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Integrative Chemical Proteomics-Metabolomics Approach Reveals Acaca/Acacb as Direct Molecular Targets of PFOA

Xiaojian Shao,[†] Fenfen Ji,[†] Yawei Wang,[‡] Lin Zhu,[†] Zhen Zhang,[†] Xiubo Du,[§] Arthur Chi Kong Chung,[†] Yanjun Hong,[†] Qian Zhao,*[,∥] and Zongwei Cai*[,†]

[†]State Key Laboratory of Environmental and Biological Analysis, Department of Chemistry, Hong Kong Baptist University, Hong Kong, China

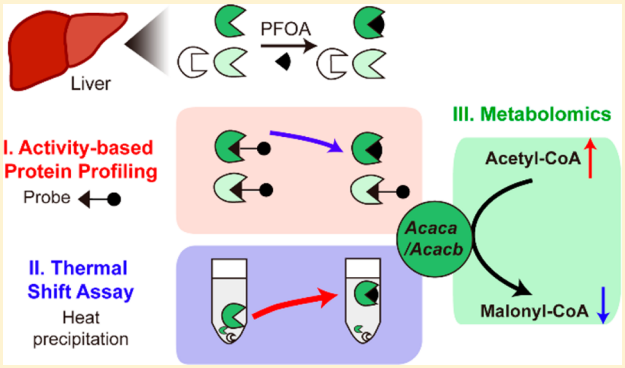
[‡]State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China

[§]College of Life Sciences, Shenzhen Key Laboratory of Microbial Genetic Engineering, Shenzhen University, Shenzhen, China

[∥]State Key Laboratory of Chirosciences, Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, Hong Kong, China

Supporting Information

ABSTRACT: Identification of the direct molecular targets of environmental pollutants is of great importance for toxicity mechanism studies. Despite numerous studies have been conducted to investigate the toxicity mechanism of perfluorinated compounds (PFCs), their direct-binding protein targets which trigger downstream toxicity effects remain largely unknown. Herein, we present a systematic chemical proteomic study to profile the target proteins of PFCs by taking PFOA as a representative. Considering its electrophilicity, PFOA could preferentially bind to reactive cysteine-containing proteins. Therefore, two complementary cysteine-targeting probes, iodoacetamide alkyne (IAA) and ethynyl benziodoxolone azide (EBX), were selected to enrich the putative target proteins in the absence or presence of PFOA. Quantitative proteomic analysis of the enriched proteins identified Acaca and Acacb as novel target proteins of PFOA. We then applied parallel reaction monitoring (PRM)-based targeted proteomics study combined with thermal shift assay-based chemical proteomics to verify Acaca and Acacb as bona fide binding targets. These findings afford a plausible explanation for the PFOA-induced liver toxicity, especially regarding abnormal fatty acid metabolism that was validated by targeted metabolomics analysis. The present study documents an integrative chemical proteomics-metabolomics platform that facilitates the authentic identification of proteins that are targeted by small molecules and its potential to be applied for toxicity mechanism studies of environmental pollutants.



The ever-increasing chemical substances introduced into the environment pose a severe risk to human health, however, most of the chemicals are not adequately assessed for their toxicity. Recently, several USA agencies have collaborated to develop Tox21 program for high-throughput toxicity evaluation of the emerging pollutants.[1] Although Tox21 affords innovative methods for toxicity prediction, it is typically focused on limited protein targets or signaling pathways.[2] Alternatively, numerous chemical proteomic strategies were developed to investigate the modes of action of small molecules by pinning down their direct protein targets. The target-centered strategies hold great potential for discovery of novel signal pathways that could be used to elucidate the toxicity mechanism of environmental pollutants. A recent study chemically immobilized 4,5-dichloro-2-octyl-4-isothiazolin-3-one (DCOIT), an emerging costal pollutant, onto beads to enrich the compound-binding proteins. DCOIT was thus verified to bind G protein alpha subunit to dysregulate mitogen-activated protein kinase (MAPK) pathway and hormone production.[3] In addition, some strategies are developed based on the fact that ligand binding increases the thermal stability and proteolysis resistance of target proteins, which include thermal shift assay (TSA) and drug affinity responsive target stability assay (DARTS).[4,5] Previous TSA experiment has demonstrated that 6-OH-BDE-47 targeted enoyl-acyl carrier protein reductase to perturb fatty acid metabolism and exert antibacterial activity.[4]

However, these approaches show limitations for precise target discovery of environmental pollutants. The immobilization of the environmental pollutants onto beads requires

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chemical modification, which could change compound activities and alter target preferences. Without depletion of highly abundant proteins and enrichment of targets, TSA and DARTS assays usually result in a huge complexity of protein samples that could hamper the identification of real protein targets.[6] Alternatively, a chemical proteomic strategy called activity-based protein profiling (ABPP) was developed. Instead of direct chemical modification of pollutant compounds, ABPP mainly utilizes chemical probes that react with mechanistically related amino acid residues to label and enrich the related proteins.[7] Since the proteins could also be occupied by reactive environmental compounds, the chemical probes could compete with reactive compounds to pinpoint the compound-binding proteins. Moreover, on-bead enrichment through the chemical probes could greatly reduce protein complexity and facilitate more accurate measurement.[8,9] Among the chemical probes, iodoacetamide alkyne (IAA) is widely accepted to target reactive cysteine-containing proteins.[10,11] Besides IAA, researchers nowadays develop several cysteine-targeted probes with distinctive target preference to profile the reactive cysteines.[12] It is therefore feasible to combine those probes for comprehensive identification of compound-binding proteins.

Perfluorooctanoate (PFOA) is an emerging environmental pollutant under consideration to be listed in persistent organic pollutants by Stockholm Convention.[13,14] Previous animal studies revealed several PFOA-induced signal receptors that were associated with liver toxicity, such as peroxisome proliferating receptor alpha (PPARα) and constitutive androstane receptor (CAR).[15−18] However, recent studies argued that these signaling pathways could not well explain the PFOA-induced toxicity effects, so novel signaling pathways warrant further investigation to unravel the modes of action of PFOA.[19] From chemical perspective, PFOA possesses a carboxyl group that could interact with reactive thiol group present in cysteine. Theoretically, it is feasible to use cysteine-targeting probes to determine the protein targets of PFOA.[20] Two cysteine-targeting probes, IAA and EBX (ethynyl benziodoxolone azide), were combined in this study to obtain authentic protein targets. EBX is a newly developed chemical probe which displays overlapping labeling patterns and unique targets when compared with IAA.[21] Therefore, a combination of the two activity-based probes could lead to more convincing target discovery.[22] The protein targets were further verified by using parallel reaction monitoring (PRM)-based targeted proteomics studies. PRM enables the distinguishing of authentic molecular targets from miscellaneous targets to improve LC-MS/MS sensitivity and reproducibility.[23−26] To further verify the target candidates, we utilized another chemical proteomic strategy, thermal shift assay (TSA), to characterize their physical interaction with PFOA. Then to validate the functional changes of protein targets discovered from chemical proteomics studies, we conducted metabolomics studies to investigate in vivo metabolic alterations that are closely related with the target proteins.

In the present study, we established an integrative chemical proteomics-metabolomics approach to determine the protein targets of environmental pollutant. Two different chemical proteomics methods, a targeted quantitative proteomics method and a targeted metabolomics study were combined to reveal and validate bona fide protein targets of PFOA. This study not only identifies novel protein targets of environmental pollutants to explain the toxicity mechanism, it also demonstrates the potential of combining multiomics approaches to solve challenging questions involving environmental toxicity.

MATERIALS AND METHODS

Chemicals. PFOA was purchased from Sigma-Aldrich. IAA and Streptavidin MagnaBind Beads were purchased from Thermo Fisher Scientific. EBX and Rhodamine-alkyne were kindly provided by Prof. Adibekian from University of Geneva. Biotin-alkyne and biotin-azide were purchased from Lumipore. Protease inhibitor cocktail and tris[1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine were purchased from Sigma-Aldrich. Sequencing-grade LysC and trypsin were both purchased from Promega. Tris(2-carboxyethyl)phosphine hydrochloride was purchased from Pierce.

Proteomic Analysis Using Gel- and MS-Based Competition Assays. The experimental details were described in Supporting Information with reference to previously described methods.[21] Briefly, mouse liver was homogenized in PBS with 1× protease inhibitor for in vitro gel-based assay; the lysates were then treated with DMSO (vehicle control) or PFOA for 30 min, followed by probe labeling with IAA or EBX for 1 h at dark. Then the proteins were tagged with fluorescent groups for in-gel assays or enriched by biotin−avidin interactions for MS-based analysis. Proteins were considered as potential PFOA targets if meeting the following criteria: inhibition ratio >50% and significance level $P < 0.05$.

Verification of Protein Targets Using PRM-Based Targeted Proteomic Strategy. PRM assays and acquisition methods were directly built based on data-dependent acquisition (DDA) data. DDA data were analyzed with Proteome Discoverer and then imported into Skyline 3.7; MS1 filtering was used to obtain 2−3 peptides of targeted proteins without missed cleavages and unique to the protein, as well as their corresponding retention time. For PRM analysis, fewer than 30 peptides were selected to maintain a reasonable cycle time. Information of the target peptides, including their precursor m/z, charge, retention time window, were exported from Skyline into Xcalibur software. Two unique peptides from streptavidin were integrated as internal standard to calibrate the peptides between experimental runs. After PRM acquisition, the data were imported into Skyline for further quantification by selecting peptides with *idotp* > 0.90 for precursor ions and *dotp* > 0.90 for product ions. The peak areas of the product ions were finally exported for further normalization with unique streptavidin peptides and the normalized intensities of the top six product ions were summed up to calculate the peptide level. Then the unique peptides were summed up to represent the protein abundance.

Target Verification Using Thermal Stability Assay. For thermal shift assays, protein lysates were treated with vehicle control and PFOA, respectively. Equal amounts of protein (100 μg) were aliquoted into Eppendorf tubes and heated at different temperatures for 3 min, followed by cooling down to room temperature. The proteins were subsequently centrifuged at 15000 g to pellet heat-precipitated proteins. The supernatants were separated by SDS-PAGE and analyzed by Western blot assay. The antibodies used were rabbit antiacetyl-CoA carboxylase 1 (Cell Signaling Technology), rabbit antiacetyl-CoA carboxylase 2 (Cell Signaling Technology), rabbit anti-Slc25a20 antibody (Abcam) and goat antirabbit IgG-HRP secondary antibody (Cell Signaling

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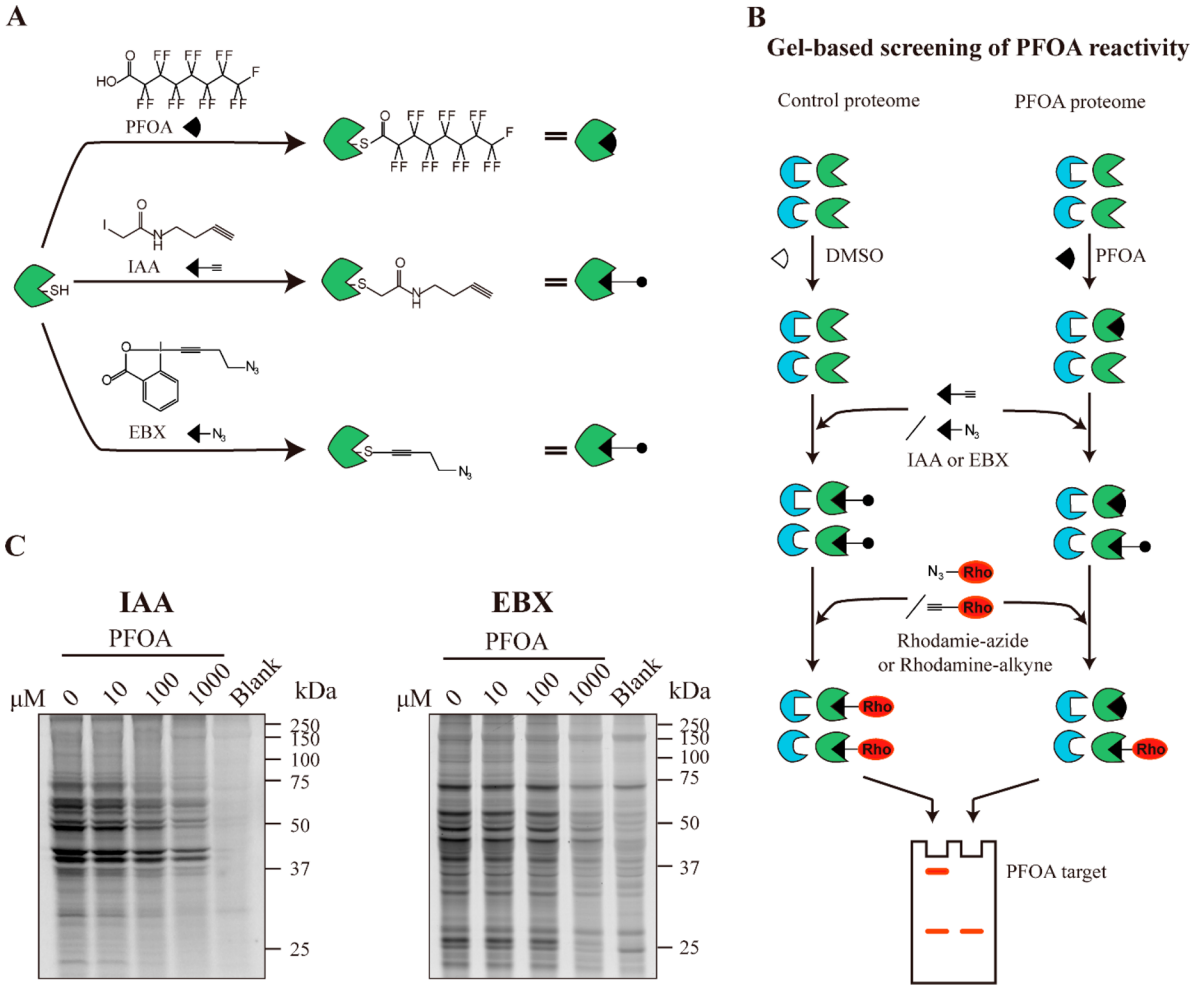

Figure 1. Gel-based fluorescence analysis of PFOA reactivity in liver proteome using IAA and EBX, respectively. (A) Possible chemical reactions of reactive cysteines with PFOA, IAA, and EBX, respectively. (B) Schematics of gel-based competition assays for profiling protein targets of PFOA. (C) In-gel fluorescent results of PFOA reactivity in mouse liver lysates with increasing PFOA gradients and blank group without chemical probe and PFOA treatment.

Technology). Western blot results were imaged with LI-Cor Odyssey system. Protein band densities were analyzed using Image-Pro Plus software. The total protein amounts in each lane of the SDS-PAGE gel were quantified for protein normalization.

Validation of Protein Targets Using Targeted Metabolomics. The experimental details are stated in Supporting Information using a previously reported protocol.[27] Briefly, female C57BL/6 mice (8−12 weeks old) were intraperitoneally injected with PFOA (300 mg/kg body weight) or vehicle control (corn oil) before treatment for 4 h. The mice were then sacrificed, and the livers were immediately removed and frozen in liquid nitrogen. Metabolites from the livers were extracted with 80% methanol and analyzed by HPLC-MS/MS. All animal procedures were in accordance with the Hong Kong Government Department of Health, the Animal Research Ethical Committee of the Chinese University of Hong Kong and consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

RESULTS AND DISCUSSION

Dual Probes Revealed Direct Molecular Targets of PFOA. Based on previous toxicokinetic studies reporting that liver was the major organ for PFOA accumulation, we utilized mouse liver proteome to profile the protein targets.[19] PFOA possesses a carboxyl group (Figure 1A), which could react with nucleophilic thiol groups present in cysteines.[28] Hence, we deployed two cysteine-targeted probes, EBX and IAA, to profile the PFOA-targeted proteins in mouse liver. The scheme for chemical proteomics studies of PFOA-targeted proteins was shown in Figure 1B. In brief, the cysteine-targeting probe, IAA or EBX, was applied to target reactive cysteine-containing proteins in the absence and presence of PFOA, respectively. Then the probe-target proteins were tagged with a Rhodamine group to show whether fluorescent signals were decreased after PFOA treatment. As shown in Figures 1C and S-1, the overall labeling patterns using IAA and EBX probes were similar to each other, which was in accordance with previous report.[21] Meanwhile, some fluorescent bands were specific to IAA or EBX labeling, confirming that these two probes are complementary in labeling reactive cysteines. Therefore, the combination of the probes allows comprehensive profiling of reactive cysteines present in liver proteome. Moreover, PFOA treatment led to clear decrease in fluorescence in both IAA and EBX labeling group, suggesting that PFOA could compete with the two probes to occupy reactive cysteines. Several fluorescent bands disappeared when the PFOA concentration reached 100

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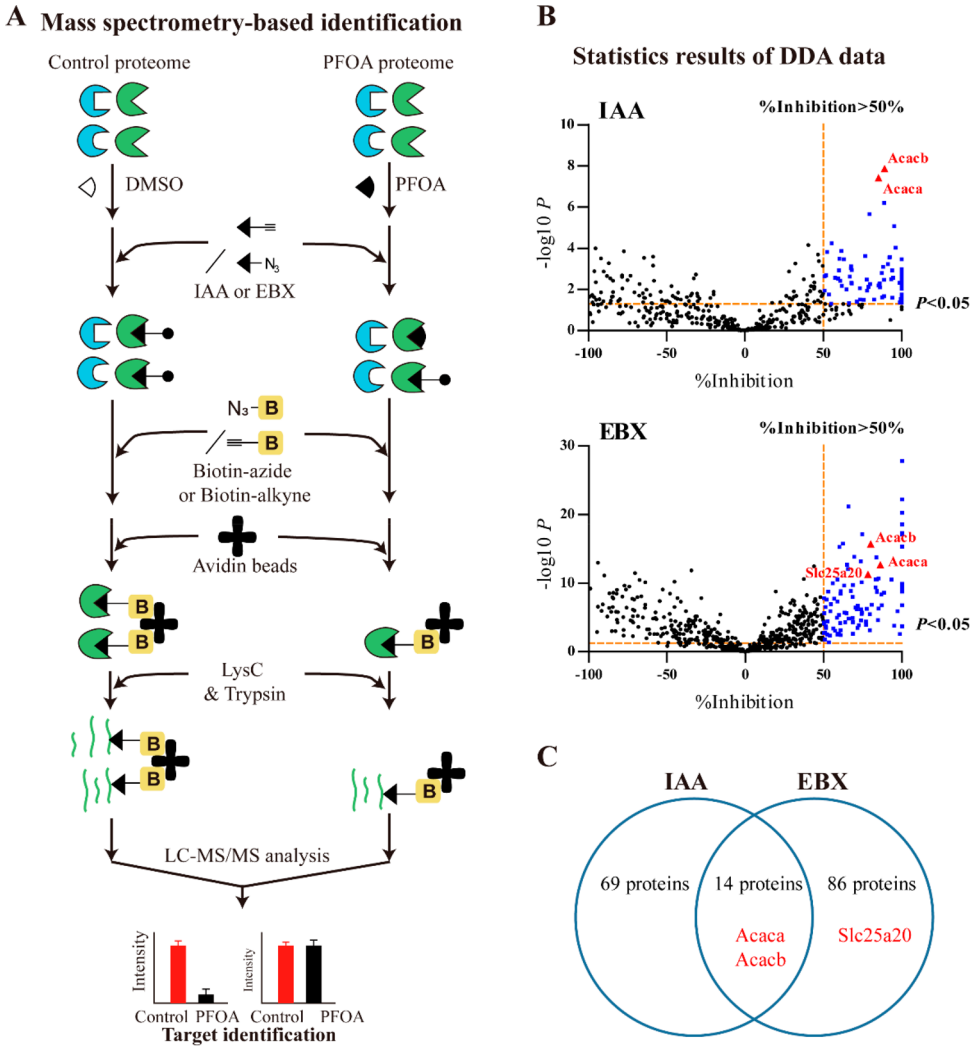

Figure 2. Mass spectrometry-based assays using IAA and EBX probes. (A) Schematics of MS-based identification of PFOA-targeted proteins. (B) Statistical volcano plots with thresholds % inhibition >50% and $P < 0.05$. (C) Venn diagram showing the protein numbers identified by using IAA and EBX, respectively.

μM while some decreased at 1000 μM, indicating that PFOA targeted a multitude of proteins in a reactivity-dependent manner.

Next, we further identified the PFOA-targeted proteins by using tandem MS analysis. The detailed experimental workflow is shown in Figure 2A.

The procedure was similar to the gel-based competition assay, but the probe-labeled proteins were tagged by biotin-alkyne or biotin-azide, followed by enrichment by streptavidin beads and digestion into peptides before LC-MS/MS analysis. Through comparing control group and PFOA group, we obtained proteins that were putatively occupied by PFOA. Protein intensity changes in response to PFOA treatment (expressed as a percentage of DMSO control samples or % Inhibition) were calculated from four experiment replicates (Figure 2B). Using a stringent cutoff (inhibition ratio >50% and statistical significance $P < 0.05$), we obtained 83 and 100 protein targets of PFOA by using IAA and EBX, respectively (Figure S-2). Among these proteins, 14 proteins were determined by both two probes (Figure 2C). The MS-based results also displayed decreases in protein labeling upon PFOA treatment, which was consistent with reduced fluorescence from gel-based results.

Quantitative Proteomics Verified the Direct Protein Targets. By combining IAA and EBX data, several potential PFOA targets were found to be involved in fatty acid metabolism, including Acaca, Acacb, and Slc25a20. Acaca and Acacb, two acetyl-CoA carboxylase isozymes, were identified to be targeted by PFOA in both groups while Slc25a20 was only identified in EBX group (Figure 2B). To further validate DDA-based results, we performed PRM-based targeted proteomic analysis. As shown in Figure 3A, a unique peptide of Acaca, VNNADDFPNLFR++, was taken as an example to elucidate the PRM-based strategy. The top six fragment ions generated from the peptide were integrated to calculate the peptide abundance in both IAA and EBX group, while the top three precursor ions were selected as reference to the peptide abundance. As a result, the fragment ions and precursor ions from the peptide were both observed to decrease after PFOA treatment, which confirmed that the fragment ions were suitable to quantify the peptides of interest. To determine the protein abundance of Acaca, 3 and 2 unique peptides were chosen in IAA and EBX group, and their precursor ions and fragment ions both evidenced a PFOA-dependent decrease (Figure S-3). The same strategy was applied to quantify Acacb and Slc25a20, further verifying those

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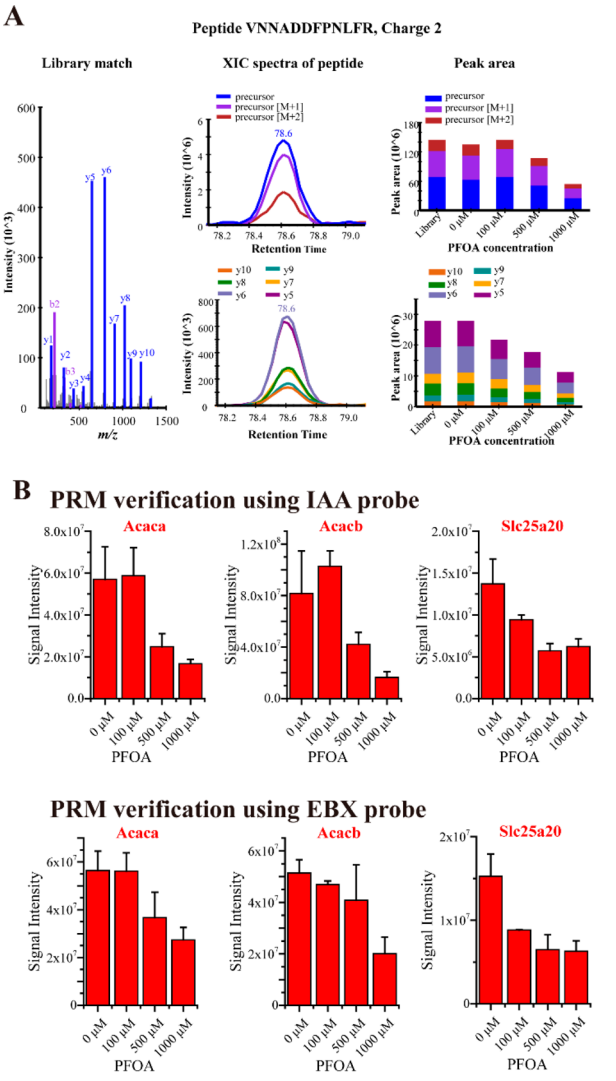

Figure 3. PRM-based quantification of PFOA-targeted proteins that are involved in fatty acid metabolism. (A) Skyline-based analysis of a unique peptide VNNADDFPNLFR++ from Acaca using precursor ions (upper) and fragment ions (lower) in IAA group. The left mass spectrum showed the library match result. (B) Quantification of Acaca, Acacb, and Slc25a20 after treatment of increasing PFOA concentrations using IAA and EBX, respectively.

as direct protein targets of PFOA (Figure 3B). Intriguingly, Slc25a20, which was only identified in EBX group using DDA quantification, was verified to decrease in PRM mode using both probes. The negative DDA result was probably caused by the relatively low abundance of Slc25a20 when using IAA probe for enrichment (data not shown), further suggesting the necessity to obtain authentic protein targets by using dual probe and PRM-based proteomics strategy.

In Vitro Validation Using Thermal Shift Assay. We used thermal shift assay (TSA) to confirm the physical interaction between PFOA and the bona fide target proteins. TSA has been widely used for target identification of bioactive molecules based on the concept that ligand binding increases protein thermal stability.[29] As shown in Figure 4A, Acaca, Acacb, and Slc25a20 all displayed a significant decrease in protein solubility after heating to a certain temperature (50, 50, and 65 °C, respectively). However, supplementation of PFOA apparently increased the protein solubility at 50, 50, and 65 °C,

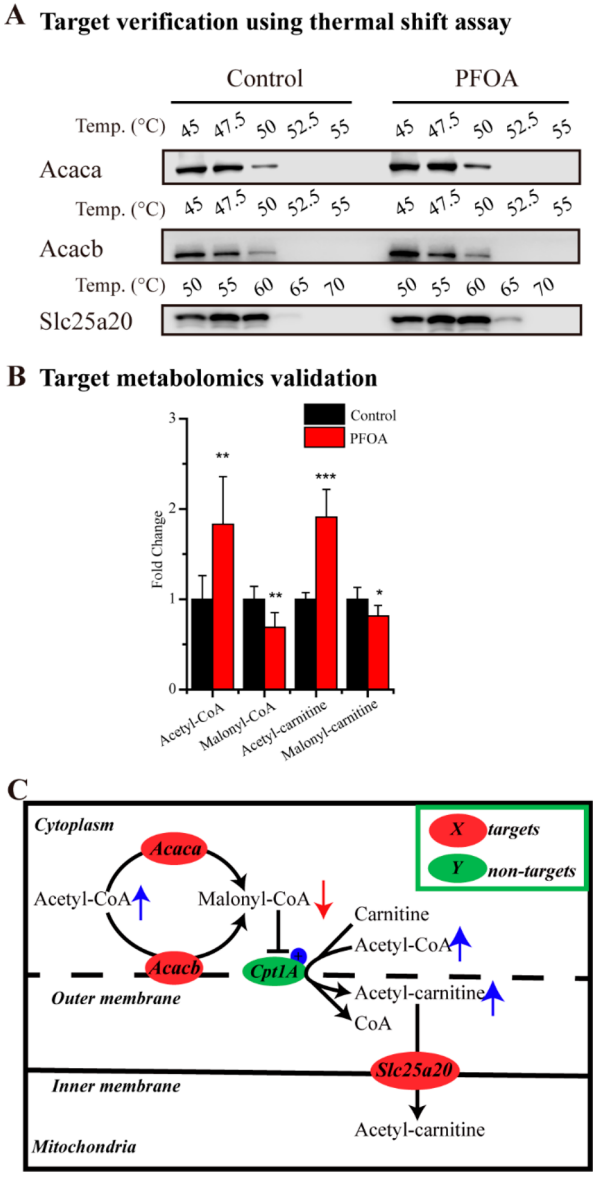

Figure 4. (A) Protein target verification using thermal shift assay, a representative result from three replicates. (B) Metabolomics validation of in vivo metabolites using PRM-based quantification of metabolites related to Acaca, Acacb, and Slc25a20 ($n = 6$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (C) Model diagram showing the PFOA-induced metabolic dysregulation. Red circles represent the protein targets of PFOA. Green circles are nontargeted proteins.

respectively. The PFOA-induced shifts of thermal stability curves could be found in Figure S-4, which indicated that PFOA supplementation could improve the protein thermal stability. The thermal shifts further confirmed the three proteins as direct PFOA targets.

In Vivo Validation Using Metabolomics Studies. For validation of the dysregulated protein functions caused by PFOA occupation, we carried out in vivo metabolomics studies. In brief, corn oil (vehicle control) or PFOA was administered into mice for acute treatment. After that, the liver tissues were collected, and the metabolites were quantified using PRM-based targeted metabolomics strategy. We acknowledge that the concentration used here was much higher than exposure levels in real situations, but the maximum doses are oftentimes applied in toxicology studies with POPs.[30]

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Acaca and Acacb are two carboxylase isoenzymes to catalyze acetyl-CoA to malonyl-CoA. As shown in Figure 4B, PFOA treatment decreased malonyl-CoA in mice and upregulated acetyl-CoA. Since the in vivo synthesis of malonyl-CoA is mainly accomplished by Acaca/Acacb, we suggested that the occupation of PFOA on Acaca/Acacb accounted for the downregulation of malonyl-CoA.[31] Malonyl-CoA synthesis is also the rate-limiting step for fatty acid synthesis, so the decreased malonyl-CoA possibly represented the downregulated fatty acid synthesis. Furthermore, malonyl-CoA is widely accepted to inhibit carnitine palmitoyl transferase 1 (Cpt1), a protein involved in fatty acid oxidation, so the decreased malonyl-CoA could represent upregulated fatty acid oxidation. Taken together, the occupation of Acaca/Acacb could possibly lead to dysregulated fatty acid metabolism.

Moreover, by surveying Slc25a20, we found that it is involved in fatty acid metabolism as an acyl-carnitine transporter located in mitochondria inner membrane. PRM quantification revealed that there was an apparent increase of acetyl-carnitine as well as decrease of malonyl-carnitine, which were highly associated with their corresponding acyl-CoAs (Figure 4B). Moreover, some acyl-carnitines were increased in PFOA-treated group (Figure S-5). These results indicated that occupation of Slc25a20 by PFOA could attribute to the blocked transportation of acyl-carnitines, further dysregulating fatty acid metabolism.

CONCLUSIONS

This integrative omics study showed that several proteins, including Acaca and Acacb, were occupied by PFOA, which was in accordance with dysregulated acyl-CoAs that were involved in fatty acid metabolism (Figure 4C). Since the dysregulated fatty acid metabolism was previously related to the hepatotoxicity, especially some symptoms like hepatomegaly.[32] Hence, this target-based strategy provides a potent explanation of PFOA-induced toxicity mechanism. Moreover, this platform integrates activity-based proteomics, thermal shift assay-based proteomics and targeted metabolomics to investigate the authentic protein targets of environmental pollutants, which affords a promising tool for exploiting the toxicity mechanism of environmental pollutants.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02995.

Supplemental text providing detailed information about the gel- and MS-based competition assays and targeted metabolomics analysis using PRM mode, as well as Figures S-1−S-5 (PDF).

AUTHOR INFORMATION

Corresponding Authors

*Tel.: +852-34117070. Fax: 34117348. E-mail: zwcai@hkbu.edu.hk.

*Tel.: +852-34008711. E-mail: q.zhao@polyu.edu.hk.

ORCID

Yawei Wang: 0000-0002-6115-4076

Arthur Chi Kong Chung: 0000-0003-1519-7224

Yanjun Hong: 0000-0003-3059-8130

Qian Zhao: 0000-0003-2244-6516

Zongwei Cai: 0000-0002-7013-5547

Notes

The authors declare no competing financial interest.

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