

# Genetic glycoengineering in mammalian cells

Received for publication, December 9, 2020, and in revised form, February 17, 2021. Published, Papers in Press, February 20, 2021, <https://doi.org/10.1016/j.jbc.2021.100448>

Yoshiki Narimatsu<sup>1,2,\*</sup>, Christian Büll<sup>1,\*</sup>, Yen-Hsi Chen<sup>2</sup>, Hans H. Wandall<sup>1</sup>, Zhang Yang<sup>1,2</sup>, and Henrik Clausen<sup>1,\*</sup>

From the <sup>1</sup>Department of Cellular and Molecular Medicine, Faculty of Health Sciences, Copenhagen Center for Glycomics, University of Copenhagen, Copenhagen, Denmark; <sup>2</sup>GlycoDisplay ApS, Copenhagen, Denmark

Edited by Gerald Hart

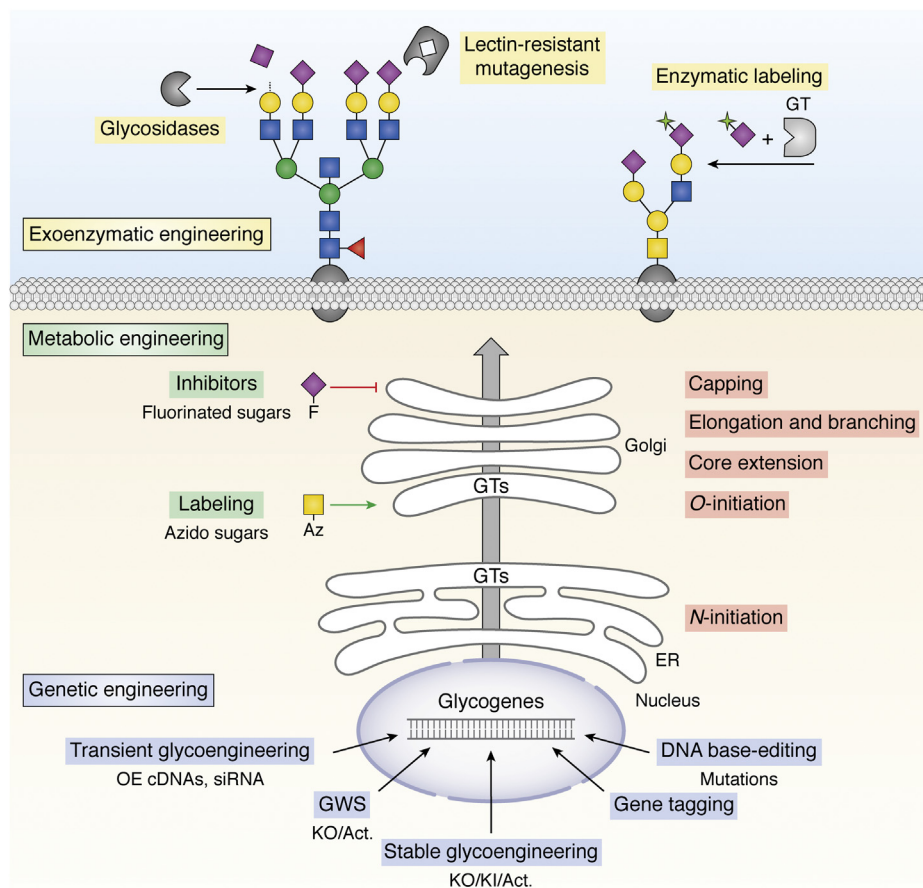
Advances in nuclease-based gene-editing technologies have enabled precise, stable, and systematic genetic engineering of glycosylation capacities in mammalian cells, opening up a plethora of opportunities for studying the glycome and exploiting glycans in biomedicine. Glycoengineering using chemical, enzymatic, and genetic approaches has a long history, and precise gene editing provides a nearly unlimited playground for stable engineering of glycosylation in mammalian cells to explore and dissect the glycome and its many biological functions. Genetic engineering of glycosylation in cells also brings studies of the glycome to the single cell level and opens up wider use and integration of data in traditional omics workflows in cell biology. The last few years have seen new applications of glycoengineering in mammalian cells with perspectives for wider use in basic and applied glycosciences, and these have already led to discoveries of functions of glycans and improved designs of glycoprotein therapeutics. Here, we review the current state of the art of genetic glycoengineering in mammalian cells and highlight emerging opportunities.

Engineering glycosylation in cells and organisms has a long history (1–3), and yet rational genetic engineering of the glycosylation capacities in mammalian cells may only be off to a start (4) (Fig. 1). The glycosylation machinery in cells involves a large and complex metabolic network of enzymes and accessory proteins that orchestrate the synthesis of different types of glycans found on glycolipids, glycoproteins, proteoglycans, and as oligosaccharides. Over 200 distinct glycosyltransferase (GT) genes contribute to glycosylation in mammalian cells, and at least 173 of these GTs function in at least 16 distinct glycosylation pathways to assemble the great diversity of glycan structures found on glycoproteins, glycolipids, and proteoglycans (5). Rational engineering of glycosylation in mammalian cells can now be performed with a high degree of confidence in predicted outcomes, and genetic engineering is one approach to studying the biology of glycans that takes origin in a single cell. However, challenges still exist with isoenzymes for which unique functions are poorly characterized and partial overlaps in properties need to be considered.

Genetic engineering of glycosylation in mammalian cells has contributed tremendously to the current view of the genetic and biosynthetic regulation of the cellular glycome and highlighted important functions of glycans in development, health, and disease (1–3, 5, 6). Early on mammalian mutant cell lines with loss of GT genes or other genes affecting glycosylation were generated by random mutagenesis and selection for lectin resistance (7–10), and later targeted knockout (KO) of GT genes in animals by homologous gene recombination strategies in embryonic stem (ES) cells opened up rational design of glycoengineered models (1–3). These models informed about GT genes that are essential for early development of the embryo and genes with important functions for normal health and diseases, but also of many GT genes that at first pass at least appeared to be dispensable for normal development and health. These studies also highlighted that some steps in the glycosylation pathways in mammalian cells are regulated by a single GT with loss of function of such genes leading to global changes in the glycan structures produced. However, these studies also revealed glycosylation steps that are regulated by multiple isoenzymes with at least partly redundant functions, where loss of function of a single isoenzyme gene may lead to no or only subtle effects on the glycans (11–15).

A major obstacle for the wider use of genetic engineering of glycosylation in mammalian cells has until recently been the lack of simple and efficient means to design and conduct rational and combinatorial engineering of genes. In lieu of this, considerable efforts have been devoted to engineering glycosylation in other eukaryotic cells such as yeast, plants, and insects where tools for genetic engineering were more readily available (16–19). Engineering of glycosylation has also been sought by a wide range of nongenetic strategies including chemical and enzymatic approaches (Fig. 1) (6, 20–23). The primary objectives for genetic engineering of glycosylation were devoted to glycoprotein therapeutics, and the first targeted KO engineering in a mammalian cell involved a tour-de-force effort performed using two rounds of targeted homologous recombination in Chinese hamster ovary (CHO) cells to knockout the two copies of the  $\alpha$ 6-fucosyltransferase (*fut8*) gene. FUT8 directs core fucosylation of N-glycans and the CHO *fut8* KO cells enabled production of afucosylated IgG with greatly improved antibody-dependent cell cytotoxicity (ADCC) (24, 25). However, the huge efforts required with this

\* For correspondence: Yoshiki Narimatsu, [yoshiki@sund.ku.dk](mailto:yoshiki@sund.ku.dk); Christian Büll, [cbull@sund.ku.dk](mailto:cbull@sund.ku.dk); Henrik Clausen, [hclau@sund.ku.dk](mailto:hclau@sund.ku.dk).



**Figure 1. Overview of glycoengineering strategies.** Basic principles for approaches available to modulate the cellular glycosylation processes and the glycome are illustrated. Extracellularly, glycans may be modulated by more or less selective endo-/exo-glycosidases (sialidases, galactosidases, PNGase, etc.) (117), and chemoenzymatic labeling methods utilizing, e.g., glycosyltransferases (GTs) may be applied to install natural or unnatural substrates on cell surface glycans (6, 337). Use of cytotoxic lectins often in combination with mutagenesis may enable selection of mutant cells with loss/gain of distinct glycosylation features (10, 338). A growing number of unnatural sugar mimetics can be applied for metabolic engineering (6, 23), including glycosylation inhibitors (i.e., fluorinated sugar analogues) (235, 339) or functionalized sugars (i.e., azido, Az, sugars) that enable conjugation chemistries for use in glycan imaging or reprogramming of their interactions (20, 160). Genetic engineering of glycosylation may be performed by overexpression (OE) of GTs using cDNA plasmid transfection and/or siRNA for silencing of endogenous GTs (6). More extensive and stable glycoengineering takes advantage of precise gene engineering for combinatorial KO/KI/Act (activation) of GTs, and this strategy is the main focus of this review. Genome-wide KO/Act screens (GWS) may be used for discovery and dissection of GTs and other genes affecting glycosylation (Table 1), and endogenous GTs may be mutated, e.g., to mimic disease mutations or enable use of unique substrates, or tagged, e.g., by insertion of antibody tags or fluorescent proteins (169).

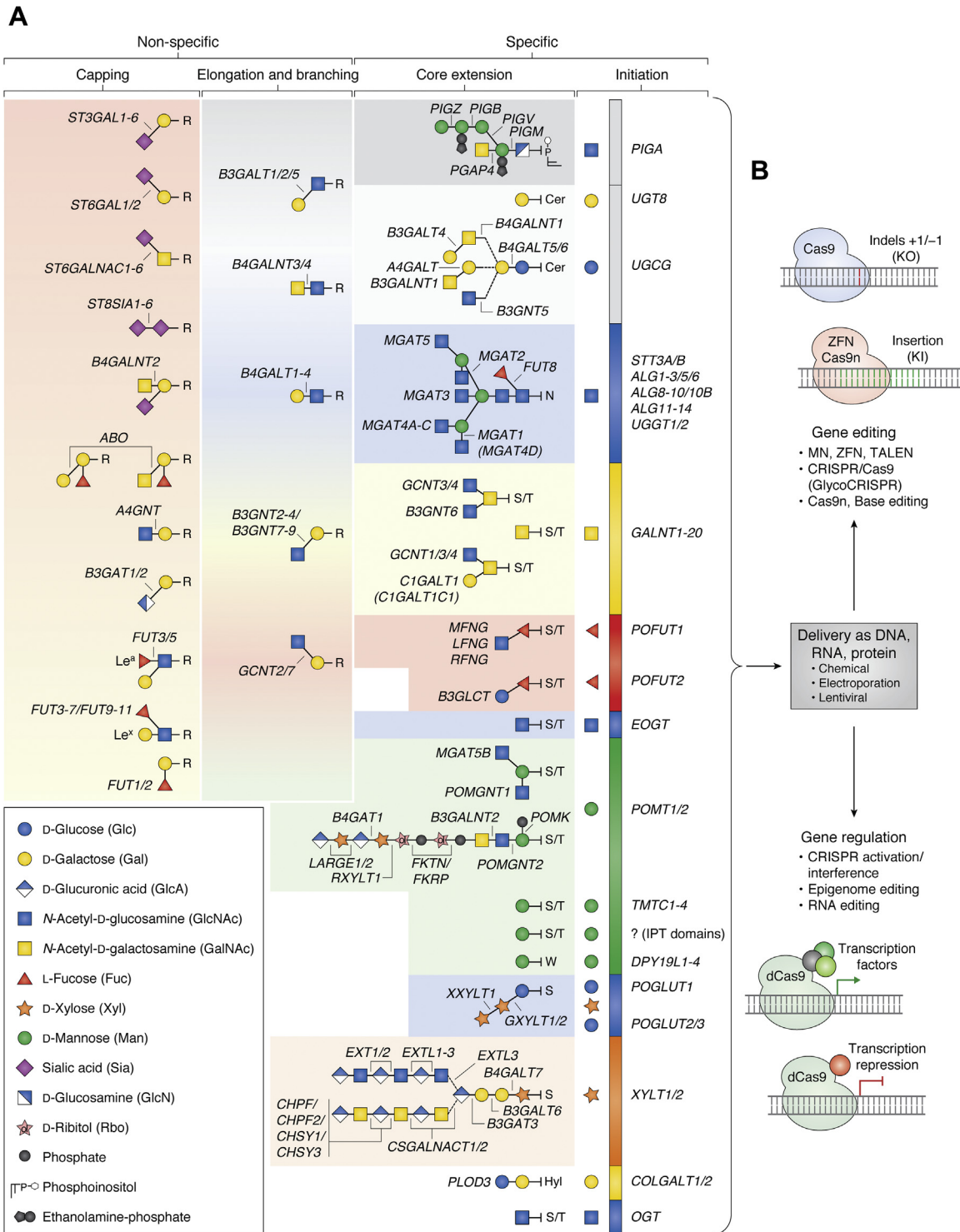
strategy limit broader use for engineering mammalian cell lines.

A new era for glycoengineering emerged with the introduction of the facile nuclease-based gene-editing strategies at the turn of the millennium (4, 27). The zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats with the CRISPR-associated protein 9 (CRISPR/Cas9) tools now allow for rational designed gene engineering events in mammalian cells with high precision, speed, and low cost. Engineering events in cells can be stacked with multiple gene KOs in combination with knock-ins (KIs) to achieve almost any desirable design of the cellular glycosylation capacities. These new opportunities are beginning to thrive, and engineering of glycosylation in cell lines is no longer only focused on meeting interests in improved glycoprotein therapeutics. Already a remarkable diversity in the use of glycoengineering has emerged. Here, we focus on these new advances and

discuss the use of strategies to comprehensively engineer and dissect glycosylation in mammalian cell lines, which we define as rational genetic engineering of glycosylation. A number of excellent reviews have already summarized and discussed progress with engineering glycosylation in different species including yeast, insects, and plants and the special opportunities these provide for production of glycoprotein therapeutics (6, 16–22, 28–30).

### Glycosylation processes in cells

The glycosylation machinery of a given cell determines the ensemble of glycan structures and types of glycoconjugates that constitute the glycome of that same cell (31). The glycosylation capacities of different cell types vary primarily through changes in expression of the GTs that directs different steps in glycosylation pathways (Fig. 2A); however, a number of other factors affect the glycosylation outcome (5, 32). GTs have different donor sugar and acceptor substrate specificities often



**Figure 2. Overview of principles and strategies for stable genetic engineering of cellular glycosylation capacities.** *A*, overview of the 16 human glycosylation pathways with predicted assignments of 173 glycosyltransferase genes to the major biosynthetic steps using the rainbow display organization (5, 199). The rainbow depiction of glycosylation pathways illustrates the major biosynthetic steps organized into pathway-specific steps (*right part*) with even colored according to the initial monosaccharide except for glycolipids and pathway nonspecific steps (*left part* with toned colors) with predicted GT genes assigned. Note that this is a simplified scheme of pathways and GT genes are assigned only to the primary predicted functions. Genetic engineering of glycosylation requires considering the properties of individual enzymes and their potential effects on the cellular glycosylation pathways. Glycosylation steps covered exclusively by one unique enzyme (nonredundant steps), e.g., core  $\alpha$ -fucosylation of N-glycans by FUT8, yield highly specific and predictable outcomes with loss/gain engineering. Steps covered by multiple isoenzymes with overlapping functions (redundant steps) may or may not yield easily predictable outcomes, and the outcome may vary in cells dependent on the expression of such isoenzymes. Most steps in elongation and branching and capping are covered by partial redundancies by multiple isoenzymes. For example, sialylation by any of the four sialyltransferase subfamilies is covered by partial redundancies, and, e.g., combinatorial KO of three genes is required to selectively eliminate  $\alpha$ 3-sialylation on N-glycans (KO of *ST3GAL3/4/6*), while KO of two genes is required to selectively eliminate  $\alpha$ 3-sialylation of core1 O-glycans (KO *ST3GAL1/2*). Glycan symbols are displayed in the Symbol Nomenclature for Glycans (SNFG) format (340). *B*, graphic depiction of current nuclease-based gene-editing tools for knockout (KO) and knock-in (KI) of genes, and emerging CRISPR-based technologies for regulating and activating gene expression.



with considerable overlaps (33), and glycosylation reactions are primarily guided by the kinetic properties of the expressed ensemble of enzymes (2, 34, 35). Glycosylation is therefore akin to a large integrated metabolic network involving a myriad of enzyme reactions, where a number of “rules” can be applied to predict the outcomes in terms of glycans being produced. While many of these rules for enzyme reactions can be applied with a high degree of fidelity, others involve less predictable outcomes. Thus, rational engineering of glycosylation in cells requires information on glycosylation processes and often experimentation and validation of outcomes.

Figure 2A organizes glycosylation into 16 distinct pathways and major biosynthetic steps with assignment of predicted roles of 173 human GTs (note that we refer to GTs by their gene names only for simplicity; capital letters refer to human GTs, and italics refer to genes) (5, 34, 36). The map provides a blueprint for rational engineering of glycosylation in mammalian cells and highlight: i) GTs that are unique and serve glycosylation pathway-specific roles (essentially all steps in initiation of glycosylation on lipids and proteins and most early steps in core extension) and GTs that serve pathway nonspecific roles (assembly of elongated and branched glycans and the final capping); and ii) glycosylation steps that are covered by single nonredundant GTs and those that are covered by partial redundancies from GT isoenzymes. Moreover, the map serves to translate cellular expression data for GTs from, *e.g.*, single-cell transcriptomics or proteomics into predicted glycosylation capacities and glycome outcomes, as recently demonstrated (34). The glycosylation capacities of immortalized mammalian cell lines such as the CHO and HEK293 cells widely used for recombinant expression of glycoproteins can be extensively manipulated by multiple KO/KI events without major effects on viability in simple cultures and performance in production and secretion (34). The only essential steps for cell viability may be complete loss of the function of the oligosaccharyltransferase (OST) complex and initiation of N-glycosylation and complete loss of the cytosolic O-GlcNAcylation orchestrated by the protein O-GlcNAc transferase (OGT) (37, 38).

A major incentive for using genetic engineering of glycosylation in studying the biology of the glycome is that this is one strategy that allows probing the glycome at the single cell level. Engineering glycosylation capacities may be used to explore biological functions of glycosylation in a cell by dissection of the glycosylation genes and enzymes required for the particular function. Knowledge of the glycosylation pathways in the cell may then be used to predict the structural features of the glycome that underlies the function. Emerging high-quality single-cell transcriptomics and proteomics data can also be used to gauge the expression of glycosylation enzymes and with some confidence used to predict the cellular glycosylation capacities and glycome outcome (5). This information may be incorporated in engineering strategies to simplify and focus the design of genetic engineering on relevant active glycosylation pathways. Currently, analytic glycome and glycomics technologies are not at this level of sensitivity, and structural analytic data sets are generally sums of glycome contributions from multiple cells often with heterogeneous

origins (39, 40). The glycome of any cell at a given state and timepoint is predicted to be less heterogeneous than current analytics suggest, and distinct glycosylation features may be highly regulated (35, 41–43). Decades of studies with glycan-binding proteins (GBPs) including lectins and antibodies have shown that the expression and distribution of a limited number of glycan epitopes or structural glycan features change characteristically during cellular maturation and differentiation, tissue formation, and in diseases (44–48). Despite progress though, we are still limited in our abilities to decipher the molecular basis of specific roles of glycosylation in biological functions, and genetic glycoengineering in mammalian cells is one promising strategy for discovery and dissection.

### The tools—precise genome-editing techniques

As pointed out above, genetic engineering of glycosylation in mammalian cells has been performed for decades. Considerable efforts have been devoted to overexpression and/or silencing of GTs (and other metabolic enzymes, transporters, hydrolases) in mammalian cells (49–54). However, overexpression of GTs may lead to markedly perturbed glycosylation (55), and gene silencing using RNA interference strategies often results in limited and variable reduction of enzyme levels with uncertain effects on glycosylation outcomes (4, 56, 57). Today, glycoengineering with precision genome-editing techniques overcomes these issues and enables stable genetic changes to the cellular glycosylation machinery. The rapidly expanding toolbox for precise genome editing offers wide opportunities for targeted KO, KI, or modulation of the expression of glyco genes to reprogram glycosylation in mammalian cells. In the following, we briefly review these tools and refer to original studies for details (58–64).

#### Targeted KO

Stable gene KO can be achieved using nuclease-based genome engineering particularly ZFNs, TALENs, and CRISPR/Cas that all can be designed to bind and cleave specific nucleotide sequences and introduce double-strand breaks (DSBs) (65, 66). Repair of DSBs through nonhomologous end joining (NHEJ) is error-prone and results in mutations or small insertions/deletions (indels) that disrupt the target gene (67) (Fig. 2B). ZFNs and TALENs have very high sequence binding specificity and low off-target effects (68); however, design options for target sites are limited and production is laborious, costly and requires expertise although premade targeting constructs are available. CRISPR/Cas gene editing only requires design of guide RNAs (gRNAs), synthetic RNAs with a 16 to 20 nucleotide genomic targeting sequence (crRNA) with a proto-spacer adjacent motif (PAM), and a Cas nuclease-recruiting sequence (tracrRNA) (58). Different Cas nucleases exist in different species each recognizing distinct PAMs, and currently this specificity is further engineered to allow broader sequence recognition (69). Most CRISPR engineering is based on the class II nucleases Cas9 from *S. aureus*, to which we refer if not otherwise stated, and more recently Cas12. Established prediction algorithms for optimal candidate gRNA designs are continuously developed; however,

candidates still need experimental validation for functional activity (70–73). Libraries of gRNAs for specific targeting of all human glycosyltransferase genes are now available, including a library of validated high efficiency single gRNAs (GlycoCRISPR) (74) and a lentiviral viral library containing ten gRNAs (predicted design) per target gene (75). Targeted gene engineering is greatly facilitated by the Indel Detection by Amplicon Analysis assay, which provides single base resolution and informs of the spectrum and frequencies of indels enabling screening for and selecting indels resulting in frameshifts and functional KOs (76, 77). Detection of indels may also be based on enzymatic mismatch cleavage assays (78, 79) and of course different sequencing strategies (80).

### Targeted KI

Site-directed and stable introduction of target genes can be achieved by nuclease-based KI. In the presence of a homologous DNA template containing target gene of interest, DSBs can be repaired *via* homology-directed repair (HDR) resulting in the genomic integration (Fig. 2B). However, HDR occurs at a lower frequency and only in the S/G2 phase compared with the dominant NHEJ repair pathway operative throughout the cell cycle (mainly G1 phase), which makes KI *via* HDR more challenging (67). Therefore, several strategies apply KI independent of the HDR pathway. For example, the obligate ligation-gated recombination (ObLiGaRe) strategy utilizes ZFNs or TALENs to create complementary DNA overhangs (sticky ends) in both the genomic target site and the donor DNA allowing its integration (60, 81). This KI strategy has already been successfully used for stable KI of GTs (34, 82).

CRISPR/Cas9-mediated KI utilizes the microhomology-mediated end joining (MMEJ) pathway to repair DSBs (with blunt ends), and foreign DNA flanked with short homologous sequences (5–25 bp) can be used for integration at target sites (83–85). CRISPR strategies have improved KI efficiency *via* HDR by virtue of Cas9 nickases (Cas9n), Cas9 nucleases with an inactivating mutation in either the HNH (Cas9H840A) or RuvC (Cas9D10A) domain (59, 62, 86). Cas9n creates single-strand breaks (nicks), and by pairing two nickases and two gRNAs for both DNA strands, DSBs with long overhangs can be created. Donor DNA flanked with complementary arms or simultaneously nicked donor DNA can then be inserted at the DSB site *via* HDR or non-HDR mechanisms with high efficiency and low off-target effects (62, 87–89). To overcome random integration of artificial DNA sequences into the genome and off-target effects, the AAVS1 locus is often used as safe-harbor site for KI (61, 90). Gene KI can make use of customizable constructs for constitutively active expression of target genes, for example, with or without tags and also holds the opportunity to insert tunable expression constructs, for example, under control of the Tet-On/Off system, and this has already been applied for glycoengineering (91).

### Regulating endogenous gene expression

The CRISPR technology is rapidly evolving by adding functionalities to Cas nucleases. Utilization of catalytically

inactive (dead) dCas9 allows to fully repress target gene expression through fusion with transcription repressors (CRISPR interference, CRISPRi) and recruitment to the promoter region of a target gene (92). Likewise, fusion with transcriptional activators such as VP64, p65, and Rta enables activation of gene expression (CRISPR activation, CRISPRa) (63, 64, 93) (Fig. 2B). CRISPRa was applied to activate the fucosyltransferase 4 and 9 genes (*fut4* and *fut9*) in a murine cell line resulting in Lewis<sup>x</sup> expression (94). Other CRISPR-based approaches for gene regulation have emerged that are potentially useful for glycoengineering. For example, dCas9 fused to base editors such as cytidine deaminase that converts cytidine to uridine allows introduction of targeted point mutations (base editing), fusion to a reverse transcriptase domain for prime editing, fusion to epigenetic modifiers (*e.g.*, P300, TET1, LSD1, DNMT3A) enables epigenome editing, RNA-targeting Cas13a nuclease enables posttranscriptional engineering of mRNA, and CRISPR tools are emerging for mutagenesis and directed evolution (27, 80, 95–97). Currently, the main obstacle for extensive glycoengineering is limitations in introducing multiple genes by targeted KI, and this obstacle may be alleviated by activation of endogenous nonexpressed genes. Another interesting opportunity for engineering is to tag endogenous GTs or their substrate proteins to enable selective monitoring of expression of enzymes or their effects on specific proteins in live cells (98).

### Delivery gene editing

A key step in genetic engineering is the delivery of editing components such as DNA, RNA, and/or protein (99, 100) (Fig. 2B). DNA plasmids are most commonly used, because of ease of availability and use, stability, and flexibility in design, but genomic insertions of plasmids may lead to off-target effects. Use of RNA circumvents this as RNA is readily translated in the cytoplasm resulting in fast protein production, and transfecting with RNA has advantages especially with primary and difficult-to-transfect cells (101). However, RNA is more costly and difficult to handle, unstable, less customizable, and expression of the encoded protein is lower and relatively short-lived. The Cas9 protein may be delivered precomplexed with gRNAs and used directly in transfection allowing for control over dosage, and this may be effective with cells difficult to transfect with DNA or RNA, but requires highly pure protein and protocol optimization for different proteins. The methods of delivery include chemical, biological, or physical means, and the efficacy of these varies greatly (27, 99, 100). For example, the human embryonic kidney (HEK293) cell is readily transfectable with cost-effective chemical reagents such as calcium phosphate, polyethylenimine (PEI) polymers, and liposomes. More difficult-to-transfect cells such as primary cells and nondividing cells may be efficiently transfected by electroporation, nucleofection, or other methods that temporarily induce pores in the plasma membrane; however, these often result in varying levels of cytotoxicity (27, 102). Lentiviral transduction may be the option of choice for difficult-to-transfect cells and enables stable integration of highly

customizable vectors. This was applied, *e.g.*, for glycoengineering of human keratinocytes to develop organotypic skin models (103), and a comprehensive lentiviral glycoengine CRISPR library has been designed (75). Notably, level and timing of Cas9 expression are difficult to control with these methods and high prolonged expression enhances off-target frequencies (77). This hurdle may be overcome by inactivating CRISPR systems that allow controlled and temporal expression levels of Cas9, and these have already been applied to reduce off-target effects of editing the human  $\alpha$ 3-sialyltransferase 4 gene (*ST3GAL4*) (104). Selection markers such as antibiotics or fluorescent reporters, *e.g.*, GFP-tagged Cas9, facilitate enrichment of transfected cells with desired nuclease expression levels. Fluorescence activated cell sorting (FACS) is useful to select and enrich for cells with desirable expression of GFP-tagged Cas9 (77, 105), as is further selection of clones with desirable changes in glycosylation and/or expression of targeted proteins by lectins and antibodies.

### Engineering of glycosylation capacities in cells

Two conceptionally opposing strategies for glycoengineering have been applied. One approach takes origin in glycan structures and seeks to identify genes required for biosynthesis and expression of specific glycans—here designated as random glycoengineering. The other approach takes origin in the glycosylation capacities of a cell and seeks to engineer the repertoire of expressed GTs to accommodate biosynthesis of glycans of interest—here designated as rational glycoengineering. These strategies are not mutually exclusive.

#### Random glycoengineering—discovery potential

Random glycoengineering was pioneered by Stanley *et al.* who generated glycosylation mutants in Chinese hamster ovary (CHO) cells, designated Lec or LEC (for loss-of-function and gain-of-function mutants, respectively). CHO cells were subjected to random mutagenesis followed by positive or negative selection with cytotoxic plant or bacterial lectins to obtain lectin-resistant mutants with loss/gain of a glycan feature. With this approach a large number of valuable CHO glycosylation mutants were isolated leading to the discovery and characterization of GTs, sugar nucleotide transporters, and other genes (10, 106). Later, more systematic studies with random insertional mutagenesis using gene trapping in haploid HAP1 cells and selection with Lassa virus binding to the matriglycan on  $\alpha$ -dystroglycan revealed several known (*e.g.*, *POMT1/2*, *B3GNT1*) and unknown (*e.g.*, *SLC35A1*, *ST3GAL4*, *B3GALNT2*) glycoenginees involved in glycosylation of  $\alpha$ -dystroglycan and underlying dystroglycanopathies (107). Random mutagenesis studies are now being substituted by whole genome screens (or select gene subsets) using CRISPR libraries (108, 109). These and other studies have uncovered unexpected genes that direct or regulate cellular glycosylation processes in interactions with bacterial toxins, viral infections, and other molecular events as summarized in Table 1. This strategy relies on the efficiency of CRISPR/Cas targeting in introducing biallelic indels (and multiallelic) in target genes

necessary to induce complete loss of function of GT enzyme activities and thus partly omitting the benefits of using haploid cell lines where mainly a single allele of genes requires targeting. Technical limitations of genome-wide CRISPR/Cas KO screens are off-target effects and false-negative hits caused by nondeleterious indels (108, 109), but also a low likelihood of identifying GTs that are members of partially redundant isoenzyme families (5). Thus, the CRISPR/Cas screening strategy has mainly identified nonredundant genes with global effects on glycosylation (Table 1). For example, genome-wide screening for genes required for avian influenza virus replication identified the Golgi CMP-sialic acid transporter (*SLC35A1*) and confirming requirement for sialic acid receptors; however, none of the many sialyltransferase genes necessary for sialylation were identified (110). A genome-wide CRISPR screen for hepatitis A virus entry identified multiple genes in the ganglioside biosynthetic pathway, including UDP-glucose ceramide glucosyltransferase (*UGCG*) and  $\beta$ 4-galactosyltransferase 5 (*B4GALT5*) directing synthesis of Gal $\beta$ 1-4Glc $\beta$ 1-Cer, and the lactosylceramide  $\alpha$ 3-sialyltransferase (*ST3GAL5*), which may only serve as the GM3 synthase (NeuAc $\alpha$ 2-3Gal $\beta$ 1-4Glc $\beta$ 1-Cer) (111). Multiple genome-wide screens have shown the importance of N-glycosylation and the OST complex for Dengue virus infection (112–114), and one screen in the HAP1 haploid cell line also identified multiple genes in the heparan sulfate pathway indicating a role of GAGs as well (115).

Genes that are not endogenously expressed in the cell line used for screening are of course disregarded, but this limitation may partly be overcome with the emerging genome-wide CRISPR activation libraries that enable gain-of-function screening. For example, a genome-wide CRISPR/dCas9 screen for inhibitory factors of  $\alpha$ 2-3Sia binding influenza A virus uncovered  $\beta$ 4-GalNAc-transferase 2 (*B4GALNT2*) and revealed that addition of a  $\beta$ 4GalNAc residue to  $\alpha$ 2-3Sia capped glycans to form the SDa epitope (Neu5Ac $\alpha$ 2-3[GalNAc $\beta$ 1-4]Gal $\beta$ 1-R) blocks binding (116). The unbiased nature of random engineering studies has demonstrated great potential for discoveries of GT genes and their functions in cellular glycosylation processes.

#### Rational genetic glycoengineering—custom design and dissection

Rational glycoengineering represents a systematic and versatile strategy for studying and dissecting biological roles of glycosylation. Advanced understanding of the genetic and biosynthetic circuits of the cellular glycosylation machinery and regulation of the glycome (Fig. 2A) (2, 5, 117, 118), combined with the facile gene-editing technologies and libraries of validated gRNAs such as the GlycoCRISPR resource (74), is enabling wide use of rational glycoengineering by KO/KI of genes. There appears to be few restrictions to the extent of reprogramming of glycosylation possible in cell lines, and extensive engineering of a majority of the known GT genes has been performed in mammalian cells (predominantly CHO and HEK293) (34, 82, 103, 119–123). Thus, it is now essentially



**Table 1**  
Genome-wide screens in mammalian cells identifying genes involved in glycosylation

Cell type	Genome-wide screen	Phenotypic selection	Identified glycozymes	Reference
Chronic myeloid leukemia cells HAP1 (haploid)	Gene trap mutagenesis	Resistance to Lassa virus entry via $\alpha$ -dystroglycan	<i>POMT1/2, B3GNT1, SLC35A1, ST3GAL4, B3GALNT2</i>	(107)
Human cervical cancer cells HeLa	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to Shiga toxin (Stx)	<i>UGCG, B4GALT5, SPTLC2, A4GALT, SPTLC1</i>	(328)
Human cervical cancer cells HeLa	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to Shiga toxin (Stx)	<i>A4GALT, B4GALT5, UGCG, GALE, SLC35A2</i>	(329)
Human bladder cancer cells 5637	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to Shiga-like toxins (Stxs) 1 and 2	<i>A4GALT, B4GALT5, SLC35A2, UGCG, SLC35A2</i>	(330)
Human colorectal adenocarcinoma cells HT-29	CRISPR/Cas9 KO screen <sup>b</sup>	Resistance to EHEC cytotoxicity (T3SS, Stx1 and Stx2)	<i>A4GALT, B4GALT5, UGCG</i>	(331)
Human cervical cancer cells HeLa	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to ricin toxin	<i>ALG5, ALG6, ALG8, MOGS, OST4, MANIA2, MAN2A1, MGAT1, MGAT2, SLC35C1, FUT4</i>	(330)
Human cervical cancer cells HeLa	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to <i>E. coli</i> subtilase cytotoxin (SubAB)-induced cell death	<i>SLC39A9, CMAS, SLC35A1, MGAT1, C1GALT1, C1GALT1C1</i>	(332)
Human cervical cancer cells HeLa	CRISPR/Cas9 KO screen <sup>a</sup>	Gal-3 cell surface localization	<i>SLC35A2, MGAT1, MANIA2, SLC39A9</i>	(129)
Human hepatocellular carcinoma cells Huh7.5.1	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to Ebola virus infection	<i>SLC30A1, GNPTAB</i>	(333)
Human cervical cancer cells HeLa and human embryonic kidney cells HEK293	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to West Nile virus infection	<i>STT3A, OST4, OSTC</i>	(334)
Chronic myeloid leukemia cells HAP1 (haploid)	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to Dengue virus infection	<i>STT3A, STT3B, RPN2, B4GALT7, B3GALT6, B3GAT3, PAPSS1, SLC35B2, DPPI1, DPM3</i>	(115)
Chronic myeloid leukemia cells HAP1 (haploid) and Human hepatocellular carcinoma cells Huh7.5.1	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to Dengue virus infection	<i>STT3A, STT3B, MGAT1, OSTC, RPN1, RPN2, OST4, B3GALT6, EXT1</i>	(113)
Human lung epithelial cells A549	CRISPR/Cas9 KO screen <sup>a</sup>	Resistance to IAV (H5N1) virus infection	<i>SLC35A1, DPM2, ALG3, ALG4, ALG12, GANAB, A4GALT, B3GAT1, B4GALNT4, CHSY1, CSGALNACT2, HS3ST6, PIGN, DPM2</i>	(110)
Human lung epithelial cells A549	CRISPR/dCas9 activation screen <sup>c</sup>	Blocking IAV (H1N1/PR8/1934) infection	<i>B4GALNT2</i>	(116)
Human colorectal adenocarcinoma cells HT-29	CRISPR/Cas9 KO screen <sup>b</sup>	Resistance to <i>V. parahaemolyticus</i> cytotoxicity (T3SS1 and T3SS2)	<i>SLC35B2, SLC35B3, HS6ST1, SLC35C1, GMD, FUT4, SLC35A2</i>	(335)
Human lymphoma cells JeKo-1	CRISPR/dCas9 activation screen <sup>c</sup>	Resistance to anti/CD3xCD20 bispecific antibody-mediated killing	<i>B4GALNT1, B3GNT4</i>	(336)

<sup>a</sup> Lentiviral GeCKO sgRNA library targeting 19,050 genes and 1864 miRNAs.

<sup>b</sup> Lentiviral AVANA sgRNA library targeting 18,675 genes.

<sup>c</sup> Lentiviral sgRNA library targeting upstream TSS of 23,430 coding isoforms.

possible to consider the blueprint of glycosylation pathways (Fig. 2A) and deconstruct and/or reconstruct any of these without substantially affecting cell viability and performance, or, for example, expression and secretion of recombinant glycoproteins (121). Rational genetic engineering may also apply to the many enzymatic modifications of glycans including epimerization and attachment of sulfate, phosphate, acetyl, methyl, and other groups (31). The glycome is further shaped by endogenous glycoside hydrolases and in particular the four mammalian neuraminidases (NEU1–4) may affect the degree of sialic acid capping (124). Design of rational glycoengineering experiments needs to consider the enzymes expressed in the cell of choice and potential genetic redundancy for biosynthetic steps provided by isoenzymes. Single-cell RNAseq transcriptomics (and to some extent proteomics) may provide information on the repertoire of expressed GTs and other relevant enzymes, and this may be useful for the engineering design considering the current knowledge of glycosylation pathways in cells (Fig. 2A). However, it is important to acknowledge that interpretation of such data still requires caution. Further advances are needed to be able to reliably predict glycosylation outcomes based on

quantitative levels of enzymes in cells, and the predicted roles of GTs in the different glycosylation pathways and biosynthetic steps need validation.

Targeting biosynthetic steps in glycosylation that are controlled nonredundantly by a single unique GT generally results in predictable global outcomes. For example, KO of *FUT8* that solely directs transfer of  $\alpha$ 1-6 fucose to the innermost GlcNAc residue of the chitobiose core of N-glycans is sufficient to eliminate this glycosylation feature on N-glycoproteins (24, 25, 125). Many steps in the initiation and immediate core extension of glycosylation pathways are controlled by nonredundant enzymes (Fig. 2A), and this is used to dissect roles of elaborated glycans on different types of glycoconjugates by targeting the earliest committed biosynthetic steps in core extension. Thus, KO of the Glc-Cer  $\beta$ 4-galactosyltransferase *B4GALT5(6)* gene(s) eliminates synthesis of elaborated glycolipids, KO of the  $\alpha$ 3-mannosyl-glycoprotein  $\beta$ 2-GlcNAc-transferase *MGAT1* gene eliminates elaboration of N-glycans, and KO of the core1 synthase *C1GALT1* gene or the private chaperone COSMC eliminates elaboration of the most common types of O-glycan (34, 103, 126–129). Rational engineering may lead to discoveries and challenge the current

understanding of genetic and biosynthetic regulation of glycosylation pathways. For example, when the—at the time—known genes controlling protein O-mannosylation (*POMT1/2*) were KO in mammalian cells followed by analysis of the O-Man glycoproteome, two new O-mannosylation pathways directed by previously unknown enzymes were discovered (130, 131). Another example of glycoengineering leading to discoveries involved the use of forward genetic screening to identify the two enzymes directing the Gal $\beta$ 1-3GalNAc $\beta$ 1-4 branch attached to the first Man residue of the glycosylphosphatidylinositol (GPI)-anchor (132). The post-GPI attachment to proteins GalNAc-transferase 4 (*PGAP4*) predicted to attach the first  $\beta$ 4GalNAc was validated by KO of the *PGAP4* gene, and a genome-wide CRISPR/Cas screen identified the  $\beta$ 3-galactosyltransferase 4 (*B3GALT4*) as the enzyme extending the  $\beta$ 4GalNAc (133). *B3GALT4* also functions as the GM1 glycolipid synthase (134, 135). A cell model was first engineered by KO of GPI transamidase component PIG-S gene (*PIGS*), encoding a subunit of the GPI-Tase that transfers GPI to proteins, to block protein transfer of GPIs and enable screening for loss of galactosylation by an antibody detecting the Gal $\beta$ 1-3GalNAc $\beta$  epitope on free GPIs. Interestingly, in the process of dissecting the putative dual role of *B3GALT4*, it was discovered that UGCG that initiates glycolipid biosynthesis is required for *B3GALT4* functions in both glycolipid and GPI biosynthesis. These studies thus not only uncovered an unexpected cross talk between two different glycosylation pathways, but also led to discovery of interaction between UGCG and *B3GALT4*. The Ribitol  $\beta$ 4-xylosyltransferase (*TMEM5*) required for biosynthesis of the core O-mannosyl matriglycan of dystroglycan was originally identified in a HAP1 screen and validated by KO showing that the Xyl $\beta$ 1-4Rbo5P structure was disrupted (136).

A “brutal” variant of rational glycoengineering is to target genes that serve glycosylation in global ways such as in synthesis and transport of nucleotide sugar donors, e.g., as demonstrated already with the CHO Lec mutants (10). KO of *SLC35A1* results in loss of all types of sialylation (137, 138). The original discovery that loss of the UDP-Glc/GlcNAc C4-epimerase (*GALE*) in the CHO lld cell model resulted in deficiencies of UDP-Gal/GalNAc and impaired glycosylation of N- and O-glycoproteins, a defect that can be reversed by exogenous addition of Gal and/or GalNAc sugars (139, 140), is now being replicated in other cells by targeted KO of *GALE/GALK1/2* to install the unique ability to regulate glycosylation by exogenous addition of sugars (141, 142). Similarly, KO of the isoprenoid synthase domain-containing protein *ISPD* required for synthesis of CDP-Ribitol blocks synthesis of the matriglycan on  $\alpha$ -dystroglycan (143, 144).

Targeting biosynthetic steps that are covered by partial redundancy from isoenzymes remains a challenge for predicting outcomes of rational glycoengineering. However, targeting isoenzymes also present unique opportunities to uncover their nonredundant functions. For isoenzymes that function in pathway specific steps, such as the many polypeptide GalNAc-transferases (*GALNTs*), selective KO/KI of individual isoenzyme genes (*GALNT1-20*) in cell models was

very useful to dissect non-redundant functions using differential O-glycoproteomics (145–147). This strategy provides deeper and more unbiased insights into substrates for *GALNTs* compared with *in vitro* enzyme assays with short peptides and has revealed important isoform-specific targets such as the O-glycosylation of the ligand-binding region of the low density lipoprotein receptor-related receptors directed exclusively by *GALNT11* (148–150). A particularly useful extension of this strategy is to install inducible expression of GT isoenzymes to explore their functions in regulating glycosylation. Thus, engineering cells with KO of an endogenous *GALNT* and reinstallation of the same isoenzyme under stringent inducible expression by KI enabled the remarkable observation that increasing levels of the *GALNT* isoenzyme produce tight regulation of nonredundant substrates without interference or change to the majority of redundant substrates (91). KO screen of *GALNTs* was used to show that *GALNT1* plays an essential role in the glycosylation of the mucin domain of the Ebola surface glycoprotein and the function of this in cell detachment and hemorrhaging (151). Engineering *GALNT* genes in a human keratinocyte skin model showed that distinct isoforms serve unique functions in epithelial formation and differentiation (103). Similarly, a characteristic *de novo* expression of the *GALNT6* isoenzyme in colon cancer was investigated by engineering *GALNTs* in a colon cancer cell line, and *GALNT6* was selectively shown to affect differentiation and growth (152). The unique functions of several other *GALNT* isoenzymes were also explored in KO studies (153–159). Moreover, glycoengineering is being used with approaches for metabolic labeling and tagging of glycosylation (22, 23, 160–162), including the azido sugars for click chemistry introduced by the Bertozzi group (163–165). Glycoengineered cells allow for screening and validation of selective binding and labeling probes in live cells as exemplified by the bump-and-hole strategy employed by Schumann and colleagues (166), in which modified UDP-GalNAc donor substrates (bumped) and *GALNTs* engineered to selectively accommodate these by an enlarged active site (hole) are used to detect isoform-specific functions in transfected cells (167–169).

In N-glycosylation the functions of the two dolichyl-diphosphooligosaccharide protein GT *STT3A* and *STT3B* subunits of the heteromeric OST complex were analyzed by proteomics studies of HEK293 cells with KO of either subunit, demonstrating distinct modification sites for the *STT3A/B* subunits (170, 171). Similarly, KO targeting was used to demonstrate that both *FKTN* and *FKRP* are Ribitol 5-phosphate transferases (172), that both procollagen galactosyltransferase 1 (*COLGALT1*) and 2 (*COLGALT2*) function in galactosylation of hydroxylysine (HYL) in collagens (173, 174), that both protein O-glucosyltransferase 2 (*POGLUT2*) and 3 (*POGLUT3*) serve the same Notch EGF11 repeat sequence (C<sup>3</sup>-XNTXGSFX-C<sup>4</sup>) different from *POGLUT1* (175), and that the substrate specificities of some of the four DPY19L1-4 C-mannosyltransferases may differ with respect to Trp substrate sites in consecutive glycosylation site motifs (e.g., the WXXWXXWXXC sequence in



thrombospondin repeats found in the netrin receptor UNC5A) (176).

However, most isoenzymes function in glycosylation pathway nonspecific steps with considerable cross talk between pathways, and these isoenzymes direct terminal structural features that determine many of the biological interactions with, *e.g.*, GBPs (Fig. 2A). This includes the enzymes assembling the elongation and branching of LacNAc disaccharides (Gal $\beta$ 1-3/4GlcNAc $\beta$ 1-R) and LacDiNAc (GalNAc $\beta$ 1-4GlcNAc $\beta$ 1-R) termini and the capping steps by the many sialyltransferase and fucosyltransferase isoenzymes (177–184). Since these glycan structures are found widely on N-glycans, O-glycans, and glycolipids, glycoengineering may affect different types of glycoconjugates and complicate analysis and assignments of functions. Redundant functions of the B4GALTs (B4GALT1-4) in galactosylation of N-glycans have been shown by combinatorial KO in CHO cells (82, 185). The many sialyltransferase isoenzymes were explored by KO revealing that ST3GAL4, and not ST3GAL3/6, is the major contributor in forming the sialyl-Lewis<sup>X</sup> (SLe<sup>x</sup>) glycan epitope that mediates selectin binding to lymphocytes and directs lymphocyte trafficking and extravasation (186). Glycoengineering was also used to demonstrate that enterovirus D68 infection requires both  $\alpha$ 2-3Sia and  $\alpha$ 2-6Sia recognition (187) and that human noroviruses require the blood group H glycan epitope directed by the secretor  $\alpha$ 2-fucosyltransferase (FUT2) (188). More emphasis on the nonredundant functions of isoenzymes is clearly needed as these most likely provide for differential regulation of important functions (5, 118). Combinatorial engineering of such isoenzymes can be used to weed out specific nonredundant functions with appropriate assays as discussed above (34).

### Novel opportunities provided by nuclease-based glycoengineering

In the following we highlight examples for how precision engineering of glycosylation is providing new opportunities for basic and translational sciences.

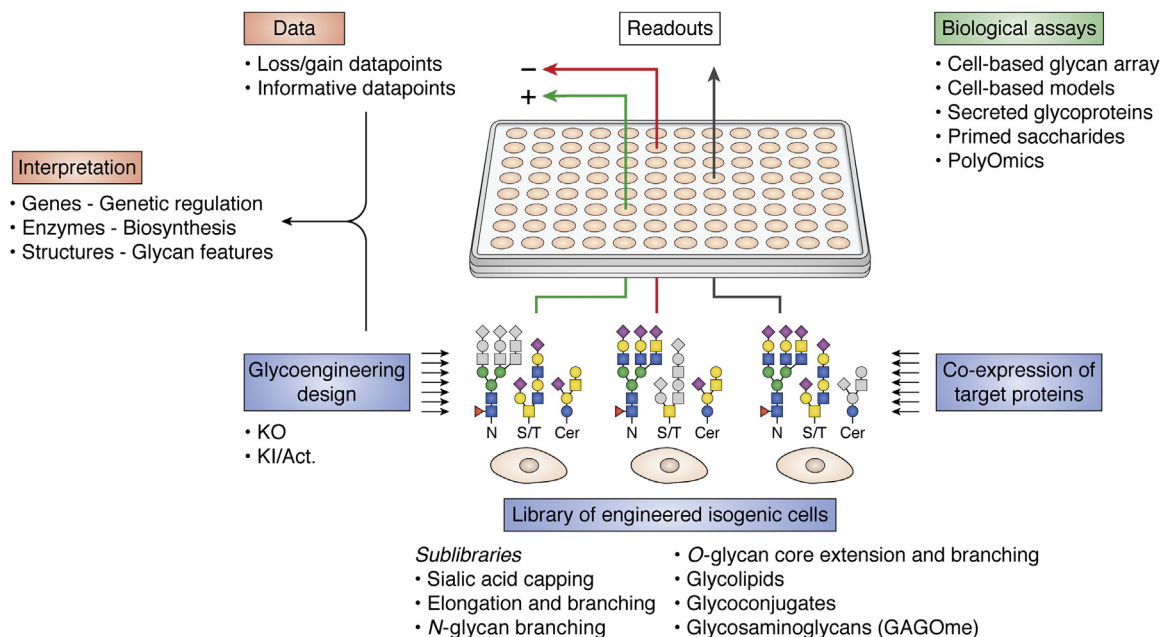
#### Cell-based glycan arrays

Glycan arrays have had huge impact on our insight into protein interactions with glycans and dissecting structural glycan features directing binding specificities of GBPs (189–194). Current glycan arrays generally comprise printed oligosaccharides, and these arrays are highly useful for dissecting structural features of glycans involved in binding of GBPs. Printed arrays with glycolipids and glycopeptides arrays or lipids are also being developed (195–198). A major advantage of printed glycan arrays is that these enable analysis of binding to distinct compounds with defined structures; however, they may not present glycans in the natural context of glycoconjugates and the cell surface. These drawbacks may at least partly be met by cell-based glycan arrays comprised of libraries of isogenic cells genetically engineered to display distinct features of the glycome (34) (Fig. 3). Mutant cells with global deficiencies in glycosylation features such as sialylation or glycosaminoglycan biosynthesis have been widely used in

the past (10, 106). It is now possible to develop libraries of stable isogenic cells that individually display the cellular glycome with loss or gain of distinct glycosylation features by systematic and combinatorial KO/KI of GT genes, and these libraries can constitute comprehensive cell-based glycan arrays. A dedicated cell-based array for glycosaminoglycans was developed in CHO cells—designated the GAGOme (120), and another library for HS GAGs in mouse lung endothelial cells was generated by a combination of immortalizing cells from KO mice and CRISPR/Cas gene editing (123). A more comprehensive array for the human glycome covering many features for glycolipids, N-glycoproteins, and GalNAc-type O-glycans was developed in HEK293 cells (34, 199).

Cell-based glycan arrays primarily inform of the glycosylation genes and biosynthetic steps involved in a queried GBP or biological function, and this information is used to predict the structural features of glycans involved (Fig. 3). Cell-based glycan arrays may reach astronomical numbers considering all possible combinations of the glycosylation genes, and organizing distinct sublibraries to cover major steps in glycosylation can help reduce workflows (34, 199). Importantly, cell-based libraries are sustainable once built and may be used not only in direct binding studies, but also with many different applications suitable for cell assays in high throughput (199). The primary use of traditional glycan arrays has been to dissect the binding properties of GBPs (189–194), and the main advantage of cell-based glycan arrays is the opportunity to dissect interactions in a more natural context using a number of different assays available with cells. One of the first studies employing a small library of human myeloid HL-60 cells designed to dissect contributions of three types of glycoconjugates carrying the SLe<sup>x</sup> selectin glycan ligands (glycolipids, N-glycoproteins, and GalNAc-type O-glycans by KO of *UGCG*, *MGAT1*, and *COSMC*, respectively) provided evidence that the types of glycoconjugates differentially promoted different phases of the rolling and adhesion cascade of leukocytes (126). A more expanded library of glycoengineered CHO cells was used to explore the glycan-binding specificities of Galectins recapitulating known preferences for N- or O-glycans (200), and studies of two Siglecs employing a library of HEK293 cells revealed distinct binding to different types of glycoconjugates (34).

Importantly, cell-based glycan libraries are providing evidence for more complex interactions with glycans that may involve a higher-order clustering or patterning guided by protein features as originally proposed by Varki (201, 202). The molecular basis for such is still unclear, but, *e.g.*, one illustrative example of a hybrid binding epitope comprised of a glycolipid (GT1b ganglioside) and a cell-membrane protein (synaptotagmin) was recently elucidated for neurotoxin B from *Clostridium botulinum* (203). These complex interactions can be probed by glycoengineering. Coreceptor involvement may also be interrogated. For example, recent findings indicating that the SARS-CoV2 viral spike protein exhibits binding to heparan sulfate (HS) GAGs (204) were explored by KO glycoengineering to demonstrate that HS does serve as a coreceptor strongly impacting binding to ACE2 and infection



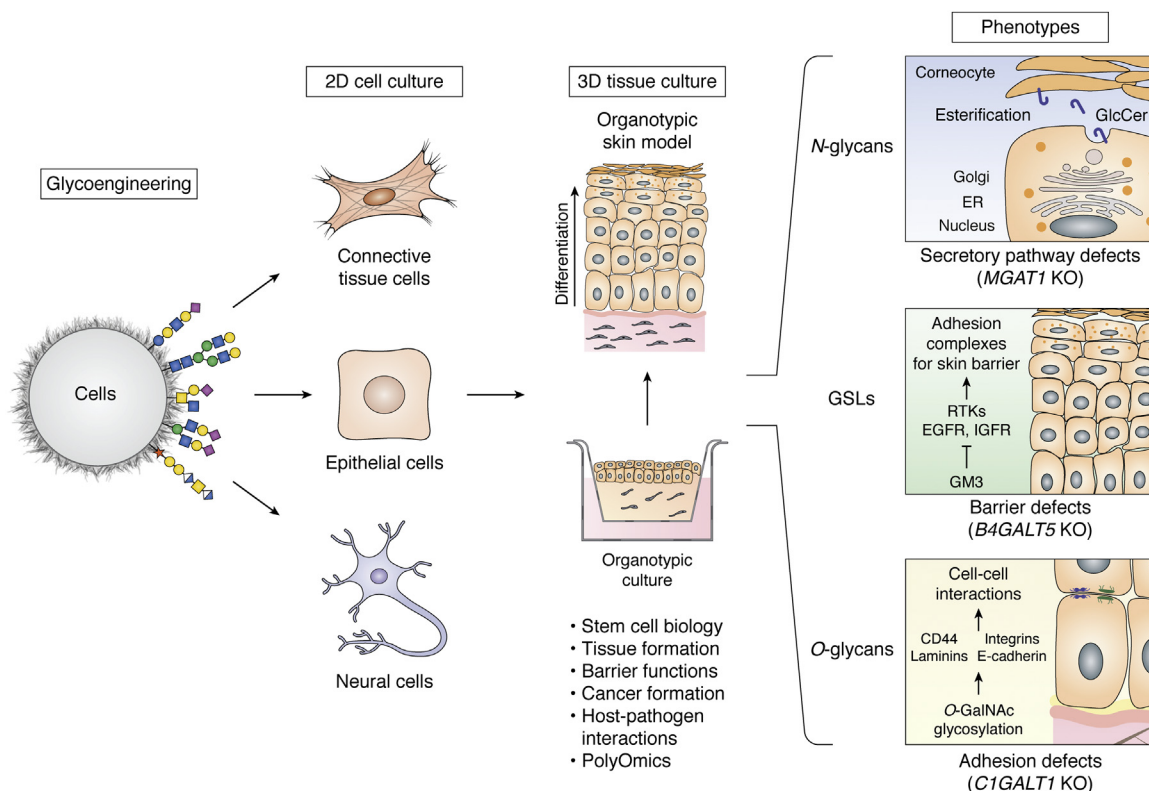
**Figure 3. Depiction of concepts of cell-based glycan arrays.** KO/KI (or activation) of GT genes in a mammalian cell line are used to generate isogenic cell lines with loss/gain of select glycosylation capacities and hence display of loss/gain of select glycan features on endogenous surface glycoconjugates. Libraries of such isogenic cell lines constitute the cell-based glycan arrays (34), and sublibraries can be designed to dissect select glycosylation steps or glycosylation pathways by combinatorial engineering of GTs (and other genes such as sulfotransferase genes modifying glycans). The design of libraries may be guided by the glycosylation pathways outlined in Figure 2A and take into account the endogenous GTs expressed in the cell. The cell line of choice, e.g., HEK293, may not express protein substrates of interest and coexpression (or activation of the endogenous gene) of one protein (or groups of) can therefore be included. The cell-based array can be probed by any biological assay suitable with live or fixed cells, but usage requires a positive signal. The positive signal may, for example, be found with the wildtype cells, induced during library screening by loss/gain of glycosylation features, or established from the start by, e.g., introducing a protein not endogenously expressed. The readouts are loss (red arrow)/gain (green arrow) of signals or data points (e.g., of binding to cells), and most datapoints will be neutral, i.e., no change in binding (gray arrow). Some neutral datapoints may still be informative in narrowing down structural features involved or not involved in interactions, especially when the genes affected are predicted to function in the same glycosylation pathway as those providing loss/gain of signals. Interpretation of the collected datapoints is performed with reference to the outlined glycosylation pathways and gene engineering matrix employed. The primary result being the GT genes required for signal (with added support from GTs genes not required), the secondary result being the biosynthetic pathway(s) and enzyme(s) required (and not), and the final result being the predicted glycan features and structures involved. This is thus different from studies with traditional glycan arrays that directly report glycan features and structures

(205). Cell-based array studies with human and avian influenza virus hemagglutinins (HAs) reproduced the established preferences for  $\alpha 2$ -6Sia and  $\alpha 2$ -3Sia (193), respectively. However, these studies also indicated that the human influenza virus HA (Hong Kong/1968 H3) showed preference for N-glycans, while the avian HA showed indiscriminate binding regardless of the type of glycoconjugate (34). We recently developed a cell-based platform for display of the densely O-glycosylated tandem repeat (TR) regions of human mucins using reporter constructs expressed in glycoengineered HEK293 cells, and this array has revealed distinct O-glycan and mucin TR specificities of viral and bacterial adhesins (34).

### 3D organotypic tissue models

While regulation of the glycome should be studied at the single cell level, the functional cues orchestrated by the glycome clearly have wider roles in tissue organization arguing for use of more complex models. The systematic glycoengineering concept for cell-based glycan arrays can be extended to cell lines and pluripotent stem cells suitable for development of organotypic cultures. Thereby, the glycoengineering strategies employed on 2D cultures can be extended to 3D cultures to explore and dissect biological

roles in more complex tissue formation (Fig. 4). This strategy was used with 3D spheres of engineered LS174T colon cancer cells to demonstrate that the repertoire of GALNTs initiating O-glycosylation may contribute to the malignant phenotype. Thus, when the characteristic colon-cancer-associated expression of GALNT6 was replicated in the LS174T model, normal cell differentiation was inhibited (152). Similarly, the characteristic expression of aberrant truncated O-glycans (Tn [GalNAc $\alpha$ 1-O-Ser/Thr] and STn [Neu5Ac $\alpha$ 2-6GalNAc $\alpha$ 1-O-Ser/Thr] in cancer was replicated in human keratinocyte cell (HaCaT) human skin models by KO of COSMC (private chaperone for the core1 synthase C1GALT1 that controls elongation of O-glycans) and used to demonstrate that the truncation of O-glycans induced oncogenic features including hyperproliferation and invasive growth (206). Another human 3D skin model (N/TERT-1) was used to address contributions of elaborated glycans on distinct types of glycoconjugates (glycolipids, N-glycans, O-GalNAc, O-Fuc, O-Glc) by KO targeting of earliest core extension steps (103), as well as probe nonredundant functions of GALNTs isoenzymes (147). Studies in these isogenic cell model systems further provide opportunities to apply wider multiomics approaches to explore changes in the



**Figure 4. Dissecting glycan functions using glycoengineered cell lines and organotypic tissue models.** Stable engineering (in 2D) of cell types (*left panels*) such as primary cells, immortalized cell line, induced pluripotent stem cells, or embryonic stem cells, with capability to differentiate into tissue-like 3D structures (*e.g.*, skin models, *right panels*), can be used to explore and dissect biological functions. Genetically engineered organotypic models simplify the interrogation and dissection of biological interactions involving glycans and enable the examination of how the individual glycoconjugates impact stem cells, tissue formation and homeostasis, cellular transformation, the interaction between different cell types, and host-pathogen interaction. For illustration phenotypic characteristics of glycoengineered human organotypic skin models with selective loss of elaborated N-glycans, glycosphingolipids, and GalNAc-type O-glycans are shown schematically (*far right panel*). Loss of complex N-linked glycans (KO *MGAT1*) affects transport and secretion of select proteins, loss of elongated glycosphingolipids (KO *B4GALT5*) affects tyrosine kinase regulation and produces barrier defects, and loss of elongated O-glycans (KO *C1GALT1*) affects differentiation and cell-cell interactions (103). Importantly, global proteomics and genomics can be integrated in the workflow to identify the molecular pathways affected by the loss/gain of individual glycoconjugates (147).

transcriptome and, *e.g.*, phosphoproteome for discovery and dissection of mechanism (147, 206).

### Glycoproteomics

Genetic glycoengineering has also advanced glycoproteomics. A major obstacle in analysis of glycosylation of proteins with proteomics strategies is the need for enrichment of glycopeptides where diversity and heterogeneity of glycans hamper uniform enrichment steps. A cocktail of lectins can enrich most N-glycopeptides (207, 208), and for GalNAc-type O-glycans the Jacalin plant lectin enriches most simple types of O-glycans (core1 based) (209, 210). Other strategies include metabolic labeling with GalNAz or alkylnyl fucose for enrichment by click chemistry (211–215) and selective capture of periodate-oxidized sialic acid containing glycopeptides on hydrazide beads (216, 217). However, genetic glycoengineering can be used to custom design glycosylation to fit to particular enrichment strategies. Simplifying the diversity of glycan structures and eliminating/reducing heterogeneities in structures may be used to enable efficient enrichment. The SimpleCell O-glycoproteomics strategy is based on this concept (127, 218). This strategy was originally employed for GalNAc-type O-glycosylation where KO of either the chaperone

*COSMC* or the *C1GALT1* synthase gene was known to abrogate O-glycosylation in most cell lines (except for cells expression the core3 synthase *B3GNT6*) (219, 220). This leaves all O-glycans truncated to the GalNAc $\alpha$ 1-O-Ser/Thr (Tn) structure and/or its  $\alpha$ 6-sialylated variant (STn) amenable for efficient capture by the VVA and Jacalin lectins (127, 221). The deepest O-glycoproteome analysis to date is with over 3000 O-glycoproteins identified and 15,000 glycosites derived from such SimpleCell lines (26, 119, 127, 221–223). The SimpleCell strategy was further designed to explore protein O-mannosylation by KO of *POMGNT1* (truncation of core M1 O-glycans to Man $\alpha$ 1-O-Ser/Thr) (224, 225), which enabled enrichment by ConA lectin (after prior removal of N-glycans) and discovery of extensive O-mannosylation of the cadherin and plexin superfamilies of receptor proteins (226). Subsequently, KO of the protein O-mannosyltransferases *POMT1/T2* resulted in discovery of two new O-mannosylation pathways directed by *TMTC1-4*, and a yet unreported gene dedicated to the cadherin and plexin receptors, respectively (130, 131). Interestingly, KO of *POMGNT1* was in fact not needed for detection of these new O-mannosylated glycoproteins since the initial O-Man residues apparently are not elongated (130) (Fig. 2A). Important to note though that the SimpleCell



glycoproteomics strategy does not provide information on structures of glycans found at identified glycosites, nor the stoichiometry of O-glycan attachments (221, 227). Recent advances in data-independent acquisition mass spectrometry (MS) may eventually overcome this drawback (222). Genetic truncation of glycosylation pathways in cells could potentially affect the earlier biosynthetic steps because of lack of competition and other unknown factors such as protein interactions (207, 228) and upstream protein functions. However, so far O-glycoproteomics studies performed with SimpleCells and WT cells have not shown substantial differences in identified glycosites (26, 147, 221, 229, 230). Regardless, the use of glycoengineering to custom design glycosylation in order to improve glycoproteomics strategies should be widely applicable and of value in exploring the different glycosylation pathways.

### Cell models for development of enzyme inhibitors

Small-molecule inhibitors of glycosylation enzymes are useful tools to dissect glycan functions in cells and animal models and have great potential for therapeutic applications (20). Global inhibitors of N-glycosylation (231, 232), GalNAc-type O-glycosylation (233, 234), sialylation (235, 236), fucosylation (235), and heparan sulfate biosynthesis (237) have been developed. However, inhibitors often lack selectivity for specific glycosylation enzymes, in particular isoenzymes, or have off-target effects (238). Screening for inhibitors of glycosyltransferases is often performed with *in vitro* assays using recombinant enzymes (233, 239), and it has been a challenge to develop cell-based assays that report direct inhibitors of GTs. Linstedt and colleagues used glycoengineered isogenic cell models to screen for GALNT isoenzyme-specific inhibitors using fluorescence biosensors that selectively report isoform-specific GALNT enzyme activities (furin cleavage of unique peptide substrates) (240). These biosensors were installed in isogenic cells with and without the *GALNT* gene of interest, and the strategy enabled discovery of one compound (T3Inh-1) that selectively binds and inhibits the GALNT3 isoenzyme *in vivo* (241). In another example, the two catalytic subunits of the OST, STT3A and STT3B, were KO engineered to screen and identify selective inhibitors of STT3B enabling regulation of N-glycan sites targeted mainly by posttranslational N-glycosylation (242). These examples demonstrate how glycoengineering of engineered mammalian cell lines now provides cell-based platforms for screening of selective inhibitors of specific biosynthetic steps and isoenzymes.

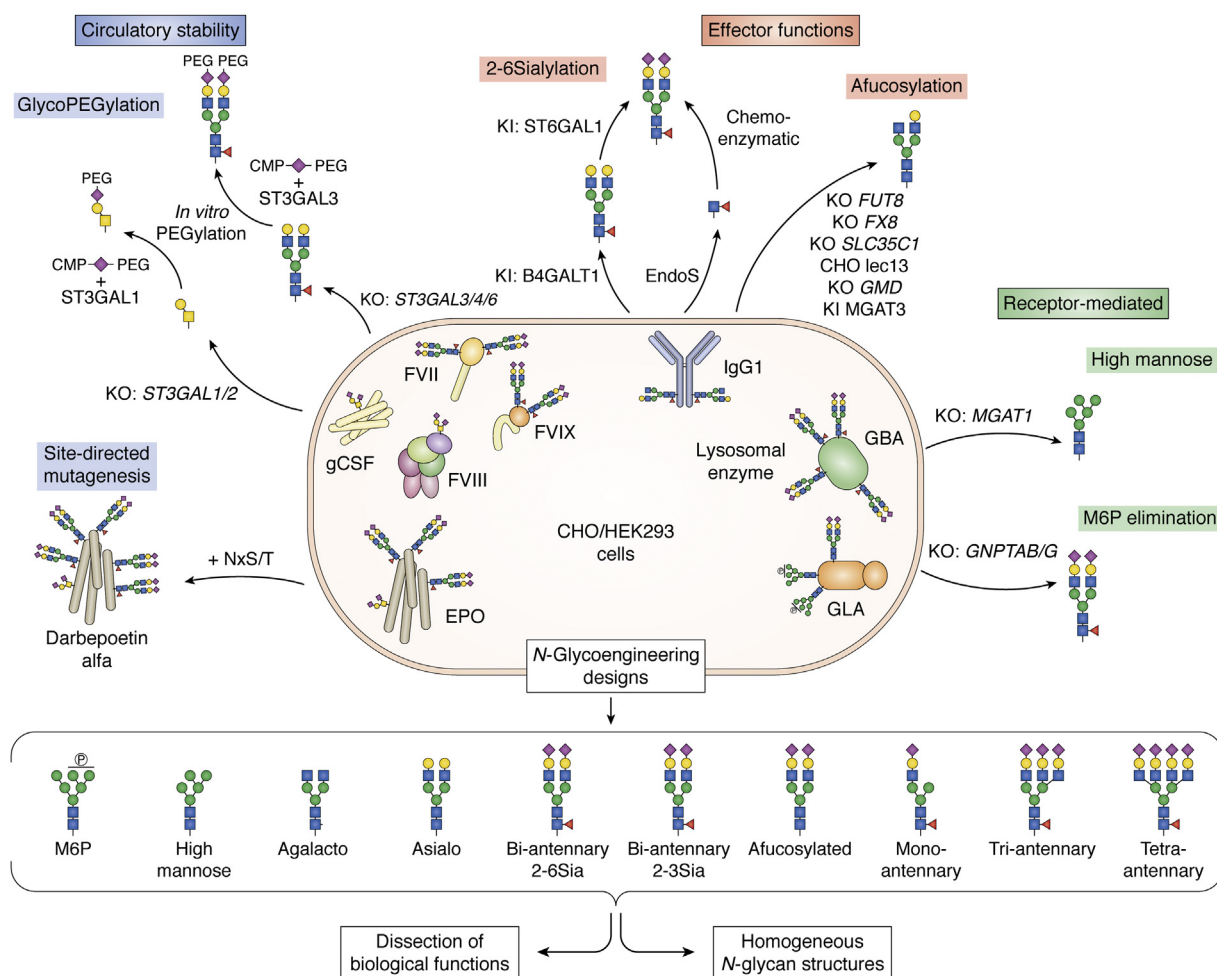
### Production of therapeutic glycoproteins in glycoengineered mammalian cells

The state of current therapeutic biologics seems to constitute a conundrum—most biologics are glycoproteins, yet glycans are not commonly considered in design and development of therapeutics. Glycosylation of therapeutic proteins may influence solubility, stability, structure and hydrodynamic size, clearance, cellular uptake, biodistribution, biological activity, and immunity, yet to day most therapeutic biologics are

produced in the classical CHO cell line with its inherent and rather simple, but human-like glycosylation capacities (19, 243–245). Decades of efforts have been devoted to engineering the glycosylation capacities in yeast (19, 246), insects (247), and plant cells (17, 248), mainly aiming to produce proteins with human-like glycosylation. Glycoengineering in mammalian cells has primarily aimed to reduce the heterogeneity (and improve consistency) in glycosylation (249, 250), increase sialylation to improve circulation (251, 252), and reduce or eliminate the core Fucose residue on IgG antibodies to enhance ADCC (253) (Fig. 5). However, glycoengineering may find much wider use. For example, CHO cells may be engineered to simplify the enzymatic glycopegylation strategy by producing substrate glycoproteins with N- or O-glycans suitable for direct transfer of sialic acids with polyethylene glycol (PEG) chains (254), and proteins may be designed with glycodomain modules and expressed with custom-designed glycans to enhance pharmacokinetics or immunogenicity (255, 256). The success with engineering CHO cells to produce afucosylated IgG therapeutic antibodies clearly stands out as the major achievement and perhaps the only widely adopted glycoengineered mammalian host cell for production of recombinant therapeutics today. Recent reviews cover these advances (16, 20, 257), and here, we limit the discussion to the opportunities provided by glycoengineering using precise gene editing in mammalian cells.

### Therapeutic IgG antibodies

A conserved N-glycan in the Fc region (Asn297) of IgG1 is critical for binding to Fc receptors (258), and the structural features of this N-glycan provide important modulation of interactions with FcγRs and complement factor C1q (259–261) (Fig. 5). Reducing or removing the core fucose of the Asn297 N-glycan results in increased binding to FcγRIIIa and enhanced ADCC (24, 262–264). This insight has been used to produce therapeutic antibodies with improved cytotoxicity in CHO cells overexpressing MGAT3 to inhibit core fucosylation (265), and in CHO cells with KO of the *fut8* gene that nonredundantly regulates core fucosylation (24, 25, 253, 266–269). The first CHO cell line engineered with *fut8* KO was generated by targeted homologous recombination (24), which is now readily reproduced with precise gene-editing strategies (270). Moreover, KO of other enzymes and transporters in *de novo* fucose biosynthesis (*GMD*, *FX*, *SLC35C1*) have enabled production of afucosylated mAbs in CHO cells (271–273). Another approach has been to express the prokaryotic GDP-6-deoxy-D-lyxo-4-hexulose reductase (RMD) to interfere with GDP-Fuc synthesis in CHO cells, which has resulted in a tunable system (GlymaxX Technology, ProBioGen) to produce IgG with different levels of α6Fuc (274). The Asn297 N-glycan is sterically hindered by the protein backbone, and as a result the N-glycan is incompletely processed with the predominant structures being biantennary (G0F, G1F, G2F) with low galactosylation and sialylation (275). However, the fine structure of the Asn297 N-glycans appears to be dynamically regulated and correlates



**Figure 5. Glycoengineering strategies for recombinant glycoprotein therapeutics.** Overview of examples of engineering designs applied to N-glycosylation in CHO and HEK293 cells to enhance circulation time of N-glycoproteins, modulate effector functions of therapeutic monoclonal antibodies (IgG1), and redirect lectin-mediated cell uptake of lysosomal replacement enzymes. Combinatorial glycoengineering of CHO and HEK293 cells has enabled production of custom-designed therapeutic glycoproteins with a variety of more homogeneous N-glycan structures that opens up wider screening and exploration of effects on pharmacokinetics, bioactivities, and immunogenicity (*bottom panel*) (82).

with diseases including inflammation, autoimmunity, infections, cancer, and following vaccination (261, 276–279). The N-glycan structure also affects the pharmacokinetic properties (280). Terminal galactose is reported to increase FcγRIIIa and C1q interactions resulting in enhanced ADCC and complement-dependent cytotoxicity (CDC) (281, 282). Sialic acid capping, in particular α2-6Sia, is indicated to decrease binding affinity of mAbs to FcγRIIIa (283) as well as C1q (284). This may mediate anti-inflammatory effects (285) and improve serum half-life (252), although contradicting studies report no effects of sialic acid on Fc conformation and recognition (286, 287). Despite this there appears to be fruitful grounds for further engineering of Fc glycosylation on therapeutic IgGs; however, given the complexity of the fine-tuning of IgG functions by N-glycosylation, this may not be straightforward and hence difficult to solve by generating simple homogeneous glycoforms. To this end another elegant strategy for fine-tuned control of IgG N-glycosylation through installation of inducible expression of glycosyltransferase genes (*FUT8*, *B4GALT1*) has been developed

(288). The engineering options for the IgG Asn297 N-glycan by simple KO/KI combinations of most of the GTs involved in N-glycan branching, galactosylation, and sialylation in CHO cells have been systematically explored (82, 289) (Fig. 5). This provides engineering designs for IgG with homogeneous biantennary N-glycans with and without galactose and fucose, although efficient sialylation (α2-3 and α2-6Sia) is only achievable on the α3-antenna. However, a monoantennary glycoform with homogeneous sialylation was achievable, and further studies are needed to evaluate effector functions of this (289). A different strategy—GlycoDelete—to simplify the IgG1 N-glycan to simple sialylated trisaccharides was engineered in HEK293 cells by KO of *MGAT1* to produce high-Man glycans combined with installation of a bacterial endo-β-N-acetylglucosaminidase (EndoT) to truncate high-Man N-glycans (290). IgG with these short N-glycans exhibits prolonged circulation time and reduced FcR binding, demonstrating the potential of glycoengineering for production of therapeutic IgG with homogeneous and custom-designed glycosylation.

**Custom-designed glycosylation of recombinant therapeutic glycoproteins**

While IgG represents a unique challenge for glycoengineering due to the protein-specific restricted glycosylation, erythropoietin (EPO) is a widely studied therapeutic glycoprotein that acquires glycosylation according to the host cell glycosylation capacity (82, 291). The three N-glycans on EPO acquire rather homogeneous tetra-antennary structures with a minor amount of polyLacNAc and efficient  $\alpha$ 2-3Sia capping when produced in CHO cells (292, 293), while a single sialylated core1 O-glycan is heterogeneously attached (~80% occupancy) (294). EPO stimulates red blood cell production and is widely used in treatment of anemia, but the native glycoprotein is rapidly cleared by glomerular filtration due to its small size (30 kDa). Pioneering work led to new generations of EPO with extended circulation, with one strategy involving introduction of two additional N-glycans (Darbepoetin alfa) to increase the hydrodynamic size and prevent clearance (295). The clearance of EPO is also highly sensitive to the sialylation state (292, 296), and sialic acid capping of glycans on glycoproteins is generally important for the serum half-life to prevent lectin-mediated uptake by the hepatic Ashwell–Morell receptor (AMR) (297–300). Batch-to-batch variations in sialylation of EPO have been a challenge (301), and secreted neuraminidases (*NEU1-3*) from CHO cells can pose issues and KO of these can improve sialylation (302). Several reports have suggested that CHO cells may add minor amounts of the immunogenic  $\alpha$ 1-3Gal epitope as well as NeuGc sialic acids (303–305), although these findings are disputed and CHO cells with KO of these genes are available (306).

The N-glycosylation capacity of CHO cells was extensively deconstructed by KO and to some extent reconstructed by limited KI of human GT genes (82, 121). Using EPO as the N-glycoprotein reporter, these studies demonstrated wide design options for antennary structures, galactosylation, polyLacNAc and  $\alpha$ 2-3/2-6 sialylation, and the ability to produce more homogeneous glycoforms (82) (Fig. 5). Redundant contributions of the many B4GALT and ST3GAL isoenzymes for efficient galactosylation and  $\alpha$ 2-3 sialylation, respectively, necessitate combinatorial KO to eliminate either of these processes (82, 185). N-glycoengineering may not be generally applicable to all N-glycoproteins and especially individual N-glycosites, since local conformational effects may influence glycosylation at some glycosites, as discussed for IgG above. Individual N-glycosites may undergo selective processing, e.g., high-Man N-glycans are often found at individual glycosites in proteins, and those have to be considered and specifically addressed in glycoengineering. Regardless, the current state of glycoengineering in mammalian cells provides considerable freedom to design and control most types of glycosylation of proteins, which provides the opportunities to produce glycoprotein variants and test these to identify the optimal design.

**Glycodesign options for enzyme replacement therapies**

Lysosomal enzymes are N-glycoproteins and select N-glycans on these acquire mannose-6-phosphate (M6P) in early Golgi,

which serves as recognition for the mannose 6-phosphate (M6P) receptors (MPRs) mediating trafficking from Golgi to the lysosome (307, 308). Lysosomal storage diseases (LSDs) including Gaucher disease with deficiency in  $\beta$ -glucocerebrosidase (GBA) and Fabry disease with deficiency in  $\alpha$ -galactosidase A (GLA) are currently treated by enzyme replacement therapies (ERTs) (308–312). The MPRs (IGF2R) also serve in endocytosis from the cell surface, and this is exploited in ERTs for uptake and delivery (309, 311, 313). When lysosomal enzymes are expressed recombinantly in mammalian cells, select N-glycans acquire M6P tags, while other N-glycans may become complex-type sialylated glycans, and the degree of M6P tagging is considered the major contribution factor for cellular uptake (309, 311, 314, 315). Several lectin receptors including the mannose receptor (MR) and AMR may also affect uptake and delivery (311, 316, 317), and the first ERT for Gaucher disease utilized this to selectively target macrophages expressing the MR by trimming N-glycans including M6P to the mannose core with enzyme digestion (318). This glycan design has also been engineered directly in CHO cells (121). However, engineering of glycans on ERTs has mainly focused on increasing M6P for improved delivery (311, 319), and systematic studies of the optimal glycan features were only performed recently using extensive combinatorial glycoengineering in CHO cells (121) (Fig. 5). Testing a panel of glycoforms of GLA *in vivo* in a Fabry mouse model revealed that M6P is not necessary for uptake and reduction of substrates in target organs, and interestingly GLA with  $\alpha$ 2-3Sia N-glycans exhibits higher circulation time than GLA with M6P (e.g., Fabrazyme), while GLA with  $\alpha$ 2-6Sia bi-antennary N-glycans is rapidly targeted to the liver. This example illustrates that testing different glycoforms of glycoproteins may be a fruitful strategy to improve and optimize design of therapeutic glycoproteins.

**Native mass spectrometry of glycoproteins**

Heterogeneous glycans pose challenges for analysis of intact glycoproteins by MS (320, 321), and considerable efforts are devoted to characterization of therapeutic glycoproteins by bottom-up MS analysis of peptide digests and profiling of released glycans. Native MS is widely used to study non-covalent protein–protein complexes, and direct MS of intact proteins is also highly suitable for analysis of the quality of recombinant proteins with posttranslational modifications (322). Native MS was applied to CHO cell produced IgG that carries the rather simple N-glycan structure with limited heterogeneity at Asn297 (323, 324). MS analysis of more complex glycoproteins with multiple N- and O-linked glycans has relied on exoglycosidases to reduce glycan heterogeneity to enable data interpretation (325, 326). However, glycoengineering of production host cells offers simpler ways to obtain glycoproteins with more homogeneous glycans suitable for intact MS analysis (82), and this strategy was used with multiple glycoforms of EPO produced in glycoengineered CHO cells (327). We envision that glycoengineering will greatly advance use of MS analysis of intact glycoproteins not only in basic science, but also in quality control of glycoprotein therapeutics.



## Conclusion and prospective

In 2014, we argued that the emergence of precision gene-editing technologies represented a small revolution for the glycobiology field—if anything an understatement (4). Precision engineering of cellular glycosylation is becoming indispensable for interrogation and dissection of biological interactions involving glycans. Arguably, today it may be simpler and more informative to gain information on the genes and biosynthetic pathways that regulate glycosylation, than to directly analyze structures of glycans. Tools and expertise for genetic engineering of cellular glycosylation are widely available to cell biologists, and the workflows employed may be integrated with general proteomics and genomics approaches. There are clearly still shortcomings that need to be addressed, and the most critical ones are to improve knowledge of the *in vivo* functions of the many GT isoenzymes and develop strategies for translating quantitative transcriptomics and proteomics data into reliable predictions of the cellular glycome. Genetic glycoengineering is currently the only method to study glycosylation at the single cell level.

Genetic glycoengineering is bringing new tools and capabilities to the glycomics field that provide innovative platforms for exploring functions of glycosylation in the natural environment of the cell such as cell-based glycan arrays and organotypic tissue models. These platforms are still being developed and knowledge of their use is limited, but results obtained with these are already providing insights into more complex interactions of GBPs with higher-order patterns of glycans on proteins. Revisiting the binding properties of glycan-binding proteins with these new resources is likely to be fruitful and adds insights into how selectivity of interactions with glycans is obtained.

Glycoengineering is poised to transform the design and production platforms for recombinant glycoprotein therapeutics. While implementation of glycoengineered host cells in the conservative pharma environment will take time, the advantages of being able to produce more homogeneous glycoforms with the added value of enabling intact analysis of biomolecules are too good to miss. We envision that the major incentive for glycoengineering of therapeutics will come from discovery of glycan designs that provide improved properties to glycoprotein therapeutics such as exemplified by afucosylated IgG therapeutic antibodies. This will require experimental testing, and it is now possible to produce and display a variety of different glycoforms of glycoproteins for use in preclinical studies.

Finally, beyond engineering of glycosylation, the precise gene-editing tools open up an entirely new level for exploring the glycosylation machinery in live cells. For example, tagging of endogenous GTs can enable live imaging of subcellular localization and changes herein in response to stimuli as well as molecular interactions. Tagging endogenous protein substrates can also be used to monitor protein-specific glycosylation in real time revealing effects on subcellular transport. In conclusion, genetic engineering of glycosylation is a fertile

playground with enormous potential for reprogramming the glycosylation processes in cells and the cellular glycome for research and therapeutic applications.

*Author contributions*—Y. N., C. B., Y. -H. C., H. H. W., Z. Y., and H. C. conceived and wrote the article.

*Funding and additional information*—This work was supported by the Lundbeck Foundation, the Danish National Research Foundation (DNRF107), the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie grant agreement No 787684 (to C. B.), the ERC Consolidator program (grant agreement No 772735 to H. W.), and the MSCA-ITN-2020 innovative training networks Imgene H2020 (to H. W.)

*Conflict of interest*—University of Copenhagen has filed a patent application on the cell-based display platform. GlycoDisplay Aps, Copenhagen, Denmark, has obtained a license to the field of the patent application. Y. N., Z. Y., and H. C. are cofounders of GlycoDisplay Aps and hold ownerships in the company. H. C. is a cofounder of GO-Therapeutics Inc and holds ownership. H. W. is a consultant for GO-Therapeutics Inc and cofounder of EbuMab ApS and holds ownership.

*Abbreviations*—The abbreviations used are: ADCC, antibody-dependent cell cytotoxicity; CHO, Chinese hamster ovary; CRISPR, clustered regularly interspaced short palindromic repeat; EPO, erythropoietin; ES, embryonic stem; GBP, glycan-binding protein; GT, glycosyltransferase; HA, hemagglutinin; HDR, homology-directed repair; KI, knock-in; KO, knockout; MMEJ, microhomology-mediated end joining; NHEJ, nonhomologous end joining; PAM, proto-spacer adjacent motif; TALEN, transcription activator-like effector nuclease; TR, tandem repeat; ZFN, zinc-finger nuclease.

## References

- Stanley, P. (2016) What have we learned from glycosyltransferase knockouts in mice? *J. Mol. Biol.* **428**, 3166–3182
- Lowe, J. B., and Marth, J. D. (2003) A genetic approach to mammalian glycan function. *Annu. Rev. Biochem.* **72**, 643–691
- Orr, S. L., Le, D., Long, J. M., Sobieszczuk, P., Ma, B., Tian, H., Fang, X., Paulson, J. C., Marth, J. D., and Varki, N. (2013) A phenotype survey of 36 mutant mouse strains with gene-targeted defects in glycosyltransferases or glycan-binding proteins. *Glycobiology* **23**, 363–380
- Steenfot, C., Bennett, E. P., Schjoldager, K. T., Vakhrushev, S. Y., Wandall, H. H., and Clausen, H. (2014) Precision genome editing: A small revolution for glycobiology. *Glycobiology* **24**, 663–680
- Schjoldager, K. T., Narimatsu, Y., Joshi, H. J., and Clausen, H. (2020) Global view of human protein glycosylation pathways and functions. *Nat. Rev. Mol. Cell Biol.* **21**, 729–749
- Griffin, M. E., and Hsieh-Wilson, L. C. (2016) Glycan engineering for cell and developmental biology. *Cell Chem. Biol.* **23**, 108–121
- Gottlieb, C., Skinner, A. M., and Kornfeld, S. (1974) Isolation of a clone of Chinese hamster ovary cells deficient in plant lectin-binding sites. *Proc. Natl. Acad. Sci. U. S. A.* **71**, 1078–1082
- Gottlieb, C., and Kornfeld, S. (1976) Isolation and characterization of two mouse L cell lines resistant to the toxic lectin ricin. *J. Biol. Chem.* **251**, 7761–7768
- Stanley, P. (1983) Lectin-resistant CHO cells: Selection of new mutant phenotypes. *Somatic Cell Genet.* **9**, 593–608
- Patnaik, S. K., and Stanley, P. (2006) Lectin-resistant CHO glycosylation mutants. *Methods Enzymol.* **416**, 159–182

11. Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126**, 855–867
12. Marth, J. D. (1996) Complexity in O-linked oligosaccharide biosynthesis engendered by multiple polypeptide N-acetylgalactosaminyltransferases. *Glycobiology* **6**, 701–705
13. Yang, W. H., Nussbaum, C., Grewal, P. K., Marth, J. D., and Sperandio, M. (2012) Coordinated roles of ST3Gal-VI and ST3Gal-IV sialyltransferases in the synthesis of selectin ligands. *Blood* **120**, 1015–1026
14. Takamatsu, S., Antonopoulos, A., Ohtsubo, K., Ditto, D., Chiba, Y., Le, D. T., Morris, H. R., Haslam, S. M., Dell, A., Marth, J. D., and Taniguchi, N. (2010) Physiological and glycomic characterization of N-acetylglucosaminyltransferase-IVa and -IVb double deficient mice. *Glycobiology* **20**, 485–497
15. Stone, E. L., Ismail, M. N., Lee, S. H., Luu, Y., Ramirez, K., Haslam, S. M., Ho, S. B., Dell, A., Fukuda, M., and Marth, J. D. (2009) Glycosyltransferase function in core 2-type protein O glycosylation. *Mol. Cell Biol.* **29**, 3770–3782
16. Van Landuyt, L., Lonigro, C., Meuris, L., and Callewaert, N. (2019) Customized protein glycosylation to improve biopharmaceutical function and targeting. *Curr. Opin. Biotechnol.* **60**, 17–28
17. Montero-Morales, L., and Steinkellner, H. (2018) Advanced plant-based glycan engineering. *Front. Bioeng. Biotechnol.* **6**, 81
18. Naegli, A., and Aebi, M. (2015) Current approaches to engineering N-linked protein glycosylation in bacteria. *Methods Mol. Biol.* **1321**, 3–16
19. Laukens, B., De Visscher, C., and Callewaert, N. (2015) Engineering yeast for producing human glycoproteins: Where are we now? *Future Microbiol.* **10**, 21–34
20. Hudak, J. E., and Bertozzi, C. R. (2014) Glycotherapy: New advances inspire a reemergence of glycans in medicine. *Chem. Biol.* **21**, 16–37
21. Kightlinger, W., Warfel, K. F., DeLisa, M. P., and Jewett, M. C. (2020) Synthetic glycobiology: Parts, systems, and applications. *ACS Synth. Biol.* **9**, 1534–1562
22. Agatemor, C., Buettner, M. J., Ariss, R., Muthiah, K., Saeui, C. T., and Yarema, K. J. (2019) Exploiting metabolic glycoengineering to advance healthcare. *Nat. Rev. Chem.* **3**, 605–620
23. Moons, S. J., Adema, G. J., Derks, M. T., Boltje, T. J., and Bull, C. (2019) Sialic acid glycoengineering using N-acetylmannosamine and sialic acid analogs. *Glycobiology* **29**, 433–445
24. Yamane-Ohnuki, N., Kinoshita, S., Inoue-Urakubo, M., Kusunoki, M., Iida, S., Nakano, R., Wakitani, M., Niwa, R., Sakurada, M., Uchida, K., Shitara, K., and Satoh, M. (2004) Establishment of FUT8 knockout Chinese hamster ovary cells: An ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. *Biotechnol. Bioeng.* **87**, 614–622
25. Malphettes, L., Freyvert, Y., Chang, J., Liu, P. Q., Chan, E., Miller, J. C., Zhou, Z., Nguyen, T., Tsai, C., Snowden, A. W., Collingwood, T. N., Gregory, P. D., and Cost, G. J. (2010) Highly efficient deletion of FUT8 in CHO cell lines using zinc-finger nucleases yields cells that produce completely nonfucosylated antibodies. *Biotechnol. Bioeng.* **106**, 774–783
26. Yang, Z., Halim, A., Narimatsu, Y., Jitendra Joshi, H., Steentoft, C., Schjoldager, K. T., Alder Schulz, M., Sealover, N. R., Kayser, K. J., Paul Bennett, E., Levery, S. B., Vakhrushev, S. Y., and Clausen, H. (2014) The GalNAc-type O-glycoproteome of CHO cells characterized by the SimpleCell strategy. *Mol. Cell. Proteomics* **13**, 3224–3235
27. Doudna, J. A. (2020) The promise and challenge of therapeutic genome editing. *Nature* **578**, 229–236
28. Wang, L. X., and Amin, M. N. (2014) Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem. Biol.* **21**, 51–66
29. Mizukami, A., Caron, A. L., Picanco-Castro, V., and Swiech, K. (2018) Platforms for recombinant therapeutic glycoprotein production. *Methods Mol. Biol.* **1674**, 1–14
30. Dicker, M., and Strasser, R. (2015) Using glyco-engineering to produce therapeutic proteins. *Expert Opin. Biol. Ther.* **15**, 1501–1516
31. Cummings, R. D. (2009) The repertoire of glycan determinants in the human glycome. *Mol. Biosyst.* **5**, 1087–1104
32. Moremen, K. W., Tiemeyer, M., and Nairn, A. V. (2012) Vertebrate protein glycosylation: Diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* **13**, 448–462
33. Moremen, K. W., and Haltiwanger, R. S. (2019) Emerging structural insights into glycosyltransferase-mediated synthesis of glycans. *Nat. Chem. Biol.* **15**, 853–864
34. Narimatsu, Y., Joshi, H. J., Nason, R., Van Coillie, J., Karlsson, R., Sun, L., Ye, Z., Chen, Y. H., Schjoldager, K. T., Steentoft, C., Furukawa, S., Bensing, B. A., Sullam, P. M., Thompson, A. J., Paulson, J. C., et al. (2019) An atlas of human glycosylation pathways enables display of the human glycome by gene engineered cells. *Mol. Cell* **75**, 394–407.e395
35. Nairn, A. V., York, W. S., Harris, K., Hall, E. M., Pierce, J. M., and Moremen, K. W. (2008) Regulation of glycan structures in animal tissues: Transcript profiling of glycan-related genes. *J. Biol. Chem.* **283**, 17298–17313
36. Joshi, H. J., Narimatsu, Y., Schjoldager, K. T., Tytgat, H. L. P., Aebi, M., Clausen, H., and Halim, A. (2018) SnapShot: O-glycosylation pathways across kingdoms. *Cell* **172**, 632–632.e2
37. O'Donnell, N., Zachara, N. E., Hart, G. W., and Marth, J. D. (2004) Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. *Mol. Cell Biol.* **24**, 1680–1690
38. Shafi, R., Iyer, S. P., Ellies, L. G., O'Donnell, N., Marek, K. W., Chui, D., Hart, G. W., and Marth, J. D. (2000) The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5735–5739
39. Cummings, R. D., and Pierce, J. M. (2014) The challenge and promise of glycomics. *Chem. Biol.* **21**, 1–15
40. Rudd, P., Karlsson, N. G., Khoo, K. H., and Packer, N. H. (2015) Glycomics and glycoproteomics. In: Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 653–666
41. Park, D. D., Xu, G., Wong, M., Phoomak, C., Liu, M., Haigh, N. E., Wongkham, S., Yang, P., Maverakis, E., and Lebrilla, C. B. (2018) Membrane glycomics reveal heterogeneity and quantitative distribution of cell surface sialylation. *Chem. Sci.* **9**, 6271–6285
42. Fujitani, N., Furukawa, J., Araki, K., Fujioka, T., Takegawa, Y., Piao, J., Nishioka, T., Tamura, T., Nikaido, T., Ito, M., Nakamura, Y., and Shinohara, Y. (2013) Total cellular glycomics allows characterizing cells and streamlining the discovery process for cellular biomarkers. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 2105–2110
43. An, H. J., Gip, P., Kim, J., Wu, S., Park, K. W., McVaugh, C. T., Schaffer, D. V., Bertozzi, C. R., and Lebrilla, C. B. (2012) Extensive determination of glycan heterogeneity reveals an unusual abundance of high mannose glycans in enriched plasma membranes of human embryonic stem cells. *Mol. Cell. Proteomics* **11**, M111.010660
44. Riley, N. M., Hebert, A. S., Westphall, M. S., and Coon, J. J. (2019) Capturing site-specific heterogeneity with large-scale N-glycoproteome analysis. *Nat. Commun.* **10**, 1311
45. Freeze, H. H., Baum, L., and Varki, A. (2015) Glycans in systemic physiology. In: Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 521–526
46. Nairn, A. V., Aoki, K., dela Rosa, M., Porterfield, M., Lim, J. M., Kulik, M., Pierce, J. M., Wells, L., Dalton, S., Tiemeyer, M., and Moremen, K. W. (2012) Regulation of glycan structures in murine embryonic stem cells: Combined transcript profiling of glycan-related genes and glycan structural analysis. *J. Biol. Chem.* **287**, 37835–37856
47. Dabelsteen, E., Vedtofte, P., Hakomori, S. I., and Young, W. W. (1982) Carbohydrate chains specific for blood group antigens in differentiation of human oral epithelium. *J. Invest. Dermatol.* **79**, 3–7
48. Bull, C., Stoel, M. A., den Brok, M. H., and Adema, G. J. (2014) Sialic acids sweeten a tumor's life. *Cancer Res.* **74**, 3199–3204
49. Mottram, L., Liu, J., Chavan, S., Tobias, J., Svennerholm, A. M., and Holgersson, J. (2018) Glyco-engineered cell line and computational

- docking studies reveals enterotoxigenic *Escherichia coli* CFA/I fimbriae bind to Lewis a glycans. *Sci. Rep.* **8**, 11250
50. Lindberg, L., Liu, J., and Holgersson, J. (2013) Engineering of therapeutic and diagnostic O-glycans on recombinant mucin-type immunoglobulin fusion proteins expressed in CHO cells. *Methods Mol. Biol.* **988**, 3–17
  51. Li, F., Wilkins, P. P., Crawley, S., Weinstein, J., Cummings, R. D., and McEver, R. P. (1996) Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. *J. Biol. Chem.* **271**, 3255–3264
  52. Lee, E. U., Roth, J., and Paulson, J. C. (1989) Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of beta-galactoside alpha 2,6-sialyltransferase. *J. Biol. Chem.* **264**, 13848–13855
  53. Shi, W. X., Chammas, R., and Varki, A. (1996) Linkage-specific action of endogenous sialic acid O-acetyltransferase in Chinese hamster ovary cells. *J. Biol. Chem.* **271**, 15130–15138
  54. Mondal, N., Dykstra, B., Lee, J., Ashline, D. J., Reinhold, V. N., Rossi, D. J., and Sackstein, R. (2018) Distinct human alpha(1,3)-fucosyltransferases drive Lewis-X/sialyl Lewis-X assembly in human cells. *J. Biol. Chem.* **293**, 7300–7314
  55. Sewell, R., Backstrom, M., Dalziel, M., Gschmeissner, S., Karlsson, H., Noll, T., Gatgens, J., Clausen, H., Hansson, G. C., Burchell, J., and Taylor-Papadimitriou, J. (2006) The ST6GalNAc-I sialyltransferase localizes throughout the Golgi and is responsible for the synthesis of the tumor-associated sialyl-Tn O-glycan in human breast cancer. *J. Biol. Chem.* **281**, 3586–3594
  56. Jackson, A. L., and Linsley, P. S. (2010) Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat. Rev. Drug Discov.* **9**, 57–67
  57. Aagaard, L., and Rossi, J. J. (2007) RNAi therapeutics: Principles, prospects and challenges. *Adv. Drug Deliv. Rev.* **59**, 75–86
  58. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821
  59. Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823
  60. Maresca, M., Lin, V. G., Guo, N., and Yang, Y. (2013) Obligate ligation-gated recombination (ObLiGaRe): Custom-designed nuclease-mediated targeted integration through nonhomologous end joining. *Genome Res.* **23**, 539–546
  61. DeKelver, R. C., Choi, V. M., Moehle, E. A., Paschon, D. E., Hockemeyer, D., Meijnsing, S. H., Sancak, Y., Cui, X., Steine, E. J., Miller, J. C., Tam, P., Bartsevich, V. V., Meng, X., Rupniewski, L., Gopalan, S. M., et al. (2010) Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res.* **20**, 1133–1142
  62. Ran, F. A., Hsu, P. D., Lin, C. Y., Gootenberg, J. S., Konermann, S., Trevino, A. E., Scott, D. A., Inoue, A., Matoba, S., Zhang, Y., and Zhang, F. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380–1389
  63. Maeder, M. L., Linder, S. J., Cascio, V. M., Fu, Y., Ho, Q. H., and Joung, J. K. (2013) CRISPR RNA-guided activation of endogenous human genes. *Nat. Methods* **10**, 977–979
  64. Chavez, A., Scheiman, J., Vora, S., Pruitt, B. W., Tuttle, M., Iyer, E. P. R., Lin, S., Kiani, S., Guzman, C. D., Wiegand, D. J., Ter-Ovanesyan, D., Braff, J. L., Davidsohn, N., Housden, B. E., Perrimon, N., et al. (2015) Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods* **12**, 326–328
  65. Zhang, H. X., Zhang, Y., and Yin, H. (2019) Genome editing with mRNA encoding ZFN, TALEN, and Cas9. *Mol. Ther.* **27**, 735–746
  66. Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., and Zhao, X. (2020) Applications of genome editing technology in the targeted therapy of human diseases: Mechanisms, advances and prospects. *Signal Transduct. Target. Ther.* **5**, 1
  67. Symington, L. S., and Gautier, J. (2011) Double-strand break end resection and repair pathway choice. *Annu. Rev. Genet.* **45**, 247–271
  68. Bogdanove, A. J., and Voytas, D. F. (2011) TAL effectors: Customizable proteins for DNA targeting. *Science* **333**, 1843–1846
  69. Walton, R. T., Christie, K. A., Whittaker, M. N., and Kleinstiver, B. P. (2020) Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science* **368**, 290–296
  70. Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G., and Zhang, F. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832
  71. Doench, J. G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E. W., Donovan, K. F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H. W., Listgarten, J., and Root, D. E. (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat. Biotechnol.* **34**, 184–191
  72. Labun, K., Montague, T. G., Gagnon, J. A., Thyme, S. B., and Valen, E. (2016) CHOPCHOP v2: A web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res.* **44**, W272–276
  73. Hanna, R. E., and Doench, J. G. (2020) Design and analysis of CRISPR-Cas experiments. *Nat. Biotechnol.* **38**, 813–823
  74. Narimatsu, Y., Joshi, H. J., Yang, Z., Gomes, C., Chen, Y. H., Lorenzetti, F. C., Furukawa, S., Schjoldager, K. T., Hansen, L., Clausen, H., Bennett, E. P., and Wandall, H. H. (2018) A validated gRNA library for CRISPR/Cas9 targeting of the human glycosyltransferase genome. *Glycobiology* **28**, 295–305
  75. Zhu, Y., Groth, T., Kelkar, A., Zhou, Y., and Neelamegham, S. (2020) A GlycoGene CRISPR-Cas9 lentiviral library to study lectin binding and human glycan biosynthesis pathways. *Glycobiology*. <https://doi.org/10.1093/glycob/cwaa074>
  76. Bennett, E. P., Petersen, B. L., Johansen, I. E., Niu, Y., Yang, Z., Chamberlain, C. A., Met, O., Wandall, H. H., and Frodin, M. (2020) INDEL detection, the 'achilles heel' of precise genome editing: A survey of methods for accurate profiling of gene editing induced indels. *Nucleic Acids Res.* **48**, 11958–11981
  77. Lonowski, L. A., Narimatsu, Y., Riaz, A., Delay, C. E., Yang, Z., Niola, F., Duda, K., Ober, E. A., Clausen, H., Wandall, H. H., Hansen, S. H., Bennett, E. P., and Frodin, M. (2017) Genome editing using FACS enrichment of nuclease-expressing cells and indel detection by amplicon analysis. *Nat. Protoc.* **12**, 581–603
  78. Mashal, R. D., Koontz, J., and Sklar, J. (1995) Detection of mutations by cleavage of DNA heteroduplexes with bacteriophage resolvases. *Nat. Genet.* **9**, 177–183
  79. Till, B. J., Burtner, C., Comai, L., and Henikoff, S. (2004) Mismatch cleavage by single-strand specific nucleases. *Nucleic Acids Res.* **32**, 2632–2641
  80. Anzalone, A. V., Koblan, L. W., and Liu, D. R. (2020) Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat. Biotechnol.* **38**, 824–844
  81. Pinto, R., Hansen, L., Hintze, J., Almeida, R., Larsen, S., Coskun, M., Davidsen, J., Mitchelmore, C., David, L., Troelsen, J. T., and Bennett, E. P. (2017) Precise integration of inducible transcriptional elements (PrIITE) enables absolute control of gene expression. *Nucleic Acids Res.* **45**, e123
  82. Yang, Z., Wang, S., Halim, A., Schulz, M. A., Frodin, M., Rahman, S. H., Vester-Christensen, M. B., Behrens, C., Kristensen, C., Vakhrushev, S. Y., Bennett, E. P., Wandall, H. H., and Clausen, H. (2015) Engineered CHO cells for production of diverse, homogeneous glycoproteins. *Nat. Biotechnol.* **33**, 842–844
  83. Nakade, S., Tsubota, T., Sakane, Y., Kume, S., Sakamoto, N., Obara, M., Daimon, T., Sezutsu, H., Yamamoto, T., Sakuma, T., and Suzuki, K. T. (2014) Microhomology-mediated end-joining-dependent integration of donor DNA in cells and animals using TALENs and CRISPR/Cas9. *Nat. Commun.* **5**, 5560
  84. Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E. J., Hatanaka, F., Yamamoto, M., Araoka, T., Li, Z., Kurita, M., Hishida, T., Li, M., Aizawa, E., Guo, S., et al. (2016) *In vivo* genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* **540**, 144–149
  85. Sakuma, T., Nakade, S., Sakane, Y., Suzuki, K. T., and Yamamoto, T. (2016) MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems. *Nat. Protoc.* **11**, 118–133



86. Nishimasu, H., Ran, F. A., Hsu, P. D., Konermann, S., Shehata, S. I., Dohmae, N., Ishitani, R., Zhang, F., and Nureki, O. (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949
87. Chen, X., Janssen, J. M., Liu, J., Maggio, I., t Jong, A. E. J., Mikkers, H. M. M., and Goncalves, M. (2017) In trans paired nicking triggers seamless genome editing without double-stranded DNA cutting. *Nat. Commun.* **8**, 657
88. Koch, B., Nijmeijer, B., Kueblbeck, M., Cai, Y., Walther, N., and Ellenberg, J. (2018) Generation and validation of homozygous fluorescent knock-in cells using CRISPR-Cas9 genome editing. *Nat. Protoc.* **13**, 1465–1487
89. Goncalves, M. A., van Nierop, G. P., Holkers, M., and de Vries, A. A. (2012) Concerted nicking of donor and chromosomal acceptor DNA promotes homology-directed gene targeting in human cells. *Nucleic Acids Res.* **40**, 3443–3455
90. Papapetrou, E. P., and Schambach, A. (2016) Gene insertion into genomic safe harbors for human gene therapy. *Mol. Ther.* **24**, 678–684
91. Hintze, J., Ye, Z., Narimatsu, Y., Madsen, T. D., Joshi, H. J., Goth, C. K., Linstedt, A., Bachert, C., Mandel, U., Bennett, E. P., Vakhrushev, S. Y., and Schjoldager, K. T. (2018) Probing the contribution of individual polypeptide GalNAc-transferase isoforms to the O-glycoproteome by inducible expression in isogenic cell lines. *J. Biol. Chem.* **293**, 19064–19077
92. Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S., and Qi, L. S. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442–451
93. Matharu, N., Rattanasopha, S., Tamura, S., Maliskova, L., Wang, Y., Bernard, A., Hardin, A., Eckalbar, W. L., Vaisse, C., and Ahituv, N. (2019) CRISPR-mediated activation of a promoter or enhancer rescues obesity caused by haploinsufficiency. *Science* **363**, eaau0629
94. Blanas, A., Cornelissen, L. A. M., Kotsias, M., van der Horst, J. C., van de Vrugt, H. J., Kalay, H., Spencer, D. I. R., Kozak, R. P., and van Vliet, S. J. (2019) Transcriptional activation of fucosyltransferase (FUT) genes using the CRISPR-dCas9-VPR technology reveals potent N-glycome alterations in colorectal cancer cells. *Glycobiology* **29**, 137–150
95. Molla, K. A., and Yang, Y. (2019) CRISPR/Cas-mediated base editing: Technical considerations and practical applications. *Trends Biotechnol.* **37**, 1121–1142
96. Knott, G. J., and Doudna, J. A. (2018) CRISPR-Cas guides the future of genetic engineering. *Science* **361**, 866–869
97. Katayama, K., Mitsunobu, H., and Nishida, K. (2019) Mammalian synthetic biology by CRISPRs engineering and applications. *Curr. Opin. Chem. Biol.* **52**, 79–84
98. Schmid-Burgk, J. L., Honing, K., Ebert, T. S., and Hornung, V. (2016) CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nat. Commun.* **7**, 12338
99. van Haasteren, J., Li, J., Scheideler, O. J., Murthy, N., and Schaffer, D. V. (2020) The delivery challenge: Fulfilling the promise of therapeutic genome editing. *Nat. Biotechnol.* **38**, 845–855
100. Lino, C. A., Harper, J. C., Carney, J. P., and Timlin, J. A. (2018) Delivering CRISPR: A review of the challenges and approaches. *Drug Deliv.* **25**, 1234–1257
101. Oh, S., and Kessler, J. A. (2018) Design, assembly, production, and transfection of synthetic modified mRNA. *Methods* **133**, 29–43
102. Dever, D. P., Bak, R. O., Reinisch, A., Camarena, J., Washington, G., Nicolas, C. E., Pavel-Dinu, M., Saxena, N., Wilkens, A. B., Mantri, S., Uchida, N., Hendel, A., Narla, A., Majeti, R., Weinberg, K. I., et al. (2016) CRISPR/Cas9 beta-globin gene targeting in human haematopoietic stem cells. *Nature* **539**, 384–389
103. Dabelsteen, S., Pallesen, E. M. H., Marinova, I. N., Nielsen, M. I., Adamopoulou, M., Romer, T. B., Levann, A., Andersen, M. M., Ye, Z., Thein, D., Bennett, E. P., Bull, C., Moons, S. J., Boltje, T., Clausen, H., et al. (2020) Essential functions of glycans in human epithelia dissected by a CRISPR-cas9-engineered human organotypic skin model. *Dev. Cell* **54**, 669–684.e667
104. Kelkar, A., Zhu, Y., Groth, T., Stolfá, G., Stablewski, A. B., Singhi, N., Nemeth, M., and Neelamegham, S. (2020) Doxycycline-dependent self-inactivation of CRISPR-cas9 to temporally regulate on- and off-target editing. *Mol. Ther.* **28**, 29–41
105. Ren, C., Xu, K., Segal, D. J., and Zhang, Z. (2019) Strategies for the enrichment and selection of genetically modified cells. *Trends Biotechnol.* **37**, 56–71
106. Esko, J. D., and Stanley, P. (2015) Glycosylation mutants of cultured mammalian cells. In: Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 627–637
107. Jae, L. T., Raaben, M., Riemersma, M., van Beusekom, E., Blomen, V. A., Velds, A., Kerkhoven, R. M., Carette, J. E., Topaloglu, H., Meinecke, P., Wessels, M. W., Lefeber, D. J., Whelan, S. P., van Bokhoven, H., and Brummelkamp, T. R. (2013) Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. *Science* **340**, 479–483
108. Kampmann, M. (2020) CRISPR-based functional genomics for neurological disease. *Nat. Rev. Neurol.* **16**, 465–480
109. Weber, J., Braun, C. J., Saur, D., and Rad, R. (2020) *In vivo* functional screening for systems-level integrative cancer genomics. *Nat. Rev. Cancer* **20**, 573–593
110. Han, J., Perez, J. T., Chen, C., Li, Y., Benitez, A., Kandasamy, M., Lee, Y., Andrade, J., ten Oever, B., and Manicassamy, B. (2018) Genome-wide CRISPR/Cas9 screen identifies host factors essential for influenza virus replication. *Cell Rep.* **23**, 596–607
111. Das, A., Barrientos, R., Shiota, T., Madigan, V., Misumi, I., McKnight, K. L., Sun, L., Li, Z., Meganck, R. M., Li, Y., Kaluzna, E., Asokan, A., Whitmire, J. K., Kapustina, M., Zhang, Q., et al. (2020) Gangliosides are essential endosomal receptors for quasi-enveloped and naked hepatitis A virus. *Nat. Microbiol.* **5**, 1069–1078
112. Lin, D. L., Cherepanova, N. A., Bozzacco, L., MacDonald, M. R., Gilmore, R., and Tai, A. W. (2017) Dengue virus hijacks a noncanonical oxidoreductase function of a cellular oligosaccharyltransferase complex. *mBio* **8**, e00939-17
113. Marceau, C. D., Puschnik, A. S., Majzoub, K., Ooi, Y. S., Brewer, S. M., Fuchs, G., Swaminathan, K., Mata, M. A., Elias, J. E., Sarnow, P., and Carette, J. E. (2016) Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. *Nature* **535**, 159–163
114. Ooi, Y. S., Majzoub, K., Flynn, R. A., Mata, M. A., Diep, J., Li, J. K., van Buuren, N., Rumachik, N., Johnson, A. G., Puschnik, A. S., Marceau, C. D., Mlera, L., Grabowski, J. M., Kirkegaard, K., Bloom, M. E., et al. (2019) An RNA-centric dissection of host complexes controlling flavivirus infection. *Nat. Microbiol.* **4**, 2369–2382
115. Labeau, A., Simon-Loriere, E., Hafirassou, M. L., Bonnet-Madin, L., Tessier, S., Zamborlini, A., Dupre, T., Seta, N., Schwartz, O., Chaix, M. L., Delaugerre, C., Amara, A., and Meertens, L. (2020) A genome-wide CRISPR-cas9 screen identifies the dolichol-phosphate mannose synthase complex as a host dependency factor for dengue virus infection. *J. Virol.* **94**, e01751-19
116. Heaton, B. E., Kennedy, E. M., Dumm, R. E., Harding, A. T., Sacco, M. T., Sachs, D., and Heaton, N. S. (2017) A CRISPR activation screen identifies a pan-avian influenza virus inhibitory host factor. *Cell Rep.* **20**, 1503–1512
117. Henriessat, B., Surolia, A., and Stanley, P. (2015) A genomic view of glycobiology. In: Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 89–97
118. Joshi, H. J., Hansen, L., Narimatsu, Y., Freeze, H. H., Henriessat, B., Bennett, E., Wandall, H. H., Clausen, H., and Schjoldager, K. T. (2018) Glycosyltransferase genes that cause monogenic congenital disorders of glycosylation are distinct from glycosyltransferase genes associated with complex diseases. *Glycobiology* **28**, 284–294

119. Narimatsu, Y., Joshi, H. J., Schjoldager, K. T., Hintze, J., Halim, A., Steentoft, C., Nason, R., Mandel, U., Bennett, E. P., Clausen, H., and Vakhrushev, S. Y. (2019) Exploring regulation of protein O-glycosylation in isogenic human HEK293 cells by differential O-glycoproteomics. *Mol. Cell. Proteomics* **18**, 1396–1409
120. Chen, Y. H., Narimatsu, Y., Clausen, T. M., Gomes, C., Karlsson, R., Steentoft, C., Sliid, C. B., Gustavsson, T., Salanti, A., Persson, A., Malmstrom, A., Willen, D., Ellervik, U., Bennett, E. P., Mao, Y., et al. (2018) The GAGOME: A cell-based library of displayed glycosaminoglycans. *Nat. Methods* **15**, 881–888
121. Tian, W., Ye, Z., Wang, S., Schulz, M. A., Van Coillie, J., Sun, L., Chen, Y. H., Narimatsu, Y., Hansen, L., Kristensen, C., Mandel, U., Bennett, E. P., Jabbarzadeh-Tabrizi, S., Schiffmann, R., Shen, J. S., et al. (2019) The glycosylation design space for recombinant lysosomal replacement enzymes produced in CHO cells. *Nat. Commun.* **10**, 1785
122. Stopschinski, B. E., Holmes, B. B., Miller, G. M., Manon, V. A., Vaquer-Alicea, J., Prueitt, W. L., Hsieh-Wilson, L. C., and Diamond, M. I. (2018) Specific glycosaminoglycan chain length and sulfation patterns are required for cell uptake of tau versus alpha-synuclein and beta-amyloid aggregates. *J. Biol. Chem.* **293**, 10826–10840
123. Qiu, H., Shi, S., Yue, J., Xin, M., Nairn, A. V., Lin, L., Liu, X., Li, G., Archer-Hartmann, S. A., Dela Rosa, M., Galizzi, M., Wang, S., Zhang, F., Azadi, P., van Kuppevelt, T. H., et al. (2018) A mutant-cell library for systematic analysis of heparan sulfate structure-function relationships. *Nat. Methods* **15**, 889–899
124. Monti, E., and Miyagi, T. (2015) Structure and function of mammalian sialidases. *Top. Curr. Chem.* **366**, 183–208
125. Wang, X., Inoue, S., Gu, J., Miyoshi, E., Noda, K., Li, W., Mizuno-Horikawa, Y., Nakano, M., Asahi, M., Takahashi, M., Uozumi, N., Ihara, S., Lee, S. H., Ikeda, Y., Yamaguchi, Y., et al. (2005) Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 15791–15796
126. Stolfa, G., Mondal, N., Zhu, Y., Yu, X., Buffone, A., Jr., and Neelamegham, S. (2016) Using CRISPR-Cas9 to quantify the contributions of O-glycans, N-glycans and glycosphingolipids to human leukocyte-endothelium adhesion. *Sci. Rep.* **6**, 30392
127. Steentoft, C., Vakhrushev, S. Y., Vester-Christensen, M. B., Schjoldager, K. T., Kong, Y., Bennett, E. P., Mandel, U., Wandall, H., Lavery, S. B., and Clausen, H. (2011) Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines. *Nat. Methods* **8**, 977–982
128. Byrne, G., O'Rourke, S. M., Alexander, D. L., Yu, B., Doran, R. C., Wright, M., Chen, Q., Azadi, P., and Berman, P. W. (2018) CRISPR/Cas9 gene editing for the creation of an MGAT1-deficient CHO cell line to control HIV-1 vaccine glycosylation. *PLoS Biol.* **16**, e2005817
129. Stewart, S. E., Menzies, S. A., Popa, S. J., Savinykh, N., Petrunkina Harrison, A., Lehner, P. J., and Moreau, K. (2017) A genome-wide CRISPR screen reconciles the role of N-linked glycosylation in galectin-3 transport to the cell surface. *J. Cell Sci.* **130**, 3234–3247
130. Larsen, I. S. B., Narimatsu, Y., Joshi, H. J., Yang, Z., Harrison, O. J., Brasch, J., Shapiro, L., Honig, B., Vakhrushev, S. Y., Clausen, H., and Halim, A. (2017) Mammalian O-mannosylation of cadherins and plexins is independent of protein O-mannosyltransferases 1 and 2. *J. Biol. Chem.* **292**, 11586–11598
131. Larsen, I. S. B., Narimatsu, Y., Joshi, H. J., Siukstaite, L., Harrison, O. J., Brasch, J., Goodman, K. M., Hansen, L., Shapiro, L., Honig, B., Vakhrushev, S. Y., Clausen, H., and Halim, A. (2017) Discovery of an O-mannosylation pathway selectively serving cadherins and proto-cadherins. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 11163–11168
132. Hirata, T., Mishra, S. K., Nakamura, S., Saito, K., Motooka, D., Takada, Y., Kanzawa, N., Murakami, Y., Maeda, Y., Fujita, M., Yamaguchi, Y., and Kinoshita, T. (2018) Identification of a Golgi GPI-N-acetylgalactosamine transferase with tandem transmembrane regions in the catalytic domain. *Nat. Commun.* **9**, 405
133. Wang, Y., Maeda, Y., Liu, Y. S., Takada, Y., Ninomiya, A., Hirata, T., Fujita, M., Murakami, Y., and Kinoshita, T. (2020) Cross-talks of glycosylphosphatidylinositol biosynthesis with glycosphingolipid biosynthesis and ER-associated degradation. *Nat. Commun.* **11**, 860
134. Amado, M., Almeida, R., Carneiro, F., Lavery, S. B., Holmes, E. H., Nomoto, M., Hollingsworth, M. A., Hassan, H., Schwientek, T., Nielsen, P. A., Bennett, E. P., and Clausen, H. (1998) A family of human beta3-galactosyltransferases. Characterization of four members of a UDP-galactose:beta-N-acetyl-glucosamine/beta-nacetyl-galactosamine beta-1,3-galactosyltransferase family. *J. Biol. Chem.* **273**, 12770–12778
135. Miyazaki, H., Fukumoto, S., Okada, M., Hasegawa, T., and Furukawa, K. (1997) Expression cloning of rat cDNA encoding UDP-galactose:GD2 beta1,3-galactosyltransferase that determines the expression of GD1b/GM1/GA1. *J. Biol. Chem.* **272**, 24794–24799
136. Many, H., Yamaguchi, Y., Kanagawa, M., Kobayashi, K., Tajiri, M., Akasaka-Many, K., Kawakami, H., Mizuno, M., Wada, Y., Toda, T., and Endo, T. (2016) The muscular dystrophy gene TMEM5 encodes a ribitol beta1,4-xylosyltransferase required for the functional glycosylation of dystroglycan. *J. Biol. Chem.* **291**, 24618–24627
137. Stanley, P., Sudo, T., and Carver, J. P. (1980) Differential involvement of cell surface sialic acid residues in wheat germ agglutinin binding to parental and wheat germ agglutinin-resistant Chinese hamster ovary cells. *J. Cell Biol.* **85**, 60–69
138. Riemersma, M., Sandrock, J., Boltje, T. J., Bull, C., Heise, T., Ashikov, A., Adema, G. J., van Bokhoven, H., and Lefeber, D. J. (2015) Disease mutations in CMP-sialic acid transporter SLC35A1 result in abnormal alpha-dystroglycan O-mannosylation, independent from sialic acid. *Hum. Mol. Genet.* **24**, 2241–2246
139. Kingsley, D. M., Kozarsky, K. F., Hobbie, L., and Krieger, M. (1986) Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell* **44**, 749–759
140. Kozarsky, K., Kingsley, D., and Krieger, M. (1988) Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4335–4339
141. Termini, J. M., Silver, Z. A., Connor, B., Antonopoulos, A., Haslam, S. M., Dell, A., and Desrosiers, R. C. (2017) HEK293T cell lines defective for O-linked glycosylation. *PLoS One* **12**, e0179949
142. Boyce, M., Carrico, I. S., Ganguli, A. S., Yu, S. H., Hangauer, M. J., Hubbard, S. C., Kohler, J. J., and Bertozzi, C. R. (2011) Metabolic cross-talk allows labeling of O-linked beta-N-acetylglucosamine-modified proteins via the N-acetylgalactosamine salvage pathway. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 3141–3146
143. Gerin, I., Ury, B., Breloy, I., Bouchet-Seraphin, C., Bolsee, J., Halbout, M., Graff, J., Vertommen, D., Muccioli, G. G., Seta, N., Cuisset, J. M., Dabaj, I., Quijano-Roy, S., Grahn, A., Van Schaftingen, E., et al. (2016) ISPD produces CDP-ribitol used by FKTN and FKRP to transfer ribitol phosphate onto alpha-dystroglycan. *Nat. Commun.* **7**, 11534
144. Riemersma, M., Froese, D. S., van Tol, W., Engelke, U. F., Kopec, J., van Scherpenzeel, M., Ashikov, A., Krojer, T., von Delft, F., Tessari, M., Buczkowska, A., Swiezewska, E., Jae, L. T., Brummelkamp, T. R., Many, H., et al. (2015) Human ISPD is a cytidyltransferase required for dystroglycan O-mannosylation. *Chem. Biol.* **22**, 1643–1652
145. Schjoldager, K. T., Joshi, H. J., Kong, Y., Goth, C. K., King, S. L., Wandall, H. H., Bennett, E. P., Vakhrushev, S. Y., and Clausen, H. (2015) Deconstruction of O-glycosylation-GalNAc-T isoforms direct distinct subsets of the O-glycoproteome. *EMBO Rep.* **16**, 1713–1722
146. Schjoldager, K. T., Vakhrushev, S. Y., Kong, Y., Steentoft, C., Nudelman, A. S., Pedersen, N. B., Wandall, H. H., Mandel, U., Bennett, E. P., Lavery, S. B., and Clausen, H. (2012) Probing isoform-specific functions of polypeptide GalNAc-transferases using zinc finger nuclease glycoengineered SimpleCells. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 9893–9898
147. Bagdonaitė, I., Pallesen, E. M., Ye, Z., Vakhrushev, S. Y., Marinova, I. N., Nielsen, M. I., Kramer, S. H., Pedersen, S. F., Joshi, H. J., Bennett, E. P., Dabelsteen, S., and Wandall, H. H. (2020) O-glycan initiation directs distinct biological pathways and controls epithelial differentiation. *EMBO Rep.* **21**, e48885
148. Kong, Y., Joshi, H. J., Schjoldager, K. T., Madsen, T. D., Gerken, T. A., Vester-Christensen, M. B., Wandall, H. H., Bennett, E. P., Lavery, S. B., Vakhrushev, S. Y., and Clausen, H. (2015) Probing polypeptide GalNAc-

- transferase isoform substrate specificities by *in vitro* analysis. *Glycobiology* **25**, 55–65
149. Wang, S., Mao, Y., Narimatsu, Y., Ye, Z., Tian, W., Goth, C. K., Lira-Navarrete, E., Pedersen, N. B., Benito-Vicente, A., Martin, C., Uribe, K. B., Hurtado-Guerrero, R., Christoffersen, C., Seidah, N. G., Nielsen, R., *et al.* (2018) Site-specific O-glycosylation of members of the low-density lipoprotein receptor superfamily enhances ligand interactions. *J. Biol. Chem.* **293**, 7408–7422
  150. Tian, E., Wang, S., Zhang, L., Zhang, Y., Malicdan, M. C., Mao, Y., Christoffersen, C., Tabak, L. A., Schjoldager, K. T., and Ten Hagen, K. G. (2019) Galnt11 regulates kidney function by glycosylating the endocytosis receptor megalin to modulate ligand binding. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 25196–25202
  151. Simon, E. J., and Linstedt, A. D. (2018) Site-specific glycosylation of Ebola virus glycoprotein by human polypeptide GalNAc-transferase 1 induces cell adhesion defects. *J. Biol. Chem.* **293**, 19866–19873
  152. Lavrsen, K., Dabelsteen, S., Vakhrushev, S. Y., Levann, A. M. R., Haue, A. D., Dylander, A., Mandel, U., Hansen, L., Frodin, M., Bennett, E. P., and Wandall, H. H. (2018) De novo expression of human polypeptide N-acetylgalactosaminyltransferase 6 (GalNAc-T6) in colon adenocarcinoma inhibits the differentiation of colonic epithelium. *J. Biol. Chem.* **293**, 1298–1314
  153. Khetarpal, S. A., Schjoldager, K. T., Christoffersen, C., Raghavan, A., Edmondson, A. C., Reutter, H. M., Ahmed, B., Ouazzani, R., Peloso, G. M., Vitali, C., Zhao, W., Somasundara, A. V., Millar, J. S., Park, Y., Fernando, G., *et al.* (2016) Loss of function of GALNT2 lowers high-density lipoproteins in humans, nonhuman primates, and rodents. *Cell Metab.* **24**, 234–245
  154. Lackman, J. J., Goth, C. K., Halim, A., Vakhrushev, S. Y., Clausen, H., and Petaja-Repo, U. E. (2018) Site-specific O-glycosylation of N-terminal serine residues by polypeptide GalNAc-transferase 2 modulates human delta-opioid receptor turnover at the plasma membrane. *Cell Signal.* **42**, 184–193
  155. Goth, C. K., Halim, A., Khetarpal, S. A., Rader, D. J., Clausen, H., and Schjoldager, K. T. (2015) A systematic study of modulation of ADAM-mediated ectodomain shedding by site-specific O-glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 14623–14628
  156. Goth, C. K., Tuhkanen, H. E., Khan, H., Lackman, J. J., Wang, S., Narimatsu, Y., Hansen, L. H., Overall, C. M., Clausen, H., Schjoldager, K. T., and Petaja-Repo, U. E. (2017) Site-specific O-glycosylation by polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-transferase T2) co-regulates beta1-adrenergic receptor N-terminal cleavage. *J. Biol. Chem.* **292**, 4714–4726
  157. de Las Rivas, M., Paul Daniel, E. J., Narimatsu, Y., Companon, I., Kato, K., Hermosilla, P., Thureau, A., Ceballos-Laita, L., Coelho, H., Bernado, P., Marcelo, F., Hansen, L., Maeda, R., Lostao, A., Corzana, F., *et al.* (2020) Molecular basis for fibroblast growth factor 23 O-glycosylation by GalNAc-T3. *Nat. Chem. Biol.* **16**, 351–360
  158. Xu, Y., Pang, W., Lu, J., Shan, A., and Zhang, Y. (2016) Polypeptide N-acetylgalactosaminyltransferase 13 contributes to neurogenesis via stabilizing the mucin-type O-glycoprotein podoplanin. *J. Biol. Chem.* **291**, 23477–23488
  159. Al Rifai, O., Julien, C., Lacombe, J., Faubert, D., Lira-Navarrete, E., Narimatsu, Y., Clausen, H., and Ferron, M. (2020) The half-life of the bone-derived hormone osteocalcin is regulated through O-glycosylation in mice, but not in humans. *Elife* **9**, e61174
  160. Prescher, J. A., and Bertozzi, C. R. (2006) Chemical technologies for probing glycans. *Cell* **126**, 851–854
  161. Belardi, B., and Bertozzi, C. R. (2015) Chemical lectinology: Tools for probing the ligands and dynamics of mammalian lectins *in vivo*. *Chem. Biol.* **22**, 983–993
  162. Hong, S., Yu, C., Wang, P., Shi, Y., Cao, W., Cheng, B., Chapla, D. G., Ma, Y., Li, J., Rodrigues, E., Narimatsu, Y., Yates, J. R., 3rd, Chen, X., Clausen, H., Moremen, K. W., *et al.* (2020) Glycoengineering of NK cells with glycan ligands of CD22 and selectins for B-cell lymphoma therapy. *Angew. Chem. Int. Ed. Engl.* **60**, 3603–3610
  163. Prescher, J. A., Dube, D. H., and Bertozzi, C. R. (2004) Chemical remodelling of cell surfaces in living animals. *Nature* **430**, 873–877
  164. Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science* **287**, 2007–2010
  165. Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. R. (2008) *In vivo* imaging of membrane-associated glycans in developing zebrafish. *Science* **320**, 664–667
  166. Choi, J., Wagner, L. J. S., Timmermans, S., Malaker, S. A., Schumann, B., Gray, M. A., Debets, M. F., Takashima, M., Gehring, J., and Bertozzi, C. R. (2019) Engineering orthogonal polypeptide GalNAc-transferase and UDP-sugar pairs. *J. Am. Chem. Soc.* **141**, 13442–13453
  167. Schumann, B., Malaker, S. A., Wisnovsky, S. P., Debets, M. F., Agbay, A. J., Fernandez, D., Wagner, L. J. S., Lin, L., Li, Z., Choi, J., Fox, D. M., Peh, J., Gray, M. A., Pedram, K., Kohler, J. J., *et al.* (2020) Bump-and-hole engineering identifies specific substrates of glycosyltransferases in living cells. *Mol. Cell* **78**, 824–834.e815
  168. Debets, M. F., Tastan, O. Y., Wisnovsky, S. P., Malaker, S. A., Angelis, N., Moeckl, L. K. R., Choi, J., Flynn, H., Wagner, L. J. S., Bineva-Todd, G., Antonopoulos, A., Cioce, A., Browne, W. M., Li, Z., Briggs, D. C., *et al.* (2020) Metabolic precision labeling enables selective probing of O-linked N-acetylgalactosamine glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 25293–25301
  169. Cioce, A., Malaker, S. A., and Schumann, B. (2020) Generating orthogonal glycosyltransferase and nucleotide sugar pairs as next-generation glycobiology tools. *Curr. Opin. Chem. Biol.* **60**, 66–78
  170. Cherepanova, N. A., Venev, S. V., Leszyk, J. D., Shaffer, S. A., and Gilmore, R. (2019) Quantitative glycoproteomics reveals new classes of STT3A- and STT3B-dependent N-glycosylation sites. *J. Cell Biol.* **218**, 2782–2796
  171. Cherepanova, N. A., and Gilmore, R. (2016) Mammalian cells lacking either the cotranslational or posttranslational oligosaccharyltransferase complex display substrate-dependent defects in asparagine linked glycosylation. *Sci. Rep.* **6**, 20946
  172. Kanagawa, M., Kobayashi, K., Tajiri, M., Many, H., Kuga, A., Yamaguchi, Y., Akasaka-Many, K., Furukawa, J. I., Mizuno, M., Kawakami, H., Shinohara, Y., Wada, Y., Endo, T., and Toda, T. (2016) Identification of a post-translational modification with ribitol-phosphate and its defect in muscular dystrophy. *Cell Rep.* **14**, 2209–2223
  173. Baumann, S., and Hennet, T. (2016) Collagen accumulation in osteosarcoma cells lacking GLT25D1 collagen galactosyltransferase. *J. Biol. Chem.* **291**, 18514–18524
  174. Hennet, T. (2019) Collagen glycosylation. *Curr. Opin. Struct. Biol.* **56**, 131–138
  175. Takeuchi, H., Schneider, M., Williamson, D. B., Ito, A., Takeuchi, M., Handford, P. A., and Haltiwanger, R. S. (2018) Two novel protein O-glucosyltransferases that modify sites distinct from POGlut1 and affect Notch trafficking and signaling. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E8395–E8402
  176. Shcherbakova, A., Tiemann, B., Buettner, F. F., and Bakker, H. (2017) Distinct C-mannosylation of netrin receptor thrombospondin type 1 repeats by mammalian DPY19L1 and DPY19L3. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 2574–2579
  177. Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M. A., Samyn-Petit, B., Julien, S., and Delannoy, P. (2001) The human sialyltransferase family. *Biochimie* **83**, 727–737
  178. de Las Rivas, M., Lira-Navarrete, E., Gerken, T. A., and Hurtado-Guerrero, R. (2019) Polypeptide GalNAc-Ts: From redundancy to specificity. *Curr. Opin. Struct. Biol.* **56**, 87–96
  179. de Vries, T., Knegtel, R. M., Holmes, E. H., and Macher, B. A. (2001) Fucosyltransferases: Structure/function studies. *Glycobiology* **11**, 119R–128R
  180. Paulson, J. C., and Rademacher, C. (2009) Glycan terminator. *Nat. Struct. Mol. Biol.* **16**, 1121–1122
  181. Becker, D. J., and Lowe, J. B. (2003) Fucose: Biosynthesis and biological function in mammals. *Glycobiology* **13**, 41R–53R
  182. Tsuji, S. (1996) Molecular cloning and functional analysis of sialyltransferases. *J. Biochem.* **120**, 1–13
  183. Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L., and Breton, C. (1999) Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. *Glycobiology* **9**, 323–334



184. Togayachi, A., Sato, T., and Narimatsu, H. (2006) Comprehensive enzymatic characterization of glycosyltransferases with a beta3GT or beta4GT motif. *Methods Enzymol.* **416**, 91–102
185. Bydlinski, N., Maresch, D., Schmieder, V., Klanert, G., Strasser, R., and Borth, N. (2018) The contributions of individual galactosyltransferases to protein specific N-glycan processing in Chinese Hamster ovary cells. *J. Biotechnol.* **282**, 101–110
186. Mondal, N., Buffone, A., Jr., Stofa, G., Antonopoulos, A., Lau, J. T., Haslam, S. M., Dell, A., and Neelamegham, S. (2015) ST3Gal-4 is the primary sialyltransferase regulating the synthesis of E-, P-, and L-selectin ligands on human myeloid leukocytes. *Blood* **125**, 687–696
187. Baggen, J., Thibaut, H. J., Staring, J., Jae, L. T., Liu, Y., Guo, H., Slager, J. J., de Bruin, J. W., van Vliet, A. L., Blomen, V. A., Overduin, P., Sheng, J., de Haan, C. A., de Vries, E., Meijer, A., et al. (2016) Enterovirus D68 receptor requirements unveiled by haploid genetics. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 1399–1404
188. Haga, K., Ettayebi, K., Tenge, V. R., Karandikar, U. C., Lewis, M. A., Lin, S. C., Neill, F. H., Ayyar, B. V., Zeng, X. L., Larson, G., Ramani, S., Atmar, R. L., and Estes, M. K. (2020) Genetic manipulation of human intestinal enteroids demonstrates the necessity of a functional fucosyltransferase 2 gene for secretor-dependent human norovirus infection. *mBio* **11**, e00251-20
189. Gao, C., Wei, M., McKittrick, T. R., McQuillan, A. M., Heimburg-Molinaro, J., and Cummings, R. D. (2019) Glycan microarrays as chemical tools for identifying glycan recognition by immune proteins. *Front. Chem.* **7**, 833
190. Rillahan, C. D., and Paulson, J. C. (2011) Glycan microarrays for decoding the glycome. *Annu. Rev. Biochem.* **80**, 797–823
191. Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M. E., Alvarez, R., Bryan, M. C., Fazio, F., Calarese, D., Stevens, J., Razi, N., Stevens, D. J., Skehel, J. J., van Die, I., Burton, D. R., et al. (2004) Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 17033–17038
192. Geissner, A., and Seeberger, P. H. (2016) Glycan arrays: From basic biochemical research to bioanalytical and biomedical applications. *Annu. Rev. Anal. Chem.* **9**, 223–247
193. Stevens, J., Blixt, O., Paulson, J. C., and Wilson, I. A. (2006) Glycan microarray technologies: Tools to survey host specificity of influenza viruses. *Nat. Rev. Microbiol.* **4**, 857–864
194. Paulson, J. C., Blixt, O., and Collins, B. E. (2006) Sweet spots in functional glycomics. *Nat. Chem. Biol.* **2**, 238–248
195. Blixt, O., Clo, E., Nudelman, A. S., Sorensen, K. K., Clausen, T., Wandall, H. H., Livingston, P. O., Clausen, H., and Jensen, K. J. (2010) A high-throughput O-glycopeptide discovery platform for seromic profiling. *J. Proteome Res.* **9**, 5250–5261
196. Mehta, A. Y., Veeraiyah, R. K. H., Dutta, S., Goth, C. K., Hanes, M. S., Gao, C., Stavenhagen, K., Kardish, R., Matsumoto, Y., Heimburg-Molinaro, J., Boyce, M., Pohl, N. L. B., and Cummings, R. D. (2020) Parallel glyco-SPOT synthesis of glycopeptide libraries. *Cell Chem. Biol.* **27**, 1207–1219 e1209
197. Mehta, A. Y., Heimburg-Molinaro, J., Cummings, R. D., and Goth, C. K. (2020) Emerging patterns of tyrosine sulfation and O-glycosylation cross-talk and co-localization. *Curr. Opin. Struct. Biol.* **62**, 102–111
198. Li, Z., and Feizi, T. (2018) The neoglycolipid (NGL) technology-based microarrays and future prospects. *FEBS Lett.* **592**, 3976–3991
199. Bull, C., Joshi, H. J., Clausen, H., and Narimatsu, Y. (2020) Cell-based glycan arrays—a practical guide to dissect the human glycome. *STAR Protoc.* **1**, 100017
200. Nielsen, M. I., Stegmayr, J., Grant, O. C., Yang, Z., Nilsson, U. J., Boos, I., Carlsson, M. C., Woods, R. J., Unverzagt, C., Leffler, H., and Wandall, H. H. (2018) Galectin binding to cells and glycoproteins with genetically modified glycosylation reveals galectin-glycan specificities in a natural context. *J. Biol. Chem.* **293**, 20249–20262
201. Cohen, M., and Varki, A. (2014) Modulation of glycan recognition by clustered saccharide patches. *Int. Rev. Cell Mol. Biol.* **308**, 75–125
202. Varki, A. (1994) Selectin ligands. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7390–7397
203. Flores, A., Ramirez-Franco, J., Desplantes, R., Debreux, K., Ferracci, G., Wernert, F., Blanchard, M. P., Maulet, Y., Youssef, F., Sangiardi, M., Iborra, C., Popoff, M. R., Seagar, M., Fantini, J., Leveque, C., et al. (2019) Gangliosides interact with synaptotagmin to form the high-affinity receptor complex for botulinum neurotoxin B. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 18098–18108
204. Kim, S. Y., Jin, W., Sood, A., Montgomery, D. W., Grant, O. C., Fuster, M. M., Fu, L., Dordick, J. S., Woods, R. J., Zhang, F., and Linhardt, R. J. (2020) Characterization of heparin and severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) spike glycoprotein binding interactions. *Antiviral Res.* **181**, 104873
205. Clausen, T. M., Sandoval, D. R., Spliid, C. B., Pihl, J., Perrett, H. R., Painter, C. D., Narayanan, A., Majowicz, S. A., Kwong, E. M., McVicar, R. N., Thacker, B. E., Glass, C. A., Yang, Z., Torres, J. L., Golden, G. J., et al. (2020) SARS-CoV-2 infection depends on cellular heparan sulfate and ACE2. *Cell* **183**, 1043–1057.e1015
206. Radhakrishnan, P., Dabelsteen, S., Madsen, F. B., Francavilla, C., Kopp, K. L., Steentoft, C., Vakhrushev, S. Y., Olsen, J. V., Hansen, L., Bennett, E. P., Woetmann, A., Yin, G., Chen, L., Song, H., Bak, M., et al. (2014) Immature truncated O-glycophenotype of cancer directly induces oncogenic features. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E4066–4075
207. Kaji, H., Yamauchi, Y., Takahashi, N., and Isobe, T. (2006) Mass spectrometric identification of N-linked glycopeptides using lectin-mediated affinity capture and glycosylation site-specific stable isotope tagging. *Nat. Protoc.* **1**, 3019–3027
208. Zielinska, D. F., Gnad, F., Wisniewski, J. R., and Mann, M. (2010) Precision mapping of an *in vivo* N-glycoproteome reveals rigid topological and sequence constraints. *Cell* **141**, 897–907
209. Darula, Z., and Medzihradsky, K. F. (2009) Affinity enrichment and characterization of mucin core-1 type glycopeptides from bovine serum. *Mol. Cell. Proteomics* **8**, 2515–2526
210. Narimatsu, H., Kaji, H., Vakhrushev, S. Y., Clausen, H., Zhang, H., Noro, E., Togayachi, A., Nagai-Okatani, C., Kuno, A., Zou, X., Cheng, L., Tao, S. C., and Sun, Y. (2018) Current technologies for complex glycoproteomics and their applications to biology/disease-driven glycoproteomics. *J. Proteome Res.* **17**, 4097–4112
211. Hang, H. C., Yu, C., Kato, D. L., and Bertozzi, C. R. (2003) A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 14846–14851
212. Dube, D. H., Prescher, J. A., Quang, C. N., and Bertozzi, C. R. (2006) Probing mucin-type O-linked glycosylation in living animals. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 4819–4824
213. Woo, C. M., Felix, A., Byrd, W. E., Zuegel, D. K., Ishihara, M., Azadi, P., Iavarone, A. T., Pitteri, S. J., and Bertozzi, C. R. (2017) Development of IsoTaG, a chemical glycoproteomics technique for profiling intact N- and O-glycopeptides from whole cell proteomes. *J. Proteome Res.* **16**, 1706–1718
214. Slade, P. G., Hajivandi, M., Bartel, C. M., and Gorfien, S. F. (2012) Identifying the CHO secretome using mucin-type O-linked glycosylation and click-chemistry. *J. Proteome Res.* **11**, 6175–6186
215. Liu, T. W., Kaji, H., Togayachi, A., Ito, H., Sato, T., and Narimatsu, H. (2012) A chemoenzymatic approach toward the identification of fucosylated glycoproteins and mapping of N-glycan sites. *Glycobiology* **22**, 630–637
216. Nilsson, J., Ruetschi, U., Halim, A., Hesse, C., Carlsohn, E., Brinkmalm, G., and Larson, G. (2009) Enrichment of glycopeptides for glycan structure and attachment site identification. *Nat. Methods* **6**, 809–811
217. Nilsson, J., Halim, A., Grahn, A., and Larson, G. (2013) Targeting the glycoproteome. *Glycoconj. J.* **30**, 119–136
218. Levery, S. B., Steentoft, C., Halim, A., Narimatsu, Y., Clausen, H., and Vakhrushev, S. Y. (2015) Advances in mass spectrometry driven O-glycoproteomics. *Biochim. Biophys. Acta* **1850**, 33–42
219. Ju, T., Aryal, R. P., Stowell, C. J., and Cummings, R. D. (2008) Regulation of protein O-glycosylation by the endoplasmic reticulum-localized molecular chaperone Cosmc. *J. Cell Biol.* **182**, 531–542
220. Wang, Y., Ju, T., Ding, X., Xia, B., Wang, W., Xia, L., He, M., and Cummings, R. D. (2010) Cosmc is an essential chaperone for correct protein O-glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 9228–9233
221. Steentoft, C., Vakhrushev, S. Y., Joshi, H. J., Kong, Y., Vester-Christensen, M. B., Schjoldager, K. T., Lavrsen, K., Dabelsteen, S., Pedersen, N. B.,

- Marcos-Silva, L., Gupta, R., Bennett, E. P., Mandel, U., Brunak, S., Wandall, H. H., *et al.* (2013) Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *EMBO J.* **32**, 1478–1488
222. Ye, Z., Mao, Y., Clausen, H., and Vakhrushev, S. Y. (2019) Glyco-DIA: A method for quantitative O-glycoproteomics with in silico-boosted glycopeptide libraries. *Nat. Methods* **16**, 902–910
223. Campos, D., Freitas, D., Gomes, J., Magalhaes, A., Steentoft, C., Gomes, C., Vester-Christensen, M. B., Ferreira, J. A., Afonso, L. P., Santos, L. L., Pinto de Sousa, J., Mandel, U., Clausen, H., Vakhrushev, S. Y., and Reis, C. A. (2015) Probing the O-glycoproteome of gastric cancer cell lines for biomarker discovery. *Mol. Cell. Proteomics* **14**, 1616–1629
224. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsunashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., *et al.* (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell* **1**, 717–724
225. Kuwabara, N., Manya, H., Yamada, T., Tateno, H., Kanagawa, M., Kobayashi, K., Akasaka-Manya, K., Hirose, Y., Mizuno, M., Ikeguchi, M., Toda, T., Hirabayashi, J., Senda, T., Endo, T., and Kato, R. (2016) Carbohydrate-binding domain of the POMGnT1 stem region modulates O-mannosylation sites of alpha-dystroglycan. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 9280–9285
226. Vester-Christensen, M. B., Halim, A., Joshi, H. J., Steentoft, C., Bennett, E. P., Levery, S. B., Vakhrushev, S. Y., and Clausen, H. (2013) Mining the O-mannose glycoproteome reveals cadherins as major O-mannosylated glycoproteins. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 21018–21023
227. Khoo, K. H. (2019) Advances toward mapping the full extent of protein site-specific O-GalNAc glycosylation that better reflects underlying glycomic complexity. *Curr. Opin. Struct. Biol.* **56**, 146–154
228. Kellokumpu, S., Hassinen, A., and Glumoff, T. (2016) Glycosyltransferase complexes in eukaryotes: Long-known, prevalent but still unrecognized. *Cell Mol. Life Sci.* **73**, 305–325
229. Yang, W., Ao, M., Hu, Y., Li, Q. K., and Zhang, H. (2018) Mapping the O-glycoproteome using site-specific extraction of O-linked glycopeptides (EXoO). *Mol. Syst. Biol.* **14**, e8486
230. Yang, S., Onigman, P., Wu, W. W., Sjogren, J., Nyhlen, H., Shen, R. F., and Cipollo, J. (2018) Deciphering protein O-glycosylation: Solid-phase chemoenzymatic cleavage and enrichment. *Anal. Chem.* **90**, 8261–8269
231. Takatsuki, A., Arima, K., and Tamura, G. (1971) Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. *J. Antibiot. (Tokyo)* **24**, 215–223
232. Lopez-Sambrooks, C., Shrimal, S., Khodier, C., Flaherty, D. P., Rinis, N., Charest, J. C., Gao, N., Zhao, P., Wells, L., Lewis, T. A., Lehrman, M. A., Gilmore, R., Golden, J. E., and Contessa, J. N. (2016) Oligosaccharyltransferase inhibition induces senescence in RTK-driven tumor cells. *Nat. Chem. Biol.* **12**, 1023–1030
233. Hang, H. C., Yu, C., Ten Hagen, K. G., Tian, E., Winans, K. A., Tabak, L. A., and Bertozzi, C. R. (2004) Small molecule inhibitors of mucin-type O-linked glycosylation from a uridine-based library. *Chem. Biol.* **11**, 337–345
234. Agarwal, K., Kaul, R., Garg, M., Shajahan, A., Jha, S. K., and Sampathkumar, S. G. (2013) Inhibition of mucin-type O-glycosylation through metabolic processing and incorporation of N-thioglycolyl-D-galactosamine peracetate (Ac5GalNTGc). *J. Am. Chem. Soc.* **135**, 14189–14197
235. Rillahan, C. D., Antonopoulos, A., Lefort, C. T., Sonon, R., Azadi, P., Ley, K., Dell, A., Haslam, S. M., and Paulson, J. C. (2012) Global metabolic inhibitors of sialyl- and fucosyltransferases remodel the glycome. *Nat. Chem. Biol.* **8**, 661–668
236. Heise, T., Pijnenborg, J. F. A., Bull, C., van Hilten, N., Kers-Rebel, E. D., Balneger, N., Elferink, H., Adema, G. J., and Boltje, T. J. (2019) Potent metabolic sialylation inhibitors based on C-5-modified fluorinated sialic acids. *J. Med. Chem.* **62**, 1014–1021
237. van Wijk, X. M., Thijssen, V. L., Lawrence, R., van den Broek, S. A., Dona, M., Naidu, N., Oosterhof, A., van de Westerlo, E. M., Kusters, L. J., Khaled, Y., Jokela, T. A., Nowak-Sliwinska, P., Kremer, H., Stringer, S. E., Griffioen, A. W., *et al.* (2013) Interfering with UDP-GlcNAc metabolism and heparan sulfate expression using a sugar analogue reduces angiogenesis. *ACS Chem. Biol.* **8**, 2331–2338
238. Tian, E., Ten Hagen, K. G., Shum, L., Hang, H. C., Imbert, Y., Young, W. W., Jr., Bertozzi, C. R., and Tabak, L. A. (2008) An inhibitor of O-glycosylation induces apoptosis in NIH3T3 cells and developing mouse embryonic mandibular tissues. *J. Biol. Chem.* **283**, 4460
239. Rillahan, C. D., Brown, S. J., Register, A. C., Rosen, H., and Paulson, J. C. (2011) High-throughput screening for inhibitors of sialyl- and fucosyltransferases. *Angew. Chem. Int. Ed. Engl.* **50**, 12534–12537
240. Song, L., Bachert, C., Schjoldager, K. T., Clausen, H., and Linstedt, A. D. (2014) Development of isoform-specific sensors of polypeptide GalNAc-transferase activity. *J. Biol. Chem.* **289**, 30556–30566
241. Song, L., and Linstedt, A. D. (2017) Inhibitor of ppGalNAc-T3-mediated O-glycosylation blocks cancer cell invasiveness and lowers FGF23 levels. *Elife* **6**, e24051
242. Rinis, N., Golden, J. E., Marceau, C. D., Carette, J. E., Van Zandt, M. C., Gilmore, R., and Contessa, J. N. (2018) Editing N-glycan site occupancy with small-molecule oligosaccharyltransferase inhibitors. *Cell Chem. Biol.* **25**, 1231–1241.e1234
243. Walsh, G. (2010) Post-translational modifications of protein biopharmaceuticals. *Drug Discov. Today* **15**, 773–780
244. Walsh, G. (2018) Biopharmaceutical benchmarks 2018. *Nat. Biotechnol.* **36**, 1136–1145
245. Carillo, S., Mittermayr, S., Farrell, A., Albrecht, S., and Bones, J. (2017) Glycosylation analysis of therapeutic glycoproteins produced in CHO cells. *Methods Mol. Biol.* **1603**, 227–241
246. Wildt, S., and Gerngross, T. U. (2005) The humanization of N-glycosylation pathways in yeast. *Nat. Rev. Microbiol.* **3**, 119–128
247. Geisler, C., Mabashi-Asazuma, H., and Jarvis, D. L. (2015) An overview and history of glyco-engineering in insect expression systems. *Methods Mol. Biol.* **1321**, 131–152
248. Strasser, R., Altmann, F., and Steinkellner, H. (2014) Controlled glycosylation of plant-produced recombinant proteins. *Curr. Opin. Biotechnol.* **30**, 95–100
249. Lalonde, M. E., and Durocher, Y. (2017) Therapeutic glycoprotein production in mammalian cells. *J. Biotechnol.* **251**, 128–140
250. van Beers, M. M., and Bardor, M. (2012) Minimizing immunogenicity of biopharmaceuticals by controlling critical quality attributes of proteins. *Biotechnol. J.* **7**, 1473–1484
251. Bork, K., Horstkorte, R., and Weidemann, W. (2009) Increasing the sialylation of therapeutic glycoproteins: The potential of the sialic acid biosynthetic pathway. *J. Pharm. Sci.* **98**, 3499–3508
252. Bas, M., Terrier, A., Jacque, E., Dehenne, A., Pochet-Beghin, V., Beghin, C., Dezetter, A. S., Dupont, G., Engrand, A., Beaufile, B., Mondon, P., Fournier, N., de Romeuf, C., Jorieux, S., Fontayne, A., *et al.* (2019) Fc sialylation prolongs serum half-life of therapeutic antibodies. *J. Immunol.* **202**, 1582–1594
253. Pereira, N. A., Chan, K. F., Lin, P. C., and Song, Z. (2018) The “less-is-more” in therapeutic antibodies: Afucosylated anti-cancer antibodies with enhanced antibody-dependent cellular cytotoxicity. *MAbs* **10**, 693–711
254. DeFrees, S., Wang, Z. G., Xing, R., Scott, A. E., Wang, J., Zopf, D., Gouty, D. L., Sjoberg, E. R., Panneerselvam, K., Brinkman-Van der Linden, E. C., Bayer, R. J., Tarp, M. A., and Clausen, H. (2006) Glyco-PEGylation of recombinant therapeutic proteins produced in *Escherichia coli*. *Glycobiology* **16**, 833–843
255. Mathiesen, C. B. K., Carlsson, M. C., Brand, S., Moller, S. R., Idorn, M., Thor Straten, P., Pedersen, A. E., Dabelsteen, S., Halim, A., Wurtzen, P. A., Brimnes, J., Ipsen, H., Petersen, B. L., and Wandall, H. H. (2018) Genetically engineered cell factories produce glycoengineered vaccines that target antigen-presenting cells and reduce antigen-specific T-cell reactivity. *J. Allergy Clin. Immunol.* **142**, 1983–1987
256. Sales, M. L. M., Kratje, R., Oggero, M., and Ceaglio, N. (2021) Bifunctional GM-CSF-derived peptides as tools for O-glycoengineering and protein tagging. *J. Biotechnol.* **327**, 18–27
257. Buettner, M. J., Shah, S. R., Saeui, C. T., Ariss, R., and Yarema, K. J. (2018) Improving immunotherapy through glycodeign. *Front. Immunol.* **9**, 2485
258. Vidarsson, G., Dekkers, G., and Rispens, T. (2014) IgG subclasses and allotypes: From structure to effector functions. *Front. Immunol.* **5**, 520

259. Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M., and Dwek, R. A. (2007) The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu. Rev. Immunol.* **25**, 21–50
260. Cobb, B. A. (2020) The history of IgG glycosylation and where we are now. *Glycobiology* **30**, 202–213
261. Jennewein, M. F., and Alter, G. (2017) The immunoregulatory roles of antibody glycosylation. *Trends Immunol.* **38**, 358–372
262. Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., and Shitara, K. (2003) The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J. Biol. Chem.* **278**, 3466–3473
263. Shields, R. L., Lai, J., Keck, R., O’Connell, L. Y., Hong, K., Meng, Y. G., Weikert, S. H., and Presta, L. G. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcγ<sub>3</sub>RIII and antibody-dependent cellular toxicity. *J. Biol. Chem.* **277**, 26733–26740
264. Ferrara, C., Grau, S., Jager, C., Sondermann, P., Brunker, P., Waldhauer, I., Hennig, M., Ruf, A., Rufer, A. C., Stihle, M., Umana, P., and Benz, J. (2011) Unique carbohydrate-carbohydrate interactions are required for high affinity binding between Fcγ<sub>3</sub>RIII and antibodies lacking core fucose. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 12669–12674
265. Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., and Bailey, J. E. (1999) Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.* **17**, 176–180
266. Gerdes, C. A., and Umana, P. (2014) GA201: A novel humanized and glycoengineered anti-EGFR antibody–response. *Clin. Cancer Res.* **20**, 1055
267. Oppenheim, D. E., Spreafico, R., Etuk, A., Malone, D., Amofah, E., Pena-Murillo, C., Murray, T., McLaughlin, L., Choi, B. S., Allan, S., Belousov, A., Passiukov, A., Gerdes, C., Umana, P., Farzaneh, F., et al. (2014) Glyco-engineered anti-EGFR mAb elicits ADCC by NK cells from colorectal cancer patients irrespective of chemotherapy. *Br. J. Cancer* **110**, 1221–1227
268. Herter, S., Birk, M. C., Klein, C., Gerdes, C., Umana, P., and Bacac, M. (2014) Glycoengineering of therapeutic antibodies enhances monocyte/macrophage-mediated phagocytosis and cytotoxicity. *J. Immunol.* **192**, 2252–2260
269. Herting, F., Friess, T., Bader, S., Muth, G., Holzlwimmer, G., Rieder, N., Umana, P., and Klein, C. (2014) Enhanced anti-tumor activity of the glycoengineered type II CD20 antibody obinutuzumab (GA101) in combination with chemotherapy in xenograft models of human lymphoma. *Leuk. Lymphoma* **55**, 2151–2160
270. Wang, Q., Chung, C. Y., Rosenberg, J. N., Yu, G., and Betenbaugh, M. J. (2018) Application of the CRISPR/Cas9 gene editing method for modulating antibody fucosylation in CHO cells. *Methods Mol. Biol.* **1850**, 237–257
271. Kanda, Y., Imai-Nishiya, H., Kuni-Kamochi, R., Mori, K., Inoue, M., Kitajima-Miyama, K., Okazaki, A., Iida, S., Shitara, K., and Satoh, M. (2007) Establishment of a GDP-mannose 4,6-dehydratase (GMD) knockout host cell line: A new strategy for generating completely non-fucosylated recombinant therapeutics. *J. Biotechnol.* **130**, 300–310
272. Louie, S., Haley, B., Marshall, B., Heidersbach, A., Yim, M., Brozynski, M., Tang, D., Lam, C., Petryniak, B., Shaw, D., Shim, J., Miller, A., Lowe, J. B., Snedecor, B., and Misaghi, S. (2017) FX knockout CHO hosts can express desired ratios of fucosylated or afucosylated antibodies with high titers and comparable product quality. *Biotechnol. Bioeng.* **114**, 632–644
273. Chan, K. F., Shahreel, W., Wan, C., Teo, G., Hayati, N., Tay, S. J., Tong, W. H., Yang, Y., Rudd, P. M., Zhang, P., and Song, Z. (2016) Inactivation of GDP-fucose transporter gene (Slc35c1) in CHO cells by ZFNs, TALENs and CRISPR-Cas9 for production of fucose-free antibodies. *Biotechnol. J.* **11**, 399–414
274. Roy, G., Martin, T., Barnes, A., Wang, J., Jimenez, R. B., Rice, M., Li, L., Feng, H., Zhang, S., Chaerkady, R., Wu, H., Marelli, M., Hatton, D., Zhu, J., and Bowen, M. A. (2018) A novel bicistronic gene design couples stable cell line selection with a fucose switch in a designer CHO host to produce native and afucosylated glycoform antibodies. *MAbs* **10**, 416–430
275. Jefferis, R. (2016) Posttranslational modifications and the immunogenicity of biotherapeutics. *J. Immunol. Res.* **2016**, 5358272
276. Seeling, M., Bruckner, C., and Nimmerjahn, F. (2017) Differential antibody glycosylation in autoimmunity: Sweet biomarker or modulator of disease activity? *Nat. Rev. Rheumatol.* **13**, 621–630
277. Alter, G., Ottenhoff, T. H. M., and Joosten, S. A. (2018) Antibody glycosylation in inflammation, disease and vaccination. *Semin. Immunol.* **39**, 102–110
278. Gudelj, I., Lauc, G., and Pezer, M. (2018) Immunoglobulin G glycosylation in aging and diseases. *Cell Immunol.* **333**, 65–79
279. Plomp, R., Ruhaak, L. R., Uh, H. W., Reiding, K. R., Selman, M., Houwing-Duistermaat, J. J., Slagboom, P. E., Beekman, M., and Wuhler, M. (2017) Subclass-specific IgG glycosylation is associated with markers of inflammation and metabolic health. *Sci. Rep.* **7**, 12325
280. Falck, D., Thomann, M., Lechmann, M., Koeleman, C. A. M., Malik, S., Jany, C., Wuhler, M., and Reusch, D. (2021) Glycoform-resolved pharmacokinetic studies in a rat model employing glycoengineered variants of a therapeutic monoclonal antibody. *MAbs* **13**, 1865596
281. Thomann, M., Reckermann, K., Reusch, D., Prasser, J., and Tejada, M. L. (2016) Fc-galactosylation modulates antibody-dependent cellular cytotoxicity of therapeutic antibodies. *Mol. Immunol.* **73**, 69–75
282. Peschke, B., Keller, C. W., Weber, P., Quast, I., and Lunemann, J. D. (2017) Fc-galactosylation of human immunoglobulin gamma isotypes improves C1q binding and enhances complement-dependent cytotoxicity. *Front. Immunol.* **8**, 646
283. Anthony, R. M., Wermeling, F., and Ravetch, J. V. (2012) Novel roles for the IgG Fc glycan. *Ann. N. Y. Acad. Sci.* **1253**, 170–180
284. Quast, I., Keller, C. W., Maurer, M. A., Giddens, J. P., Tackenberg, B., Wang, L. X., Munz, C., Nimmerjahn, F., Dalakas, M. C., and Lunemann, J. D. (2015) Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. *J. Clin. Invest.* **125**, 4160–4170
285. Kaneko, Y., Nimmerjahn, F., and Ravetch, J. V. (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* **313**, 670–673
286. Crispin, M., Yu, X., and Bowden, T. A. (2013) Crystal structure of sialylated IgG Fc: Implications for the mechanism of intravenous immunoglobulin therapy. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E3544–E3546
287. Dekkers, G., Treffers, L., Plomp, R., Bentlage, A. E. H., de Boer, M., Koeleman, C. A. M., Lissenberg-Thunnissen, S. N., Visser, R., Brouwer, M., Mok, J. Y., Matlung, H., van den Berg, T. K., van Esch, W. J. E., Kuijpers, T. W., Wouters, D., et al. (2017) Decoding the human immunoglobulin G-glycan repertoire reveals a spectrum of Fc-receptor- and complement-mediated-effector activities. *Front. Immunol.* **8**, 877
288. Chang, M. M., Gaidukov, L., Jung, G., Tseng, W. A., Scarcelli, J. J., Cornell, R., Marshall, J. K., Lyles, J. L., Sakorafas, P., Chu, A. A., Cote, K., Tzvetkova, B., Dolatshahi, S., Sumit, M., Mulukutla, B. C., et al. (2019) Small-molecule control of antibody N-glycosylation in engineered mammalian cells. *Nat. Chem. Biol.* **15**, 730–736
289. Schulz, M. A., Tian, W., Mao, Y., Van Coillie, J., Sun, L., Larsen, J. S., Chen, Y. H., Kristensen, C., Vakhrushev, S. Y., Clausen, H., and Yang, Z. (2018) Glycoengineering design options for IgG1 in CHO cells using precise gene editing. *Glycobiology* **28**, 542–549
290. Meuris, L., Santens, F., Elson, G., Festjens, N., Boone, M., Dos Santos, A., Devos, S., Rousseau, F., Plets, E., Houthuys, E., Malinge, P., Magistrelli, G., Cons, L., Chatel, L., Devreese, B., et al. (2014) GlycoDelete engineering of mammalian cells simplifies N-glycosylation of recombinant proteins. *Nat. Biotechnol.* **32**, 485–489
291. Goh, J. B., and Ng, S. K. (2018) Impact of host cell line choice on glycan profile. *Crit. Rev. Biotechnol.* **38**, 851–867
292. Fukuda, M. N., Sasaki, H., Lopez, L., and Fukuda, M. (1989) Survival of recombinant erythropoietin in the circulation: The role of carbohydrates. *Blood* **73**, 84–89
293. Seeberger, P. H., and Cummings, R. D. (2015) Glycans in biotechnology and the pharmaceutical industry. In: Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 729–741



294. Lai, P. H., Everett, R., Wang, F. F., Arakawa, T., and Goldwasser, E. (1986) Structural characterization of human erythropoietin. *J. Biol. Chem.* **261**, 3116–3121
295. Elliott, S., Lorenzini, T., Asher, S., Aoki, K., Brankow, D., Buck, L., Busse, L., Chang, D., Fuller, J., Grant, J., Hernday, N., Hokum, M., Hu, S., Knudsen, A., Levin, N., *et al.* (2003) Enhancement of therapeutic protein *in vivo* activities through glycoengineering. *Nat. Biotechnol.* **21**, 414–421
296. Delorme, E., Lorenzini, T., Giffin, J., Martin, F., Jacobsen, F., Boone, T., and Elliott, S. (1992) Role of glycosylation on the secretion and biological activity of erythropoietin. *Biochemistry* **31**, 9871–9876
297. Aquino, D., Wong, R., Margolis, R. U., and Margolis, R. K. (1980) Sialic acid residues inhibit proteolytic degradation of dopamine beta-hydroxylase. *FEBS Lett.* **112**, 195–198
298. Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., and Ashwell, G. (1971) The role of sialic acid in determining the survival of glycoproteins in the circulation. *J. Biol. Chem.* **246**, 1461–1467
299. Ashwell, G., and Kawasaki, T. (1978) A protein from mammalian liver that specifically binds galactose-terminated glycoproteins. *Methods Enzymol.* **50**, 287–288
300. Sorensen, A. L., Clausen, H., and Wandall, H. H. (2012) Carbohydrate clearance receptors in transfusion medicine. *Biochim. Biophys. Acta* **1820**, 1797–1808
301. Li, H., and d’Anjou, M. (2009) Pharmacological significance of glycosylation in therapeutic proteins. *Curr. Opin. Biotechnol.* **20**, 678–684
302. Ha, T. K., Hansen, A. H., Kildegaard, H. F., and Lee, G. M. (2020) Knockout of sialidase and pro-apoptotic genes in Chinese hamster ovary cells enables the production of recombinant human erythropoietin in fed-batch cultures. *Metab. Eng.* **57**, 182–192
303. Bosques, C. J., Collins, B. E., Meador, J. W., 3rd, Sarvaiya, H., Murphy, J. L., Dellorusso, G., Bulik, D. A., Hsu, I. H., Washburn, N., Sipsey, S. F., Myette, J. R., Raman, R., Shriver, Z., Sasisekharan, R., and Venkataraman, G. (2010) Chinese hamster ovary cells can produce galactose- $\alpha$ -1,3-galactose antigens on proteins. *Nat. Biotechnol.* **28**, 1153–1156
304. Noguchi, A., Mukuria, C. J., Suzuki, E., and Naiki, M. (1995) Immunogenicity of N-glycolylneuraminic acid-containing carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. *J. Biochem.* **117**, 59–62
305. Ghaderi, D., Taylor, R. E., Padler-Karavani, V., Diaz, S., and Varki, A. (2010) Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. *Nat. Biotechnol.* **28**, 863–867
306. Willems, A. P., Sun, L., Schulz, M. A., Tian, W., Ashkov, A., van Scherpenzeel, M., Hermans, E., Clausen, H., Yang, Z., and Lefeber, D. J. (2019) Activity of N-acylneuraminase-9-phosphatase (NANP) is not essential for de novo sialic acid biosynthesis. *Biochim. Biophys. Acta* **1863**, 1471–1479
307. Coutinho, M. F., Prata, M. J., and Alves, S. (2012) Mannose-6-phosphate pathway: A review on its role in lysosomal function and dysfunction. *Mol. Genet. Metab.* **105**, 542–550
308. Futerman, A. H., and van Meer, G. (2004) The cell biology of lysosomal storage disorders. *Nat. Rev. Mol. Cell Biol.* **5**, 554–565
309. Desnick, R. J., and Schuchman, E. H. (2012) Enzyme replacement therapy for lysosomal diseases: Lessons from 20 years of experience and remaining challenges. *Annu. Rev. Genomics Hum. Genet.* **13**, 307–335
310. Barton, N. W., Brady, R. O., Dambrosia, J. M., Di Bisceglie, A. M., Doppelt, S. H., Hill, S. C., Mankin, H. J., Murray, G. J., Parker, R. I., Argoff, C. E., Grewal, R. P., and Yu, K.-T. (1991) Replacement therapy for inherited enzyme deficiency—macrophage-targeted glucocerebrosidase for Gaucher’s disease. *N. Engl. J. Med.* **324**, 1464–1470
311. Solomon, M., and Muro, S. (2017) Lysosomal enzyme replacement therapies: Historical development, clinical outcomes, and future perspectives. *Adv. Drug Deliv. Rev.* **118**, 109–134
312. Platt, F. M., Boland, B., and van der Spoel, A. C. (2012) The cell biology of disease: Lysosomal storage disorders: The cellular impact of lysosomal dysfunction. *J. Cell Biol.* **199**, 723–734
313. Ghosh, P., Dahms, N. M., and Kornfeld, S. (2003) Mannose 6-phosphate receptors: New twists in the tale. *Nat. Rev. Mol. Cell Biol.* **4**, 202–212
314. Furbish, F. S., Steer, C. J., Barranger, J. A., Jones, E. A., and Brady, R. O. (1978) The uptake of native and desialylated glucocerebrosidase by rat hepatocytes and Kupffer cells. *Biochem. Biophys. Res. Commun.* **81**, 1047–1053
315. Lee, K., Jin, X., Zhang, K., Copertino, L., Andrews, L., Baker-Malcolm, J., Geagan, L., Qiu, H., Seiger, K., Barngrover, D., McPherson, J. M., and Edmunds, T. (2003) A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. *Glycobiology* **13**, 305–313
316. Doebber, T. W., Wu, M. S., Bugianesi, R. L., Ponpipom, M. M., Furbish, F. S., Barranger, J. A., Brady, R. O., and Shen, T. Y. (1982) Enhanced macrophage uptake of synthetically glycosylated human placental beta-glucocerebrosidase. *J. Biol. Chem.* **257**, 2193–2199
317. Achord, D. T., Brot, F. E., Bell, C. E., and Sly, W. S. (1978) Human beta-glucuronidase: *In vivo* clearance and *in vitro* uptake by a glycoprotein recognition system on reticuloendothelial cells. *Cell* **15**, 269–278
318. Brady, R. O., Murray, G. J., and Barton, N. W. (1994) Modifying exogenous glucocerebrosidase for effective replacement therapy in Gaucher disease. *J. Inher. Metab. Dis.* **17**, 510–519
319. Tiels, P., Baranova, E., Piens, K., De Visscher, C., Pynaert, G., Nerinckx, W., Stout, J., Fudalej, F., Hulpiau, P., Tannler, S., Geysens, S., Van Hecke, A., Valevska, A., Verweken, W., Remaut, H., *et al.* (2012) A bacterial glycosidase enables mannose-6-phosphate modification and improved cellular uptake of yeast-produced recombinant human lysosomal enzymes. *Nat. Biotechnol.* **30**, 1225–1231
320. Rose, R. J., Damoc, E., Denisov, E., Makarov, A., and Heck, A. J. (2012) High-sensitivity orbitrap mass analysis of intact macromolecular assemblies. *Nat. Methods* **9**, 1084–1086
321. Yang, Y., Franc, V., and Heck, A. J. R. (2017) Glycoproteomics: A balance between high-throughput and in-depth analysis. *Trends Biotechnol.* **35**, 598–609
322. Leney, A. C., and Heck, A. J. (2017) Native mass spectrometry: What is in the name? *J. Am. Soc. Mass Spectrom.* **28**, 5–13
323. Parsons, T. B., Struwe, W. B., Gault, J., Yamamoto, K., Taylor, T. A., Raj, R., Wals, K., Mohammed, S., Robinson, C. V., Benesch, J. L., and Davis, B. G. (2016) Optimal synthetic glycosylation of a therapeutic antibody. *Angew. Chem. Int. Ed. Engl.* **55**, 2361–2367
324. Rosati, S., Rose, R. J., Thompson, N. J., van Duijn, E., Damoc, E., Denisov, E., Makarov, A., and Heck, A. J. (2012) Exploring an orbitrap analyzer for the characterization of intact antibodies by native mass spectrometry. *Angew. Chem. Int. Ed. Engl.* **51**, 12992–12996
325. Wohlschlagler, T., Scheffler, K., Forstenlehner, I. C., Skala, W., Senn, S., Damoc, E., Holzmann, J., and Huber, C. G. (2018) Native mass spectrometry combined with enzymatic dissection unravels glycoform heterogeneity of biopharmaceuticals. *Nat. Commun.* **9**, 1713
326. D’Atri, V., Novakova, L., Fekete, S., Stoll, D., Lauber, M., Beck, A., and Guilleme, D. (2019) Orthogonal middle-up approaches for characterization of the glycan heterogeneity of etanercept by hydrophilic interaction chromatography coupled to high-resolution mass spectrometry. *Anal. Chem.* **91**, 873–880
327. Caval, T., Tian, W., Yang, Z., Clausen, H., and Heck, A. J. R. (2018) Direct quality control of glycoengineered erythropoietin variants. *Nat. Commun.* **9**, 3342
328. Majumder, S., Kono, M., Lee, Y. T., Byrnes, C., Li, C., Tuymetova, G., and Proia, R. L. (2020) A genome-wide CRISPR/Cas9 screen reveals that the aryl hydrocarbon receptor stimulates sphingolipid levels. *J. Biol. Chem.* **295**, 4341–4349
329. Yamaji, T., Sekizuka, T., Tachida, Y., Sakuma, C., Morimoto, K., Kuroda, M., and Hanada, K. (2019) A CRISPR screen identifies LAPTM4A and TM9SF proteins as glycolipid-regulating factors. *iScience* **11**, 409–424
330. Tian, S., Muneeruddin, K., Choi, M. Y., Tao, L., Bhuiyan, R. H., Ohmi, Y., Furukawa, K., Furukawa, K., Boland, S., Shaffer, S. A., Adam, R. M., and Dong, M. (2018) Genome-wide CRISPR screens for Shiga toxins and ricin reveal Golgi proteins critical for glycosylation. *PLoS Biol.* **16**, e2006951
331. Pacheco, A. R., Lazarus, J. E., Sit, B., Schmieder, S., Lencer, W. I., Blondel, C. J., Doench, J. G., Davis, B. M., and Waldor, M. K. (2018)

- CRISPR screen reveals that EHEC's T3SS and Shiga toxin rely on shared host factors for infection. *mBio* **9**, e01003-18
332. Yamaji, T., Hanamatsu, H., Sekizuka, T., Kuroda, M., Iwasaki, N., Ohnishi, M., Furukawa, J. I., Yahiro, K., and Hanada, K. (2019) A CRISPR screen using subtilase cytotoxin identifies SLC39A9 as a glycan-regulating factor. *iScience* **15**, 407–420
333. Flint, M., Chatterjee, P., Lin, D. L., McMullan, L. K., Shrivastava-Ranjan, P., Bergeron, E., Lo, M. K., Welch, S. R., Nichol, S. T., Tai, A. W., and Spiropoulou, C. F. (2019) A genome-wide CRISPR screen identifies N-acetylglucosamine-1-phosphate transferase as a potential antiviral target for Ebola virus. *Nat. Commun.* **10**, 285
334. Zhang, R., Miner, J. J., Gorman, M. J., Rausch, K., Ramage, H., White, J. P., Zuiani, A., Zhang, P., Fernandez, E., Zhang, Q., Dowd, K. A., Pierson, T. C., Cherry, S., and Diamond, M. S. (2016) A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. *Nature* **535**, 164–168
335. Blondel, C. J., Park, J. S., Hubbard, T. P., Pacheco, A. R., Kuehl, C. J., Walsh, M. J., Davis, B. M., Gewurz, B. E., Doench, J. G., and Waldor, M. K. (2016) CRISPR/Cas9 screens reveal requirements for host cell sulfation and fucosylation in bacterial type III secretion system-mediated cytotoxicity. *Cell Host Microbe* **20**, 226–237
336. Decker, C. E., Young, T., Pasnikowski, E., Chiu, J., Song, H., Wei, Y., Thurston, G., and Daly, C. (2019) Genome-scale CRISPR activation screen uncovers tumor-intrinsic modulators of CD3 bispecific antibody efficacy. *Sci. Rep.* **9**, 20068
337. Mbua, N. E., Li, X., Flanagan-Steet, H. R., Meng, L., Aoki, K., Moremen, K. W., Wolfert, M. A., Steet, R., and Boons, G. J. (2013) Selective exo-enzymatic labeling of N-glycans on the surface of living cells by recombinant ST6Gal I. *Angew. Chem. Int. Ed. Engl.* **52**, 13012–13015
338. Esko, J. D., and Stanley, P. (2009) Glycosylation mutants of cultured cells. In: Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W., Etzler, M. E., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 627–637
339. Esko, J. D., Bertozzi, C., and Schnaar, R. L. (2015) Chemical tools for inhibiting glycosylation. In: Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 701-712
340. Varki, A., Cummings, R. D., Aebi, M., Packer, N. H., Seeberger, P. H., Esko, J. D., Stanley, P., Hart, G., Darvill, A., Kinoshita, T., Prestegard, J. J., Schnaar, R. L., Freeze, H. H., Marth, J. D., Bertozzi, C. R., *et al.* (2015) Symbol nomenclature for graphical representations of glycans. *Glycobiology* **25**, 1323–1324