

Glycan Metabolism

A validated gRNA library for CRISPR/Cas9 targeting of the human glycosyltransferase genome

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Abstract

Over 200 glycosyltransferases are involved in the orchestration of the biosynthesis of the human glycome, which is comprised of all glycan structures found on different glycoconjugates in cells. The glycome is vast, and despite advancements in analytic strategies it continues to be difficult to decipher biological roles of glycans with respect to specific glycan structures, type of glycoconjugate, particular glycoproteins, and distinct glycosites on proteins. In contrast to this, the number of glycosyltransferase genes involved in the biosynthesis of the human glycome is manageable, and the biosynthetic roles of most of these enzymes are defined or can be predicted with reasonable confidence. Thus, with the availability of the facile CRISPR/Cas9 gene editing tool it now seems easier to approach investigation of the functions of the glycome through genetic dissection of biosynthetic pathways, rather than by direct glycan analysis. However, obstacles still remain with design and validation of efficient gene targeting constructs, as well as with the interpretation of results from gene targeting and the translation of gene function to glycan structures. This is especially true for glycosylation steps covered by isoenzyme gene families. Here, we present a library of validated high-efficiency gRNA designs suitable for individual and combinatorial targeting of the human glycosyltransferase genome together with a global view of the predicted functions of human glycosyltransferases to facilitate and guide gene targeting strategies in studies of the human glycome.

Key words: gRNA design, IDAA, gene editing, glycoengineering, glycome, glycosylation

Introduction

The glycome comprising all glycan structures found on different glycoconjugates including glycosphingolipids, glycoproteins and

proteoglycans of human cells is vast (Cummings 2009). Glycans serve many and diverse roles in the biology of cells and organisms and part of interactions with the environment (Varki 2017).

Studying the glycome requires detailed information on individual glycan structures and their glycoconjugate context, and in part due to analytic constraints, heterogeneity and complexity often present as factors limiting the deciphering and appreciation of the many fundamental roles of glycosylation. Analysis of the glycome of cells is generally limited to profiling strategies of glycans released from individual glycoconjugates, which to a large extent sacrifices information on the origin of individual glycans with respect to proteins and positions on proteins – the glycosites (Thaysen-Andersen and Packer 2014). In contrast, glycoproteome strategies defining glycosites often sacrifice information on the structures of the attached glycans (Levery et al. 2015).

While the glycome of cells and organs is large and heterogeneous, the biosynthetic and genetic basis for the glycome is considerably less complex (Hansen et al. 2015). Some 200–250 human glycosyltransferases orchestrate the biosynthesis of the diverse types of glycoconjugates and glycan structures produced in human cells (Henrissat et al. 2009). These genes are classified in the CAZy database into 44 GT families (Lombard et al. 2014). This is not considering the additional diversity brought by enzymatic modifications of glycans by sulfation, phosphorylation, acetylation and epimerization, which are carried out by another 100 or more enzymes. Nevertheless, the glycosyltransferase genome, the GTf-genome, is quite limited compared to the glycome, and although the individual enzymes have highly specific functions in biosynthetic pathways their functions in common and repeated structural features produce the great structural diversity of the glycome. The current knowledge of the GTf-genome and the roles of individual enzymes is quite advanced, making it possible with a reasonable degree of confidence to assign individual GTf genes to specific biosynthetic steps (Hansen et al. 2015). Thus, a large part of the GTf-genome has unique functions and can be assigned to the biosynthesis of specific glycoconjugates and glycan structures, and these are mainly involved in initiation, immediate core extension and/or branching of distinct types of glycoconjugates (Figure 1). The remainder of the GTf-genome has broader functions with roles in the biosynthesis of several different types of glycoconjugates, and these enzymes are mainly involved in elongation and capping of glycans. Importantly, the GTf-genome consists of a number of families with closely homologous genes shown (or predicted) to encode isoenzymes with related properties such as, e.g., galactosyl and sialyltransferases (Tsuji et al. 1996; Amado et al. 1999; Narimatsu 2006), and while the contributions of these to the glycome is still poorly understood, it is clear that many of these isoenzymes have at least partly overlapping functions and contribute functional redundancy. Thus, with our current understanding of the genetic and biosynthetic regulation of the human glycome it is not possible to assign every single GTf gene unambiguously to a specific glycan structure. However, we can assign a large part of the GTf-genome unambiguously to distinct glycosylation pathways and even structures, while the many families of isoenzymes may be assigned to more broad glycosylation features shared among multiple glycoconjugates.

Genetic dissection of biological functions of glycans has a long history in the glycosylation field (Conzelmann and Kornfeld 1984; Patnaik and Stanley 2006). In particular knockout of GTf genes in mice and other model organisms have greatly advanced appreciation of essential functions of particular types of glycosylation (Lowe and Marth 2003). However, for detailed dissection and identification of specific structure–function relationships it is advantageous to use more simple cell systems as a first step. For a long time genetic dissection of GTf function in higher eukaryotic cell lines was not

possible, but recently, elegant studies with haploid cell lines have uncovered entire glycosylation pathways required for specific biological functions (Jae et al. 2013), and with the advent of precise gene editing including ZFNs, TALENs and CRISPR/Cas9 (Chandrasegaran and Carroll 2016), it is now possible and rather simple to use a gene editing approach as a tool for dissection of glycosylation in cell lines (Steenfot et al. 2014). Evaluating the role of any particular glycosyltransferase gene generally requires complete loss of function, and explains why techniques that do not reliably achieve complete suppression of transcription (such as RNA silencing) have not been favored in the field. Similarly, complete bi-allelic (and sometimes multiallelic) knockout of most GTf genes are needed to abrogate glycosylation features and evaluate glycome and biological effects.

A key factor for broad use of precise gene targeting is efficiency and specificity, and the CRISPR/Cas9 gene editing tool is currently the most efficient and cost-effective (Chandrasegaran and Carroll 2016). However, the efficiency in CRISPR/Cas9 gene targeting relies on design of gRNAs, and although several prediction tools have been developed including Deskgen (Doench et al. 2014, 2016), CHOPCHOP (Montague et al. 2014), E-CRISP (Heigwer et al. 2014) and CRISPR design (Hsu et al. 2013), the performance of predicted gRNAs vary from no/low to high efficiency (Cong et al. 2013; Hart et al. 2017; Shi et al. 2015). Thus, there is still a need for experimental trial and error to identify gRNA designs for efficient mono and/or bi-allelic gene targeting (Yang et al. 2015a; Lonowski et al. 2017; Metzakopian et al. 2017).

In order to facilitate GTf-genome wide dissection and discovery, we have therefore designed and experimentally tested a collection of more than 600 gRNAs for all known human GTf-genes. We tested four gRNAs for each glyco gene with a high throughput workflow involving FACS and Indel Detection by Amplicon Analysis (IDAA) (Lonowski et al. 2017), which enabled us to select one validated gRNA for each gene with high multi-allelic cutting efficiency and predictable small indels resulting in coding frameshifts. To facilitate broad use of this resource, we provide a comprehensive protocol for CRISPR/Cas9 gRNA delivery, screening, sorting and selection of gene targeted cells, as well as a predicted framework for interpretation of effects of single and combinatorial targeting of GTf genes on the cellular glycome.

Results

Overview of the GTf-genome and predicted roles in glycosylation pathways

To date 208 human glycosyltransferase genes have been identified and their catalytic properties characterized to some detail or in a few examples predicted based on close sequence similarities (Table 1) (Hansen et al. 2015). In Figure 1 we present a graphic view of 167 human GTf-genes with their predicted functions in the known glycosylation pathways found in human cells. Glycosylation pathways are organized into pathway-specific (vertical colored boxing) and non-pathway-specific (horizontal colored boxing) sequential biosynthetic steps of glycosylation that includes initiation, core extension, branching and elongation, and terminal capping. The underlying glycan structures characteristic for each type of glycosylation and those common to multiple glycosylation pathways are presented in Supplementary data, Figure S1. The organization provides important guidance for predicted consequences of gene editing of human GTfs for the glycosylation capacity of cells. Thus,

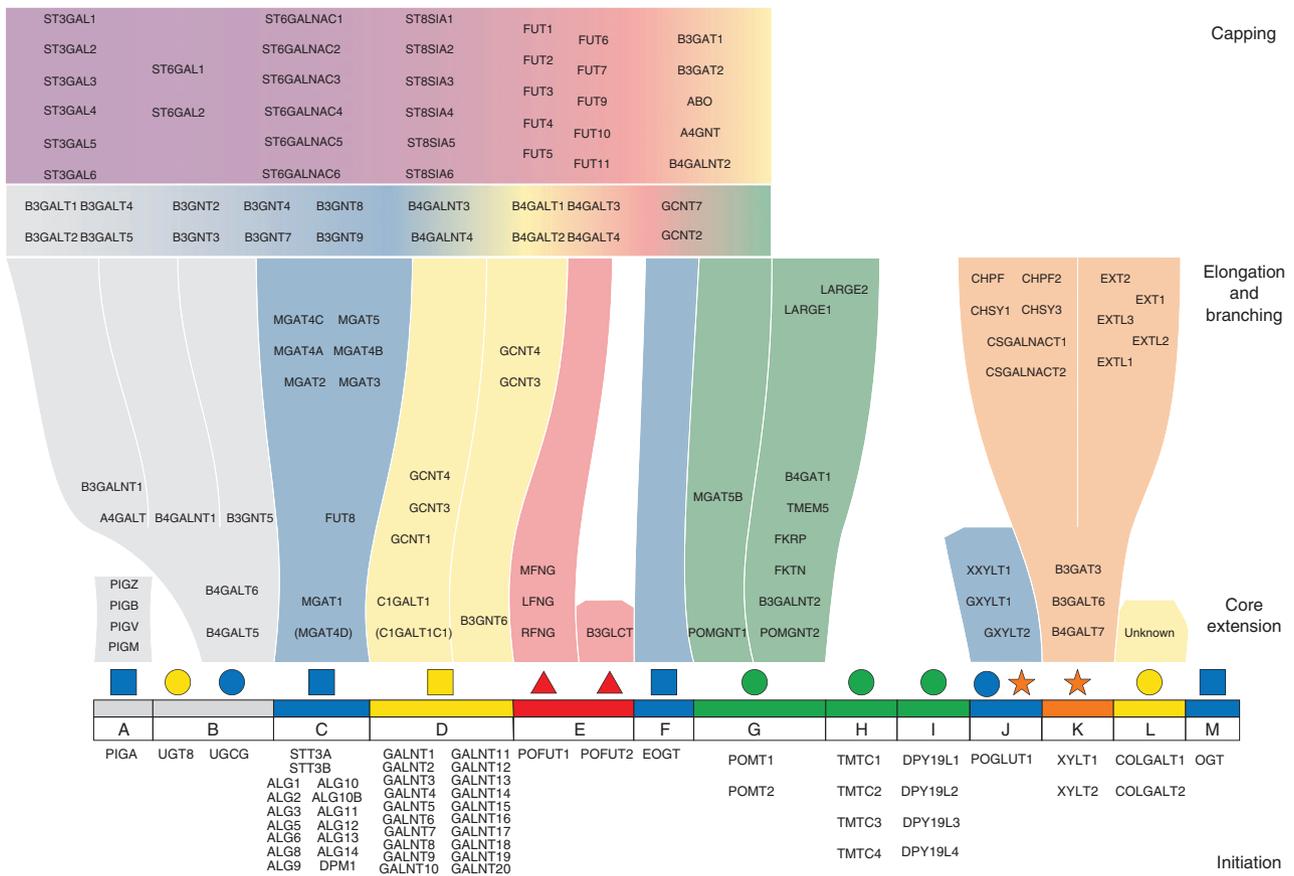


Fig. 1. Graphic rainbow depiction of predicted assignments of the 167 human GTf-genes involved in biosynthesis of protein or lipid glycan structures. Glycosylation pathways from left to right are: (A) GPI-anchor, (B) glycolipids (two pathways), (C) N-linked glycans, (D) O-GalNac mucin-type, (E) O-Fuc type (two pathways), (F) O-Man type (POMT-directed), (G) O-Man type (TMTC-directed), (H) C-Man type, (I) O-Glc type, (J) O-Xyl type (proteoglycans), (K) O-Gal type (collagen) and (L) two types of O-GlcNAc (extracellular and cytosolic). The horizontal first rainbow level illustrates GTf-genes predicted to perform glycosylation pathway-specific functions in the initiation, core extension and branching/elongation steps of the indicated types of glycosylation. The two horizontal rainbow levels illustrate GTf-genes with predicted pathway-unspecific functions in the elongation or capping steps that are common among the multiple glycosylation pathways beneath. Glycan symbols are drawn according to the SNFG format (Varki et al. 2015).

targeting pathway-specific GTf-genes is predicted to only affect one type of glycoconjugate, while targeting non-pathway-specific GTfs is predicted to affect multiple types. Moreover, the organization illustrates the homologous GTf-gene families encoding isoenzymes with potential overlapping functions, where it is important to consider combinatorial targeting for complete abrogation of glycosylation.

Construction of a human GTf-genome gRNA library

The design of gRNAs is important for the efficiency of gene editing (Yang et al. 2015a; Lonowski et al. 2017; Metzakopian et al. 2017). The target site for indels should obviously be selected to eventually disrupt protein function, and in many large scale screening studies the first coding exon(s) is selected to ensure truncated protein products without having to discriminate individual genes and target functional domains (Doench et al. 2014; Shalem et al. 2014; Wang et al. 2014). Here, we chose the same strategy partly due to the finding that the first exons are the least homologous regions of most GTf-genes including close paralogs. We first tested several gRNA prediction algorithms, and the Desktop Genetics DESKGEN Cloud gRNA design platform (<https://www.deskgen.com/landing/>) was selected for a pilot study with 48 gRNA designs. DESKGEN provides both on-target as well as off-target scores with

assignment of potential off-target sites and similarity with the target sequence (number and position of mismatches). This is valuable for targeting close homologs in isoenzyme gene families, such as the β 4galactosyltransferase family as previously reported (Duda et al. 2014). As shown in Supplementary data, Figure S2 the pilot study suggested that the on-target scores did influence the cutting-efficiency and the range of 31–50 was optimal.

We proceeded with design and testing of 3–4 gRNAs for all human GTf-genes with a high throughput workflow as illustrated in Figure 2. gRNAs were designed to target the first third of the coding region within an early exon with DESKGEN parameters set for on target score >30, and with off target score set >80 with no predicted off targets with 0 or 1 mismatches and minimal number off targets with 2 or 3 mismatches with gRNA target sequence (Supplementary data, Table SI). gRNAs were tested with human embryonic kidney HEK293T cells and the cutting efficiency and indel profile of each gRNA was characterized by IDAA (Yang et al. 2015a; Lonowski et al. 2017). We and others have shown that an effective gRNA design may induce highly similar indel profiles across most of the commonly used cell lines including iPSC's (Paquet et al. 2016; Lonowski et al. 2017). We used a GFP-tagged CRISPR/Cas9 nuclease to evaluate Cas9 expression and enrich for different expression

Table I. GlycoCRISPR: the list of validated gRNA target to human glycosyltransferase

¹ CAZy family	Gene name (HGNC)	gRNA sequence	² Target exon	³ Major indel	¹ CAZy family	Gene name (HGNC)	gRNA sequence	² Target exon	³ Major indel	¹ CAZy family	Gene name (HGNC)	gRNA sequence	² Target exon	³ Major indel
N-glycosylation					Glycosaminoglycan (GAG)					Redundant (Elongation)				
GT33	ALG1	GCAATGCTAGGCAGACCTGG	4/13	+1	GT14	XYLT1	ACAACAGCAACTTCGCACCC	3/12	+1	GT31	B3GALT1	TCAGCCACCTAACAGTTGCC	1/1	+1
GT4	ALG2	TGTTCCAGGCTGGCTAGACGG	2/2	-12/-8/+1	GT14	XYLT2	GACAGTTTCAGCAGGGCGACG	2/11	+1	GT31	B3GALT2	CCTGTGACATACACTTTCGG	1/1	+1
GT58	ALG3	GATCTATCACCAGACCTGCA	3/9	+1	GT7	B4GALT7	TGACCTGCTCCCTCTCAACG	3/6	+1	GT31	B3GALT4	GGAAGCTTGACAGTGGTCCCG	1/1	-1
GT2	ALG5	GTAGGTGAGTCCCATATGCT	2/10	+1	GT31	B3GALT6	CTTCGAGTTCGTGCTCAAGG	1/1	+1	GT31	B3GALT5	TTTCCCCACGCTGTCGGGA	1/1	+1
GT57	ALG6	AGACTTTCGCCGGTTGATCG	8/14	+1	GT43	B3GALT3	TCGGAGTTCGGCTTGCAGCT	2/5	+1	GT7	B4GALT1	GGAGTCTCCACCGCTGCA	1/6	+1
GT57	ALG8	GTCCGTCGCCGAGCAATGG	1/13	+1	GT7	CSGALNACT1	GGTACCCCTCCTTCCCCGTG	1/7	+1	GT7	B4GALT2	AGTAGAGGATGACGGCCACG	2/7	+1
GT22	ALG9	CGTTGATAGCCATGACTGGA	6/15	+1	GT7	CSGALNACT2	GTATTATCAAGCCCTCCTAC	1/7	+1	GT7	B4GALT3	TCCCTGATCTCGGCCAAATA	1/6	+1
GT59	ALG10	CTGCCATTTGGATCTTTGGA	2/3	+1	GT7	CHPF	GGAACACCACGCTCCAGC	2/4	+1	GT7	B4GALT4	ACCCACGAAAGTAGTTACTGG	1/6	+1
GT59	ALG10B	GCAGGAATGGCGCAGCTAGA	1/3	+1	GT7	CHPF2	CAGTGAAGTAGAGTAAACCGA	2/4	+1	GT31	B3GALT2	GTTCACAGTATGCCTCGGGAG	1/1	-1
GT4	ALG11	GCAGCAGTCTGATTTCCCAA	2/4	-2/+1	GT7	CHSY1	GTACATCAAAGGAGACCCGT	2/3	-11/-1/+1	GT31	B3GALT3	GCAGCCACCGCGATCCCCG	1/2	-2/-1
GT22	ALG12	GCGAAAGCACGTAACCCGG	2/9	+1	GT7	CHSY3	TCTCGGCTAAAGATCATGCC	2/3	+1	GT31	B3GALT4	GTATCCTTGGAAACAGCCTGA	2/2	-1/+1
GT1	ALG13	AATGGCCCGAAAGAGCAAG	4/27	-7/+1	GT64	EXT1	GTAGAACCCTGGAGCCCTCGA	1/11	+1	GT31	B3GALT7	GGTCCGCAAGAACTGACCA	1/2	+1
GT1	ALG14	AAGATACTGAGAGACTCCCG	1/4	-2/-1	GT64	EXT2	TCGGCTGGCAGCCTAACAAAC	1/13	+1	GT31	B3GALT8	GGAGTGTGAGCAGTGTGAC	1/1	+1
GT2	DPM1	GTAGTCTCGCAGGCTCTCGG	1/9	+1	GT64	EXTL1	GTAGAAGCGGAGCCCTCAA	1/11	+1	GT31	B3GALT9	AACCAGCCGCAAGTGCCTG	1/1	+1/+2
GT23	FUT8	ACCTTGCTGTTTTATATAGG	1/9	+1	GT64	EXTL2	CAGTACCAGTAAATATACG	1/4	+1	GT7	B4GALT3	CATAGATTCGCACAACCCTG	5/20	-1/+1
GT13	MGAT1	CCCTCAGTCAGCGCTCTCGA	1/1	+1	GT64	EXTL3	GGTGGGGAACGAGCTGTGCG	1/5	+1	GT7	B4GALT4	CAGTGAGACCGACGGCCGG	2/20	-1/+1
GT16	MGAT2	GTTCGGCGTCCAGCAACCGT	1/1	+1	O-GlcNAc					GT14	GCNT1	ATAGCAGGTAGCTTCATCAA	1/3	+1
GT17	MGAT3	TCCTCGGCCGCTTGTGGG	1/1	+1	GT61	OGT	GCAACCTATTCTTCTCTAAC	12/22	+1	GT14	GCNT2	GGTCAAATAGATGACGTAGG	1/1	+1
GT54	MGAT4A	CTTTGCTTGGTATACTACA	1/15	+1	GT41	EOGT	GTTTGCAGCTATGTGACAT	2/15	+1	GT14	GCNT3	ACTGCTCCAGGATTTCTCGG	1/3	+1
GT54	MGAT4B	GGAGAGCCTCAAGCGCTCCA	2/15	+1	O-Fucose					Redundant (Capping)				
GT54	MGAT4C	TGTGGCAGCTAGGTAGCGAT	2/3	+1	GT65	POFUT1	AAAGCTGCTAAACCGTACCT	2/7	-5/+1	GT11	FUT1	CAGGGTGTGCGGAATACCG	1/1	+1
GTnc	MGAT4D	GGTATTTCCACTGTTAACAG	4/11	+1	GT68	POFUT2	GGAAGGCTTCAACCTGCGCA	2/9	+1	GT11	FUT2	AGTGCTAGCCTCAACATCAA	1/1	+1
GT18	MGAT5	GCTGTCTGACTCCAGCGTA	1/16	+1	GT31	MFNG	GCGCAAGCGGTGAAAGCCC	1/8	-2	GT10	FUT3	TGTCCTGAGCAGGATCAGGA	1/1	+1
GT66	STT3A	ATGTTGTTCTTAGCATAGA	6/14	+1	GT31	LFNG	GATGAAGCGGTCACTACTCCA	3/8	+1	GT10	FUT4	CCTCCACGCTGCGGACGCG	1/1	+1
GT66	STT3B	TTGGGTGTATCACTAGCTGC	7/16	+1	GT31	RFNG	GCCACCCCTGGACCTCTCGG	4/8	+1	GT10	FUT5	GGCAGTGAACCTGTCACCG	1/1	+1
GT24	UGGT1	CTACTATCATGCAATATTGG	4/41	+1	GT31	B3GLCT	GTTTAGCCAGCTGATGAAGG	5/15	-1/+1	GT10	FUT6	GGACCCATTAGGGTACACAG	1/1	-2/+1
GT24	UGGT2	TTGCGAGCTCGGCTCCGGGA	1/39	+1/+2	O-Mannose					GT10	FUT7	TAGCGGGTGCAGGTGTGCT	2/2	+1
O-GalNAc					GT39	POMT1	GAGCTCCAACACTATCTGGT	4/19	+1	GT10	FUT9	GTGAACGGTCCGTTGTGAGA	1/1	-2
GT27	GALNT1	TCCCACCTGTACACTCACAAT	4/11	+1	GT39	POMT2	GTTTCGAGGCGGTTCGGTGGT	1/21	+1	GT10	FUT10	GGGACCCACAGAGCATAATG	2/4	-1/+1
GT27	GALNT2	GTGAAACGTGATCACCACGC	4/16	+1	GT13	POMGNT1	GAGGGACACATGGCCCTTCG	6/21	-10/-1/+1	GT10	FUT11	CGCGCAGCTCTGGGACGCG	1/3	+2
GT27	GALNT3	TATGGAAGTAACCATAACCG	4/10	+1	GT61	POMGNT2	ACTGAGGATCGACTACCCGA	1/1	-1/+1	GT29	ST3GAL1	TCCAAGTCGATGGTCTTGAA	3/6	+1
GT27	GALNT4	AACAGTGGCCTATATCTTCG	1/1	+1	GT18	MGAT5B	CCACCAAGGTACCTTCTGTG	2/17	+1	GT29	ST3GAL2	GTGGCTGTCAAACAGTCCG	1/6	+1
GT27	GALNT5	GATGACTTCGATTACTGGAC	4/10	-1	GT31	B3GALNT2	GAAACGTGATAAGAAGCACCC	2/12	+1	GT29	ST3GAL3	GATTCTAGCCCACTTGCAG	5/11	+1
GT27	GALNT6	GAAGAGCAAGTGGACCCCGC	1/10	-2	GTnc	FKTN	GAGTCTATCCGCTAGCCCG	4/9	+1	GT29	ST3GAL4	TTACCCGCTTCTTATCACTC	7/10	-4/+1
GT27	GALNT7	ATGCCCAACCAGGGCGGCAA	2/12	+1	GTnc	FKRP	GCACCAGGACGGTACACCG	1/1	-8/-1/+1	GT29	ST3GAL5	ATTTGAGCACAGGTATAGCG	4/7	+1
GT27	GALNT8	GTAGTCTCGCGTGTCCGGGA	2/11	+1	GT49	B4GAT1	CATCGCCACCAGCATGACGG	1/2	+1	GT29	ST3GAL6	TGAGAATCACTGTGCTATTA	6/9	+1
GT27	GALNT9	CGCACTGTCCGGATCCGAG	5/11	+1	GT49	LARGE	CCTGGAGGTGCGCATGCGCG	2/14	+1	GT29	ST6GAL1	TGTATCCTCAAGCAGCACCC	1/5	+1
GT27	GALNT10	CTCTCTCAGCATCGGTCTATG	3/12	+1	GT49	LARGE2	CCAGAGCTCCGAGATGTCAG	4/13	+1	GT29	ST6GAL2	AGCTGGGTACAGGCTCAGCG	1/5	+1
GT27	GALNT11	TATGCTTATCAGTGACCGCT	2/11	+1	GTnc	TMTC1	GACCTGCCAGTCATAGCACA	6/18	+1	GT29	ST6GALNAC1	ACGGTGTGAGAGAACACCA	2/9	+1
GT27	GALNT12	TTCTCTAGCAGGATATCCG	2/10	+1	GTnc	TMTC2	GCCCTGAACCATGCCATTGGA	2/12	+1	GT29	ST6GALNAC2	GAGCCCCCGCAGCCATACG	3/9	+1
GT27	GALNT13	TTAATACGTGCCGCTCTTCG	4/11	+1	GTnc	TMTC3	ACTGCTGGACAGTTTCTCCG	5/13	+1	GT29	ST6GALNAC3	GTATCCATAGTGAGTTGCG	2/5	+1
GT27	GALNT14	CTTACAGGACTACACGCGGG	4/15	+1	GTnc	TMTC4	GCTGCGTGCAAAACACACAA	1/17	-2/+1	GT29	ST6GALNAC4	TGTGTGTGAGACGACGCG	3/5	+1
GT27	GALNT15	GCTGGCTGAGGTGCTCCACG	2/10	+1	C-Mannose					GT29	ST6GALNAC5	CTAGTGTACAGCAGCTCCG	2/5	+1

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GT27	GALNT16	GTAATGGCGGGTGTCCCGGA	2/15	+1	GT98	DPY19L1	TCTCCCTTTCCAATGTTGAG	3/22	+1	GT29	ST881ALNAC6	TCTTCCATTACGGCTCCCTG	3/6	-1/+1
GT27	GALNT17	AGTTCACATTGACCTCGCAG	5/12	-2/-1	GT98	DPY19L2	AGCCAGTCTAAGGGGCGCG	1/22	-9/+1	GT29	ST881A1	CGTTGGGCGCCGGTAGAGC	1/5	+1
GT27	GALNT18	TGAGATAGAAGAGTACCCGC	5/11	-1/+1	GT98	DPY19L3	GCTGGTACTCAGTGGTACA	5/18	+1	GT29	ST881A2	TGCCATCGTGGGCAACTCGG	4/6	+1
GT27	GALNT19	AGTTGGCCACCTCGGCGCG	1/11	+1	GT98	DPY19L4	TGCTTGCACGGTTACTAG	3/19	+1	GT29	ST881A3	GATGAGCGATAAAATCAGCA	1/4	+1
GT27	GALNT20	ATACTCTGTTCACTCACAG	5/8	+1	O-Galactose					GT29	ST881A4	AGATGCGCTCCATTAGGAAG	1/5	+1
GT31	C1GALT1	GTAAAGCAGGGTACATGAG	2/3	+1	GT25	COLGALT1	GAAGAGTTTGTACCATTCGG	2/12	+1	GT29	ST881A5	ATACAGGATCTGTTGCAGCA	1/8	+1
GT31	C1GALTC1	GTAGTGATGATGCTCATGG	1/1	+1	GT25	COLGALT2	GTAGAAAGTCAGCTTGTCCG	5/12	+1	GT29	ST881A6	GGAGCGTCTCAGCGCTGGC	2/8	+1
GT14	GCNT1	TAGTCGTGAGTGTCCACCG	1/1	+1	Hyaluronan					GT43	B3CAT1	GCTAGGATGTCCCGTCTCTT	1/4	+1
GT14	GCNT3	ACTGTTTCAGGGGTACCCGA	1/1	-1/+1	GT2	HAS1	CATGGTCGACATGTTCCGCG	2/5	+1	GT43	B3CAT2	AAAGAAGCGGGTGAAGCG	1/4	-1/+1
GT14	GCNT4	GCAGCCATAGGGTAAAAAC	1/1	-2/-1	GT2	HAS2	TGAAAAGGCTAACCTACCCCT	1/3	-5/-2/-1	GT32	A4CNT	CTACGGAACAGGAGACAAA	1/2	-1/+1
GT31	B3GNT6	GCGGGACCTTGGCGCTGG	1/1	-7/+1	GT2	HAS3	GGTGGTGGATGGCAACCGCC	1/3	-1/+1	GT6	ABC	AATGTGCCCTCCAGCAAT	6/7	+1
Glycosphingolipid (GSL)					Glycogen					GT12	B4GALNT2	CCGTGGACTGGGTACCCAAA	4/11	-2/-1/+1
GT21	UGCG	TTAGGATCTACCCCTTTCAG	2/9	+1	GT8	GYG1	GACCAGGGCACCTTTGGCGT	2/8	-2/-1	Unknown				
GT1	UGT8	TGGTACTGTTAAAGATCCCT	1/5	-1	GT8	GYG2	GAGAGTCCAACAGTGAAGCT	4/11	+1	GT2	B3CNTL1	CAGTCCACAACGCTGAACCG	2/12	+1
GT7	B4GALT5	TTCGGAGTGCTTATGCCAAG	2/9	+1	GT3	GYS1	GACGAAGGCGAAGGTGACAG	2/16	-7/+1	GT25	CEBAM	GCTGCCCCCTCAGCCCGCA	5/12	+1
GT7	B4GALT6	CTCTTTATGGTACAAGCTCG	2/9	+1	GT3	GYS2	ACTGTAAGAAGAATGCTTCG	5/16	+1	GT4	GLD1	GCTCTTCATCTCTATAGGGG	2/8	-2/-1
GT32	A4GALT	GTCTGCACCCGTTCATCAT	1/1	+1	GPI anchor					GT6	GLD1	GGGAAGGGACTTTCGACAGG	3/5	+1
GT31	B3GALNT1	CGTTCATCACATGTAGTG	1/1	+1	GT4	PIGA	CCGTACCCATAATATATGCA	1/5	+1	GT8	GLD1	GCTCTCCGACATGCAGTAGA	3/9	+1
GT31	B3GNT5	CTCTTAAGCACACCTCAGCG	1/1	-7/+1	GT22	PIGB	TACCTTGTACTTCAACACCC	1/12	-2/+1	GT8	GLD2	GCTATTGATGGCAGCCATAG	3/9	-12/+1
GT12	B4GALNT1	ACCGGGATGTGTGCGTAGCG	1/10	+1	GT50	PIGM	CCCCTCCGTGACGAAGCGCG	1/1	+1	GT4	GLD1	TCAGAGTGTGATACACTG	4/9	+1
GT6	GBGT1	GTTGGCGCCCATCGTCTCCG	5/6	+1	GT76	PIGV	AGTTGGTCCACAAGCCTGA	2/3	+1	GT90	KDPC1	CTGAATATAGAAATAGCGGG	1/10	+1
					GT22	PIGZ	GCACATAGCCCTCTGCGGA	1/2	+1	GT90	KDPC2	GATTTGACTACGACTTTGAA	2/8	-1/+1
O-Glucose														
GT8	GXYLT2	ACCCGAGCTCTGGATCCACC	2/7	+1										
GT90	POGLUT1	GAGGATCTAACTCCTTCCG	3/11	+1/+2										
GT8	XXYLT1	GCAGTGAGCGCAGCGGACG	1/4	+1										

¹GT family according to the CAZy database (www.cazy.org).

²The targeted exon out of the total numbers of exons assigned according to UniprotKB/Swiss-Prot information.

³Indication of the indel profile obtained by IDAA. Predominant indel present >30% of total is indicated. For lower indel representations, only the three most predominant indel events are indicated.

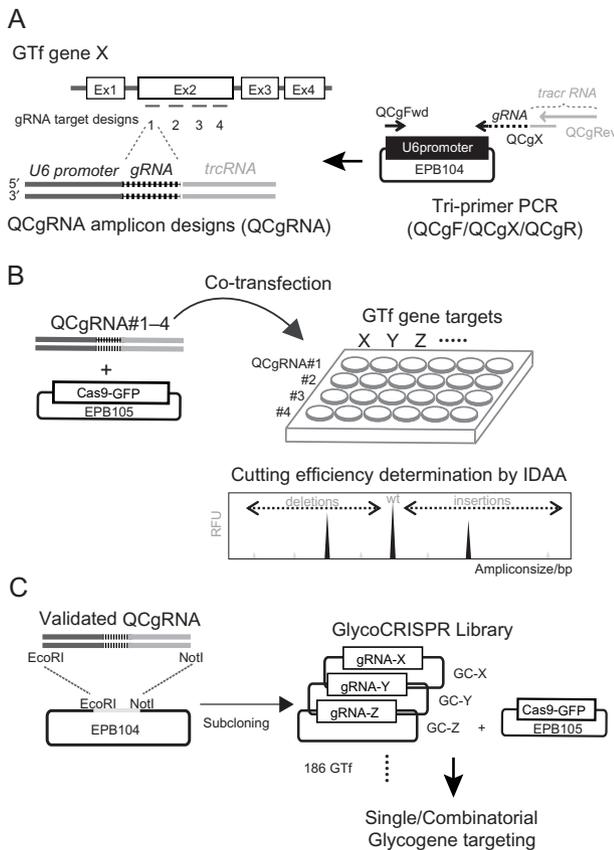


Fig. 2. Schematic workflow for GlycoCRISPR library construction and validation. **(A)** Desktop Genetics DESKGEN Cloud gRNA design platform was used for gRNA design and the most 5'-exons of the GTf-genes were preferentially selected as target site. Amplicons encoding the GTf specific gRNA sequence were amplified using the invariant QCgFwd and QCgRev primers and the variable QCgX primers. The generated QuickChange gRNA amplicon expression cassettes (QCgRNA) were generated by a single tri-primer PCR reaction and a plasmid template only encoding the U6 promoter sequence. **(B)** Individual QCgRNA amplicons were co-transfected with Cas9-GFP plasmid into HEK293 cells and seeded into 24-wells. Two days post-transfection crude lysates were used for amplification across the respective GTf gRNA targeted loci using our tri-primer labeling methodology for single reaction fluorescence labeling of amplicons, followed by indel profiling by IDAA. **(C)** IDAA allows for simultaneous profiling and quantification of indel events, and gRNA designs giving rise to greatest indel formation efficiencies were subcloned into the EPB104 plasmid. The selected constructs were included in the validated GlycoCRISPR toolbox with GC-“gene names”, for use in individual or combinatorial engineering experiments.

levels as a measure of transfection efficiency by FACS (Figure 2B). Cellular transfection of all gRNA designs tested was initially based on our amplicon delivery (QCgRNA) method that only requires co-delivery of Cas9 plasmid with a PCR derived gRNA encoding template to cells (Lonowski et al. 2017). The QCgRNA delivery method avoids laborious and time-consuming cloning, screening and sequencing of gRNA plasmid designs, and allows for fast evaluation of the indel formation efficiency of individual gRNA designs.

IDAA profiles were obtained for unsorted, medium and high GFP positive FACS sorted cells as illustrated in Figure 3. Cutting efficiencies were calculated based on individual indel IDAA peak sizes relative to total accumulated peak sizes (including wildtype-allele). For the validated gRNA library we selected cutting efficiencies >30% derived from medium sorted cells and preferentially with

small out of frame causing indels, and in most cases it was possible to select gRNA designs inducing ± 1 bp indels as these are by far the most common and ensure in-exon frameshifts (Table I). Cutting efficiencies and indel profiles for all gRNAs are listed in Supplementary data, Table SI. All primers and conditions for the target-specific IDAA assays are designed and validated as listed in Supplementary data, Table SII.

A sustainable community resource

To enable wide distribution of the gRNA library to the scientific community, we cloned the selected optimal QCgRNA for each GTf-gene into the EPB104 plasmid backbone (available from Addgene, <https://www.addgene.org/>, see “materials and methods” section for detailed information), and for ease we designated these GC-“gene name” for the GlycoCRISPR library (Figure 2C). We previously demonstrated that delivery of gRNAs as amplicons or plasmids produced similar cutting efficiencies and indel profiles (Lonowski et al. 2017). Moreover, all parameters for the IDAA based screening and clone selection are listed in Supplementary data, Table SII. The pre-designed IDAA assay is a simple protocol that can be used in most labs without major investments, and it is now also becoming commercially available as a custom service by several vendors.

Multiplex gene targeting example

Access to validated plasmid gRNA targeting constructs also enables rational multiplex targeting, which is often required to engineer glycosylation capacities of cells due to isoenzymes with partially overlapping functions as illustrated in Figure 1 (Hansen et al. 2015). The quantitative indel profiling by IDAA further enables easy and fast screening and selection of clones with desirable multiple gene editing events. To illustrate this we used two optimal gRNA plasmids to simultaneously target two polypeptide GalNAc-transferase isoenzyme genes, *GALNT2* and *T3* in HEK293 cells (Figure 4). We monitored efficiencies at the two target sites by IDAA profiling and immunocytology after FACS enrichment and single cell cloning. IDAA profiling of the transfected pool clearly demonstrated $+1$ indel formation for both target sites ($\sim 20\%$), and FACS enrichment resulted in substantial increase in the $+1$ indel peak. Only one example of a single cloned cell is shown with a clear bi-allelic $+1$ indel in *GALNT2* and compound heterozygote bi-allelic $-1/+1$ indel in *GALNT3*, however, most tested single cells in fact contained bi-allelic indels in both targeted genes. While IDAA profiling of heterogeneous cell pools does not provide unambiguous information of the allelic targeting state of the individual cells, we demonstrate by immunocytology using monoclonal antibodies specific for GalNAc-T2 or T3 that the FACS enriched pool contains about 50% cells without immunoreactivity suggesting that most targeting events in fact are bi-allelic for both genes in a subpopulation of cells.

Discussion

Here we provide a ready to use resource for efficient and validated gene targeting in the GTf-genome with a comprehensive set of plasmid targeting constructs, optimized screening and selection protocols, and a predicted framework to guide engineering of the glycosylation capacity of human cell lines. While gene targeting is becoming a house-hold procedure in most cell biology labs there are still obstacles to overcome and efforts that can be minimized. In particular we have identified the following factors that limit dissection of complex biosynthetic pathways like glycosylation: (i) need for

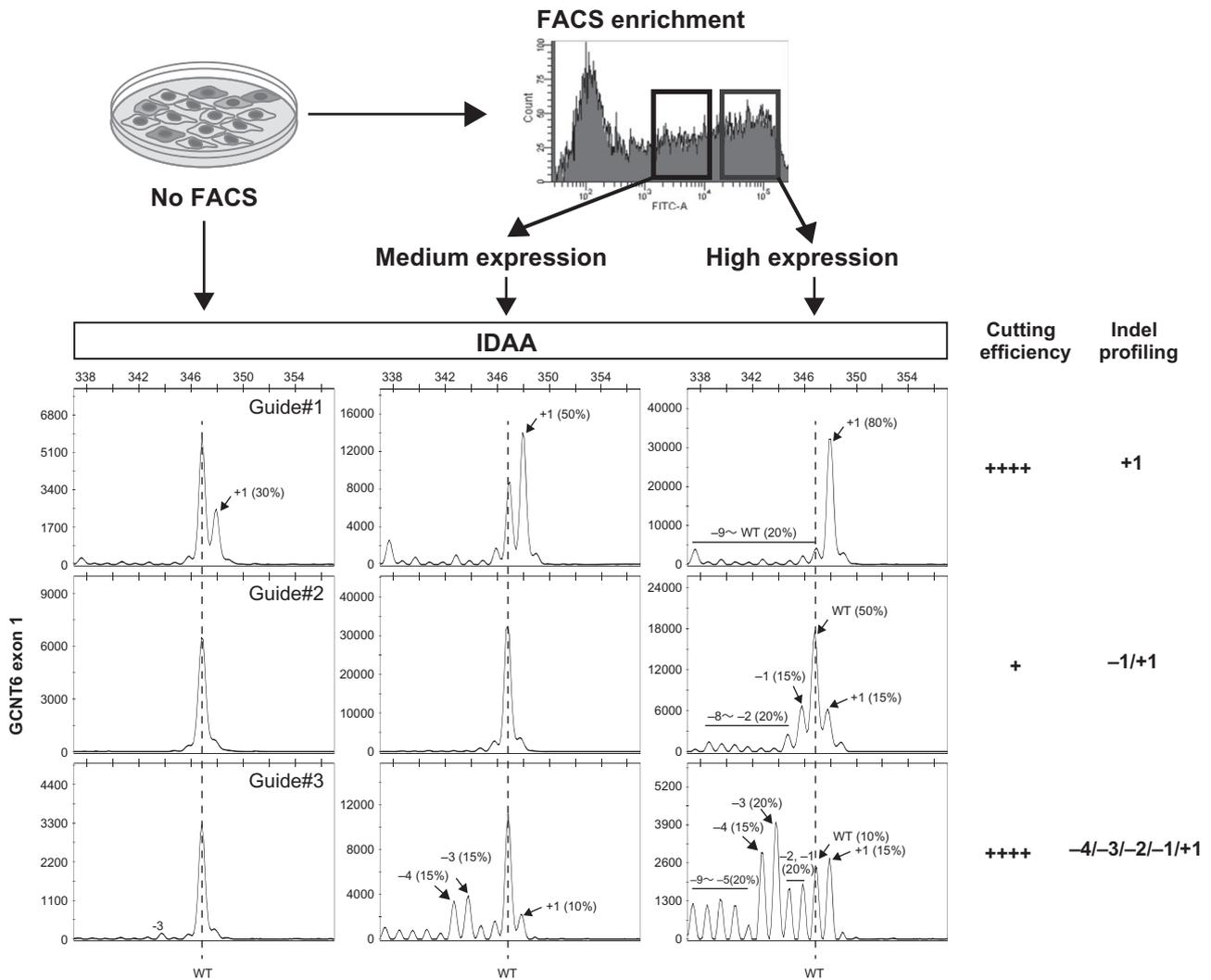


Fig. 3. Schematic illustration of the gRNA selection and validation strategy using FACS enrichment and IDAA monitoring. Shown are IDAA profiles for three different QCgRNAs designs (Guide #1–3) monitored in the original pool of transfected HEK293T cells (48 h posttransfection), and after FACS enrichment for cells with medium or high Cas9-GFP expression. The target gene was *GCNT6*, and Guide#1 displayed the highest cutting efficiency with a rather homogenous +1 indel, which was easily detectable even in the original cell pool. Guide#2 displayed low efficiency essentially only detectable after enrichment for high GFP expression, while Guide#3 displayed high efficiency but rather heterogeneous indel formation.

testing multiple guides to identify efficient gRNAs despite improved prediction algorithms; (ii) need for expertise in design of targeting regions in GTf genes to ensure functional inactivation; (iii) need for facile screening and isolation of gene targeted cells with mono and/or bi-allelic inactivating indels; and (iv) need for expertise with translation of individual gene functions to the complex biosynthetic pathways of glycosylation. We believe the developed resource, that we name GlycoCRISPR, meets most of these needs. Importantly the framework for design and interpretation of gene targeting of the complex glycosylation pathways will continue to improve with wider use.

The efficiency and ease of the CRISPR/Cas9 tool for gene targeting makes this the clear choice for broad genome screening strategies, and predesigned gRNA targeting constructs for all human genes as well as entire CRISPR libraries designed for whole genome targeting are now available (Miles et al. 2016). However, the study of glycosylation in cells can be greatly optimized and facilitated by access to individual gRNA targeting constructs for all GTf-genes

that are pre-validated for high efficiency and simple indel formation (+/−1 bp) ensuring loss of function by in-exon frameshifts (Figure 3). Recent studies show that a given gRNA design gives rise to highly similar CRISPR/Cas9 induced indels in most commonly used cell lines, such as K562, HEK293 and iPSC (Paquet et al. 2016; van Overbeek et al. 2016; Lonowski et al. 2017). Thus, the use of validated gRNAs facilitates screening and cloning especially in combinatorial engineering experiments. Genetic targeting strategies to decipher functions in glycosylation pathways present special considerations and limitations. The phenotypic read-out is rarely the enzyme or protein encoded by the targeted gene as, e.g., illustrated in Figure 4, but rather changes in glycosylation and glycan structures. Complete inactivation of targeted genes is almost always required to affect glycosylation detectably (Stentoft et al. 2014), and it is often necessary to target multiple genes to affect glycosylation covered by overlapping isoenzymes and/or alternative pathways such as galactosylation, fucosylation and sialylation (Yang et al. 2015b). Probes to detect loss and gain of specific glycan structures

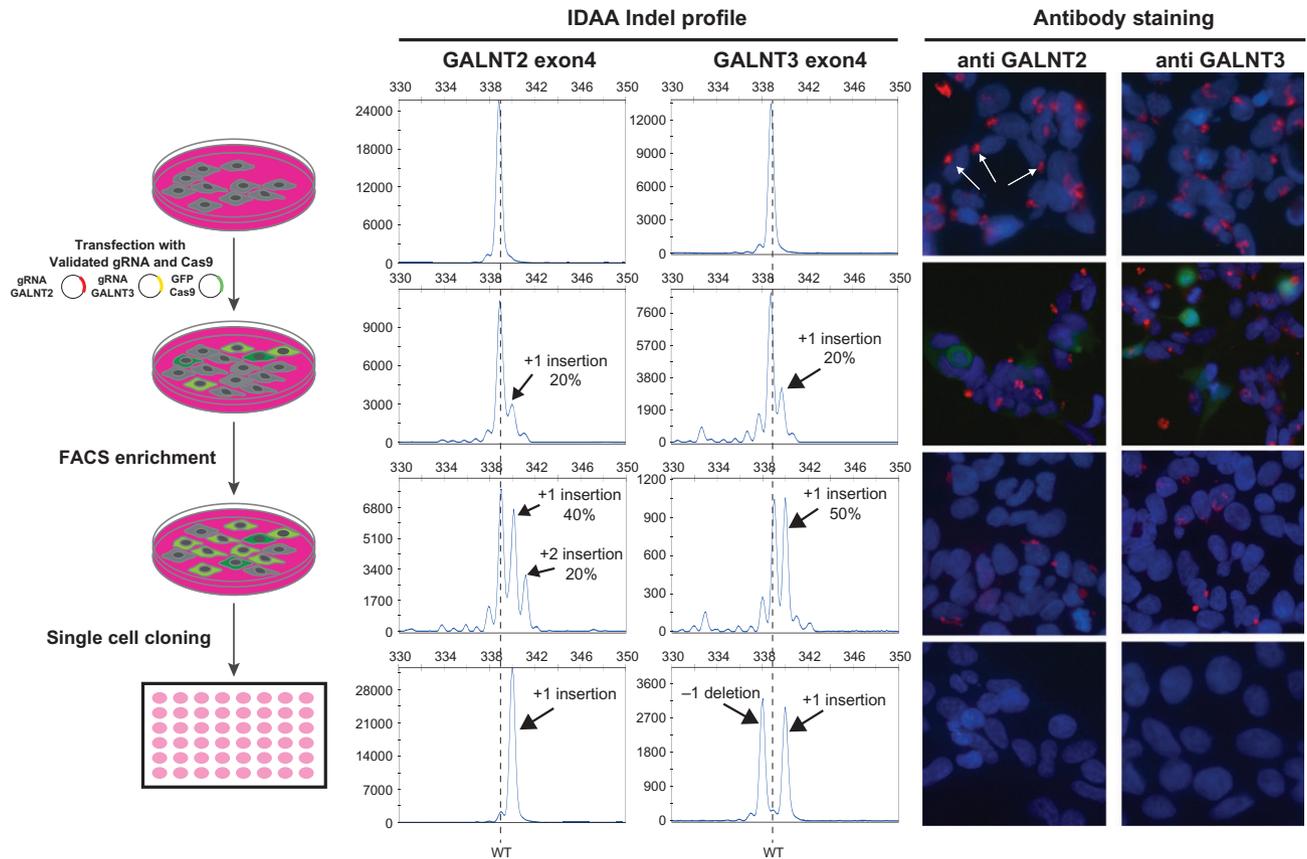


Fig. 4. Multiplex targeting of two GTf-gene genes in HEK293 cells monitored by IDAA and immunocytology. Simultaneous targeting of *GALNT2* and *T3* with validated GC-*GALNT2/3* plasmids and Cas9-GFP was monitored before transfection (upper panels), in the transfected cell pool (72 h posttransfection), after FACS enrichment for medium GFP expression, and after single cell cloning (lower panels). IDAA profiling demonstrated clear detection of indels in the cell pool with increase after enrichment, and in one final clone tested absence of wild type alleles. Immunocytology at each stage confirmed selective loss of protein expression in subpopulations of cells, and complete loss of expression of both enzymes in the final single cloned cell. GalNAc-T2 and T3 are endogenously expressed in HEK293 wild type cells with supranuclear Golgi-like localization (red color) as indicated by white arrows. Cas9-GFP expression (green) can be seen in the transfected cell pool after 72 h.

greatly simplify the task (Stentoft et al. 2011). This was elegantly illustrated by the whole genome screen for the Lassa virus glycan receptor in the THP-1 haploid cell line, which identified a large number of GTf-genes and other related enzymes acting in successive steps in biosynthesis of the uniquely complex glycan receptor for laminin on alpha-dystroglycan (Jae et al. 2013). In most cases probes are not available though, and in general comprehensive analysis of the glycome and glycoproteomes of cells are too daunting tasks to be used for screening and selection in larger gene engineering experiments (Thaysen-Andersen and Packer 2014; Rudd et al. 2017). Thus, access to a single validated gRNA targeting construct for every GTf-gene with facile IDAA protocols to confirm expected predefined indels that ensure frameshifts and inactivation, facilitates screening and cloning of single cells, and enables multiplex gene targeting as illustrated in Figure 4.

Targeting GTf-genes presents unique problems with prediction and interpretation of the outcome. Human cells have at least 15 distinct glycosylation pathways for the different types of glycoconjugates and the step-wise biosynthesis of these involve complex and often entangled biosynthetic steps. Figure 1 and Supplementary data, Figure S1 present a predicted global framework for the genetic regulation of these glycosylation pathways, which illustrates GTf-genes that are predicted to affect specific glycosylation pathways

and GTf-genes that are predicted to serve multiple pathways, or for which it currently is difficult to assign pathway specificity. The latter group is represented by GTf-genes mainly involved in elongation and capping of glycans. The framework further highlights the many families of paralogous genes encoding isoenzymes with related and potentially overlapping functions.

Clearly, this global view is predicted based on current knowledge and should be considered a project that will have to evolve over time and with additional data. Genetic dissection of glycosylation pathways with ZFNs, TALENs and CRISPR/Cas9 is only at its infancy (Stentoft et al. 2014), but it is clear that these tools are transforming glycosciences and opening up new levels of understanding of the genetic and biosynthetic regulation of glycosylation, new approaches to glycoproteomics, unprecedented capabilities for discovery and molecular dissection of biological functions of glycans, and eventually providing us with design control over cellular glycosylation. Thus, targeting genes that truncate one or more distinct glycosylation pathways is a fruitful strategy to produce homogeneous glycoproteomes suitable for simple enrichment strategies needed for sensitive glycoproteomics studies (Stentoft et al. 2011; Radhakrishnan et al. 2014; Vester-Christensen et al. 2013), as well as to define the involvement of individual types of glycoconjugates or glycosylation pathways in biological processes (Stolfa et al.

2016). Moreover, targeting individual isoenzymes expressed in a cell may provide insight into the non-redundant functions of these by comparative analysis of isogenic cell systems, as recently done with the large family of polypeptide GalNAc-transferase genes that initiate GalNAc-type O-glycosylation (Schjoldager et al. 2015). The strategy is also highly fruitful for discovery of novel GTf-genes, as illustrated by the discovery that the two POMTs do not serve to O-mannosylate cadherins and protocadherins (Larsen et al. 2017b), and further leading to discovery of four genes designated TMTC1-4 as novel protein mannosyltransferases (Larsen et al. 2017a). Finally, the gene engineering tools are making progress in custom engineered host cells like the Chinese hamster ovary (CHO) cell for recombinant production of therapeutic glycoproteins with defined glycosylation (Yang et al. 2015b).

In summary, we present a ready to use CRISPR/Cas9 gene targeting resource with pre-designed and validated gRNAs and complete protocols for use. Moreover, we present a first generation global map of the genetic regulation of glycosylation pathways in human cells that will facilitate design and interpretation of genetic engineering of glycosylation.

Materials and methods

gRNA design and gRNA amplicon expression cassettes (QCgRNA)

Three to four gRNA sequences for each of 186 human GTf-genes (Figure 1 and Table I) were designed using DESKGEN (<https://www.deskgen.com/landing/>). The gRNAs were incorporated into amplicon gRNA expression cassettes (QCgRNA) through a tri-primer amplification protocol as outlined in Figure 2. The tri-primer amplification included a universal forward primer (QCgFwd), a reverse primer (QCgRev) and gRNA encoding reverse primer (QCgX). The two downstream reverse primers QCgFwd and QCgRev overlap and in the latter case encode the invariant tracrRNA sequence. The QCgX primer encodes the variable gRNA sequence designed to be specific for the respective GTf gene targets. Importantly, the template used for QCgRNA amplification lacks the complete gRNA and tracrRNA sequences. Thus, QCgRNA tri primer amplification using EPB104 as template allows for specific gRNA design amplification determined by the variable QCgX primer included in the PCR assay. The QCgRNA amplicons include the U6 promoter upstream of the gRNA sequence and scaffolding tracrRNA elements were generated essentially as previously described (Lonowski et al. 2017). Only in a few cases (5) were adjustments needed to the standard conditions. All gRNA sequences and primers used are listed in Supplementary data, Table SI.

Screening gRNA designs by QCgRNA

HEK293T cells were used for gRNA design screening and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 2 mM L-glutamine. 1×10^5 cells were seeded into 24 well plates one day before the transfection. Two microliter unpurified QCgRNA amplicon was co-transfected with 1 μ g Cas9-2A-GFP (EPB105) using polyethyleneimine (PEI), as outlined in detail previously (Lonowski et al. 2017). Cells were harvested 48 h post-transfection and a fraction sorted and 50,000 cells with medium or high GFP expression collected as a pool. FACS was performed using a FACSAriaTM III cell sorter, using procedures recommended by the manufacturer (BD Bioscience, USA). The collected cell pool and unsorted pool were lysed with 30 μ L QuickExtractTM (Epicentre), and crude lysates analyzed for presence of indels by IDAA as

described in the following section "IDAA profiling". The best performing gRNA preferentially induced ± 1 bp indels for each GTf-gene target was selected, and the QCgRNA amplicon expression cassette sub-cloned into the pEPB104 plasmid (Addgene #68369) using EcoRI/KpnI restriction endonuclease sites. The validated GlycoCRISPR gRNA plasmid collection has been deposited at Addgene (<https://www.addgene.org/>, deposit#75008) where individual gRNA plasmids can be obtained (deposit#106682-106867).

IDAA profiling

To evaluate the in-del profile induced by the designed gRNAs we used the IDAA protocol recently reported (Lonowski et al. 2017). In brief, PCR was performed on 1 μ L crude cell pool lysates in 25 μ L volume with 5% DMSO, using TEMPase Hot Start DNA Polymerase (Amplicon, Denmark), and following concentrations 0.5 μ M:0.05 μ M:0.5 μ M of the three primers (universal 5'-labeled primer FamF:geneXF:geneXR). Primer sequences are supplied in Supplementary data, Table SIII. PCR was performed with a touchdown thermocycling profile based on an initial 72°C annealing temperature ramping down by 1°C/cycle to 58°C, followed by an additional 25 cycles using 58°C annealing temperature. Denaturation and elongation was performed at 95°C for 45 s and 72°C for 30 s, respectively. The tri-primer principle enables uniform labeling of amplicons, and thus allows for size discrimination based on fragment analysis using standard fragment analytical equipment. Efficient discrimination of fragments down to single base size is only supported by denaturing capillary electrophoretic conditions, which is supported by the ABI3130 instruments and later versions. 0.5 μ L of the PCR reaction or dilutions hereof was mixed with 0.05 μ L LIZ500 size standard (ABI/Life Technologies, USA), formamide and applied to fragment analysis using an ABI3500XL instrument (ABI/Life Technologies, USA) as recommended by the manufacturer. Raw data output was analyzed using Gene Mapper (ABI/Life Technologies, USA).

Multiplex GTf-gene targeting

HEK293 cells (2×10^5) were seeded in six well plates, and one day after 1 μ g of GC-GALNT2 and GC-GALNT3 validated plasmids were co-transfected with 1 μ g of Cas9-PBKS plasmid using Lipofectamine 3000. Cells were harvested after one day, and a fraction sorted for GFP by FACS. After one week of culture the FACS sorted cell pool was further single-sorted into 96-well plates and screened by IDAA and immunocytology again.

Immunocytology

HEK293 cells grown on sterile cover slides for one day were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 3% BSA, and incubated with monoclonal antibodies to human GalNAc-T2 (UH4 4C4) and T3 (UH5 2D10) followed by rabbit anti-mouse IgG TRITIC-conjugated antibodies (Dako), and mounted with Vectashield with DAPI (Vector labs) as described previously (Mandel et al. 1999; Steentoft et al. 2013). Fluorescence microscopy was performed using a Zeiss Axioskop 2 plus with an AxioCam MR3.

Supplementary data

Supplementary data is available at *Glycobiology* online.

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Conflict of interest statement

Authors declare conflict of interest related to ownership in GlycoDisplay ApS and patent applications submitted by University of Copenhagen.

Abbreviations

Gtf, glycosyltransferase; CRISPR, clustered regularly interspaced short repeats; gRNA, guide RNA; ZFN, zinc finger nucleases; TALEN, transcription activator-like effector nucleases; HEK293T, human embryonic kidney 293T; IDAA, indel detection by amplicon analysis; FACS, fluorescence activated cell sorting; QCgRNA, quick change guide RNA; HEK293 human embryonic kidney 293; iPSC, induced pluripotent stem cell.

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