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REVIEW

Sialylation of N-glycans: mechanism, cellular compartmentalization and function

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Abstract Sialylated N-glycans play essential roles in the immune system, pathogen recognition and cancer. This review approaches the sialylation of N-glycans from three perspectives. The first section focuses on the sialyltransferases that add sialic acid to N-glycans. Included in the discussion is a description of these enzymes' glycan acceptors, conserved domain organization and sequences, molecular structure and catalytic mechanism. In addition, we discuss the protein interactions underlying the polysialylation of a select group of adhesion and signaling molecules. In the second section, the biosynthesis of sialic acid, CMP-sialic acid and sialylated N-glycans is discussed, with a special emphasis on the compartmentalization of these processes in the mammalian cell. The sequences and mechanisms maintaining the sialyltransferases and other glycosylation enzymes in the Golgi are also reviewed. In the final section, we have chosen to discuss processes in which sialylated glycans, both N- and O-linked, play a role. The first part of this section focuses on sialic acid-binding proteins including viral hemagglutinins, Siglecs and selectins. In the second half of this section, we comment on the role of sialylated N-glycans in cancer, including the roles of β1-integrin and Fas receptor N-glycan sialylation in cancer cell survival and drug resistance, and the role of these sialylated proteins and polysialic acid in cancer metastasis.

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The synthesis of sialylated glycans on proteins and lipids in the secretory pathway is catalyzed by twenty Golgi localized sialyltransferases (STs) with distinct substrate and linkage specificity [reviewed in Harduin-Lepers et al. (2005)]. As highlighted in the introduction to this thematic issue (Gabius and Roth 2017), glycan structures, including those that contain sialic acid (Sia), encode an amazing array of information that is essential for health and can be altered in disease. In this review, we will provide a general overview of N-glycan sialylation, the structure and function of the STs that catalyze these reactions, the mechanisms of their Golgi localization, substrate recognition and the function of sialic acid on N-glycans in health and disease. Please also see the article by A. P. Corfield in this issue for general information on protein N-glycosylation (Corfield 2017), as well as the article by J. Kopitz concerning the basics of lipid glycosylation (Kopitz 2017). We would also like to direct your attention to a recent special issue of Trends in Biochemical Sciences entitled *The Magic of the Sugar Code* with forward by Gabius (2015) for a broad overview of all forms glycosylation and their function. Finally, there are numerous excellent reviews referenced throughout the text on all aspects of N-glycan sialylation that we encourage the reader to consider. We also want to sincerely apologize to the authors of many fine papers that we did not have the room to include.

Sialyltransferases: structure and function

This discussion will focus on the STs involved in the modification of N-glycans in mammalian cells including the ST6Gal-I and ST6Gal-II, ST3Gal-IV and ST3Gal-VI, and the polysialyltransferases (polySTs), ST8Sia-II and ST8Sia-IV (see Fig. 1 for the structures formed by these
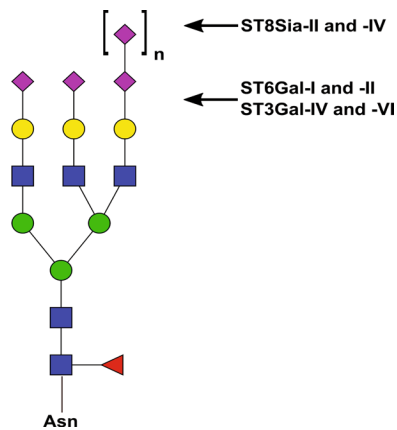
Sialyltransferases	N-glycan Structure Made
ST3Gal-IV and VI	Neu5Acα2,3Galβ1,4GlcNAc
ST6Gal-I and II	Neu5Acα2,6Galβ1,4GlcNAc
ST8Sia-II and IV	(Neu5Acα2,8)$_n$Neu5Acα2,6Galβ1,4GlcNAc

Fig. 1 N-glycan structures synthesized by α2,3-, α2,6- and α2,8-sialyltransferases. In this review, we have focused on the sialyltransferases that add Sia to N-glycans. The ST3Gal enzymes may also modify O-glycans and glycolipids, while the role of ST6Gal-II in modifying protein-bound N-glycans has not been unequivocally demonstrated. The polysialyltransferases, ST8Sia-II and ST8Sia-IV, synthesize polySia chains of 8 to greater than 400 units long on a preexisting Sia that is many times α2,6-linked. Shown is a triantennary N-glycan as a model and does not imply that the activity of these enzymes in any way is restricted to this type of N-glycan. Pink diamond, Neu5Ac; *yellow circle*, galactose (Gal); *blue square*, *N*-acetyl-glucosamine (GlcNAc); *green circle*, mannose (Man); *red triangle*, fucose (Fuc)

STs on N-glycans). We will discuss these enzymes' substrates and general function, their common domain structure and conserved sequences, the molecular structure of the ST6Gal-I, the ST catalytic mechanism and mechanism underlying the protein specificity of polysialylation.

ST6Gal-I and ST6Gal-II

In the early 1970s, a number of laboratories identified ST activities in several mammalian tissues (Bartholomew et al. 1973; Carlson et al. 1973a, b; Hudgin and Schachter 1972). In 1977, an α2,6-ST from bovine colostrum that used Galβ1,4GlcNAc as an acceptor was isolated and characterized (Paulson et al. 1977a, b). This enzyme was later called ST6Gal-I. Cloning of this enzyme's coding sequence from a rat liver cDNA library revealed its amino acid sequence and confirmed that the soluble forms found in body fluids, such as colostrum, breast milk and blood,

are proteolytically truncated versions of those found in the cell (Weinstein et al. 1987). Subsequent work suggested that this ST is cleaved in a late Golgi or post-Golgi compartment (Kitazume-Kawaguchi et al. 1999; Ma et al. 1997) and that several proteases could be involved, including cathepsin D and BACE (Kitazume et al. 2001; Lammers and Jamieson 1988). The ability of soluble glycosyltransferases to catalytically function outside the Golgi was questioned until recently (see discussion below). A second α2,6-ST, ST6Gal-II, was identified, cloned and characterized (Takashima et al. 2002). While this enzyme can use the Galβ1,4GlcNAc structure of oligosaccharides as an acceptor, its shows poor activity with this structure on glycoproteins and glycolipids, and consequently, its in vivo function is unclear.

The expression of ST6Gal-I is tissue specific, and its expression is regulated by multiple transcriptional promoters (Svensson et al. 1992; Wang et al. 1993; Wen et al. 1992). A constitutive, ubiquitous promoter maintains ST6Gal-I expression at low but steady levels, particularly in liver. However, an inducible, liver-specific P1 promoter was shown to drive high ST6Gal-I expression during inflammation and the observed increase in cleaved and secreted ST6Gal-I in the blood (Appenheimer et al. 2003). This inducible pool was shown to be important as a regulator of myelopoiesis/hematopoiesis (Jones et al. 2010; Nasirikenari et al. 2014); however, the source of CMP-Sia that would serve as a donor in these serum ST reactions was unclear. Recent work has demonstrated that activated platelets release the CMP-Sia that serves as the donor for circulating ST6Gal-I, allowing for the remodeling of the glycans of hematopoietic progenitor cells (Lee et al. 2014). Additional in vivo support for the biosynthetic role of circulating ST6Gal-I comes from a recent study showing that ST6Gal-I-deficient B cells express IgGs that are modified with α2,6-sialylated glycans and these are synthesized by circulatory ST6Gal-I released from the liver and CMP-Sia from activated platelets (Jones et al. 2016).

ST3Gal-IV and ST3Gal-VI

These STs add a single Sia in an α2,3-linkage to terminal Gal residues of glycoproteins and glycolipids. ST3Gal-IV has been reported to sialylate Galβ1,4(3)GlcNAc found on N-glycans, as well as core-2, core-3 or core-4 O-glycans that carry GlcNAc (Yang et al. 2012), and Galβ1,3GalNAc terminated structures in glycoproteins or glycolipids (Kitagawa and Paulson 1994; Sasaki et al. 1993). The ST3Gal-VI enzyme uses Galβ1,4GlcNAc acceptors on glycoproteins and glycolipids, but has additional specificity for the glycolipid moieties of its acceptors. As both enzymes use the type II lactosamine structure (Galβ1,4GlcNAc), they were proposed to be involved in the synthesis of the

sialyl Lewis X (sLe^X) determinant on leukocyte E-, L- and P-selectin ligands (Kitagawa and Paulson 1994). These ligands are required for leukocyte binding to selectins on endothelial cells, and their slow rolling and tethering prior to extravasation. Please see the discussion of selectins and their ligands below, and the article in this issue by Mayer et al. for a review of C-type lectins (Mayer et al. 2017). For additional insights into lectin structure, function and analysis, please see the article by Manning et al. in this issue (Manning et al. 2017), and other excellent reviews published elsewhere (Gabius et al. 2015, 2016; Solis et al. 2015).

ST8Sia-II and ST8Sia-IV

The demonstrated role of polysialic acid (polySia) in cell migration and plasticity in the brain prompted the search for the polySTs [reviewed in Colley et al. (2014), Rutishauser (2008), Schnaar et al. (2014)]. Originally called STX and PST-1, ST8Sia-II and ST8Sia-IV polymerize long chains of α2,8-linked Sia that can extend from 8 to over 400 residues at the termini of both N-linked and O-linked glycans (Sato and Kitajima 2013). Structural analysis of the polysialylated N-glycans of bovine neural cell adhesion molecule (NCAM) shows that polySia is added to hybrid, bi-, tri- and small numbers of tetra-antennary complex glycans, with terminal α2,6-linked Sia as the preferred acceptor (von Der Ohe et al. 2002; Wuhrer et al. 2003). Expression of neuropilin-2 (NRP-2) and the polySTs in COS-7 cells revealed that ST8Sia-IV exclusively polysialylates NRP-2 and that polySia is found on sialylated core-1 and core-2 O-glycans (Rollenhagen et al. 2013). What sets polysialylation apart from other glycosylation reactions is that it is protein specific, is found on very few glycoprotein substrates in mammalian cells and, as such, requires the polySTs to recognize and bind these substrates prior to modification of their glycans (Colley 2010; Colley et al. 2014). Interestingly, the two polySTs do show substrate preferences. For example, while the neural cell adhesion molecule, NCAM, can be polysialylated by both polySTs, NRP-2 is exclusively polysialylated by ST8Sia-IV, and SynCAM-1 is exclusively polysialylated by ST8Sia-II, in the cells evaluated so far (Galuska et al. 2010; Rollenhagen et al. 2012, 2013).

The expression and roles of polySia as part of the capsule of neuroinvasive bacteria and during the development of the mammalian nervous system have been appreciated for some time and are comprehensively documented in the book *Polysialic Acid-From Microbes to Man* edited by J. Roth, U. Rutishauser and F. A. Troy II (Roth et al. 1993). In mammals, polySia is found on N- and O-linked glycans and is essential for cell migration and plasticity during nervous system development and to maintain these processes

in select areas of the adult brain such as the hippocampus, olfactory bulb and hypothalamus [reviewed in Colley et al. (2014), Rutishauser (2008), Schnaar et al. (2014)]. More recent work has demonstrated a role for polySia in regeneration of damaged neurons (El Maarouf et al. 2006; El Maarouf and Rutishauser 2010; Zhang et al. 2007a, b), and in liver development and regeneration (Tsuchiya et al. 2014). In addition, many cancer cells upregulate polySia, and its expression correlates with increased invasion and metastasis (Colley et al. 2014; Falconer et al. 2012). For sometime, polySia was believed to exert its effects exclusively through an anti-adhesive mechanism; however, more recently, its ability to impact signaling, either directly by controlling protein–protein interactions or indirectly by serving as a reservoir for signaling molecules, has been appreciated (Colley et al. 2014; Schnaar et al. 2014). Interestingly, polySia is also being used as a less immunogenic and more biodegradable substitute for polyethylene glycol (PEG) to enhance the stability and circulating half-life of therapeutic proteins [reviewed in Bader and Wardwell (2014), Colley et al. (2014)], and as part of nanoparticles for drug delivery (Zhang et al. 2014, 2016). Please see below for a discussion of the protein specificity of polysialylation, its role cancer, and the article in this issue by Higuero and colleagues that discusses the role of glycans, including polySia, in CNS and PNS development and function (Higuero et al. 2017).

Sialyltransferase domain organization and conserved sequence motifs

The elucidation of the primary structure of the ST6Gal-I (Weinstein et al. 1987), and the cloning of other ST coding sequences [reviewed in Harduin-Lepers et al. (2005)], allowed the definition of the domain structure of STs and the identification of conserved sequences. The STs, and indeed all Golgi glycosyltransferases involved in N-linked glycosylation, are type II membrane proteins that possess short N-terminal cytoplasmic tails followed by transmembrane (TM) regions (signal anchors), and proteolytically sensitive stem or stalk regions that tether a large lumenal catalytic domain to the membrane (Paulson and Colley 1989) (Fig. 2b). Conserved sequences called sialylmotifs have been identified in the ST family that play roles in CMP-Sia and glycan acceptor recognition, and in the catalytic mechanism (Fig. 2a) [reviewed in Audry et al. (2011), Datta and Paulson (1997)]. Mutational analyses by Datta and Paulson and others demonstrated that the large sialylmotif (SML) participates in the binding of CMP-Sia donor (Datta and Paulson 1995), while the small sialylmotif (SMS) participates in interactions with both CMP-Sia and acceptor substrates (Datta et al. 1998), and the very small sialylmotif (SMVS) is involved in the catalytic
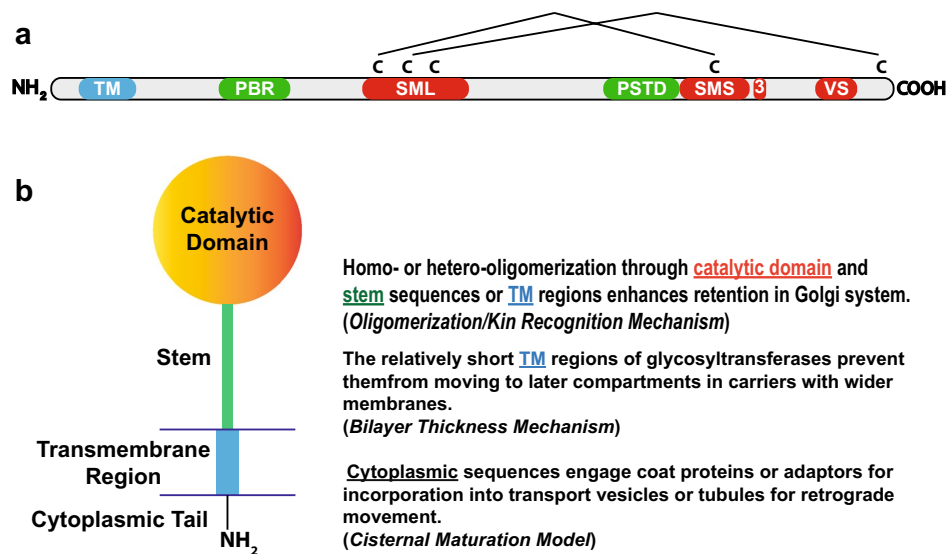
Fig. 2 Sialyltransferase conserved sequences, domain structure and sequences involved in Golgi localization. **a** The positions of the conserved sequences found in all STs, the sialylmotifs, are shown using a generic polyST structure. SML, large sialylmotif; SMS, small sialylmotif; 3, motif 3; VS (or SMVS in text), very small sialylmotif. These sequences are involved in glycan substrate and CMP-Sia donor binding as well as catalysis. A key catalytic His residue mentioned in the text is the first residue in the SMVS. Also shown are two sequences conserved in the polySTs, the polybasic region (PBR) and polysialyltransferase domain (PSTD), which are involved in substrate recognition (via protein–protein interactions—PBR) and that are predicted to form a basic surface or groove for the growing polySia chain (PBR and PSTD). The disulfide formed between the SML and the SMS is conserved across all STs, while the disulfide bond linking the C-terminus is essential for the polySTs. **b** Sialyltransferase domain structure (*left*) and Golgi localization sequences (*right*). STs are type II membrane proteins with short, N-terminal cytoplasmic tails followed by relatively short transmembrane (TM) regions, proteolytically sensitive stem regions followed by large catalytic domains that contain the sialylmotifs and face the Golgi lumen. Golgi glycosylation enzymes are localized by multiple mechanisms. Shown is a summary of sequences and mechanisms involved in Golgi localization

reaction (Datta 2009; Jeanneau et al. 2004). Jeanneau and colleagues identified another sialylmotif called motif 3 and provided support for its involvement in acceptor recognition and catalytic efficiency in the ST3Gal-I enzyme (Jeanneau et al. 2004). The polySTs have two additional conserved sequences (Fig. 2). The polybasic region (PBR) is found at the stem-catalytic domain border and includes selected basic residues involved in substrate recognition and binding (Foley et al. 2009; Zapater and Colley 2012). The polyST domain (PSTD) is a 32-amino acid region adjacent to the small sialylmotif (Nakata et al. 2006). Basic residues in this region are required for catalytic activity and are proposed to play a role in forming an extended binding groove for the growing polySia chain (Foley et al. 2009; Nakata et al. 2006). Structural evidence supporting the roles of these conserved ST and polyST sequences is described below.

Sialyltransferase structure and catalytic mechanism

To date, the X-ray crystal structures of three STs with well-defined substrate specificity have been solved including that of CstII, a bifunctional α2,3/α2,8-ST from *Campylobacter jejuni* (Chiu et al. 2004), porcine ST3Gal-I, which transfers Sia in an α2,3-linkage to Gal residues on O-glycans (Rao et al. 2009), and human (Kuhn et al. 2013) and rat (Meng et al. 2013) ST6Gal-I. The ST6Gal-I adopts a glycosyltransferase-A (GT-A) variant twofold that consists of a seven stranded twisted β sheet flanked by 14 α-helices (Kuhn et al. 2013; Meng et al. 2013). Both the CstII and ST3Gal-I also adopt a GT-A fold (Chiu et al. 2004; Rao et al. 2009), and all three enzymes share high levels of similarity in their β sheets, which contain the conserved sialylmotifs. These three STs exhibit less similarity in their helical and loop segments and in the arrangement of the final strand of the β sheet; all seven β strands are parallel in CstII and ST3Gal-I, while the last strand (β7) of ST6Gal-I is inserted into the sheet in an antiparallel orientation due to an α-helical insertion between it and the preceding strand (β6) (Kuhn et al. 2013; Meng et al. 2013). The catalytic domain of ST6Gal-I also has a large N-terminal extension not found in ST3Gal-I that may be involved in substrate binding (Kuhn et al. 2013). All six cysteines in the ST6Gal-I are engaged in disulfide bonds (Kuhn et al. 2013; Meng et al. 2013). Two disulfide bonds are critical for activity (Datta et al. 2001; Hirano et al. 2012): One between Cys[181] and Cys[332] links sialylmotifs SML and SMS and is conserved across all STs, and the other between Cys[350] and Cys[361] holds a loop above the

active site that forms a "lid" over the nucleotide sugar-binding pocket (Meng et al. 2013). The former disulfide bond is also conserved in ST3Gal-I (Rao et al. 2009). The latter disulfide bond is unique to ST6Gal-I, although ST3Gal-I maintains a "lid" above the active site in the absence of a disulfide bond (Meng et al. 2013; Rao et al. 2009). This "lid" in both STs is unstructured in the absence of CMP-Sia but forms a more ordered structure in the presence of CMP-Sia (Meng et al. 2013). Evidence for two critical disulfide bonds has also been reported for the polyST, ST8Sia-IV, and in this case, a single cysteine remains unpaired (Angata et al. 2001) (see Fig. 2a).

The CMP-Sia-binding pocket of ST6Gal-I is located in a well-defined cavity formed by SML residues (Kuhn et al. 2013). Meng and colleagues performed modeling and molecular dynamics simulation to evaluate CMP-Sia donor and acceptor substrate-binding sites (Meng et al. 2013). They found that the majority of the interactions between CMP-Sia and the enzyme arise from interactions with the nucleotide portion rather than with the monosaccharide portion, consistent with the previously observed binding affinities for these groups (Blume et al. 2006; Meng et al. 2013). These interactions include hydrogen bonds with the hydroxyl groups of the ribose and the phosphate group of CMP, and hydrophobic stacking between the ribose ring and Phe[208]. The observed extension of the glycerol chain and C5 N-acetyl group of the Sia toward the solvent explains why CMP-Sia modified at O9 and the N-acetyl group can be tolerated by STs (Meng et al. 2013).

The structure reported by Kuhn and coworkers includes a glycan from the partner molecule inserted into the ST6Gal-I active site (Kuhn et al. 2013). This structure, and the modeling and molecular dynamics simulations of donor and acceptor binding by Meng et al. (2013), provides insights into the specificity of Sia addition to substrates. Consistent with the inverting S_N2-like direct displacement reaction mechanism of STs, a base is required for deprotonation of a galactose (Gal) residue for the addition of Sia (Audry et al. 2011). SMVS residues, His[370] in the ST6Gal-I and His[319] in the ST3Gal-I, are shown to be in proximity of the acceptor substrates in the respective crystal structures and are likely to serve in this capacity (Kuhn et al. 2013; Meng et al. 2013; Rao et al. 2009). In fact, the regiospecificity of Sia addition is explained by the orientation of the acceptor oxygen on Gal (O3 for the ST3Gal-I and O6 for the ST6Gal-I) toward the respective catalytic His, while the other hydroxyl groups of the Gal are "locked" into their positions by specific hydrogen bonds in and around the enzyme active site. Moreover, mutation of these catalytic His residues in these proteins, as well as the analogous residues in polySTs, ST8Sia-II and ST8Sia-IV, results in

complete inactivation of these enzymes (Jeanneau et al. 2004; Kitazume-Kawaguchi et al. 2001; Zapater and Colley 2012).

The polySTs and protein-specific polysialylation

The polySTs are unusual in that, while they can modify typical sialylated N- and O-linked glycans, polySia is found on a limited number of mammalian glycoproteins. These include, the neural cell adhesion molecule, NCAM (Finne et al. 1983; Hoffman et al. 1982; Rothbard et al. 1982), the synaptic cell adhesion molecule, SynCAM-1 (Galuska et al. 2010), neuropilin-2 (NRP-2) (Curreli et al. 2007), the C–C chemokine receptor type 7 (CCR7) (Kiermaier et al. 2016), E-selectin ligand-1 (Werneburg et al. 2016), the α subunit of the voltage-dependent sodium channel (James and Agnew 1987; Zuber et al. 1992), CD36 scavenger receptor in human milk (Yabe et al. 2003), and the polySTs themselves, which are capable of autopolysialylation (Close et al. 2000; Mühlenhoff et al. 1996). The protein specificity of polysialylation was suggested by the limited number of polyST substrates and the finding that free oligosaccharides, or proteins like fetuin that are not physiologically polysialylated, are poor polyST substrates in vitro (Angata et al. 2000).

Using NCAM as a model substrate, and subsequent studies with NRP-2, our laboratory has determined that polysialylation is indeed protein specific in that the polySTs must recognize the substrate through an initial protein–protein interaction that allows it to dock and then modify glycans in an adjacent domain [reviewed in Colley (2010), Colley et al. (2014) and Bhide et al. (2016)] (Fig. 3). For NCAM, the first fibronectin type III repeat (FN1) serves as the polyST recognition and docking site for the polysialylation of two N-glycans in the adjacent Ig5 domain (Close et al. 2003). Removing the FN1 domain from the full length NCAM molecule eliminates its polysialylation and its ability to bind to ST8Sia-IV (Mendiratta et al. 2005; Thompson et al. 2011). Likewise, the meprin-A5 protein-μ tyrosine phosphatase (MAM) domain of NRP-2 is required for the recognition and polysialylation of the O-glycans in the adjacent linker region (Bhide et al. 2016). Acidic residues in FN1 are key for NCAM polysialylation, and our data indicate that they bind to specific basic residues in the PBR of the polySTs (Mendiratta et al. 2005, 2006; Thompson et al. 2011, 2013). Similarly, mutation of specific acidic residues in the MAM domain of NRP-2 results in reduction in NRP-2 polysialylation by ST8Sia-IV. For NCAM polysialylation, after binding to the FN1 domain, ST8Sia-IV makes secondary contacts with the Ig5 domain that appear to stabilize the interaction to further promote polysialylation (Thompson et al. 2013). Similar secondary contacts
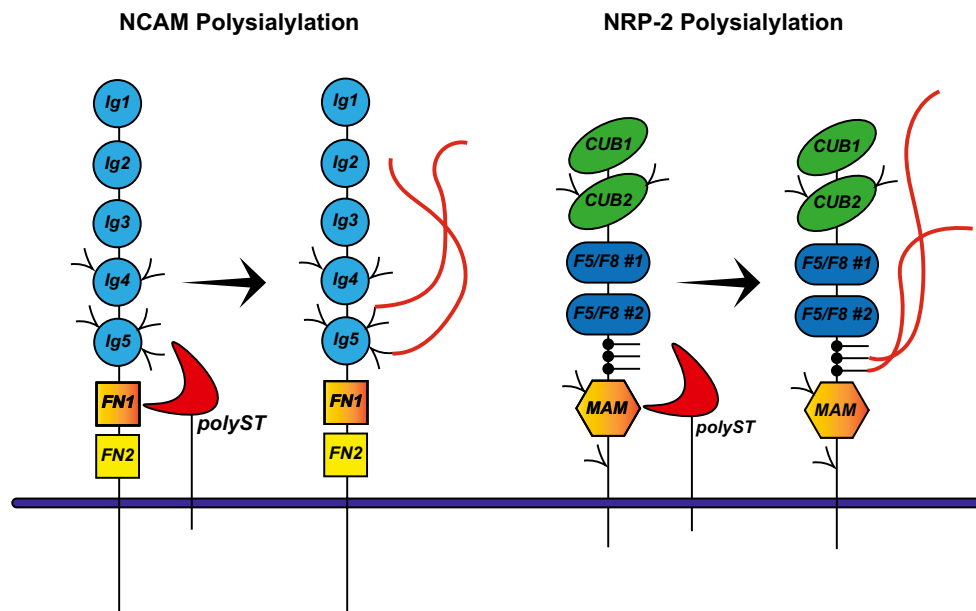
NCAM Polysialylation   **NRP-2 Polysialylation**

Fig. 3 The protein-specific polysialylation of NCAM and NRP-2. The polysialylation of NCAM and NRP-2 requires that the polySTs recognize and bind an acidic patch or surface on the domain adjacent to the domain or region that carries the glycans to be polysialylated. NCAM consists of five immunoglobulin domains (Ig1–Ig5) and two fibronectin type III repeats (FN1 and 2). NRP-2 consists of two complement homology (CUB) domains, two coagulation factor V/VIII homology (F5/8) domains and one Meprin-A5 protein-μ tyrosine phosphatase (MAM) domain. N-glycans are shown as Vs, O-glycans

are shown as horizontal lines, and polySia chains are shown in red. For NCAM polysialylation, either ST8Sia-II or ST8Sia-IV recognize and dock on an acidic patch on the first fibronectin type III repeat (FN1) to allow the polysialylation of two N-glycans in the adjacent Ig5 domain (*left*). Likewise, for NRP-2, acidic residues of the MAM domain are required for ST8Sia-IV to polysialylate O-glycans in the adjacent linker region between the MAM domain and the second F5/8 domain (F5/8 #2)

have not been identified for NRP-2. In summary, studies of NCAM and NRP-2 polysialylation have established a two-step paradigm for protein-specific polysialylation that includes substrate recognition and docking of the enzyme on one domain and polysialylation of glycans on an adjacent domain (Fig. 3).

Recently, structural models of ST8Sia-IV were published by two groups that address the protein specificity of polysialylation and mechanism of polySia chain elongation (Volkers et al. 2015; Zhou et al. 2015). Three-dimensional modeling suggested that the previously made mutations in basic residues of the conserved PBR and PSTD sequences that resulted in loss of NCAM polysialylation may have altered the enzyme secondary structure and this hindered substrate recognition (Zhou et al. 2015). On the other hand, biophysical studies from our laboratory suggest a direct electrostatic interaction between residues in the NCAM FN1 domain and ST8Sia-IV PBR (Bhide et al. 2015). In another study, the structure of ST8Sia-IV was modeled based on the X-ray crystal structure of another α2,8-sialyltransferase, ST8Sia-III. This modeling, and docking of the previously solved NCAM Ig5-FN1 structure from our laboratory (Foley et al. 2010), suggested that the PBR and PSTD regions together form an extended basic groove

providing both substrate recognition and an electropositive surface to guide the growing polySia chain (Volkers et al. 2015).
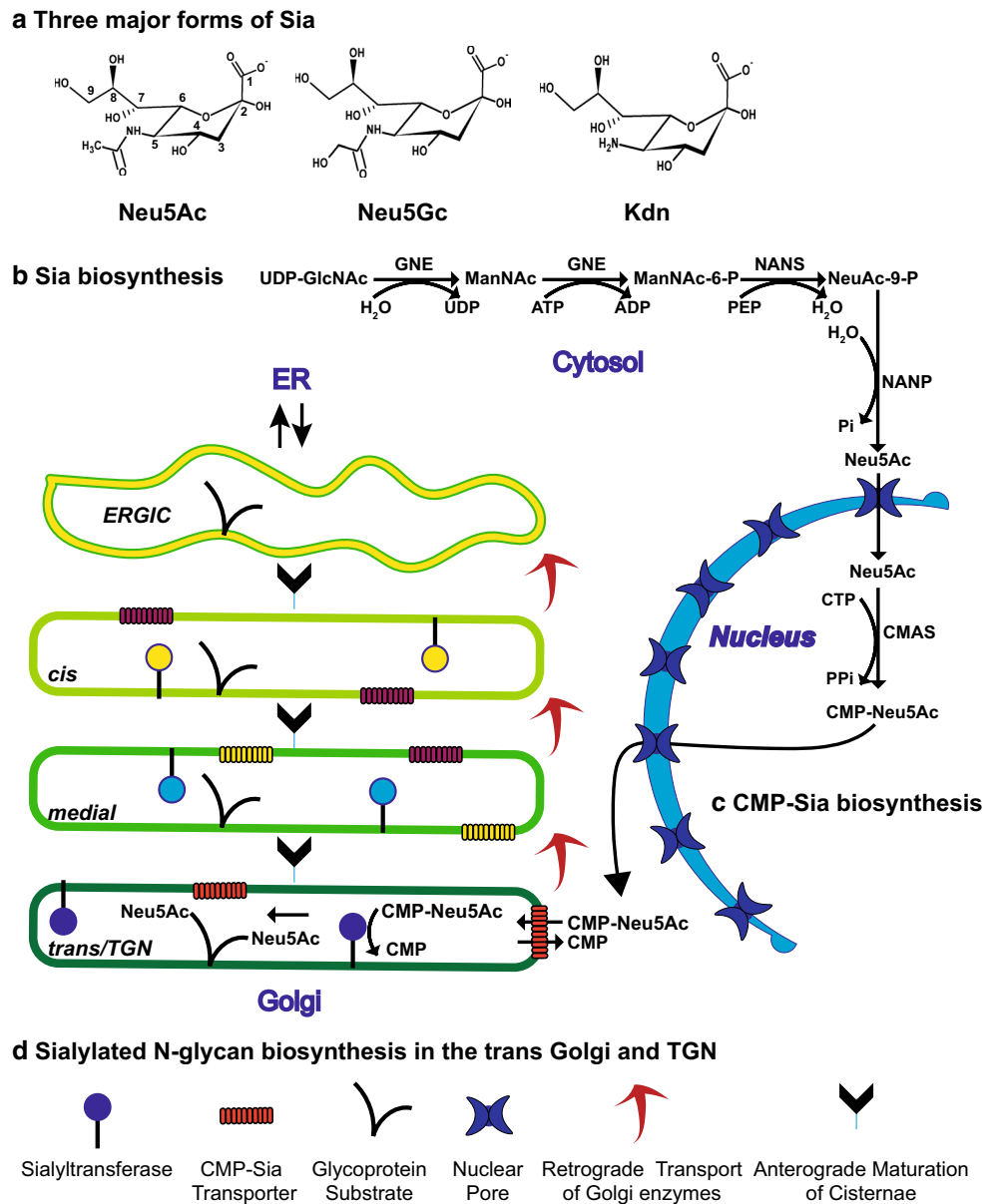
The cell biology of sialylation

Glycan sialylation in the secretory pathway requires that STs, their CMP-Sia nucleotide sugar donor and the appropriate substrates are colocalized in the Golgi (Fig. 4d). In this section, we will discuss the synthesis of Sia, CMP-Sia and sialylated glycans, including the unique localization of the CMP-Sia synthetase enzyme in the vertebrate nucleus, its transport into the Golgi via the CMP-Sia transporter (CST) and the mechanisms localizing the STs and other glycosyltransferases in the Golgi.

The biosynthesis of sialic acids

The most abundant Sias in mammals are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) (Fig. 4a). Two to three million years ago, humans lost the ability to synthesize Neu5Gc owing to a 92 base pair deletion in the cytidine

Fig. 4 Sialic acid structure and the compartmentation of Sia, CMP-Sia and sialylated N-glycan biosynthesis. **a** Three major forms of Sia are shown. **b** The pathway for Neu5Ac biosynthesis in the cytosol. GNE, UDP-GlcNAc 2-epimerase/ManNAc Kinase; NANS, *N*-acetylneuraminic acid synthase; NANP, *N*-acetyl-neuraminic acid phosphatase. **c** The pathway for CMP-Sia biosynthesis in the nucleus is shown. Both Neu5Ac and CMP-Neu5Ac are small enough to flow into and out of the nucleus through nuclear pores. CMAS, CMP-sialic acid synthetase. **d** The pathway for sialylated N-glycan biosynthesis in the trans Golgi and TGN. According to the cisternal maturation model, Golgi enzymes are localized/retained in the Golgi by continuous retrograde transport in COPI-coated vesicles or tubules and cisternae-containing cargo proteins are "matured" by the sequential introduction of glycosylation enzymes. The sialyltransferase reaction in the Golgi *trans* cisternae and TGN is highlighted. CMP-Sia, like other nucleotide sugar donors, is transported into the Golgi by a specific CMP-Sia transporter (CST). It is then used in the transferase reaction with the release of CMP which is transported to the cytosol by the CST in exchange for CMP-Sia

a Three major forms of Sia

Neu5Ac   Neu5Gc   Kdn

b Sia biosynthesis

c CMP-Sia biosynthesis

d Sialylated N-glycan biosynthesis in the trans Golgi and TGN

Sialyltransferase   CMP-Sia Transporter   Glycoprotein Substrate   Nuclear Pore   Retrograde Transport of Golgi enzymes   Anterograde Maturation of Cisternae

monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH) gene causing a frameshift and elimination of activity (Chou et al. 1998). However, Neu5Gc from dietary sources such as red meat can be incorporated in human sialoglycoproteins and gangliosides and this incorporation has recently been shown to promote inflammation and tumor progression (Samraj et al. 2015). Kdn (2-keto-3-deoxy-D-glycerol-D-galacto-nononic acid) is another form of Sia that has a hydroxyl group instead of acetyl amino group at C5 (Fig. 4a). It is overexpressed in numerous cancers, possibly due to hypoxia-mediated changes in expression level of Kdn processing enzymes [reviewed in Pearce and Läubli (2016)]. Acetylated Sias play important roles in embryogenesis, development and immunological processes (Mandal et al. 2015). Additionally, sulfated Sias,

found on gangliosides in mammals, have been reported but not well studied (Kitajima et al. 2015).

The biosynthesis of Neu5Ac is a highly regulated process and begins with the glycolysis product, fructose-6-phosphate, which is diverted to the synthesis of hexosamines by the enzyme glutamine fructose 6-phosphate amidotransferase (Hinderlich et al. 2015; Varki and Schauer 2009). Following the acetylation of the free amine group of glucosamine-6-phosphate ($GlcNH_2$-6-P) to form *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P), an epimerization reaction converts GlcNAc-6-P to GlcNAc-1-phosphate (GlcNAc-1-P) which is then condensed with UTP to form UDP-GlcNAc. Subsequently, the action of the bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc Kinase (GNE), commits UDP-GlcNAc to the Sia biosynthesis pathway (Fig. 4b).

In the first step, GNE catalyzes the hydrolysis of UDP-GlcNAc to UDP-ManNAc, which is then epimerized to form free ManNAc (Fig. 4b). CMP-Sia is a negative feedback inhibitor of the epimerization step, and its levels serve to regulate the amount of Sia synthesized by the cell (Weiss et al. 1989). In the second GNE-catalyzed step, ManNAc is phosphorylated to form ManNAc-6-phosphate. Consistent with its role as the "master regulator" of Sia biosynthesis, eliminating GNE expression in mice leads to a complete loss of sialylation and embryonic lethality, highlighting the importance of Sia in development (Schwarzkopf et al. 2002). Mutations in *Gne* have also been shown to be responsible for sialuria, a rare disease in which Sia accumulates due to a loss of GNE feedback inhibition by CMP-Sia; this disease is characterized by delayed skeletal muscle development and microcytic anemia (Seppala et al. 1999; Weiss et al. 1989). In addition, other mutations in *Gne* have been shown to cause a rare, autosomal recessive form of progressive muscular dystrophy called GNE myopathy or hereditary inclusion body myopathy (HIBM) (Eisenberg et al. 2001).

Free cytosolic Sia (Neu5Ac) is generated from Man-NAc-6-P in two steps (Fig. 4b). First *N*-acetylneuraminic acid synthase (NANS) catalyzes the condensation of Man-NAc-6-P with phosphoenol pyruvate to produce Neu5Ac-9-phosphate (Neu5Ac-9-P). Second, *N*-acetylneuraminic acid phosphatase (NANP) removes the phosphate group from Neu5Ac-9-P to generate Neu5Ac (Varki and Schauer 2009). Notably, biallelic mutations in NANS gene were recently shown to be responsible for severe developmental delay and skeletal dysplasia in infants (van Karnebeek et al. 2016). The levels of free Sia in both prokaryotes and eukaryotes are likely controlled by cytosolic sialate pyruvate-lyases that hydrolyze Neu5Ac to ManNAc and pyruvate (Schauer et al. 1999).

The biosynthesis and transport of CMP-Sia

Cytidine monophosphate-*N*-acetylneuraminic acid (CMP-Sia) is the nucleotide sugar donor used in all ST reactions. Free Sia is activated by the transfer of cytidine monophosphate (CMP) from CTP to the hydroxyl group at C2 by CMP-Sia synthetase (CMAS) (Fig. 4c) (Sellmeier et al. 2015). Importance of CMP-Sia was demonstrated in CMAS-knockout mice where it was shown to cause kidney failure and death three days after birth (Weinhold et al. 2012). Unlike other vertebrate sugar activating enzymes that are localized in the cytosol, the CMAS is localized in the nucleus (Sellmeier et al. 2015). Mouse CMAS possesses a key monopartite nuclear localization signal (K^{198}RPRR) that also contains residues important for catalytic activity. Mutagenesis studies demonstrated that activity and nuclear localization are separable and that

nuclear localization is not required for activity [reviewed in Sellmeier et al. (2015)]. Why CMAS enzymes from all vertebrate species studied are localized in the nucleus in the steady state is unclear, but not surprisingly, they also have nuclear export signals that allow them to enter the cytoplasm for import into the Golgi. Interestingly, the CMAS from *Drosophila melanogaster* is a membrane protein localized in the Golgi (Viswanathan et al. 2006). The reasons for this differential localization remain unknown.

Following the synthesis of CMP-Sia in the nucleus, it makes its way into the cytoplasm and then is transported into the Golgi cisternae to be used by STs (Fig. 4d). Nucleotide sugars that serve as donors in glycosyltransferase reactions are imported into the Golgi lumen by specific transporters (reviewed in Caffaro and Hirschberg (2006); Hirschberg et al. (1998); Zhao and Colley (2008)). The CMP-Sia transporter (CST) is a type III (multi-spanning) transmembrane protein (10 TM regions) localized in *medial–trans* Golgi, which functions as an antiporter to import CMP-Sia into the Golgi in exchange for CMP (Zhao et al. 2006). A CHO cell mutant, Lec2, has a defect in CST function and is widely used to study transporter function and the role of sialylation in glycoprotein and glycolipid function (Eckhardt et al. 1998). The creation of CST mutants and chimeric proteins using regions from the UDP-Gal transporter has identified specific TM regions and residues critical for the specificity and mechanism of CMP-Sia transport [reviewed in Hadley et al. (2014), Zhao and Colley (2008)]. While a four-amino acid sequence (IIGV) at the carboxy-terminus of the CST is required for its ER export (Zhao et al. 2006), little is known about the Golgi localization mechanism of mammalian nucleotide sugar transporters. Interestingly, a GDP-Man transporter in yeast, Vrg4p, possesses membrane proximal lysine residues in its carboxy-terminal tail (KQKK) that bind to COPI coats on transport vesicles to maintain its Golgi localization (Abe et al. 2004). A similar carboxy-terminal motif is not found in the mammalian CST.

The biosynthesis of sialylated N-glycans in the Golgi

The N-linked glycosylation of proteins is initiated in the endoplasmic reticulum (ER) with the *en bloc* transfer of a preformed oligosaccharide structure to the consensus sequence Asn-X-Ser/Thr. This structure is subsequently processed by glycosidases and glycosyltransferases in the ER and Golgi [reviewed in Moremen et al. (2012)] (please see the articles by Corfield (2017) and Roth and Zuber (2017) in this theme issue for a review of this process). While the glycosylation enzymes in the Golgi are localized in the cisternae essentially in the order in which they act to modify the new glycans, there is distinct overlap (Rabouille et al. 1995). Sias are typically added to N-glycans on

proteins in the *trans* most cisternae and *trans* Golgi network (TGN) of the Golgi. In fact, the TGN (then called the *trans* tubular network) was first visualized in rat hepatocytes by Roth and colleagues (Roth et al. 1985) using an antibody against the ST6Gal-I. In these cells, the ST6Gal-I was found in both the *trans* cisternae and TGN; however, later work demonstrated that the distribution of this ST diverged in different cell types (Roth et al. 1986). Specifically, these investigators demonstrated that the ST6Gal-I is localized in the *trans* cisternae of the Golgi in intestinal goblet cells, but across all Golgi cisternae, with the exception of the "fenestrated first *cis* cisterna" in adjacent intestinal absorptive cells.

Glycosylation enzyme localization in the Golgi

Studies by many groups over the last 20 years have concluded that multiple mechanisms serve to localize glycosylation enzymes in the Golgi [reviewed in Banfield (2011), Colley (1997), Derby and Gleeson (2007)]. Following the cloning of the coding sequences of several glycosyltransferases, including the ST6Gal-I, investigators began to evaluate what sequences were required for the localization of these enzymes in the Golgi. At that time (mid-1980s), the prevailing view was that the Golgi was comprised of a series of "stable" cisternae that contained specific glycosylation enzymes (Kornfeld and Kornfeld 1985). In the "stable compartments/vesicular transport model," cargo from the ER entered the Golgi at its *cis* face, traversed the cisternae in vesicular carriers and exited to post-Golgi compartments from the *trans* face. Each cisterna or group of cisternae was defined by the presence of specific glycosylation enzymes. Cargo proteins were envisioned to move in an anterograde fashion between compartments in COPI-coated vesicles, and glycoproteins modified sequentially by the glycosylation enzymes resident in each cisterna (Glick and Luini 2011; Glick and Nakano 2009). At this time, investigators searched for sequences in the Golgi glycosyltransferases that allowed their retention in the Golgi cisternae.

This stable compartments/vesicular transport mechanism of movement through the Golgi came into question when researchers noticed that cells could transport cargo that is too large to fit into small transport vesicles (Bonfanti et al. 1998; Mironov et al. 2001), and evidence suggested that COPI vesicles functioned in retrograde rather than anterograde transport (Rabouille and Klumperman 2005). This brought the field back to the cisternal progression or "cisternal maturation model" that was supported by the early studies of morphologists [reviewed in Mollenhauer and Morré (1991)] and has been supported by recent studies by several groups [for example, Losev et al. (2006), Matsuura-Tokita et al. (2006)]. In this mechanism, a new cisterna forms at the *cis* face of the Golgi by the combined anterograde transport of protein cargo from the ER in COPII-coated vesicles and the retrograde transport of *cis* Golgi enzymes in COPI-coated vesicles. This cisterna and its cargo "mature" as *medial* and then *trans* Golgi enzymes are transported in a retrograde fashion from earlier cisterna. At the level of the TGN, cargo is sorted into different carriers for constitutive or regulated secretion, transport to the endosome/lysosome system, or for return to earlier portions of the Golgi. It is possible that both mechanisms function in the same cell and that tubules as well as vesicles participate in movement of membrane and protein between compartments (Glick and Luini 2011; Mollenhauer and Morré 1991). With the reemergence of the cisternal maturation model, researchers began to consider the possibility that the localization of Golgi glycosyltransferases depended upon their incorporation into retrograde transport vesicles.

On this backdrop, investigators have generally concluded that Golgi glycosylation enzymes are localized in the Golgi via multiple mechanisms that may include sequences in different parts of the protein, namely the TM region (bilayer thickness mechanism), lumenal sequences (oligomerization/kin recognition mechanism) and cytoplasmic tail (COPI vesicle incorporation) (Fig. 2c) [reviewed in Banfield (2011)]. The ST6Gal-I has been used extensively as a model for these studies. Munro first noted that Golgi proteins on average had shorter TM regions than plasma membrane proteins (Munro 1991, 1995a) and that the width of the membrane increases along the secretory pathway, from ER to Golgi to plasma membrane, due to an increase in the concentration of cholesterol in the membrane (Bretscher and Munro 1993). These observations led him to propose the "bilayer thickness model" that suggested that Golgi proteins were not able to partition into carriers moving toward the plasma membrane because of their shorter TM regions (Munro 1998). This model was tested by altering the TM region of the full length ST6Gal-I and creating chimeric proteins. It was concluded that the 17-amino acid TM region and flanking sequences are critical for Golgi localization and that the length rather than amino acid composition of this region was essential (Dahdal and Colley 1993; Munro 1991, 1995b). On the other hand, it was also observed that fusing the ST6Gal-I cytoplasmic tail and TM region onto two different reporter proteins was not able to completely retain these chimeric proteins in the Golgi, suggesting that the enzyme's lumenal sequences might mediate another process that increased retention efficiency (Dahdal and Colley 1993).

Machamer and her colleagues provided support for an oligomerization-based mechanism for the *cis* Golgi localization of the coronavirus E1 protein (Swift and Machamer 1991; Weisz et al. 1993). Their oligomerization model was extended and elaborated upon for

Golgi glycosylation enzymes by Nilsson and colleagues (Nilsson et al. 1994, 1996) who provided evidence that enzymes residing in the same cisterna, such as the *medial* Golgi enzymes, GlcNAcT1 and mannosidase II, form "kin" complexes. These observations gave rise to the "kin recognition hypothesis" where hetero-oligomers form between enzymes in the same Golgi cisterna via their TM and lumenal sequences to prevent their incorporation into transport vesicles and movement to later compartments (Nilsson et al. 1993). Work by a number of other laboratories implicated oligomerization as a potential mechanism for glycosylation enzyme Golgi localization (Chen et al. 2000; Fenteany and Colley 2005; Opat et al. 2000). Differences in trafficking behavior between two isoforms of the ST6Gal-I, STtyr and STcys, which have a single-amino acid difference in their catalytic domain at position 123, suggested that oligomerization is a major factor in the stable Golgi localization of this ST. Both isoforms are equally active (Chen et al. 2003); however, the STcys is stably Golgi localized, while the STtyr is transiently Golgi localized and moves to a post-Golgi compartment where it is cleaved and secreted (Ma et al. 1997). Stable Golgi localization was correlated with pH-dependent oligomer formation; 100% of the STcys, and only 13% of the STtyr formed Triton-insoluble oligomers at pH 6.3 (pH of late Golgi), and neither isoform formed oligomers when membranes were solubilized at pH 8.0 (Chen et al. 2000). More recent studies provided support for the kin recognition model by demonstrating that Golgi N-linked and O-linked glycosylation enzymes form pH-dependent oligomers (Hassinen et al. 2011). Using FRET and FRAP experiments, this group found that the *medial* Golgi enzymes GnTI and GnTII, and the *trans* Golgi enzymes GalT-1 and ST6Gal-I, individually form homodimers in the ER and then form cisterna-specific, pH-dependent, hetero-oligomers in the Golgi (GnT-I/GnT-II and GalT-I/ST6Gal-I) (Rivinoja et al. 2012).

A more complete picture of the mechanisms involved in ST6Gal-I Golgi localization was revealed when we simultaneously evaluated the role of the cytoplasmic tail, TM region and oligomerization in STcys Golgi localization (Fenteany and Colley 2005). Individually altering the sequence or length of the TM region or deleting the core amino acids of the cytoplasmic tail did not alter the stability of STcys Golgi localization or its oligomerization. However, making both of these changes simultaneously led to impaired oligomerization and movement of the STcys to compartments where it was cleaved and secreted. We concluded that the ST6Gal-I STcys isoform is localized by mechanisms independently mediated by its cytoplasmic tail and TM region and that oligomerization was likely a secondary event that resulted after a concentration of this protein in the Golgi.

With the reemergence of the cisternal maturation mechanism for protein transport through the Golgi, the cytoplasmic tails of glycosylation enzymes would be expected to play important roles in their incorporation into retrograde COPI transport vesicles. Golgi glycosylation enzymes have been found in COPI vesicles, but how they are incorporated into these vesicles was not clear because their cytoplasmic tails lacked known COPI-binding motifs (Nilsson et al. 2009). Work done by Banfield and colleagues identified a peripheral membrane protein, Vps74p, that binds as a sorting receptor to COPI coats and to a pentameric sequence in the cytoplasmic tails of yeast Golgi glycosyltransferases (Tu et al. 2008). The mammalian homolog of Vps74p, GOLPH3, was later shown to bind to coatomer (Tu et al. 2012) and the cytoplasmic tails of both the core-2 GlcNAcT-I (C2GnT-I) (Ali et al. 2012) and the protein O-linked mannose-1,2-GlcNAcT-1 (POMGnTI) (Pereira et al. 2014) to control their Golgi localization. A LLRRR motif in the cytoplasmic tail of C2GnT-1 was shown to be required for GOLPH3 binding (Ali et al. 2012). Further studies demonstrated that GOLPH3 allows the incorporation of both C2GnT-1 and ST6Gal-I, but not GalT-1, into COPI-coated vesicles (Eckert et al. 2014). GOLPH3 is a phosphatidylinositol 4-phosphate-binding protein and is enriched in the TGN due to the prevalence of this phospholipid in this compartment. Consequently, it has been hypothesized that GOLPH3 interactions and COPI vesicle incorporation serve to prevent glycosyltransferases from escaping the Golgi and moving to the cell surface (Eckert et al. 2014). As GOLPH3-binding sequences, which consist of conjoined hydrophobic and basic sequences (LKKK in ST6Gal-I (predicted), LLRRR in C2GnT-1), are not found in all Golgi enzymes, it has been suggested that the alterations in the expression of GOLPH3 may also variably regulate Golgi enzyme localization and the types of glycans expressed by cells (Eckert et al. 2014).

The integrity of the secretory pathway and glycosylation are also tightly linked via the COG complex. The role of the COG complex in maintaining Golgi structure and efficient glycosylation was appreciated when several congenital disorders of glycosylation (CDGs) were identified that were the result of mutations in various COG subunits [reviewed in Reynders et al. (2011), Rosnoblet et al. (2013)]. The COG complex is an eight-subunit heteromeric complex organized in two lobes (A and B) that serves as a tethering complex for the interaction of incoming vesicles with target membranes in COPI-mediated retrograde transport in the Golgi and between the Golgi and ER. Mutations in COG subunits lead to changes in Golgi structure, deficits in glycan synthesis, and alterations in retrograde trafficking and the localization and/or stability of Golgi proteins, including glycosylation enzymes [reviewed in Rosnoblet et al. (2013)]. Mislocalization of several Golgi

glycosylation enzymes has been observed when lobe A and B subunits are depleted. Notably, Peanne et al. (2011) observed that the stability of GalT1 and ST6Gal-I was compromised in cells depleted of lobe B subunits (Peanne et al. 2011). However, more recent work suggests that each COG subunit is essential for the complex's function (Bailey Blackburn et al. 2016).

Removal of sialic acid from glycoconjugates

Four neuraminidases in mammalian cells, NEU1, 2, 3 and 4, have been shown to cleave α-linked Sia residues from glycoconjugates. These enzymes, also called sialidases, exhibit differences in cellular localization and substrate specificity [reviewed in Miyagi and Yamaguchi (2012), Monti et al. (2010), Pearce and Läubli (2016)]. They are characterized by two types of conserved sequences: the aspartate box (-Ser-X-Asp-X-Gly-X-Thr-Trp-), which can appear multiple times in each enzyme's sequence, and the RIP sequence (-Phe/Tyr-Arg-Ile-Pro-) (Miyagi and Yamaguchi 2012). NEU1 is found in the lysosome and on the cell surface and is the most highly expressed of this sialidase family, but shows the least conservation with the other members. It exclusively acts on glycoproteins and preferentially cleaves α2,3 linkages over α2,6 or α2,8 linkages (Miyagi and Tsuiki 1984). Interestingly, in lysosomes it is found in a complex with carboxypeptidase protective protein/cathepsin A (PPCA) and β-galactosidase that is key for its activity and localization (Bonten et al. 2009; d'Azzo et al. 1982; Galjart et al. 1988). Mutations in NEU1 DNA lead to the lysosomal storage disease, sialidosis, while a defect in PPCA leads to a combined deficiency in both NEU1 and β-galactosidase activity, called galactosialidosis (d'Azzo and Bonten 2010). NEU1 traffics to the cell surface in exovesicles following cell stimulation (lysosomal exocytosis), and interestingly, NEU1 itself controls lysosomal exocytosis by controlling LAMP1 sialylation (Machado et al. 2015). In addition, NEU1, released from cells in exosomes, was shown to trigger loss of polySia from the surface of Ra2 microglial cells or Neuro2A neuroblastoma cells co-cultured with Ra2 cells in response to an inflammatory stimulus (lipopolysaccharide). Chemical inhibition of NEU1 using Sia analog GSC-649 or its knockdown rescued this shedding of polySia (Sumida et al. 2015). In addition, NEU1 is believed to control several aspects of the immune response by the desialylation of key molecules, such as Toll-like receptor 4 (TLR4) and adhesion molecules involved in the recruitment of leukocytes to inflammatory sites (Miyagi and Yamaguchi 2012). NEU2 is localized in the cytosol, has a broad cleavage specificity, has a neutral pH optimum and has been suggested to act on cytosolic free glycans arising from non-lysosomal breakdown (Miyagi and Yamaguchi 2012). NEU3 is localized to plasma membrane and exclusively hydrolyzes Sias on gangliosides, preferring either the α2,3- or α2,8-linkages found in GM3 and GD3 gangliosides. Interestingly, the ability of NEU3 to convert GT1b and GD1a to monosialic GM1 plays a role in axon growth and regeneration (reviewed in (Higuero et al. 2017) in this issue). See also the article by Kopitz (2017) in this issue for more information on glycolipids and the article by Kaltner et al. (2017) in this issue on the functional consequences of galectin binding to glycolipids once Sia is removed. Two isoforms of NEU4 have been reported, and they are believed to direct the localization of NEU4 to lysosomes, mitochondria or the ER in different tissues. NEU4 is believed to act on gangliosides as well as sialyl Lewis^x (sLe^x) and sialyl Lewis^a (sLe^a) antigens (Läubli and Borsig 2010b; Miyagi and Yamaguchi 2012; Pearce and Läubli 2016).

While all four neuraminidases have been implicated in cancer progression, NEU1 and NEU3 have been most well studied (Miyagi et al. 2012; Miyagi and Yamaguchi 2012; Pearce and Läubli 2016). One group provided evidence that NEU1 is a positive regulator of the epithelial-to-mesenchymal transition (EMT) and cancer progression in pancreatic cancer via its desialylation of the EGF receptor (Gilmour et al. 2013). In contrast, another group provided evidence that NEU1 is a negative modulator of metastasis in colorectal cancer (Uemura et al. 2009). The latter observation appears to be more in line with the upregulation of STs in cancer and the resulting anti-apoptotic and pro-metastatic effects of increased sialylation (see below). Most recently, d'Azzo and colleagues demonstrated that the low NEU1 levels in metastatic sarcomas promote lysosomal exocytosis of lysosomal hydrolases which in turn enhances ECM degradation and invasion (Machado et al. 2015). The ganglioside-specific sialidase activity of NEU3 has been demonstrated to stimulate a number of signaling pathways to increase proliferation and block apoptosis in cancer (Pearce and Läubli 2016). The roles of NEU2 and NEU4 in cancer are summarized in (Miyagi and Yamaguchi 2012; Pearce and Läubli 2016).

Roles of sialic acid in health and disease

Sia is a unique monosaccharide. With its negative charge at physiological pH, it is comparable to a phosphate group, albeit larger, and also can be modified at several positions by acetyl, sulfate and other groups (Schauer 2009; Varki and Schauer 2009). As a result, Sia can impact protein conformation and oligomerization, and the interactions of proteins with other proteins and the extracellular matrix. Not surprisingly, Sia expression is dynamic, changes during development and is altered in numerous diseases including immune disorders and cancer (Dall'Olio et al. 2014; Pearce and Läubli 2016; Varki and Gagneux 2012). Sialylated
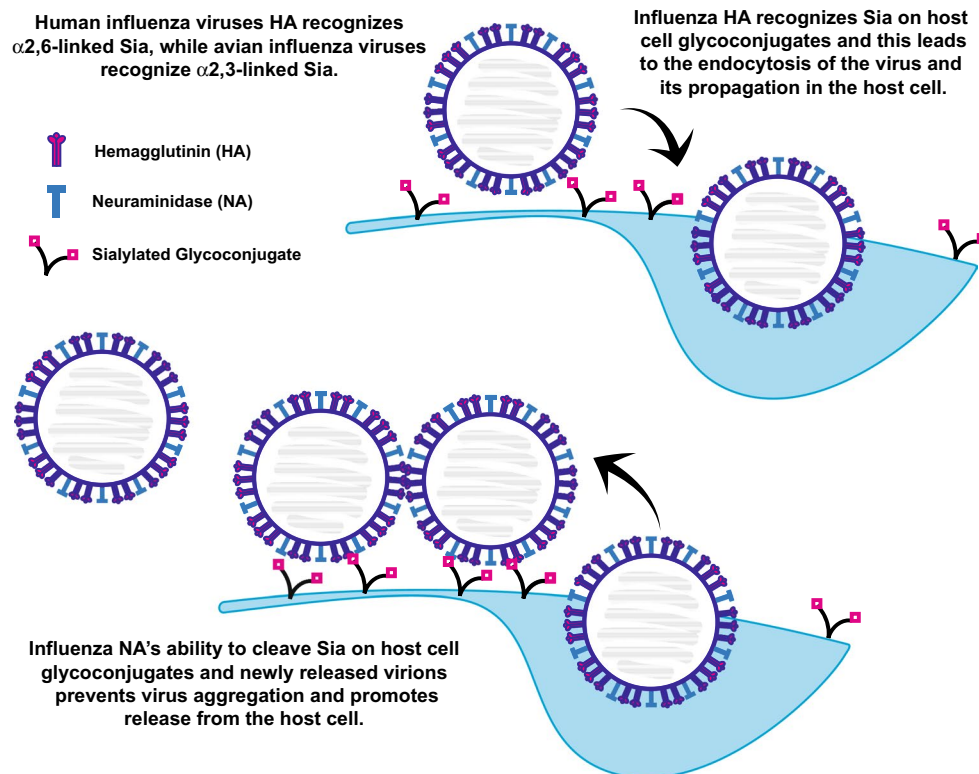
Fig. 5 Influenza virus hemagglutinin and neuraminidase recognize Sia residues on host cell glycoconjugates. The hemagglutinin (HA) proteins on the influenza virus membrane recognize Sia residues on host cell surface glycoconjugates. This interaction allows the endocytosis of the virus and its propagation inside the host cell. Release of the newly made virions from the host cell can be hampered by HA–Sia interactions between the host cell surface glycoconjugates and the virus. Sia-mediated interactions between viruses can also cause aggregation at the host cell surface. The viral neuraminidase (NA) cleaves Sia allowing the release of the virus from the host cell and the dispersion of aggregates. Many influenza drugs are designed to block the activity of the NA. Influenza viruses that infect humans recognize α2,6-linked Sia, while influenza viruses that recognize avian species recognize α2,3-linked Sia

glycans are binding partners for lectins that control key processes in health and disease (selectins, Siglecs, viral cell attachment proteins), impact the ligand binding and signaling of receptors (β1-integrin) and regulate cell migration during development and in cancer. These functions of Sia, with a particular focus on those involving sialylated N-linked glycans, will be discussed below.

Sia and its binding proteins in viral infection

Sia on both glycoproteins and glycolipids (gangliosides) is used as an entry receptor by many different viruses including influenza viruses, adenoviruses, coronaviruses, rotaviruses, toroviruses and reoviruses [reviewed in Matrosovich et al. (2015), Neu et al. (2011), Stencel-Baerenwald et al. (2014)]. Binding to host cell receptors, followed by membrane fusion (either plasma membrane or endosomal membrane), allows viruses to deposit their genetic material in host cells and replicate (Fig. 5). The influenza A and B viruses have two Sia recognition molecules: the hemagglutinin (HA) that recognizes the sialylated glycoconjugate on the host cell surface and possesses sequences essential for membrane fusion, and the neuraminidase (NA), an acetylesterase that cleaves Sia and is critical for virion release following viral replication (Fig. 5). In contrast, for influenza C the ability to recognize its receptor (9-O-acetylated Sia), to fuse with the membrane and to cleave Sia is all combined in a hemagglutinin-esterase-fusion (HEF) protein (Matrosovich et al. 2015). Other viruses, such as coronaviruses, toroviruses and Newcastle disease virus have hemagglutinin-esterase (HE) or hemagglutinin-neuraminidase (HN) proteins, respectively (Neu et al. 2011). Both the influenza viruses and type 3 reoviruses use sialylated N-glycans as receptors (Stencel-Baerenwald et al. 2014). For type 3 reoviruses, a cell attachment protein, σ1, recognizes Sia allowing tethering to the host cell membrane and secondary binding by the junctional adhesion molecule, JAM-A (JAM-1) (Matrosovich et al. 2015; Stencel-Baerenwald et al. 2014). The structure of the reovirus σ1 in complex with α2,3-, α2,6- and α2,8-sialyllactose has been recently solved (Reiter et al. 2011). Below, we discuss in more detail Sia binding by influenza HA and NA.

The influenza HA is a homotrimeric receptor, is responsible for both host recognition and membrane fusion and is essential for viral entry and replication (Fig. 5) (Skehel and Wiley 2002). The HA proteins from influenza viruses that infect humans and birds recognize distinct Sia linkages, with avian viruses binding preferentially to α2,3-linked Sia and human viruses binding preferentially to α2,6-linked Sia (Rogers and Paulson 1983; Rogers et al. 1983). Avian viruses are believed to be transmitted orally, while human viruses enter through the respiratory track (Wilks et al. 2012). Consistent with this notion, α2,3-linked Sia is found in bird's intestinal epithelium and only in the lower respiratory tract of humans, while α2,6-linked Sia is enriched on bronchial epithelium of the human upper respiratory tract (Shinya et al. 2006). Others have demonstrated a further distinction between avian and human viruses and shown that avian viruses infect ciliated cells while human viruses infect non-ciliated cells in the airway epithelium (Matrosovich et al. 2004a, 2007). In 1988, Weis and colleagues reported the structure of the HA in complex with Sia and identified the Sia-binding pocket and key hydrogen bonds stabilizing this interaction (Weis et al. 1988).

Influenza pandemics that lead to significant loss of life typically result when a highly virulent avian-like virus infects humans (Taubenberger and Kash 2010; Wilks et al. 2012). This can result from the oral transmission of the avian virus to humans, and/or reassortment of genetic segments when an avian and human virus infects the same host (for example, swine) that leads to a "human-specific" HA being paired with other proteins from an avian strain, or the accumulation of mutations in avian HAs that make them "human adapted" (Taubenberger and Kash 2010; Wilks et al. 2012). To this point, an interesting study by Chandrasekaran et al. (2008) evaluated the sialylated structures recognized by human-adapted H1N1 and H3N2 viruses in contrast with the avian influenza virus H5N1. Evaluation of the glycan structures on a human upper respiratory epithelial cell line revealed a preponderance of α2,6-sialylated structures, many with multiple lactosamine repeats. Structural evaluation showed that all three monosaccharides in the Neu5Acα2,3Galβ1,4GlcNAc structure form contacts with the HA-binding site and that these sugars form a cone-like structure. In contrast, the binding contacts of a Neu5Acα2,6Galβ1,4GlcNAc structure allow more flexibility and suggest that this glycan can assume not only a cone-like structure but also an umbrella-like structure in the HA-binding pocket. They also predicted that the length and branching of the oligosaccharide will influence the HA contacts made by α2,6-sialylated structures permitting longer α2,6-Sia terminated glycans to form an umbrella-like structure, while shorter α2,6-Sia terminated glycans to form a cone-like structure. Reevaluation of HA–glycan co-crystal structures, as well as direct binding assays,

demonstrated that the two human-adapted HAs (H1 and H3) had accumulated mutations that allowed them to make more contacts with α2,6-Sia in the umbrella-like form, and to bind to upper respiratory tract tissues, and in particular the apical membranes of tracheal tissue sections that are enriched in α2,6-sialylated glycans with longer branches (Chandrasekaran et al. 2008). However, a simple alteration of HA specificity in the avian virus is likely not the whole story since other viral proteins including the NA are key regulators of infection and virulence (Matrosovich et al. 2004b; Taubenberger and Kash 2010).

Influenza virions bud from the surface of the host cell, but many times aggregate or get trapped by binding to surface Sia. They are released from the cell by a tetrameric NA that is also found on the viral capsid (Fig. 5) (Matrosovich et al. 2015; Wilks et al. 2012). NA inhibitors such as oseltamivir (Tamiflu), zanamivir (Relenza) and peramivir (Rapivab) have been developed to treat influenza infections (Burnham et al. 2013). Studies of drug-resistant influenza strains have demonstrated that a balance between the contrasting activities of HA and NA dictates viral growth, transmission and host adaptation (Baigent and McCauley 2001; Gaymard et al. 2016b). For example, oseltamivir-resistant strains isolated after 2007 exhibited a particular mutation in NA, which coexisted with a new variant of HA, leading to a virus with better "viral fitness" likely due to a readjusted HA–NA balance (Gaymard et al. 2016a; Rameix-Welti et al. 2008).

Siglecs and their role in the immune response

Siglecs are another family of Sia-binding lectins that are implicated in a variety of immune cell diseases (Macauley et al. 2014; O'Reilly and Paulson 2009). Here we will briefly introduce the Siglec family and describe how the interaction of two family members (Siglec-2 or CD22 and Siglec-10 or Siglec-G in mouse) with their sialylated ligands modulates the B cell response and autoimmunity (Fig. 6).

Siglecs are type I membrane proteins and immunoglobulin (Ig) superfamily (IgSF) proteins with multiple Ig domains including a single N-terminal V-set Ig domain for Sia ligand binding linked to 1–17 C2-set Ig domains (see Fig. 6a for examples) (Macauley et al. 2014; Varki and Crocker 2009). As lectins of the IgSF, Siglecs are also categorized as I-type lectins (Varki and Crocker 2009). A number of Siglecs have been identified in humans that fall into two groups. The first group is conserved across mammals and includes Siglec-1 (sialoadhesin), Siglec-2 (CD22), Siglec-4 (MAG) and Siglec-15. The second group is equivalent to the CD33 (Siglec-3) family (five identified in mice, nine identified in humans). This second group of Siglecs is not ubiquitously found in all mammals and shows rapid
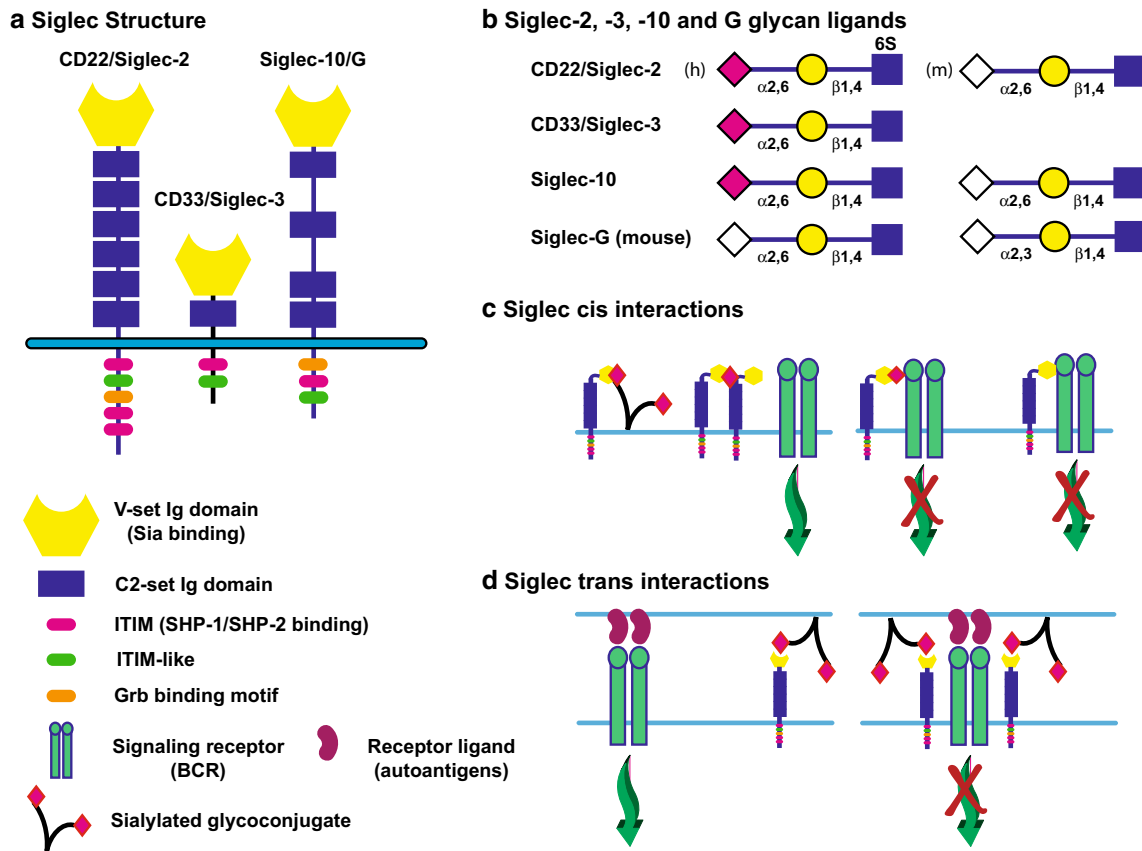
Fig. 6 Siglecs and their role in the regulation of the immune response. **a** Siglecs share a common structure with an N-terminal V-set Ig domain that recognizes Sia, followed by a variable number of C2-set Ig domains, a transmembrane region and cytoplasmic tail. The cytoplasmic tail of Siglecs contains a number of binding sequences. Inhibitory Siglecs contain immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences that allow SHP-1 and SHP-2 binding and inhibition of signaling, in addition to other signaling molecule-binding sequences. Activating Siglecs (not shown) possess a basic residue in their TM regions that allow the binding of DAP12, an immunoreceptor tyrosine-based activation motif (ITAM) adaptor protein that promotes the activation of signaling. Examples of Siglecs that bind sialylated N-glycan structures are shown. **b** The sialylated ligands recognized by selected Siglecs are shown. Siglec-10 (human) and

Siglec-G (mouse) are considered orthologs; however, the human protein recognizes only α2,6-linked Neu5Ac (*pink diamond*) or Neu5Gc (*white diamond*) structures, while the mouse Siglec recognizes both α2,3- and α2,6-linked Sia in the Neu5Gc form. **c** Siglecs can bind ligands in *cis*. An inhibitory Siglec can be sequestered away from an activating receptor by binding to other sialylated ligands, including itself (*left*). Conversely, binding to an activating receptor directly or via Sia residues on its glycans can inhibit its signaling (*right*). **d** Siglecs can bind ligands in *trans*. These interactions can either sequester the Siglec away from an activating receptor (*left*), or if sialylated ligands are close to the ligands for the activating receptor, this interaction may enhance the ability of the Siglec to inhibit the activating receptor (*right*). Please see Varki and Crocker (2009), Macauley et al. (2014) for more details on Siglec structure and function

evolution by gene duplication, conversion, exon loss and exon-shuffling events (Angata et al. 2004; Padler-Karavani et al. 2014). Siglecs exhibit specificity for distinct sialylated glycan structures; however, when similar specificities exist, the comparable Siglecs may be expressed in different cell types and/or as part of an inhibitory/activating Siglec pair (see below). For a complete list of glycan structures bound by various human and mouse Siglecs, and the cell types in which they are expressed, please refer to Macauley et al. (Macauley et al. 2014).

Siglecs function in the innate and adaptive immune system to allow the discrimination between "self" and "non-self" (Macauley et al. 2014). As Sia-binding proteins, they

effectively recognize "self" markers on the surfaces of cells in a *cis* (same cell) or *trans* (adjacent cell/organism) fashion, leading to the modulation of the immune response and preventing autoimmunity (Fig. 6c, d). Pathogens like group B *Streptococcus* exploit this mechanism for immune response downregulation by coating themselves in "self"-like sialylated glycans. The cytoplasmic tails of what are known as "inhibitory" Siglecs have immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which recruit phosphatases SHP-1 and SHP-2 to negatively regulate downstream signaling pathways (Fig. 6a) (Avril et al. 2004; Paul et al. 2000). In contrast, "activating" Siglecs have a positively charged residue in their transmembrane region that

can be bound by the immunoreceptor tyrosine-based activation motif (ITAM) adaptor protein, DAP12, leading to recruitment of the SYK kinase to activate downstream signaling pathways (Blasius et al. 2006; Macauley et al. 2014). The evolution of "activating" Siglecs (Siglec-14, Siglec-15 and Siglec-16) and their murine counterpart (Siglec-H) was suggested to be a response to pathogens exploiting inhibitory Siglecs to suppress host immune response. In support of this idea, most humans express two pairs of Siglecs, with each pair exhibiting the same glycan-binding activity but with different functional activities. For example, Siglec-5 (inhibitory) is expressed with Siglec-14 (activating), while Siglec-11 (inhibitory) is expressed with Siglec-16 (activating) (Macauley et al. 2014).

Peripheral B cells express both CD22 (Siglec-2) and Siglec-10 (Siglec-G in mice) that work together to prevent the generation of self-reactive B cells and autoantibodies by inhibiting the signaling downstream of the B cell receptor (BCR), thereby contributing to a process called B cell tolerance [reviewed in Macauley et al. (2014), Paulson et al. (2012)]. Human CD22 recognizes Neu5Acα2,6Galβ1,4(6S)GlcNAc (6S indicates sulfation of C6 hydroxyl of GlcNAc residue), while mouse CD22 recognizes the unsulfated structure containing Neu5Gc (Fig. 6b). Human Siglec-10 recognizes Neu5Acα2,6Galβ1,4GlcNAc, while mouse Siglec-G recognizes both Neu5Gcα2,6Galβ1,4GlcNAc and Neu5Gcα2,3-Galβ1,4GlcNAc (Fig. 6b) [reviewed in Macauley et al. (2014)]. Both of these Siglecs are inhibitory and possess ITIMs and other signaling molecule-binding sites in their cytoplasmic tails.

Experiments in mouse models have established the role of CD22 and Siglec-G in immune system regulation. CD22-knockout mice exhibit a hyper-responsive immune system due to an inability to inhibit BCR-induced Ca^{2+} signaling (Nitschke 2005; Nitschke et al. 1997; O'Keefe et al. 1996; Otipoby et al. 1996; Sato et al. 1996). Siglec-G-knockout mice exhibit an enhanced B1 cell lifespan and an altered BCR repertoire (Jellusova et al. 2010a). A double knockout of both of these Siglecs in mice leads to systemic autoimmunity (Jellusova et al. 2010b). On the other hand, the ST6Gal-I-knockout mouse that lacks CD22 ligands exhibits reduced signaling upon B cell stimulation (Hennet et al. 1998). A double knockout of CD22 and ST6Gal-I restores normal B cell signaling linking the diminished B cell signaling of the ST6Gal-I-knockout mouse to the ligand-binding function of CD22 (Collins et al. 2006). Interestingly, these investigators showed that there was an increase in CD22 colocalization with the BCR in clathrin-rich microdomains and a reduced CD22 homo-oligomerization in mice lacking ST6Gal-I, CD22 ligands, and exhibiting reduced BCR signaling, suggesting that CD22 interactions with the BCR are not dependent upon the presence of α2,6-Sia (Collins et al. 2006; Ghosh et al.

2006). More recently, Müller and colleagues showed that mice expressing CD22 with a mutated ligand-binding domain exhibited reduced BCR-induced Ca^{2+} signaling and this could be explained by the inability of the mutant CD22 to bind cell surface α2,6-Sia ligands in *cis* (including those on adjacent CD22 molecules) and its increased association with and inhibition of the BCR (Fig. 6c) (Müller et al. 2013). On the other hand, expressing Siglec-G with a mutated ligand-binding domain in mice led to an increase in B1 cell Ca^{2+} signaling and expansion of the B1 cell population, and this was linked to a decrease in the Siglec-G–BCR interaction (Fig. 6c) (Hutzler et al. 2014). Based on these and other experiments, the authors propose that sialylated glycans displayed by the IgM portion of the BCR are typically bound by Siglec-G to control BCR signaling (Fig. 6c). Several other studies have also suggested the possibility that simultaneous interaction of the BCR with autoantigens and CD22/Siglec-G with *trans* ligands on the same cell may bring these inhibitory Siglecs into proximity with the BCR in this "immunological synapse" to dampen self-reactive B cell signaling (Fig. 6d) reviewed in Macauley et al. (2014). In sum, a complex series of interactions between CD22 and Siglec-10/G and their ligands, including the BCR, coordinate to control B cell signaling and the autoimmune response.

Selectins and the roles they play in the immune system and cancer

Selectins are carbohydrate-binding proteins that minimally recognize the sialyl Lewis X structure (sLe^X) (Neu5Acα2,3Galβ1,4[Fucα1,3]GlcNAcβ1-R) on N- and O-glycans of specific ligands (Fig. 7). They mediate the rolling adhesion of cells such as leukocytes, platelets and hematopoietic progenitors along vascular surfaces as they are recruited to locations of injury or infection [reviewed in Lowe (1997, 2003), McEver (2015), McEver and Zhu (2010), Sperandio et al. (2009)]. Selectins are Ca^{2+}-dependent lectins and, as such, are considered C-type lectins. Please see the article by Mayer and colleagues in this thematic issue for a review of C-type lectins (Mayer et al. 2017). They are type I membrane proteins that consist of an N-terminal lectin domain, an EGF-like module, followed by 2–9 consensus repeats, a transmembrane region and a short cytoplasmic tail (McEver 2015; McEver and Zhu 2010). There are three selectins that differ in the number of consensus repeats and their expression patterns (Fig. 7a). P-selectin is stored in endothelial cell Weibel–Palade bodies and platelet α-granules and moves to the cell surface upon cell stimulation with thrombin, complement or histamine (McEver 2015). E-selectin is constitutively expressed on skin and bone endothelial cells, but in other tissue types, its expression on post-capillary venules is induced by
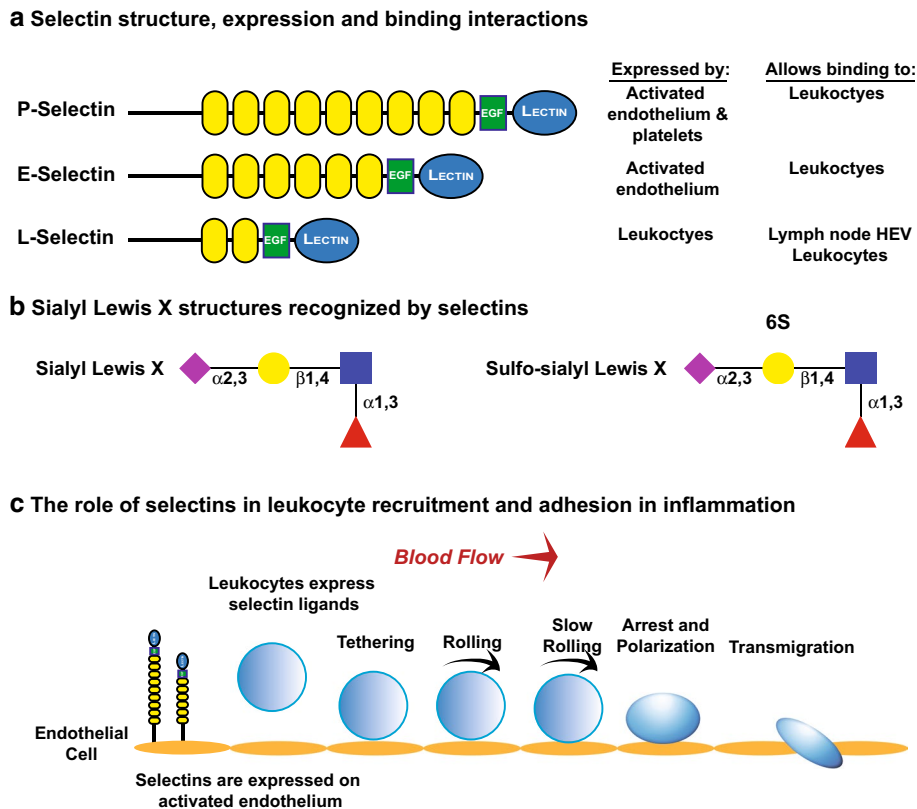
a Selectin structure, expression and binding interactions

b Sialyl Lewis X structures recognized by selectins

c The role of selectins in leukocyte recruitment and adhesion in inflammation

Fig. 7 Selectins mediate binding to glycans capped with sialyl Lewis X structures to mediate cells interactions in inflammation, lymphocyte homing and metastasis. **a** Structures of P-, E- and L-selectins, cells on which they are expressed, and the binding they mediate. **b** Sialyl Lewis X and sulfo-sialyl Lewis X structures. Note that while all selectins recognize sialyl Lewis X, sulfo-sialyl Lewis X is primarily expressed by peripheral lymph node addressins (PNAds) recognized by L-selectin. **c** Selectin function in leukocyte recruitment and adhesion in inflammation (McEver and Zhu 2010). P- and E-selectins are expressed by activated endothelium and mediate recruitment, tethering and initial stages of leukocyte rolling on the endothelium. Signaling through selectin ligands on the leukocytes stimulates a conformational change in integrins expressed by leukocytes, leading to weak binding of their receptors on the endothelium and slowing their rolling. Release of chemokines from endothelium stimulates signaling from chemokine receptors on the leukocyte membrane, and these signals stimulate the conversion of integrins to forms with high affinity for ligands, and these high-affinity interactions lead to leukocyte arrest. Ultimately, the leukocyte migrates through the endothelium to the site of inflammation

pro-inflammatory cytokines like TNFα or IL-1β. L-selectin is constitutively expressed on the surface of leukocytes (Läubli and Borsig 2010b; McEver and Zhu 2010). Selectin–ligand interactions are involved in the recruitment of leukocytes to sites of inflammation, the homing of naïve lymphocytes to secondary lymphoid organs, the recruitment of hematopoietic stem cells to the bone marrow and platelets to sites of hemorrhage. In addition, expression of selectin ligands by cancer cells facilitates their metastasis and survival (Läubli and Borsig 2010b). We will describe some of these processes in more detail below.

The sialylated and fucosylated sLe^x structure capping N- and O-glycans is a key component of selectin ligands (Fig. 7b) (Lowe et al. 1990; Phillips et al. 1990). Several pieces of evidence show that ST3Gal-IV and ST3Gal-VI are the α2,3-sialyltransferases involved in sLe^X synthesis. Combinatorial knockout of both STs led to a decrease in neutrophil binding to E- and P-selectins, selectin-dependent rolling, and lymphocyte homing (Yang et al. 2012). Other work suggests that ST3Gal-IV plays the major role in the synthesis of sLe^X structures that mediate selectin-dependent adhesion and rolling. For example, it was recently demonstrated that ST3Gal-IV knockout results in significant reduction in the synthesis of sLe^a and sLe^x structures in HL-60 cells and in neutrophils derived from human hematopoietic stem cells. These cells show significant impairment in rolling and adhesion to the endothelial cells (Mondal et al. 2015). Fucosylation is also crucial for the function of all the selectins, as mice deficient in α1,3-fucosyltransferase, FucT-VII, exhibit impaired recruitment and extravasation of leukocytes to sites of inflammation (Lowe 1997; Maly et al. 1996).

Below, we will briefly review the role of selectins in inflammation and in lymphocyte homing [reviewed in

McEver (2015), McEver and Zhu (2010), Rosen (2004)]. Leukocytes are recruited to the site of inflammation in a process that begins an interaction between endothelial cell selectins (predominantly P- and E-selectins) and their ligands on a leukocyte (Fig. 7c). These interactions allow the selectin ligands to transduce signals that convert leukocyte integrins to conformations with low affinity for their endothelial cell receptors such as ICAM-1 (ligand for αLβ2 integrin) and VCAM-1 (ligand for α4β1 integrin). These weak interactions allow the leukocytes to slowly roll on the surface of vascular wall and enable the release of immobilized chemokines from the endothelial cell membrane to stimulate signaling through chemokine G protein coupled receptors on the leukocyte membrane. These signals convert integrins to a high-affinity ligand-binding conformation that allows the arrest of cell rolling. Details of the signaling pathways involved in this process are found in (McEver 2015). The final step of leukocyte homing is transmigration through the vascular endothelium to the site of inflammation. Some of the well-characterized proteins carrying selectin ligands include P-selectin glycoprotein ligand-1 (PSGL-1), CD44 and E-selectin ligand-1 (ESL-1) [reviewed in Läubli and Borsig (2010b), McEver and Zhu (2010)]. Notably, PSGL-1 is the primary ligand for P- and L-selectins on leukocytes and optimal selectin binding requires not only the sLe^X structure but also contacts with amino acids and sulfated tyrosines in the N-terminal region of PSGL-1 (McEver and Zhu 2010). On the other hand, E-selectin binds preferentially to PSGL-1, CD44 and E-selectin ligand-1 (ESL-1), but its binding to PSGL-1 does not require the presence of sulfate (McEver and Zhu 2010).

L-selectin is involved in the process of lymphocyte homing to secondary lymphoid organs, such as the lymph nodes, via interactions with selectin ligands on high endothelial venules (HEV) (Butcher and Picker 1996; Rosen 2004). In the case of naïve T cells, entry into the secondary lymphoid organs allows them to encounter antigens, while in the case of memory T cells that have already been primed with antigen, L-selectin allows them to localize in these lymphoid organs where they will proliferate once antigen is encountered again. L-selectin ligands on the high endothelial venules are collectively called peripheral lymph node addressins (PNAds) and include CD34, GlyCAM-1 and podocalyxin (Rosen 2004). Sulfated sLe^X structures on both O- and N-glycans on these PNAds are key for L-selectin binding (Fig. 7b) [reviewed in McEver and Zhu (2010)].

Selectin interactions are deregulated in various diseases such as atherosclerosis, sepsis and cancer (Läubli and Borsig 2010b). The formation of atherosclerotic lesions is facilitated by the involvement of selectins, mainly P-selectin, on platelets (Collins et al. 2000; Dong et al. 2000). In addition, selectin knockout or inhibition prevents acute

inflammation and tissue damage in mice (Mangell et al. 2007; Singbartl et al. 2000; Tedder et al. 1995) and reduces atherosclerotic lesions (Collins et al. 2000; Dong et al. 2000). Glycans capped with sLe^X structures are enriched on the surface of cancer cells, and their presence is correlated with increased cancer progression and poor prognosis (Kannagi et al. 2004; Läubli and Borsig 2010b; Pinho and Reis 2015). Work done by several groups suggests that cancer cells employ selectin–ligand interactions to adhere to and extravasate through the endothelium and that ablation or inhibition of selectins reduces metastasis and tumor growth [for example, Biancone et al. (1996), Borsig et al. (2002), Büll et al. (2014a), Kim et al. (1998), Läubli and Borsig (2010a, b), Läubli et al. (2006)]. Notably, changes in sialylation have been observed following the induction of EMT, a process that allows cancer cells to break away from tumors, migrate and invade. For example, induction of EMT in colon cancer cells by EGF or basic FGF led to the upregulation of ST3Sia-I, ST3Sia-III and ST3Sia-IV that are involved in the synthesis of sLe^X and sLe^a structures that serve as ligands for E-selectin, a selectin implicated in mediating the extravasation of circulating tumor cells into tissues for metastatic colonization (Sakuma et al. 2012). Low molecular weight heparins are potent inhibitors of metastasis, and a single dose of heparins attenuates lung metastasis, mainly via P-selectin inhibition (Borsig et al. 2002; Koenig et al. 1998). Likewise, fucosylated polysaccharide nanoparticles are potent inhibitors of P-selectin, as well as tumor growth and metastasis (Shamay et al. 2016).

Hypersialylation of β1-integrin and Fas receptor in cancer cells and their role in the increased motility and resistance to cell death and chemotherapy

It has been long appreciated that sialylated glycans on the surface of cancer cells are upregulated and contribute to increased aggressiveness, metastasis and invasion of a tumor by increasing cancer cell motility, extravasation from the circulation into tissues and resistance to cell death [reviewed in Büll et al. (2014a, b), Dall'Olio et al. (2014), Lu and Gu (2015), Pearce and Läubli (2016)]. In addition, hypersialylation of cancer cell surfaces enhances a cancer's resistance to chemotherapy. In particular, ST6Gal-I has been reported to be upregulated in various cancers as a consequence of oncogenic Ras activation [for example, Le Marer et al. (1992), Seales et al. (2003, 2005b)]. ST6Gal-I expression has been correlated with the expression of stem cell markers in colon, pancreatic and ovarian cancers and directly shown to promote metastasis and survival/resistance to drug treatment (Schultz et al. 2013, 2016; Swindall et al. 2013). Below, we discuss how upregulation of ST6Gal-I by cancer cells and the increased sialylation of

β1-integrin and the death receptor, FasR, controls cancer cell ECM adhesion and cell motility and resistance to apoptosis, respectively.

Bellis and colleagues have demonstrated that hyper-sialylation of β1-integrin in cancer cells impacts adhesion to extracellular matrix (ECM) and cell motility as well as resistance to cell death [reviewed in Schultz et al. (2012)]. Integrins are heterodimeric transmembrane receptors capable of bidirectional signaling. They can modulate intracellular signaling as a result of changes in the ECM, termed "outside-in signaling," and can change their affinity for various ECM proteins-based various intracellular signaling events, termed "inside-out signaling" (Kim et al. 2011). The conformation and dimerization of integrins is important for their activity and influences cell adhesion, membrane detachment, migration, invasion and anchorage-dependent growth. The expression of oncogenic Ras leads to the upregulation of ST6Gal-I and the hypersialylation of the N-glycans of β1-integrin that is part of the integrin receptor for collagen (α1β1 and α2β1), fibronectin (α5β1) and laminin (α3β1) (Pochec et al. 2003; Pretzlaff et al. 2000; Seales et al. 2003; Semel et al. 2002; Shaikh et al. 2008). The sialylation of β1-integrin N-glycans alters the integrin receptor affinity for these ECM molecules. For example, hypersialylation of β1-integrins in ovarian adenocarcinoma cells results in increased cell motility by enhancing collagen binding and migration toward collagen (Christie et al. 2008; Seales et al. 2005a). These effects are believed to be caused by sialylation-mediated stabilization of β1-integrin in a conformation that has higher affinity for the particular ECM ligands (Shaikh et al. 2008).

Additional work by this group and others highlights the ability of cell surface α2,6-sialylation to block apoptosis and anoikis (cell death related to detachment from other cells or the ECM) (Amano et al. Amano et al. 2003, 2012; Liu et al. 2011; Sanchez-Ruderisch et al. 2011; Swindall and Bellis 2011). Early studies showed that the sialylation of T cell CD45 by ST6Gal-I blocks galectin-1 clustering of CD45 and resulting cell death (Amano et al. 2003). Please see the article in this thematic issue on galectins by Kaltner and colleagues for additional information on this family of proteins (Kaltner et al. 2017). More recently, Swindall and Bellis showed that α2,6-sialylation of FasR blocks binding of Fas-associated adaptor molecule (FADD) to the FasR death domain, thus inhibiting the formation of the death-inducing signaling complex (DISC) (Swindall and Bellis 2011). Internalization and additional DISC formation was also hindered further blocking apoptotic signaling. Other studies demonstrated that the sialylation of α5β1 fibronectin receptor blocks galectin-1 binding and the induction of anoikis (Amano et al. 2012; Sanchez-Ruderisch et al. 2011). Interestingly, Amano et al. (2012) showed

that p16^{INK4a}, an anoikis-related tumor suppressor, acts to upregulate the α5β1 integrin and galectin-1 and simultaneously reduces sialylation by downregulating GNE and NANS, key enzymes in the Sia biosynthesis pathway (see Fig. 4b). Along these lines, overexpression of ST6Gal-I confers resistance to chemotherapy agents such as cisplatin, irinotecan and gemcitabine (Schultz et al. 2013, 2016; Swindall et al. 2013).

PolySia and metastasis

The expression of polySia has been strongly correlated with late stage, highly metastatic cancers including small cell and non-small cell lung carcinoma (SCLC and NSCLC), neuroblastoma, pancreatic cancer, pituitary tumors, Wilms' tumor, rhabdomyosarcoma (Falconer et al. 2012) and, most recently, breast cancer (Wang et al. 2016). It is likely that the ability of polySia to abrogate cell adhesion and regulate signaling combine to exert its effects in cancer. Surprisingly, few studies have directly evaluated the role of polySia in cancer metastasis and those that did presumed that the polySia was modifying NCAM and influencing NCAM-mediated adhesion or signaling. Pioneering work by Scheidegger and colleagues compared two subclones of the NCI-H69 SCLC line and found that while both formed tumors when injected subcutaneously into nude mice, only the polySia expressing subclone formed significant numbers of intracutaneous metastases (Scheidegger et al. 1994). NCAM was expressed in these cells and was believed to be the only polysialylated protein. Later work by the Figarella-Branger group supported the role of polySia in cancer metastasis. They demonstrated that TE671 rhabdomyosarcoma cells generated lung metastases when injected into nude mice and that these could be decreased by repeated injections of EndoN, an enzyme that specifically cleaves polySia (Daniel et al. 2001). This group also showed that rat pituitary transplantable tumors that expressed high levels of polySia-NCAM were invasive and metastasized, while those that did not were benign (Daniel et al. 2000). In 2000, Tanaka et al. evaluated 57 NSCLC clinical cases and found that those tumors expressing polySia, but not NCAM, were more aggressive and metastatic than those expressing polySia-NCAM. Notably, ST8SiaII expression was highly correlated with tumor progression and later-stage tumors (Tanaka et al. 2000). The expression of NCAM was not correlated with tumor progression in this study, suggesting that the impact of polySia on carrier function rather than the sole presence of polySia might be more critical for tumor progression to later stages. As other polyST substrates like NRP-2, SynCAM-1 and ESL-1 have links to cancer, their polysialylation in NSCLC is of significant interest as a tumor marker and target for therapies.

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