Glycosylation as a regulator of site-specific metastasis

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Abstract

Metastasis is considered to be responsible for 90% of cancer-related deaths. Although it is clinically evident that metastatic patterns vary by primary tumor type, the molecular mechanisms underlying the site-specific nature of metastasis are an area of active investigation. One mechanism that has emerged as an important player in this process is glycosylation, or the addition of sugar moieties onto protein and lipid substrates. Glycosylation is the most common post-translational modification, occurring on more than 50% of translated proteins. Many of those proteins are either secreted or expressed on the cell membrane, thereby making glycosylation an important mediator of cell-cell interactions, including tumor-microenvironment interactions. It has been recently discovered that alteration of glycosylation patterns influences cancer metastasis, both globally and in a site-specific manner. This review will summarize the current knowledge regarding the role of glycosylation in the tropism of cancer cells for several common metastatic sites, including the bone, lung, brain and lymph nodes.

Keywords

INTRODUCTION

Metastasis is estimated to be responsible for 90% of cancer mortality[1], and therefore represents a pressing clinical issue. However, in spite of significant research efforts, this process remains poorly understood, and there are few treatment options for patients who present with metastatic disease.

Common sites of metastasis include the liver, lymph nodes, bone, lung, and brain. One autopsy study of a cohort of patients with metastatic cancer of many types found that 59% had liver metastases; 53% had non-regional lymph node metastases; 44% had lung; 38%

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had bone, and just over 20% had brain metastases[2]. Rates for each specific secondary site differ among primary tumor types[2]. One particularly perplexing issue is that of site specificity: that is, why do secondary tumors preferentially develop in particular distant organs? A plethora of hypotheses of have been proposed, including differences in chemotactic factors, adhesive factors, and immune infiltrate, as well as host factors such as age and overall health status [3, 4], but the organotropism of metastasis remains a highly active and poorly understood area of research.

Glycosylation, the addition of sugar moieties onto various substrates, is currently a subject of much interest for its potential role in metastasis. There are thousands of glycosylated entities in cells, many of which are either secreted or present on the cell membrane [5]. Additionally, many cell-cell adhesion molecules, and molecules involved in intercellular communication, are highly glycosylated[6], making glycosylation of substantial interest when studying metastasis and the many cell-cell interactions it requires. Glycosylation can be modified at many points, as summarized in Figure 1. Alterations to all of these points have been identified in both primary and metastatic disease. Altered glycosylation is particularly important during the metastatic cascade. These more general roles have been covered extensively in previous reviews (see, for example [5, 7–9]), and will be discussed only briefly here. More recently, glycosylation has emerged as an important regulator of the organotropism of metastasis. The impact of aberrant glycosylation on cancer development is quite broad and consideration of all putative roles is beyond the scope of a single article. Therefore, this article focuses mainly on altered glycosylation in bone (section 2.1), lung (section 2.2), brain (section 2.3) and lymph node (section 2.4) metastasis, for which the evidence of a role for glycosylation in tumor progression is best developed. Other secondary sites are briefly discussed (section 2.5); however, there is a paucity of studies focusing on glycosylation in metastasis to other organs. Finally, the role of glycosylation in circulating tumor cell biology is briefly discussed in section 2.6. In particular, we emphasize scenarios for which there is evidence of a glycosylation-dependent mechanism rather than simply observations of differential glycoprotein abundance. Within these parameters, we summarize current knowledge and identify possible targets for future study regarding the role of glycosylation in directing the site specificity of metastasis.

1. OVERVIEW OF GLYCOSYLATION AND ITS REGULATION

Glycosylation can occur on many types of biological molecules, including lipids, DNA and small organic molecules, but proteins are the most common substrates [10, 11] and the major focus of this review.

Glycosylation can take many forms. Nine of the 20 amino acids found in proteins can be potentially glycosylated, and there are 10 major monosaccharide building blocks found in mammals, all of them manufactured from glucose, which can be utilized in glycan chains [12] (Table 1). A glycosylation event can be characterized based on the type of sugar attached, the location of the attachment, the substrate receiving the sugar, and the enzyme performing the reaction. There are four main types of glycan addition to form glycoproteins: N-glycosylation, O-glycosylation, addition of glycosylphosphatidylinositol (GPI) anchors, and addition of glycosaminoglycan chains to form proteoglycans[10].

Glycans can themselves be modified by phosphorylation, sulfation, methylation, acylation, acetylation, and pyruvylation [13]. The typical processing of proteins into glycoproteins occurs in the endoplasmic reticulum (ER) and Golgi complex [11, 14]. Depending on the type of addition, the exact sequence and process of addition will vary. Selected cancer-relevant glycosylation events are summarized in Table 1 and Figure 1. The precise biochemical details of glycosylation are outside the scope of this review, but have been covered extensively elsewhere; for example, see [8, 11, 13, 15–17].

The effects of glycosylation are highly context-specific, depending on the organ or tissue in which the process occurs and the developmental stage of the organism. Additionally, there is substantial variability in glycosylation-related events between species [18]. Interestingly, many glycan deletions have little effect *in vitro*, but are lethal in a whole organism, indicating that important glycan functions are largely interaction-dependent rather than intrinsic [19]. Since the addition and processing of glycans is quite complex, it can be difficult to localize the exact cause of a change in glycosylation, but it is clear that a relatively small alteration in any step of the process can have serious consequences for the cell.

Alterations in glycosylation have emerged as a significant player in malignant transformation and tumor progression. These changes have been extensively reviewed elsewhere [20–24]. Briefly, abnormal glycan expression assists with invasion through the basement membrane; comprises a significant portion of cancer-associated extracellular matrix alterations [25]; contributes to tumor-induced immunosuppression [26] and drug resistance [7, 27]; increases growth factor sensitivity[22]; promotes epithelial-to-mesenchymal (EMT) programs [25, 28, 29]; encourages the formation of "tumor emboli" (cancer cell-platelet complexes) which help tumor cells survive in circulation [20]; enables cancer cells to engage in "rolling" behavior characteristic of lymphocytes[22], which is required for extravasation; and facilitates their interaction with the destination microenvironment[7, 20, 25, 30]. Selected glycoproteins and glycan structures which are strongly associated with metastasis are summarized in Table 2 for reference.

2. THE ROLE OF GLYCOSYLATION IN SITE-SPECIFIC METASTASIS

Locations of metastasis vary greatly according to primary tumor type [31, 32]. Over the years, many hypotheses have been advanced regarding the reasons behind metastatic preferences, including Ewing's "mechanical trapping" theory [33] and Paget's "seed and soil," which suggests that tumors must find a hospitable environment in order to grow [34], as well as more recent ideas about establishment of a premetastatic niche [4, 32], inflammation [4], and the contribution of host factors such as age, genetic factors, and co-morbid health conditions[4]. These theories are not mutually exclusive and we now appreciate that multiple factors can influence the patterns of metastasis. Still, identification of targetable regulators has proved elusive. Recent research has identified glycosylation as a potentially important mediator of site-specific metastasis, which raises the possibility of defined therapeutic interventions. This section provides an overview of those findings in the context of several common metastatic destination organs (summarized in Figure 2). In order to provide a more comprehensive discussion and identify possible commonalities

between tumor types, we focus on metastatic destination rather than tumor of origin. The role of glycosylation specifically within various primary tumor types has been extensively reviewed; see, for example, [35, 36] (brain), [37] (breast), [38, 39] (lung), [40] (pancreatic) and [41] (prostate), among others.

2.1 Glycosylation and bone metastasis

Bone metastasis is a frequent metastatic event for many cancer types, both early and late. It is also a source of significant patient morbidity due to pain, tumor-induced fracture and mobility loss [42]. As originally noted by Stephen Paget in the original 'seed and soil' hypothesis [34], and confirmed in more recent studies, breast cancer has particular affinity for bone[42]. We now know that altered glycosylation appears to play a significant role in multiple stages of metastasis to bone. Several studies have identified specific enzymes or glycoproteins that associate with development of bone metastases in clinical cohorts. Many such studies have been mostly concerned with identification of biomarkers; for example, Freire *et al.* (2006) identified ppGalNAc-T6, one of a family of enzymes that catalyze the first step in mucin-type O-glycan synthesis, as a negative prognostic marker for disease-free survival when found in the bone marrow of early-stage, node-negative breast cancer patients. There were no mechanistic analyses, but the researchers hypothesized that the expression of ppGalNAc-T-6 might play a role in altered O-glycan expression, especially the Tn antigen, in breast cancer [43]. Another example is bone sialoprotein (BSP), a secreted and heavily sialylated and phosphorylated protein produced by osteoblasts, osteoclasts, osteocytes, and chondrocytes. Normally, it promotes initial mineral crystal formation in bone and teeth. A form of BSP exhibiting truncated O-glycosylation (hypoBSP) is particularly associated with breast cancer metastasis [44]. In rat mammary cancer models, hypoBSP was strongly expressed in skeletal lesions but only weakly in primary tumors. A neutralizing antibody specific for hypoBSP induced remission in 80% of animals, with activity restricted to skeletal lesions. Additionally, in a cohort of 11 primary tumors and skeletal metastases from breast cancer patients, bone metastases had a higher average hypoBSP staining score than primary tumors [44], suggesting that this aberrant glycoform is biologically and therapeutically relevant for breast cancer skeletal metastases. These studies provide evidence that aberrant expression of several glycosylation-related species (a glycoprotein, a glycosyltransferase, and a glycan, respectively; see Figure 1) has a measurable impact on bone metastatic behavior. However, these studies, and others like them, often lack a mechanistic component, so it remains unclear how the abnormal glycosylation phenotype contributes to site-specific metastatic tendencies.

A small number of studies have probed the mechanism underlying the contributions of glycosylation to bone metastasis. These contributions are diverse, affecting all different stages of metastasis, and may vary according to the origin of the primary tumor. For example, heparanase, the enzyme which cleaves the proteoglycan heparan sulfate (see Table 2) increases homing of multiple myeloma cells to the bone in mice [45], which led to speculation that it may contribute to bone metastasis of solid tumors as well. It was observed that in a breast cancer model, heparanase did not directly increase bone metastasis, but instead elevated osteolytic activity [46]; the authors of that study hypothesized that release of extra growth factors from the resorbed bone may create a pro-tumor pre-metastatic niche

within the bone. In prostate cancer bone metastasis, sialylation appears to be extremely important for progression. Global levels of $\alpha2,3$-sialylation are elevated in bone-metastatic as compared to a non-invasive prostate cancer cell lines, and hypersialylation of the $\alpha2$ subunit of integrin $\alpha2\beta1$ increases cell adhesion capacity *in vitro*. Simultaneously, however, bone-metastatic prostate cancer cells express an abnormally glycosylated form of the glycosphingolipid GM1 called asialo-GM1 (AsGM1), which lacks an $\alpha2,3$-sialylation mark. Mechanistic work reveals that AsGM1, but not fully-sialylated GM1, complexes with integrin $\alpha2\beta1$ [47] via the same sialylated moiety that promotes adhesive interactions [48], and the formation of these complexes promotes invasive cell behavior [47]. Further *in vitro* work suggests that hypersialylated $\alpha2\beta1$ is important for initial attachment of the prostate cancer cells, while the AsGM1-integrin complexes participate in longer-term tethering interactions to hold them there[48]. In contrast, a recent study in breast cancer [49] showed that MDA-MB-231 cells engineered (via forced expression of ST6GalNAc1) to express high levels of sialyl-Tn (sTn) (see Table 2), display reduced bone metastasis, and less osteolytic activity *in vivo*. *In vitro*, they show reduced capacity for adhesion to fibronectin or collagen I, and reduced interaction with bone marrow stem cells. S-Tn is carried primarily on integrin $\beta1$, thus in contrast to the facilitative role played by elevated sialylation in the context of integrin $\alpha2\beta1$ in prostate cancer, the presence of excessive sTn antigen impairs the adhesion capabilities of the MDA-MB-231 cells, possibly through causing integrin dysfunction. In sum, these studies demonstrate that alterations in glycosylation can have mechanistic impacts at various stages of metastasis. However, these effects are highly nuanced; even a modification which is traditionally considered pro-metastatic (such as hypersialylation [24]) may not be in all contexts, and the pertinent mechanisms vary according to tumor type.

One glycoprotein whose relevance for bone metastasis has been exhaustively investigated is E-selectin, a glycoprotein expressed on endothelial cells that is primarily known for its involvement in leukocyte rolling and adhesion [50]. Interactions of E-selectin with its ligand sialyl-Lewis[X] have been observed to promote metastasis for decades [50–53]. A 2008 study in prostate cancer, which primarily (90%+) metastasizes to the bone, showed that extravasation of cancer cells is mainly mediated by E-selectin ligands expressed by the tumor cells, interfacing with E-selectin on the bone endothelial cells; preferred ligands include PSGL-1 and sLe[X] [54]. Another study found that sialyl-Lewis[X] (sLe[X]; see Table 2) is associated with bone metastasis in estrogen receptor (ER)-positive, but not ER−, breast cancer. In *in vitro* mechanistic studies, adherence of the ER+ cancer cells to endothelial cells under shear stress conditions is mediated by sLe[X]-E-selectin interaction; the authors hypothesize that this interaction may be important for bone metastasis given that E-selectin is constitutively expressed in the bone [55]. ER− cells, despite expressing sLe[X] and of the enzymes required for its synthesis at higher levels than ER+ tumors, appear to interact with other selectins primarily via heparan sulfate (HS) chains, which the cells also express at elevated levels, and do not bind to E-selectin under those *in vitro* conditions. The authors hypothesize that the context of glycan expression (i.e., ER+ vs. ER−) may be important for determining its relevance to tumor biology, and further, that ER+ tumors may express sLe[X] primarily on glycoproteins, while ER− tumors may incorporate it primarily into glycolipids, which would alter the functional consequences of its expression for the cell.

Entry of cancer cells into the bone marrow takes place through the sinusoidal vasculature, which expresses E-selectin and SDF-1 (the ligand for CXCR4) at high levels. Price *et al.* [56] identified genes associated with late relapse of breast cancer, which predominantly metastasizes to the bone, in a patient cohort. They identified seven genes, including several glycoproteins (CXCR4, CD44, SELPLG (PSGL-1), Lysosome-Associated Membrane Glycoprotein 1 [LAMP1], and the E-selectin ligand Golgi glycoprotein 1 [GLG1]) and glycosyltransferases (β-1,4-Galactosyltransferase 6 [B4GALT6], FUT4). This group was specifically interested in the trafficking of breast cancer cells through the sinusoidal vasculature during early bone metastasis. They find that E-selectin-mediated interactions are critical for initial entry of the cancer cells into the bone and interaction with the vasculature. In contrast to the findings from Julien *et al.* [55], E-selectin influenced bone homing in both ER+ and ER− breast cancer, though they note that ER+ and ER− cells express different subsets of E-selectin ligands. Importantly, this group confirmed E-selectin-mediated trafficking of breast cancer cells in the bone in human-in-mouse models derived from patient tumors, suggesting that these mechanisms are in fact relevant to the human disease process.

Esposito *et al.* (2019) further investigated the ability of E-selectin/Glg1 interactions to promote bone metastasis in mice [57]. E-selectin-null mice showed decreased bone metastatic burden from a bone-metastatic subline of human MDA-MB-231 breast cancer cells, but lung metastases were unaffected. In human ER− breast cancer, tumor Glg1 status correlated with bone metastasis-free survival, but not with metastasis-free survival to lung, liver, or brain. In the mouse model, Fut3 or Fut6-driven fucosylation of Glg1 was identified as the relevant glycosylation event on Glg1 for bone metastasis. The authors speculate that the importance of E-selectin is independent of its classic role in arresting tumor cells within the blood, and instead relates to induction of specific signaling cascades when the tumor cells interact with the bone vasculature. A complex feedback loop involving E-selectin binding, Glg1 fucosylation by Fut3/6, and Wnt pathway activation appears necessary to permit colonization and tumor expansion [57]. This provides an excellent example of the complexity of changes that can result from a relatively simply change in glycosylation activity, as well as demonstrating that glycosylation can in fact act in an organ-specific manner to promote metastasis. Of note, and in contrast to many earlier studies, these data suggest that the role of the E-selectin/Glg1 interaction is important for the later stages of metastasis, specifically colonization and outgrowth. One consideration in evaluating these studies is that there are species differences in selectin-ligand interactions; in particular, mouse E-selectin shows a wider diversity of potential ligands than human E-selectin[18]. For this reason, this mechanism will require additional investigation to verify clinical relevance.

It is clear that altered glycosylation can influence bone metastasis, both in terms of interaction with the bone vasculature for early-stage extravasation and seeding, and later colonization and outgrowth. To date, studies have identified a number of possible targets in various tumor types, including altered expression of glycoproteins or glycosyltransferases, and expression of abnormal glycan epitopes. As demonstrated by Esposito *et al.*, relatively small changes in just a few glycosylation events and/or glycosyltransferase expression can have significant, multifaceted consequences for cancer cell behavior. Moreover,

glycosylation-related aberrations may represent druggable targets. However, there exists substantial heterogeneity in the literature (which likely reflects biological heterogeneity in addition to technical differences), and detailed mechanistic investigations have only begun in the last few years. Additional substantial mechanistic work is necessary to rigorously establish targetable alterations.

2.2. Glycosylation and lung metastasis

The lung microenvironment is fertile soil for both primary lung tumors and metastatic tumors from other organs. Studies have identified a number of glycoproteins (e.g. lipocalin-2, podoplanin, tenascin C, von Willebrand factor) whose overexpression is associated with lung-specific metastases [58–61]. However, it has not yet been established whether altered glycosylation of these glycoproteins is relevant to their role in metastatic progression.

Procollagen C-proteinase enhancer protein (PCOLCE) is a potential mediator of lung metastasis identified using osteosarcoma models. It is a secreted glycoprotein with a known role in ECM remodeling, and is associated with poor prognosis in osteosarcoma. Osteosarcoma cells with shRNA-mediated PCOLCE knockdown showed reduced invasion and migration but not proliferation *in vitro*, and reduced lung metastasis in an orthotopic mouse model [62]. Importantly, the authors of this study identified an N-glycosylation site at Asn29 on PCOLCE which appeared essential for protein stability and secretion into the ECM. When osteosarcoma cells were transfected with either wild-type (WT) or mutant PCOLCE lacking the glycosylation site, those that received the mutant form showed impaired migratory and metastatic phenotypes. This is a relatively rare example of how glycosylation of a specific residue impacts metastatic function, but represents a good model of the type of mechanistic study needed to fully understand the role of protein modifications. However, it lacks clarity on whether this modification is important only for lung metastasis.

Other potential mediators of lung metastasis have been identified using different tumor models. The presence of β1,6-N-linked-oligosaccharides on the cell surface is a change linked with invasion and metastasis in many tumor types that preferentially metastasize to the liver and the lung[63, 64]. The site preference may be related to the possibility for several other modifications on the oligosaccharide branches (addition of Lewis antigen, α2,3-sialic acids, polylacNAc, and others) that have affinity for receptors found in the lung, such as E-selectin and galectins. Using a B16 melanoma model, which was previously shown to metastasize specifically to the lungs independent of mechanical or anatomic cues [65, 66], Krishnan *et al.* [67] investigated the role of β1,6-N-linked-oligosaccharides in lung-specific melanoma metastasis. In B16F10 cells, the lysosomal protein LAMP1, was identified as the substrate for a polylacNAc modification that led to interaction with galectin-3 on lung vasculature [67]. Further investigation revealed that it is polylacNAc substitution specifically on β(1,6)-N-oligosaccharides, and not on O-glycans or T/Tn antigens, that is responsible for the increased binding of galectin-3 to highly metastatic cells and lung metastatic capacity [68]. Interestingly, some previously identified cancer-associated species, such as sLe[X] (in contrast to [69]) and E-selectin (in contrast to [70]), did not influence lung metastasis in these studies [67]. It should also be noted that a subsequent

study by this group found that whole-body loss of galectin-3 failed to attenuate lung metastasis. The authors suggest that, since galectin-3 is important for the maturation and function of many immune cell types, knock-out mice have impaired antitumor immunity which prevents any decrease in lung tumor burden [71]; more nuanced studies would be necessary to dissect the exact role of galectin-3 in lung-specific metastasis. Together, the studies highlighted here indicate that specific glycosylation events regulate lung metastasis, via several mechanisms that include interactions with the vasculature and general ECM remodeling. However, a major weakness of much of this work is that the data do not establish whether these candidates mediate lung metastasis specifically, or the metastatic process more generally. Further studies investigating this question will be needed in order to fully understand the biology of lung-specific metastasis, as well as to identify appropriate targets for therapeutic development.

As with bone metastasis, the roles of selectins and their ligands in lung metastasis are some of the more thoroughly investigated glycosylation-relevant mechanisms. Very early studies on the mediators of lung colonization also suggested that E-selectin/sLe^X interactions were important for a lung colonization phenotype [72], though this should be interpreted with substantial caution given the age and technical limitations of this study. A more recent study linked E-selectin to promotion of breast to lung metastasis [70]. This, however, contrasts with the findings of Esposito *et al.* [57] who subsequently suggested that lung metastasis is unaffected by E-selectin deletion as described above. This is likely to be due to a difference in models; while Esposito *et al.* compared E-selectin knock-out and wild-type mice, Jiang *et al.* [70] observed overexpression of E-selectin on lung vasculature following LPS-induced systemic inflammation, and attenuation of this overexpression prevented an inflammation-associated increase in breast cancer lung metastasis. Since E-selectin expression is quite low in the lungs under standard physiological conditions [57, 70], it may be that it does not influence lung metastasis in absence of significant inflammation. Other researchers developed a therapeutic agent targeting sLe$^{X/A}$, called per-O-acetylated GlcNAcβ1,3Galβ-O-naphthalenemethanol (AcGnG-NM)[69, 73], which they tested in a mouse model[69]. AcGnG-NM acts as a synthetic decoy of an intermediate in sLe^X biosynthesis, thereby preventing cells from decorating glycoproteins with sLe^X and thus attenuating the selectin-ligand interaction that contributes to metastatic spread. Murine Lewis lung carcinoma (LLC) cells positive for sLe$^{X/A}$ bound to constructs containing P-selectin but not L- or E-selectin, and this was blocked by AcGnG-NM. Mice subcutaneously injected with LLC cells and subsequently treated with AcGnG-NM demonstrated a 3-fold decrease in the micrometastatic burden in the lung. Since the primary tumor size was not affected, this appears to be a metastasis-specific impact[69].

Sialylation represents an additional glycosylation-related process which is modified in both lung and bone metastasis. Because of the pleiotropic roles of sialylation in metastasis [24, 27], Büll *et al.* [74] tested the efficacy of P-3Fax-Neu5Ac, a peracetylated analogue of sialic acid that acts as an inhibitory glycomimetic, for reducing metastasis in a mouse model of melanoma. They found a significant reduction of lung metastases after tail vein injection in the treatment group, either when using cancer cells pre-treated with the inhibitor, or when mice were treated with melanoma-targeted (anti-TRP1) nanoparticles carrying the inhibitor. The nanoparticle-conjugated formulation demonstrated prolonged inhibition of sialylation

in vitro. In vivo, mice were pre-treated with nanoparticles one hour before receiving tumor cells via tail vein injection, and treated a second time with the nanoparticles approximately 16h later; this brief treatment course was sufficient to reduce lung colonization at 14 days post-injection. In this model, they did not observe metastases in other organs, so it is difficult to know whether this effect is truly lung-specific, but it does demonstrate the potential of targeting abnormal glycosylation to treat metastasis. In a follow-up experiment, the researchers investigated whether there was a difference between growth inhibition in melanotic and amelanotic (which downregulate TRP-1) lesions; they found that treatment with the targeted nanoparticles carrying the glycomimetic specifically inhibited development of melanotic lesions, and that the glycomimetic increased both TRP-1 expression and nanoparticle uptake in the melanotic lesions, suggesting a potential positive feedback loop that could be therapeutically useful.

In addition to identification of glycosylated targets, progress has been made in identifying critical enzymes. Polypeptide N-acetyl galactosaminyl transferases (GALNTs) are a family of enzymes responsible for O-linked N-acetyl galactosamine (GalNAc) addition. Song *et al.* (2016) identified GALNT14 as a mediator of breast to lung metastasis[75] and dissected multiple ways that GALNT14 activity could enhance metastatic behavior. According to patient microarray data, GALNT14 is the only GALNT family member whose expression in primary breast tumors was significantly associated with distant metastasis-free survival. Specifically, patients with GALNT14 expression in their primary tumors had higher risk of metastasis to lung but not brain or bone. Knockdown of GALNT14 in lung-seeking breast cancer sublines reduced both O-GalNAc glycosylation and metastatic spread to the lungs in a mouse model, which could be rescued by restoring GALNT14 expression. Overall, the authors suggested that KRas/PI3K stimulated, c-Jun driven upregulation of GALNT14 results in a survival advantage during the micrometastatic phase by making cells resistant to anti-metastatic BMP signaling, potentially via inactivating O-GalNAcylation of the BMP receptor. As the tumor reaches the macrometastatic phase, GALNT14 enhances CXCL1 production through an as yet unknown post-translational mechanism which recruits macrophages to help form a permissive environment, and also renders the cancer cells more receptive to macrophage-derived FGF growth signaling [75]. Importantly, this paper demonstrates that change in one glycosylation regulator can contribute to multiple stages of metastasis tailored to the microenvironment of a specific organ.

Overall, the current data indicate that aberrant glycosylation is relevant for lung metastasis. Some mediators – notably E-selectin and sialylation – are undoubtedly shared with other metastatic sites. However, it does appear that the precise interaction partners and mechanisms underlying these effects are frequently organ-specific, which again supports the hypothesis of site-specific metastatic mechanisms, and gives credence to the idea that the highly contextual nature of glycosylation and its alterations make it a promising target of study for this purpose.

2.3. Glycosylation and brain metastasis

Brain metastasis represents a serious clinical challenge due to significant associated morbidity and mortality and lack of effective treatment options. Its incidence is on the

rise, likely as a consequence of better primary tumor control and improved imaging techniques[76]. Unlike other metastatic sites, where the study of the impact of glycosylation remains in its early stages, glycosylation and glycogene changes are well-established in the brain setting; it was established in the 1980s and 1990s that brain tumors show aberrant ganglioside expression and shedding (summarized in [77]), and altered glycosphingolipid expression. Initial studies focused on primary brain tumors. These are reviewed by Moskal *et al.* [35] and will not be repeated here. A number of glycoproteins also seem to mediate brain metastasis (see, for example, [78–82]). As noted for glycoproteins that mediate metastasis at other sites, however, altered glycosylation has not yet been specifically linked to their pro-metastatic functions.

More recent studies have begun to elucidate roles for glycosylation enzymes in brain metastasis. Pangeni *et al.* [83] identified GALNT9 (encodes GALNAC-T9, a transferase responsible for the initial step in mucin O-glycan synthesis [Table 1, Figure 1]) as heavily methylated in brain metastases but infrequently methylated in primary breast tumors. Loss of protein expression was associated with worse prognosis for breast cancer patients. This suggests that impairment of O-glycosylation, the result of GALNT9 loss, may play a role in breast-to-brain metastasis. Another enzyme, the α2–6 sialyltransferase ST6GALNAC5, was identified in metastatic breast cancer cells in a landmark paper by the Massagué group [84]. Affinity testing using a lectin that binds α2,6-sialylated groups revealed substantial binding in both mammary tumors and brain lesions formed by brain-metastatic BrM2 cells in mice, as well as in 6 out of 12 of human brain metastatic patient samples tested, but low or no binding in patient lung metastasis samples. Modulation of ST6GALNAC5 confirmed that its role is exclusive to brain metastasis, and that it specifically impacts infiltration of cancer cells through the blood-brain barrier. Importantly, transduction of a lung-metastatic derivative of MDA-MB-231 breast cancer cells with ST6GALNAC5 resulted in increased brain micrometastases, suggesting that ST6GALNAC5 is important for the initial invasion of the brain, while other unknown mediators are necessary for growth into macrometastases [84]. Thus, those authors suggest that glycogenes with restricted expression patterns might be important organ-specific mediators of metastatic colonization and emphasizes that mediators may be stage- as well as organ-specific. It should be noted that another group attempted to verify this finding using an *in vitro* BBB model derived from human cell lines and the BrM2 cell line [85]; in that model, BrM2 cells and cells engineered to overexpression ST6GALNAC5 specifically demonstrated reduced adhesion to the BBB and no change in transmigration capacity. The authors suggest that this is due to the use of a human-on-human model rather than a human-in-mouse model as in the Massagué study, and hypothesize that the relevance of ST6GALNAC5 may stem from adaptation of the human cancer cells to the mouse brain environment. Regrettably, this hypothesis was not investigated further, nor were any other potential mediators identified.

Interestingly, given the importance of E-selectin/ligand interactions at other metastatic sites, there is evidence that NSCLC cells produce a variety of cytokines and other factors which destroy the endothelial cell glycocalyx to expose adhesion molecules, including E-selectin, during brain metastasis[86]. The endothelial glycocalyx is a dense layer of transmembrane and membrane-bound species bound to the endothelium by various glycoproteins and proteoglycans; it forms a "mesh" in which various adhesion molecules such as E- and

P-selectin are normally embedded. Brain-metastatic lung cancer cells rapidly destroy this layer upon invasion, exposing E-selectin. The cancer cells, which tend to express high levels of E-selectin ligands such as sLe^X antigens, can then more effectively interface with the endothelial cells for transmigration of the BBB. The authors additionally speculate that long-term exposure to cancer cell-derived cytokines drives increased expression of E-selectin by the vasculature, which would further facilitate brain metastasis [86]. Of note, different cell subtypes expose and interact with different selectins. While both adenocarcinoma and squamous cell models expose E-selectin, adenocarcinoma cell models additional expose P-selectin. A second group investigating NSCLC [87] found that the interaction between Le^X and E-selectin is critical for NSCLC brain metastasis. Importantly, considering the known species-specific differences in selectin/ligand interactions [18], they verify the expression of E-selectin and Le^X (which is quite rare in the normal human cortex) in brain metastasis tissue samples from patients, supporting a role for this interaction in human disease as well as animal models.

The blood-brain barrier (BBB) represents a unique challenge for brain-metastatic cancer cells, and a number of studies have shown that altered glycosylation is particularly important for cancer cell transmigration of the BBB. This barrier, composed of a basal lamina, endothelial cells, astroglia, pericytes and perivascular macrophages, separates the brain and its internal extracellular fluid from the normal systemic blood flow[88]. It is characterized by high electric resistance, a lack of permeability, and the presence of many tight junctions and complex adhesion junctions [89]. Adding to the complexity, the so-called "brain-tumor barrier" (BTB) is in many ways distinct from the non-pathologic BBB. Tumor-associated changes to the microenvironment and the vasculature result in loss of selectivity between the blood and extracellular fluid, and abnormal, non-uniform permeability that can facilitate further cancer cell invasion and/or inhibit effective drug delivery [90]. In order to form a brain metastasis, cancer cells must breach this barrier; there have been several studies suggesting that glycosylation changes may be important for endowing cancer cells with this ability. One paper found that inhibition of the synthesis of G_{M1} glycosphingolipid decreased adhesive abilities of MDA-MB-231 and MCF-7 cells to a human cord-blood derived model of the BBB by approximately 40%, suggesting that glycosphingolipids may play a role in cancer cell adhesion to the BBB [91]. Although not in a cancer setting, another group investigating retention differences of IgG antibodies against amyloid beta (for treatment of Alzheimers) found that the presence of an α2,6-sialylation on the F_{AB} chain of an IgG antibody drug known as mAb 4G8 played a key role in retention of the drug by inhibiting efflux back across the BBB. Removal of that glycan restored efflux to normal levels [92]. This suggests that α2,6-sialylation may play an important role in the breaching of the BBB, in addition to the role in brain-specific colonization identified by Massagué[84]. Additionally, the glycan species Le^X and sLe^X have been identified as important facilitators of NSCLC invasion through the BBB [93], similar to their well-documented roles in extravasation in other contexts[5]. Several integrins, such as α5β3, α5β8 [94], and VLA-4 [95], are also strongly associated with brain metastases, likely by contributing to adhesive tumor-endothelial cell interactions that destabilize the BBB. It should be noted, however, that the role of glycosylation in these processes has not yet been confirmed, and further that several of these mechanisms are not brain-exclusive (see [95–97].

Because glycosylation is such a well-established factor in brain tumor growth, there has been significant interest in specifically identifying the relevant glycan species. This is technically demanding, due to the difficulty of separating and studying the various glycan isomers[89]. The Mechref group performed a series of studies examining the role of glycosylation in breast-to-brain metastasis [88, 89, 98]. First, they performed NGS transcriptome and gene ontology analysis on a panel of cell lines including a primary brain cancer and five metastatic breast cell lines [98]. Twelve glycosylation-related genes were found to be differentially expressed in a brain-seeking line as compared to the rest of the panel, including genes relevant to sialylation, fucosylation, synthesis of complex glycan structures, and glycan anchors. The upregulated genes included the previously-identified[84] sialyltransferase ST6GALNAC5, as well as the related ST6GAL1, and several genes within the integrin pathway (IGTA2, IGTA3, IGTA6)[98]. Subsequent studies by this group focus on the development of precise mass-spectrometry methods for identification of specific glycan species that are present in altered abundance in brain-seeking metastatic breast cancer. These studies identify 46 isomers of 23 glycan species with unique expression patterns in brain-seeking MDA-MB-231BR cells; of those, 24 were sialylated, supporting the critical role of this modification for brain metastasis. Further, α2,6-sialylated N-glycans were particularly enriched in both the MDA-MB-231BR and CRL-1620, a primary brain cancer [89]. The authors speculate that this indicates that these glycans are particularly important for both the breaching of the BBB and subsequent colonization. A second study restricted to membrane N-glycans demonstrated that the most important global change in metastatic cell lines seemed to be N-glycan sialylation, while altered fucosylation was second-most common[88]. Such detailed biochemical analyses confirm the role of sialylation in brain metastasis and identify certain glycans for further investigation with regard to their specific biological role.

One final characteristic of brain metastatic tumors which deserves mention is their penchant for metabolic abnormalities, which have potential to produce glycosylation abnormalities due to alterations in available metabolites for glycosylation reactions. Brain-metastatic tumors tend to display altered sphingolipid metabolism [99], altered amino acid metabolism [100], and some are highly glycolytic [100]. A very recent study also suggests that fatty acid synthesis is upregulated in brain-metastatic breast cancer cells, and is in fact required for successful brain metastasis, though this study relied primarily on intracranial injection models and therefore reveals more about metabolic requirements for growth within the brain than for brain-specific metastasis [101]. However, that finding is supported by the identification of altered lipid metabolism downstream of PI3K as an important mediator of specifically of outgrowth, rather than dissemination, of brain-metastatic tumors[102]. Brain-metastatic tumors are also more sensitive to perturbations in environmental metabolite availability than metastatic cells at other sites. For example, one study found that brain metastatic breast cancer cells were more sensitive to hyper- or hypoglycemia than bone-metastatic cells. In response to glucose deprivation, they experience substantial changes in cell morphology, decreases in colony formation capacity, and downregulation of proteins which are normally overexpressed, including secreted VEGF and integrin β3 [103]. Demonstrating the link between metabolic and glycosylation abnormalities, this study also showed that both bone- and brain-metastatic derivatives of MDA-MB-231 cells expressed

exclusively an immature, unglycosylated form of VEGFR2 under normal physiologic glucose conditions, while the parental cells expressed a mix of a fully-glycosylated and unglycosylated form of the protein. The metastatic derivatives required hyperglycemic conditions to produce the fully glycosylated form[103]. This avenue of investigation is fairly new, and much work remains to be done regarding both metabolic abnormalities, and any potential link to glycosylation, in the brain metastatic context. However, the early data are encouraging, especially given the sensitivity of brain metastatic cells to metabolic changes, which might represent a vulnerability for these otherwise difficult tumors.

In summary, the role of abnormal glycosylation in the context of brain metastasis is, in many ways, much more developed than for other metastatic sites, though many controversies and open questions remain. It is interesting to note, again, that several mechanisms are common between the brain and other metastatic sites, such as mediators of BBB transmigration which also participate in extracranial extravasation, E-selectin-mediated interactions, and sialylation. Indeed, Valiente *et al.* [80] note that it is likely that some of the mechanisms identified in brain-metastatic tumors are relevant for other sites, but that the unique selective pressures of the brain microenvironment select for cells which display more exaggerated versions of those phenotypes. Given the sheer number of glycosylation-relevant mediators of primary and metastatic brain tumor growth, it seems likely that this is the case for glycosylation-related phenotypes. The current picture is both promising and complex, and as with all research on brain metastasis, the limitations of our current model systems (which rely largely on *in vitro* BBB surrogates and mouse models, which do not easily or fully recapitulate human brain metastasis [104]) should be considered. Altogether, however, abnormal glycosylation is clearly a frequent, biologically consequential event in brain metastasis with substantial potential for therapeutic targeting.

2.4. Glycosylation and lymph node metastasis

Lymph node metastasis is a common early event in cancer and is considered a predictor of poor prognosis. Many studies have identified altered glycosylation events in lymphatic metastases. It is known that L-selectin-mediated interactions between leukocytes and lymphatic vessels are critically important for migration of leukocytes into the lymph nodes[105], and that even moderate reduction of L-selectin can substantially impair leukocyte trafficking[26]. Interaction between lymphocytes and the lymph node high endothelial venule (HEV) appears to depend specifically on interaction between L-selectin and O- and N-glycans carrying sulfated glycans such as 6-sulfo-sLe^X [16, 105]. A few studies have suggested that L-selectin expression on cancer cells may facilitate lymphatic metastasis, although this appears to be tumor-type specific (reviewed in [106]).

Because of the clinical relevance of lymphatic metastasis, many studies have been conducted to identify potential markers, which have revealed that altered glycosylation is commonly associated with lymphatic metastasis across a variety of tumor types. A 1991 study [107] found that breast cancer patients whose primary tumors stained positive for HPA lectin, which binds N-acetyl-galactosamine (GalNAc), are more likely to develop axillary lymph node metastases. Similarly, increased β1,6-branched oligosaccharides correlate with lymph node metastasis and overall poor prognosis in a breast cancer cohort [108]. A more

recent study using MALDI-IMS to detect N-glycan species identified F(6)A4G4Lac1, a core-fucosylated tetra-antennary glycan containing a single polylactosamine arm, as associated with lymphatic metastasis and poor prognosis in breast cancer patients and animal models [109]. A few studies in breast cancer have provided more mechanistic data. One *in vitro* study using co-culture of lymphatic endothelial cells with either a weakly metastatic (MCF7) or highly metastatic (MDA-MB-231) breast cancer cell line identified "metastasis-specific" genes which were uniquely upregulated in the lymphatic endothelial cells in response to co-culture with the highly metastatic cell line. Among them were MAN1A1, a mannosidase, and MGAT4A, a glycosyltransferase (see Table 2). In both that model and a similar model using weakly- or highly-metastatic prostate cancer cell lines, E-selectin was also upregulated [110]. Another study probing primary tumor samples from patients with invasive ductal carcinoma of the breast found that O-GlcNAc (see Table 1 and Figure 1) levels were globally reduced in tumors from patients with lymph node metastases. This was accompanied by a reduction in OGT protein expression (the enzyme responsible for O-GlcNAcylation), but no change in OGA, which removes the mark. Within the metastatic group, tumors from patients who had >3 positive lymph nodes demonstrated reduced global O-GlcNAc as compared to those with only 1–3 positive lymph nodes. Which proteins species carried O-GlcNAc modifications also varied between the metastatic and non-metastatic groups; interestingly, the authors note that proteins involved in glycolysis and the pentose-phosphate pathway were particularly likely to display discordant O-GlcNAc status [111].

Similar correlative studies have been conducted in other tumor types as well. In endometrial cancer, primary tumor samples from node-positive patients had lower levels of a complex core-fucosylated N-glycan species (named (Hex)2(HexNAc)2(Deoxyhexose)1+ (Man)3(GlcNAc)2) than primary tumor samples from node-negative patients [112]. In head and neck squamous cell carcinoma, increased expression of the ganglioside-specific sialidase (a glycosyltransferase enzyme that removes sialic acid) NEU3 correlated with lymph node metastasis [113]. Elevated expression of the glycosyltransferase B4GALT1 correlates with lymph node metastasis in pancreatic cancer, specifically through increased N-glycosylation of CDK11[P110], which stabilizes the CDK and promotes tumor progression and chemoresistance [114]. In oral squamous cell carcinoma, expression of the pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase (G6PD) in the primary tumor is associated with lymphatic metastasis. Mechanistic investigation using G6PD-deficient cells demonstrated reduced lymphatic metastases in an orthotopic mouse model, and revealed that G6PD loss results in elevated expression of the glycosyltransferase MGAT3, which specifically increased the bisecting GlcNAc-branched N-glycosylation of E-cadherin. This suggests that the opposite – reduction of MGAT3 and reduced bisecting GlcNAc-brached N-glycosylation of E-cadherin – might encourage lymphatic metastasis in the tumor setting [115]. Concordantly, in a mouse model of hepatocellular carcinoma, miRNA-mediated downregulation of MGAT3, and the resultant decrease in bisecting β1,4-GlcNAc-branched N-glycosylation, is associated with elevated lymphatic metastasis [116].

These studies strongly suggest that altered glycosylation is relevant in lymphatic metastasis of a variety of tumor types. However, most of them are simply correlative, with little or no mechanistic exploration, and whether any of these events are explicitly required

for lymph node metastasis is not assessed. Additionally, these studies do not explore the impact of these alterations of the frequency of metastasis at other sites. Given that lymphatic metastasis is often an intermediate event between primary tumor growth and the establishment of distant metastases, it must be considered that the altered glycosylation events identified by these studies may simply be associated with general metastatic or invasive behavior of tumor cells, rather than lymph node-specific organotropism.

One recent study has identified a glycosylation-dependent mechanism specific to lymphatic metastases. Gu *et al.* [117] investigated the role of B cells in the promotion of breast cancer lymphatic metastasis. They find that orthotopic mammary tumors in mouse models induce B-cell accumulation in the tumor-draining lymph nodes, and that pathogenic IgG from those B cells promotes lymphatic, but not bone, lung, or primary tumor growth. Pathogenic IgG is found to bind HSP4A. Interestingly, tumor-associated HSP4 is aberrantly N-glycosylated; removing this N-glycosylation abrogates the interaction of pathogenic IgG and HSP4A. The binding event between N-glycosylated HSP4 and pathogenic IgG activates the CXCR4/SDF1α pathway via NF-κB. Of note, CXCR4/SDF1α has been previously implicated in the attraction of tumor cells to distant organs during metastasis[118]. Indeed, breast cancer patients with lymph node metastases showed higher levels of serum anti-HSP4 IgG than either non-metastatic cases or healthy controls. Overall, this paper intriguingly demonstrates that interaction between an abnormal glycoform of HSP4A and pathogenic IgG activates pro-metastatic signaling and specifically promotes a premetastatic niche in the lymph nodes.

2.5 Glycosylation at other secondary sites

There is some evidence that glycosylation may impact metastasis to other secondary organs in addition to the four outlined above, but there exists a paucity of studies on the topic. Peritoneal metastasis in ovarian cancer is dependent on P-selectin/sLe^X interactions, which slow the cancer cell "rolling" sufficiently as compared to non-metastatic cells to allow metastasis to take place [119]. A study in mice injected subcutaneously with human neuroblastoma samples found that ovarian metastases, but not liver metastases, displayed elevated E-selectin binding capacity which was specifically ablated by removal of α2,3-sialylation, which is more common on glycolipids and suggests that glycolipid-mediated selectin interactions might be key for this metastatic event [120]. Several older studies have suggested that interaction of E-selectin with β1,6-branched N-oligosaccharides carrying sLe antigens is important for liver metastasis [71], and particularly that modulation of sLe antigen addition through manipulation of the fucosyltransferases responsible for the final addition can modulate liver metastasis in animal models of liver metastasis after intrasplenic injection[52]. A more recent study investigated the impact of galectin-3 on the creation of the pre-metastatic niche and consequent liver metastasis. Reticker-Flynn *et al.* [121] established a spontaneous mouse metastasis model using cells derived from KRas-mutant, p53-knockout lung adenocarcinoma cells. The livers of tumor-bearing mice exhibited increased galectin-3 levels that was derived from recruited myeloid cells, even before detectable metastasis. Galectin-3 has particular affinity for the T-antigen (see Table 2) but is unable to bind more extensively glycosylated forms of the protein. Comparison of glycosyltransferase expression between metastatic and nonmetastatic cell lines showed that GCNT3, an enzyme responsible for the addition of a β(1,6)-GlcNAc to the T antigen,

is under-expressed in metastatic cell lines, while ST6GalNAc4, which is responsible for the addition of an α(2,6)-NeuAc to sialyl-T antigens, is overexpressed. These changes prevent branching (GCNT3) and cap elongation (ST6GalNAc4), leading to increased presentation of the T antigen without expression changes. Genetic manipulation confirmed that reintroduction of GCNT3 or knockdown of ST6GalNAc4 reduced galectin-3 binding. Importantly, ST6GalNAc4 knock-down cells showed a 95% decrease in liver metastases after splenic injection in mice. Overall, the recruitment of galectin-3+ myeloid cells to the pre-metastatic niche might represent a mechanism which aids with both extravasation and colonization [121]. It is important to note, however, that although this study utilized a lung-to-liver metastasis model, organ specificity was not directly tested, so although it appears that this mechanism is relevant to liver metastasis, it remains unknown whether it is recapitulated in other sites.

There has also been interest in characterizing altered glycosylation within hematologic malignancies. Although most of these cancers do not "metastasize" in the typical sense, there are examples of aberrant glycosylation leading to increased spread. Common glycosylation alterations in hematologic malignancies match those reported in solid tumors, including fucosylation, sialylation, and abnormal bisecting GlcNAc, exposure of Tn antigen, and alterations in MUC1 expression (reviewed in [122]). Notably, MGAT3, the enzyme responsible for the addition of bisecting GlcNAc residues, has been reported to be elevated in blast-crisis stage chronic myeloid leukemia and multiple myeloma, and experimental upregulation led to increased spleen colonization by K562 leukemia cells [123]. Multiple myeloma (MM), a cancer of plasma cells that is known to traffic into the bone marrow and participate in metastasis-like behavior throughout the axial skeleton, exhibits altered glycosylation that in many cases mirrors that of bone-metastatic solid tumors. As noted earlier (see 'Bone Metastasis'), elevated expression of heparanase, the enzyme that cleaves heparan sulfate (see Table 2), increases homing of MM cells to the bone in mice [45]. Additionally, altered sialylation plays a substantial role in the bone-homing behavior of multiple myeloma cells. Two sialyltransferases, ST3GAL1 and ST3GAL6, are associated with poor prognosis in MM patients[124]. ST3GAL6, which participates in the synthesis of sLe^X, (see Table 2), is highly expressed in MM. Knockdown of ST3GAL6 interferes with adhesion and migration *in vitro* and bone marrow homing and survival by MM cells *in vivo*, putatively because the presence of sLe^X glycans on selectin ligands such as P-selectin glycoprotein ligand 1 (PSGL-1) is necessary for interaction with E- and P-selectins on the bone vasculature [125]. Because E-selectin is strongly expressed on bone vasculature, the authors hypothesize that this interaction is primarily E-selectin mediated, which parallels the E-selectin-mediated interaction observed in breast-to-bone metastasis [57]. That same selectin ligand, PSGL-1, is essential for hematogenous metastasis of lymphomas [122]. Interestingly, application of the same glycomimetic E-selectin inhibitor which showed potential for reducing bone metastasis in the Esposito paper [57] also reduces bone homing in mouse models of multiple myeloma [124]; this raises the possibility that some glycosylation-dependent bone-homing mechanisms may be relevant across otherwise disparate tumor types. A follow-up paper [126] suggests that pharmacological, global inhibition of sialylation (both by administration of the inhibitor *in vivo* and pre-treatment of multiple myeloma cells to avoid toxicity) reduces homing of multiple myeloma cells

to the bone and improves animal survival in a normally-aggressive mouse model and acts synergistically with bortezomib, the standard of care for multiple myeloma. Desialylation reduces E-selectin-mediated affinity, as well as the interactions between integrin α4β7 and MADCAM1 and integrin α4β1 and VCAM1, primarily through desialylation of the α4 subunit, which are additionally important for the homing process. Interestingly, administration of the sialylation inhibitor *in vitro* reversed stromal cell-mediated, but not endothelial cell-mediated, resistance to bortezomib. Together, these studies demonstrate that there exist intriguing commonalities in glycosylation-dependent mechanisms between solid tumor metastasis and the spread of hematologic cancers.

2.6. Glycosylation in circulating tumor cells

Circulating tumor cells (CTCs) – tumor cells which have escaped from solid-organ tumor sites and entered the bloodstream – are a subject of intense interest, for clinical applications (both prognostic/diagnostic, and intervention-focused) as well as for their role in the biology of metastasis [127]. CTCs that are able to survive in circulation for long enough to reach a distant site and extravasate are the cells from which distant metastases develop. Recently, there has been recognition of the role that glycosylation plays in the biology and behavior of circulating tumor cells. CTCs are thought to use selectin/ligand interactions similar to physiological leukocyte rolling in order to interact with blood vessels[22]. Interestingly, several studies have shown that cancer cells express distinct fucosyltransferaes (FUTs), which are critical enzymes for the production of selectin ligands, as compared to leukocytes[128]. A huge number of studies have probed the expression of selectin ligands and the landscape of cancer cell-endothelial cell interactions. Which selectin and ligand predominate is largely tumor-type specific, and due to space constraints, the nuances of this will not be discussed here; many excellent reviews have been published, including [128, 129]. Importantly, considering the prevalence of animal research, selectin-ligand interactions are also highly species specific; one study which performed a panel of analyses investigating the interaction of various human cancer cell lines with human or mouse E-selectin concluded that (1) different ligands are functional in static vs. dynamic flow conditions; (2) mouse and human E-selectin interact with different ligands, and (3) mouse E-selectin interacts with a wider variety of ligands than does human E-selectin[18]. Taken together, these results suggest that meticulous experimental design which considers both flow conditions and species compatibility will be necessary to elucidate the most clinically-relevant selectin/ligand binding events.

Several studies have shown that cancer cells are capable of using alternative ligands for selectin binding. For example, hypo-glycosylated MUC1 has been found to interact with E- and P- (but not L-) selectin [130], and HS and chondroitin sulfate (CS) – both GAGs (see Table 2) – have been shown to act as P- and L-selectin ligands; in particular, over-sulfated forms of both have been found in breast cancer cells and display higher-affinity selectin interactions than their normally-sulfated counterparts due to ionic interactions. Patient data shows that overexpression of CS-sulfating enzymes correlates with increased risk of distant metastasis [131]. Finally, CD44, which is highly associated with cancer, can act as an E-selectin ligand under dynamic flow conditions. The CD44s isoform is expressed by leukocytes and can act as an E-selectin ligand in that context, but the glycosylation marks

present on that isoform differ significantly from those present on cancer-associated CD44. One group has specifically identified CD44v3 and CD44v4/5, as E-selectin ligands on human breast cancer cells[132]. In sum, cancer cells are capable of leveraging a number of non-canonical selectin ligands to aid their survival in the bloodstream and their interaction with endothelial cells, platelets, and others as they engage in hematogenous metastasis.

More recently, the role of abnormal blood flow in tumor biology has also been a subject of interest. Abnormal blood flow is a common characteristic in and around tumors due to changes in vascular permeability and vessel geometry. It is more common in organs with substantial vessel branching and turning, such as the lungs and liver, which also happen to be common metastatic sites. The luminal side of endothelial cells is coated in a "glycocalyx" composed of many glycosylated species, including sialic acids and proteoglycans. "Disturbed flow" is known to wear down the glycocalyx over time. One group has demonstrated that under abnormal blood flow conditions, both pro-survival clustering behavior and endothelial cell interactions of CTCs are increased[133]. This was found to be due to global erosion of the glycocalyx; however, it was not directly dependent on increased E-selectin exposure, as previously published by Rai *et al.* [86]. Overall, it is clear that CTC survival and interaction with endothelial cells are highly dependent on glycosylation; hopefully, further studies will elucidate more tumor-specific mechanisms that can be leveraged for cancer therapy.

GLYCAN TARGETING AS A THERAPEUTIC APPROACH

Given its wide-ranging impact on cancer development and progression, targeting glycosylation for therapeutic purposes is an active area of research. Several recent reviews provide extensive detail [8, 134–136]; some relevant therapies are highlighted here. Preclinically, fluorinated analogs of sialic acid and fucose can be used to inhibit sialyl- and fucosyltransferases, and have been shown to reduce sLe[X] expression and leukocyte rolling behavior[137]. Soyasaponin-I [138] and AL10 [139], both sialyltransferase inhibitors, inhibited cancer cell metastasis in mouse models of breast cancer and lung cancer, respectively. Additionally, a small-molecule inhibitor of the O-glycosyltransferase ppGalNAc-T-3 has been identified[140]. ppGalNAc-T-3 has been broadly implicated in metastasis; in *in vitro* studies, the inhibitor was capable of blocking invasive behavior by breast cancer cells[140]. Finally, there is substantial interest in using antibodies against cancer-associated glycoforms to improve drug targeting, and a growing recognition that which protein glycoform a cell expresses can have consequences for therapeutic response[135].

On the clinical side, there have been a handful of efforts to harness cancer-associated abnormal glycosylation. One such effort, an sTn-KLH vaccine for metastatic breast cancer (KLH is a carrier added for immunogenicity), was abandoned due to lack of clinical benefit[141]. However, recent results have been more promising. Despite the failure of the sTn-KLH vaccine, interest in developing glycan vaccines for cancer has not abated; a number of vaccines against cancer-associated glycans (for example: MUC1, GD2/GD3, CEA,sLe[A]) in early clinical trials for a variety of cancer types as of this writing. So far, none have been outstandingly effective, but some have demonstrated modest positive results[142].

GMI-1271, the E-selectin antagonist employed by both Esposito [57] and Price[56], has completed phase I safety trials in healthy patients, and is currently in testing for use in acute myeloid leukemia and multiple myeloma [135]. In the neuroblastoma setting, there are a number of trials investigating antibodies to GD2, a disialoganglioside found on nearly all neuroblastoma tumors, as monotherapy and in combination with various immunotherapeutic strategies [23, 143]. The same is true across many cancer types for MUC1, a heavily glycosylated mucin protein that is frequently overexpressed in cancer and associated with metastasis [135], as well as a number of other cancer-associated glycans[142]. For a comprehensive discussion of the state of the art of glycan-directed therapies, see Smith *et al.* [142]. It should also be noted that there is significant interest in identifying CTC-specific glycan epitopes for more sensitive, specific CTC identification as a noninvasive surveillance strategy. The success of these attempts has been mixed, and work is ongoing [144–146]. Altogether, development of glycosylation-related therapies is rapidly advancing; however additional basic and translational research will be needed in order to identify and exploit effective targets, particularly in the case of site-specific metastasis.

CONCLUSIONS

Glycosylation is a ubiquitous and highly impactful form of post-translational modification that is getting increasing attention as a contributor to both primary tumor and metastatic biology. Although modifications in glycans were at first thought to be random, it has become clear that they are actually highly controlled and, due to their prevalence on the cell surface and in the ECM, can impact a wide variety of cell-cell communication processes, as well as intrinsic processes. Modifications in various aspects of glycosylation, including alterations in glycosyltransferase activity, glycoprotein expression, and the specific glycan species present (see Figure 1), have demonstrated relevance throughout the metastatic cascade, and particularly with respect to the later stages such as colonization and outgrowth. Abnormal glycosylation is diagnostically, prognostically, and increasingly therapeutically relevant to treatment of cancer. Metastasis already represents a significant morbidity and mortality burden for cancer patients; as treatments for primary tumors continue to improve and patients live longer, it will become increasingly important to also control metastatic outgrowths. One persistent mystery of metastasis is how, exactly, cancer cells develop a predilection for particular secondary organ sites. This problem is receiving a substantial influx of attention, particularly as advances in technology enable improved tracking and analysis of organotropism [102]. As summarized in Figure 2, alterations in glycosylation - due to changes in glycosyltransferase activity, levels of specific glycan marks, and/or the glycosylation status of critical proteins - is a consequential mediator of organ-specific metastasis. Recent investigations have recognized mediators that are shared across organ sites, as well as glycosylation events that appear highly specific for a given organ microenvironment. However, particularly outside of the brain, our understanding of the specific mechanistic role of differential glycosylation in organotropism remains in its early stages. Future work should focus on delineating additional organ-specific, mechanistically-relevant glycosylation changes and determining which of these could be therapeutically exploited.

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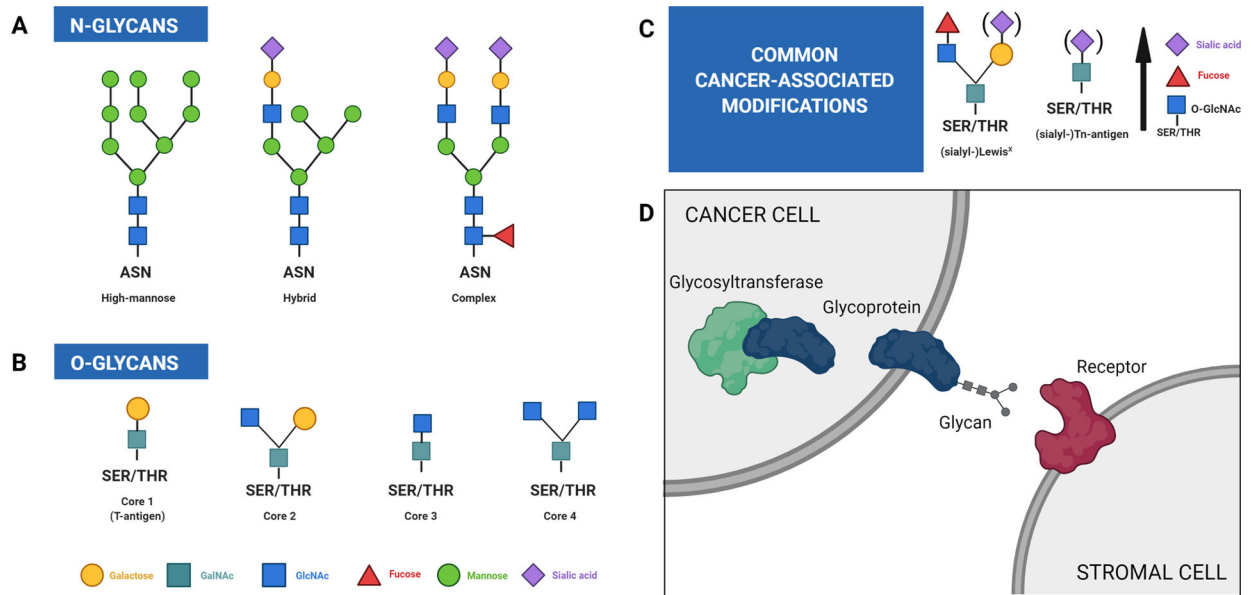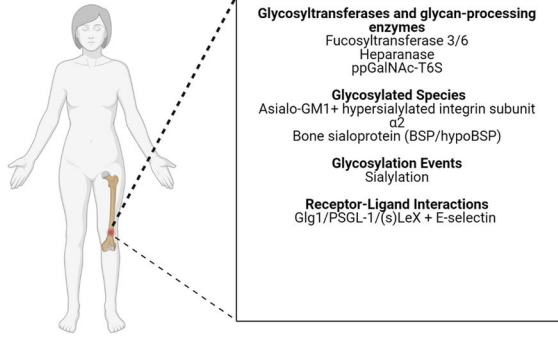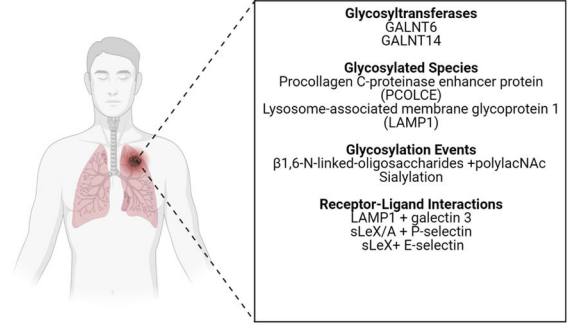
Figure 1: An introduction to glycan structure and glycosylation reactions.
Glycosylation is a non-templated addition and there exist a vast array of possible glycan structures. Glycans can be divided into two classes, N-glycans and O-glycans. (A) N-glycans can be subdivided into three main classes, as shown here. (B) O-glycans typically consist of one of the four core structures shown here, which can be further extended and modified. Note that unmodified core 1, also known as the T-antigen, is considered a truncated glycoform and is frequently associated with cancer. (C) Several glycosylation alterations of particular importance to this review. See Table 2 for additional detail. (D) Glycosylation is a multistep process with many modifiable points. Examples include changes in the expression or activity level of glycosyltransferases, expression levels of glycoproteins, the compositions and amounts of glycans added, and the receptors with which those glycans can interact. The availability of the necessary substrates and the overall metabolic condition of the cell can also influence which glycosylation events take place. Because of the interconnected nature of this system, a perturbation at any one point has the potential to impact everything downstream, and a relatively small initial change can exert dramatic effects on the cell. Figure adapted and complied from "Glycans 1" by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates. Figure edited with BioRender.com
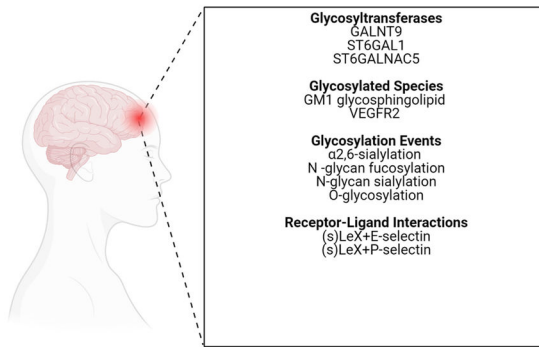
a | **Bone Metastasis**

Glycosyltransferases and glycan-processing enzymes
Fucosyltransferase 3/6
Heparanase
ppGalNAc-T6S

Glycosylated Species
Asialo-GM1+ hypersialylated integrin subunit α2
Bone sialoprotein (BSP/hypoBSP)

Glycosylation Events
Sialylation

Receptor-Ligand Interactions
Glg1/PSGL-1/(s)LeX + E-selectin

b | **Lung Metastasis**

Glycosyltransferases
GALNT6
GALNT14

Glycosylated Species
Procollagen C-proteinase enhancer protein (PCOLCE)
Lysosome-associated membrane glycoprotein 1 (LAMP1)

Glycosylation Events
β1,6-N-linked-oligosaccharides +polylacNAc
Sialylation

Receptor-Ligand Interactions
LAMP1 + galectin 3
sLeX/A + P-selectin
sLeX+ E-selectin

c | **Brain Metastasis**

Glycosyltransferases
GALNT9
ST6GAL1
ST6GALNAC5

Glycosylated Species
GM1 glycosphingolipid
VEGFR2

Glycosylation Events
α2,6-sialylation
N -glycan fucosylation
N-glycan sialylation
O-glycosylation

Receptor-Ligand Interactions
(s)LeX+E-selectin
(s)LeX+P-selectin

d | **Lymphatic Metastasis**

Glycosyltransferases
B4GALT1
MAN1A1
MGAT3
MGAT4A
NEU3
OGT

Glycosylated Species
CDK11P110
HSP4A

Glycosylation Events
β1,6-branched oligosaccharides
Complex core-fucosylated N-glycans (several)
GalNAc
O-GlcNAc

Receptor-Ligand Interactions
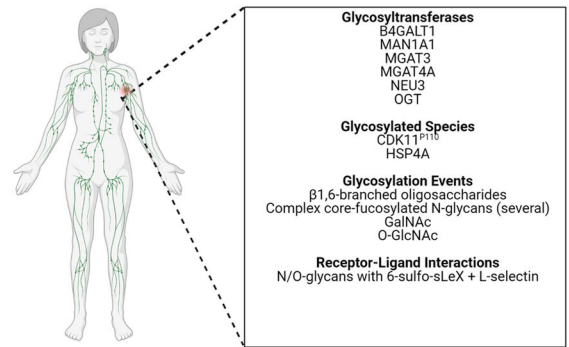N/O-glycans with 6-sulfo-sLeX + L-selectin

Figure 2: Summary of glycosylation-associated factors identified thus far as mediators of site-specific metastasis.

Mediators are classified according to type of alteration observed. Figure adapted and compiled from "Brain Callout (Human)," "Lung Cancer Callout," "Metastasis to Bone Disrupts Bone Homeostasis," and "Lymph Node Locations" by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates. Figure edited with BioRender.com

Table 1.

Selected glycosylation events

Type	Synthesis	Key enzymes	General structure	Notes and cancer relevance
O-glycosylation [5, 10, 15, 20, 22]	*Location:* Golgi complex *Timing:* Post-translational *Sequence dependence:* No consensus sequence, although a Ser/Thr residue is required and a nearby proline seems to be preferred *Initial Addition:* Addition of monosacchride onto the terminal oxygen of serine or threonine	*GALNT:* Makes first addition of a GALNAc residue via α linkage to Ser/Thr for a mucin-type O-glycan *C1GALT1 + COSMC:* transferase + chaperone protein responsible for the formation of core 1 O-glycans *GCNT-1/-2/-3:* responsible for the synthesis of core 2 structures from core 1 Many other O-glycosyltransferases with various affinities and enzyme activities	Common core structures designated #1–4, with 1 and 2 being the most common *Core 1:* the addition of β1–3Gal to O-GalNAc *Core 2:* addition of β1–6GlcNAc to the core 1 GalNAc *Core 3* and *Core 4* are larger, more complex structures and are thought to repress tumor growth	Subdivided into mucin-type (GalNAc) and non-mucin (various moieties; includes O-GlcNAc, O-glucose, O-fucose, O-xylose, and O-mannose). O-GlcNAc: the only reversible glycan modification; competes with phosphorylation at some regulatory sites. See Table 2. Elevated core 2 levels are associated with cancer, presumably because extravasation-promoting selectin ligands can be built from core 2 structures. Elevated levels of C1GALT1 and COSMC are associated with advanced tumors and metastasis.
N-glycosylation [5, 11, 16, 22]	*Location:* Lumen of the endoplasmic reticulum *Timing:* Cooccurs with translation *Consensus sequence:* Asn-X-Ser/Thr motif, X ≠ proline; (Rarely) Asn-XCys *Initial addition:* Large oligosaccharide precursor, Gl3Man9GlcNAc2, which is later trimmed by a variety of glycosidases, glycosyltransferases, and mannosidases. In mammals, attached via GlcNAcβ1-Asn linkage.	*Oligosaccharyltransferase (OST):* The enzyme responsible for synthesis of the N-glycan oligosaccharide precursor *Dolichel phosphate (Dol-P):* The lipid carrier on which the N-glycan precursor is initially synthesized	Stereotypical core includes three mannose residues and two N-acetylglucosamine resides. (Written as: Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn-X-Ser/Thr) Branching patterns can be generally classified as (1) *oligomannose:* elongation by mannose resiudes (2) *complex:* elongation by GlcNAc structures and (3) *hybrid:* elongation by both on different parts of the core structure	Increased size and branching of N-glycans particularly β1,6-type branching, is associated with malignant phenotypes. May help form lattices to enhance growth factor signaling and promote the formation of sialyl-Lewis[X] antigens (see Table 2; sLe[X]). Altered N-glycosylation can affect RTK signaling and membrane retention.
Sialylation [10, 17, 22, 24]	*Location:* Synthesis takes place in the cytoplasm; activation (via addition of CMP)	20 sialyltransferases, specific for the linkage type and glycan being added *ST8SIA* family, 6	9-carbon structures carrying a negatively charged carboxylate on C1 and having a 3-carbon non-cyclic sidechain (C7–C9) Parent compounds: 2-keto-deoxynonulosonic acid	Characteristic feature of mammalian glycans. Critical roles in

Type	Synthesis	Key enzymes	General structure	Notes and cancer relevance
	takes place in the nucleus *Linkage:* Added to galactose or N-acetylgalactosa mine using α2–3 or α2–6 linkages, and can be part of a polysialic chain using α2,8 linkages. Both O- and N-glycans are typically terminated with a sialic acid at the nonreducing end.	members: attachment of the C-2 carbon of a sialic acid to the C-8 of a second sialic acid *ST3GAL* family, 6 members: C-2 to the C-3 *ST6GAL*, 2 members: C2 to *ST6GALNAc*, 6 members: C-2 to C-6 position of galactose to the C-6 of *N*-acetylgalactosamine NEU genes: encode sialidases (remove sialic acid)	(Kdn) neuraminic acid (Neu; found only in glycosidic linkages) *N*-acetylneurami nic acid (Neu5Ac)	immune function and modulation and cell-cell communicati on. Capable of "masking" cell surface glycans from recognition by other cell types. Highly electronegat ive; can mediate electrostatic repulsion between cells, which has been implicated in both metastasis and neuronal development Sialylation globally increases with malignant transformati on and is correlated with increased metastatic spread. α2–6GalNAc linkages become prominent, which has a variety of signaling effects including suppression of Fas and galectin-3 apoptotic signaling, increased FAK and integrin activity, cell motility, and chemothera py resistance. O-acetylation on C-9 of sialic acid can increase or decrease depending on cancer type. Presence of Neu5Gc (not made in humans; obtained via dietary sources, e.g. red meat) provokes chronic, tumorpromoting inflammatio n and angiogenesis due to immune reaction.

Table 2.

Cancer-relevant glycan structures

Glycan Type	General Structure	Role in Cancer
Glycosaminoglycans (GAGs) and proteoglycans [10, 20, 22]	*GAGs*: Long, unbranched chains of polysaccharides with molecular weights of >15 KDa. Frequently modified by sulfation. *Proteoglycans*: Created by the addition of GAGs onto proteins	Key roles in creating the extracellular matrix (ECM) and maintaining the eukaryotic cell membrane. GAGs can tether ligands to RTKs, resulting in constitutive activation independent of protein/RNA levels. Increased cleavage of the proteoglycan heparan sulfate is implicated the promotion of angiogenesis and metastasis. Cleaved ectodomains from syndecan-I can promote bone metastasis.
Hyaluronan [22]	Large, negatively-charged polysaccharide made up of repeating [GlcAβ1–3GlcNAcβ1–4] units	Frequently highly expressed by both tumor-associated stroma and tumor cells. Can increase tissue hydration to facilitate tumor cell migration and influence tumor-matrix interactions. Known activator of CD44 signaling; essential to activate various cancer hallmark pathways, including EMT, PI3K/AKT, and MAP Kinase.
Lectins [2, 15, 20, 23, 24, 105]	Glycan-binding proteins. Main types include siglecs, galectins, and selectins. Sialic-acid binding immunoglobulin-like lectins (siglecs) are found on B cells, macrophages, and NK cells. Physiologic role is to dampen the immune response by masking sialoglycans on cell surfaces from other cells. Selectins (E, L, and P) have glycan ligands containing sulfate, fucose, and sialic acid.	Siglecs can assist in tumor immune evasion and therefore are a therapeutic target of interest. Galectins bind galactose and form lattices via β1,3- or β1,4-linkages to N-acetylglucosamine; these lattices can help modulate receptor-ligand interaction. Selectin expression can influence platelet adhesion (P), homing and development of leukocytes (L) and recruitment of immune cells in response to inflammation (E and L).
Mucins [5, 15]	O-GalNAc glycans, synthesis details found in Table 1	Physiological expression is restricted to the apical (lumen) side of cells; as cell polarity is lost in malignant transformation, expression becomes dysregulated. Thought to interfere with adhesive cell-cell interactions via steric and electrostatic mechanisms, which may contribute to metastasis. Several mucin-type O-glycosyltransferases have been documented as diagnostic and prognostic markers of lung and pancreatic cancer (GALNT3), contribute to breast cancer (GALNT6), and influence death receptor glycosylation in NSCLC, melanoma, and pancreatic cancer (GALNT14). CA125, commonly used as a cancer marker, is a heavily-glycosylated mucin.
O-GlcNAc [12, 22]	The only reversible glycan modification. Addition occurs in cytoplasm.	Has regulatory capacity similar to phosphorylation and sometimes competes for phospho-sites. Generally elevated in cancer. O-GlcNAc is found on a number of cancer-associated proteins, including cyclin D1, c-Myc, NF-κB/p65, and SNAIL. Contributes to enhancement of cancer cell metabolism.
(sialyl)-Lewis[X/A] (sLe[X/A]) [22, 72, 105]	Produced by fucosylation of O-GalNAc core 1 (Le[A]) or O-GalNAc core 2 (Le[X]). Can be exposed by loss of the AB blood group	Overexpressed in many cancers. One of the earliest metastasis-associated glycans to be identified. Important for selectin-lectin interaction allowing extravasation (physiologically used by leukocytes; co-opted by migratory tumor cells). Expression contributes to the formation of "tumor emboli", platelet-tumor complexes that help protect circulating tumor cells.
(sialyl)-T antigen (sT) [22, 23]	O-glycan core 1 structure without any extensions. Can be modified by sialylation.	Is considered a truncated glycoform. Rarely present in normal tissue but frequent in tumors.
(sialyl)-Tn antigen (sTn) [15, 22, 23]	A single GalNAc residue attached to Ser/Thr. Can be modified by sialylation; sialyl-Tn can be O-acetylated as well. Sialylated by ST6GALNAC1-4, which are α2-6 sialyltransferases, as well as a family of α2-3 sialyltransferases that act on the Galβ1-3 residues of O-GalNAc glycans.	Is considered a truncated glycoform. Rarely present in normal tissue but frequent in tumors. Presence in cancer may be related to elevated levels of C1GALT1 and COSMC (see Table 1).