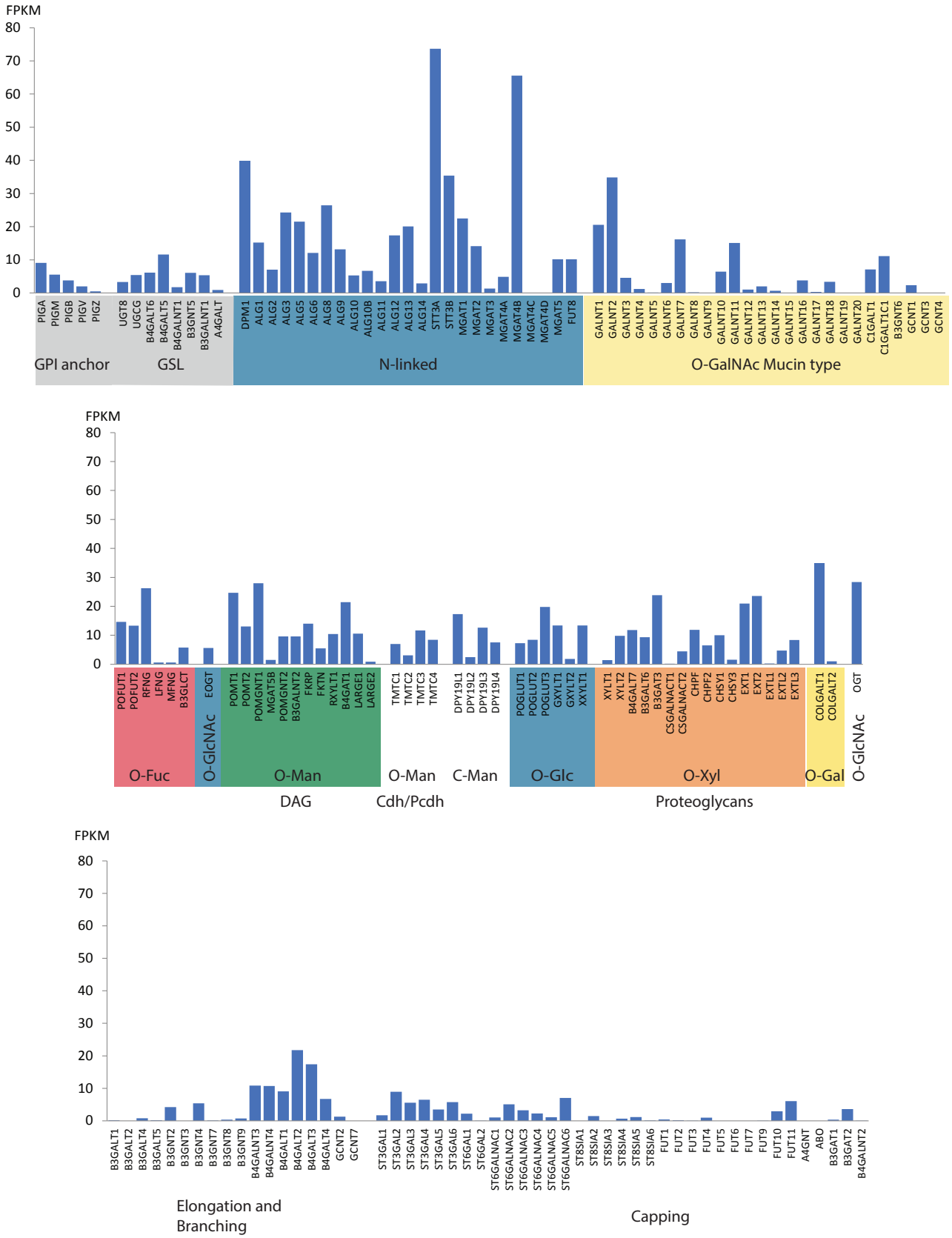


Figure S1. Summary of RNAseq Analysis of Glycosyltransferase Genes in HEK293 wildtype Cells, Related to Figure 1 Genes are organized according to pathways and the rainbow diagram in **Figure 1**.

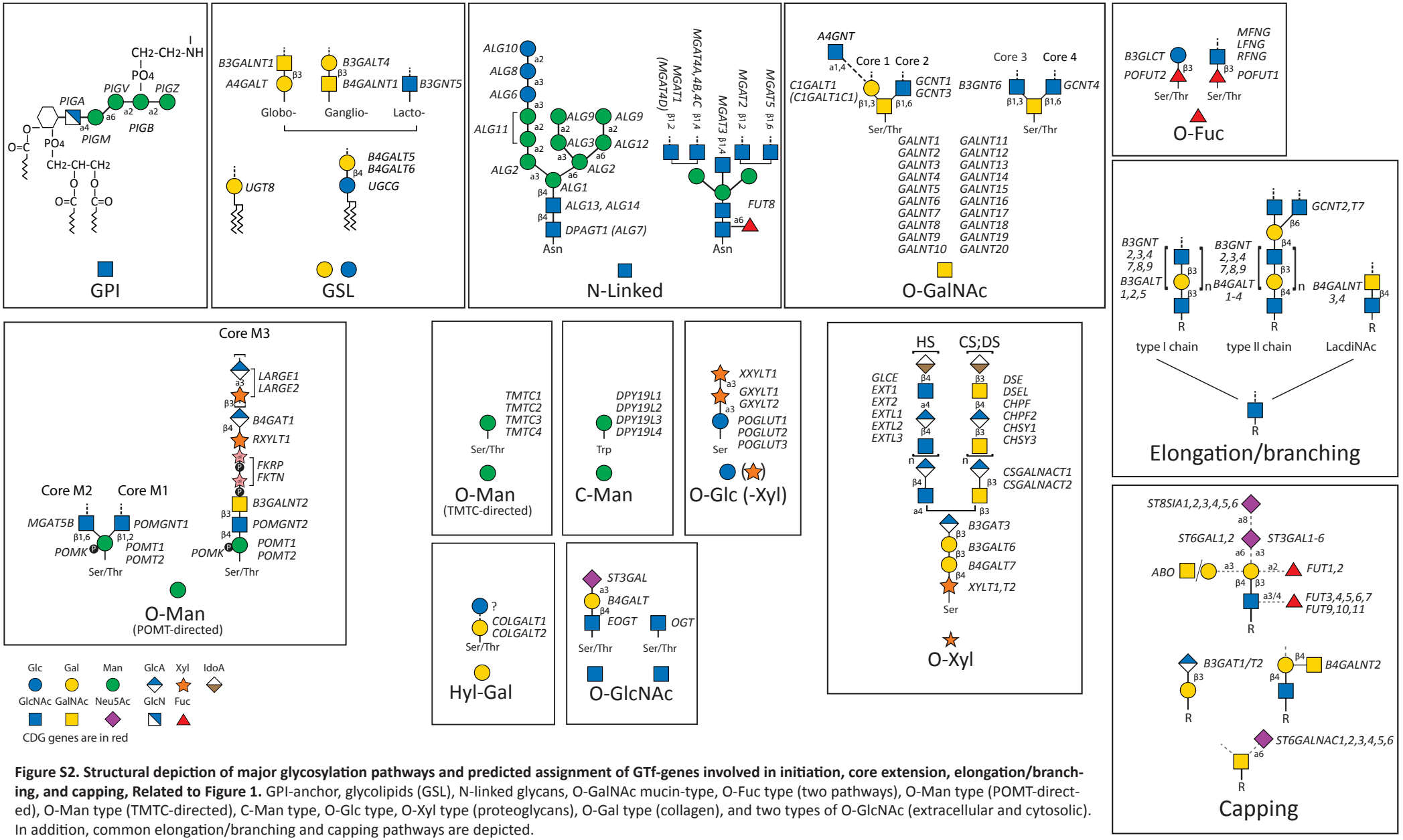
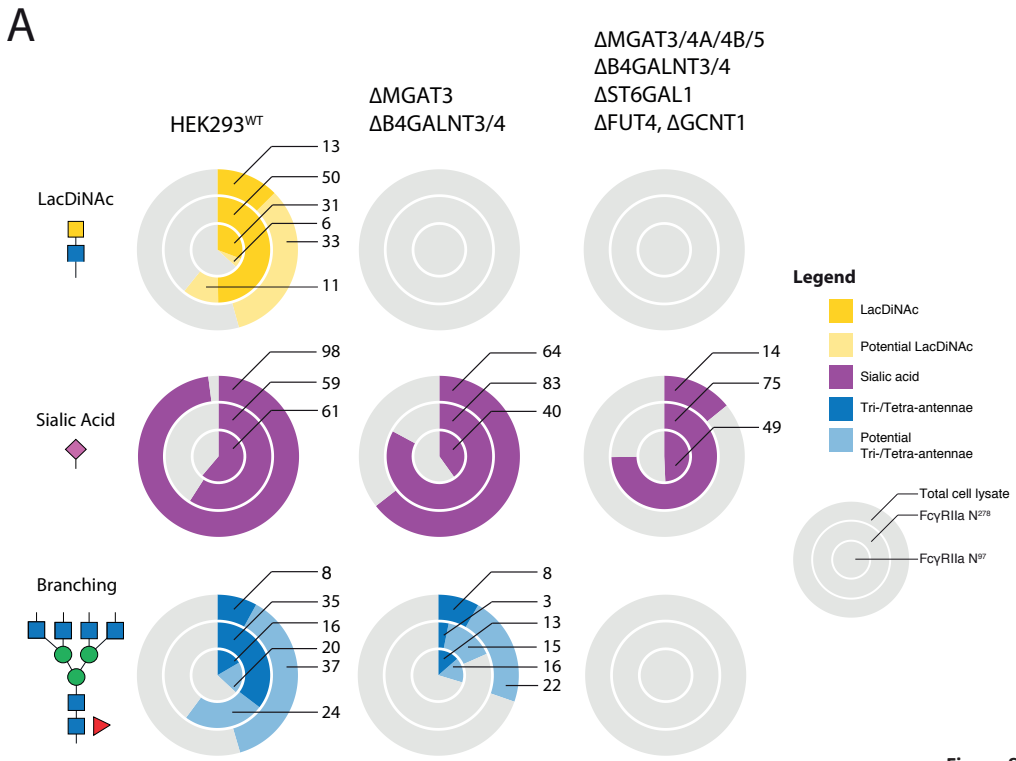
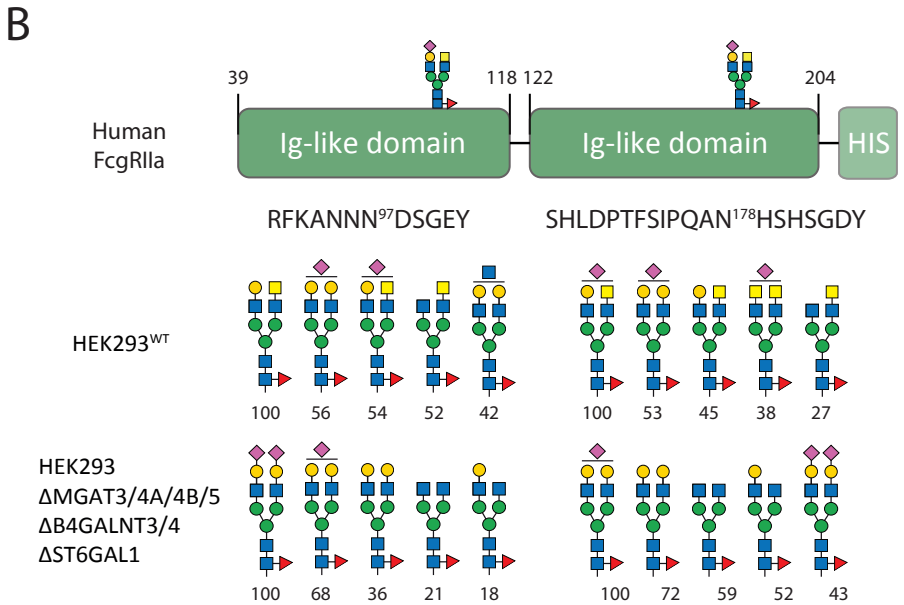


Figure S2. Structural depiction of major glycosylation pathways and predicted assignment of GTf-genes involved in initiation, core extension, elongation/branching, and capping, Related to Figure 1. GPI-anchor, glycolipids (GSL), N-linked glycans, O-GalNAc mucin-type, O-Fuc type (two pathways), O-Man type (POMT-directed), O-Man type (TMTC-directed), C-Man type, O-Glc type, O-Xyl type (proteoglycans), O-Gal type (collagen), and two types of O-GlcNAc (extracellular and cytosolic). In addition, common elongation/branching and capping pathways are depicted.





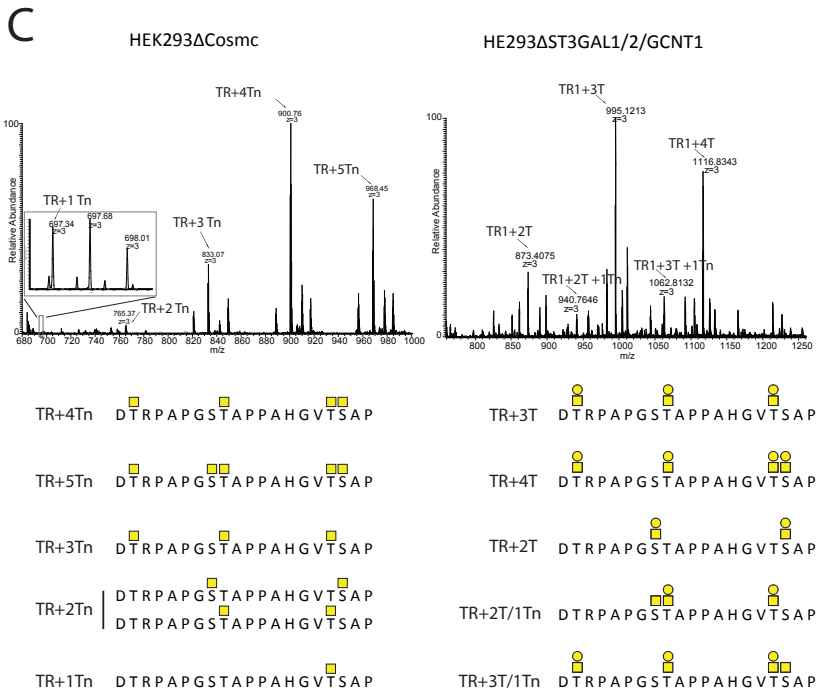


Figure S3. LC-MS Analysis of N- and O- Reporter Constructs, Related to Figure 2

(A) Graphic summary of N-glycan profiles of total cell lysate (TCL) and site-specific N-glycan analysis of secreted FcgRIIa reporter expressed in HEK293 WT and engineered isogenic cells as indicated by KO design (Δ). Ring charts illustrate the abundance of the indicated glycan features (LacDiNAc, sialic acid, and N-branching) of N-glycans in TCL (outer circle) and at the two N-glycosites (N97 and N178) of secreted FcgRIIa (middle and inner circle, respectively). The presence of LacDiNAc (top), sialic acid (middle) and tri/tetraantennary branched N-glycans are shown for structures with abundance higher than 10% of the most abundant structure. LacDiNAc and branching features were predicted from the compositional analysis, and most asignments were validated by predictable outcomes of the gene engineering performed. For the TCL results we excluded all high mannose and hybrid structures. (B) Illustration of the site-specific N-glycan analysis of the secreted FcgRIIa reporter expressed in HEK293 WT and an engineered isogenic cell without capacity for LacDiNAc and α2-6SA glycosylation. N-glycan structures are shown with their relative abundance normalized to the most abundant structure. (C) Full MS spectra of MUC1 tandem repeats (TR) released by endo-Asp from the MUC1 reporter expressed in engineered HEK293 with KO of COSMC or combinatorial KO of ST3GAL1/2/GCNT1. The 5 most intense MUC1-TRs glycopeptide precursors were applied to MS/MS ETD analysis and identified glycosites and O-glycan structures illustrated. Tn (GalNAc) and T (Gal-GalNAc). All annotated glycan structures are described in Supplementary Table S3.

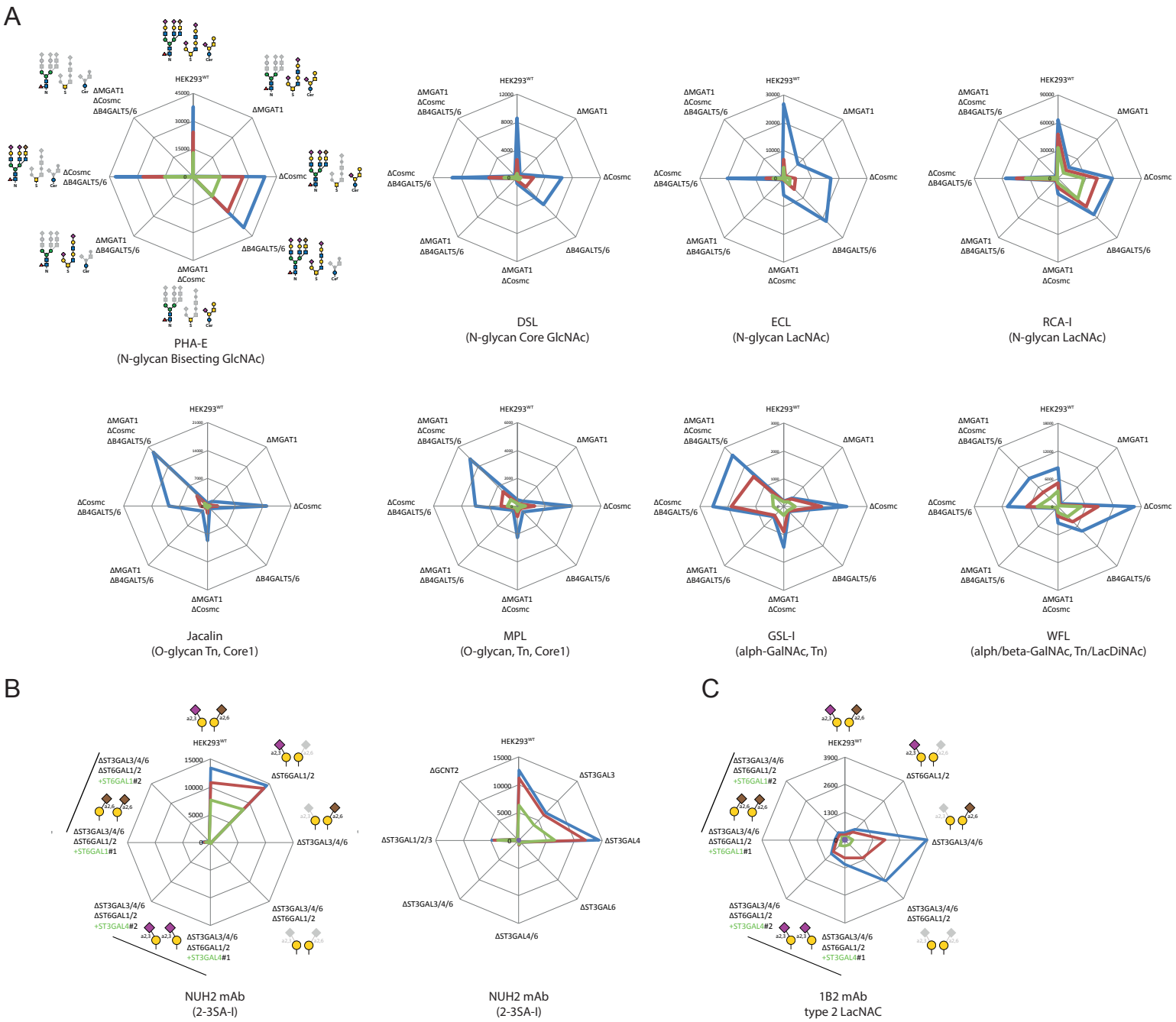


Figure S4. Flow Cytometry Analysis With Plant lectins and mAbs, Related to Figure 3

(A) Analysis of the glycoconjugate sublibrary 1 with eight lectins (PHA-E, RCA-1, ECL, DSL, GSL-I, WFL, Jacalin and MPL) at different concentrations (2, 1, 0.5 µg/mL color coded).
(B-C) Analysis of the Capping sublibrary 7 including ΔGCNT2 with mAbs NUH2 and 1B2.

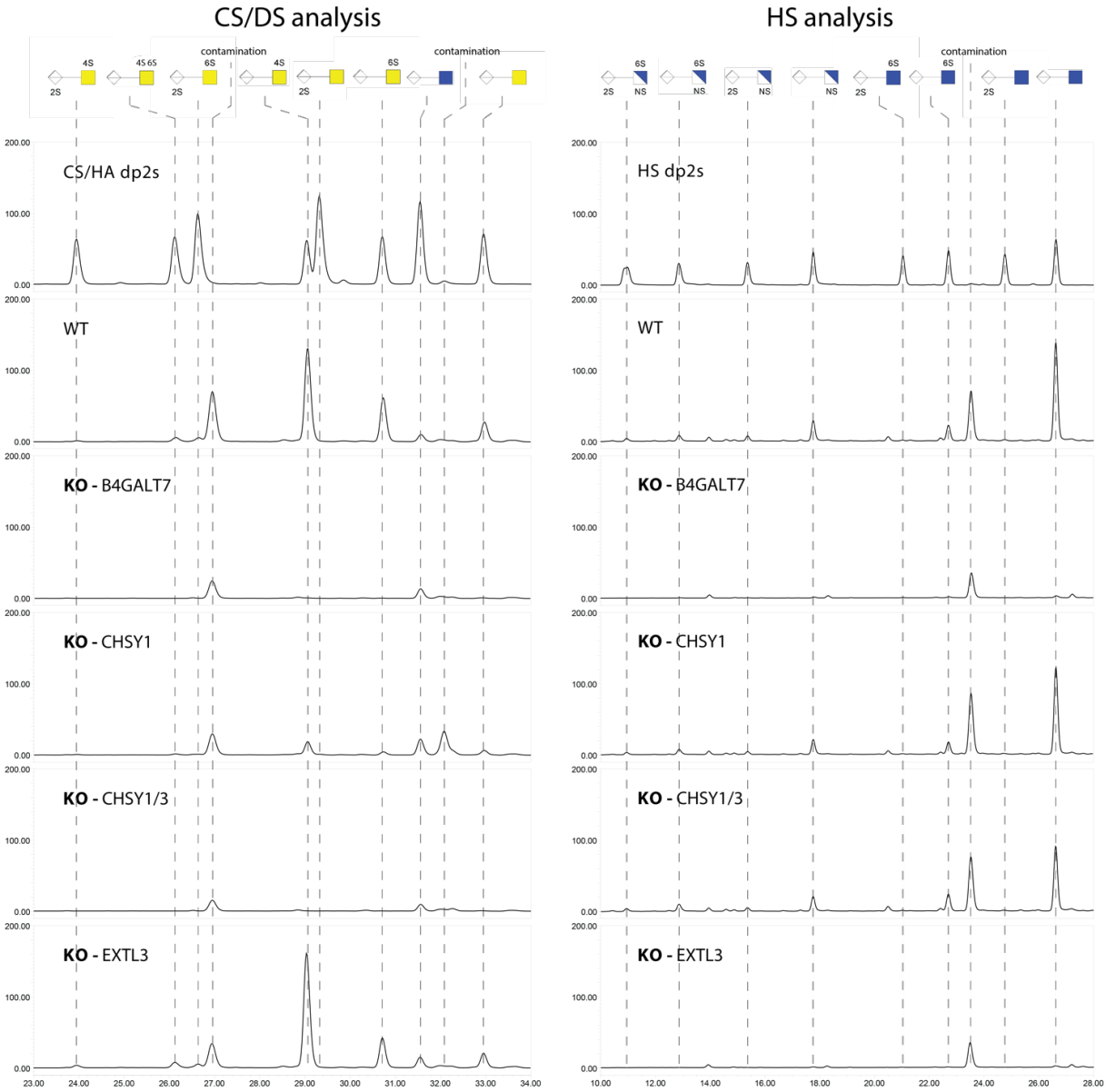


Figure S5. HPLC Disaccharide Analysis of Isolated GAGs From Total Cell Lysates, Related to Figure 2. HPLC traces for CS/DS (left panels) and HS (right panels) are shown. Equal amounts of AMAC-labelled disaccharides based on cell quantity were injected. Top row shows peaks for disaccharide standards (20 pmol injected for each). The four examples of GAG gene engineering performed to eliminate GAG biosynthesis (KO B4GALT7), to eliminate CS/DS biosynthesis (KO CHSY1/CHSY3), and to eliminate HS biosynthesis (KO EXTL3) are shown.

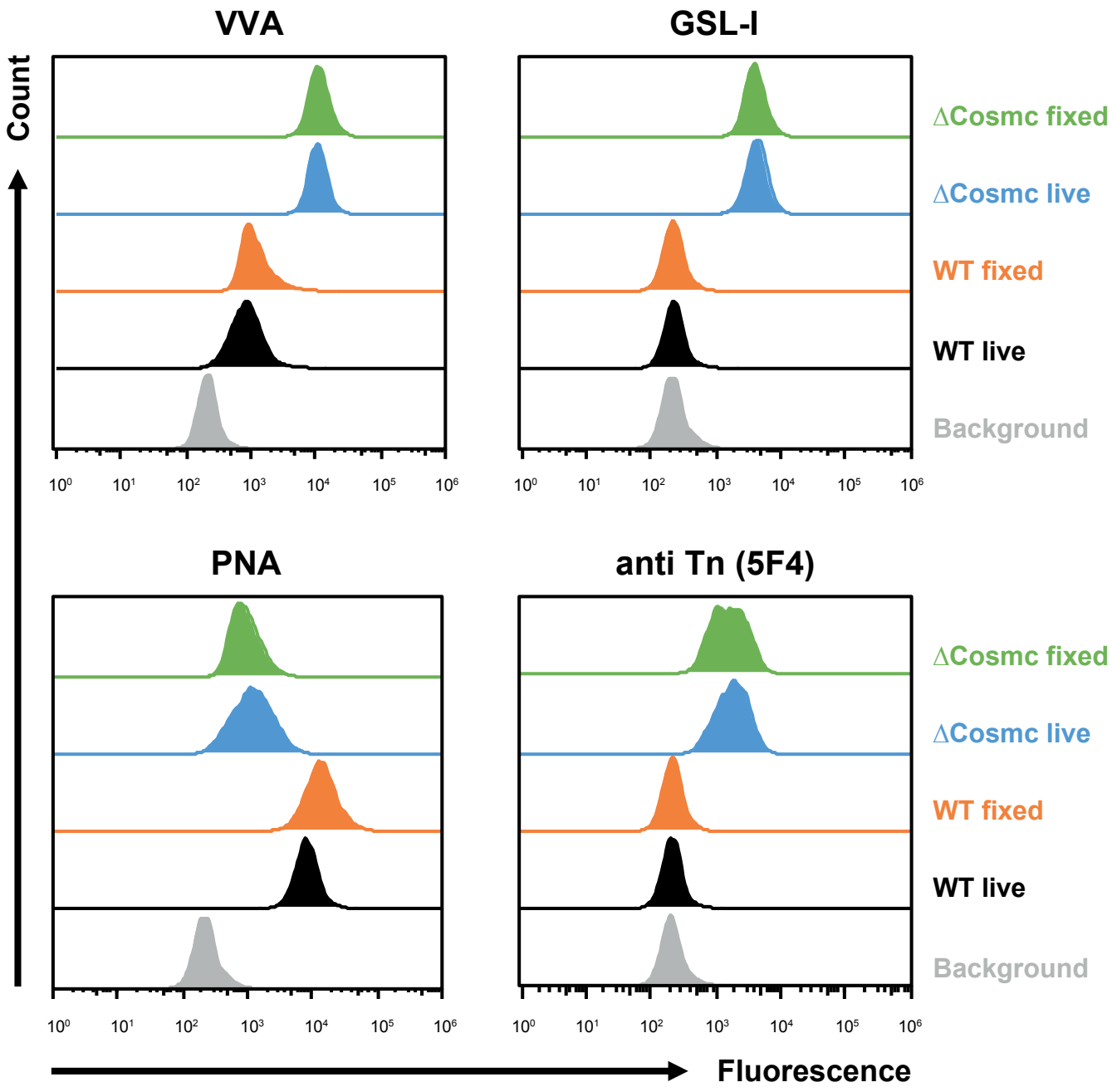


Figure S6. Representative histograms show lectin and antibody binding to fixed and live HEK293^{WT} and HEK293$^{\Delta COSMC}$ cells, Related to Figure 2.

Flow cytometry analysis of live and fixed cells. HEK293^{WT} and HEK293$^{\Delta COSMC}$ cells were fixed with 2% paraformaldehyde for 10 minutes at room temperature, thoroughly washed, and frozen in freeze medium (DMEM containing 50% fetal bovine serum and 10% DMSO). Cryopreserved cells were thawed and stained with biotinylated lectins VVA, GSL-I and PNA or anti-Tn antibody (5F4) followed by conjugation to streptatvidin-AF647 or anti-mouse Ig-AF647, respectively. Corresponding live cells were used as control.