Revealing the human mucinome

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Mucin domains are densely O-glycosylated modular protein domains found in various extracellular and transmembrane proteins. Mucin-domain glycoproteins play important roles in many human diseases, such as cancer and cystic fibrosis, but the scope of the mucinome remains poorly defined. Recently, we characterized a bacterial O-glycoprotease, StcE, and demonstrated that an inactive point mutant retains binding selectivity for mucin-domain glycoproteins. In this work, we leverage inactive StcE to selectively enrich and identify mucin-domain glycoproteins from complex samples like cell lysate and crude ovarian cancer patient ascites fluid. Our enrichment strategy is further aided by an algorithm to assign confidence to mucin-domain glycoprotein identifications. This mucinomics platform facilitates detection of hundreds of glycopeptides from mucin domains and highly overlapping populations of mucin-domain glycoproteins from ovarian cancer patients. Ultimately, we demonstrate our mucinomics approach can reveal key molecular signatures of cancer from in vitro and ex vivo sources.

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Mucin domains are modular protein domains that adopt rigid and extended bottle-brush-like structures due to a high density of O-glycosylated serine and threonine residues. Mucin-type O-glycans are characterized by an initiating α-N-acetylgalactosamine (α-GalNAc) monosaccharide that can be further elaborated into several core structures through complex regulation of glycosyltransferases. As a result, mucin domains serve as highly heterogeneous swaths of glycosylation that exert both biophysical and biochemical influence. For instance, this includes the ability to redistribute receptor molecules at the glycocalyx and to drive high avidity binding interactions. In the canonical mucin (MUC) family, mucin domains often occur as tandem repeats, creating heavily glycosylated superstructures. Canonical mucins are central to many functions in health and disease, and have long been associated with human cancers, e.g., MUC1 and MUC16 (also known as CA-125). Dysregulation of mucin domain expression and aberrant mucin domain glycosylation patterns have been implicated in disease pathologies, especially in tumor progression, where mucins modulate immune responses and also promote proliferation through biomechanical mechanisms.

Mucin domains also exist in proteins outside of the 21 canonical mucins (Fig. 1A). For example, CD43 on the surface of leukemia cells selectively interacts with the glyco-immune checkpoint receptor Siglec-7 through its N-terminal mucin domain; mucin domain-containing splice variants of CD44 (CD44v) serve as cancer cell markers relative to the ubiquitously expressed standard isoform; CD45 mucin domains act as suppressors of T-cell activation; mucin domain O-glycosylation on PSGL-1 is required for leukocyte-endothelial interactions; and aberrant regulation of mucin domains in podocalyxin and Syndecan1 are implicated in a variety of cancers.

In all of these cases, shared functional attributes of mucin domains impart structural and biophysical properties relevant to their biology. Thus, instead of the more traditional categorization of the glycoproteome into N- and O-glycoproteins (both of which are represented by mucin-domain glycoproteins), it is logical to parse the glycoproteome into the mucinome, a family of glycoproteins whose mucin domains make them functionally related. However, even as the tools to capture the broadly defined N- and O-glycoproteome continue to improve, mucin domains remain enigmatic and difficult to characterize. As such, a comprehensive list of all proteins with a mucin domain does not exist. This lack of a well-defined mucinome leaves a critical blind spot in our ability to interrogate mucin domain functions across molecular biology.

Toward this goal, enzymes derived from microorganisms known to colonize mucosal environments have shown promise for developing tools specifically suited to characterize mucin-domain glycoproteins. We recently characterized a panel of such enzymes, termed O-glycoproteases, and showed that each of them harbor a selectivity toward mucins as well as unique peptide- and glycan-based cleavage motifs. Using catalytic point mutants, we also demonstrated that select O-glycoproteases can retain binding specificity for mucin domains; these were then used as mucin-selective staining reagents for Western blots, immunohistochemistry, and flow cytometry. One particular enzyme of interest is secreted protease of C1 esterase inhibitor (StcE) from enterohemorrhagic Escherichia coli, which recognizes mucin domains decorated with a variety of O-glycan modifications. This gives StcE both the selectivity needed to specifically bind mucin domains and the breadth to bind diverse mucin domain subtypes that vary in glycosylation patterns. Indeed, StcE has shown great utility for selective release of mucin fragments from biological samples and for improving mass spectrometry (MS)-based analysis of mucin domains.

We reasoned that the catalytically inactive point mutant of StcE (StcE447D) could function as a universal mucin enrichment tool for mucin domain discovery, similar to how inactive O-glycosidases and engineered sialidases can enrich broadly for O-glycosylated and sialylated glycoproteins, respectively. Here we show that StcE447D-conjugated beads selectively enrich mucin-domain glycoproteins from complex cancer cell lysates and from crude ovarian cancer patient ascites fluid. As part of this workflow, we developed a mucin-domain candidacy algorithm to assign confidence scores to proteins that have a high likelihood of containing a mucin domain. Additionally, we detected hundreds of glycopeptides derived from mucin domains in the StcE447D-enriched samples. Ultimately, we demonstrate that this mucinomics platform can define key molecular signatures of cancer in both in vitro and ex vivo systems and is a valuable approach to unravel the role of mucin domains in health and disease.

**Results**

**Mucin enrichment and definition strategy to describe the mucinome.** Our previous work indicated that a catalytically inactive point mutant of StcE (StcE447D) retains its binding specificity for mucin domains while leaving them intact for subsequent analysis. Through a straightforward reductive amination approach, we conjugated StcE447D to POROS-AL beads to generate a solid phase support material to use for enrichments. To optimize our enrichment protocol, we added StcE447D-conjugated beads to OVCAR3 supernatant followed by an anti-MUC16 Western blot for detection. We tuned several parameters of the enrichment, including binding time, bead-to-substrate ratio, flow for enrichment technique. StcE447D beads were conjugated using reductive amination to POROS-AL 20 beads, followed by capping in Tris-HCl (1). Complex samples (lysate, ascites) were added to the beads and allowed to bind overnight (2), washed, and eluted by boiling in protein loading buffer (3). Samples were fractionated via one-dimensional gel electrophoresis and digested in-gel using trypsin (4); the gel electrophoresis chamber was created with BioRender.com.

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**Fig. 1 Mucinomics platform for enrichment of mucin-domain glycoproteins in complex samples.** A The mucinome comprises a variety of proteins that have a densely glycosylated mucin domain. Mucin domains are found in canonical mucins, mucin-domain glycoproteins, and even multi-pass transmembrane proteins. B Workflow for enrichment technique. StcE447D beads were conjugated using reductive amination to POROS-AL 20 beads, followed by capping in Tris-HCl (1). Complex samples (lysate, ascites) were added to the beads and allowed to bind overnight (2), washed, and eluted by boiling in protein loading buffer (3). Samples were fractionated via one-dimensional gel electrophoresis and digested in-gel using trypsin (4); the gel electrophoresis chamber was created with BioRender.com.
Fig. 2 Mucin-domain candidacy algorithm for confident assignment of mucin-domain glycoproteins. A Known mucins in HeLa lysate enrichment. HeLa lysate was subjected to the enrichment procedure described in Fig. 1 and known mucin-domain glycoproteins (MUC1, MUC13, MUC16, DAF, and SDC1) were labeled. Source data are in Supplementary Data 3. Significance testing was performed using a two-tailed t-test with 250 randomizations to correct for multiple comparisons, an FDR of 0.01, and a 50 value of 2. B Mucin-domain glycoprotein candidate annotation. A mucin-domain candidacy algorithm was created to assign Mucin Scores to indicate confidence that a given protein contains a mucin domain. First, predicted O-GalNAc sites were generated by the NetOGlyc4.0 tool, curated lists of phosphosites were downloaded from PhosphoSitePlus and Uniprot, and cellular localization GO terms were downloaded. The mucin-domain candidacy algorithm then removed predicted O-GalNAc sites overlapping with known phosphosites, calculated the proportion of threonine to serine residues (T/S-ratio), evaluated protein subcellular localization, and checked for frequency and density of predicted O-GalNAc sites. These metrics were used to calculate a Mucin Score, which could then be used to evaluate mucinome enrichment. The entire human proteome was processed with the mucin-domain candidacy algorithm; using manually curated benchmarks, 357 proteins have mucin domains (~2% of human proteome). C Mucinome of HeLa lysate. The results in A were processed with the mucin domain definition program, and mucin-domain glycoproteins were labeled according to the Mucin Score. Red signifies a score of >2 (high confidence), orange 2-1.5 (medium confidence), and yellow 1.5-1.2 (low confidence). Known mucin-domain glycoproteins labeled in A are still labeled in green. Source data are in Supplementary Data 3. Significance testing was performed using a two-tailed t-test with 250 randomizations to correct for multiple comparisons, an FDR of 0.01, and an 50 value of 2.
number of false positive mucin candidates after downstream processing. Note that O-GalNAc and phosphorylation sites are not known to have a high degree of overlap, as the former is generally extracellular whereas the latter is often intracellular.

Following O-GalNAc site prediction and phosphosite filtering, the algorithm asked four questions of each protein: (1) Was the protein predicted to be extracellular, secreted, and/or transmembrane? (2) Were there at least 9 predicted O-glycosylation sites within a stretch of 50 residues? (3) Was the distance between any given pair of O-glycosites less than 12% of the entire mucin domain (i.e., are glycosites <6 residues away from each other in a 50 residue sequence)? and (4) Was the ratio of threonine to serine residues skewed toward threonine? Each of these benchmarks were determined through expert curation of known mucin sequences, which are further described in Methods. Using a point system based on the answers to these questions, the algorithm ultimately assigned a Mucin Score to each protein in the human proteome. By manually assessing outputs, we determined that a score of >2 was a high confidence mucin-domain glycoprotein, between 2 and 1.5 was a medium confidence mucin-domain glycoprotein, and between 1.5 and 1.2 was a low confidence mucin-domain glycoprotein. Proteins with a score lower than 1.2 were not considered mucin-domain glycoproteins. Levels of confidence also capture the idea that a mucin domain may not be a binary concept; there may be gradients of O-glycosylation density and patterns that contribute to mucin-like attributes. See Supplementary Data 1 for the mucin candidate algorithm output and Supplementary Data 2 for the mucin candidate algorithm output and Supplementary Data 3. Significance testing was performed using a two-tailed t-test with 250 randomizations to correct for multiple comparisons, an FDR of 0.01, and an SO value of 2 for all volcano plots.

Inactive O-glycoproteases enrich mucin-domain glycoproteins from various cancer cell lines. Given that the HeLa lysate enrichment was successful, we decided to expand the approach to other cancer-associated cell lines, including SKBR3 (breast), OVCAR3 (ovarian), K562 (leukemia), and Capan2 (colorectal). The corresponding volcano plots are shown in Fig. 3A–D (see Supplementary Data 3 for Perseus processing files). As before, red dots signified a score of >2 (high confidence), orange dots 2–1.5 (medium confidence), and yellow dots 1.5–1.2 (low confidence). Strongly enriched mucin-domain glycoproteins were labeled with their gene names associated with specific proteins.

The Upset plot in Fig. 4A compares commonly observed mucin-domain glycoproteins across the cell lines. The total number of enriched mucin-domain glycoproteins from each cell line is shown on the bottom left (blue horizontal bars). If a group of mucin-domain glycoproteins was only seen in one cell line, only one gray dot is darkened; the number of proteins that are only seen in that cell line are shown in bar graph form above. For instance, 9 mucin-domain glycoproteins were only detected in the K562 cell line. Overlap between samples are shown by multiple darkened gray dots and a line connecting them; as an example, 2 mucin-domain glycoproteins were only detected in both the SKBR3 and OVCAR3 cell lines. A total of seven mucin-domain glycoproteins were seen in all five cell lines; these proteins are shown above the Upset plot. The putative mucin domain (orange, as calculated by the mucin-domain candidacy algorithm), transmembrane domains (purple), and annotated N-glycan sites (green) are noted on each of the proteins.

To better understand how many of the proteins contained previously undescribed mucin domains, we compared our dataset to the SimpleCell dataset from Clausen and colleagues 51, which is one of the most comprehensive study on O-glycosites to date (albeit with truncated O-glycan species). To consider a
mucin-domain glycoprotein in this comparison, more than 1 glycopeptide had to be detected from within the assigned mucin domain. Additionally, if the protein was a canonical (e.g. MUC15) or confirmed (e.g. Gp1ba) mucin-domain glycoprotein, these were considered as previously described/known proteins. Several of the proteins (4/7) found in all five cell lines were previously known to have a mucin domain, including: Mucin-1 (MUC1), dystroglycan (DAG1), agrin (AGN), and complement decay factor (CD55, DAF). However, we discovered that three of the overlapping proteins have previously undescribed mucin domains: low-density lipoprotein receptor 8 (LRP8), major facilitator superfamily domain 6 (MSFD6), and porimin (PORIM). MSFD6 is a multi-pass transmembrane protein that is implicated in antigen processing and presentation of exogenous peptide antigens via MHC class II, whereas porimin is involved in oncotic cell death characterized by vacuolization and increased membrane permeability.

Extending this analysis to all of the enriched mucin-domain glycoproteins, we found that approximately one-quarter (~31%) of the identified proteins had a mucin domain. Additionally, the most enriched cellular component (CC) GO terms were associated with membranes, cell surfaces, extracellular space, and increased membrane permeability. The percentage of mucin-domain glycoproteins bearing shortened O-glycan structures.

In another extension of our mucinomics workflow, we performed an enrichment using a different O-glycoprotease. While StcE does not demonstrate drastic glycan specificity, we have characterized several other O-glycoproteases with varying glyco-proteolytic specificities. BT2444 is a O-glycoprotease of particular interest from Bacteroides thetaiotaomicron that cleaves N-terminally to serine and threonine residues bearing truncated O-glycans, such as the cancer-associated T- and Tn-antigens (Gal-GalNAc and GalNAc, respectively). We reasoned that a point mutant of BT2444 (BT2444E575A) could also enrich mucin-domain glycoproteins bearing shortened O-glycan structures. Thus, we conjugated BT2444E575A to beads and performed an analogous enrichment using HeLa lysate with and without sialidase pretreatment, with results shown in Supplementary Fig. 2. Without sialidase treatment, only six mucin-domain glycoproteins were significantly enriched in the elution, suggesting that not many mucin-domain glycoproteins bear truncated O-glycans in HeLa cells. We then pre-treated HeLa lysate with 100 nM sialidase overnight and repeated this procedure, which resulted in the enrichment of 13 mucin-domain glycoproteins. Though not as robust as StcE enrichment, this proof-of-principle procedure demonstrates that other O-glycoproteases could be used to enrich and identify cancer-associated glycoforms of mucin-domain glycoproteins.

We next asked how selective our mucin-domain-centric platform is when compared to lectin (i.e., glycan-centric) enrichments commonly used for O-glycoproteomics. Jacalin has preference for mucin-type O-glycans including GalNAc and GalNAc-Gal; thus, we conjugated Jacalin to POROS-AL beads and performed enrichments on HeLa cell lysate with and without sialidase pretreatment, with results shown in Supplementary Fig. 3. To be sure, Jacalin does enrich most of the mucin-domain glycoproteins, but as demonstrated by the large number of enriched non-mucin proteins, it is clear that Jacalin is less specific for mucin-domain glycoproteins. This point is further illustrated in Supplementary Fig. 4. The Jacalin (+/−sialidase) pulldown resulted in the enrichment of 205 and 273 proteins, respectively. The percentage of mucin-domain glycoproteins within this subset is only 16–17%, meaning that 171 and
230 non-mucin proteins were enriched in the two samples. Using the same HeLa lysate, StcE\textsuperscript{E447D}-conjugated beads enriched a total of 75 proteins, 28\% of which were mucin-domain glycoproteins. Thus, StcE\textsuperscript{E447D} is approximately two-fold more selective for mucin-domain glycoproteins. Further, we detected only 54 non-mucin proteins in this enrichment, compared to the 230 in the Jacalin pulldown, representing a >4-fold reduction in non-mucin proteins. While Jacalin did enrich more mucin-domain glycoproteins, selectivity is especially important when considering potential goals of characterizing mucin-domain O-glycopeptides; non-mucin proteins, and their associated unmodified peptides, will outcompete the glycopeptides for ionization and detection.

We then investigated the non-mucins that were enriched by the StcE\textsuperscript{E447D} cell line enrichments to understand if there was an unexpected selectivity for features other than mucin domains or if it was likely due to non-specific binding. We calculated how many of the non-mucins were commonly found between cell lines, as demonstrated by the Upset Plot in Supplementary Fig. 5. Here, the majority of enriched proteins were found in only one cell line, suggesting that these proteins were primarily non-specifically binding to the beads. On the other hand, 5 proteins were found in all cell lines, and 7 were found in at least 4 cell lines (Supplementary Data 5; Master_NonMucin tab). Of these 12 proteins, 6 are potential mucin-domain glycoproteins with Mucin Scores that did not meet our initial thresholds but have several predicted O-glycosylation sites. The other proteins are likely to be (a) abundantly expressed and non-specifically binding (e.g. myosin) and/or (b) previously undescribed glycan or mucin-binding proteins. Taking this one step further, we performed cellular component GO term enrichments for all of the non-mucins. The highest protein counts were “extracellular exosome” (87) and “integral component of membrane” (80); “perinuclear region of cytoplasm” was far less abundant at a protein count of 15 (Supplementary Data 5).

Additionally, we explored which assigned mucin-domain glycoproteins were repeatedly not enriched by our technique. As with the enriched non-mucins, we generated an Upset Plot to determine which of our assigned mucin-domain glycoproteins were not enriched reproducibly (Supplementary Fig. 6). Here, five proteins were consistently not enriched across all five cell lines and five across at least four cell lines. The majority of these proteins were intracellular cytoplasmic proteins that were likely overscored as mucin-domain glycoproteins due to their presumed phosphorylation/O-GlcNAc sites that were predicted by NetO-Gly4.0 as O-GalNAc sites (Supplementary Data 6). We tried to account for these proteins by removing annotated phosphosites from the NetO-Gly4.0 glycosite assignments, though, we note that phosphosite databases are likely incomplete. Taken together, we believe that these analyses demonstrate that our approach provides satisfactory selectivity for mucin domains.

Mucinomics platform allows for identification of ovarian cancer patient mucinome. Following the establishment of our mucin domain enrichment approach in cell lines, we next wanted to test the mucinomics platform on clinically relevant patient samples. Ovarian cancer ranks fifth in cancer deaths among women and is often diagnosed in stage III or IV, leading to a poor prognosis. This is due, in part, to the fact that the only clinically relevant biomarker is CA-125, a peptide epitope of MUC16, but the exact structural definition of this antigen continues to be elusive. Previously, we showed that StcE could digest MUC16 from crude ovarian cancer patient ascites fluid\textsuperscript{46}, leading us to reason that our enrichment technique could be used to selectively isolate MUC16 and other mucin-domain glycoproteins from ascites fluid as a potential diagnostic strategy. As such, we performed mucinomics enrichment with StcE\textsuperscript{E447D} beads on five de-identified patient samples (OC235, OC234, OC114, OC109, and OC107). As seen in Fig. 5A–E, the grand majority of putative mucin-domain glycoproteins were significantly enriched in the elution (see Supplementary Data 7 for Perseus processing information); in all but one of the experiments (OC114), MUC16 (denoted in purple) was significantly enriched. The enrichment in these experiments was even more successful than in the cell lines; in four out of five patient samples (excluding OC235), zero mucin-domain glycoproteins were “enriched” in the crude ascites fluid. This is also demonstrated by the selectivity calculations depicted in Supplementary Fig. 7, as well as the non-mucin proteins investigated in Supplementary Fig. 8 and Data 8. For the full list of enriched mucin-domain glycoproteins, see Supplementary Data 8. The enrichment was likely more successful due to the presence of fewer interfering proteins found in biofluids. Again, we compared our results to the SimpleCell dataset and found approximately half (~54\%, 33 of 61) of the mucin-domain glycoprotein candidates (those that previously annotated mucin domains) to be enriched; these are detailed in Supplementary Data 4.

Figure 5F compares overlap between the ascites samples with a Venn diagram of all enriched mucin proteins. Each sample is represented by a different color box, and the overlap between samples is given by a number within the boxes. Notably, 26 mucin-domain glycoproteins were enriched in all five samples, demonstrating substantial overlap between patients. The 26 overlapping proteins and their MucinScores are listed in Supplementary Table 1. Again, as expected, the most enriched cellular component GO terms for the mucin-domain glycoproteins were associated with membranes, lumen, extracellular matrix, and the basement membrane (Fig. 5G). As before, the mucinome list contains some known mucin-domain glycoproteins, such as CD44, podocalyxin (PODXL), and agrin (AGRN).

In addition, the list contains previously undescribed mucin-domain glycoproteins, such as thymosin beta-4 and Trem-like transcript 2 protein. This further underscores the need for tools, like the strategy described here, to help define members of the mucinome. Additionally, we detected adhesion G protein-coupled receptor L1 (ADGRL1) as enriched in all five samples, further enforcing our conviction that this protein contains a mucin domain. While our patient cohort is currently too small to make any clinical claims, we believe that these overlapping mucin-domain glycoproteins could represent a better diagnostic and/or prognostic indicator for ovarian cancer. Future efforts will be devoted to expanding the study to a larger number of patients and comparing the results to patient outcomes, with the goal of developing a rapid mucin-fingerprinting approach using this mucinomics platform.

StcE\textsuperscript{E447D}-enrichment also captures O-glycopeptides from mucin domains. Characterization of intact O-glycopeptides was not an original goal when designing these experiments, but we reasoned that StcE\textsuperscript{E447D}-enrichment should function as a \textit{de facto} glycopeptide enrichment by selecting for highly O-glycosylated mucin-domain glycoproteins at the protein (i.e., pre-proteolysis) level. We observed a large number of spectra in our ascites enrichments bearing the “HexNAc fingerprint”, that is, oxonium ions specific to glycopeptides, which prompted us to search our data for intact glycopeptides. Generally, electron-driven dissociation is better suited for characterizing O-glycopeptides because it can provide O-glycosite localization\textsuperscript{37,38}. This is especially true for O-glycopeptides derived from mucin-domain glycoproteins, which will likely have multiply glycosylated sequences\textsuperscript{59–61}. Even so, collision-based fragmentation can still
provide O-glycopeptide identifications that include peptide sequence and the total glycan mass modification, though details about number of glycans or glycosite positions (and by extension, fine details about glycan structure) are usually inaccessible. Previous glycomic work suggests that some of these structures may include large, highly fucosylated and sialylated complex and hybrid N-glycans in addition to highly sialylated core-1 and -2 O-glycans with a smaller amount of sulfated core-2 O-glycan structures. We collected only higher-energy collision dissociation (HCD) spectra through this study, limiting our ability to thoroughly characterize O-glycopeptides. Additionally, given that we performed in-gel tryptic digestion, it is unlikely that we were able to extract the intact mucin domains from many of our samples, nor were we able to fully characterize mucin domains of interest. Attempts to use StcE for in-gel digests resulted in limited digestion efficiency, and alternative methods to couple StcE proteolysis to this enrichment strategy are currently under investigation. Regardless, we searched our ascites data using O-Pair Search, a recently developed open-modification glycopeptide spectral match algorithm. Strongly enriched proteins are labeled with gene names associated with specific proteins. MUC16 is labeled in purple. Fig. 6A. Venn diagram comparing mucinomics results from five cell lines. Each sample is shown as a different color (red, orange, purple, green, and blue); 26 mucin proteins were enriched in all five samples. Graphic was generated using Intervene BattlePlot. G GO terms associated with mucin-domain glycoproteins. Enriched CC GO terms using DAVID are shown with protein count indicated on the x-axis. Source data are in Supplementary Data 7 and 8. Significance testing was performed using a two-tailed t-test with 250 randomizations to correct for multiple comparisons, an FDR of 0.01, and an S0 value of 2 for all volcano plots.

Intriguingly, we discovered several O-glycopeptides on proteins that had previously uncharacterized mucin domains, as demonstrated in Fig. 6A. Here, the putative mucin domain is indicated by an orange box, annotated N-glycan sites are shown with green dots, and approximate location of the O-glycopeptides detected are shown using red dots. These proteins did not have any annotated O-glycosites in the SimpleCell dataset or in Uniprot, thus these O-glycopeptides represent novel modifications on the mucin-domain glycoproteins. The presence of several identified O-glycopeptides in the regions assigned to be putative mucin domains by our mucin-domain candidacy algorithm also strengthens our claim that the proteins do, in fact, have mucin domains. Additionally, we detected a large number of glycopeptides from MUC16, which is a key step toward better structural definition of this important cancer antigen. The total glycan compositions for these peptides included N1, H1N1, N2, H1N2, N3, H1N1A1, H2N2, H1N2A1, H1N1A2, H2N2A1, and H2N2A2, where H is hexose, N is HexNAc, and A is Neu5Ac. The ratio of 138/144 in all of these cases was ~1, suggesting that the glycans are primarily core 1 (i.e., do not contain GlcNAc). Together, this would suggest that the compositions N2, H1N2, N3, H2N2, H1N1A1, H2N2A1, and H2N2A2 were multiply glycosylated peptides.

Next, we wanted to compare the glycoprotein sources of glycopeptides detected in the elution versus the crude cancer patient ascites fluid. As demonstrated in Fig. 6B, only 3% of glycopeptide spectral matches (glycoPSMs) originated from mucin-domain glycoprotein identifications in the unenriched ascites fluid, while 60% of glycopeptides from the elution came from mucin-domain glycoproteins. Further, 82% of all glycoPSMs in the elution were O-glycopeptides (rather than N-glycopeptides), compared to only 17% in ascites fluid (Fig. 6C). Supplementary Fig. 9 (data available in Data 9 and 10) shows the number of N- and O-glycopeptides detected in n number of experiments (where unique glycopeptide is defined as sequence peptide sequences and total mass combination), suggesting a significant biological variance in glycopeptide species between patients despite high protein-level overlap observed in Fig. 5F. We note that there is some level of ambiguity in glycopeptide identifications, given that 2 fucose residues may be assigned as a single sialic acid and vice versa. Regardless, to visualize the degree
of uniqueness/overlap between glycopeptides identified in ascites and enriched samples, we constructed glycopeptide-glycan networks shown in Fig. 6D, E, which are modified versions of previous protein-glycan visualizations introduced in Riley et al.24 In these networks, unique glycopeptide identifications are arranged vertically as nodes in the middle of the network (black nodes in both panels). Unique glycan masses are then organized as nodes in the semi-circles on either side of the glycopeptide identifications, with each semi-circle representing the same glycan masses. In other words, gray nodes on the left of each network and color nodes on the right show the same glycan masses and are mirror images of each other. If glycan masses map to the same glycopeptide identifications, that means identifications are shared between the ascites (left, gray) and enriched (right, color) conditions. Otherwise, glycopeptide-glycan connections that only appear on one side of the network are unique to that condition. In Fig. 6D, the majority of N-glycopeptides were identified in ascites rather than enriched samples, with relatively few N-glycopeptides mapping uniquely to the enriched samples. Conversely, Fig. 6E shows that the majority of O-glycopeptides were detected more often than N-glycopeptides in StceE447D-enriched ascites fluid, the inverse of the non-enriched ascites fluid. Supplementary Data 11 and 12 provide total glycan mass compositions (outer nodes) identities and the unique glycopeptide list (middle nodes) for N-glycopeptide (D) and O-glycopeptide (E) networks, respectively. Source data are in Supplementary Data 10–13 and in Source Data.
important contributors to health and disease, a mucin-domain candidacy algorithm to address this problem. We chose to build this candidacy algorithm on the hallmark mucin domain feature of serine and threonine O-glycosylation, as predicted by NetOGlyc4.0, while not focusing on other sequence characteristics such as proline frequency. While the enrichment feature of this mucinome workflow appears robust, we note that the mucin-domain candidacy algorithm is imperfect; yet, it serves a functional purpose for evaluating mucin-domain glycoprotein enrichments. Identification of mucin-domain glycoproteins more abundantly detected in cell lysates rather than the elution could also indicate that certain mucin domains remain under-glycosylated depending on cellular state or cell type, meaning our mucinomics approach could be used to screen the mucin status of proteins under a variety of conditions. Additionally, our mucin-domain candidacy algorithm could improve substantially from enhanced O-glycosite and mucin domain prediction tools. That said, prediction of mucin-type O-glycosites, much less mucin domains, remains challenging due to the complex regulation of O-glycosites by a poorly resolved family of glycosyltransferases. Future iterations could also explore other O-glycosite prediction algorithms beyond NetOGlyc4.0, such as ISOGlyP26.

Though we have identified a subset of putative mucin-domain glycoproteins determined by the candidacy algorithm, we did not detect nearly 300 of these proteins. This can be likely attributed to a number of reasons: first, we only explored 5 types of epithelial cancer cells; many other cancers and subsets of the same cancers are likely to express a different subset of mucin-domain glycoproteins. Also, we primarily used whole-cell lysates in this study, biasing toward membrane-tethered glycoproteins; given that mucin-domain glycoproteins can also exist as purely secreted biomolecules rather than membrane-tethered, it is possible that we missed a large number of mucin-domain glycoproteins only found in the secretome of cells. Further, it is entirely possible that the dense glycosylation in the mucin-domain glycoproteins renders them inaccessible to the in-gel digestion strategy used here. Current efforts are focused on optimizing the elution of the mucin-domain glycoproteins to enable in-solution digestion approaches. Finally, though previous experiments have suggested otherwise, it is possible that StcE enriches only a certain subset of mucin-domain glycoproteins from the samples. Interestingly, during the review process of this manuscript, Nason, Büll, et al. reported that the C-terminal domain of StcE can confer mucin-binding properties irrespective of the active site29, meaning that the selectively of StcE enrichment is not purely based on the O-glycosylated TXT motif that dictates its protease activity. This generates interesting new directions to explore complexities of O-glycopeptide binding harbored by catalytically inactive O-glycopeptide mutants.

Regardless, with this mucinomics platform, we enriched mucin-domain glycoproteins from several cancer-associated cell lines and crude ovarian cancer patient ascites fluid. We demonstrated high mucin overlap between ovarian cancer patients, and the enrichment strategy allowed us to detect hundreds of glycopeptides from the mucin proteins, with a substantial increase in O- over N-glycopeptides. We also identified many proteins previously unknown to contain a mucin domain, thus demonstrating the utility of this technique in discovering new mucin-domain glycoproteins. Future efforts will be devoted to expanding our patient cohort in order to determine whether the ovarian cancer mucinome can be used as a diagnostic and/or prognostic indicator.

Though this work represents a significant step forward in understanding mucin domains, several open questions remain. To begin, mucin domains are known to regulate interactions at cell surfaces via biological effects at the cell-surface interactions. However, these roles are likely extremely dynamic, and may depend on various glycan structures (alone or in combination), expression of the mucin domain, and the overall cellular milieu. Further, the role of an individual mucin domain is unlikely to be identical across all of the mucin-domain glycoproteins. Thus, future studies should be devoted to understanding the role that discrete mucin domains are playing in cellular function. We predict that these mucin domains will fall into subgroups with categorical roles in health and disease.

Additionally, while we have identified a large number of mucin-domain glycoproteins from cell lines and ascites fluid, many other mucin-domain glycoproteins are likely present in different cell types and in other applications. In particular, the immune cell mucinome is of incredible interest and may represent a class of new ‘checkpoint inhibitors’ with both glycan and peptide components to investigate16. Further, while we chose to focus our efforts on the cancer mucinome, several other mucinomes have yet to be studied in diseases known to involve dysregulated mucins. These mucinopathies include, but are not limited to, inflammatory bowel disease, cystic fibrosis, chronic obstructive pulmonary disease (COPD), Sjögren’s syndrome, and dry mouth/eyes. Ultimately, we believe our mucinomics strategy will find utility in several settings and will prove to be an invaluable tool for glycobiochemists and biochemists alike.

Methods

O-glycopeptidase cloning, expression, and purification. StcE and BT4244 were expressed as previously described39,40. Briefly, Natalie Strynadka (University of British Columbia) kindly provided the plasmid pET28b-StcE-Δ35-NHis39, Robert Hirt (Newcastle University) kindly provided the plasmid pRSETA-BT424433. pET28b-StcEE447D, Δ35-NHis and pRSETA-BT4244E575A were generated using the following primers: StcE447D_for 5′-TAAGATGACGTTGGTCATAATTATG-3′, and BT4244E575A_rev 5′-TCAGTCATGACGTTGGTCATAATTATG-3′. StcE447D rev 5′-ACTCATTTCCACAAATGTTG-3′, BT4244E575A for 5′-CCAG TCTATGCAAATGGCATG-3′, and BT4244E575A rev 5′-TCCCAACCGGT TCTCCTG-3′. StcE447D was expressed and purified as previously described40. BT4244E575A was expressed in BL21(DE3) E. coli (New England Biolabs) grown in Luria broth (LB) with 100 μg/mL ampicillin at 37 °C, 225 rpm. The culture was induced at OD 0.6–0.8 with 1 mM IPTG and grown overnight at 30 °C. Cell pellets were lysed in pH 7.4, and elution was performed with a linear gradient to 150 mM imidazole. For BT4244, fractions containing pure protein were concentrated using Amicon Ultra 10 kDa MWCO filters (Millipore Sigma), dialyzed into PBS, pH 7.4, and stored at −80 °C. BT4244E575A was further purified by size exclusion chromatography using a Superdex 200 Increase 10/300 GL column (Cytiva Life Sciences) in PBS, pH 7.4, and fractions containing pure protein were stored at −80 °C.
Cell culture. Cells were maintained at 37 °C and 5% CO2. HeLa cells (ATCC CCL-2) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). ORA-2 cells (ATCC HTB-80) were cultured in McCoy’s 5A supplemented with 10% FBS and 1% P/S. SKBR3 cells (ATCC CRL-3344 and HTB-30, respectively) were cultured in RPMI supplemented with 10% FBS and 1% P/S. OVCAR-3 cells (ATCC HTB-161) were cultured in RPMI supplemented with 20% FBS, 0.01 mg/mL bovine insulin, and 1% P/S. To prepare lysate for pulldowns, cells plated in 70% confluence, washed three times with DPBS, then lysed in 500 µL of RIPA buffer (Thermo Fisher Scientific) supplemented with EDTA-free protease inhibitor cocktail (Roche) and 0.1% benzamidine (Millipore Sigma). Lysates were stored at −80 °C prior to pulldown.

Bead derivatization. An aliquot containing approximately 2 mg of StcEE447D (1 mL of 1.93 mg/mL) was added to 7–8 mg of POROS-AL beads, along with 1 µL of 4% Flt-fl (FLRT2) as a control. After the reaction proceeded overnight, with shaking, the beads were washed three times with 500 µL of ultrapure water, spinning at 8500 rpm for 5 min each time. To cap all excess aldehyde sites on the beads, 200 µL of Tris-HCl with 1 µL of 80 mg/mL NaN3H, was added to the beads. The reaction shook at room temperature for 2 h. Excess beads were stored at 4 °C overnight, with shaking, then dried in a vacuum concentrator and rehydrated with 0.1 µg of trypsin in 50 µL of solution. The beads were then boiled at 95 °C for 5 min, spun for 2 min at 8500 rpm, then lysed in 500 µL of RIPA buffer (Thermo Fisher Scientific) supplemented with EDTA-free protease inhibitor cocktail (Roche) and 0.1% benzamidine (Millipore Sigma). Lysates were stored at −80 °C prior to pulldown.

Enrichment of mucin-domain glycoproteins from cell lysates and ascites fluid. Cell lysates were clarified by centrifuging for 20 min at 18,000 x g, and concentrated using standard protocols (Thermo Easyspin). At this point, all experiments, the ideal ratio of lysate to beads (w/v) was determined to be 500 µg/100 µL, where 100 µL of the conjugated beads corresponded to 700 µg of beads in solution. The beads were pelleted at 8500 rpm for 5 min and the supernatant was removed. Then, 5 µL of 0.5 M EDTA and 500 µg of cell lysate was added to the beads and incubated at 4 °C overnight, with shaking. The reaction was performed six times, in tandem. After binding, the beads were spun at 8500 rpm for 5 min, and the supernatant was saved (“FT” or flow-through). Then, the beads were washed three times with 250 µL of PBS buffer containing 5 µL of 0.5 M EDTA. After the last wash, 32 µL of 4X protein loading buffer was added to the beads. For unenriched (control) samples, 30 µg of lysate was added to 10 µL of 4X loading buffer. All samples were then boiled at 95 °C for 5 min, spun for 2 min at 13,000 x g, and frozen for at least 1 h. The samples were then thawed and loaded onto 4–12% Criterion XT Bis-Tris precast gels (Bio-Rad), and run in 1x MOPS buffer. After electrophoresis, the slices were reconstituted in 150 µL of solvent A and added to the plate three times. The washes were done with three washes with 150 µL of solvent A, elution three times using 100 µL of 0.1% FA in 80% ACN (“loading pump solvent”). Subsequently, the C18 nano pre-column was switched in line with the C18 nanoscale separation column (75 µm x 250 mm EASYSpray containing 2 µm C18 beads) for gradient elution. The column was held at 45 °C using a column heater in the EASY-Status software. The instrument method used an MS1 resolution of 60,000 at FWHM 200 m/z, an AGC target of 3e5, and a mass range from 350 to 1,500 m/z. Dynamic exclusion was used, with a repeat exclusion of 12 s, exclusion duration of 10 s. Only charge states 2–6 were selected for fragmentation. MS2s were generated at top speed for 3 s. HCD was performed on all selected precursor masses with the following parameters: isolation window 2 m/z, 30% collision energy, orbitrap detection (resolution of 30,000), and an AGC target of 1e4 ions.

Mucin-domain candidacy algorithm. To build the mucin-domain candidacy algorithm, the entire human proteome was first downloaded from Uniprot (20,365 entries) and parsed into FASTA files containing 150 entries each (a total of 136 files). Each file was individually uploaded to the NetOGlyc4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) for O-glycosite prediction51. NetOglyc4.0 results were saved as .csv files for further processing, with 20,121 entries returning usable output data. NetOglyc output was then filtered for any predictions that used the supplemental datatables, which features a score of 0 that can be calculated through the description below. Cellular component (CC) GO terms for the human proteome were also downloaded from Uniprot, and phosphosite annotations were downloaded from Uniprot and PhosphositePlus52,53. Predictions from NetOGlyc were then screened for any predictions that did not include O-glycosylation sites, filtered for any proteins with predicted O-GalNAc sites, and scored. Predictions with predicted O-GalNAc sites resulted in removal of the predicted O-GalNAc site from consideration. To annotate proteins as extracellular, secreted, and/or transmembrane, cellular component localization terms from Uniprot were checked for each protein entry. A protein was annotated as “extracellular” if its CC GO terms contained the phrases “Cell Membrane”, “Cell Membrane protein”, “Secreted”, “extracellular”, or “Extracellular”. Proteins also received the “extracellular” distinction if they contained GO accessions of 0005887, 0016021, or 0005576. Because many proteins have multiple locations, “extracellular” proteins were further denoted as “exclusively extracellular” if their GO terms did NOT include “cell”, “cellular”, or “cellular”. Next, predicted O-glycosites were iterated over to determine if a given protein would pass our “mucin test”, which consisted of two calculations. First, we required a protein to have at least nine predicted O-glycosites within a 50-residue region. If a protein qualified for this benchmark, we applied our “12% rule” to determine the number of resiudes that separated any two given O-glycosites within this 50-residue region. The 12% rule applied to a 50-residue region meant that fewer than 6 residues could separate any given pair of O-glycosites. Both the “9 sites within 50 residues” metric and the “12% rule” were derived through hand annotation of known and thoroughly studied mucins mostly curated by the Mucin Biology Group (http://www.medgen.duke.edu/mucinbiology/databases/dh/Mucin- human-2015.htm)[8,9]. Although this could be considered both too stringent or too relaxed depending on perspective, empirical testing showed these rules (in conjunction with the other metrics discussed) to be reasonably reliable in properly annotating known mucin domains. Exploration of these “mucin test” metrics in particular is an interesting area for future studies looking to employ a mucin-domain candidacy algorithm. Finally, a threonine to serine ratio (T/S-ratio) was calculated for the predicted O-glycosites, mainly as a metric to discriminate
O-GalNAc sites (slight threonine preference) from phosphosites (slight serine preference) due to the proclivity of NetOglyc4.0 to predict dense regions of O-GalNAc sites in proteins that are actually intercellular. Bar graphs in Fig. 6B, C were made using OriginPro 2022 and show the average value of the five data points shown indicated along with standard deviations. The glycopeptide-glycan networks in Fig. 6D, E were created in R 3.5.1 using the igraph library.

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

The raw mass spectrometry data generated in this study have been deposited in the PRIDE database under accession code PXD024995. The SimpleCell dataset from Clauens and colleagues was obtained from Steenoft et al. (Supplemental Table 2 in that publication). The proteomics data generated from the mucinome enrichments of cell lysates and ascites fluid, the outputs from the mucin candidacy algorithm, the glycan databases used for glycopeptide searches, the glycoproteomics data generated from the mucinome enrichments of ascites fluid, data to make the N- and O-glycopeptide networks, and data to recreate figures are provided in the Supplementary Data files as indicated in the text. Source data are provided with this paper.

**Code availability**

Code for the mucinome candidacy algorithm is available as Supplementary Software 1.

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**Unmodified peptide MS data analysis (MassQuant).** Raw data were processed using MaxQuant version 1.6.3.4, and tandem mass spectra were searched with the Andromeda search algorithm. Oxidation of methionine and protein N-terminal acetylation were specified as variable modifications, while carbamidomethylation of cysteinyll residues was set as a fixed modification. A precursor ion search tolerance of 20 ppm and a product ion mass tolerance of 20 ppm were used for searches, and two missed cleavages were allowed for full trypsin specificity. Peptide spectral matches were made against a target-decoy human reference proteome database downloaded from Uniprot, FBS contamination was not examined for the lysate samples. Peptides were filtered to a 1% FDR and a 1% protein FDR was applied according to the target-decoy method. Proteins were identified and quantified using at least one peptide (razor + unique), where razor peptide is defined as a non-unique peptide assigned to the protein group with the most other peptides (Occam’s razor principle). Proteins were quantified and normalized using MaxLQF31 with a label-free quantification (LFQ) minimal ratio count of 1. LFQ intensities were calculated using the match between runs feature, and MS/MS spectra were required for LFQ comparisons. For quantitative Article comparisons, protein intensity values were log2-transformed before further analysis, and missing values were imputed from a normal distribution with width 0.3 and downshift value of 1.8 (that is, default values) using the Perseus software suite.48 A Boolean value “IsAMucin” was also appended to each protein, with the value set as true if the Mucin Score was greater than 1. Mucin Scores and IsAMucin were input manually for LFQ comparisons. The raw mass spectrometry data generated in this study have been deposited in the PRIDE database under accession code PXD024995. The SimpleCell dataset from Clauens and colleagues was obtained from Steenoft et al. (Supplemental Table 2 in that publication). The proteomics data generated from the mucinome enrichments of cell lysates and ascites fluid, the outputs from the mucin candidacy algorithm, the glycan databases used for glycopeptide searches, the glycoproteomics data generated from the mucinome enrichments of ascites fluid, data to make the N- and O-glycopeptide networks, and data to recreate figures are provided in the Supplementary Data files as indicated in the text. Source data are provided with this paper.

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**Glycopeptide MS data analysis (O-Pair Search).** For glycopeptide analysis, samples were loaded into MetaMorphose in groups of 8, related to one individual replicate (e.g. “lysalte 1 slice-1”). The human proteome was loaded into the database downloaded from Uniprot June, 2016), and a “glycan” search task was selected. For each group of 8 raw files, an N- and an O-glycan search was performed separately. Parameters for the O-Glycopeptide Search were as follows: O-glycan database “Oglycan.db” (the default 12-glycan database46), keep top 50 candidates, Dissociation type “HCID” and child scan “null”, 4 maximum O-glycan allowed, with OxoniumIonFit on. For the N-Glycopeptide Search, all parameters were the same except the “NGlycan182.db” database was used. These glycan databases are available in Supplementary Data 9. For general peptide parameters, the following features were used: tryptic cleavage, maximum missed 2 cleavages, maximum 2 modifications per peptide, with a peptide length of 5–60. Precursor mass tolerance was set to be 20 ppm, product mass tolerance at a retention value at 20%, peptide intensity value was calculated using the match between runs feature, and MS/MS spectra were required for LFQ comparisons. The raw mass spectrometry data generated in this study have been deposited in the PRIDE database under accession code PXD024995. The SimpleCell dataset from Clauens and colleagues was obtained from Steenoft et al. (Supplemental Table 2 in that publication). The proteomics data generated from the mucinome enrichments of cell lysates and ascites fluid, the outputs from the mucin candidacy algorithm, the glycan databases used for glycopeptide searches, the glycoproteomics data generated from the mucinome enrichments of ascites fluid, data to make the N- and O-glycopeptide networks, and data to recreate figures are provided in the Supplementary Data files as indicated in the text. Source data are provided with this paper.
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Author contributions
S.A.M. and C.R.B. designed research; S.A.M., N.M.R., D.J.S., and K.P. performed research; V.K. and O.D. contributed human clinical samples; S.A.M., N.M.R., D.J.S., and K.P. analyzed data. S.A.M., N.M.R., and C.R.B. wrote the paper with input from all authors.

Competing interests
S.A.M., D.J.S., K.P., and C.R.B. are coinventors on a Stanford nonprovisional utility patent application that has been filed and is pending in the US (number US20220003777) related to the use of inactive mucinases to enrich mucin-domain glycoproteins. C.R.B. is a co-founder and Scientific Advisory Board member of Lycia Therapeutics, Palleon Pharmaceuticals, Enable Bioscience, Redwood Biosciences (a subsidiary of Catalent), and InterVenn Biosciences, and a member of the Board of Directors of Eli Lilly & Company. O.D. has participated in advisory boards for Tesaro, Merck, and Geneos. O.D. is a speaker for Tesaro and AstraZeneca. The remaining authors declare no competing interests.

Additional information
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