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Investigation of Glycosylphosphatidylinositol (GPI)–Plasma Membrane Interaction in Live Cells and the Influence of GPI Glycan Structure on the Interaction

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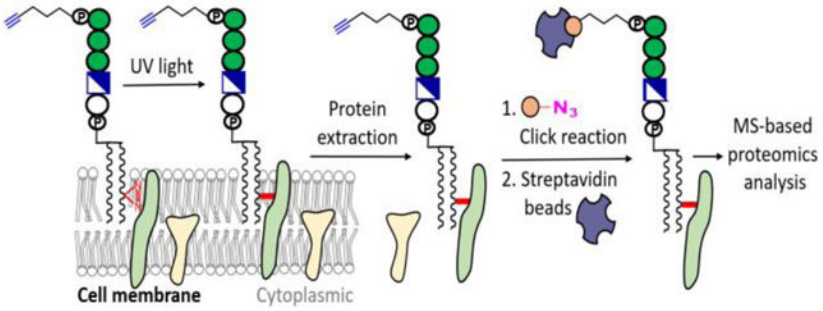
Abstract

Glycosylphosphatidylinositols (GPIs) need to interact with other components in the cell membrane to transduce transmembrane signals. A bifunctional GPI probe was employed for photoaffinity-based proximity labelling and identification of GPI-interacting proteins in the cell membrane. This probe contained the entire core structure of GPIs and was functionalized with photoreactive diazirine and clickable alkyne to facilitate its crosslinking with proteins and attachment of an affinity tag. It was disclosed that this probe was more selective than our previously reported probe containing only a part structure of the GPI core for cell membrane incorporation and an improved probe for studying GPI-cell membrane interaction. Eighty-eight unique membrane proteins, many of which are related to GPIs/GPI-anchored proteins, were identified utilizing this probe. The proteomics dataset is a valuable resource for further analyses and data mining to find new GPI-related proteins and signalling pathways. A comparison of these results with those of our previous probe provided direct evidence for the profound impact of GPI glycan structure on its interaction with the cell membrane.

Graphical Abstract



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Supporting Information
The authors have cited additional references within the Supporting Information.[63]

A bifunctional GPI derivative containing the entire conserved core structure of GPIs was proved to be a useful probe to target the cell membrane and tag GPI-related membrane proteins to facilitate protein isolation and proteomics analysis.

Keywords

glycolipid; glycosylphosphatidylinositol; cell membrane; interaction; photoaffinity; labelling; proteomics

Introduction

The plasma membrane is the central hub for cells to communicate with the extracellular world to fulfill their functions, which involves the interaction of extracellular cues with membrane-associated biomolecules.[1] Glycosylphosphatidylinositol-anchored proteins (GPI-APs, Figure 1) are among such biomolecules and display diverse functions.[2] GPIs are ubiquitous glycolipids[3] that attach to the protein C-terminus to facilitate protein anchorage to the cell (Figure 1). It has been shown that GPI anchors are essential for the functions of GPI-APs mediated by the interaction of GPIs with specific molecules in the cell membrane. For example, deleting the GPIs of CD55 and CD59 can cause hemolysis,[4] and mutating the yeast gene *PER1* involved in GPI lipid remodeling interrupts GPI association with the lipid rafts.[5] Therefore, GPIs are not only membrane protein anchors but also modulators directly involved in cellular signaling and other biological activities.

However, the lipids of GPIs (typically 16–20 carbons long) are not long enough to span the membrane bilayer. Thus, they need to interact with other components in the membrane, such as proteins and lipids, to transduce transmembrane signals.[2c, 6] However, it is difficult to identify these molecules due to the heterogeneity of cell membrane, complex structure of GPIs/GPI-APs, and naturally low abundance of GPI-APs. We aim to develop new methods to examine GPI-cell membrane interaction to help understand their functions.

We recently profiled GPI-interacting membrane proteins using a GPI probe **1** (Figure 1),[7] which contained a photoreactive group and clickable alkyne in the lipid and glycan to enable photoactivated crosslink with GPI-interacting proteins and affinity-based isolation of crosslinked proteins for proteomics study. Probe **1** helped reveal a series of GPI-related membrane proteins. However, comprising only a part of GPIs (Figure 1), **1** may not fully represent natural GPIs. To address this problem, we have designed probe **2** (Figure 1) containing the entire conserved core structure of GPIs and the photoreactive and clickable functionalities and utilized it to study GPI-cell membrane interaction. Comparing the proteins identified with **1**, **2**, and **3** (negative control) would provide novel information about the interaction between GPIs and the cell membrane and the impact of GPI glycan structure on the interaction.

Results and Discussion

Probes for the study of GPI-plasma membrane interaction.

To identify GPI-interacting membrane proteins, it is essential to have probes that can label the target proteins to enable their separation from other proteins. To this end, we designed bifunctional probe **2**.[8] The diazirine in its phosphatidyl moiety is activable by UV light to generate reactive carbenes for covalent link to nearby proteins,[9] thereby labeling the proteins. The alkyne in the glycan of **2** can serve as a molecular handle to install affinity tags (e.g., biotin) by click chemistry to facilitate the isolation of cross-linked proteins for proteomics study.[10] Similarly functionalized glycolipid **3** will be used as the control to identify nonspecifically crosslinked proteins. The diazirine and alkynyl groups in **2** are relatively small–not much larger than the replaced groups in the lipid and glycan of natural GPIs. Therefore, we expected them to have minimal impacts on the structure and organization of the cell membrane. Moreover, using a phosphoalkyne to replace phosphoethanolamine (PEA) at the mannose (Man) III 6-O-position, to which proteins are attached in natural GPI-APs, will prevent the probe from participating in GPI-AP synthesis, thereby excluding the labeling of GPI-APs to reduce background and increase specificity.

In stark contrast to **1**, probe **2** contains the entire conserved core structure of GPIs[11] and is a drastically improved mimic of natural GPIs. It has been shown that the glycan of GPIs is pivotal for their biological functions. For instance, GPI glycan is the signal for GPI-AP transport from endoplasmic reticulum (ER) to Golgi;[12] altering the GPI part of prion proteins (PrPs) can affect the cell membrane microenvironment and cellular signaling.[13] Thus, there is a need to study GPIs with different glycans. Furthermore, we expect that comparing the results of **1** and **2** will help reveal the impact of GPI glycan on GPI-cell membrane interaction to gain more insights into the mechanisms of GPI/GPI-AP-mediated signaling.

Experimental design for identifying GPI-interacting membrane proteins.

First, we will engineer cells using **2** and **3**. Studies have proved the spontaneous and effective incorporation of exogenous lipids/glycolipids, including GPIs, by cells.[6a, 7, 14] We anticipated that cells would take up **2** and **3** from the media and incorporate them into their membranes. Next, as shown in Figure 2, we will expose the cells to 365 nm UV light to promote the crosslinking of probes with membrane proteins. Photoactivated labeling of proteins for proteomics study has been successfully applied to different cells and systems.[9b, 15] Thereafter, the cells will be lysed, and the lysates will be treated with azide-biotin to add biotin as an affinity tag by Cu-catalyzed alkyne-azide cycloaddition (CuAAC), a bioorthogonal click reaction.[10a, 16] Finally, the tagged proteins will be separated using streptavidin-beads and applied to mass spectrometry (MS)-based proteomics analysis.

Cellular incorporation of the probes.

Fluorescence labeling and analysis were used to validate cellular incorporation of the probes in the plasma membrane. In these experiments, HeLa cells were first treated with **2**, **3** or **1**, followed by azide-biotin for CuAAC and then streptavidin-A488 (green) or -Cy5 (purple) for fluorescent labeling.[7, 16] Next, the labeled cells were studied with fluorescence

microscopy. Under the cell culture conditions established for **1**, i.e., incubation with probe at 37 °C for 4 h,[7, 16] we observed a significant increase in green fluorescence A488 for cells treated with **2** and **3**, compared to the control that was treated with phosphate-buffered saline (PBS) (Figure 3). These results indicate the effective cellular incorporation of **2** and **3**, which was also supported by the results of flow cytometry analysis of the labeled cells (Figure S1, SI).

To optimize the conditions for cell incorporation, we treated HeLa cells with **1, 2**, and **3** for varied lengths of time (1, 2, 3, 4, 6, 8 h), before reaction with azide-biotin, treatment with streptavidin-A488, and then imaging. The results (Figure S2A, SI) showed a steady increase in cellular fluorescence with elongated incubation time up to 4 h, when the maxima were reached, followed by a gradual decline. The mean fluorescence intensities of these cells were also calculated to validate the same (Figure S2B, SI). These results are consistent with previous observations.[16–17] Another discovery was that cells treated with **2** exhibited consistently higher fluorescence intensity than cells treated with **1** and **3**, which may be attributed to either more effective incorporation of **2** or the presence of an additional alkynyl group in its structure.

Our previous work suggested that besides the plasma membrane, **1** and **3** were also incorporated in the membranes of intracellular organelles.[7] To determine if **2** was more selective than **1** and **3** to target the plasma membrane, we compared their distributions in cells using fluorescence labeling. To this end, we examined first the cellular localization of **2**. After HeLa cells were incubated with **2** for 1, 2, 3, and 4 h and labeled with A488, they were fixed, permeabilized, and then treated with Cy5-tagged ERp72, GOLPH2, EEA1, and LAMP1 antibodies to stain endoplasmic reticulum (ER), Golgi, early endosome, and lysosome, respectively. Next, the cells were studied with a microscope using the appropriate fluorescent channels. The results (Figure 4; Figures S3–S6, SI) revealed the different localizations of the two fluorescent labels, suggesting low colocalization of **2** with intracellular organelles. Therefore, probe **2** may be incorporated preferably into the plasma membrane and remains there at least during the initial 4 h.

To verify the favored incorporation of **2** in the cell membrane and compare it with **1** and **3**, we examined the localizations of **1**, **2**, and **3** within cells. To this end, we treated HeLa cell with **1**, **2** or **3** for 4 h followed by A488 labeling and then specific C10045 staining of the cell membrane. The cells were studied with a microscope and appropriate fluorescent channels. Our results suggest (Figure 5A) that although cell internalization occurred for all probes, C10045 and A488 fluorescence overlap was significantly higher for **2** than **1** and **3**. This was also verified by the significantly larger Pearson correlation coefficient for **2** than for **1** and **3** (Figure 5B). Therefore, **2** was demonstrated to be more selective than **1** and **3** to target the plasma membrane.

Identification of GPI-interacting proteins in the cell membrane.

HeLa cell was used for this study because its proteomics data and membrane proteins interacting with **1** were available. This would facilitate comparative studies to uncover the impact of GPI glycan structure on GPI-cell membrane interactions. These experiments

were conducted according to the procedure outlined in Figure 2. Accordingly, HeLa cells were treated with **2** or **3** and then UV light. The cells were lysed, and the lysates were incubated with azide-biotin to biotinylate probe-tagged proteins by CuAAC. Total proteins were extracted by methanol or acetone precipitation. SDS-PAGE analysis of the precipitated proteins indicated the labeling of many proteins by probes **2** and **3** (Figure S7, SI). For MS-based analysis of the crosslinked proteomes, biotinylated proteins were separated with streptavidin beads and applied to MS study. Each experiment was repeated three times. Our proteomics results showed that **3** and **2** labeled 3164 and 3197 proteins, respectively, among which 3109 were found with both probes (Figure 6A). The volcano plot in Figure 6B shows the distribution of all proteins identified with **2**, utilizing **3**-labeled proteins as the reference. In total, 88 unique proteins were identified with **2**, among which 12 were found in all three experiments, 16 in any two experiments, and the remaining in one experiment (Figure 6A). The identities of all 88 proteins, along with their cellular locations and putative biological functions, were inspected (Table S1, SI). In addition, we have also identified 27 significantly enriched (≥ 4 folds, $P \leq 0.05$) proteins with **2** (Table S2, SI), compared to **3**-labeled proteins.

Although **2** showed better localization in the cell membrane than **1**, its cellular internalization is inevitable (Figures 4 and S3–S6, SI), thus its crosslinkage with ER and Golgi membrane proteins was expected (Table S1, SI). However, aligned with the observation of favorable localization of **2** in the cell membrane, a smaller number of unique proteins was identified using **2** than using **1**. Importantly, many of these proteins are associated with GPI/GPI-AP–related pathways or signaling mediators that engage GPI-APs (Table 1). Particularly, several proteins, e.g., Fas-associated death domain (FADD) protein – an apoptotic adaptor in membrane-cytoskeletal rearrangement,[18] are related to Cdc42. Since Cdc42d plays a role in many signaling pathways and is a key regulator of clathrin-independent GPI-AP endocytosis, Cdc42-related proteins in Table 1 are expected to be involved in GPI-AP signaling, which can be an interesting area of future studies.

Among the remaining proteins in Table 1, dystroglycan 1 (DAG1) – a transmembrane protein interacting with GPI-APs such as C4.4a and prion protein, is involved in cell membrane-extracellular matrix organization, cell interaction and metastasis, membrane assembly, and GPI-regulated signaling.[19] OSBP2 (oxysterol-binding protein 2) and OSBPL11 play a role in ceramide (Cer) transport protein complex formation responsible for vesicular Cer trafficking. Although there is no conclusive evidence linking OSBPs to GPI-APs, studies have proved the interdependence of Cer and GPI trafficking.[20] Thus, OSBPs are anticipated to engage in GPI-AP trafficking. Beclin-1 (BECN1) is a trans-Golgi membrane protein involved in the phosphatidylinositol-3-kinase (PI3K) pathway.[21] It interacts with prion protein to enhance PI3K activity and their colocalization in the lipid rafts, regulating vesicular trafficking of GPI-APs.[22] BECN1-interacting vacuolar protein sorting (VPS)-associated proteins [21a] were also captured, indicating that BECN1 may engage VPS proteins to activate GPI-AP trafficking.

V-Type proton ATPase is a transmembrane proteolipid subunit C protein colocalized with Golgi-resident GPI-APs (GREGs) for structural and functional regulation of Golgi compartments.[23] v-ATPase resides in the lipid rafts to participate in many signaling pathways,[24] suggesting its role in GPI signaling. Another piece of evidence supporting

the correlation of v-ATPase with GPI-APs is that PbS21, a GPI-AP, can specifically target epithelial cells having elevated v-ATPase.[25] EHD4 is a membrane ATPase in early endosomes to regulate membrane organization, tubulin formation, and GPI-AP trafficking.[26] EHD4 is an interesting protein as it was also crosslinked with **1**, indicating a new mechanism of membrane ATPases in GPI-AP trafficking that is unknown yet.

Yes-associated protein 1 (YAP/YAP1) is a transcriptional regulator in the Hippo signaling pathway interacting with GPI-APs.[27] It has been shown that THY1, a basal epidermis GPI-AP, could inhibit YAP in a Src-kinase dependent manner to regulate cell-matrix and cell-cell interaction[28] and YAP deregulates GPI-AP expression or its interaction with other signaling partners within the lipid rafts via regulating the Hippo signaling pathway.[29] YAP-regulated GPI-AP arrangement and localization occur via the mechanocytoskeleton signaling mechanism, which may be linked to GPI-AP-mediated actin cytoskeleton remodeling,[30] and thus modulate other cellular mechanisms. PKAC, AP2S1, and EHD4 are proteins crosslinked with both **1** and **2**, suggesting their recognition of some common motifs of phosphatidylinositol (PI), thus their binding or interaction with GPIs is independent of the GPI glycan structure.

Overall, our results suggest that **2** can cross-react with membrane proteins in live cells. Moreover, it is more selective and thus more useful than **1** to target and identify GPI/GPI-AP-related proteins in the plasma membrane. For example, less proteins were pulled down with **2** than with **1**. More importantly, 29 of the 88 unique proteins identified with **2**, but only 12 proteins identified with **1**, were related to GPIs/GPI-APs. Among all proteins identified, only three were the same for **1** and **2**, suggesting that **1** and **2** may have distinct localizations/organizations in the cell and organelle membranes to interact with distinct sets of proteins. Because **1** and **2** contained the same PI and were only different in their glycans, these results suggest the significant impacts of the GPI glycan structure on the properties of GPIs/GPI-APs and their capabilities to target specific cell membrane proteins.

The different efficacy and specificity of **2** and **1** to target GPI/GPI-AP-related proteins in the cell membrane may be attributed to their distinct glycans, as the glycan can affect GPI interaction with membrane proteins and then impact the activity of GPI-APs.[6c, 31] Probe **2** with the entire core structure of GPIs can mimic natural GPIs better than **1**. This may lead to more selective and stronger interactions of **2** with the targeted membrane proteins. Moreover, **1** and **2** may face different trafficking fates. Probe **1** resembles one of the biosynthetic precursors of GPIs on the cytoplasmic face of ER membrane that is recognized and translocated onto the lumen side by flippase.[32] On the other hand, **2** is the end product of GPI biosynthesis before its attachment to target proteins, thus it is not recognized by flippase. After endocytosis, **1** may be transported to Golgi and other organelles through ER, while **2** may tend to reside on ER. More importantly, **1** is smaller than **2** and mimics a GPI biosynthetic precursor and, thus, is likely to be more prone to cellular internalization. All the factors combined may determine that **2** would be more effective and specific than **1** to target GPI-interacting proteins in the cell membrane.

Bioinformatics study.

We also performed bioinformatics analysis of the proteins identified with **2**. Gene ontology analysis regarding biological functions shows that many of the proteins are molecular adaptors or have GDP-binding activities (Figure 7A). The result is not surprising as molecular adaptors and GTPases often reside at the cell membrane–cytoskeleton junction to regulate GPI-AP–mediated signaling.[2d, 58] Several proteins are related to activities like GTP-binding, endocytosis, vesicle trafficking or cytoskeleton rearrangement. This is also expected as GPI-APs are transiently located in the cell membrane with a high turnover rate and, thus, frequently endocytosed, trafficked, and translocated.[59] Ontology analysis regarding biological processes reveals that most of the proteins are related to signaling, cell adhesion, regulation, vesicle trafficking, and lipid metabolism (Figure 7B), which supports the wide scope of biological functions of GPIs and GPI-APs.

Conclusion

GPI-APs are important molecules on the cell surface and need to interact with other membrane components for signal transduction and various biological activities. However, it is difficult to study and identify GPI-interacting molecules in the cell membrane. To circumvent the problem, we have developed a photoaffinity-based proximity labeling strategy to study GPI-cell membrane interaction using a bifunctional probe **2** that contains the entire core structure of natural GPIs, as well as a photoreactive group and an alkyne for its crosslinking with proteins and attachment of an affinity tag. Using fluorescence microscopy, we have proved that **2** can be effectively incorporated by HeLa cells into the plasma membrane and is more selective than our previous probe **1** containing only a partial structure of the GPI core. This result demonstrates that **2** is probably a more useful tool for investigating GPI-cell membrane interactions. Using **3** as the control, we have identified 88 unique and 27 enriched proteins with **2**. Bioinformatic analysis of these proteins disclosed that most of them are membrane-associated. Notably, many of the unique proteins have been described to be related to GPIs/GPI-APs in cell signaling, vesicular trafficking, and other bioactivities. Particularly, these proteins are mainly related to GTP/GDP-binding and molecular adaptors/transducers, as well as vesicle transport/endocytosis/cytoskeletal rearrangement, and may play a role in extracellular signal transduction through GPIs. Some proteins were identified with both **1** and **2**, indicating that they might be involved in signaling events jointly associated with PIs, GPIs, or both. On the other hand, the functions of many identified proteins are unclear, in which case we cannot exclude the potential of their association with GPIs/GPI-APs. All these proteins deserve further investigation as they represent a promising avenue to find novel GPI signaling and trafficking pathways or mechanisms. This work makes it possible, for the first time, to examine and directly validate the impact of glycan structure on the biological property of GPIs by comparing the proteomics results obtained with **1**[7] and **2** that are different only in their glycan. Our results have also shown that **2** is a more specific and useful probe to target the cell membrane and study GPI/GPI-AP–cell membrane interaction.

Besides glycan, the lipid of GPIs also has a direct impact on their organization in and interaction with the cell membrane and, thus, their functions.[60] The N-acyl group in the

Cer of **1**-**3** is stearic, the most common N-acyl group found in natural GPIs. Another factor that may influence the results is the location of the photoreactive group in the lipid of probes. In this research, we chose to put the diazirine at the acyl C12-position, which is in the middle of the cell membrane outer layer. To explore the impact of lipid structure of GPIs on their activities, new GPI probes with varied lipid structures and diazirine locations should be developed, while these studies can be conducted by the means established here. This represents another future direction of this research.

Although HeLa cell was chosen in this study, probes and methods developed herein are applicable to other cells. Systematic studies and comparison of GPI-interacting membrane proteins in different cells using the same probes and protocols will shed light on the mechanisms of GPI-APs in various events and their relationships with diseases. For example, comparing the results from different cancer and normal cells will help reveal new cancer markers and signaling pathways. Moreover, the proteomics dataset generated in this study should be a useful resource for further analyses and data mining to disclose new GPI/GPI-AP-interacting proteins.

Experimental Section

General Methods.

All commercial chemicals were of analytical grade and used without further purification unless otherwise noted. Ethanol, copper sulphate, sodium ascorbate, paraformaldehyde (PFA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and poly-L-lysine were purchased from Sigma Aldrich. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and the penicillin-streptomycin solution were from ATCC. Mammalian cell lysis buffer was from Gold Biotechnology. Dulbecco's phosphate buffer (DPBS), DAPI, CellMask™ cell membrane stain C10045, Streptavidin-A488, and agarose resin were from Thermo Fisher Scientific. tris-Hydroxypropyltriazolylmethylamine (THPTA), Cy5-azide, and biotin-azide (i.e., biotin-PEG3-azide) were from Click Chemistry Tools. RPMI 1640 medium was purchased from Lonza. Monoclonal antibodies for organelle markers ERp72 (ER), GP73/GOLPH2 (Golgi), EEA1 (early endosomes), and CD107a/LAMP1 (lysosomes), and corresponding secondary antibody (Coralite Plus 647 anti-mouse IgG1) were from Proteintech. Fluorescence imaging was performed using an Olympus IX71 inverted fluorescence microscope with a light emitting diode (LED) light source, 40X0.75 NA and 60X1.25 NA (oil objective) plan apochromatic objectives, DAPI, GFP, and Cy5 fluorescence channels, and a DP23M colour camera. Analyses of fluorescent images were performed using Olympus Cellsens standard 3 software and FIJI/ImageJ software. Probes **1**, **2**, and **3** were synthesized by reported methods.[7–8, 61] For bioinformatics analysis, Panther bioinformatics software was used following the publisher's instruction.[62] For different statistical analysis and plots, GraphPad Prism 9 software was used.

Cell Culture.

HeLa cells were cultured in high-glucose DMEM media containing 10% (v/v) FBS, 100 g/mL streptomycin, and 100 U/mL penicillin. The cell culture was maintained in an

incubator at 37 °C in a 5% CO_2 and water-saturated atmosphere. The third passage of HeLa cells were utilized in different biological experiments.

Fluorescence Microscopic Analysis of Cell Incorporation of Probes.

HeLa cells were seeded onto a poly-L-lysine (1% solution in DPBS)-coated 35 mm tissue culture dish and allowed to grow to ~50% confluence. The cells were washed with DPBS three times and incubated in RPMI medium (1 mL) containing 10 μM of **2** or **3** (10 μL of 1 mM stock solution in DMSO). After 4 h of incubation, the cells were washed with DPBS three times and then incubated with 4% PFA in DPBS at rt for 15 min. The fixed cells were thoroughly rinsed and incubated with the Click Master Mix (biotin-PEG3-azide10 μM, THPTA 100 mM, sodium ascorbate 100 mM, and $CuSO_4$ 15 mM) at rt for 1 h.[7] The cells were washed with DPBS (1 mL) three times, 500 mM aq. NaCl solution three times, and then deionized (DI) water. Cells were incubated with streptavidin-A488 (1 mL, 1:1000 dilution of 1 mg/mL stock solution with DPBS) in dark at rt for 30 min. The cells were washed with DPBS and incubated with DAPI (50 nM in DPBS, 1 mL) at rt for 5 min. Finally, the cells were washed with DPBS and subjected to fluorescence imaging. For time-dependent imaging experiments, cells were treated with **1**, **2**, and **3** for different lengths of time, and all remaining steps were the same as described.

Labelling Proteins in HeLa Cell Using the Probes.

HeLa cells (~1×10^6) were seeded on a 100 mm tissue culture dish and allowed for growing to ~90% confluence. The cells were harvested, pelleted, and resuspended serum-free media (5 mL) with a final cell count of 5×10^6. The cells were equally divided into three tubes, centrifuged, washed with DPBS, and pelleted. Each cell pellet was resuspended in 2.5 mL of serum-free media containing 200 μM of a probe or PBS and transferred to a 35 mm tissue culture dish. After 3 h of incubation at 37 °C, cells were washed with DPBS three times, resuspended in DPBS (1 mL), and exposed to 365 nm UV light (Spectroline, UV lamp ENF-280C, 120 V, 60 Hz, 0.20 Amps) at 4 °C for 15 min. Then, cells were collected in a 1.5 mL centrifuge tube, washed with DPBS three times, and pelleted via centrifugation (800g, 6 min, 4 °C). The cells were applied to the next step or stored at −80 °C until use.

MS Analysis of Labelled Proteins.

The cell pellets obtained above were treated with the lysis buffer (500 μL) containing 5.0 μL of protease inhibitor Halt™ Protease inhibitor cocktail (Thermo Fisher Scientific) with a Sonika probe sonicator (6 pulses, 60% duty cycle, 30 S each, Amp 10). The lysate was centrifuged at 14,000g and 4 °C for 5 min to collect the supernatants. The supernatant protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) following manufacturer's instructions. The proteins (50 μg) were aliquoted to a 1.5 mL tube, followed by adding freshly prepared solutions of biotin-azide (1 mM in DMSO, 5 μL) and $CuSO_4$ (150.9 mM in H_2O, 0.7 μL), and then a mixture of tris[(1-benzyl-4-triazolyl)methyl]amine (TBTA, 43 mM in 1:4 DMSO/*t*-BuOH, 0.23 μL) and tris(2-carboxyethyl)phosphine (TCEP, 272.6 mM in H_2O, 0.4 μL). DPBS was added to reach a volume of 50 μL. The mixture was vortexed at rt for 1 h before adding 50 μL of ice-cold MeOH. Cold DPBS (50 μL) was added, followed by cold MeOH (150 μL), $CHCl_3$

(50 μL), and water (300 μL) to get a ratio of 4/1/7 (v/v/v, MeOH/$CHCl_3$/buffer). The cloudy solution was vortexed vigorously and centrifuged at 21,000g (4 °C, 20 min). The protein fraction was separated from the aqueous and organic layers, washed with cold MeOH (3 x), and pelleted. The pellets were dried at rt to remove residual MeOH and resuspended in 1X DPBS (300 μL). The solution was incubated with streptavidin-agarose beads at rt for 1 h with end-to-end rotation. Then, the beads were separated through centrifugation (15,000g, 30 min) and washed with DPBS (3 × 2 mL), 0.2% SDS in DPBS (3 × 2 mL), and DPBS (3 × 2 mL). Finally, the proteins on beads were subjected to protein digestion.

The suspension of protein beads in DPBS was centrifuged at 4000 rpm for 1 min. The supernatant was removed, and the beads were washed with 50 mM ammonium bicarbonate (ABC, 100 μL) three times. After ProteoMAX Surfactant (0.2%, 20 μL, Promega) prepared in 50 mM ABC was added, the mixture was vortexed for 15 min. DTT (0.5 M, 1 μL) was added, which was followed by incubation at 56 °C for 20 min and cooling to rt. Thereafter, 2.7 μL of 0.55 M iodoacetamide was added, and the mixture was incubated in the dark at rt for 20 min. A trypsin solution (1.0 μg/ μL, 1 μL) was added, and the mixture was incubated at 50 °C for 1 h. Following digestion, 1 μL of a 10% TFA solution was added to quench the enzymatic reaction. The sample was centrifuged at 10,000 rpm for 3 min, and the supernatant was transferred to an HPLC vial for MS analysis.

Proteomics analysis of the digested protein samples was performed using nano-liquid chromatography tandem mass spectrometry (Nano-LC/MS/MS) on a Thermo Scientific Q Exactive HF Orbitrap mass spectrometer equipped with an EASY Spray nanospray source operated in positive ion mode. The LC system was UltiMate™ 3000 RSLCnano equipped with a trapping column (PharmaFluidics mPAC™ C18, 5 μm pillar diameter, 10 mm length, 2.5 μm interpillar distance), and the chromatographic separation column was PharmaFluidics mPAC™ 50 cm (C18, 5 μm pillar diameter, 50 cm length, 2.5 μm interpillar distance). Mobile phase A was water with 0.1% formic acid; mobile phase B was acetonitrile with 0.1% formic acid. Mobile phase A with 0.1% trifluoracetic acid was used to dissolve the digested protein samples. After the sample (5 mL) was injected into the trapping column, it was eluted with mobile phase A plus 1%B at a flow rate of 10 μL/min for 3 min to desalt and concentrate peptides. Then, the peptides were eluted off the trap to the separation column. The column temperature was maintained at 40 °C and eluted with a gradient of: A plus 1%B 0–3 min, 5%B 3–15 min, 20%B 15.1–100 min, 45%B 100–123 min, 95%B 123–130 min, 95%B 130–135 min, 1%B 135–135.1 min, and 1%B from 135.1 min until the end of the run (150 min). The flow rate was at 750 μL/min for the first 15 min and then reduced to 300 μL/min, which was maintained thereafter. Peptides were eluted off the column directly into the Q Exactive system.

MS/MS spectra were acquired according to standard conditions established in the lab. The EASY Spray source operated with a spray voltage of 1.5 KV and capillary temperature of 200 °C. The spectrometer scan sequence was based on the original TopTen™ method. The analysis was programmed for the full scan recorded between 375–1575 Da at 60,000 resolution and MS/MS scan at 15,000 resolution to generate product ion spectra used to determine amino acid sequence in consecutive instrument scans of the fifteen most abundant peaks in the spectrum. The AGC Target ion number was set at 3e6 ions for the full scan and

2e5 ions for the MS/MS mode. Maximum ion injection time was 50 ms for the full scan and 55 ms for the MS/MS mode. Micro scan number was 1 for both the full and MS/MS scans. The HCD fragmentation energy (N)CE/stepped NCE was 28 with an isolation window of 4 *m/z*. Dynamic exclusion was enabled with a repeat count of 1 within 15 s to exclude isotopes. A Siloxane background peak at 445.12003 was used as the internal lock. HeLa cell protein digest standard was used to evaluate the integrity and performance of the column and spectrometer. If the number of protein IDs from the HeLa standard fell below 2,700, the instrument was cleaned, and new columns were installed.

All the MS/MS spectra were analysed using Sequest (version IseNode in Proteome Discoverer 2.4.0.305, Thermo Fisher Scientific). Sequest was set up to search Homo sapiens (NcbiAV TaxID = 9606) (v2017–10-30) assuming the digestion enzyme as trypsin. Sequest was searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Sequest as a fixed modification. Met-loss of methionine, met-loss+Acetyl of methionine, oxidation of methionine, and acetyl of the N-terminus were specified in Sequest as variable modifications. Precursor ion intensity label free quantitation was done using Proteome Discoverer (vs 2.4.0.305, Thermo Fisher Scientific). Two groups (B33p4 vs Hp4) were compared using a “non-nested” study factor. Normalization was derived by using all peptides. Protein abundance was calculated by summed abundances, meaning by summing the sample abundances of connected peptide groups. Fisher’s exact test (pairwise ratio-based) was used to calculate p-values with no missing value imputation included. Adjusted p-values were calculated by the Benjamini-Hochberg method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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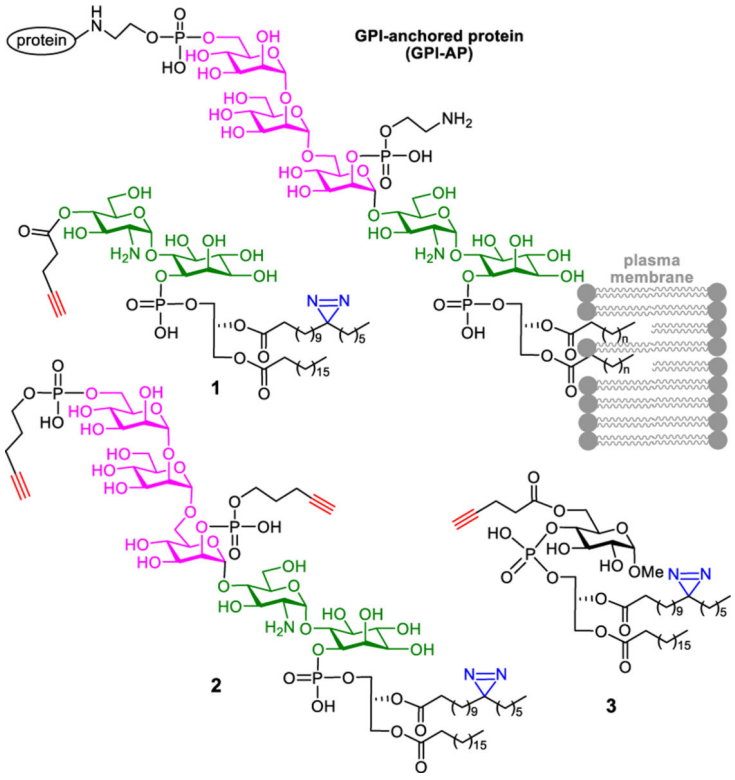

Figure 1.

A representative GPI-AP associated with the cell membrane, which carries the conserved core structure of GPI anchors, and structures of probes **1**-**3** used to study GPI-interacting membrane proteins. Probe **1** contains the core pseudodisaccharide moiety (green) of GPIs; **2** contains the entire core structure of GPI anchors, and **3** has a functionalized phosphatidyl moiety linked to an α-D-glucoside, which serves as the negative control.

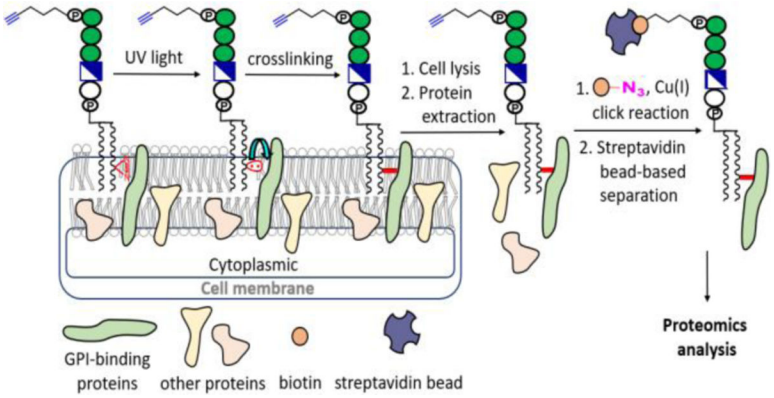

Figure 2:

Illustration of the experimental procedure for labeling, pull-down, and identification of GPI-interacting membrane proteins in live cells using **1**, **2**, and **3**. Upon incubation with a probe, cells can incorporate the probe into the plasma membrane. UV light irradiation of the engineered cells generates a carbene that can cross-react with proteins binding or next to it to form covalent linkages. The cells are lysed, and total proteins are extracted from the lysates and subjected to CuAAC with azide-biotin to biotinylate crosslinked proteins. The biotinylated proteins are separated from other proteins using streptavidin beads and finally applied to proteomic analysis.

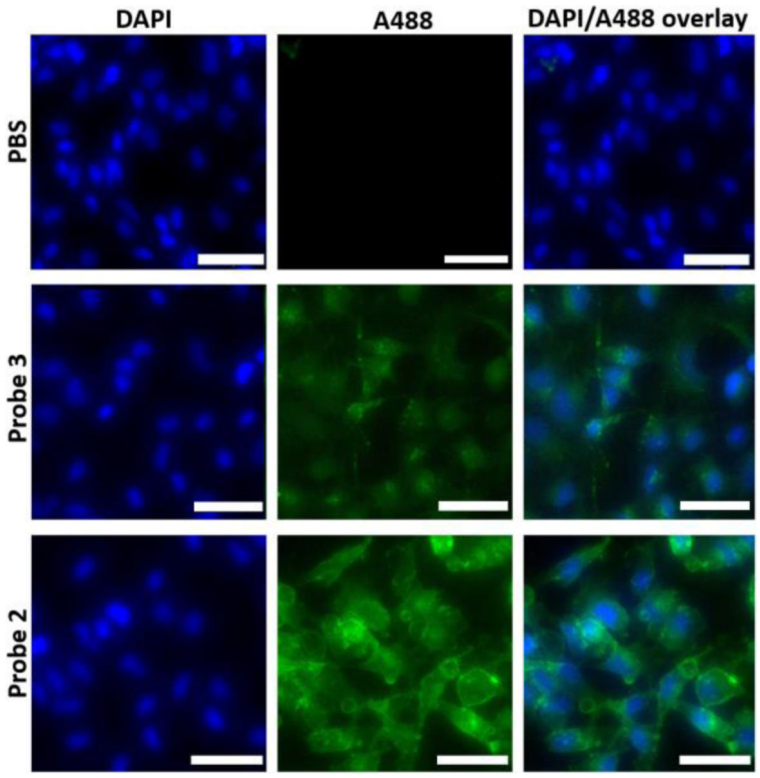

Figure 3:

4′,6-Diamidino-2-phenylindole (DAPI) fluorescence (blue), A488 fluorescence (green), and DAPI/A488 overlay images of Hela cells treated with PBS or with probe **2** or **3** at 37 °C for 4 h, and then with azide-biotin and streptavidin-A488 to stain the probe and with DAPI to stain the cell nucleus. The scale bars are 50 μm.

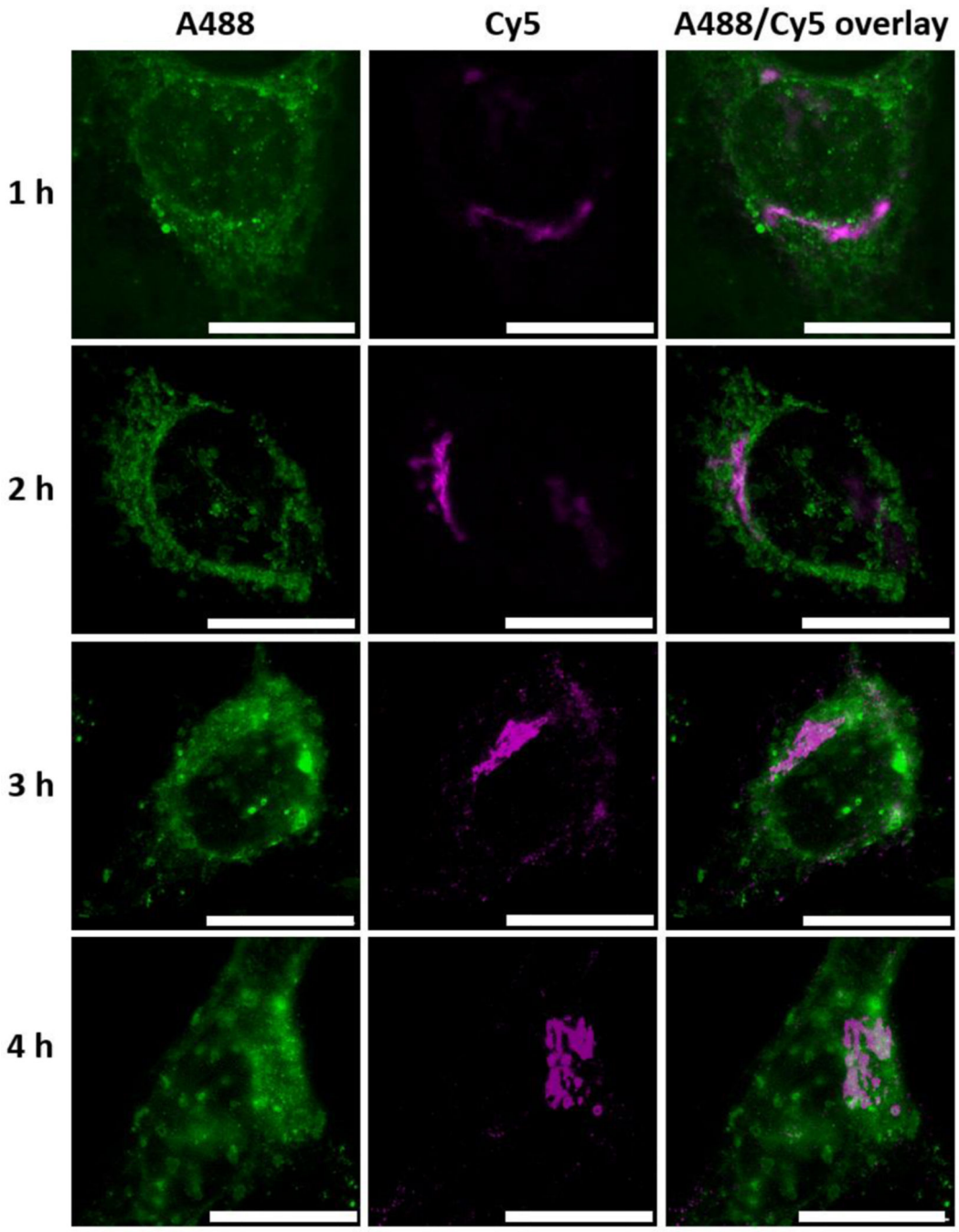

Figure 4.

Fluorescent images of HeLa cells treated with probe **2** for different time (1, 2, 3, and 4 h), and then with azide-biotin and streptavidin-A488 to stain the probe (green), followed by cell fixation, permeabilization, and then treatment with Cy5-tagged anti-GOLPH2 antibody to stain Golgi (purple), and their overlay. The scale bars are 20 μm.

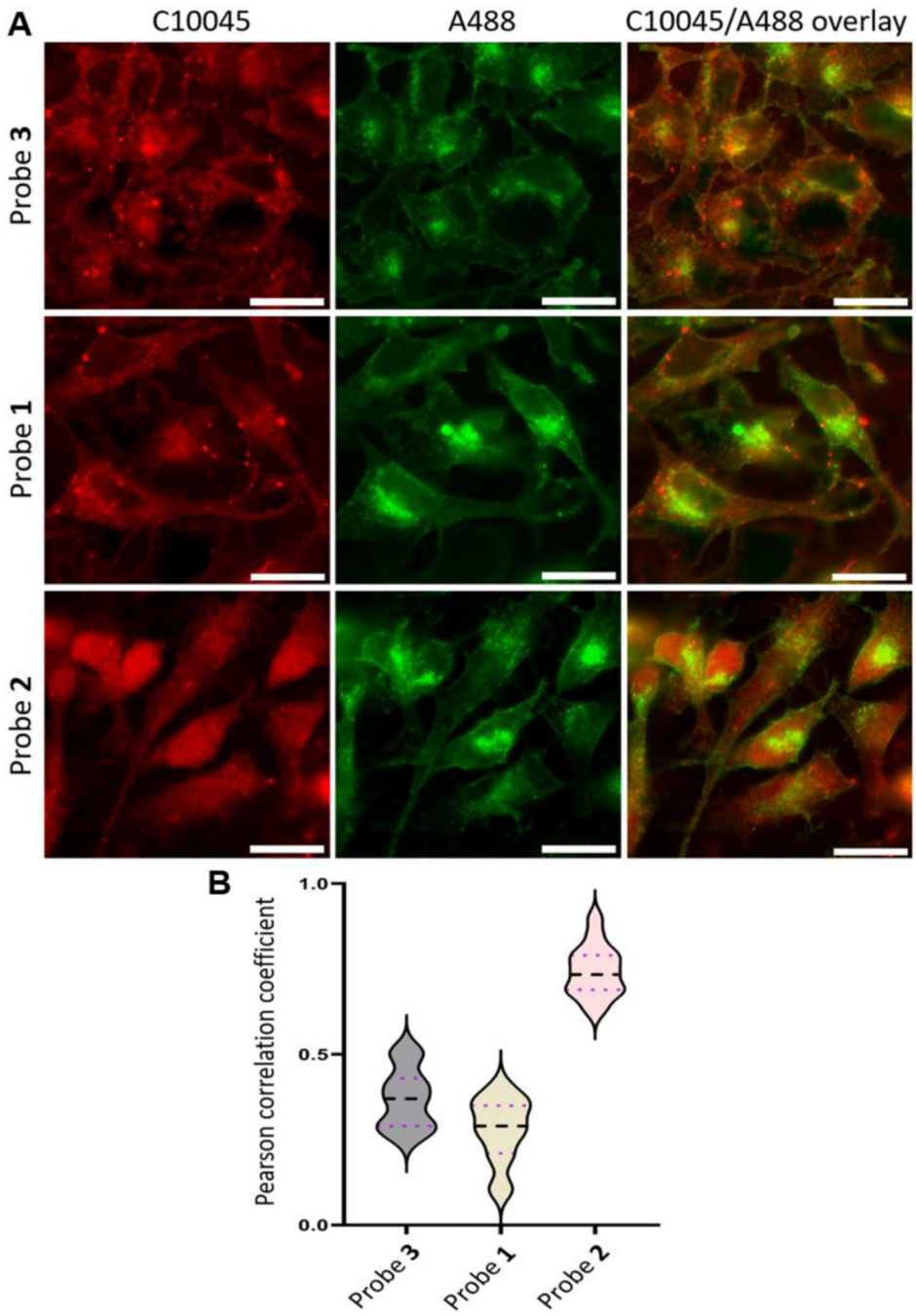

Figure 5.

(**A**) Fluorescence images of HeLa cells treated with **1**, **2**, or **3** for 4 h, and then azide-biotin and streptavidin-A488 to stain the probe (green), followed by CellMask™ C10045 to stain the plasma membrane (red), and their overlays. The scale bars are 20 μm. (**B**) Violin plots of the Pearson correlation coefficients for 15 stained cells from three independent fluorescent micrographs, showing the colocalization levels of green and red fluorescence.

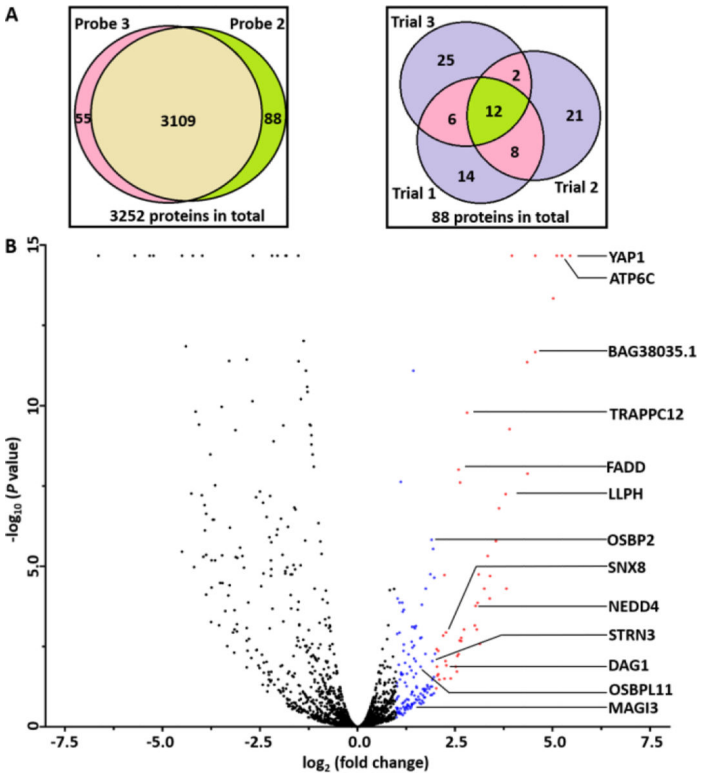

Figure 6:

(**A**) Venn diagrams showing the number of proteins identified with **2** and **3** (left) and unique proteins identified with **2** (right) in three experiments. (**B**) Volcano plot displaying the distribution of all proteins identified with **2** presented as Log_2FC (fold change) vs $Log_{10}P$, using proteins pulled down by **3** as controls. Color dots indicate significantly ($P < 0.05$) enriched proteins by ≥4 folds (red) or ≥2 folds (blue); other proteins are presented in black dots. The marked proteins are a few examples that are related to GPI-APs as reported in the literature.

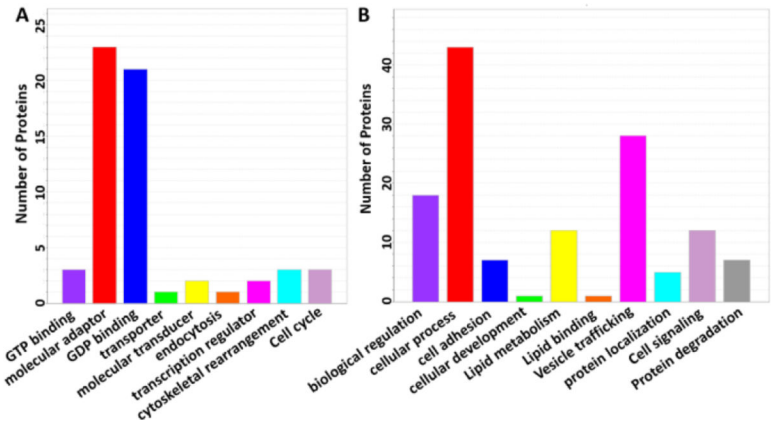

Figure 7:

Gene ontology analysis of the unique proteins identified using **2**, with respect to their (**A**) biological functions and (**B**) engaged biological processes. The horizontal axis denotes biological function or process, and the vertical axis denotes the number of proteins within each category.

Table 1.

GPI-AP-related proteins identified with probe **2**.

Uniprot IDs	Proteins	Literature reported functions and association with GPI-Aps
Q13158	FADD	An adaptor protein that interacts with Cdc42 to regulate GPI-AP endocytosis.[18]
P46934	NEDD4	An E3 ubiquitin-protein ligase that regulates protein lysosomal degradation or endocytic trafficking,[33] including GPI-AP trafficking through interacting with activated Cdc42 protein complex ACK1.[34]
O14976	GAK	A trans-Golgi network protein interacting with Cdc42 to mediate endocytosis of GPI-APs.[35]
P01112	HRAS	A GTPase that plays a role in the trafficking of various lipid-modified proteins.[36] It interacts with Cdc42 and/or Rac1 to transfer the fibroblast cells into malignant cells.[37]
Q14118	DAG1	A transmembrane protein interacting with GPI-APs (e.g., C4.4a) to initiate cellular signaling[38] or with actin and tubulin to regulate extracellular signal transduction mediated by prion protein.[39]
Q9BXB4	OSBPL11	Oxysterol-binding proteins are involved in ceramide transfer protein complex (CERT) formation and indirectly regulate vesicle transfer of GPI-APs in the trans-Golgi network.[40]
Q969R2	OSBP2	
Q14457	BECN1	A membrane protein in the trans-Golgi network, which undergoes localization in the lipid rafts upon interaction with prion protein, a GPI-AP, to trigger PI-related pathways.[22]
P27449	ATP6C	A subunit of the V-ATPase transmembrane complex that pumps protons from cytoplasm to the lumen of organelles.[23b] V-ATPase is frequently found to colocalize with GREG, a Golgi-resident GPI-AP, that plays a role in maintaining Golgi structure via various signaling pathways.[23a, c]
P46937	YAP1	A cell junction protein as transducer of Hippo signaling pathway related to GPI-AP deregulation.[27, 29]
Q9BRT6	LLPH	An long-term synaptic facilitation factor homolog that binds and communicates with other proteins associated with GPI-APs and glycosphingolipids.[36, 41]
Q13033	STRN3	A peripheral membrane protein as a scaffolding or signaling partner for GPI-APs through the Striatin-interacting phosphatase and kinase (STRIPAK) complex.[42]
P49459	UBE2A	A cytoplasmic ubiquitin-conjugating enzyme, which interacts with GPI-APs to mediate proximity-dependent protein degradation for quality control.[43]
Q5TCQ9	MAGI3	A membrane associated kinase containing the PDZ domain[41a] found in several plant GPI-APs (e.g., COBRA) that promotes the interaction of proteins with other similarly scaffolding proteins.[44]
Q9ULC3	RAB23	A small GTPase coupled to the cytoplasmic side of the plasma membrane, which engages GPI-APs to facilitate the transport of protein-containing vesicles towards the ER.[45]
Q96CV9	OPTN	An endosomal membrane protein mediating clathrin-independent endocytic trafficking of GPI-APs.[46]
Q9Y5X2	SNX8	An early endosomal membrane protein involved in endosomal trafficking of GPI-APs.[47]
Q8WVT3	TRAPPC12	A trafficking protein for the ER-Golgi intermediate compartment, which interacts with the SEC protein complex to initiate COPII vesicle-mediated GPI-AP trafficking.[48]
A0A0A6YYL4	CORO7-PAM16	A coronin family protein implicated in several processes of actin rearrangement.[49] Coronin proteins bind to GPIs to mediate proximity actin rearrangement necessary for signal transduction.[50]
Q9Y4C2	TCAF1	A membrane protein that regulates the activity of cell membrane cation channel TRPM8, resulting in acto-myosin-mediated lateral diffusion of GPI-APs.[51]

Uniprot IDs	Proteins	Literature reported functions and association with GPI-Aps
Q92643	PIGK	A GPI transamidase that recognizes and attaches GPIs to proteins at the ER lumen.[52]
Q9UBQ0	VPS29	Vacuolar protein sorting-associated proteins that are component proteins of membrane-associated retromer complex required for endosome-to-Golgi retrograde protein transport.[6b, 53]
Q96RL7	VPS13A	
O75351	VPS4B	
Q8NHV4	NEDD1	A cytoskeletal protein responsible for microtubule nucleation, which interacts with smGPI-1 (a GPI-AP) to form STRIPAK complex that initiates cytoskeletal rearrangement during GPI signal transduction.[54]
P42892	ECE1	An endothelin-converting enzyme possessing GPI-targeting property.[55]
P17612	PKAC*	A cAMP-dependent protein kinase that regulates GPI-AP endocytosis and intracellular trafficking.[56]
P53680	AP2S1*	Component protein of adaptor protein complex-2 (AP-2) that plays a role in vesicle-mediated trafficking of membrane proteins.[26b, 57]
Q9H223	EHD4*	EH domain-containing protein 4 is a membrane-bound ATP primarily found in the membrane of early endosomes. It regulates GPI-AP trafficking and signaling, but the mechanism is unclear.[26]

* These proteins were also identified using probe **1**.