sensitivity, especially in early-stage disease, and insufficient specificity for diagnosis as they can show elevated amounts in non-neoplastic conditions. For instance, MUC1 and MUC16 levels show lack of sensitivity in early detection of breast cancer [260] and ovarian cancer [261] respectively. Combined detection of different markers has shown somewhat better diagnostic specificity and sensitivity [262,263], but use of a single marker is simpler to interpret and remains the preferred choice in clinics [264–266]. Thus, more recent attempts involve targeting cancer-associated O-glycoforms. The principle was proven with sandwich ELISAs and antibody capture array assays that incorporate measurement of various O-glycoforms of MUC16 in circulation. S-Tn-glycosylated MUC16 showed improved specificity for ovarian cancer compared to measuring MUC16 levels alone [267,268], although the sensitivity did not increase significantly, limiting the use in early diagnosis. This might be resolved by further differentiation of distinct MUC16 O-glycosite forms.

### 5.3. Biomarker detection in tissue biopsies

Part of diagnosis, treatment management and intra/postoperative assessment requires staining solid tissue biopsies and tumor imaging. Immuno-staining of biopsies is currently the primary method for cancer diagnosis. To improve sensitivity, proximity ligation assays (PLA) have been applied on tissue sections [269]. As PLA allows combined detection of both protein and glycan features that are in proximity, it was used to detect different O-glycoforms of mucins. Using PLA, S-Tn-glycosylated MUC2 was observed to be upregulated in all intestinal metaplasia and most gastric carcinoma cases [270]. Tn- and S-Tn-MUC1 glycoforms were identified in a large proportion of mucinous ovarian carcinomas [271]. More recently, improved specificity (100%) and sensitivity (~80%) was achieved through PLA reactions for Tn- and S-Tn-MUC16 and MUC1 glycoforms for tissue screening in ovarian serous neoplasia [272].

Tumor imaging requires radiopharmaceuticals such as immuno-radionuclides that requires specific antibodies or antibody fragments to assess tumor properly. Several antibodies targeting Tn-, S-Tn-, TF- and S-TF-glycosylated proteins have been developed and used in both rodents and humans [273]. Interestingly, a humanized mAb CC49, that targets S-TF and S-Tn on TAG-72, has been found useful radio-guided immunosurgery in colorectal cancer [274] and is currently in clinical trial.

### 5.4. Detection of tumor-specific auto-antibodies in patients

The discovery that tumor formation elicits autoantibodies has prompted attempts to use them as biomarkers for tumor diagnosis [275]. Autoantibodies might possibly arise at early stages of the disease, so they could potentially be used to diagnose malignancy when there are no detectable tumors and clinical signs. These antibodies can also

indicate the localization of a tumor lesion if the targeted protein is organ-specific [276,277].

To identify these autoantibodies, assays based on expressed cDNA libraries [275], protein and peptide arrays [278–280], and phage arrays [281] have been developed that aim at detecting different tumor types, including breast [280,282–284], colorectal [285] and lung cancer [286–288],These approaches focus predominantly on screening the proteome and were found to bind to several intracellular and cell membrane proteins such as p53 [289] and MUC1 [290] respectively.

Early evidences from Springer indicate the existence of Tn and TF glycan-specific immune responses resulting in anti-glycan antibodies [28]. Cancer-specific human IgM autoantibodies that targeted glycan epitopes [291] and protein O-glycoforms [292] were later identified. Recently, sera from prostate, breast and ovarian cancer patients were screened for autoantibodies produced against tumor-associated O-glycans with a panel of O-glycopeptides. Glycopeptide-specific IgG against Tn-, S-Tn-, TF-, and core 3-glycosylated MUC1 glycopeptides could be detected in a number of patients [293]. A subsequent study with a larger array of O-glycopeptides of MUC1 and MUC4 screened the sera of colorectal cancer patients and healthy individuals in a blind case-controlled manner, and IgG autoantibodies against S-Tn- and Core3-MUC1 were detected in colorectal patients with relatively high specificity. The sensitivity increased when the data was combined with the p53 autoantibody signature, improving early colorectal cancer detection [294]. Autoantibody arrays could also be used in cancer prognosis. A number of breast cancer patients with elevated autoantibodies against S-Tn- and Core3-MUC1 show increased survival rates [295].

### *5.5. Targeting O-GalNAc glycans for immunotherapy in cancer therapeutics*

mAb-based treatment has been among the most successful therapeutic strategies for cancer in the last 20 years [296]. Current cancer immunotherapies involve the use of unconjugated antibodies with intrinsic cytolytic activity, radionuclide-conjugated antibodies (radioimmunotherapy) and toxin-conjugated antibodies (immunotoxins). The unconjugated IgG antibodies can bind with high affinity to target tumor cells and induce their destruction through complement release (complement dependent cytotoxicity (CDC)) or killing by NK cells (antibody-dependent cell-mediated cytotoxicity (ADCC)).

Extensive efforts have been made to develop specific antibodies and therapeutic vaccines to evoke the immune system to target tumor-associated O-glycans. A variety of antibodies have been produced against tumor-associated O-glycoforms Tn, S-Tn and mucins. Some show potential applications in therapy, exhibiting ADCC-dependent and independent cytolytic activities, and several have been evaluated in vivo [297–303]. For instance, anti-S-Tn 3P9, an IgM mAb derived from human colorectal adenocarcinoma SW1116 cell immunization, inhibited proliferation and migration of S-Tn expressing colon carcinoma cells and blocks tumor growth by inducing apoptosis [304]. Various mAbs, such as KM3413, MLS128 and GOD3-2C4 have been also shown to inhibit the cell growth of cancer cell lines expressing Tn antigen. GOD3-2C4 also inhibits the growth of lung carcinoma xenografts in vivo, altogether suggesting the potential applications of these mAbs in therapy [303,305].

As for other therapeutic antibodies, anti-glycan antibodies have to target a highly tumor-specific antigen that is not expressed in normal cells to avoid toxicity. An immunotoxin antibody specific for $Le^y$ showed dose-dependent toxicities, which is likely due to targeting of normal tissues where low levels of $Le^y$ could be observed [306–308].

### *5.6. Therapeutic vaccines against O-GalNAc glycans*

Vaccines for the prevention and treatment of cancer have been proposed over the years, albeit being controversial for a long time. Cancer preventive vaccines serve to reduce cancer risk in healthy individuals and the three approved by FDA include Gardasil and Cervarix for cervical cancer, and hepatitis B vaccine for liver cancer. Cancer treatment vaccines act as immunotherapy for cancer patients. Sipuleucel-T (Provenge®) is the first FDA-approved cancer treatment vaccine used for metastatic prostate cancer [309]. Other vaccines such as Vitespen (Oncophage®) for kidney cancer and gp100 peptide vaccine for melanoma treatments are in clinical trials [310,311]. These studies validate therapeutic vaccines as a feasible approach for cancer treatment.

Early tests by Springer involved vaccination with TF/Tn-RBC. Results suggest elicited anti-glycan immune responses and improved patient survival after vaccination [34,312]. Since then, many attempts were carried out to develop vaccines with these glycan antigens. Studies have shown that immunization with S-Tn or Tn-containing proteins/peptides induces glycan specific antibodies in mouse and human [313–317]. Slower tumor growth in mice and cancer progression in patients were observed in some studies [315,316]. The vaccine S-Tn conjugated to KLH protein (Theratope®) stimulates anti-S-Tn antibody production [318] which correlates with disease-free survival [319]. However, these observed immune responses were not robust enough to trigger T cell-mediated immunity; hence, the clinical trials with Theratope remain inconclusive.

To improve the immunogenicity of the antigens, carbohydrate-based vaccines (e.g. GM2, Globo H, $Lewis^y$, TF(c), Tn(c), S-Tn(c), etc) or glycopeptide-based vaccines (e.g. Tn-MUC1, Tn(c)-MUC1) conjugated on different carriers (e.g. KLH, BSA, polystyrene) have been compared for their ability to trigger immune response in mice [317,320]. In the initial study, Tn-MUC1 induced high titers against breast cancer cell line MCF7 [317]. The subsequent study revealed that the carbohydrate-based vaccines induced mainly short-term IgM responses while MUC1 glycopeptides led to long-term IgG responses, particularly the glycoform with triple Tn (Tn(c)-MUC1) [320]. Increasing the number of O-glycans on MUC1 glycopeptide seems to further improve the immunogenicity of the vaccines as the glycopeptides with five Tn showed the strongest response. Vaccination of MUC1 glycopeptides with complete O-glycan occupancy elicited high IgG levels to the MUC1 from human breast cancers but not to MUC1 from normal cells [191].

Although numerous attempts have been made, most vaccines remain insufficient to induce strong T-cell dependent pathways for prolonged immunity against these tumor antigens. None of the vaccines against tumor-associated O-glyco-antigens have been approved for the clinics at present. Further research to characterize glycoforms of O-glycoproteins and to understand how the immune system reacts to these antigens would aid design of a vaccine with better immunogenicity and potency.

### *5.7. Relevance of the GALA pathway in cancer diagnosis and therapeutics*

As described above, up until recently, high Tn in tumors was thought to reveal a general shortening of O-GalNAc glycans, leading to a focus on exploiting these short glycans for therapeutic purposes. By contrast, in the context of GALA, Tn structures are mostly present in the ER and thus not directly accessible by antibodies or the immune system. On the other hand, GALA is promoting glycosylation initiation events that occur otherwise at low frequency, therefore suggesting the possibility that tumors display relatively tumor-specific glycoforms of secreted and cell surface proteins. The challenge will be to identify the newly formed glycosites and to characterize the glycan attached to them. With this approach, it might be possible to identify leads for novel cancer biomarkers.

As the expression of GALNTs can be tissue- or cell type-specific, the GALA induced O-glycoproteomes could be to some extent tissue-specific as well. Further research comparing these O-glycoproteomes could thus point to tissue and/or disease specific glycosites. These glycosites could be used to generate new antibodies for improving diagnosis as well as providing new therapeutic targets. Similarly, potential

novel GALA-specific glycopeptides could provide greater specificity in cancer vaccines. In addition, since specific GALNTs appear correlated with specific cancer subtypes and may be driving factors, it may be interesting to consider drug therapies targeting specific GALNT isoforms themselves.

It would be important, however, to explore the normal physiological role of the GALA pathway, as it is likely that GALA is a tightly regulated pathway co-opted by cancer cells. In the long run, the efficient targeting of O-GalNAc glycans for therapeutic purposes will also require further fundamental advances in our understanding of the regulatory mechanisms of both the initiation and extension of O-GalNAc glycosylation.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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