Regulation of protein N-glycosylation is essential in human cells. However, large-scale, accurate, and site-specific quantification of glycosylation is still technically challenging. We here introduce SugarQuant, an integrated mass spectrometry-based pipeline comprising protein aggregation capture (PAC)-based sample preparation, multi-notch MS3 acquisition (Glyco-SPS-MS3) and a data-processing tool (GlycoBinder) that enables confident identification and quantification of intact glycopeptides in complex biological samples. PAC significantly reduces sample-handling time without compromising sensitivity. Glyco-SPS-MS3 combines high-resolution MS2 and MS3 scans, resulting in enhanced reporter signals of isobaric mass tags, improved detection of N-glycopeptide fragments, and lowered interference in multiplexed quantification. GlycoBinder enables streamlined processing of Glyco-SPS-MS3 data, followed by a two-step database search, which increases the identification rates of glycopeptides by 22% compared with conventional strategies. We apply SugarQuant to identify and quantify more than 5,000 unique glycoforms in Burkitt’s lymphoma cells, and determine site-specific glycosylation changes that occurred upon inhibition of fucosylation at high confidence.
With an increasing understanding of protein N-glycosylation and its site-specific regulation in physiology and pathology\(^1,2\), a pressing need is arising for large-scale mass spectrometry (MS)-based identification and quantification of intact N-glycopeptides. Conventional analytical approaches often release the glycan moiety chemically or enzymatically from proteins before performing MS analysis of either the resulting oligosaccharides or the de-glycosylated peptides\(^3\), thus deliberately discarding information about site specificity. Instead, analysis of intact N-glycopeptides (i.e., peptides that derive from endoproteolytic digestion of glycosylated proteins, but still carry the intact glycan moieties) is essential to determine the linkage between protein and glycan, and to profile the microheterogeneity of glycosylation at each site\(^4,5\). Significant improvements have been made with regard to methods for intact glycopeptide characterization, including glycopeptide enrichment\(^6-8\), optimized MS acquisition strategies\(^8\), advanced fragmentation techniques\(^9,10\), and database search algorithms\(^11,12\). These have helped address technical difficulties resulting from heterogeneous and complex glycopeptide structures and their less informative fragmentation behavior in tandem mass spectrometry (MS2). Nevertheless, these developments have largely focused on increasing glycopeptide identification rates rather than on reliable, global glycopeptide quantification.

Both iTRAQ and TMT isobaric labeling have been used for large-scale quantitative glycoproteomic studies\(^12,13\). Despite the advantages of isobaric labeling in quantitative intact glycoproteomics (Supplementary Note 1), standard MS2-based methods for the analysis of labeled glycopeptides often suffer from reduced identification rates and impaired accuracy of quantification\(^14\). The recently developed synchronous precursor selection (SPS)-MS3 approach has improved quantitative accuracy in isobaric mass tag labeling experiments by reducing co-selected precursor interference at the cost of scan speed\(^15,16\), but its application to glycoproteomics has not yet been explored. Quantification of intact glycopeptides on a large scale remains technically challenging, and software tools for quantitative data processing are still lacking.

Here we introduce SugarQuant, an integrated workflow for large-scale, global glycopeptide identification and quantification (Fig. 1). SugarQuant comprises (i) lysis of cells in the presence of SDS, (ii) protein extraction, (iii) protein concentration and endoproteolytic digestion using protein aggregation capture (PAC)\(^17,18\), (iv) multiplex TMT labeling, (v) N-glycopeptide enrichment by zwiterionic HILIC (ZIC-HILIC) followed by (vi) basic reverse phase (bRP) prefractionation, and (vii) LC-MS\(^3\) analysis using Glyco-SPS-MS3, which generates high-resolution MS2 and MS3 product ion scans. Data processing using the GlycoBinder tool (viii) combines MS2 and MS3 fragment ions for N-glycopeptide identification, and extracts TMT reporter-ion intensities from MS2 scans for each identified N-glycopeptide-spectrum-match (GPSM). GlycoBinder consolidates redundant GPSMs with their quantitative values, and reports multi-dimensional quantification results for unique glycoforms, glycosylation sites, and glycan compositions in an accessible table-based format. As a proof of concept, we apply SugarQuant to quantitatively map the protein glycosylation patterns in Burkitt’s lymphoma cells treated with varying doses of 2-deoxy-2-fluoro-L-fucose (2FF), a guanosine diphosphate fucose analog that inhibits cellular fucosylation. We demonstrate the capabilities of SugarQuant for site-specific determination of reduced N-fucosylation following 2FF treatment, which is missed by conventional MS2 analysis. Our findings uncover 2FF-sensitive N-glycosylation sites and reveal 2FF-mediated changes in N-glycosylation on key effectors in B-cell receptor-mediated signaling.

**Results**

**PAC enables fast preparation of TMT-labeled glycopeptides.** We developed a PAC-based workflow for fast preparation of TMT-labeled N-glycopeptides (Fig. 1). Using human lymphoma cell lysates (DG75 and Daudi), we evaluated and optimized cell lysis conditions, PAC bead types, ZIC-HILIC enrichment, and TMT chromatography to achieve a shorter handling time and good recovery of N-glycopeptides (Supplementary Note 2). Cell lysis in the presence of 4% SDS recovered 9% more unique proteins and 20% more unique glycoforms than the use of 1% RapiGest, a MS-compatible cleavable detergent (Supplementary Fig. 1a). PAC allowed efficient removal of SDS with reduced sample preparation time and led to 13.8% and 26% more identified glycoforms and glycosites compared to conventional aceton- one precipitation, respectively (Supplementary Fig. 1b). In particular, additional prefractionation of ZIC-HILIC enriched N-glycopeptides by bRP chromatography before LC-MS analysis resulted in the identification of >1.5 times more N-glycopeptides when compared to repeated direct LC-MS injections of the same sample amount obtained after ZIC-HILIC, even when using prolonged chromatographic gradients for the latter (Supplementary Fig. 1c). Importantly, bRP chromatography offers high resolution, as evidenced by 94.1% of glycopeptides which were exclusively identified in at most two fractions (Supplementary Fig. 1d) whereas almost one-third of glycopeptides were identified in five replicates when using replicate injections, and only 26.7% of glycopeptides were unique to a single run (Supplementary Fig. 1e). bRP prefractionation is thus suited to maximize the productivity of LC-MS acquisition time.

We then introduced TMT-labeling into the workflow to allow for highly multiplexed relative quantitation. In its most recent implementation, TMT enables up to 16-fold multiplexing, which greatly increases sample throughput and allows for sophisticated experimental setups\(^19\). We confirmed that ZIC-HILIC enriches TMT-labeled N-glycopeptides, either from purified human IgM or from whole cell lysate, with comparable efficiency and specificity as their unlabeled counterparts (Supplementary Fig. 1f). The optimized workflow (Supplementary Fig. 1g) including cell lysis, PAC-based clean-up and proteolysis, TMT-labeling, ZIC-HILIC enrichment, and off-line prefractionation of N-glycosylated peptides prior to LC-MS, enables one-day sample preparation for large-scale multiplex quantitative glycoproteomics.

**Glyco-SPS-MS3 for improved identification and quantification of TMT-labeled N-glycopeptides.** Continuous developments of MS methods in past decades\(^10,20\) have greatly improved the characterization of glycopeptides, but revealed shortcomings in the quantitation of their TMT-labeled counterparts. We therefore evaluated both MS2- and MS3-based approaches available on Orbitrap Tribid mass spectrometers with regard to their performance for the identification and quantitation of glycopeptides.

We used higher-energy collisional dissociation (HCD) on both unlabeled and labeled N-glycopeptides obtained from human serum IgM (Supplementary Fig. 2) to determine the influence of collision energy on the information content of the MS/MS spectra of TMT-labeled glycopeptides. On a purely identification-based level, the highest number of labeled glycopeptides was identified at 30–35% NCE (Normalized Collision Energy), as compared to an optimum value of 25–30% for non-labeled glycopeptides (Supplementary Fig. 2). We next evaluated the production of different structural moiety-specific fragment types (i.e. fragments related to peptide sequence and to glycan composition, and TMT reporter ions) at different NCE settings (Fig. 2 and Supplementary Fig. 3). We note that the most intense glycan product ions are observed at NCE <35%, peptide fragment ions at NCE
~40–45%, and TMT reporter ions at NCE >45% (Fig. 2a). Such a wide range of NCEs for the various types of fragment ions poses a challenge for achieving both identification and quantification of N-glycopeptides when using HCD-MS2 only. Our observations corroborate earlier published datasets12,13 that showed near one-order less intense reporter ions for labeled glycopeptides compared to labeled linear peptides in HCD-MS2, even with NCE as high as 45% (Supplementary Fig. 4). Possibly the breakage of the more labile glycosidic bonds absorbs most of the collisional energy and thus limits the generation of reporter ions14. Low reporter ion intensities will compromise TMT-based quantification sensitivity and reproducibility. In addition, MS2-based methods suffer from impaired quantitative accuracy due to interference caused by co-isolation21.

Based on previous MS3 strategies for glycopeptide characterization22,23 and the recently developed SPS-MS3 method15,16, we generated an MS acquisition workflow for the analysis of TMT-labeled N-glycopeptides, namely Glyco-SPS-MS3 (Fig. 2b and Supplementary Note 3). It applies HCD at low-NCE (~25%) to produce intense glycan Y-series ions carrying the intact peptide backbone. The 10 most abundant fragment ions in the range of 700–2,000 m/z are then co-selected and co-fragmented with higher NCE (35–40%) HCD in MS3 to generate abundant peptide b-/y-ions as well as TMT reporter ions (Supplementary Fig. 5). The dominant glycan Y ions observed in glycopeptide MS2 scans inherently bear TMT tags, precluding the possibility of selecting an untagged peptide y-ion for SPS-MS3 quantification as frequently happens when analyzing regular tryptic peptides without lysine residues. Besides, glyc an Y ions often produce better peptide backbone fragmentation than the corresponding intact glycopeptides, which improves overall peptide sequence coverage. Glyco-SPS-MS3 reconciles the divergent collision energy requirements of different fragment ion types, and results in increased quantitative accuracy by removing MS2-co-isolated interferences. As an adjustment to the originally proposed SPS-MS3 workflow, we chose to detect both MS2 and MS3 fragment ions with high resolution and high accuracy in the Orbitrap, and use HCD in MS2 as well as MS3 (Fig. 2b and Supplementary Fig. 6). This allows subsequent combination of MS2 and MS3 fragment ions from the same N-glycopeptide precursor in post-acquisition processing in a straightforward manner (see below). Synchronous selection of multiple MS2 fragments for MS3 analysis further boosted the overall sensitivity (Supplementary Fig. 7).

A shortcoming of any MS3-based method is the longer duty cycle. However, our results showed that increased automatic gain...
control (AGC) target values and prolonged ion injection time (IT) benefited N-glycopeptide identification in a standard MS2 analysis in Orbitrap Tribid mass spectrometers (Supplementary Fig. 8a). Those settings improve N-glycopeptide identification but significantly slow down the overall scan speed in a MS2 analysis. Accordingly, the AGC and IT in Glyco-SPS-MS3 were adjusted (Supplementary Fig. 8b–e) in order to maintain a cycle time of ~3 s, comparable with MS2 without compromising the identification of N-glycopeptides. We note that Glyco-SPS-MS3 triggered 30% fewer precursors than MS2, but reached up to 3.6 fold GPSM identification rates depending on the NCEs used (Supplementary Fig. 9).

Next, we evaluated various single or stepped NCE (sNCE) settings in the Glyco-SPS-MS3 versus standard MS2 methods to optimize both the signal-to-noise (S/N) ratios of the TMT reporter ions as well as the numbers of identified GPSMs and glycoforms. In addition, peptide and glycan subscore distributions were analyzed to better understand the effect of NCE settings (Fig. 2c and Supplementary Fig. 10). While MS2 with sNCE moderately outperformed Glyco-SPS-MS3 (Fig. 2c, bottom panel), Glyco-SPS-MS3 consistently reached up to 3.6 fold GPSM identification rates depending on the NCEs used (Supplementary Fig. 9).

Fig. 2 Glyco-SPS-MS3 method for confident glycopeptides identification and accurate quantification. a A heatmap showing normalized intensities of different product ion types (see Supplementary Fig. 3 for nomenclature) of 12 TMT-labeled IgM glycopeptides detected in MS2 experiments using various NCEs. Optimal NCEs for individual product ion types were highlighted with blue boxes. b Schematic workflows of the original SPS-MS3 as compared with Glyco-SPS-MS3. Ions being selected for MS2 or MS3 fragmentation are in blue. TMT reporter ions are in red. MS detectors and fragmentation modes used in the methods are shown (IT for ion trap and OT for Orbitrap). c Comparisons of the distributions of TMT-reporter S/N ratios (upper panel, n = 116, 306, 181, 226, 206, and 218), pGlyco total score of GPSMs (middle, n = 336, 504, 188, 233, 219, and 227) and the number of identifications (bottom) obtained using MS2 or Glyco-SPS-MS3 methods with varied NCE settings. n = 2 independent measurements of TMT-labeled IgM glycopeptides. Boxplots show the median (centerline), mean (squares), first and third quartiles (box edges) and 1.5× the interquartile range (whiskers). Outliers are not shown. d Determined reporter ion ratios of IgM glycopeptides from the IgM-yeast mixture using MS2 or Glyco-SPS-MS3 methods. Channels with and without yeast interference are shown in blue and red, respectively. Predicted ratios are indicated. Source data are provided as a Source Data file.
GlycoBinder for single-step quantitative N-glycoproteomics data-processing. There is an increasing number of open software tools which use specialized scoring algorithms designed to achieve reliable glycopeptide identification. However, none of these supports database search of MS3 spectra and multiplexed quantification with isobaric mass tags. To make full use of the benefits of Glyco-SPS-MS3 for LC-MS-based identification and quantification of labeled N-glycopeptides, we developed GlycoBinder, an R-based tool. It combines existing computational tools to automatically extract and merge MS2 and MS3 fragments obtained on the same precursor ion into pseudo-fraction ion spectra, and to conduct glycopeptide identification by database search. Specifically, GlycoBinder uses MConvert to convert MS2 and MS3 spectra into mgf format, and further uses RawTools to generate a table listing the respective scan numbers of all MS3 scans and their parent MS2 scans. GlycoBinder then merges all MS2 and MS3 fragment ions accordingly into a predefined mass tolerance (1 ppm by default). GlycoBinder executes pParse to re-assign the mono- isotopic peak of each precursor. Subsequently, GlycoBinder searches the merged pseudo-fraction spectra with corrected precursor m/z-values for N-glycopeptide identification using the algorithm pGlyco, which supports command-line execution and FDR control on both peptide and glycan levels. At the end, GlycoBinder utilizes RawTools again to extract TMT reporter intensities from raw MS files and then supplements each GPSM with corresponding quantitative values.

We evaluated the performance of GlycoBinder for data analysis of TMT-labeled N-linked glycopeptides derived from DG75 cells in a human database search (in total 48 Glyco-SPS-MS3 raw files). GlycoBinder with the use of pParse identified 6% more GPSMs than without pParse. The Thermo Proteome Discoverer (PD) platform, which enables similar MS2-MS3 spectra merging (see Methods section), identifies 9% or 15% less GPSMs when compared with GlycoBinder with or without pParse, respectively (Fig. 3b). In terms of quantification of multiplexed N-glycopeptides, we confirmed that the reporter intensities extracted by RawTools are consistent with the values reported by PD (Fig. 3c).

We next investigated whether N-glycopeptide identification by GlycoBinder can be improved by searching against more specialized databases. We used a reviewed human proteome database from Swiss-Prot ("Human-reviewed", 20,303 entries, March 2018), a curated glycoprotein database from Swiss-Prot (Glycoprotein-reviewed, 4824 entries, March 2018), an in-house "B-cell-Specific glycoprotein" database with 974 glycoproteins that were repetitively identified in our inventory studies, and finally a non-related database consisting of a random selection of 1000 proteins (Random-1000) from the Human-reviewed database (Methods, Supplementary Data 1). GlycoBinder identified similar numbers of unique glycoforms from the Human-reviewed and the Glycoprotein-reviewed databases (4381 vs. 4335, respectively) at 2% FDR with 80% glycopeptide overlap. In contrast, the B-cell-specific database contains only 974 proteins—a 95% decrease compared to the Human-reviewed—GlycoBinder achieved 10% more identifications on this project-specific database than on the Glycoprotein-reviewed database. The search against the Random-1000 database resulted in the identification of only 243 of unique glycoforms, suggesting that using a smaller database does not spontaneously cause overfitting of the database search. The specificity of the database rather than its size is crucial for the improvement of identification.

A highly specific, experimentally based database carries the disadvantage that only glycosylation sites on known glycoproteins will be identified and quantified. Therefore, we implemented a two-step database search, in which we first search the raw data against the entire human protein database (here: Human-reviewed). Identified glycoproteins are subsequently used for a second database search of the same raw data (Fig. 3a). With this approach, we aimed to rescue low-stoichiometry glycoforms from the glycoproteins identified in the first search. Indeed, the two-step search boosted the identifications to 5367 unique glycoforms and 855 glycosites, representing respectively a 22.5% and an 8.5% increase as compared with the single search using the Human-reviewed database. More than 90% of the glycosites were identified in both the first and second searches (Supplementary Fig. 11b). In addition, our results indicated that the two-step search helped to identify more lower-scored glycoforms that were missed in the first search, at a fixed 2% FDR (Supplementary Fig. 11c).

In summary, GlycoBinder not only supports standard database searches with user-defined FASTA protein sequence databases, but also allows an automated two-step search for glycopeptide identification with considerably higher sensitivity. In addition, GlycoBinder uses the annotated result from pGlyco 2 software and propagates the corresponding GPSMs to different levels of quantification, including unique glycosylation sites, unique glycoforms, and unique glycan compositions (Fig. 4a and Supplementary Note 4). The propagation of quantitative values of GPSMs is...
performed in the same manner as in quantitative proteomics, where peptide quantifications are propagated to the protein level. To maximize the utility’s value, GlycoBinder also supports raw files acquired with common MS2 methods for those who have no access to the MS3-capable instruments. GlycoBinder is available on GitHub (https://github.com/IvanSilbern/GlycoBinder).

**Multiplexed quantification of N-glycopeptide on fucosylation-inhibited human Burkitt’s lymphoma cells.** To test the suitability of the SugarQuant workflow for large-scale glycosylation analysis, we applied it to the analysis of global fucosylation changes in human Burkitt’s lymphoma cells under the influence of 2-deoxy-2-fluoro-L-fucose (2FF). Fucosylated glycans play vital roles in a variety of biological and pathological processes, and aberrant fucosylation is associated with human diseases, such as the increase of core fucosylation in cancers. De-fucosylation using fucose analogs like 2FF suppresses cell proliferation and migration in human liver cancer cells, suggesting a possible way to retard tumor development. However, it remains elusive whether 2FF has any effect on the glycoprotein quantities per se, which might in turn affect the protein site-specific glycosylation in (cancer) cells. We applied SugarQuant to the quantitative analysis of glycopeptides derived from 2FF-treated human Burkitt’s lymphoma cells (DG75) in order to gain a deeper understanding of the underlying mechanism of 2FF-treatment and discover 2FF-sensitive glycosylation sites (Fig. 4b). DG75 cells were treated with 2FF at different concentrations (60–600 µM) for 3 days. The overall decrease in protein fucosylation was confirmed by western blotting against a biotinylated fucose-specific lectin (Aleuria Aurantia lectin, AAL, Supplementary Fig. 12a). Proteins were extracted and digested using the PAG method, and the resulting peptides were labeled with TMT6plex. Labeled glycopeptides were enriched with ZIC-HILIC, and further separated by bRP and analyzed by LC-MS using the Glyco-SPS-MS3 or the MS2 method. Databases were searched and N-glycopeptides were annotated by using GlycoBinder. We found that the standard MS2 method (Supplementary Table 2) identified slightly more glycoforms (6%), glycosites (3%), and glycans (2%) than the Glyco-SPS-MS3 method (Supplementary Fig. 12b). In contrast to the 13–30% of decreased identification rates commonly observed in proteomics or phosphoproteomics by switching from MS2 methods to SPS-MS3, SugarQuant reached a comparable level of sensitivity in glycoproteomics as compared to MS2, in spite of the slower acquisition rate. Importantly though, Glyco-SPS-MS3 outperformed the MS2 method to precisely determine the decrease of TMT-labeled glycoforms under 2FF treatment at all concentrations (Fig. 4c and Supplementary Fig. 13). Our results showed good reproducibility (Pearson correlation $r = 0.59–0.98$) among

---

**Fig. 3** GlycoBinder enables streamlined MS3 data handling for advanced intact glycopeptide identification and accurate multiplexed quantification. 

**a** The schematic workflow of GlycoBinder. 

**b** Different spectra-merging workflows produced different GPSM identification rates. TMT-labeled N-glycopeptides derived from DG75 cells were analyzed by Glyco-SPS-MS3 (48 raw files). All combined mgf files were searched using pGlyco 2 against a human database under 2% FDR. 

**c** Correlation of reporter ion intensities extracted by RawTools and PD. Each circle represents the extracted reporter ion from a scan. A total of 14,081 reporter ions were compared.

**d** Comparison of the two-step search with single searches using various databases. 

---

**Table 2** shows the number of GPSMs identified by pGlyco 2.0 search (FDR<2%).

---

**Fig. 4** Glyco-SPS-MS3 performance compared to other workflows. 

**a** The comparison of identified glycoforms, glycosites, and glycans between different workflows. 

**b** Comparison of sensitivity rates and specificities between different workflows.

---

**Fig. 5** shows the peak list merging process of GlycoBinder.

---

**Fig. 6** shows the workflow of GlycoBinder.

---

**Fig. 7** shows the comparison of peptide identifications and quantifications between different workflows.

---

**Fig. 8** shows the comparison of glycopeptide identifications and quantifications between different workflows.

---

**Fig. 9** shows the comparison of glycan identifications and quantifications between different workflows.

---

**Fig. 10** shows the comparison of protein identifications and quantifications between different workflows.

---

**Fig. 11** shows the comparison of phosphopeptide identifications and quantifications between different workflows.

---

**Fig. 12** shows the comparison of phosphoglycopeptide identifications and quantifications between different workflows.

---

**Fig. 13** shows the comparison of phospho-N-glycopeptide identifications and quantifications between different workflows.
than 27% of all glycosites, with an average of 6.3 glycoforms per glycosite. The majority of the glycoforms bore glycans containing 8 monosaccharide moieties with molecular masses of 2–3 kDa (Supplementary Fig. 15b, c). Of the 528 glycoproteins identified, however, only 13% have three or more different glycosites (Supplementary Fig. 15d).

Using the propagation function implemented in GlycoBinder, we extended the TMT-based quantification to the glycosite- and glycan-centric levels (Fig. 4d, e). Our results show a substantial reduction in numbers of fucosylated glycans under 2FF treatment, concomitant with a 2FF concentration-dependent increase of non-fucosylated glycans (Fig. 4e). At 480 µM 2FF, the quantities of 321 glycoforms on 138 glycosites within 105 proteins changed significantly (Z score > 2 or < −2) and 48 glycans from 46 glycoproteins are under-glycosylated under 2FF treatment, and 42 of these glycosites contain significantly changed glycoforms (Supplementary Data 3 and 4). Annotation of glycoproteins shows that the 2FF-affected glycoproteins are tightly associated with cell surface molecules with highly confident functional connections between them, including CD molecules, integrins, and targets in various cancers (Fig. 5a and Supplementary Data 5). The majority of those lymphoma-related proteins are enzymes or monosaccharide moieties with molecular masses of 2–3 kDa (Supplementary Fig. 15b, c). Of the 528 glycoproteins identified, however, only 13% have three or more different glycosites (Supplementary Fig. 15d).

Using the propagation function implemented in GlycoBinder, we extended the TMT-based quantification to the glycosite- and glycan-centric levels (Fig. 4d, e). Our results show a substantial reduction in numbers of fucosylated glycans under 2FF treatment, concomitant with a 2FF concentration-dependent increase of non-fucosylated glycans (Fig. 4e). At 480 µM 2FF, the quantities of 321 glycoforms on 138 glycosites within 105 proteins changed significantly (Z score > 2 or < −2) and 48 glycans from 46 glycoproteins are under-glycosylated under 2FF treatment, and 42 of these glycosites contain significantly changed glycoforms (Supplementary Data 3 and 4). Annotation of glycoproteins shows that the 2FF-affected glycoproteins are tightly associated with cell surface molecules with highly confident functional connections between them, including CD molecules, integrins, and targets in various cancers (Fig. 5a and Supplementary Data 5). The majority of those lymphoma-related proteins are enzymes or monosaccharide moieties with molecular masses of 2–3 kDa (Supplementary Fig. 15b, c). Of the 528 glycoproteins identified, however, only 13% have three or more different glycosites (Supplementary Fig. 15d).

Using the propagation function implemented in GlycoBinder, we extended the TMT-based quantification to the glycosite- and glycan-centric levels (Fig. 4d, e). Our results show a substantial reduction in numbers of fucosylated glycans under 2FF treatment, concomitant with a 2FF concentration-dependent increase of non-fucosylated glycans (Fig. 4e). At 480 µM 2FF, the quantities of 321 glycoforms on 138 glycosites within 105 proteins changed significantly (Z score > 2 or < −2) and 48 glycans from 46 glycoproteins are under-glycosylated under 2FF treatment, and 42 of these glycosites contain significantly changed glycoforms (Supplementary Data 3 and 4). Annotation of glycoproteins shows that the 2FF-affected glycoproteins are tightly associated with cell surface molecules with highly confident functional connections between them, including CD molecules, integrins, and targets in various cancers (Fig. 5a and Supplementary Data 5). The majority of those lymphoma-related proteins are enzymes or monosaccharide moieties with molecular masses of 2–3 kDa (Supplementary Fig. 15b, c). Of the 528 glycoproteins identified, however, only 13% have three or more different glycosites (Supplementary Fig. 15d).

Using the propagation function implemented in GlycoBinder, we extended the TMT-based quantification to the glycosite- and glycan-centric levels (Fig. 4d, e). Our results show a substantial reduction in numbers of fucosylated glycans under 2FF treatment, concomitant with a 2FF concentration-dependent increase of non-fucosylated glycans (Fig. 4e). At 480 µM 2FF, the quantities of 321 glycoforms on 138 glycosites within 105 proteins changed significantly (Z score > 2 or < −2) and 48 glycans from 46 glycoproteins are under-glycosylated under 2FF treatment, and 42 of these glycosites contain significantly changed glycoforms (Supplementary Data 3 and 4). Annotation of glycoproteins shows that the 2FF-affected glycoproteins are tightly associated with cell surface molecules with highly confident functional connections between them, including CD molecules, integrins, and targets in various cancers (Fig. 5a and Supplementary Data 5). The majority of those lymphoma-related proteins are enzymes or monosaccharide moieties with molecular masses of 2–3 kDa (Supplementary Fig. 15b, c). Of the 528 glycoproteins identified, however, only 13% have three or more different glycosites (Supplementary Fig. 15d).
MHC class II molecules (Supplementary Fig. 16). For example, the fucosylated glycan epitope, Lewis\textsuperscript{x}, is expressed on Hodgkin’s Reed–Sternberg cells (often carried by CD98 and ICAM-1, both are 2FF-affected glycoproteins) and is an established diagnostic marker for patients with Hodgkin’s lymphoma\textsuperscript{34,35}.

The glycoproteins affected by 2FF treatment include major players in B-cell receptor (BCR)-mediated signaling. For instance, in the human receptor-type tyrosine-protein phosphatase C (PTPRC), which is a tyrosine phosphatase involved in the regulation of B-lymphocyte activation, SugarQuant identifies and quantifies in total 192 glycoforms on 6 glycosites (Fig. 5b). While fucosylated glycoforms are significantly reduced on Asn-234, 337, and 531, only the glycosite Asn-531 exhibits decreased glycosylation occupancy (given that no 2FF-induced proteome change was detected (Supplementary Data 6)). Also, 19 glycoforms of two glycosites on CD79B, which is the B-cell antigen receptor complex-associated protein beta chain for initiation of the signal transduction cascade activated by the B-cell antigen receptor complex, were also quantified by SugarQuant (Fig. 5c). Both quantified CD79B glycosites contain 2FF-induced decreases of fucosylated glycoforms, but none of them shows significantly changed glycosylation occupancy. Our results suggest that 2FF site-specifically regulates glycosylation of human Burkitt’s lymphoma cells on important BCR effectors, which may in turn influence downstream BCR signaling.

**Discussion**

We present here a developed SugarQuant pipeline for multiplexed quantification of site-specific N-glycoproteomics. SugarQuant utilizes a PAC-based method to ensure sufficient solubilization of membrane-associated glycoproteins, complete and fast removal of
detergents, efficient TMT-labeling, and selective glycopeptide enrichment with reduced handling time and sample loss. PAC-based sample preparation has been used in a substantial range of proteomics experiments that extends from in-depth analyses of cell enrichment with reduced handling time and sample loss. PAC-Methods the capability of SugarQuant for accurate multi-dimension and propagate GPSMs for glycoform-, glycosite-, and glycan-centric quantification. We successfully applied the SugarQuant to determine site-specific glycosylation changes in fucosylation-inhibited DG75 cells. Our results not only represent the present largest glycoproteomics data of human lymphoma cells but also demonstrate the capability of SugarQuant for accurate multi-dimension quantification.

Methods

Cell culture and fucosylation inhibition. DG75 and Duadi cells were cultured in RPMI medium (Invitrogen) supplemented with 10–20% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen), penicillin/streptomycin (Invitrogen), and 1-glutamine (Invitrogen) at 37 °C with 5% CO2. DG75 cells (DSMZ no.: ACC 83) were provided by Prof. Dr. Thorsten Zenz at the National Centre for Tumor Diseases (NCT) in Heidelberg, Germany; Duadi cells (DSMZ no.: ACC 78) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH in Braunschweig, Germany. Cells were grown to ~90% confluence before harvesting and provided as cell pellets aliquoted at ~1 × 107 cells per tube. For fucosylation inhibition, cells with an initial concentration of 0.2 × 106 cells per mL in medium were treated with various concentrations of 2-fluoro-L-fucose (2FF) (Carbosynth Limited) in dimethyl sulfoxide (DMSO). All the four lysis buffers were supplemented with 1× Halt Protease and Phosphatase Inhibitor (Thermo Scientific). All the samples were digested for 15 min (15 s on, 15 s off) using Bioruptor at 4 °C. After centrifugation at 14,000 × g for 10 min using a benchtop centrifuge. The samples were cleaned up with Oasis HBL columns. All the samples were sonicated for 1 hr before harvesting and provided as cell pellets aliquoted at ~1 × 107 cells per tube. After incubation for 72 h, cell pellets were collected after washing three times with cold PBS, then stored at ~80 °C until use.

Preparation of peptides using conventional approaches. Cell pellets were lysed with either one of the four buffers: Buffer 1 consisted of 4% SDS (w/v), 50 mM HEPES, pH 8.0; Buffer 2 consisted of 8 M urea in 50 mM Triethylammonium bicarbonate (TEAB); Buffer 3 consisted of 0.1% (w/v) RapiGest SF surfactant (Waters) in 50 mM TEAB; and Buffer 4 consisted of 1% (w/v) RapiGest in 50 mM TEAB. All the four lysis buffers were supplemented with 1× Halt Protease and Phosphatase Inhibitor (Thermo Scientific). All the samples were digested for 15 min (15 s on, 15 s off) using Bioruptor at 4 °C. After centrifugation at 14,000 × g for 15 min, the supernatants were collected and the protein concentration were determined using Pierce BCA Protein Assay Kit (Thermo Scientific). All the proteins were reduced and alkylated with 10 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 500 mM stock, Sigma) and 20 mM iodoacetamide at 37 °C for 60 min in the dark. For samples in buffer 1, 3 times sample volume of cold (−20 °C) acetonitrile was added, and mixed samples were stored at −20 °C for at least 3 h or overnight to precipitate the samples. The samples were then centrifuged for 10 min at 14,000 × g, and the supernatants were removed carefully without disturbing the protein pellets. The protein pellets were resuspended in 1% Rapigest in 50 mM TEAB by thorough pipetting or vortexing. The proteins were digested using trypsin at an enzyme to protein ratio of 1:50 at 37 °C overnight. For samples in buffer 2, we diluted the samples 8-fold with 50 mM ammonia bicarbonate (ABC). After overnight trypsin digestion, the resulted peptides were desalted by Oasis HLR columns (Waters). For samples in buffer 3, trypsin were directly added for overnight digestion. For samples in buffer 4, we did the same as the samples in buffer 3, except that we diluted 10-fold with 50 mM TEAB before digestion.

PAC-based preparation of TMT-labeled peptides from cells. Cell pellets were lysed in buffer 1. Cell protein extraction, reduction, and alkylation were the same as mentioned above. We compared three different types of magnetic beads, including Sera-Mag SpeedBeads with a hydrophilic surface (GE Healthcare, cat.no. 45152101010250, Magnetic Carboxylate Modified), Sera-Mag SpeedBeads with a hydrophobic surface (GE Healthcare, cat.no. 45152105050250, Magnetic Carboxylate Modified) and MagReSyn HILIC (ReSynBio, cat.no. MR-HLC005) with a mixed-mode functional surface. The magnetic beads were rinsed twice with water on a magnetic rack prior to use. The beads were added to protein lysates at the optimal working ratio of 10:1 (wt/wt, beads to proteins). The required minimum bead concentration is 0.5 µg/µL in order to provide sufficient surface for the immobilization of aggregated proteins. We then added acetonitrile to protein (70% final percentage of 1% (vol/vol). The samples were allowed to stay off the rack for 10 min at room temperature (RT), followed by resting on the magnetic rack for 2 min at RT. The supernatant were discarded and the beads were then washed for three times with 80% (vol/vol) ethanol. Beads were resuspended in 50 mM TEAB containing sequencing grade modified trypsin (1:50 of enzyme to protein concentration) and incubated at 37 °C for 4 hr. We then loaded the beads on a ZIC-HILIC column (3.5 µm particles, 1.0 mm inner diameter, and 150 mm in length). The gradient was 2% B for 5 min, to 34% B in 37 min, to 60% B in 8 min, to 90% B in 1 min, held at 90% B for 5.5 min, to 2% B in 0.5 min, and then held at 2% B for 7 min (64 min total runtime). Peptides were detected at 214 nm and 58 fractions were collected along with the LC-separation in a time-based mode from 6 to 64 min. Fractions were then pooled into eight concatenated fractions.

Preparation of TMT-labeled IgM peptides. Human IgM purified from human serum (Sigma, Ig2680) was reconstituted at the final concentration of 1 mg/mL in 0.05 M Tris-HCl, 0.2 M sodium chloride, 15 mM sodium azide, pH 8.0. The proteins were heated at 95 °C for 10 min. After cooling to RT, protein reduction, alkylation and digestion were the same as mentioned above. The digested samples were acidified by adding TFA to a final concentration of 1% (vol/vol). The insoluble particles in the samples were removed by centrifuging at 14,000 × g for 10 min using a benchtop centrifuge. After cleaning up with Oasis HLB columns, the resulting peptides were dried in a SpeedVac concentrator. TMT labeling procedure (Thermo Scientific) was performed according to the manufacturer’s instruction.

IgM and yeast peptide interference model. Yeast proteins prepared from S. cerevisiae cells were purchased from Promega (Cat. V7341). Yeast proteins in 6.5 M urea/50 mM Tris-HCl (pH 8) at a protein concentration of 10 mg/mL were thawed on ice. Protein reduction and alkylation were the same as mentioned above. Dilute the urea to 1 M using 50 mM Tris-HCl (pH 8). Trypsin (Promega) was added at a trypsin:protein ratio of 1:50 at 37 °C. After overnight incubation, the samples were acidified by adding TFA to a final concentration of 1% (vol/vol). The insoluble particles in the samples were removed by centrifuging at 14,000 × g for 10 min using a benchtop centrifuge. The samples were cleaned up with Oasis HLB columns. The resulted peptides were dried in a SpeedVac concentrator.

We separately labeled IgM digests with individual TMT6 reagents and pooled them together afterward with the ratio of 10:4:1:4:1:10. In contrast, yeast peptides were labeled with only the first three channels of TMT6 reagents (126, 127, and 128) and mixed equally (Fig. 2d). Then we spiked the pooled IgM peptides into the yeast peptide mixture with an equal amount. The mixed samples were then cleaned up with Oasis HLB columns. The samples were analyzed by either Glyo-SPS-MS3 or standard MS2 method (see below).

Lectin blotting. DG75 cell pellets with and without 2FF treatment were lysed in 4% SDS, 0.1 M Tris-HCL, pH 8.0. Protein concentrations were determined using BCA. For each condition, 20 µg proteins were reconstituted in 1× NuPAGE LDS Sample Buffer (Invitrogen) and separated on 4–12% NuPAGE Novex Bis-Tris Minigels (Life Technologies). The proteins separated in gel were transferred onto a nitrocellulose membrane (GE Healthcare). The membrane was blocked with 5% (w/v) bovine serum albumin (Sigma) in TBST overnight at 4 °C. The membrane was then incubated with biotinylated lectins AAL (vector laboratories; 3 µg/mL in blocking buffer) for 1 h at room temperature. The membrane was washed three times using TBST containing HRP-conjugated streptavidin (Abcam) in 1× TBST for 1 h. After washing, the signal was visualized using a chemiluminescence detection system (ECL, GE Healthcare) and detected on X-ray film.
LC-MS analysis. TMT-labeled or unlabeled glycopeptides were resuspended in 5% ACN, 0.1% FA, and subjected for LC-MS/MS analysis using Orbitrap Fusion Tribrid or Lumos Mass Spectrometers (Thermo Scientific), both coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Scientific). IgM glycopeptides were firstly concentrated on a C18 trap column (3 cm long; inner diameter, 100 μm; outer diameter, 360 μm) for 3 min and then separated on a home-made analytical column (ReproSil-Pur 120 C18-AQ, 1.9 μm pore size, 75 μm inner diameter, Dr. Maisch GmbH, Germany) at 50 cm (only for Supplementary Fig. 11) for one-hour gradient at a flow rate of 300 nL/min. Mobile phase A and B were 0.1% (vol/vol) formic acid (FA) and 80% ACN, 0.08% FA, respectively. The gradient started at 5% B at 3 min, increased to 50% B in 42 min, and then to 70% B in 4 min. After washing for 90% B, the column was re-equilibrated with 5% B. For glycopeptides from DG75 cells, no trap column was used. After sample loading with 2% B in 49 min, the gradient increased to 5% B in 4 min, continued to 38% B in 147 min, and then to 60% B in 16 min, followed by the above-mentioned washing and re-equilibration steps. For proteomics analysis, a trap column was used. The gradient started at 8% B at 3 min, increased to 40% B in 50 min, and then to 60% B in 13 min, followed by the above-mentioned washing and re-equilibration steps.

MS parameters evaluated in Glyco-SPS-MS3 were listed in Supplementary Table 1. The instrument settings optimized for Glyco-SPS-MS3 and those used for common MS2 acquisition were summarized in Supplementary Table 2. For proteomics analysis on Orbitrap Fusion, the following settings were used. MS1 settings: Detector Type-Orbitrap, Orbitrap Resolutions-120 K, Scan Range-350–1,550, Maximum injection time-50 ms, AGC target-4×106, RF Lens-60%, and Data Type-Profile. MS2 settings: Isolation mode-Quadrupole, Isolation window-1.6 m/z, Scan range mode-Auto normal, Mass bin-110, Activation type-HCD, Collision energy (%)—40, Detector type-Orbitrap, Orbitrap Resolution-60 K, Maximum injection time-60 ms, AGC target-5×106, and Data Type-Profile.

For the evaluation of IgM glycopeptide HCD fragmentation, each selected precursor in MS1 + MS2 scans was subject to eight consecutive MS2 scans with NCE15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50%, respectively (Fig. 2a and Supplementary Figs. 2 and 3). The intensities of product ions detected under different NCEs were extracted and normalized to the total normal ion current of the respective spectra. For the evaluation of detector type and fragmentation type in MS2 methods, each selected precursor from IgM samples was subject to 4 sequential MS2 scans, including Orbitrap_HCD, Ion trap_HCD, Orbitrap_CID and Ion trap_CID (Supplementary Fig. 5).

LC-MS data processing. For intact glycopeptide identification and quantification, raw files were processed via GlycoBinder (Supplementary Note 4), which is available on GitHub (https://github.com/IvanSilbers/GlycoBinder). Parameters used for pGlyco 2 include fully specific tryptic digestion with maximal two missed cleavage and mass tolerance for precursors and fragment ions of 10 and 20 ppm, respectively. We considered cysteine carbamidomethylation (+57.02146 Da) as a fixed modification, and methionine oxidation (+15.99492 Da) as a variable modification.

For IgM-Yeast interference model, the raw MS files were processed using GlycoBinder and searched against a protein database including sequences from all known S.cerevisiae ORFs. FDR < 2% for both the first and second database search was used.

For processing Glyco-SPS-MS3 results using PD, the nodes of "Spectrum selector" and "Spectrum grouper" were used for converting and combining spectra from MS2 and MS3, respectively, with the following settings. Precursor mass criterion: same singly charged mass; Precursor mass tolerance: 0.1 ppm; Max. RT difference: 0.04 min; Allow mass analyzer mismatch: False; Allow MS order mismatch: True. The resulting mgf files were used for pGlyco 2 searches. Alternatively, the mgf files can be used for glycopeptide identification and quantification using the Byonic node in the PD platform.

Proteomics results were verified with built-in Sequest HT and Percolator nodes using the following settings: the "Human reviewed" database, fully specific tryptic digestion, maximum two missed cleavages, mass tolerance for precursors and fragment ions of 10 and 20 ppm, respectively, cysteine carboxymethylation and TM/TE on peptide N-termini and lysine residues as fixed modifications, methionine oxidation as a variable modification, and target FDR as 1%.

Data analysis. GlycoBinder reports directly the integrated TMT-reporter ratios of unique glycoforms, glycosites, and glycan compositions in separate text files. To account for quantitative errors introduced before TMT-labeling, TMT-ratios of glycoforms determined in each glycoproteomics experiment on DG75 cells were normalized by the median TMT ratio of respective proteomics analysis. Significant regulation is defined using Z score >2 or <−2. Unless mentioned elsewhere, all figures were made using Excel 2016.

For the sunburst plot, we investigated and annotated the protein functions of 2FF-affected glycoproteins manually via surveying literature relevant to the reported gene and protein names (including alternative names) and words of "cancer", "lymphoma" or "B cell" (Supplementary Data 5). We summarized the information about the biological function(s) and subcellular localization of all proteins and classified them accordingly into multiple categories. For example, the first inner layer contains the groups of lymphoma-related, drug target in other cancer, enzymes and others. The number of proteins in each category is proportional to the size of the corresponding categorical area in the sunburst plot. The interaction networks of proteins were done by STRING (version 11.0) [50]. Boxplot, violin plot, and density plot were created using OriginPro 2020 (9.7.0.185). In the boxplots, central lines and squares in plotted boxes indicate the median and mean, respectively. The upper and lower ends of box shows the 75th and 25th percentiles. The extreme lie shows 1.5× the interquartile range.

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

Data availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [53] partner repository with the dataset identifier PXD018349.

Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT and Proteomics results were processed via PD with built-in Sequest HT. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability. The developed GlycoBinder is available on GitHub (https://github.com/IvanSilbers/GlycoBinder).

Received: 1 May 2020; Accepted: 23 September 2020; Published online: 19 October 2020

References

1. Reily, C., Stewart, T. J., Renfrow, M. B. & Novak, J. Glycosylation in health and disease. Nat. Rev. Nephrol. 15, 548–566 (2019).

2. Lassen, J.-A. et al. Malignant glycoproteomics technologies provide unique structural insights into the N-glycoproteome and its regulation in health and disease. Mol. Cell. Proteomics 15, 1773–1790 (2016).

3. Song, X. et al. Oxidative release of natural glycans for functional glycomics. Nat. Methods 13, 528–534 (2016).

4. Li, Q., Xie, Y., Wong, M. & Lebrilla, C. B. Characterization of cell glycocalyx with mass spectrometry methods. Cells 8, 882 (2019).

5. Fang, P. et al. Multilayered N-glycoproteome profiling reveals highly heterogeneous and dysregulated protein N-glycosylation related to Alzheimer's disease. Anal. Chem. 92, 867–874 (2020).
6. Kaji, H. et al. Lectin affinity capture, isoyme-coded tagging and mass spectrometry to identify N-linked glycoproteins. Nat. Biotechnol. 21, 667 (2003).
7. Mysling, S., Palmisano, G., Hajrup, P. & Thaysen-Andersen, M. Utilizing ion-pairing hydrophilic interaction chromatography solid phase extraction for efficient glycoconjugate enrichment in glycoproteins. Anal. Chem. 82, 5958–5969 (2010).
8. Xiao, H., Sun, S., Suttuprasukul, S. & Wu, R. Global and site-specific analysis of protein glycosylation in complex biological systems with Mass Spectrometry. Mass Spectrom. Rev. 999, 1–24 (2019).
9. Wuhrer, M., Catalina, M. I., Deelder, A. M. & Hokke, C. H. Glycoproteomics based on tandem mass spectrometry of glycopeptides. J. Chromatogr. B 849, 115–128 (2007).
10. Riley, N. M., Hebert, A. S., Westphall, M. S. & Coon, J. J. Capturing site-specific heterogeneity with large-scale N-glycopeptide analysis. Nat. Commun. 10, 1311 (2019).
11. Ruhaak, L. R., Xu, G., Li, Q., Goonatilleke, E. & Lebrilla, C. B. Mass spectrometry approaches to glycomic and glycoproteomic analyses. Chem. Rev. 118, 7886–7930 (2018).
12. Sun, S. et al. Comprehensive analysis of protein glycosylation by solid-phase extraction of N-linked glycanas and glycosyl-containing peptides. Nat. Biotechnol. 34, 84–88 (2016).
13. Stadlmann, J. et al. Comparative glycoproteomics of stem cells identifies new players in ricin toxicity. Nature 549, 538–542 (2017).
14. Lee, H. J. et al. Abundance-ratio-based semiquantitative analysis of site-specific N-linked glycopeptides present in the plasma of hepatocellular carcinoma patients. J. Proteome Res. 13, 2328–2338 (2014).
15. Ting, L., Rad, R., Gygi, S. P. & Haas, W. MS3 eliminates ratio distortion in isotopic multiplexed quantitative proteomics. Nat. Methods 8, 937–940 (2011).
16. McAlister, G. C. et al. Multinotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. Anal. Chem. 86, 7150–7158 (2014).
17. Hughes, C. S. et al. Ultrasensitive proteome analysis using paramagnetic bead technology. Mol. Syst. Biol. 10, 757 (2014).
18. Tanveer, S. B. et al. Protein aggregation capture on microparticles enables multipurpose proteomics sample preparation. Mol. Cell. Proteomics 18, 1027–1035 (2019).
19. Thompson, A. et al. TMTpro: design, synthesis, and initial evaluation of a proline-based isobaric 16-pex tandem mass tag reagent set. Anal. Chem. 91, 15941–15950 (2019).
20. Hu, H., Khatri, K. & Zaia, J. Algorithms and design strategies towards automated glycoproteomics analysis. Mass Spectrom. Rev. 36, 475–498 (2017).
21. Savitski, M. M. et al. Measuring and managing ratio compression for accurate iTRAQ/TMT quantitation. J. Proteome Res. 12, 3586–3598 (2013).
22. Zeng, W. F. et al. pGlyco: a pipeline for the identification of intact N-glycopeptides by using HCD-and CID-MS/MS and MS3. Sci. Rep. 6, 25102 (2016).
23. Wu, S. W., Liang, S. Y., Pu, T. H., Chang, F. Y. & Khoo, K. H. Sweet-Heart—an integrated suite of enabling computational tools for automated MS2/MS3 sequencing and identification of glycopeptides. J. Proteom. 84, 1–16 (2013).
24. Holman, J. D., Tabb, D. L. & Mallick, P. Employing ProteoWizard to convert raw mass spectrometry data. Curr. Protoc. Bioinformatics 46, 13 24 11-19 (2014).
25. Kovách, K. A. et al. RawTools: rapid and dynamic interrogation of Orbitrap data files for mass spectrometer system management. J. Proteome Res. 18, 700–708 (2019).
26. Yuan, Z. F. et al. pPars: a method for accurate determination of monosotopic peaks in high-resolution mass spectra. Proteomics 12, 226–235 (2012).
27. Liu, M. Q. et al. pGlyco 2.0 enables precision N-glycopeptomics with comprehensive quality control and one-step mass spectrometry for intact glycopeptide identification. Nat. Commun. 8, 438 (2017).
28. Noble, W. S. Mass spectrometrists should search only for peptides they care about. Nat. Methods 12, 605–608 (2015).
29. Bantscheff, M., Schirle, M., Sweetman, G., Rick, J. & Kuster, B. Quantitative mass spectrometry in proteomics: a critical review. Anal. Bioanal. Chem. 389, 1017–1031 (2007).
30. Miyoshi, E., Moriwaki, K. & Nakagawa, T. Biological function of fucosylation in cancer biology. J. Biochem. 143, 725–729 (2008).
31. Zhou, Y. et al. Inhibition of fucosylation by 2-fluorofucose suppresses human liver cancer HepG2 cell proliferation and migration as well as tumor formation. Sci. Rep. 7, 11563 (2017).