A chemoproteomics approach to profile phospholipase D-derived phosphatidyl alcohol interactions

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ABSTRACT

Alcohol consumption leads to formation of phosphatidylethanol (PEth) via the transphosphatidylation activity of phospholipase D (PLD) enzymes. Though this non-natural phospholipid routinely serves as a biomarker of chronic alcoholism, its pathophysiological roles remain unknown. We use a minimalist diazirene alkyne alcohol as an ethanol surrogate to generate clickable, photoaffinity lipid reporters of PEth localization and lipid–protein interactions via PLD-mediated transphosphatidylation. We use these tools to visualize phosphatidyl alcohols in a manner compatible with standard permeabilization and immunofluorescence methods. We also use click chemistry tagging, enrichment, and proteomics analysis to define the phosphatidyl alcohol interactome. Our analysis reveals an enrichment of putative interactors at various membrane locations, and we validate one such interaction with the single-pass transmembrane protein basigin/CD147. This study provides a comprehensive view of the molecular interactions of phosphatidyl alcohols with the cellular proteome and points to future work to connect such interactions to potential pathophysiological roles of PEth.

INTRODUCTION

Alcohol use is responsible for approximately 5.1% of the global burden of disease, and it is the leading cause for death among those 15-49 years of age.[1] It is associated with many diseases, including addiction, malnutrition, alcoholic liver disease, dementia, and several cancers.[2-4] Ethanol is itself rather inert, needing high concentrations to directly perturb membrane and protein functions. Instead, metabolites of ethanol contribute to important aspects of the pathophysiology of alcohol consumption.[5]

The phospholipid phosphatidylethanol (PEth) is one such ethanol metabolite. It is produced through the transphosphatidylation activity of ubiquitous phospholipase D (PLD) enzymes. PLDs catalyze nucleophilic substitution of the head group of the abundant phospholipid phosphatidylcholine (PC). It accepts either water, which generates phosphatidic acid, a lipid with important signaling functions, or short, aliphatic primary alcohols, which generates phosphatidyl alcohols. Incorporation of primary alcohols such as ethanol into phosphatidyl alcohols is a conserved function of PLDs across plants, bacteria, and animals.[6] PEth can accumulate to quite high levels (up to 0.1% of total blood phospholipids in heavy drinkers).[7] Owing to the specificity of its biosynthesis and its long half-life (~days in human blood), PEth is widely used as a clinical biomarker of chronic alcoholism.[7] Despite the widespread prevalence of PEth, the effects of this ethanol-derived lipid metabolite on cellular function remain largely unknown.

Existing studies on the functions of phosphatidyl alcohols have relied on low throughput, candidate-based approaches. First, some studies have investigated effects of PEth on the in vitro, catalytic activities of enzymes known to be implicated in the pathology of alcohol use (e.g., protein kinase C, PI-specific phospholipase C).[8-11] Such studies, which found modest effects, have typically used in vitro assays at very high, non-physiological lipid concentrations. Alternatively,

application of ethanol to cells has revealed numerous pleiotropic effects, but specificity to PEth and physiological relevance is unclear for these approaches.[12-14] In particular, ethanol-induced phenotypes, even when they were found to be dependent upon PLD activity, were not linked to engagement of specific molecular targets by PEth. To reveal the biological mechanisms by which PEth may affect physiological function, and how such mechanisms may contribute to the pathological effects of alcohol abuse, a comprehensive view of the molecular interactions of PEth with endogenous biological molecules is urgently needed.

Here, we describe a chemoproteomics approach to map the protein interactors of phosphatidyl alcohols. Previously, we had discovered that PLD enzymes can accept bioorthogonally functionalized primary alcohols in transphosphatidylation reactions, leading to a method we termed **Im**aging **P**LD **A**ctivity with **C**lickable Alcohols via **T**ransphosphatidylation (IMPACT).[15-17] Building on these findings, here we used a bifunctional primary alcohol to generate a clickable photoaffinity phosphatidyl alcohol probe in situ, via the action of endogenous PLD enzymes. Chemoenzymatic production of the lipid photoaffinity probe in this manner, i.e., in live cells, enables it to have the same subcellular localizations as other phosphatidyl alcohols such as PEth, that are generated by PLD transphosphatidylation.

The photocrosslinking diazirene group within the lipid probe enables UV light-mediated covalent labeling of protein interactors, and the terminal alkyne within the probe enables downstream detection following a click chemistry tagging step. This method, termed crosslinking IMPACT, or XL-IMPACT, enables both visualization of sites of phosphatidyl alcohol production and identification of phosphatidyl alcohol-interacting proteins. Herein, we demonstrate both types of applications of XL-IMPACT. We identify hundreds of putative phosphatidyl alcohol interactors and validate the interaction with the single-pass transmembrane protein basigin/CD147. Overall,

this work highlights how the permissiveness of a key lipid metabolic enzyme to functionalized, non-natural substrates enables the identification of the putative protein interactome of a disease-related lipid metabolite.

RESULTS AND DISCUSSION

We reasoned that the minimalist diazirine alkyne alcohol (DAA)[18], typically used to functionalize small-molecule probes for chemoproteomics applications, could itself be a likely transphosphatidylation substrate of PLDs, because it is an aliphatic primary alcohol similar to other PLD transphosphatidylation substrates (e.g., hexynol). The expected transphosphatidylation product, phosphatidyl diazirene alkyne alcohol (PDAA), would have the same overall charge and polarity as PEth and other phosphatidyl alcohols derived from hydrophobic, aliphatic primary alcohols, thus making it an ideal chemoproteomics probe for these species (**Figure 1A**).

Thus, we began by treating cells with DAA under stimulation with the phorbol ester PMA, which greatly increases endogenous PLD activity. Lipid extracts of the labeled cells were tagged via Cu-catalyzed azide-alkyne cycloaddition (CuAAC) with a BODIPY-azide fluorophore. Fluorescence-coupled HPLC analysis revealed a peak with a retention time of 7 minutes whose presence depended upon PLD activity, as it was absent from extracts from cells treated with the PLD inhibitor FIPI (**Figure 1B**). LC–MS analysis of lipid extracts further confirmed the presence of the PDAA probe. As phosphatidyl alcohols encompass a class of compounds with differing lipid tail lengths and degrees of unsaturation, we were encouraged to see a lipid tail distribution of PDAA species that closely mirrors that of other PLD transphosphatidylation products,[16] as well as the PLD substrate PC (**Figure 1C**).

We then assessed if PDAA could be crosslinked to proteins. After generating PDAA in

cells as above, we performed crosslinking by irradiating with UV light (365 nm). Cell lysates were generated and tagged via CuAAC with a biotin-azide probe, following by protein precipitation to remove excess biotin-azide and affinity enrichment using precleared streptavidin-conjugated resin. Analysis of these samples revealed labeling of many proteins (**Figure 2A**). Importantly, the use of FIPI during the DAA labeling step resulted in very little labeling, indicating that the labeling was indeed due to PDAA, whose formation requires PLD activity, and not free DAA.

Though the key motivation for the development of XL-IMPACT was the identification of phosphatidyl alcohol interactome, it is also a useful variant of IMPACT for visualization of the localization of these lipids in situ. The localization of signaling lipids can play important roles in determining their effects on signaling pathways.[19-20] Yet, a challenge with visualizing lipids is their dynamic behavior, both from diffusion within a bilayer and trafficking between different membranes.[21] Even standard cell fixation methods, which rely on crosslinking of primary amines or treatment with organic solvents, do not covalently immobilize most lipids, leading to artifacts or loss of signal when imaging lipid localization in fixed samples.[22] By design, XL-IMPACT involves covalent tethering of the target lipids to nearby proteins, opening up the possibility of using traditional cell fixation procedures, compatible with detergent permeabilization for immunofluorescence labeling of intracellular epitopes, to visualize the localization of PDAA lipids.

We found that labeling cells via XL-IMPACT followed by fixation and permeabilization led to a retention of XL-IMPACT fluorescence on intracellular organelles. Here, we treated cells with DAA and PMA as before, followed by rinsing, UV irradiation, fixation with methanol, and CuAAC tagging with a green fluorophore (AFDye488-azide). Critically, methanol fixation typically removes most lipids, but using XL-IMPACT we observed fluorescence at several

locations, including the ER and nuclear envelope and the Golgi complex (**Figure 2B**). Again, the fluorescence was dependent upon PLD activity, as it was eliminated by treatment with FIPI during the DAA labeling step and also depended upon UV crosslinking. We obtained similar results with paraformaldehyde fixation followed by permeabilization with Triton X-100, albeit with slightly higher levels of background, potentially due to incomplete rinse-out of unreacted probe or disruption of lipid bilayers by paraformaldehyde fixation (**Figure S1**).

Because this protocol enables lipid visualization in permeabilized cells, it is in principle compatible with antibody-based immunofluorescence labeling of intracellular epitopes. Therefore, we performed colocalization studies with markers of different organelles after XL-IMPACT labeling to demonstrate the feasibility of this approach and to establish the localizations of XL-IMPACT fluorescence (**Figures S2** and **S3**). We found that XL-IMPACT fluorescence substantially colocalized with markers of the ER and the Golgi complex, and to a lesser extent with other components of the endomembrane system such as exosomes, endosomes, and lysosomes. These results highlight the utility of using a bifunctional tagging approach with clickable and photocrosslinking functionalities to enable visualization of lipid probes using methods optimized for protein visualization, as has been demonstrated previously for several classes of endogenous lipids.[23-26]

To identify proteins that interact with phosphatidyl alcohols, we performed bottom-up proteomics analysis of XL-IMPACT-labeled proteomes enriched as described above (**Figure 2A**). We identified more than 500 proteins across three biological replicates, including 99 proteins that were identified in all three replicates (**Figure 3A**). Normalized protein abundances were log-transformed, and Student's t-tests were performed to check statistical significance. Of the 99 proteins, virtually all were enriched in the XL-IMPACT group over the FIPI control, and 50 of

these were statistically significantly enriched based on a cutoff of $p < 0.05$ on a Student's t-test and at least a two-fold enrichment in the XL-IMPACT vs. FIPI-treated samples (**Figure 3B**).

To understand broad trends for this large putative PDAA interactome, we performed gene ontology (GO) analysis (**Figure 3C**). Encouragingly, among the 20 most significant GO groups, most are locations or functions enriched in membranes. This result is also consistent with recent reports indicating a preference for diazirene-based probes for labeling membrane-proximal residues.[27-28] Interestingly, more than half of the labeled proteins are related to exosomes, which is reinforced by the partial colocalization of XL-IMPACT fluorescence with a marker of this organelle (**Figure S2**). Whereas the ER, a major site of XL-IMPACT labeling (**Figures 2B** and **S3**) does not appear among the most significantly enriched GO groups, gene set enrichment analysis (GSEA) of the proteomics data revealed that ER-residing proteins are significantly more likely to be the most highly enriched proteins among the detected interactome (**Figure 3D**). This result suggests that, whereas the number of ER-residing proteins are not the most enriched compared to a random sample of proteins, the proteins that are detected have relatively high abundances, accounting for the strong signal in the imaging.

Finally, we selected one of the most highly enriched proteins as a candidate for detailed examination to validate the findings from the high-throughput proteomics results. We focused our attention on basigin (BSG/CD147) for several reasons: it was one of the top hits, it can function in processes occurring in the ER and in exosomes,[29-30] and it has been rarely detected by others in similar streptavidin-pulldown proteomics negative control samples,[31] meaning that it is more likely to be a specific interactor. We first expressed HA-tagged BSG in cells, carried out XL-IMPACT labeling with biotin-azide tagging, and then performed anti-HA immunoprecipitation to enrich BSG-HA. Gratifyingly, a streptavidin blot of these samples indicated that BSG-HA was

biotinylated (**Figure 4A**). When we instead enriched the XL-IMPACT-labeled proteome using streptavidin-conjugated agarose and then performed an anti-HA blot, we again observed biotinylation of BSG-HA (**Figure 4B**). In both experiments, XL-IMPACT labeling of BSG-HA was FIPI-sensitive and dependent upon UV irradiation. Finally, we performed a similar streptavidin-agarose enrichment of non-transfected, XL-IMPACT-labeled cells and were able to detect biotinylation on endogenous BSG (**Figure 4C**).

BSG is a single-pass transmembrane protein that appears as two different glycosylated forms, a high-glycosylation form with a molecular weight of ~50-75 kDa, and a low-glycosylation form with a molecular weight of ~37 kDa.[32] The high-glycosylation form is considered to be the mature form, residing on post-Golgi compartments (predominantly at the cell surface). By contrast, the low-glycosylation form is considered to be the immature form, and it has a largely ER-based, intracellular localization. Interestingly, XL-IMPACT preferentially labels the low-glycosylation form, revealed by examination of the extent of labeling of the enriched forms of the protein compared to its levels in lysate (**Figures 4B–C**). In the BSG-HA experiment, XL-IMPACT labeling was visible only on the low-glycosylation form, which is present at non-physiologically high amounts due to BSG-HA overexpression, i.e., roughly equivalent to the high-glycosylation form (**Figure 4B**). In the endogenous BSG experiment, the high-glycosylation form was much more abundant than the low-glycosylation form in lysate, as expected, but XL-IMPACT labeled each form roughly equivalently, again indicating a preference for the low-glycosylation form (**Figure 4C**).

These data are consistent with the primarily intracellular localization pattern of PDAA (**Figure 2B**) and other similar phosphatidyl alcohols, as we have demonstrated previously.[15-17] These studies validate BSG as a bona fide interactor of PDAA and highlight the power of XL-

IMPACT for coupling the visualization of PLD-derived phosphatidyl alcohol lipids to the identification of interactomes of these lipids using chemoproteomics. Further, they point to the possibility that engagement of BSG and other proteins may be a mechanism by which ethanol-derived PEth lipids affect cell physiology.

CONCLUSION

In this study, we developed a chemoproteomics approach for probing the direct protein interactors of phosphatidyl alcohol lipids. By taking advantage of the substrate tolerance of PLD enzymes, our approach uses in situ, chemoenzymatic synthesis to generate clickable, photoaffinity lipid analogs that mirror the environment of phosphatidyl alcohols such as PEth that are produced by PLDs following ethanol consumption. Because it involves covalent crosslinking of lipid analogs to nearby proteins, XL-IMPACT enables both the visualization of lipid localizations compatible with standard fixation and permeabilization methods and the identification of protein interacting partners of phosphatidyl alcohol lipids. These results set the stage for future studies to unravel mechanisms underlying how interactions of these alcohol-derived lipids with cellular proteins may mediate pathophysiological effects in diseases associated with alcohol abuse.

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and Fromme laboratories for use of equipment. The authors declare no competing financial interests.

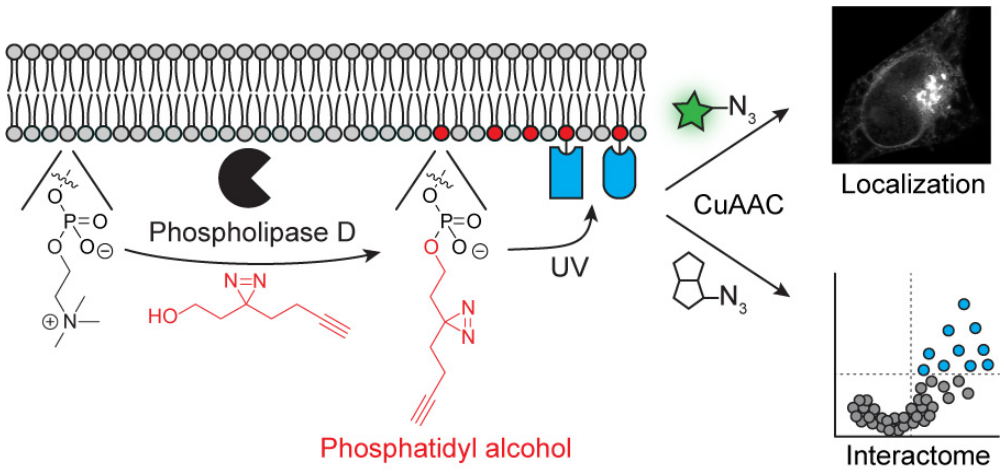
AUTHOR CONTRIBUTIONS

W.Y. performed all experiments. W.Y. and J.M.B. conceived of the project idea, designed experiments, analyzed data, and wrote the manuscript.

ASSOCIATED CONTENT

Supporting information is available free of charge: Materials and methods, XL-IMPACT imaging with paraformaldehyde fixation (Figure S1), Colocalization of XL-IMPACT with organelle markers (Figures S2 and S3), and Raw proteomics data (Table S1).

GRAPHICAL TOC ENTRY



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FIGURES

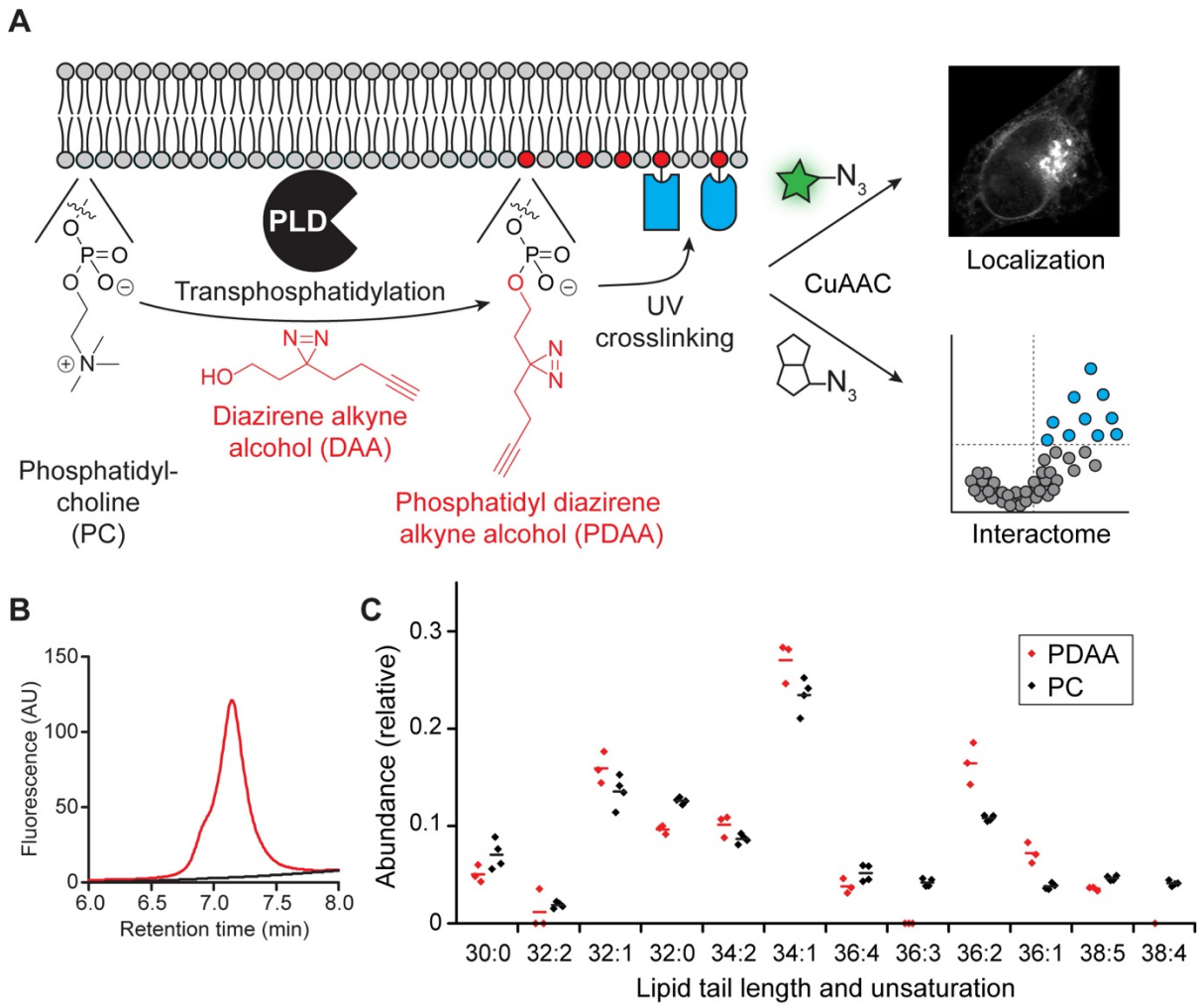

Figure 1. XL-IMPACT is a strategy to generate clickable, photocrosslinkable phosphatidyl alcohol probes in cells via phospholipase D activity. (A) Schematic of the XL-IMPACT approach, wherein the minimalist diazirene alkyne alcohol (DAA) is a PLD transphosphatidylation substrate to generate phosphatidyl DAA (PDAA) lipids that can be covalently tethered to nearby proteins after UV irradiation to enable visualization or enrichment of lipid–protein interactions following CuAAC tagging. (B) HPLC analysis of extracts from cells labeled via XL-IMPACT. HeLa cells were treated with the PLD inhibitor FIPI (black) or vehicle (red) for 30 min followed

by DAA and the phorbol ester PMA for 1 h. Lipid extracts were tagged via CuAAC with a BODIPY-azide probe and then analyzed by fluorescence-coupled HPLC. Shown is the peak corresponding to the BODIPY-tagged PDAA at a retention time of ~7 min. (C) LC–MS analysis of lipid extracts from cells labeled with XL-IMPACT. Cells were treated as in (B), except that the CuAAC tagging step was omitted, and lipid extracts were analyzed directly by LC–MS. PDAA and phosphatidylcholine (PC) species were quantified, identified by acyl tail composition (length:number of double bonds).

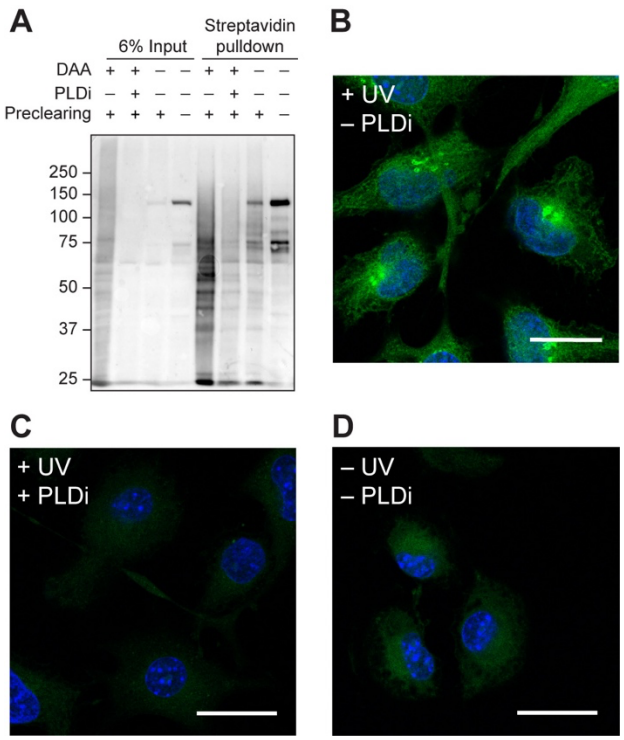

Figure 2. XL-IMPACT enabling crosslinking to putative PDAA-binding proteins. (A) HeLa cells were treated with the PLD inhibitor FIPI (PLDi) or vehicle for 30 min followed by DAA and the phorbol ester PMA for 1 h. After a rinse, photocrosslinking was performed with UV illumination (365 nm) for 30 min, followed by lysis. A portion of the sample was used to preclear streptavidin-agarose resin to remove endogenously biotinylated proteins, as indicated. Lysates were then tagged with biotin-azide via CuAAC, and biotinylated proteins were enriched with streptavidin-agarose resin. Shown is a streptavidin blot, with total lysate at left (input) and enriched samples at right. Molecular weight markers are in kDa. (B–D) Confocal microscopy images of HeLa cells labeled with XL-IMPACT. Cells were labeled, and UV photocrosslinking was performed as described above. Cells were then fixed with methanol, CuAAC tagging with AFDye488-azide was carried out, and confocal microscopy was performed. Green: XL-IMPACT fluorescence; Blue: DAPI. Scale bars: 20 μm.

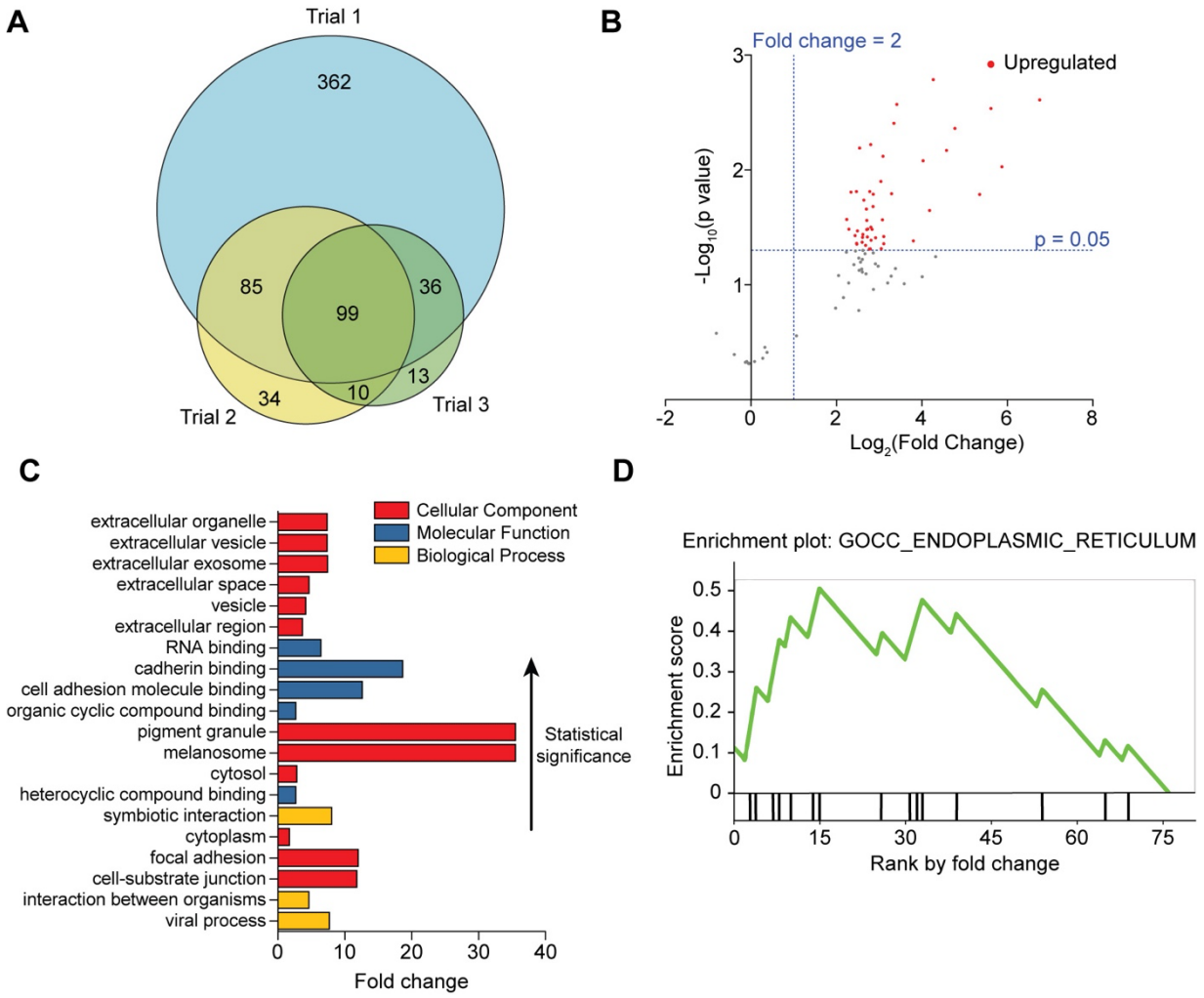

Figure 3. Identification of PDAA-binding proteins using XL-IMPACT and bottom-up proteomics. (A) Venn diagram showing all proteins identified in the proteomics analysis across three replicate trials. (B) Volcano plot of proteins that were detected in all three replicate experiments. The x-axis shows the $\log_2$(fold change), where fold change represents the ratio of protein abundance in the DAA vs. DAA+PLDi (negative control) samples, and y-axis shows the $-\log_{10}$(p value) (n=3). (C) Gene Ontology (GO) analysis of proteins detected in all three replicates, showing the top 20 groups, ordered by statistical significance (p value), with highest significance at the top, and with x-axis showing the fold change indicating extent of enrichment of proteins from that data set compared to the entire proteome. (D) Gene Set Enrichment Analysis (GSEA) of

ER-localized proteins detected in all three replicates, with higher fold change to the left, indicating that, among all the PDAA interactors identified, those that localize to the ER are among the most highly enriched in our dataset.

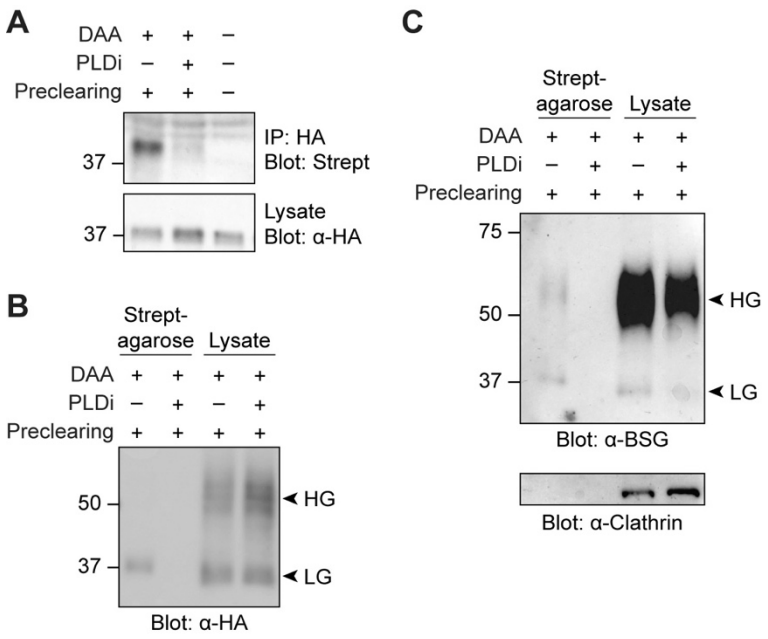

Figure 4. Basigin (BSG/CD147) interacts with the phosphatidyl alcohol probe PDAA. Cells were transfected with BSG-HA (A–B) or not transfected with any plasmids (C), and XL-IMPACT was performed by incubation with PLDi (FIPI) or vehicle for 30 min, followed by DAA and PMA for 1 h, rinsing, UV crosslinking, and CuAAC tagging with biotin-azide. Extracts were then enriched using either anti-HA agarose (A) or streptavidin-agarose (B–C). Shown in (A) is a streptavidin blot of anti-HA-enriched proteomes, showing that XL-IMPACT labels BSG-HA. An anti-HA blot of whole-cell lysate indicates similar levels of BSG-HA in all samples. Shown in (B) is reciprocal anti-HA blot of the streptavidin-enriched proteome, showing that XL-IMPACT predominantly labels the ~37 kDa form of overexpressed BSG-HA. Shown in (C) is an anti-BSG blot of streptavidin-enriched proteomes, showing that XL-IMPACT can label both the ~55 kDa (high glycosylation, HG) form and ~37 kDa (low glycosylation, LG) forms of endogenous BSG, with a preference for the LG form. An anti-clathrin blot is included as a loading control for whole cell lysates. Molecular weight markers are in kDa.