Supplementary Materials for

Allosteric regulation the lid domain of PCK2 as a novel strategy for modulating mitochondrial dynamics

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1. Chemical and reagents

Cucurbitacin B (CuB, $C_{32}H_{46}O_8$) and dihydrocucurbitacin B (DHCB, $C_{32}H_{48}O_8$) were purchased from DeSiTe Biological Technology (Chengdu, Sichuan, China). Opti-MEM, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin were purchased from Macgene Biotechnology (Beijing, China). N-hydroxysulfosuccinimide (sulfo-NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), immobilon western chemiluminescent HRP substrate, and Lipofectamine 2000 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sorlabio (Beijing, China). Kinase buffer was purchased from Cell Signaling Technology (Beverly, MA, USA). ATP assay kit, Dil, Mito-tracker, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), and RIPA lysis buffer were purchased from Beyotime (Nanjing, Jiangsu, China). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Yeasen (Shanghai, China). Lactate kit and PEPCK assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). EasyPure Genomic DNA kit and BCA protein assay reagent kit were purchased from Transgen (Beijing, China). PVDF membrane was purchased from Millipore (Bedford, MA, USA). Pronase was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Primary antibodies Mitofusin2 (#ab124773), DRP1 (#ab184247), and PCK1 (#ab28455) were purchased from Abcam (Cambridge, UK). PCK2 (8565S), HRP conjugated goat anti-rabbit IgG, and HRP conjugated goat anti-mouse IgG were purchased from Cell Signaling Technology.

2. Synthesis of CuB bifunctional probe

CuB probe was synthesized by an esterification reaction. In brief, CuB (100 mg), dicyclohexylcarbodiimide (DCC) (44.33 mg), 3-(3-(But-3-yn-1-yl)-3H-diazirin-3-yl) propanoic acid (35.69 mg) and 4-dimethylaminopyridine (DMAP) (21.97 mg) were added into dichloromethane (100 mL), respectively. The reaction was stirred at room temperature for 24 h and subsequently quenched in water (100 mL). The organic phase was separated and washed with saturated sodium chloride for three times. After filtration, the residue was purified by preparative thin-layer chromatography (ethyl acetate: petroleum ether, 1:1) to obtain the desired product. Analytical data. HRMS *m/z* calculated for $C_{40}H_{54}O_9N_2$ $[M+Na]^+$ 729.3722, calculated 729.3721.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.06 (d, J = 15.6 Hz, 1H), δ 6.49 (d, J = 15.6 Hz, 1H), δ 5.79 (d, J = 6.1 Hz, 1H), δ 5.47 (dd, J = 13.6 Hz, J = 5.5 Hz, 1H), δ 5.34 (m, 1H), δ 4.36 (m, 1H), δ 3.24 (d, J = 14.1 Hz 1H), δ 2.82 (d, J = 12.9 Hz, 1H), δ 2.69 (m, 1H), δ

2.50 (d, *J* = 7.1 Hz 1H), *δ* 2.41 (m, 1H), *δ* 2.29 (m, 1H), *δ* 2.13 (m, 2H), *δ* 2.12 (m, 2H), *δ* 2.01-1.99 (m, 6H), *δ* 1.93 (m, 2H), *δ* 1.83 (m, 1H), *δ* 1.82 (s, 2H), *δ* 1.70 (m, 3H), *δ* 1.66 (m, 2H), *δ* 1.57 (s, 3H), *δ* 1.53 (s, 3H), *δ* 1.52 (m, 1H), *δ* 1.44 (s, 3H), *δ* 1.42 (s, 1H), *δ* 1.31 (s, 3H), *δ* 1.29 (s, 3H), *δ* 1.08 (s, 3H), *δ* 0.98 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) *δ* 205.7, 202.6, 171.5, 170.4, 152.2, 139.7, 130.0, 120.8, 120.4, 82.8, 79.5, 78.3, 73.8, 71.5, 69.4, 58.3, 51.4, 50.8, 48.8, 48.2, 45.5, 42.5, 34.4, 33.9, 32.3, 32.1, 29.8, 28.9, 28.2, 26.6, 26.1, 25.7, 25.0, 24.1, 22.1, 21.4, 20.0, 19.0, 14.3, 13.4.

3. Cell culture

Human hepatocellular carcinoma Huh7 cells and HEK293T cells were obtained from the Cell Bank of Peking Union Medical College (Beijing, China). Lentivirus-mediated PCK2 knockout Huh7 cells were established by CRISPR/Cas9 system (Hanbio Biotechnology, Shanghai, China). PCK2 knockout efficiency was confirmed by western blot. All the cells were routinely cultured in DMEM, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin, and maintained in a humidified 5% CO_2 atmosphere incubator at 37 °C.

4. Surface plasmon resonance (SPR) analysis

SPR analysis was performed on Biacore T200 system (GE Healthcare) at 25 °C. Briefly, CM5 sensor chip (GE Healthcare) was activated by sulfo-NHS/EDC. Then, PCK2 was coupled onto CM5 sensor chip at a concentration of 500 μg·mL^{-1} in acetic acid (pH 4.0). For screening, the compounds in library were dissolved in DMSO and detected with a final concentration of 50 μM. For kinetic analysis, CuB was diluted in various concentrations from 0.098 to 50 μM and injected at a flow rate of 30 μL·min^{-1} with the running buffer PBS-P (10 mM phosphate buffer containing 2.7 mM KCl, 137 mM NaCl, 0.05% surfactant P20, and 5% DMSO). Time parameters of combination and dissociation were set as 60 s and 120 s, respectively. Data were analyzed with Biacore evaluation software (T200 Version 2.0). K_a, K_d, and K_D values were calculated by fitting kinetic data using the 1: 1 Langmuir binding model.

5. Cell viability assay

Cell viability was evaluated by MTT assay. Briefly, cells were seeded into 96-well plates and treated with various concentrations of CuB (0.05-100 μM) for 48 h. Then, 0.5 mg·mL^{-1} MTT solution was added and incubated at 37 °C for 4 h in the dark. Formazan crystals were solubilized with 100 μL DMSO and absorbance was measured at 570 nm using a microplate reader (Austria GmbH

5082 Grodlg, Tecan, Männedorf, Switzerland).

Cell viability was also measured by Incucyte S3 platform (Sartorius, Göttingen, Germany). Briefly, cells were seeded in 96-well plates and treated with indicated CuB. Subsequently, imaged cells using phase contrast channel in the Incucyte S3 platform at intervals of 3 h using a 10 × objective. Incucyte S3 image analysis software was set to detect the edges of the cells and to determine their confluence in percentage, and then normalized by the cell confluence at 0 h.

6. Drug affinity responsive target stability (DARTS) assay

DARTS assay was performed to detect the interaction between CuB and PCK2. Briefly, cells were collected and lysed with lysis buffer (0.4% Triton X-100, 400 mM NaCl, 100 mM Tris·Cl, pH 7.5, 20% glycerol) containing pronase and phosphatase inhibitor cocktail. Then, cell lysates were diluted with TNC buffer (50 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris·Cl, pH 8.0) at 1:10 and further incubated with different concentrations of CuB (0, 5, 10, 20, 50, 100 μM) or DMSO at 4 °C for 4 h. After that, pronase (2 $\mu g \cdot mL^{-1}$) was added and incubated for 10 min at 37 °C, followed by western blot.

7. Cellular thermal shift assay (CETSA)

For CETSA in lysates, cells were harvested and freeze-thawed five times with liquid nitrogen in kinase buffer. The supernatant was treated with 20 μM CuB or DMSO for 4 h at 4 °C, and then heated for 3 min at indicated temperature from 42 °C to 64 °C. For CETSA in intact cells, cells were treated with 20 μM CuB or DMSO for 2 h, and heated for 3 min at indicated temperature from 42 °C to 64 °C. Then, cells were freeze-thawed as mentioned above to obtain cell lysates. The expression of PCK2 was detected with western blot.

8. Pull-down assay

Huh7 cells were treated with CuB bifunctional probe with the concentration of 10 μM for 6 h. Meanwhile, an excess amount of CuB for competitive binding was added into medium. Then, the cells were washed with PBS, UV irradiated for 10 min, and the click reaction with biotin-PEG3-azide was allowed to proceed. Subsequently, the labeled proteins were washed with 500 μL methanol. The proteins were resuspended in SDS/PBS followed by incubation with streptavidin beads at 4 °C for overnight. Then, the beads were washed with PBS containing 0.1% Triton X-100. The beads-captured proteins were obtained and separated through SDS-PAGE for further western blot using PCK1 or PCK2 antibody.

9. Identification of CuB-binding site on PCK2

LC-MS/MS was conducted to identify the CuB-binding site on PCK2. Briefly, 5 μg recombinant PCK2 protein was incubated with CuB in PBS overnight at 4 °C. The resultants were separated by SDS-PAGE, visualized by silver staining, and further analyzed by EASY-nLC II system and LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). Data was processed with Proteome Discoverer (1.4) software and SEQUEST search engine (Thermo Fisher Scientific). Analysis criteria was followed as below: taxonomy, human; enzyme, trypsin; missed cleavage sites, 2; variable modifications, methionine oxidation (+15.99 Da), cysteine carbamidomethylation (+57.02 Da), cysteine binding with PCK2 (+558.71 Da); precursor mass tolerance as 10 ppm, fragment mass tolerance as 0.6 Da; and the false discovery rate (FDR) at 0.01.

10. Transfection of plasmids

The constructed plasmids were premixed with Lipofectamine 2000 in Opti-MEM and then added into the HEK293T cells. The medium was replaced with fresh DMEM medium after 4 h. The cells were continually cultured at 37 °C in a humidified 5% CO_2 atmosphere incubator for 48 h prior to further experiments.

11. Tryptophan fluorescence quenching assay

Tryptophan fluorescence quenching assay was conducted with a fluorescence spectrophotometer (PerkinElmer, Waltham, MA, USA). Briefly, PCK2 protein was incubated with various concentrations of CuB (1-20 μM) in a quartz plate at 25 °C. Fluorescence was monitored by excitation wavelength at 370 nm and emission wavelengths from 300 to 550 nm with slit width of 1 nm. Fluorescence intensity was corrected by the blank solution emission spectra.

12. JC-1 staining assay

Mitochondrial membrane potential (ΔΨm) was measured through JC-1 staining. Briefly, cells were incubated with 10 μg$\cdot$mL^{-1} JC-1 for 30 min at 37 °C in the dark. Then, cells were washed with PBS three times. Images were obtained with IX73 fluorescence microscope (Olympus, Tokyo, Japan). JC-1 monomers emit green fluorescence with a maximum at 529 nm (green), whereas JC-1 aggregates emit red fluorescence with a maximum at 590 nm.

13. Reactive oxygen species (ROS) assay

Fluorescent probe DCFH-DA was used to assess the generation of intracellular reactive oxygen species. Briefly, cells were incubated with 10 μM

DCFH-DA for 10 min at 37 °C in the dark. Then, cells were washed twice with PBS, and images were captured by IX73 fluorescence microscopy (Olympus) at an excitation wavelength of 480 nm and an emission wavelength of 525 nm.

14. Mito-tracker staining assay

Cells were cultured in confocal petri dishes for at least 12 h. After treatment, cells were stained with 200 nM Mito-tracker solution for 30 min at 37 °C in the dark, and then washed twice with PBS. Images were obtained using LSM880 confocal microscope (Zeiss, Oberkochen, Germany) at excitation/emission wavelength of 579 nm/599 nm.

15. Mitochondrial DNA (mtDNA) analysis

Mitochondrial DNA content was evaluated using quantitative real-time PCR (qRT-PCR). After treatment, cells were collected and total DNA was extracted using EasyPure Genomic DNA kit. The procedure of qRT-PCR was as follow: pre-degeneration at 95 °C for 5 min, followed by 40 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. mtND1 and RPL13A primer pairs were used. mtND1: forward: CACCCAAGAACAGGGTTTGT; reverse: TGGCCATGGGTATGTTGTTAA. RPL13A: forward: CGCCCTACGACAAGAAAAAG; reverse: CCGTAGCCTCATGAGCTGTT. The CT value of mtND1 was normalized to the CT value of RPL13A.

16. PCK2 activity assay

PCK2 activity was evaluated by detecting the rate of oxaloacetate (OAA) converted into phosphoenolpyruvate (PEP) in the presence of GTP. Briefly, PCK2 protein was incubated with indicated concentrations of CuB (0, 5, 10, 20 μM). Then, 50 mM HEPES (pH 7.5), 1 mM ADP, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 500 μM GTP, 150 μM NADH, 8 U malic dehydrogenase, and 0.1 μg PCK2 protein were mixed and immediately monitored after 1 min at 340 nm using a microplate reader (PerkinElmer). One unit of PCK2 activity corresponded to the consumption of 1 nmol NADPH min^{-1} at 37 °C and then was normalized by the total protein content.

$$\text{PCK2 (U mg}^{-1}) = 3215 \times (A_{1\,min} - A_{0\,min})$$

17. Molecular docking

Full sequence of human PCK2 protein (PCKGM_HUMAN, UniProt ID: Q16822) was downloaded from UniProt (http://www.uniprot.org/). The crystal structure of (PDB ID: 2GMV) was chosen as a structural template for the homology modeling of PCK2, because it shares a similar enzyme function and

the highest sequence identities (%) with human PCK2. The 3D structure of the CuB was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The molecular docking was performed using the Maestro software suite 2018 (Schrödinger, NY, USA). The Cys63 of PCK2 was chosen as the ligand pocket.

18. Lipid metabolomics analysis

Lipid metabolomics was implemented on XploreMET platform (Metabo-Profile, Shanghai, China). The sample preparation was performed as previous report with minor modifications[3]. Briefly, pre-chilled zirconium oxide beads and ammonium acetate in methanol containing internal standards were added to each sample for lipid extraction. After incubation, the supernatant was diluted with extraction solvent for LC-MS analysis. The final concentration of internal standards i.e., C0-D3, C2-D3, C4-D3, C6-D3, C8-D3, C10-D3, C16-D3, C18-D3, lysoPC a C9:0, PC aa C28:0, PC aa C40:0, SM C6:0 was 1.25 $\mu g \cdot mL^{-1}$. Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters, Milford, MA, USA) was used to quantitate the metabolite. The UPLC was performed on a flow injection analysis (FIA) system. The elution program, using methanol (5 mM ammonium acetate) as mobile phases, was applied with a flow rate of 0.03 $mL \cdot min^{-1}$ for 0-0.5 min, 0.03-0.2 $mL \cdot min^{-1}$ for 0.5-1 min, 0.2 $mL \cdot min^{-1}$ for 1-2 min, 0.2-0.03 $mL \cdot min^{-1}$ for 2-2.1 min, and 0.03 $mL \cdot min^{-1}$ for 2.1-3 min. MS analysis was performed with the following parameters: capillary, 3.0 Kv; source temperature, 150 °C; desolvation, 350 °C; desolvation gas flow, 500 $L \cdot h^{-1}$. The raw data generated by UPLC-MS/MS were processed with LipidMET (v1.0). To elucidate potential significant metabolic pathways, MetaboAnalyst 5.0 (http://www. metaboanalyst.ca) was carried out for further metabolic pathway enrichment analysis.

19. Statistical analysis

Data were shown as means ± SD. The *student's t-test* was employed for statistical analysis using GraphPad Prism software. The *P* value < 0.05 was considered statistically significant.

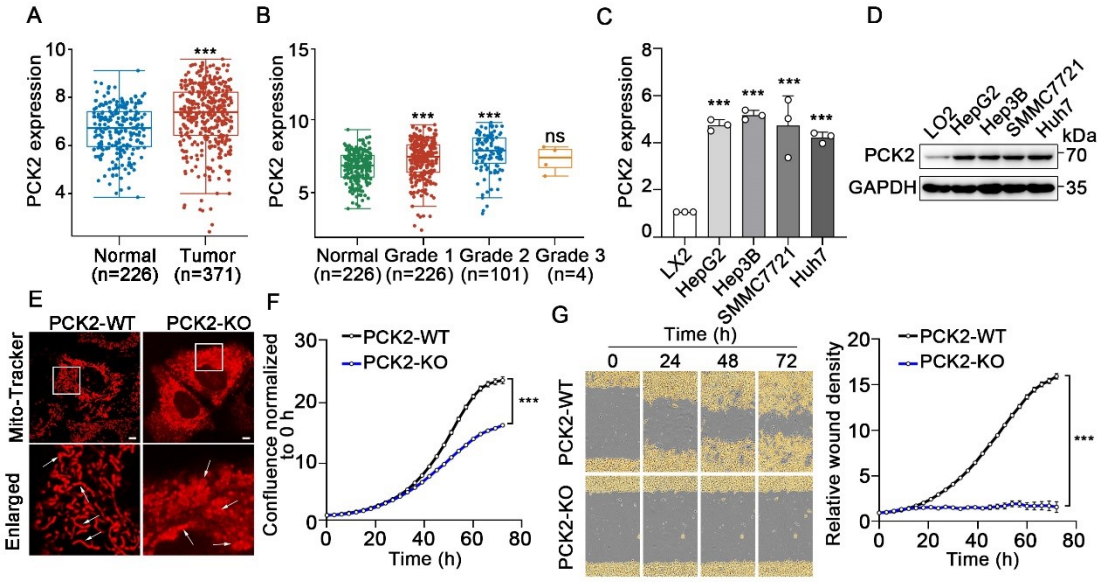

Figure S1. PCK2 is a key determinant of its oncogenic role in hepatocellular carcinoma (A) The levels of PCK2 were markedly increased in LIHC tissues compared to adjacent normal tissues determined by TCGA database. (B) PCK2 expression was highly associated with advanced tumor stage. (C) The mRNA levels of PCK2 across several hepatocellular carcinoma cell lines. (D) The protein levels of PCK2 across several hepatocellular carcinoma cell lines. (E) PCK2 knockout caused mitochondrial fragmentation in Huh7 cells, which was visualized by Mito-tracker staining (scale bar = 10 μm). (F) PCK2 knockout attenuated the proliferation of Huh7 cells. (G) PCK2-KO inhibited the migration of Huh7 cells using Incucyte S3 normalized to 0 h wound confluence. ***P < 0.001, compared with the PCK2-KO or PCK2-WT in Huh7 cells. Data was expressed as means ± SD.

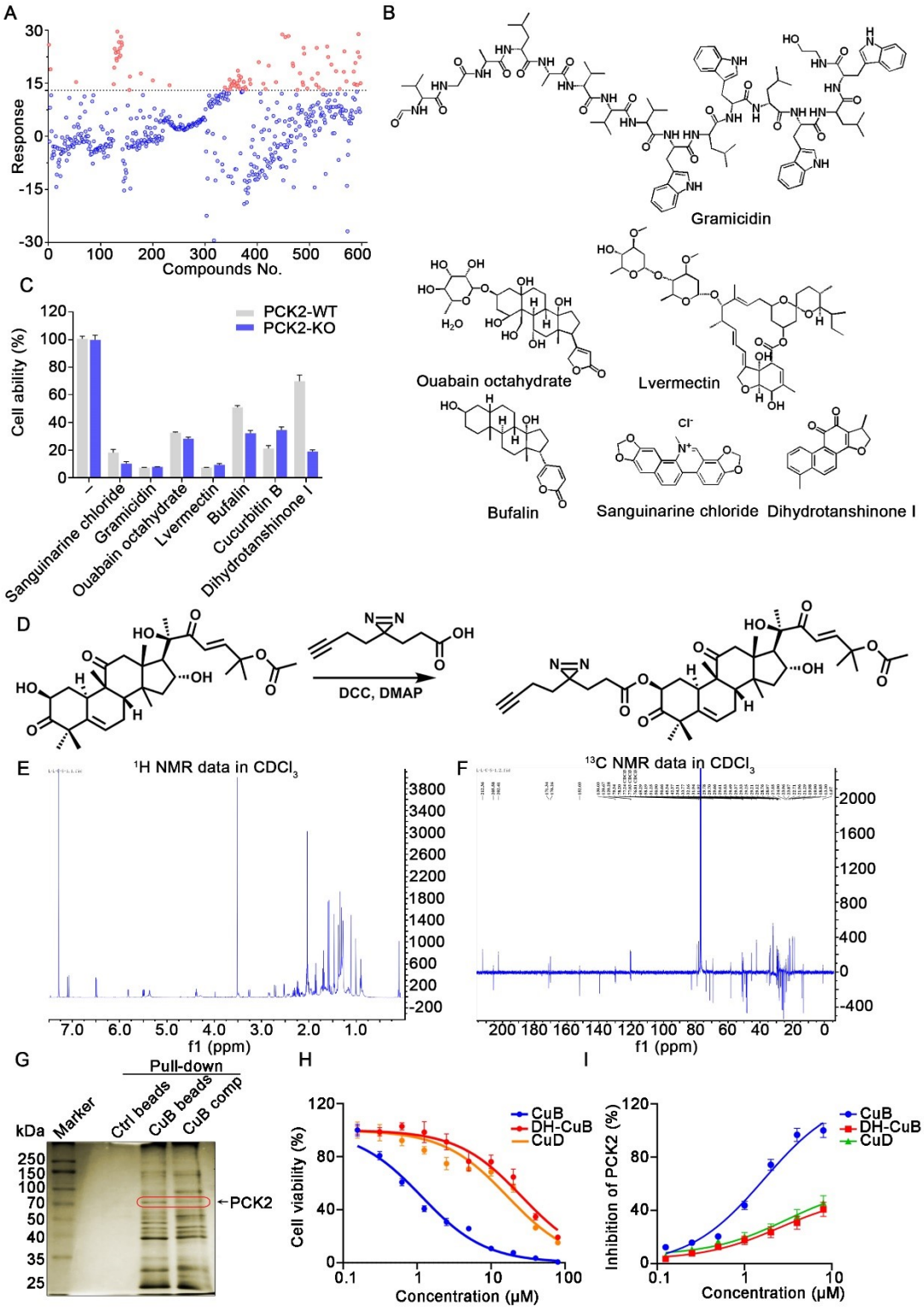

Figure S2. PCK2 is a direct cellular target of CuB. (A) SPR screening for small-molecule that could directly bind with PCK2 at 10 μM. Black line indicates the criteria (response: 14). Red points indicate the “hit” candidates. (B) Chemical structures for those candidates could inhibits Huh7 cell proliferation. (C)

Cytotoxicity evaluation against PCK2-WT or PCK2-KO Huh7 cells of seven candidates at 10 μM. (D) Schematic representation of the synthesis of CuB bifunctional probe. (E) ^{1}H NMR spectrum of CuB probe in CDCl3. (F) ^{13}C NMR spectrum of CuB probe in CDCl3. (G) Identification of binding proteins by pull-down assay with CuB probe using silver staining and LC-MS/MS. (H) The anti-proliferative effects of CuB, DH-CuB and CuD were determined by cck8 assay. (I) Effect of CuB, DH-CuB, and CuD on PCK2 enzymatic activity in vitro.

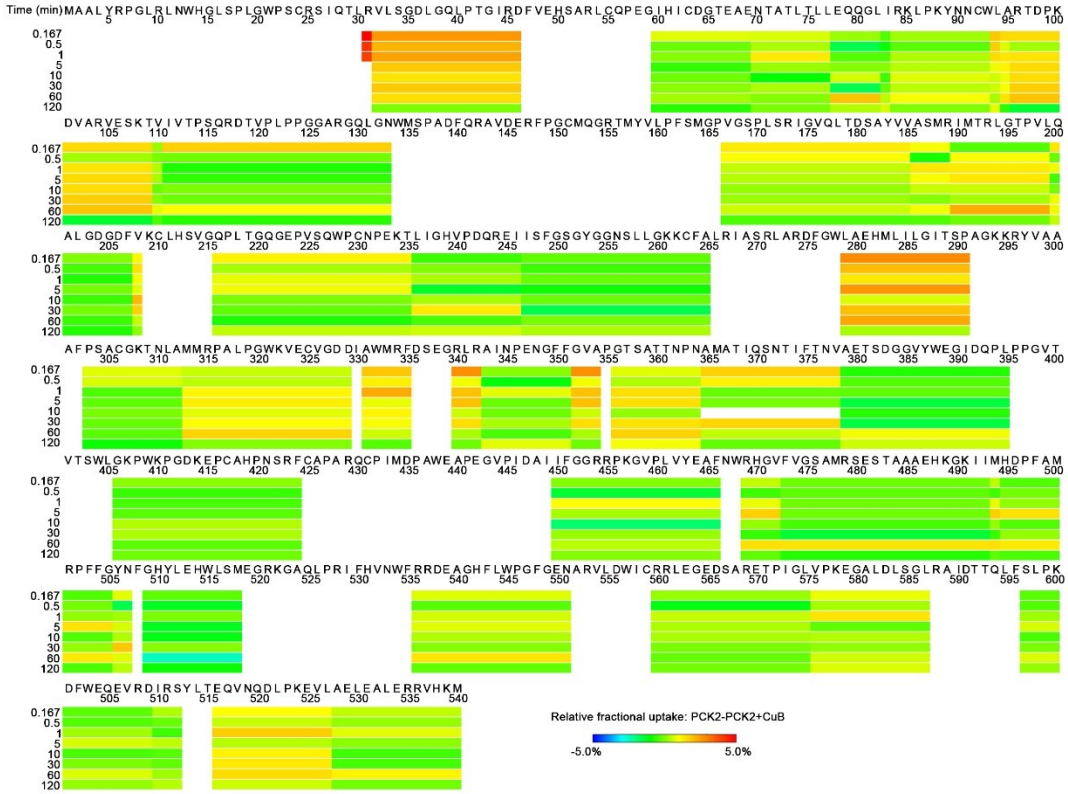

Figure S3. CuB allosterically regulates PCK2 conformation by HDX-MS profile.

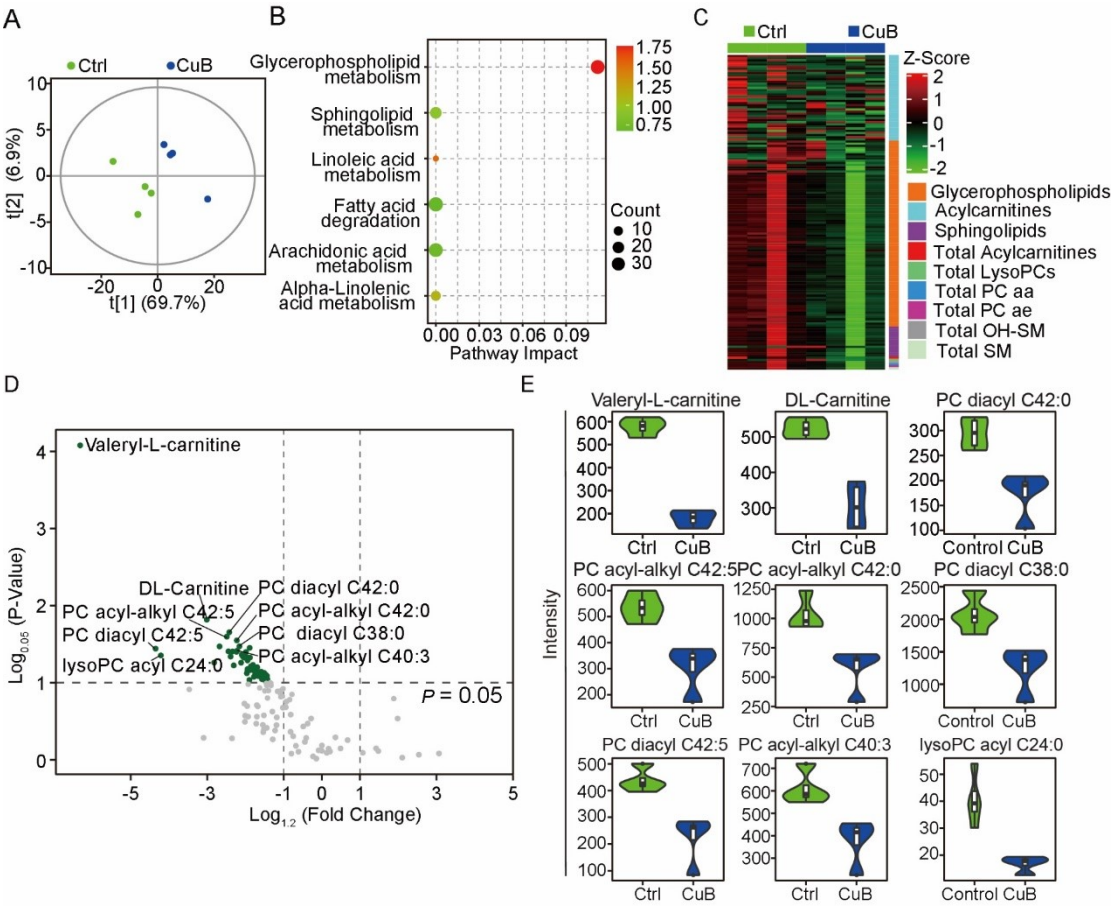

Figure S4. CuB disrupts phosphatidylcholine metabolism to destroy cellular membrane integrity (A) Principal component analysis (PCA) revealed the segregation between control and CuB-treated groups. (B) Heatmap depicted 147 differentially abundant lipid metabolites. Red color indicated upregulated metabolites; green color indicated downregulated metabolites. (C) Bubble chart displayed the enrichment pathways of differential metabolites. X-axis represented the pathway impact and Y-axis represented -$\log_{10}$ (*P* value). (D) Volcano plot was mapped to distinguish differential metabolites. X-axis represented fold change ($\log_{1.2}$fold change) and Y-axis represented p value. (E) Violin plots listed the top nine differential metabolites.

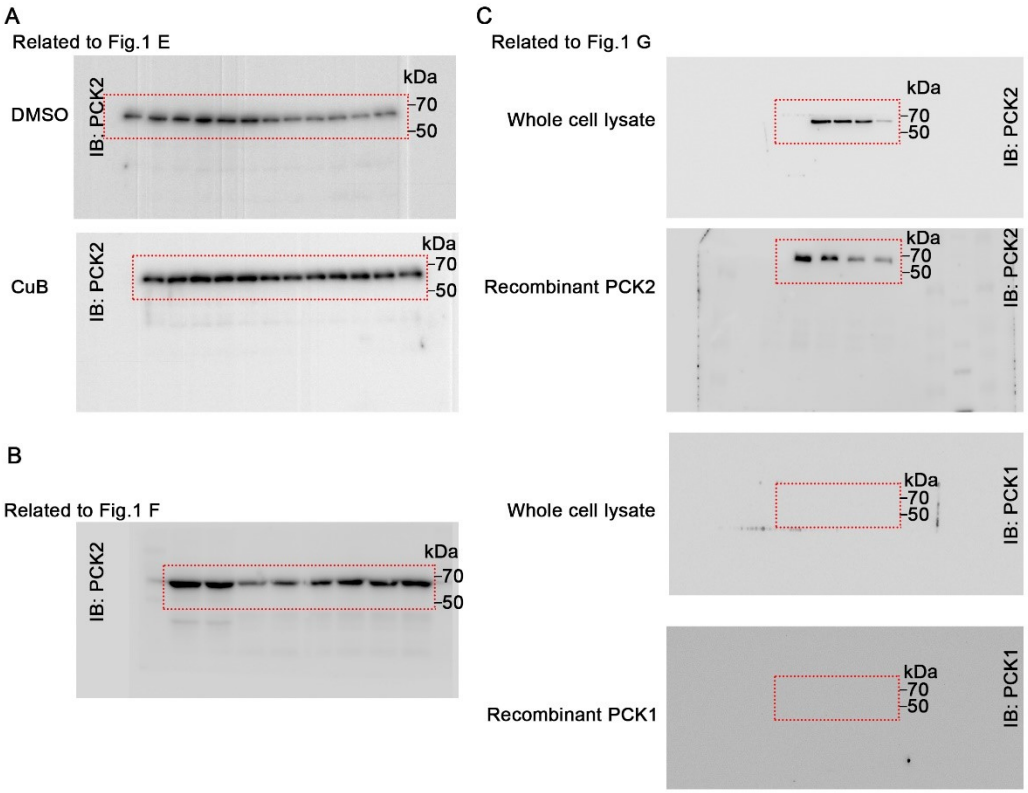

Figure S5. Whole uncropped images of the original western blots.

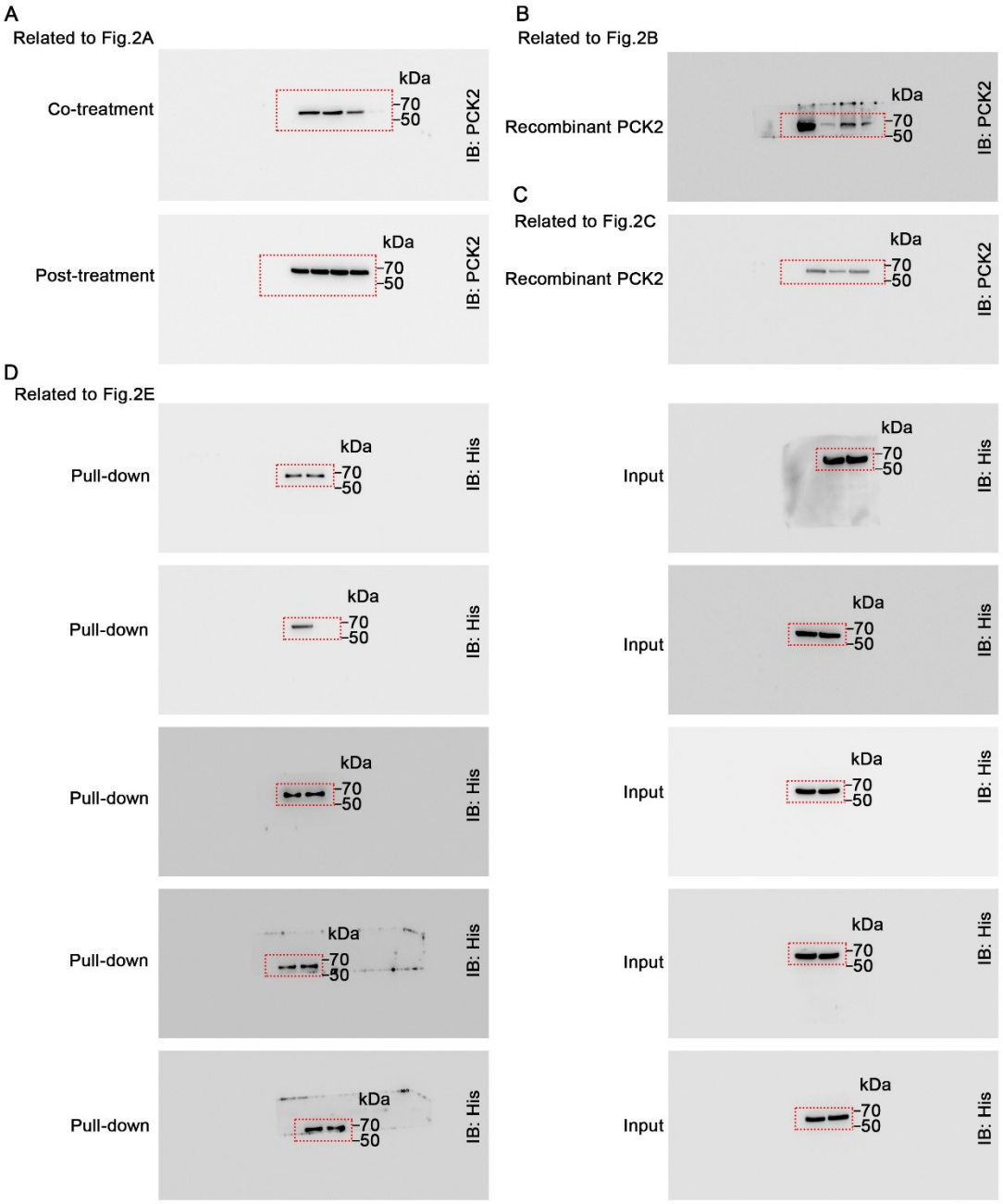

Figure S6. Whole uncropped images of the original western blots.

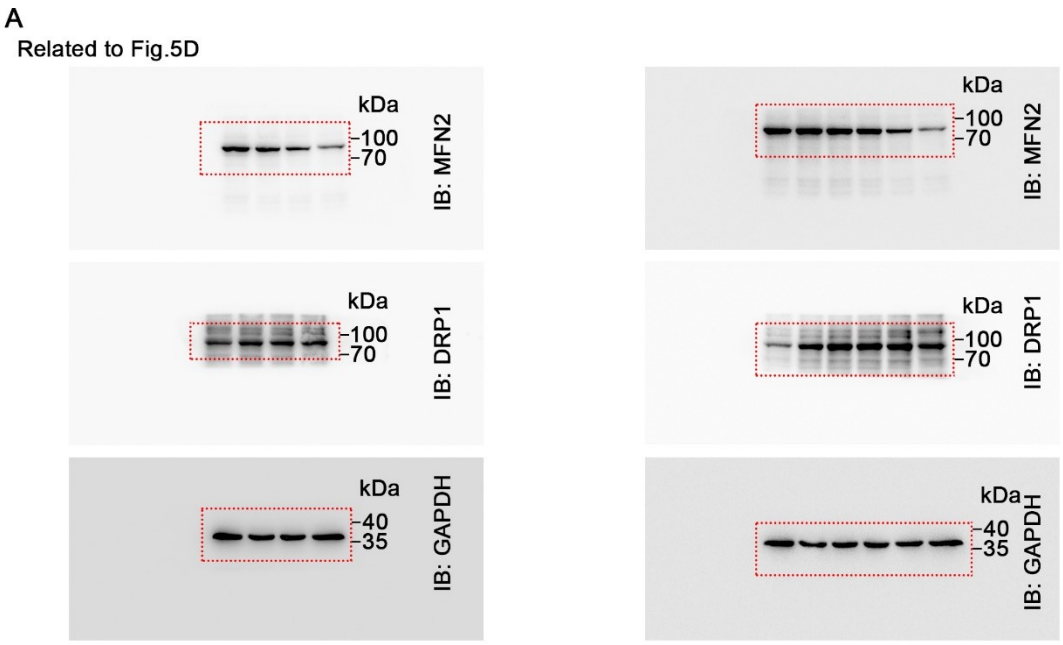

Figure S7. Whole uncropped images of the original western blots.