Display of the human mucinome with defined O-glycans by gene engineered cells

Rebecca Nason1,11, Christian Büll1,11, Andriana Konstantinidi1, Lingbo Sun1, Zilu Ye1, Adnan Halim1, Wenjuan Du2, Daniel M. Sørensen1, Fabien Durbesson3, Sanae Furukawa1, Ulla Mandel1, Hiren J. Joshi1, Leo Alexander Dworkin1, Lars Hansen1, Leonor David4,5, Tina M. Iverson6, Barbara A. Bensing7, Paul M. Sullam7, Ajit Varki8, Erik de Vries2, Cornelis A. M. de Haan2,2, Renaud Vincentelli3, Bernard Henriassat1,3,9, Sergey Y. Vakhrushev1,1, Henrik Clausen1,10 & Yoshiki Narimatsu1,10

Mucins are a large family of heavily O-glycosylated proteins that cover all mucosal surfaces and constitute the major macromolecules in most body fluids. Mucins are primarily defined by their variable tandem repeat (TR) domains that are densely decorated with different O-glycan structures in distinct patterns, and these arguably convey much of the informational content of mucins. Here, we develop a cell-based platform for the display and production of human TR O-glycodomains (~200 amino acids) with tunable structures and patterns of O-glycans using membrane-bound and secreted reporters expressed in glycoengineered HEK293 cells. Availability of defined mucin TR O-glycodomains advances experimental studies into the versatile role of mucins at the interface with pathogenic microorganisms and the microbiome, and sparks new strategies for molecular dissection of specific roles of adhesins, glycoside hydrolases, glycopeptidases, viruses and other interactions with mucin TRs as highlighted by examples.
NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-24366-4
ARTICLE

nature’s overarching solution for fulfilling the need for symbiosis with a vast community of microorganisms—our microbiomes—are mucins\(^{13,14}\). Mucins in the gut constitute the primary barrier as well as the ecological niche for the microbiome. Dynamic replenishment of mucin layers provides a constant selection of the resident microbiome through adhesive interactions, and degradation of mucin O-glycans by members of the microbiota supply nutrients\(^{6-8}\). Mucins are a large family of heavily glycosylated proteins that line all mucosal surfaces and represent the major macromolecules in body fluids\(^9\). Mucins clear, contain, feed, direct, and continuously replenish our microbiomes, limiting unwanted co-habitation and representing harmful pathogenic microorganisms\(^7\). Recent studies show that specific mucins can disperse biofilms and disrupt bacterial aggregation\(^8\), but the molecular basis of these effects remains largely obscure. Mucin O-glycans present the essential binding opportunities and informational cues for microorganisms via adhesins, however, our understanding of these features is essentially limited to results from studies with simple oligosaccharides without the protein context of mucins and the higher-order features presented by dense O-glycan motifs. Mucins are notoriously difficult to isolate due to their size and heterogeneity, and production by recombinant expression in cell lines is impeded due to difficulties with the assembly of full coding expression plasmids often resulting in heterogeneous products\(^9\).

State-of-the-art technologies to capture the informational content of mucins are confined to studies with synthetic and isolated O-glycans\(^{10}\), synthetic and chemoenzymatically produced short glycopeptides\(^{11}\), and synthetic glycopeptides as well as non-natural polymers\(^{12,13}\); all of which are rare commodities that do not reflect the complex information captured in distinct human mucins by their display of patterns and structures of O-glycans. With the advent of the facile nuclease-based gene engineering technologies, it has become possible to engineer mammalian cells with combinatorial knockout (KO) and knockin (KI) of glycosylation genes to display subsets and distinct features of the glycome on the cell surface or on secreted reporter proteins in order to probe biological interactions dependent on glycans\(^{14-16}\). Genetic engineering provides opportunities for interpretation and dissection of the glycosylation genes, biosynthetic pathways, and structural features required for identifiable interactions with the cell library\(^{17}\). Importantly, such cell-based display strategies allow for the presentation of glycans in the natural context of glycoproteins and the cell surface, and this has provided the first experimental evidence for the existence of higher-order binding motifs consisting of O-glycans in dense patterns\(^{15}\).

There are at least 18 distinct mucin genes encoding membrane or secreted mucins\(^{18}\). The large gel-forming secreted mucins may form oligomeric networks or extended bundles through inter- and intramolecular disulfide bridges in the C- and N-terminal cysteine-rich regions\(^2\). A common characteristic of all mucins is that the major part of their extracellular region comprises a variable number of imperfect tandem repeated (TR) sequences (also called PTS sequences) that carry dense O-glycans (Fig. 1), with the notable exception of MUC16 that contains a large, densely O-glycosylated N-terminal region without TRs\(^{19,20}\). Arguably, the main cues for the microbiota lie in the TR regions that display O-glycans of diverse structures and positions in unique patterns. Interestingly, the TR regions appear poorly conserved throughout mucins, yet our microbiomes, limiting unwanted co-habitation and representing harmful pathogenic microorganisms. The TR regions appear poorly conserved throughout mucins, yet our microbiomes, limiting unwanted co-habitation and representing harmful pathogenic microorganisms. Dissecting selected mucin microorganisms revealed that the TR regions are imperfect in sequence but present characteristic patterns of O-glycans. The mucin TRs and their glycocodes may be considered the informational content of mucins and thus comprise the mucinome. The TR mucinome provides a much greater potential binding epitome than the comparatively limited repertoire of binding epitopes comprised of simple oligosaccharide motifs available in humans\(^3\).

Here, we sought to capture the molecular cues contained in human mucin TRs and enable molecular dissection of these cues. We developed a cell-based platform for the display and production of representative mucin TRs with defined O-glycans. We reasoned that most of the features of human mucin TRs could be displayed in shorter segments of 150–200 amino acids, and used a GFP-tagged expression design containing representative TRs from different mucins to produce a library of the cell membrane and secreted mucin TR reporters in human embryonic kidney (HEK293) cells with distinct programmed O-glycosylation capabilities. Strikingly, we found that these mucin TR reporters could readily be produced as highly homogeneous molecules with essentially complete O-glycan occupancies and with distinct O-glycan structures in amounts that enabled us to characterize the simplest reporters by intact mass spectrometry (MS), and hence circumvent the longstanding obstacles with protease digestion and bottom-up analysis of mucins\(^{24,25}\). We demonstrate the utility of the cell-based mucin display through probing and dissecting selective mucin TR binding specificities of microbial adhesins, and the power of making defined mucin TRs available through analysis of the substrate specificities of microbial glyco pepidases, as well as by demonstrating selective binding by an influenza virus. These findings widely open the mucin and microbiome fields for studies with libraries of homogeneous mucin TR glycodomains, and for entirely new approaches to test and dissect the biophysical properties and the informational content of human mucins. Moreover, the ability to produce large quantities of mucin TR glycodomains provides a substantial first step toward sustainable manufacturing of natural mucin polymers and exploration of their therapeutic opportunities.

Results

Engineering strategy for the display of the human mucinome. Figure 1 presents an overview of the concept for the cell-based display and production of human mucin TR reporters with programmed O-glycan structures. The mucin TR reporters were designed pairwise for either secretion or cell membrane integration through the inclusion of the C-terminal SEA and transmembrane domain of MUC1, and they all included N-terminal GFP, and FLAG tags\(^{15}\). We generated a comprehensive set of TR reporters containing approximately 200 amino acids from the TR O-glycodomains of most human secreted and membrane-bound mucins (Fig. 1). The entire sequences selected as representative for each of the human mucin TR O-glycodomains are shown in Supplementary Fig. 1 and Table 1, which also illustrates that mucin TRs are imperfect in sequence but present characteristic patterns of O-glycans. Most of the mucins contain multiple TR sequences and for the mucins MUC2, MUC3, MUC5B, and MUC6 multiple TR reporters were expressed and analyzed.

The transmembrane TR reporters were expressed transiently in glycoengineered HEK293 cells that do not appear to express endogenous mucins, and the secreted reporters were expressed stably\(^{15,26}\). We took advantage of our previously reported O-glycoengineering strategy to establish designs for homogeneous
O-glycosylation capacities that result in the attachment of defined O-glycan structures (Fig. 1). The gene engineering of HEK293 cells included designs for O-glycans designated Tn (KO C1GALT1), STn (KO COSMC/KI ST6GALNAcI), T (KO GCNT1, KO ST3GAL1/2, ST6GALNAC2/3/4), monosialyl-T (ST) (KO GCNT1, ST6GALNAC2/3/4), as well as DiST comprised of a mixture of mSTa and disialyl-T (dST) (KO GCNT1) (Supplementary Table 2). We engineered capacity for core3 (GlcNAcα1-3GalNAcα1-O-Ser/Thr) O-glycosylation by using AAVS1 locus targeted KI of the core3 synthase (B3GNT6) on top of KO of COSMC to eliminate competition from the core1 synthase. To further customize the O-glycan occupancy on mucin TRs, we used KO of GALNT4 and GALNT7/10. The current state of the HEK293 O-glycoengineered cell libraries is listed in Supplementary Table 2.

Validating the cell-based mucin TR display platform. We previously verified the general glycosylation outcomes of most of the glycoengineering performed in HEK293 cells. Here, we tested a subset of transiently expressed membrane-bound TR reporters with lectins and monoclonal antibodies (mAbs) with well-characterized specificities for distinct O-glycan structures (Supplementary Fig. 2a, b). There was a substantial window of signal difference in flow cytometry for HEK293 cells with and without expression of the GFP-tagged mucin TR reporter. Thus, the engineered glycosylation capacity for Tn, T, and STn O-glycans are shown with symbols drawn according to the Symbol Nomenclature for Glycans (SNFG) format98.
O-glycosylation could be shown both with the cell population not expressing the mucin TRs (GFP negative) and the transfected cell population expressing these (GFP positive), albeit with higher intensities when mucin TRs were expressed.

We also probed the mucin TR reporters with a panel of mAbs directed to human mucin TR regions, most of which are known to be affected by O-glycosylation either because glycosylation interferes with or blocks binding to the protein core (e.g., mAb to MUC1 such as SM3 or 5E10) or because O-glycans are required for the binding (e.g., mAbs to Tn-MUC1 (5E5), Tn-MUC2 (PMH1), and Tn-MUC4 (3B1)) (Supplementary Fig. 2c). The observed reactivity patterns were in agreement with the reported specificities of the tested mAbs. We also screened a larger panel of available anti-MUC1 mAbs for reactivity with different glycoforms of MUC1, which confirmed specific reactivities with defined glycoforms (Tn-MUC1 for 5E5 and 2D9; T-MUC1 for 1B9)29, while the binding of antibodies to the PDTR peptide region (SM3, HMGF1, and 5E10) revealed different glycoform selectivities (Supplementary Fig. 2d).

**Structural analysis of secreted mucin TRs.** Secreted TR reporters stably expressed in glycoengineered HEK293 cells were isolated by Ni-chromatography and assessed by SDS-PAGE analysis, which showed that the GFP-tagged proteins migrated as distinct rather homogeneous bands (Supplementary Figs. 3 and 4). We used LysC digestion to liberate the intact TR O-glycodomains and C4 and C18-HPLC to purify these for further analysis (Supplementary Fig. 5). For direct intact mass analysis of mucin TRs, we used pretreatment with neuraminidase to reduce complexity and facilitate deconvolution and interpretation.

The dense O-glycosylation of mucin TRs in most cases blocks cleavage by peptidases limiting conventional glycoproteomics strategies. However, the MUC1 TRs are cleavable by endoprotease-Asp-N (AspN) in the PDTR sequence24–36, and we, therefore, used the MUC1 reporter for full characterization (Fig. 2). The MUC1 reporter contains 34 predicted O-glycosites and includes six 20-mer TRs and a C-terminal TR, where the last GVTSA sequence proceeds into the 6xHis tag. We used LysC to cleave the purified GFP-tagged reporter and isolated the TR O-glycodomain for LC–MS intact MS analysis (Fig. 2a). The simplest Tn glycoform (HEK293KO CIGALT)1 revealed a rather small range of incremental masses corresponding to HexNAc (203.08) centered around the predicted protein size (m/z 14,902.14) with 28-35 HexNAc residues, while the T (HEK293KO GCNT1, ST3GAL1/2,ST6GALNAC2/3/4) and ST (HEK293KO GCNT1) glycoforms after neuraminidase treatment generated the same narrow range of predicted 28-35 Hex-HexNAc disaccharides. In contrast, the STn glycoform (HEK293KO CIGALT1 KI ST6GALNAC1) analyzed after treatment with neuraminidase produced a slightly broader range of detectable glycoforms from 18-35 HexNAcs, suggesting that ST6GALNAC1 competes partly with the completion of GalNAcO-glycosylation by GalNt1, in agreement with previous studies.37,38 Analysis of the MUC1 TR reporters after AspN digestion revealed that the predominant 20-mer glycopeptides derived from Tn-MUC1 and STn-MUC1 were those with 4-5 O-glycans per TR (Fig. 2b). For the bottom-up analysis, we were required to use pretreatment with neuraminidase because the sialylated glycoforms were poorly digested by AspN. For the MUC1 reporters with higher glycan complexity (T-MUC1 and ST-MUC1) the most abundant glycopeptide variants appeared to be shifted toward 3–4 O-glycans per TR (Fig. 2b), however, this result may be biased by inefficient AspN digestion since the intact MS analysis did not show the same tendency (Fig. 2a).

As an illustrative example for the ability to engineer the occupancy of O-glycans in mucin TRs, we analyzed the MUC1 TR reporter produced in cells with and without KO of GALNT4 (HEK293KO COSMC, GALNT4) (Supplementary Fig. 6). KO of GALNT4 resulted in reduced HexNAc incorporation with the major proteoform centered around 28 HexNAc residues indicating loss of 6–7 O-glycans, which is in agreement with previous in vitro studies and predicted to represent the loss of glycosylation in the PDTR sequence motifs. Finally, we confirmed the glycoengineering by O-glycan profiling of released O-glycans from the MUC1 TR reporters with four different O-glycan designs by MALDI-TOF analysis (Supplementary Fig. 7).

The promising results obtained with intact MS analysis of the MUC1 TR glycodomains prompted intact MS analysis of the simplest Tn glycoforms of MUC2, MUC5AC, MUC7, MUC13, and MUC22 TR glycodomains, which showed similarly high occupancy of available glycans with rather homogeneous patterns (Fig. 3). For most mucin TRs the proteoform with the highest number of HexNAc residues correlated with the number of potential O-glycosites with the most abundant proteoforms centered close to or a little lower than this. However, for the MUC22 TR reporter the highest abundant peaks were centered around 68–71 with 83 potential O-glycosites suggesting lower occupancy.

Interestingly, we noted minor molecular species with an apparent excess of one HexNAc compared to the total number of Ser/Thr potential O-glycosites, and we anticipate that this is due to a very low degree of HexNAc-HexNAc disaccharide incorporation. This may provide a basis for the existence of GalNAc-GalNAc O-glycans as reported in human meconium40. Similarly, we observed the appearance of low amounts of fucose on mucin TRs expressed in HEK293 cells with KO of GCNT1 and ST3GAL1/2, presumably related to minor capping with blood group H (Supplementary Fig. 7).

**Dissection of the binding specificities of Streptococcal Siglec-like adhesins.** We previously used the cell-based glycan array platform to dissect the binding specificities of two Siglec-like binding regions (BRs) of serine-rich repeat adhesins expressed by oral streptococci, and demonstrated distinct binding specificities of Streptococcus gordonii (HsaBR) for mSTa and Streptococcus mitis (10712BR) for disialylated core2 O-glycans displayed on a reporter containing the mucin-like domain of GPI1ba (Fig. 1).15,31,42,43 We also found evidence that these adhesins showed binding selectivity for mucin TRs expressed in HEK293WT, and to explore these findings further we here included binding studies with the preferred O-glycan structures on different mucin TRs (Fig. 4a). We compared the expression of TRs with core2 O-glycans (HEK293WT) and mSTa O-glycans (HEK293KO GCNT1, ST6GALNAC2/3/4), which replicated highly select mucin TR binding specificities, and also surprisingly uncovered that O-glycosylation of some mucin TRs do not follow the general glycosylation capacities. Importantly, membrane expression levels were comparable between mucin TR reporters and between isogenic cell lines as confirmed by anti-FLAG staining (Supplementary Fig. 8a). Thus, we found that the selective binding of HsaBR to MUC2α1, MUC7, and GPI1ba was unaffected by limiting O-glycans to mSTa (KO ST6GALNACs), while binding, especially to GPI1ba, was markedly increased. Moreover, the binding of 10712BR to GPI1ba was abolished by limiting O-glycans to mSTa since this removes the preferred core2 O-glycan structures. This suggests that the mucin TR sequences to some extent affect the structural outcome of O-glycosylation with respect to modifications of the innermost GalNAc residue, i.e., core2 branching by addition of β1-6GalNAc (directed by GCNT1) and/or sialylation by addition of sialic acid (α2-6Neu5Ac) (directed by ST6GALNACs). The most remarkable finding was that the MUC2 TRs (especially the MUC2 region...
Fig. 2 Mass spectrometry analysis of secreted MUC1 TR reporter O-glycoforms. 

**a** Deconvoluted intact mass spectra of secreted, purified MUC1 reporter produced in HEK293KO C1GALT1, HEK293KO COSMC, KI ST6GALNAC1, HEK293KO GCNT1, ST3GAL1/2, and HEK293KO GCNT1 cells. Reporters were treated with neuraminidase to remove sialic acids and reduce complexity, and digested by Lys-C followed by HPLC C4 isolation yielding the 157 amino acid MUC1 TR O-glycodomain fragment. All MUC1 O-glycoforms (Tn and T) showed a rather homogeneous mass comprising 32–35 HexNAc/HexHexNAc residues, and with 33 or 34 HexNAc/HexHexNAc being the most abundant peak. For all intact mass spectra experimentally determined and theoretically calculated masses were composed in Supplementary Table 4.

**b** Site-specific O-glycopeptide LC-MS/MS analysis of MUC1 reporters after AspN digestion.
designated #1) had a relatively high proportion of core1 O-glycans with only α2-3 linked sialic acids (mSTa) installed in HEK293WT cells, while most other mucin TRs displayed a mixture of core2 and dST (Fig. 4b).

We also tested another Siglec-like BR, Streptococcus gordonii GspBBR, which like HsabR binds to ST, but showed a very different binding pattern with the highest binding to MUC7 and MUC22 TRs (Fig. 4a). Similar to HsabR, this binding was increased when these reporters were expressed in HEK293KO GCNT1, ST6GALNAC2/3/4 with homogenous mSTa O-glycosylation. Collectively, our results support the notion that the Siglec-like adhesins recognize specific O-glycans, and that this recognition is co-determined by the context provided by the mucin TR sequence. While further studies are still needed, it is likely that recognition of clustered patches or patterns of multiple O-glycans is involved. Simple multivalent presentation of O-glycans does not seem to be a major determining factor since all the TR reporters studied are predicted to present multiple closely spaced O-glycans. Mucin TRs and mucin-like domains such as the stem region of GP1bα are characterized by dense O-glycosylation, and the positions and patterns of the O-glycan decoration is determined by the peptide sequence (distribution of Ser/Thr residues) and the specificities of the available polypeptide GalNAc-transferases (GALNT1-20) that control the initiation of O-glycosylation. Analysis of the amino acid sequences used for the TR reporters derived from human mucins and GP1bα did not reveal simple common sequence motifs shared among those providing binding for the Siglec-like adhesins, and thus, the data do not allow us to define the recognition motifs in further detail.

Characterization of the mucin-degrading activity of the glycoprotease StcE. Secreted protease of CI esterase inhibitor (StcE) from Enterohemorrhagic Escherichia coli (EHEC) O157:H7 is a zinc metalloprotease with a remarkable ability for cleaving densely O-glycosylated mucins and mucin-like glycoproteins. StcE is thought to serve in colonic mucin degradation facilitating EHEC adherence to the epithelium and subsequent infection. Recent studies demonstrated that StcE has selective substrate specificity for S/T-X-S/T motifs with a requirement for O-glycans at the first S/T residue. Moreover, a catalytically inactive mutant of StcE (StcEE447D) has glycan-binding properties for the dST core1 O-glycan as evaluated by printed glycan arrays, but also exhibits general binding properties for mucins. We used our purified TR reporters and those displayed on cells to further explore the fine substrate specificity of the recombinantly purified StcE glycoprotease and to dissect its reported mucin-binding properties (Fig. 5). First, we found that StcE efficiently cleaved isolated MUC2 and MUC5AC reporters with core2 (HEK293WT) and Tn O-glycans already at low concentrations of 10-40 ng/mL (1:2500–500, StcE: TR reporter ratio), while the STn glycoform was essentially resistant to cleavage (Fig. 5b and Supplementary Fig. 9a). Next, we explored the selectivity of StcE with the 20 membrane-bound mucin TRs displayed on HEK293WT cells by monitoring loss of the N-terminal FLAG tag by flow cytometry using fluorescent anti-FLAG tag antibodies (Fig. 5a). StcE efficiently cleaved most of the mucin TRs with the notable exception of MUC1 and MUC20, as well as the control TR reporter.
designed with a single O-glycosite (Fig. 5c and Supplementary Fig. 9c, d). Dose-titration analysis in both assays showed low ng/mL cleavage for most mucin TR reporters, while no cleavage of the MUC1 TR reporter was found even at 10 µg/mL (Supplementary Fig. 9b, d). StcE was shown previously to cleave the entire MUC1 expressed on cancer cells, but this may be due to cleavage outside the TR region as the proposed StcE cleavage motif (S/T-X-S/T) is absent from the well-conserved TRs. Finally, we dissected the effect of O-glycan structures on StcE cleavage using the MUC2 and MUC5AC TR reporters, and found that core1 and core2 O-glycans including Tn are efficiently cleaved, while STn, as well as core3 O-glycans, efficiently blocked proteolysis (Fig. 5d and Supplementary Fig. 9e).

Characterization of the mucin-binding properties of StcE. Next, we explored the intriguing suggestion that StcE plays a role in adherence of EHEC to the intestinal epithelium by binding mucins. A catalytically inactive mutant of StcE (StcE<sup>E447D</sup>) was recently shown to exhibit broad binding to mucins in tissue sections and suggested to recognize isolated DST O-glycans on a glycan array. Examination of the 3-D structure of StcE revealed that the protein contained a C-terminal domain (here designated X<sub>409</sub>) opposite to the catalytic metalloprotease domain (M<sub>66</sub>), which we predicted could represent an evolutionarily mobile binding module. In line with previous findings, deletion of this X<sub>409</sub> module did not affect the catalytic activity of StcE with the WT MUC2 reporter (Fig. 6a, b and Supplementary Fig. 10a), and the X<sub>409</sub> module alone did not exhibit enzymatic activity (Fig. 6b). However, deletion of the X<sub>409</sub> module completely abrogated StcE binding to mucin-producing cells in human colonic tissues, whereas the X<sub>409</sub> alone (GFP-tagged) reproduced the strong tissue staining properties of the intact StcE<sup>WT</sup> (Fig. 6c). Note also that the binding of active StcE, inactive StcE<sup>E447D</sup>, as well as X<sub>409</sub> alone fully recapitulated the tissue binding found previously with the inactive StcE<sup>E447D</sup> mutant. Interestingly, the StcE/X409 staining appeared to overlap with MUC2 expression in the human colon as shown by
co-localization of staining with a mAb directed against Tn-MUC2 (PMH1) (Supplementary Fig. 10b).\(^{30}\) The X409 module displayed strong binding to normal human colon and stomach tissues and colon and stomach cancers. While we found low binding to other normal tissues including the pancreas and breast, strong binding to the counterpart cancer tissues was observed (Supplementary Fig. 10c). Probing the X409 module with the cell-based mucin TR display revealed that this binding module bound to select human mucin TRs (carrying core2 O-glycans), and e.g., not to MUC1 (Fig. 6d). Moreover, the strong binding to MUC2 and MUC5AC was only slightly influenced by the O-glycan structures attached to the TRs, although weaker binding to TRs carrying Tn, core3, and especially STn O-glycans was observed (Fig. 6e). These results clearly demonstrate that the X409 module mediates the mucin-binding properties of StcE, while the catalytic unit of StcE may mediate selective binding to dST O-glycans\(^{49}\). Here, we did not pursue this glycan-binding property of the catalytic unit of StcE given the focus on the mucin TRs, but a recent preliminary report describing the use of the full StcE\(^{E447D}\) mutant for affinity isolation of O-glycoproteins, suggests that the enzyme enriches not only mucins but also a variety of other O-glycoproteins\(^{54}\). Given that StcE, StcE\(^{E447D}\), and X409 alone exhibited the same highly selective tissue binding to mucin-producing cells, it is likely that the dST glycan-binding properties of the catalytic unit of StcE found with a high density of glycans printed on glass slides\(^{49}\), does not contribute substantially to binding to tissues. In conclusion, our results show that X409 does not directly impact the catalytic activity of StcE, and it is likely that this domain serves to target StcE to mucins through its highly selective mucin-binding properties\(^{47,49}\). The X409 module may be exploited to probe for the expression of mucins in normal and cancer tissues.
**Analysis of influenza virus receptor interaction.** Influenza A virus (IAV) employs a trimeric hemagglutinin (HA) that recognizes sialoglycans to bind and infect host cells, and a neuraminidase (NA) to cleave sialic acid and destroy the HA receptors for virus release. IAV interacts with mucins lining the intranasal, tracheal, and lung surfaces, and HA binding and NA cleavage of mucins have been suggested to play an important role in the penetration of the mucus layer and infection of respiratory tissues. Here, we addressed if the sequence and glycosylation of four mucin TRs produced in HEK293WT or HEK293KO GCNT1 influenced IAV HA-NA activity by analyzing binding and dissociation kinetics of the laboratory mouse-adapted influenza strain A/Puerto Rico/8/1934 (H1N1) (PR8) virions (α2-3Neu5Ac specificity) to loaded sensors using biolayer interferometry.
Biolayer interferometry enables the interactional study of the PR8 virions with glycans presented in their native protein-linked form. In the presence of the NA inhibitor OsC58, virus particles bound to sensors saturated with MUC2, MUC5AC, MUC7, and MUC1 TRs carrying core2 O-glycans (produced in HEK293WT) with similar kinetics (Fig. 7a). This binding was markedly slowed with mucin TRs carrying core1 O-glycans only (produced in HEK293KO GCNT1), with the largest reduction observed for MUC5AC. NA activity-dependent self-elution of virions bound to similar levels to the different mucin TR coated sensors, was monitored after removal of the OsC NA inhibitor (Fig.7a). This self-elution reflects the HA-NA-receptor balance as it depends on HA avidity and NA activity for the receptors present on the sensor surface and on the receptor density55. Self-elution was slowest from MUC1-coated sensors, remarkably despite the fact that the MUC1 TR reporter contains fewer O-glycans compared to the other TR reporters. Self-elution from mucin TR reporters with core2 O-glycans was faster compared to their counterparts with core1 O-glycans, and still the slowest release was observed with MUC1 TR reporter (Fig. 7a). Next, the different mucin TR reporters were assessed for their ability to compete with PR8 virion binding to sensors coated with recombinant soluble lysosomal-associated membrane glycoprotein 1 (LAMP1), which carries multiple N-glycans with α2-3 and α2-6 linked sialic acids and serves as a potent IAV receptor59. Overall, mucin TR reporters with core2 O-glycans displayed stronger competition with LAMP1 binding than TRs carrying only core1 O-glycans (Fig. 7b). The MUC1 TR with core2 O-glycans was most effective and the MUC5AC with only core1 O-glycans was least effective in competing with LAMP1 (Fig. 7b). Similar results were obtained by mucin-driven elution.
overexpression15. We can therefore not assess the effects of using site-specific from elongation60,61. However, using MUC1 as a model it was biased toward higher ef
formed with the simplest Tn O-glycosylation, which may be increase in proteoforms with lower occupancy (Fig. 2). Over-
expression of ST6GALNAC1 in cell lines overrides the O-glycan
increase of ST6GALNAC1 in cell lines override the O-glycan
interfere with O-glycan occupancy, while biased toward higher ef
which demonstrated that the TRs of the mucins tested are O-
glycoforms, and importantly discovering that the normal core3 O-glycosylation pathway in the colon actually inhibits StcE digestion of MUC2 (Figs. 5 and 6). Studies with IAV also suggest that binding to and release from mucins are not simply determined by the number of O-glycans and sialic acids, but partly driven by cues encoded by the TR sequences and O-glycan structure (Fig. 7). The mucin TR reporter production platform also provided unprecedented insights into the O-glycosylation of mucins and enabled intact MS analysis to demonstrate surprising efficiency in the initiation of O-glycosylation by the polypeptide GalNAc-Ts (Figs. 2 and 3). Mucins arguably represent the last frontier in the analytics of glycoproteins. Most mucins are extremely large and heterogeneous glycoproteins that are resistant to conventional glyco-proteomics strategies dependent on proteolytic fragmentation and sequencing24,25,33, and despite increasing knowledge of O-glycosites20, identification of actual sites of glycosylation in mucins is essentially limited to MUC134–36, lubrinic25, and the large N-terminal mucin-like region of MUC1620. The platform developed here for the production of secreted mucin TR reporters enabled us to produce representative fragments of the large mucin TR regions and to analyze these by intact MS analysis, which demonstrated that the TRs of the mucins tested are O-glycosylated to near completion at all putative Ser/Thr glycosites with high fidelity (Figs. 2 and 3). This analysis was only performed with the simplest Tn O-glycosylation, which may be biased toward higher efficiency in the incorporation of GalNAc residues by the many GALNTs in the absence of competition from elongation60,61. However, using MUC1 as a model it was possible to analyze the more complex ST and STn glycoforms, after removal of sialic acids, to demonstrate that elongation by the corel synthase (CIGALTL1) in this cell model did not significantly interfere with O-glycan occupancy, while α2-6 sialylation by ST6GALNAC1 did appear to slightly interfere as observed by an increase in proteoforms with lower occupancy (Fig. 2). Over-expression of ST6GALNAC1 in cell lines overrides the O-glycan elongation process leading to accumulation of the cancer-associated STn O-glycan37,38. We engineered homogenous expression of STn in HEK293 cells by eliminating O-glycan elongation (KO COSMC) and by introducing ST6GALNAC1 using site-specific KI, which does not lead to substantial overexpression15. We can therefore not assess the effects of expression of ST6GALNAC1 on O-glycan elongation, but it is interesting to note that even without overexpression the efficiency of O-glycosylation initiation was slightly affected (Fig. 2a). ST6GALNAC1 may compete with the initiation of O-glycosylation orchestrated by the many GALNTs that utilize C-terminal lectin modules for binding to GalNAc residues and efficient incorporation of GalNAc at all glycosites. The STn O-glycan is generally not expressed in normal cells62–64, but interestingly high expression of 9-O-Acetylated STn is selectively found in the normal colon63,65; showing that the capacity for STn O-glycosylation is present and may compete with the normal core3 pathway directed by B3GNT6. Further expansion of the cell-based glycan array through the engineering of the sialic acid acetyltransferase will allow us to address 9-O-acetylation of STn.

For a long time mucins have represented a black box in exploring the molecular cues that serve in intrinsic interactions with glycan-binding proteins and in extrinsic interactions with microorganisms66. Dissection of interactions with simple O-glycan structures found on mucins have benefited tremendously from the development of printed glycan arrays67,68, and these have for decades served as essential tools in exploring the interactions of glycans and proteins69. However, mucins and their large variable TR domains present O-glycans in different densities, and patterns which are likely to provide more specific interactions and instructive cues. Mucin TRs differ markedly in sequence, length, and numbers within closely related mammals70, and this divergence in TRs may have evolved to accommodate specific recognition of higher-order patterns and clusters of O-glycans15. We previously provided evidence for this by use of the cell-based glycan array demonstrating that two distinct streptococcal Siglec-like adhesins bind selectively to O-glycans presented on distinct mucin-like domains in O-glycoproteins and mucins15. This prompted us to develop the cell-based mucin display combining TR reporters with glycoengineered HEK293 isogenic cells (Fig. 1), which enabled us to demonstrate that three adhesins (HsaBR, GSP_BR, and NCT10712_24a) bind to different O-glycan structures and to distinct subsets of mucin TRs (Fig. 4a). Moreover, the select binding of the HsaBR adhesin to O-glycans on MUC2 could be installed in the NCT10712_24a adhesin by mutating a loop found in HsaBR71. The distinct binding specificities of these adhesins have an impact on their role in endocardial infection and adherence to platelets and aortic valves41. More recently, the mucin display was also used to demonstrate that several SiglecS selectively recognize sialylated O-glycans in the context of select mucin TRs72. The mucin display platform can clearly be used to discover and dissect interactions with clusters and patterns of O-glycans on mucins, and the results presented strongly suggest that the informational content of mucin TRs is great and as of yet unexplored.

The mucin display platform is also ideal for the discovery and exploration of mucin-degrading enzymes such as the pathogenic glycoprotease StcE46–49. EHEC is a food-derived human pathogen able to colonize the colon and cause gastroenteritis and bloody diarrhea. Strains of the O157:H7 serotype carry a large virulence plasmid pO157:H7 that directs the secretion of StcE46,51. StcE is predicted to provide EHEC with adherence to the normal core3 pathway directed by B3GNT6. Further expansion of the cell-based glycan array demonstrating that two distinct streptococcal Siglec-like adhesins bind selectively to O-glycans presented on distinct mucin-like domains in O-glycoproteins and mucins15. For a long time mucins have represented a black box in exploring the molecular cues that serve in intrinsic interactions with glycan-binding proteins and in extrinsic interactions with microorganisms66. Dissection of interactions with simple O-glycan structures found on mucins have benefited tremendously from the development of printed glycan arrays67,68, and these have for decades served as essential tools in exploring the interactions of glycans and proteins69. However, mucins and their large variable TR domains present O-glycans in different densities, and patterns which are likely to provide more specific interactions and instructive cues. Mucin TRs differ markedly in sequence, length, and numbers within closely related mammals70, and this divergence in TRs may have evolved to accommodate specific recognition of higher-order patterns and clusters of O-glycans15. We previously provided evidence for this by use of the cell-based glycan array demonstrating that two distinct streptococcal Siglec-like adhesins bind selectively to O-glycans presented on distinct mucin-like domains in O-glycoproteins and mucins15. This prompted us to develop the cell-based mucin display combining TR reporters with glycoengineered HEK293 isogenic cells (Fig. 1), which enabled us to demonstrate that three adhesins (HsaBR, GSP_BR, and NCT10712_24a) bind to different O-glycan structures and to distinct subsets of mucin TRs (Fig. 4a). Moreover, the select binding of the HsaBR adhesin to O-glycans on MUC2 could be installed in the NCT10712_24a adhesin by mutating a loop found in HsaBR71. The distinct binding specificities of these adhesins have an impact on their role in endocardial infection and adherence to platelets and aortic valves41. More recently, the mucin display was also used to demonstrate that several SiglecS selectively recognize sialylated O-glycans in the context of select mucin TRs72. The mucin display platform can clearly be used to discover and dissect interactions with clusters and patterns of O-glycans on mucins, and the results presented strongly suggest that the informational content of mucin TRs is great and as of yet unexplored.

The mucin display platform is also ideal for the discovery and exploration of mucin-degrading enzymes such as the pathogenic glycoprotease StcE46–49. EHEC is a food-derived human pathogen able to colonize the colon and cause gastroenteritis and bloody diarrhea. Strains of the O157:H7 serotype carry a large virulence plasmid pO157:H7 that directs the secretion of StcE46,51. StcE is predicted to provide EHEC with adherence to the gastrointestinal tract and the ability to penetrate through the mucin layers via its impressive mucin-degrading properties73. StcE cleaves the C1 esterase inhibitor glycoprotein (C1-INH) that contains a highly O-glycosylated mucin-like domain and is required for complement activation46. StcE was previously shown to cleave several mucins including MUC1, MUC7, and MUC1647,48,50,74, and the cleavage required O-glycosylation and accommodated complex O-glycan structures48,75. The gut microbiome is contained in a network of the gel-forming mucin MUC2 that forms the loose outer mucin layer, and a dense inner layer of MUC2 forms a barrier and prevents the microbiota from reaching the underlying colonic epithelium76,77. We found that

artic
StcE efficiently binds to and cleaves MUC2 TRs, which would enable StcE to destroy the MUC2 networks and provide access to the epithelium (Fig. 5). However, we also discovered that the normal core3 O-glycosylation of MUC2 in the human colon83,84, as well as the truncated cancer-associated STn O-glycosylation efficiently block cleavage by StcE. Currently, the molecular basis for the substrate selectivity of StcE with respect to glycoforms is unclear, but in this respect, it is perhaps relevant that the catalytic unit of StcE appears to exhibit high binding specificity for the dST core1 O-glycan85. Core3 O-glycosylation is restricted to the gastrointestinal tract in humans, and in the mouse, MUC2 is mainly O-glycosylated with core1 and core2 O-glycans that are also commonly found outside the gastrointestinal tract in humans86,87,88. Moreover, we found that the human MUC2 TR sequence was not amenable for core2 O-glycosylation in cells that introduced core2 O-glycans on other mucin TRs. Importantly, the core1 O-glycans installed on MUC2 TRs instead did not acquire the dST O-glycan with α2-6 sialylation as other mucin TRs (Fig. 4b), and we found that this modification (dST/STn) blocked StcE cleavage (Fig. 5b,d). The normal core3 O-glycosylation pathway is downregulated in colorectal cancer83, and truncated O-glycans including STn are found in colitis and other bowel inflammatory diseases84. Furthermore, mice deficient in the core3 b3galnt6 gene exhibit enhanced susceptibility to colorectal cancer and colitis.82,85 These findings are highly relevant for the understanding of the functions of MUC2 in the intestine as a barrier and in the containment of the gut microbiome78. Coincidently, the α2-6 sialyltransferase ST6GALNAC1 is quite selectively and highly expressed in the intestine, and the expression pattern mimics that of the CASD1 enzyme that O-acetylates sialic acids and blocks the action of most sialidases86. Expression of 9-O-acetylated STn is remarkably specific for normal intestine53, and while STn is generally considered a cancer-associated type of O-glycosylation, in fact, deacetylation of normal intestine demonstrates wide reactivity for STn in normal goblet cells producing MUC253. We, therefore, propose that the O-glycosylation of MUC2 in goblet cells has co-evolved with MUC2 to provide protection from StcE-like mucin-degrading glycopeptidases by introducing the core3 pathway with ST6GALNAC1 mediated α2-6 sialylation and 9-O-acetylation by CASD1, and by the design of the TR sequence that inhibits core2 branching. We further predict that the unusual accumulation of 9-O-acetylated STn in goblet cells may be a result of overexpression of ST6GALNAC1, which could selectively block core1 synthesis and favor core3 synthesis.

The mucin display platform further enabled us to dissect the mucin-binding properties of StcE and discover a distinct mucin-binding module X409 on StcE (Fig. 6). Recently, the mucin-binding properties of StcE were explored with a catalytically inactivated mutant enzyme following the concept that inactivated hydrolases often can be used as binding reagents52. However, the results presented here demonstrate that the mucin-binding properties of StcE are conferred exclusively by the distinct X409 binding domain placed in the C-terminal region (Fig. 6). Importantly, the mucin-binding properties of X409 are not driven by a particular O-glycan structure suggesting a more complex interaction with the mucin TR backbone and innermost monosaccharide residues of attached O-glycans (Fig. 6e). Carbohydrate-binding modules (CBMs) are found widely on microbial glycoside hydrolases, glycosyltransferases, and other carbohydrate-active enzymes; in most cases, they serve to localize these enzymes to their substrates, but sometimes participate in modulating the enzymatic functions87,88. Among eukaryotic glycosyltransferases, only the GALNTs directing O-glycosylation have appended CBMs, which orchestrate distant glycosylation by coordinating partially GalNAc-glycosylated substrates into the catalytic site89. The identified X409 mucin-binding module on StcE clearly directed the binding to O-glycosylated mucin TRs with a preference for more complex O-glycosylation and interestingly slightly lower binding to the Tn and STn glycoforms. Further studies are needed to explore how X409 selectively binds mucin TRs with diverse O-glycan structures, and to determine if this binding domain may be classified as a CBM.

The sialic acid receptor specificity of influenza virus HA is essential for virus transmission90, while the receptor destroying activity of NA that cleaves off sialic acid is required for release and propagation of the virus91. We previously demonstrated that the cell-based display of the human glycome could be used reliably to dissect the α2-3 and α2-6 sialic acid-binding specificities of influenza HAs and provide information on the underlying glycoconjugate nature91. Here, we extended these studies with PR8, a mouse-adapted IAV displaying α2-3Neu5Ac linkage specificity, and mucin TRs to explore the role of mucins in HA binding and NA release. IAV encounters mucins in secretions and on cell surfaces, and penetration of the mucus layer is a prerequisite for infection of respiratory tissues92. Interestingly, we found specific interactions of PR8 IAV with MUC1 and interactions of this α2-3Neu5Ac binding virus depended on the presence of core2 O-glycans. MUC1 was previously suggested to interact with IAV binding limiting to host epithelial and subsequent infection, and synthetic MUC1 peptides decorated with STn or ST reduced IAV infection of MDCK cells in vitro92. Our findings suggest the selective binding of PR8 to MUC1 with preferred binding specificities demonstrating the ability of the mucin display to address mucin and glycan context of influenza virus binding with an opportunity to produce decoy reporters for interference studies.

In summary, the cell-based mucin display platform provides a unique resource, that for the first time will enable deeper exploration of the nature and functions of human mucins. We illustrated this with classical examples of binding studies with microbial adhesins and substrate analysis of a microbial glycopeptidase that provided clear evidence of selectivities for different human mucins and their O-glycans. The finding that defined mucin TR modules can be produced with programmable O-glycans will enable the microbiome community to integrate mucins in studies at a level and detail not previously envisioned. In this respect, recent reports have demonstrated that sparse and heterogenous isolated human MUC1AC and O-glycans, therefore, have unique properties and trigger the downregulation of virulence genes and the disintegration of biofilms93.

**Methods**

**Cell culture.** HEK293-6EWT (obtained through a license agreement with Dr. Yves Durocher, Bioprocédès Institut de recherche en Biotechnologie, Montréal) and all isogenic clones were cultured in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 2 mM GlutaMAX (Gibco) in a humidified incubator at 37 °C and 5% CO₂. HEK293-6E were also grown in suspension in serum-free F17 culture media (Invitrogen) supplemented with 0.1% Kolliphor P188 (SIGMA) and 4 mM GlutaMax at 37 °C and 5% CO₂ under constant agitation (120 rpm). All glycoengineered isogenic HEK293 cells used in this study are listed in Supplementary Table 2 and are available as part of the cell-based glycan array resource49.

**CRISPR/Cas9-targeted KO in HEK293 cells.** CRISPR/Cas9 KO was performed using the GlycoCRISPR resource containing validated gRNAs libraries for targeting of all human glycosyltransferases84. In brief, HEK293 cells grown in 6-well plates (NUNC) to ~70% confluency were transfected for CRISPR/Cas9 KO with 1 μg of gRNA and 1 μg of GP-tagged Cas9-PBKS using lipofectamine 3000 (Thermo Scientific) following the manufacturer’s protocol. Twenty-four hour post-transfection, cells were bulk-sorted based on GFP expression by FACS (SONY SH800). After one week of culture, the bulk-sorted cells were single cell-sorted into 96-well plates. KO clones were screened by Indel Detection by Amplicon Analysis (IDAA)49 with the primers amplifying gRNA targeting sites and final clones were further verified by Sanger sequencing. All the gRNA and the primers used in this study were listed in Supplementary Table 3.
Human mucin TR reporters. The transmembrane mucin TR reporters were designed by fusion of human MUC1 signal peptide (amino acids 1–62, Uniprot P109213) with 6×His, Flag, GFP, multiple cloning sites, and the membrane anchoring domain of human MUC1 (amino acids 1042–1138) (Fig. 1 and Supplementary Table 1)\(^\text{1,2,3}\). Exchangeable mucin TR inserts of 150–200 amino acids were synthesized as TrueValue constructs with in-frame BamHI and NotI sites (Geneva, USA). The secreted TR plasmids containing secret mucin TR reporters were digested with XhoI and NotI restriction sites and a 6×His tag/STOP oligo (5’-GGCCGCGCCATCACCACCATG CATGATAGGCCGCTAG-3’, NotI/XhoI restriction sites underlined). We also included a TR reporter design containing six 11-mer sequences with a single O- glycosylation site (AEEAAPAAPPAAE,) to serve as a control for the patterns of O- glycans found in mucin TRs (Supplementary Fig. 1a and Supplementary Table 1).

Transient transfection with mucin TR reporters. Transmembrane GFP-tagged mucin TR reporters were transiently expressed in engineered HEK293 cells. Briefly, cells were seeded in 24-wells (NUNC) and transfected at ~70% confluence with 0.5 µg of plasmids using Lipofectamine 3000. Cells were harvested 24 h post-transfection and used for assays followed by flow cytometry analysis.

Production and purification of recombinant mucin TR reporters. The secreted reporters were stably expressed in isogenic HEK293-6E cell lines selected over two weeks of culture in the presence of 0.32 µg/mL G418 (Sigma-Aldrich) and two rounds of FACS enrichment for GFP expression. A stable pool of cells was seeded at a density of 0.25 × 10^6 cells/mL and cultured for 5 days on an orbital shaker in F17 medium (Gibco) supplemented with 0.1 Kolliphor P188 (Sigma-Aldrich) and 2% Glucose. Supernatants containing secret mucin TR reporter was clarified (3000 x g, 10 min), mixed 3:1 (v/v) with 4× binding buffer (100 mM sodium phosphate, pH 7.4, 2 mM NaCl), and run through a nickel-nitrilotriacetic acid (Ni-NTA) affinity resin column (Qiagen), pre-equilibrated with washing buffer (25 mM sodium phosphate, pH 7.4, 500 mM NaCl, 20 mM imidazole). The column was washed twice to remove any non-specifically bound proteins.

The reaction was terminated by addition of 200 µL ice-cold MQ water followed by desalting by cation-exchange chromatography (Dowex AG 50W 8X). Borate salts were converted into methyl borate esters by adding 1% acetic acid in methanol and evaporated under N2 gas. Permethylated O-glycans were derivatized from C4 HPLC incubated o/n at 4 °C in 50 mL carbonate-bicarbonate buffer (pH 9.6). Plates were blocked with PLI-P buffer (PO4, Na/K, 1% Triton-X 200, 1% BSA, pH 7.4) and incubated with mAbs 3C9, 5F4 and TKH2 (undiluted (0.5 µg/mL) (Lectenz Bio) diluted in PBA (1× PBA containing 1% BSA (w/v)) for 1 h, followed by washing and staining with Alex Fluor 647-conjugated streptavidin (1:1000) (Invitrogen by Thermofisher Scientific) for 20 min. Stainings with mAbs specific to mucin glycoforms produced in mice were performed by incubating cells for 30 min at 4 °C with supernatants from the respective hybridomas followed by staining with Alex Fluor 647-conjugated goat anti-rabbit (1:1000) (Invitrogen by Thermofisher Scientific) for 1 h. Cells were stained with GST-tagged streptococcal adhesins at 10 mM concentration diluted in PBA for 1 h on ice, followed by incubation with rabbit polyclonal anti-GST antibodies (1:500) (ThermoFisher) for 1 h and subsequent staining with Alex Fluor 647-conjugated goat anti-rabbit (1:1000) (Invitrogen by Thermofisher Scientific) for 1 h. All cells were resuspended in PFA for flow cytometry analysis (TOTAL Header). Mean fluorescent intensity (MFI) of the binding of streptococcal adhesins to GFP positive (expressing mucin TR reporters) and negative (not expressing) populations was quantified using FlowJo software (FlowJo LLC).

ELISA. ELISA assays were performed using MaxiSorp 96-well plates (Nunc) coated with dilutions of purified mucin TR reporter starting from 100 ng/mL or fractions derived from C4 HPLC incubated o/n at 4 °C in 50 µL carbonate-bicarbonate buffer (pH 9.6). Plates were developed with PCL-P buffer (PO4, Na/K, 1% Triton-X 100, 1% BSA, pH 7.4) and incubated with mAb C9, SF4 and TKH2 (undiluted (100 µg/mL), biotiated-lecins VVA (0.5 µg/mL), PNA (0.2 µg/mL), MAL II (2.0 µg/mL)) (Vector Laboratories) or Pan Lectenz (1.0 µg/mL) (Lectenz Bio) diluted in PBA (1x PBA containing 1% BSA (w/v)) for 1 h, followed by washing and staining with Alex Fluor 647-conjugated streptavidin (1:1000) (Invitrogen by Thermofisher Scientific) for 20 min. TNFα standard was developed with TMB substrate (Dako) and reactions were stopped by the addition of 0.5 M H2SO4 followed by measurement of absorbance at 450 nm. Positive reaction was developed with TMB substrate (Dako) and reactions were stopped by the addition of 0.5 M H2SO4 followed by measurement of absorbance at 450 nm.

StC proteolytic activity and binding assays. E. coli codon optimization, gene synthesis and cloning of the recombinant StCε (residues 39-898), StCε4477D (residues 39-898 with mutation of E447D), StCε5x409 (residues 39-796), and X409 (residues 797-898) were outsourced to Twist Bioscience (USA). The genes were cloned into a pET28-based vector (Kanamycin) on the direct 3’ end of a MHHHHHHHHSHENLYFQG linker. The plasmids were transformed in T7 Express (NEB) bacterial strains, grown at 37 °C for 24 h, induced with 1 mM IPTG, and

Data analysis. Glycopeptide compositional analysis was performed from m/z features extracted from LC-MS data using in-house written SymbioWare software\(^\text{26}\). For m/z feature recognition from full MS scans Minora Feature Recognition software (Perseus, Sweden) was used. The list of precursor ions (m/z, charge, peak area) was imported as ASCII data into SymbioWare and compositional assignment within 3 ppm mass tolerance was performed. The main building blocks used for the compositional analysis were: NeuAc, Hex, HexNAc, dHex, and the theoretical mass increment of the most prominent O-glycan structure was added to the theoretical mass of the potential glycosylate lipid, each glycosite was ranked for the top 10 most abundant candidates and each candidate structure was confirmed by doing targeted MS/MS analysis followed by manual interpretation of the corresponding MS/MS spectrum. For intact mass analysis raw spectra were deconvoluted to zero-charge by BioPharma Finder Software (ThermoFisher Scientific, San Jose) using default settings. Glycotopeformers were annotated by in-house written SymbioWare software\(^\text{27}\) using average masses of Hexose, N-acetylhexosamine, and the known backbone mass of mucin TR reporter sequence.

ELISA assays were performed using MaxiSorp 96-well plates (Nunc) coated with dilutions of purified mucin TR reporter starting from 100 ng/mL or fractions derived from C4 HPLC incubated o/n at 4 °C in 50 µL carbonate-bicarbonate buffer (pH 9.6). Plates were developed with PCL-P buffer (PO4, Na/K, 1% Triton-X 100, 1% BSA, pH 7.4) and incubated with mAbs C9, SF4 and TKH2 (undiluted (100 µg/mL), biotiated-lecins VVA (0.5 µg/mL), PNA (0.2 µg/mL), MAL II (2.0 µg/mL)) (Vector Laboratories) or Pan Lectenz (1.0 µg/mL) (Lectenz Bio) diluted in PBA (1x PBA containing 1% BSA (w/v)) for 1 h, followed by washing and staining with Alex Fluor 647-conjugated streptavidin (1:1000) (Invitrogen by Thermofisher Scientific) for 20 min. Stainings with mAbs specific to mucin glycoforms produced in mice were performed by incubating cells for 30 min at 4 °C with supernatants from the respective hybridomas followed by staining with Alex Fluor 647-conjugated goat anti-mouse IgG (1:1000) (Invitrogen by Thermofisher Scientific) for 1 h. Cells were stained with GST-tagged streptococcal adhesins at 10 mM concentration diluted in PBA for 1 h on ice, followed by incubation with rabbit polyclonal anti-GST antibodies (1:500) (ThermoFisher) for 1 h and subsequent staining with Alex Fluor 647-conjugated goat anti-rabbit (1:1000) (Invitrogen by Thermofisher Scientific) for 1 h. All cells were resuspended in PBA for flow cytometry analysis (TOTAL Header). Mean fluorescent intensity (MFI) of the binding of streptococcal adhesins to GFP positive (expressing mucin TR reporters) and negative (not expressing) populations was quantified using FlowJo software (FlowJo LLC).
cultures were continued at 16 °C overnight. Cells were harvested by centrifugation and lysed in buffer A (30 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 8) supplemented with 0.5% BSA. Lysates were clarified by centrifugation at 10,000 g for 30 min and incubated with 5 µg/mL 6xHis-tagged StcEWT, StcEΔ in PBA at 37 °C. After 1 h, cells were washed with PBA, stained with APC-tagged membrane TR reporters were used. Cells were incubated with different concentrations of X409-GFP, HEK293 cells expressing ECFP (Enhanced Cyan Fluorescent Protein) tagged membrane TR reporters were used. Cells were incubated with different concentrations of X409-GFP for 1 h at 4 °C followed by staining with APC-conjugated anti-FLAG antibody. X409-GFP labeling to anti-FLAG positive cells was quantified using FlowJo software. For histology analysis, deparaffinized tissue microarray sections58 were microwave treated for 20 min in sodium citrate buffer. Hansson, G. C. Mucus and mucins in diseases of the intestinal and respiratory tract. J. Intern. Med. 285, 479–490 (2019).

Johansson, M. E. V. and Hansson, G. C. Immunological aspects of intestinal mucus and mucins. Nat. Rev. Immunol. 16, 639–649 (2016).

Sommerburg, J. L., Angelent, L. T. & Gordon, J. I. Getting a grip on things: how do communities of bacterial symbionts become established in our intestines? Nat. Immunol. 5, 569–573 (2004).

5. McLaughlin, K., Schluter, J., Rakoff-Nahoum, S., Smith, A. L. & Foster, K. R. Host selection of microbiota via differential adhesion. Cell Host Microbe 19, 550–559 (2016).

6. Johansson, M. E. V., Sjövall, H. & Hansson, G. C. The gastrointestinal mucus system in health and disease. Nat. Rev. Gastroenterol. Hepatol. 10, 352–361 (2013).

7. Werlang, C., Cárcamo-Oyarce, G. & Ribbeck, K. Engineering mucus to study and influence the microbiota. Nat. Rev. Mater. 13, 144–145 (2018).

8. Co, J. Y. et al. Mucins trigger dispersal of Pseudomonas aeruginosa biofilms. npj Biofilms Microbiomes 4, 23–28 (2018).

9. Link, T. et al. Bioprocess development for the production of a recombinant MUC1 fusion protein expressed by CHO-K1 cells in protein-free medium. J. Biotechnol. 110, 51–62 (2004).

10. Kudelka, M. R. et al. Cellular O-glycome reporter/amplification to explore glycans of living cells. Nat. Methods 7, 618–686 (2010).

11. Blixt, O. et al. A high-throughput O-glycopeptide discovery platform for serological profiling. J. Proteome Res. 9, 5250–5261 (2010).

12. Kramer, J. R., Onoaa, B., Bustamante, C. & Bertozzi, C. R. Chemically tunable mucin chimeras assembled on living cells. Proc. Natl Acad. Sci. USA 112, 12574–12579 (2015).

13. Petrou, G. & Crouzier, T. Mucins as multifunctional building blocks of biomasers. Biomater. Sci. 6, 2282–2297 (2018).

14. Chen, Y.-H. et al. The GAGome: a cell-based library of displayed glycosaminoglycans. Nat. Methods 15, 881–888 (2018).

15. Narimatsu, Y. et al. An atlas of human glycosylation pathways enables display of the human glycome by gene engineered cells. Mol. Cell 75, 394 (2019).

16. Narimatsu, Y. et al Genetic glycoengineering in mammalian cells. J. Biol. Chem. https://doi.org/10.1002/jbc.201.100448 (2021).

17. Bull, C., Joshi, H. J., Claussen, H. & Narimatsu, Y. Cell-based glycarray analysis—a practical guide to dissect the human glycome. STAR Protoc. 1, 100017 (2020).

18. Corduff, A. P. Mucins: an essential relevant glycan barrier in mucosal defence. Biochim. Biophys. Acta 1850, 236–252 (2015).

19. Marcos-Silva, L. et al Characterization of binding epitopes of CA125 monoclonal antibodies. J. Proteome Res. 13, 3349–3359 (2014).

20. Steenott, C. et al Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. EMBO J. 32, 1478–1488 (2013).

21. Lang, T., Hansson, G. C. and Samuelsson, T. Gel-forming mucins appear early in metazoan evolution. Proc. Natl Acad. Sci. USA 104, 16209–16214 (2007).

22. Hollingsworth, M. A. & Swanson, B. J. Mucins in cancer: protection and control of the cell surface. Nat. Rev. Cancer 4, 45–60 (2004).

23. Cummings, R. D. The repertoire of glycan determinants in the human glycome. Mol. Biosyst. 5, 1087–1104 (2009).

24. Levery, S. B. et al Advances in mass spectrometry driven O-glycoproteomics. Biochim. Biophys. Acta 1850, 33–42 (2015).

25. Ali, L. et al. The O-glycomap of lubricin, a novel mucin responsible for joint lubrication, identified by site-specific glycan analysis. Mol. Cell. Proteom. 13, 3396–3409 (2014).

26. Narimatsu, Y. et al Exploring regulation of protein O-glycosylation in isogenic human HEK293 cells by differential O-glycoproteomics. Mol. Cell Proteom. 18, 1396–1409 (2019).

27. Steenott, C. et al. Mining the O-glycoproteome using zinc-finger nuclease glycoengineered SimpleCell lines. Nat. Methods 8, 977–982 (2011).

28. Burchell, J., Taylor-Papadimitriou, J., Boshell, M., Gendler, S. & Dahug, T. A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. Int. J. Cancer 44, 691–696 (1998).

29. Tarp, M. A. et al. Identification of a novel cancer-specific immunodominant glycopeptide epitope in the MUC1 tandem repeat. Glycobiology 17, 399–407 (2007).

30. Reis, C. A. et al. Development and characterization of an antibody directed to an alpha-N-acetyl-D-galactosamine glycosylated MUC2 peptide. Glycoconj. J. 15, 51–62 (1998).

31. Remmers, N. et al. Aberrant expression of mucin core proteins and o-linked glycans associated with progression of pancreatic cancer. Clin. Cancer Res. 19, 1891–1913 (2013).

32. Dalziel, M. et al. The relative activities of the C2GnT1 and ST3Ga1 glycosyltransferases determine O-glycan structure and expression of a tumour-associated epitope on MUC1. J. Biol. Chem. 276, 11007–11015 (2001).

33. Khoo, K.-H. Advances toward mapping the full extent of protein site-specific O-GalNAc glycosylation that better reflects underlying glycosomic complexity. Curr. Opin. Struct. Biol. 56, 146–154 (2019).

34. Goletz, S. et al. A sequencing strategy for the localization of O-glycosylation sites of MUC1 tandem repeats by PSD-MALDI mass spectrometry. Glycobiology 7, 881–896 (1997).

35. Hanisch, F. G., Green, B. S., Reisman, R. & Peter-Katalinic, J. Localization of O-glycosylation sites of MUC1 tandem repeats by QTOF ESI mass spectrometry. J. Mass Spectrom. 33, 358–362 (1998).
Acknowledgements
This work was supported by the Lundbeck Foundation, the Novo Nordisk Foundation, the European Commission (GlycoImaging H2020-MSCA-ITN-721297, BioCapture H2020-MSCA-ITN-722171), the Danish National Research Foundation (DNRF107), the Mizutani Foundation (to Y.N.), the European Union’s Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie grant agreement No 787684 (to C.B.), and the National Institutes of Health grant (R01GM32373 to A.V. and GM137458 to T.M.I.).

Author contributions
R.N., C.B., H.C., and Y.N conceived and designed the study; A.K., L.S., Z.Y., A.H., D.M.S., S.F., U.M., L.H., S.Y.V., H.J.J., and L.A.D contributed with experimental data and interpretation; T.M.I., B.A.B., P.M.S., and A.V. contributed to the streptococcal adhesin studies; L.D., R.V., F.D., and B.H. contributed to the glycomucinase studies; R.N., C.B., H.C., and Y.N. wrote the manuscript, and all authors edited and approved the final version.

Competing interests
The University of Copenhagen has filed a patent application relating to X409 mucin-binding peptides (EP application EP21177857.6, pending). R.N., C.B., Y.N., B.H., and H.C. are named inventors. GlycoDisplay Aps, Copenhagen, Denmark, has obtained a license to the patent application. Y.N. and H.C. are co-founders of GlycoDisplay Aps and hold ownerships and financial interest in the company. The remaining authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24366-4.

Correspondence and requests for materials should be addressed to H.C. or Y.N.

Peer review information Nature Communications thanks Matthew Macauley, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021