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Published in:
Current Opinion in Structural Biology

Link to article, DOI:
10.1016/j.sbi.2016.08.008

Publication date:
2016

Document Version
Peer reviewed version

HHS Public Access

Author manuscript

Curr Opin Struct Biol. Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

Curr Opin Struct Biol. 2016 October ; 40: 104–111. doi:10.1016/j.sbi.2016.08.008.

Modulating carbohydrate-protein interactions through glycoengineering of monoclonal antibodies to impact cancer physiology

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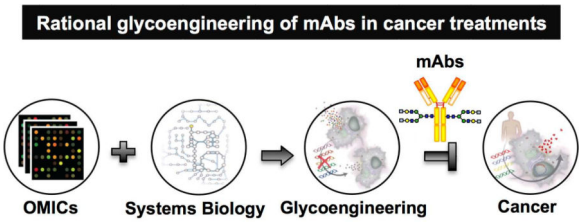
Abstract

Diverse glycans on proteins help impact cell and organism physiology along with drug activity. Since many protein-based biotherapeutics are glycosylated and these glycans have biological activity, there is a desire to engineer glycosylation for recombinant protein-based biotherapeutics. Engineered glycosylation can impact the recombinant protein efficacy and also influence many cell pathways by first changing glycan-protein interactions and consequently modulating disease physiologies. However, its complexity is enormous. Due to recent advances in glycoengineering, modulating protein-glycan interactions become more amenable to therapeutic approaches. Here, we discuss how engineered glycans contribute to therapeutic monoclonal antibodies (mAbs) in the treatment of cancers, how these glycoengineered therapeutic mAbs affect the transformed phenotypes and downstream cell pathways, and how systems biology can help in the next generation mAb glycoengineering process by aiding in data analysis and guiding engineering efforts to tailor mAb glycan and ultimately drug efficacy, safety and affordability.

Graphical Abstract



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Conflict of interest statement

The authors declare no conflict of interest.

Introduction

Monoclonal antibodies (mAbs) are the major category of glycoprotein-based therapeutic drugs, approved by the US Food and Drug Administration (FDA) [1]. Furthermore, they have attained considerable success in many therapies, including cancer, over the past three decades [2]. Despite remarkable advances in contemporary biopharmaceutical technologies, many challenges remain in efficiently manufacturing effective and affordable antibody-based drugs. Since glycosylation essentially impacts the therapeutic efficacies of mAbs [3], it is desirable to control glycoforms on therapeutic mAbs for the next generation mAb development. With fast growing innovative engineering and cutting-edge technologies, glycoengineering provides a promising method to tune the activities of therapeutic mAbs [4,5]. An effective glycoengineered mAb usually modulates specific interactions between designed glycans and target proteins, thereby impacting the activity of downstream pathways that control cancer physiology. Conversely, a wrong glycan can induce unwanted side effects and even adverse immunogenic response [6]. For example, some colorectal cancer patients develop hypersensitivity to the FDA approved mAb cetuximab [7].

Several intriguing and unsolved questions in mAb glycoengineering include the following. Which glycan structures will provide the optimal mAb? How can we efficiently and reliably engineer a consistent glycoform on mAbs? Challenges in answering these questions stem from our limited understanding regarding the intricate relationships between glycans, proteins, and host cell physiologies. Furthermore, even when desired glycoforms are known, it has been difficult to unravel all of the factors that influence glycosylation and to control the complex system.0020Systems biology provides a powerful toolbox for integrating heterogeneous omics data and for deciphering the mechanisms and interactions between molecules and pathways, using network analysis, mathematical modeling, and simulation [8,9]. An abundance of omics technologies have been developed to aid in studying glycoengineering and expression systems (e.g., [10]), but the application of omics data and systems biology in glycoengineering is still in its infancy. Here we review the state-of-art knowledge of glycan-protein interactions in the context of FDA-approved therapeutic mAbs and then summarize several innovative technologies that can help control the glycoforms on mAbs. Finally, systems biology-based glycoengineering approaches are explored with an emphasis on how systems biology can be used to advance anti-tumor mAb development toward a predictable glycoengineering era.

Glycan-protein interaction, therapeutic antibody, and cancer physiology

Glycosylation helps to modulate interactions between mAbs and antigens or Fcγ receptors (Figure 1A), and impact the efficacy and safety of a biotherapeutic drug. The glycan-protein interactions of FDA-approved therapeutic mAbs in various cancer settings and their subsequent effects reported in the literature are summarized in Table 1.

Fab-antigen interaction

Fab glycans have several roles in modulating interactions with receptors and glycoengineering can help reduce negative interactions leading to immunogenicity. Early research demonstrated that engineering N-linked oligosaccharides on the Fab region can

enhance the antigen-binding affinity of mAbs [11]. However, a comprehensive understanding of the glycans on Fab region is still lacking. One example of their role is the allergenic responses to therapeutic antibody cetuximab [7]. It was produced by murine myeloma cells (SP2/0), which adds an additional α1,3-galactose (the α-Gal epitope) on the N-linked oligosaccharide at Asn88 of the Fab region (Figure 1A). Unfortunately, the human IgE recognizes the non-human α-Gal epitope and leads to downstream immune responses, such as hypersensitivity reactions (anaphylaxis) after drug treatments. Therefore, extra efforts must be made to glycoengineer the drug to reduce α-Gal content and solve the immunogenicity problem (e.g., [12]). Moreover, the Fab-antigen binding affinity could be affected by targeting receptors. Recent mutagenesis studies showed that the potential glycosylation sites (Asn16, Ans25, Asn41, and Asn83) on Programmed death 1 (PD1) could impact antigen-binding affinity (e.g., nivolumab and pembrolizumab) by influencing its local structure [13]. Physiologically, PD1 is an inhibitory receptor that suppresses T cell responses to avoiding auto-immunity. Indeed, many factors can affect Fab-antigen binding in cancer treatment, and glycoengineering can be applied in mAb design either to optimize Fab-antigen binding affinity or to eliminate immunogenicity problem.

Beyond immunogenicity, Fab-binding can target cancer by modulating glycan-protein interactions. This occurs by either directly targeting glycans (Fab-glycan interaction) or indirectly modulating downstream protein-glycan interactions to treat cancer. At least four types of glycan-protein interactions involving the Fab have been explored. First, the Fab can interact with a target glycan, such as seen with Alemtuzumab which directly targets a glycosylphosphatidylinositol (GPI) anchor on the CAMPATH-1 (CD52) antigen to treat chronic myeloid leukemia. CD52 plays a dominant role in mediating cell depletion via apoptosis [14]. Second, the Fab can target siglec-sialic acid interactions of inhibitory siglecs (e.g., CD22) that normally prevent B-cell over-stimulation by inhibiting B-cell receptor signaling [15]. For example, the anti-CD22 antibody (epratuzumab) targets siglec-sialic acid interactions between B cells with endothelial cells, leading to downstream B-cell receptor signaling suppression [16]. Third, the Fab can interfere with angiogenesis by blocking interactions between glycans and VEGF, a key inducer for several downstream signaling pathways (e.g., MAPK and PI3K/AKT [17]) that lead to vascular and stromal cell ablation [18]. Bevacizumab binds VEGF to interrupt interactions between VEGF and heparin sulfate proteoglycans (HSPGs), leading to VEGF-VEGFR interaction blockade [19]. Fourth, mAbs can modulate Gal1-N-linked glycan interactions. Specifically, anti-VEGF refractory tumors can secrete excess Galectin-1 (Gal1). This enables tumor immune escape and metastasis by interacting with the β1,6-GlcNAc branching N-glycans on the endothelial cell receptors [20]. Anti-Gal1 antibodies can block the interaction between Gal1 and the N-glycan on VEGFR, leading to angiogenesis inhibition. While some mAbs might not require a glycosylation on the Fab-antigen binding site, to successfully interrupt downstream glycan-protein interactions, engineering glycosylation can improve their pharmacological properties (e.g., stability and effector functions) for maintaining optimal efficacy (see discussion below).

Fc-FcγR interaction

Fc binding with the Fcγ receptor (FcγR) on immune cells is crucial to mAb efficacy in governing downstream immunological responses of complement-dependent cytotoxicity (CDC) [21] and antibody-dependent cellular cytotoxicity (ADCC) [22] activities, which are immune mechanisms for killing target cells. In essence, the interaction between Fc and FcγR is a glycan-glycan interaction that is significantly affected by Fc-glycosylation (at Asn297). Indeed, mAbs with aglycosylated Fc regions lose effector functions, and mAbs with glycosylated Fc can activate downstream effector mechanisms. However, different engineered glycoforms significantly alter the efficacies of effector functions; for example, removal of the α1,6-linked core fucose from IgG-Fc glycans results in better effector functions compared to the IgG with fucosylated Fc glycans [23]. The N-glycans attached to the FcγRIIIa mediate the interaction with nonfucosylated IgG1-Fc, thereby stabilizing the Fc-FcγRIIIa complex, while fucosylation of the Fc N-glycans otherwise inhibits the interaction due to the steric hindrance. NMR analysis shows that defucosylation facilitates the active conformation of the Fc Tyr296 and the formation of a high-affinity complex; however, presence of fucose inhibits complex formation by reducing the flexibility of Tyr296. Other modifications to glycans further impact function. For example., bi-antennary N-glycans that are terminated with alpha-2,6-linked sialic acids is an optimal structure to enhance effector functions by strengthening interactions with the FcγRIIIa [24]. Intriguingly, effector functions are highly correlated with structural stability [25,26]. Glycosylated mAbs exhibit better structural stability and aglycosylated mAbs tend to unfold [27]. Indeed, a recent study indicated that an Fc Nglycan substantially stabilized the conformation of the Fc C'E loop by interacting with self-protein. Interestingly, the C'E loop stability is correlated with the Fc-binding affinity [28]. Moreover, an Fc with bisecting or high galactosylation N-glycans may open up the horseshoe-shaped conformation of the Fc fragment, which is favored by Fc-FcγR interaction, resulting in the enhanced effector functions [29]. In addition, different glycoforms also impact the pharmacokinetic behavior of mAbs [30]. Indeed, glycosylated mAbs can significantly increase their serum half-life, due to the prevention of proteolytic degradation [31]. Indeed, conformational changes of aglycosylated mAbs increase protease accessibility to the hinge region [32]. Notably, terminal sugars affect proteolytic resistance; glycoengineered mAb terminated with GlcNAc (G0 glycoforms) showed at least two times greater resistance to papain digestion than those terminated with sialic acid (G2S2 glycoform) and beta-galactose (G2 glycoform) [33].

Two glycoengineered mAbs have been approved by the FDA — mogamulizumab and obinutuzumab. Mogamulizumab targets CC chemokine receptor 4 (CCR4) to inhibit CCR4-mediated signal transduction pathways for T-cell leukemia/lymphoma treatment [34]. The antitumor activity strongly relies on ADCC, which is enhanced by glycoengineered afucosylated-Fc [35]. Obinutuzumab targets CD20 to treat chronic lymphocytic leukemia by directly inducing tumor cell apoptosis. Obinutuzumab demonstrated superior antitumor activity than rituximab, a leading FDA-approved CD20-targeting mAb. The superiority of obinutuzumab comes from the enhanced ADCC by glycoengineering afucosylated N-glycans with bisecting GlcNAc [36]. Inspired by these successful examples, more than 20 glycoengineered mAbs are currently in clinical trials.

Remarkably, the structure of glycans and their locations are the two most prominent properties governing the interaction between mAb and receptor, three generally desired properties of glycosylation on mAbs are summarized. First, removal of the N-glycan on Fab (Asn88) could avoid immunogenicity. Second, modulation of N-glycans on the Fc (Asn297) can enhance structural stability and Fc binding affinity. Third, three structures for the N-glycan on Fc (Asn297) have shown to enhance effector functions (ADCC/CDC): the bi-antennary N-glycans terminated with two alpha-2,6-linked sialic acids (ADCC) [24], the afucosylated Fc (ADCC) [23], and the high galactosylated Fc (CDC) [42]. These properties have been considered extensively while glycoengineering mAbs, but it is possible that many more desirable structures and locations on mAbs remain undiscovered.

Glycoengineering to control glycoforms on therapeutic proteins

While there is a vast diversity of glycans in mammalian cells, far fewer are commonly on mAbs (Figure 1B). Novel technologies and knowledge allow us to modify the glycan for more effective mAbs for cancer treatments. Glycoengineered mAbs can enhance therapeutic efficacy (antigen binding affinity and effector functions) and safety (immunogenicity). They also influence other properties (e.g., pharmacokinetics (PK) and pharmacodynamics (PD)) on biotherapeutics, which has been covered elsewhere recently (see [37–39]).

There are three major considerations when glycoengineering mAbs for cancer therapy. First, modified glycans should minimize immunogenicity. Most protein expression systems are nonhuman, and if the non-human sugars and linkages are added to the mAbs (e.g., fucose or xylose in plants [40]), severe adverse drug reactions can occur (e.g., "Cytokine Storm" of TGN142 [41] and anaphylaxis of cetuximab [7]). Indeed, several glycoforms from heterologous expression systems (see Figure 1B (ii)) have been identified to be immunogenic to human. Thus, incompatible expression systems need to be humanized by knocking out relevant transferases (Figure 1C (i)), or human-compatible expression systems (e.g., Chinese hamster ovary cells) must be selected to create safe and bioactive glycoforms.

A second consideration for glycoengineering in cancer therapeutics is the need to enhance effector functions, such as ADCC and CDC. Many studies have reported that N-linked glycans at Asn297 in the Fc region are crucial to ADCC. Glycoengineering by depleting fucosylation or increasing N-glycan branching (e.g. by over-expressing the GnTIII gene) can improve ADCC. These glycoforms increase binding affinity to Fc-FcγRIIIa [25]. The CDC effector function can also be modulated through glycoengineering. Specifically, high galactosylation can improve effector function of CDC, and this can be achieved by process engineering of adding uridine, manganese chloride, and galactose in media [42]. The increased galactosylation can improve the binding affinity between the mAb and complement (C1q) [43].

A third consideration is that glycoengineering can enhance antigen-binding affinity. mAb affinity has been widely studied, and efforts use tools like phage-display libraries, combined with point mutations, to engineer the protein for improving antigen-binding affinity [44]. However, glycoengineering mAbs by introducing more N-glycosylation sites using protein

engineering can improve antigen-binding affinity. For example, adding an N-glycan at Asn58 on Fab region can increase antigen-binding affinity by 50 fold [11].

Systems biology for predictive and rational glycoengineering of mAbs

In the past decade, considerable efforts have aimed to control glycosylation through media optimization, chemical treatments, or genetic changes (e.g., [45]). Despite many successful examples, it is still challenging to obtain desired quality and quantity of mAbs. However, omics technologies and systems biology modeling promise to aid in the glycoengineering of improved anti-tumor mAbs [10] (Figure 2). Indeed, systems biology provides platforms to integrate omics data with a holistic perspective, thereby guiding glycoengineering while accounting for competing pathways and processes [46]. Moreover, computational models in systems biology can be used for predicting knock-in or knockout strategies to obtain desired glycoforms [47].

Systems biology holds great promise in advancing at least three glycoengineering processes. First, it can be used for reliable cell line development. Cell line development is often a bottleneck step in protein-based drug manufacturing (usually taking more than a year). Many cellular processes must be considered during cell line development, since they influence protein quality and glycosylation. These include metabolism, protein secretion, and cell growth/apoptosis [48]. Computational models can be developed to understand how to control these processes in expression systems, as we can predict growth characteristics and production capabilities of mAbs [49,50]. Second, with increased understanding of the molecular pathways influencing mAb production, models can be used for predictable glycoengineering. The complexity of glycosylation greatly hampers the glycoengineering process. Computational models have been developed that enables us to predict glycoforms under different glycoengineering strategies [47,51]. The third area where systems biology can aid in glycoengineering is for its use in assessing the physiological effects of glycoengineering. Some glycoengineering designs have proven toxic to host cell lines; for example, the OCH1 deletion [52] and double knockouts of PMT-family genes [53] in yeast were shown to significantly reduce host-cell fitness. However, there has not been a systematic study concerning how glycoengineering affects host cells and their protein secretion system. In the future, we can combine several omics data (e.g., transcriptomics [54,55] and glycomics [56]) and relevant phenotypes (e.g., [57]) to analyze them with systems biology approaches. These results can provide insights into how key metabolic and signaling pathways are modulated by alterations in glycosylation [58]. We are also hopeful that future research should be pursued in developing reliable prediction models for predicting outcomes of glycosylation alterations on mAbs. Ultimately, a clear blueprint of what glycosylation to be engineered on a mAb with desire properties of low cost, safety and effectiveness will be revealed.

Conclusions

While therapeutic effects of mAbs are profoundly influenced by how the glycans impact cancer physiology, the detailed mechanistic relationships are largely unknown. By analyzing the FDA-approved mAbs in oncology indications, deeper insights will be obtained into how

glycosylation and glycan-protein interactions impact on cancer physiology. In the future, systems biology technologies will aid in glycoengineering as it will allow us to better control and engineer glycans at the system level, thus, facilitating the development of novel therapeutic mAbs with increased efficacy, safety, and affordability.

Acknowledgments

This work was supported by generous funding from the Novo Nordisk Foundation provided to the Center for Biosustainability at the Technical University of Denmark (grant no. NNF16CC0021858), and from NIGMS (grant no. R35 GM119850). AWTC was also partially supported by a Postdoctoral fellowship from the Ministry of Science and Technology of Taiwan (grant no. 104-2917-I-564-028).

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Highlights

- Glycan-protein interactions modulate tumor cell killing.
- Different glycans can significantly alter the glycan-protein interactions.
- Glycan structure and location on mAb are essential properties need careful control.
- Glycoengineering can modify glycans on therapeutic mAbs with desired glycoforms.
- Systems biology can advance mAb glycoengineering toward a rational design era.

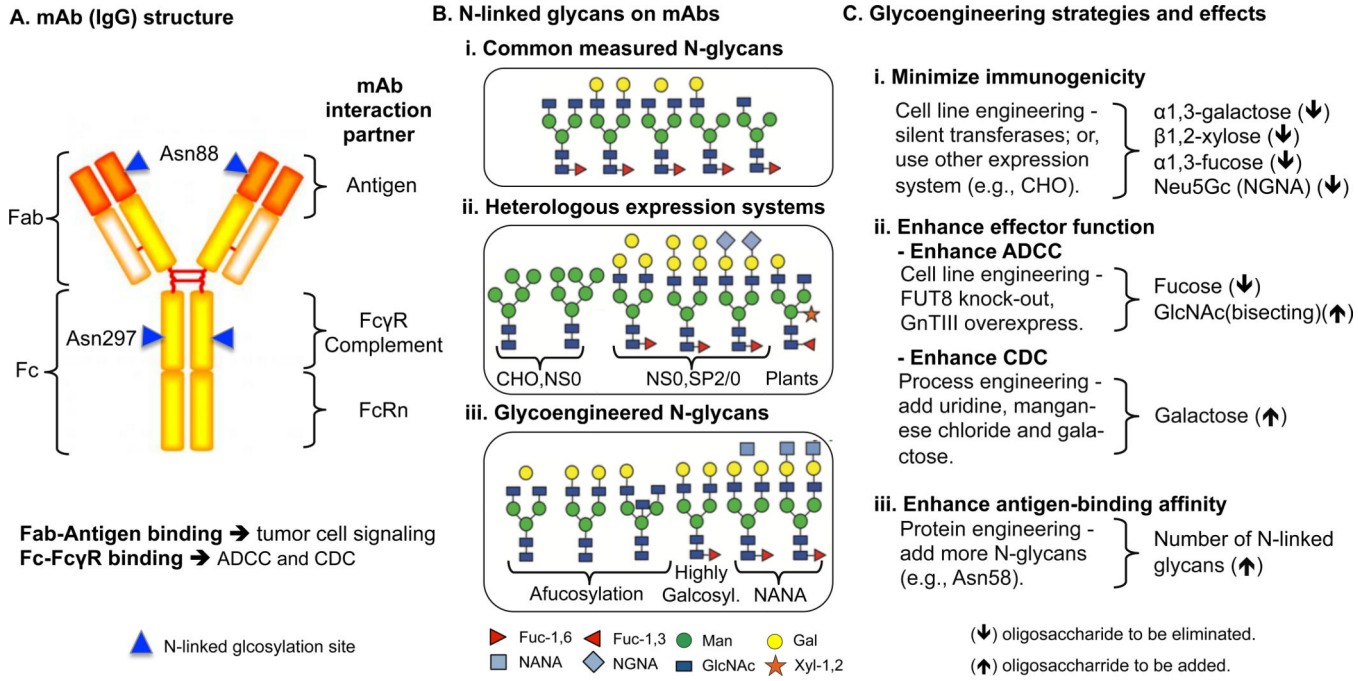

Figure 1. Therapeutic mAb structure, glycoforms, and glycoengineering strategies for generating desired glycoforms

(A) The structure of an IgG with interaction-partner binding regions and N-linked glycosylation sites (highlighted in blue triangles) are annotated. (B) The dominant N-linked glycans on mAbs can vary depending on the host and product. However, (i) common glycans on therapeutic mAbs have been measured. (ii) MAbs expressed in heterologous expression systems introduce non-human compatible sugars and linkages, leading to immunogenicity and low serum half-life. (iii) Glycoengineering aims to make mAbs with N-glycans that are human compatible and exhibit enhanced mAb efficacy and safety. (C) Many glycoengineering efforts aim to enhance the drugs and achieve any of the three effects (i-iii) by modifying glycans on mAbs. NANA: N-glycolylneuraminic acid (hyper-sialylation). Data of (B) in this figure was adapted from [34].

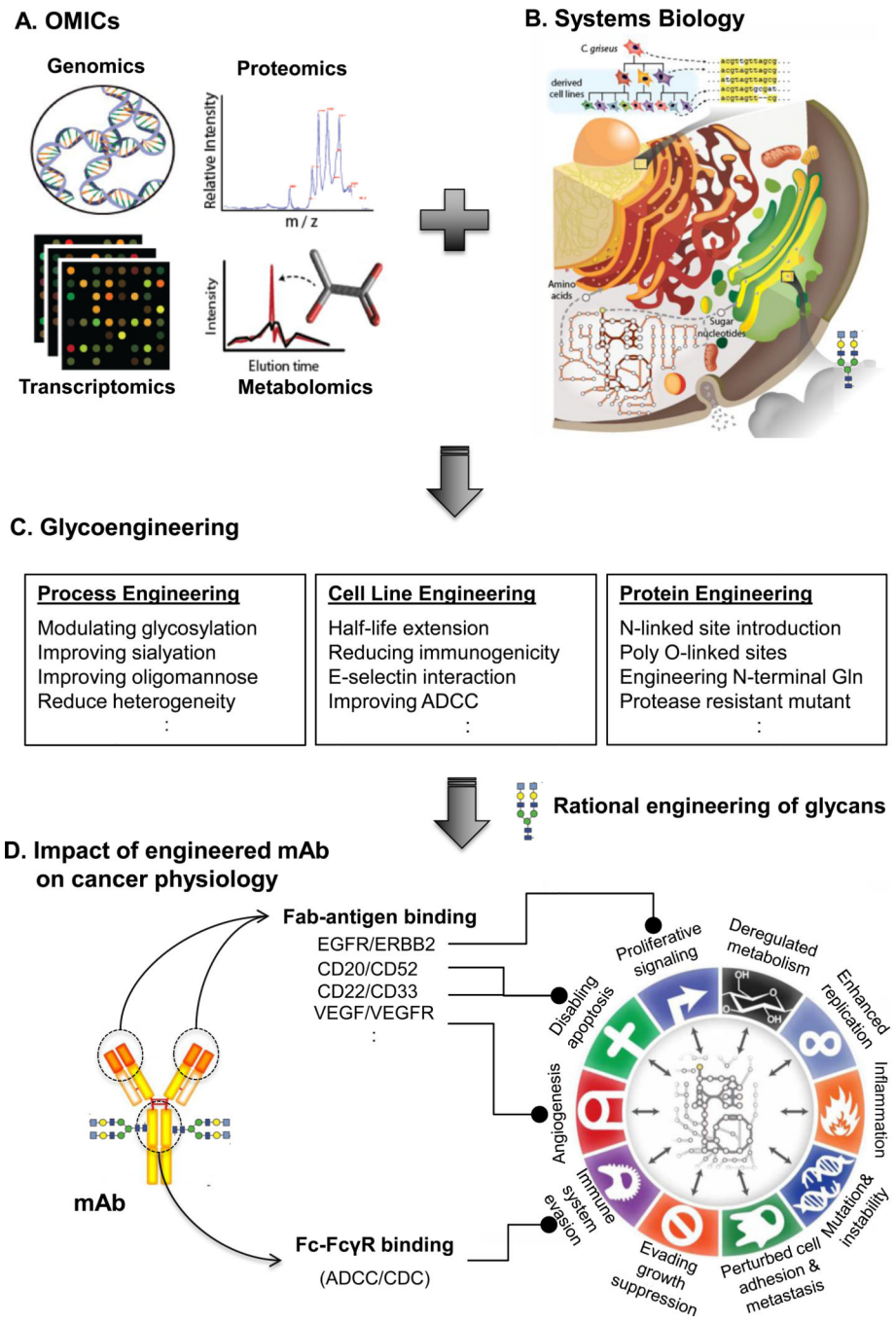

Figure 2. Omics data and systems biology provide novel tools for rational glycoengineering of therapeutic mAbs and to assess the impact of glycoengineered mAbs on cancer

(A) Omics data can be acquired to identify how to glycoengineer mAb production cells. (B) Computational models of the pathways influencing glycosylation and protein production can serve as a platform for interpreting omics data and (C) guiding the rational design of glycans on therapeutic mAbs. (D) The impact of engineered mAbs on cancer physiology can further be analyzed using systems biology techniques, especially assessing the glycan-protein interactions [9].

Table 1

Cancer physiology impacted by the FDA approved therapeutic mAbs through glycan-protein interactions

Targeting mechanism	Impacted physiologies in tumors	State-of-art knowledge of glycan-protein interactions		Examples of FDA-approved product (Receptor; First approved indications)
Fab-antigen binding	Tumor cell proliferation reduced (PI3K-AKT, MAPK); Apoptosis of tumor cells.	**1**	Additional N-glycan (e.g., Asn88 on cetuximab) can induce immunogenicity.	Cetuximab (EGFR; Head and neck, colorectal cancer) Alemtuzumab (CD52; Chronic myeloid leukemia) Epratuzumab (CD22; Acute lymphocytic leukemia)
		2	Alemtuzumab can directly interact with the glycan (GPI-anchor).	
		3	Epratuzumabcan can modulate glycan-protein interactions between B-cell (siglec) and Endothelial-cells (sialic acid).	
	Angiogenesis inhibited	**1**	Bevacizumab can block downstream VEGFHSPGs interactions.	Bevacizumab (VEGF; Colorectal cancer)
		2	Gal1-Endothelial cell interactions provide new opportunities for developing mAbs to inhibit tumor growth and metastasis.	
	T cell activation and tumor cell killed	Fab-antigen binding affinity is affected by N-glycans on receptors (e.g., Asn16, Ans25, Asn41, and Asn83 at PD1).		Pembrolizumab (PD1; Ipi-refractory melanoma) Nivolumab (PD1; Metastatic melanoma)
Fc-FcγR binding	Tumor cell killed by immune cells (ADCC/CDC)	**1**	Asn297 is crucial to the Fc-FcγR binding affinity and effector functions (ADCC/CDC), which is correlated with structural stability.	Mogamulizumab (CCR4; T-cell leukemia/lymphoma) Rituximab (CD20; Chronic lymphocytic leukemia) Obinutuzumab (CD20; Chronic lymphocytic leukemia)
		2	Mogamulizumab is the first glycoengineered mAb with afucosylated-Fc.	
		3	Obinutuzumab is glycoengineered with afucosylated with bisecting GlcNAc N-glycans, and showing superior antitumor activity than nonglycoengineered rituximab.	