Supporting Information

Photoaffinity labelling-based chemoproteomic strategy identifies PEBP1 as the target of ethyl gallate against macrophage activation

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Table of contents

I. Supplementary materials and methods

1. Chemicals and reagents

Ethyl gallate ($C_9H_{10}O_5$) was purchased from Yuanye Bio-Technology (Shanghai, China) and confirmed with MS, ^{13}C-NMR and ^{1}H-NMR data. The purity is above 98 % by HPLC analysis. 5-TAMRA-azide was purchased by Jena Bioscience (Jena, Thuringia, Germany). 4,6-diamidino-2-phenylindole (DAPI), tris (2-carboxyethyl) phosphine (TCEP), tris [(1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl] amine (TBTA), lipopolysaccharides (LPS), and 3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl) propanoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biotin-PEG3-azide was obtained from AAT Bioquest (Mercury Drive Sunnyvale, CA, USA). Pronase, *E. coli* DH5α and *E. coli* BL21 (DE3) were purchased from Solarbio (Beijing, China). Anti-PEBP1 antibody (ab76582) and anti-p-PEBP1 (Ser153) antibody (EP2845Y) were obtained from Abcam (Cambridge, Cambs, UK). Anti-p-NF-κB p65 (Ser536) antibody (3033S), anti-NF-κB p65 (8242S), anti-IKKβ antibody (8493P), anti-p-IKKα/β antibody (2694T), anti-IkBα antibody (4814T), anti-p-IKB-α (Ser32) antibody (2859T), and anti-HA-Tag (3724) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Raf-1 (66592-1-Ig) antibody, HRP-conjugated goat anti-rabbit IgG antibody (SA00001-2), and anti-GAPDH HRP-conjugate antibody (60004-1-Ig) were purchased from Proteintech (Chicago, IL, USA). NO assay kit was purchased from Nanjing Jiancheng Bio-Engineering Institute (Nanjing, Jiangsu, China). BCA protein assay reagent was purchased from TransGen Biotech (Beijing, China). TNF-α and IL-6 ELISA kits were purchased from ExCell Biology (Shanghai, China).

2. The synthesis of ethyl gallate probe (AD-EG)

The synthetic route of alkyne/diazirine-double labelled EG probe (AD-EG) is shown in Figure 1A. 1-benzoyl-4-piperidinemethanamine HCl (120 μL) and 3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl) propanoic acid (80.5 mg) were dissolved in dichloromethane for 24 h. Next, EG (143.2 mg) and NaH (34.6 mg) were dissolved in tetrahydrofuran. Next, added 164.5 mg N,N'-Dicyclohexylcarbodiimide (DCC) and 105.6 mg 4-dimeth-ylaminopyridine (DMAP) to the above reaction mixture and stirred in darkness at 25 °C for 24 h, purified through silica gel column chromatography, and ultimately eluted with ethyl acetate: petroleum ether (1 : 10) to

get AD-EG as white solid with 28.3 % yield. The structure of AD-EG was verified by MS, ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.53 (d, J = 1.9 Hz, 1H), 7.38 (d, J = 1.9 Hz, 1H), 4.35 (q, J = 7.1 Hz, 2H), 2.47 (t, J = 7.3 Hz, 2H), 2.20 (t, J = 7.7 Hz, 2H), 2.07 – 2.03 (m, 5H), 1.84 (t, J = 7.7 Hz, 2H), 1.74 (d, J = 7.3 Hz, 1H), 1.68 (t, J = 7.4 Hz, 2H), 1.39 (t, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 177.16, 170.73, 165.85, 145.27, 140.11, 137.81, 122.51, 116.31, 114.48, 82.48, 69.56, 69.32, 61.23, 32.16, 28.50, 27.70, 14.28, 13.25, 1.03, 0.00.

3. Cell culture

RAW264.7 mouse macrophages were provided from Peking Union Medical College Cell Bank (Beijing, China). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % FBS and 1 % penicillin-streptomycin solution at 37 °C with 5 % CO_2.

4. MTT assay

The cells were seeded at a density of 5 × 10^4 /well in 96-well plates overnight, and next LPS (1 μg/mL) was added with or without EG (12.5, 25 and 50 μM). After 24 h, 0.5 mg/mL MTT solution was added and cells were incubated under a temperature of 37 °C for 4 h. Formazan crystals were dissolved in 200 μL DMSO. Utilizing a microplate reader (Tecan Austria GmbH, Männedorf, Switzerland), the formazan concentration was assessed through detecting the absorbance at 570 nm.

5. Nitric oxide (NO) assay

The generation of NO was determined with NO assay kit. RAW264.7 cells were treated with 1 μg/mL LPS with or without EG (12.5, 25 and 50 μM) for 24 h. Subsequently, the cell supernatant was mixed with Griess reagent for 10 min at 1 : 1 ratio for incubation at room temperature. The value of absorbance was determined at 540 nm through microplate reader (Tecan Austria GmbH, Männedorf, Switzerland).

6. Enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-6 were detected using ELISA assay. Different concentrations of EG (12.5, 25 and 50 μM) were applied to RAW264.7 cells in the presence of 1 μg/mL LPS. For the further determination, the cell supernatant was collected at various time points (4 h for TNF-α assay, 8 h for IL-6 assay) using commercial ELISA kits. Briefly, the IL-6/TNF-α standard solutions and samples were added into 96-well plates that were coated with specific monoclonal antibody. Subsequently, the plates were exposed to biotin-anti-conjugated cytokine antibodies and tested with streptavidin-peroxidase-conjugated anti-rabbit antibodies. Ultimately, the stop solution was added and the ODs were measured at 450 nm on a microplate reader (Tecan Austria GmbH, Männedorf, Switzerland). The concentration of TNF-α/IL-6 was detected in accordance with the standard curve fitted through IL-6/TNF-α standard solutions.

7. In-gel fluorescence labelling

RAW264.7 cells were inoculated with various concentrations of AD-EG (25, 50 and 100 μM) for 4 h. Next, the cells were washed two times using PBS and they were exposed to UV (365 nm) for 10 min. Cells were lysed in NP-40 buffer (Beyotime, Shanghai, China) supplemented 1 % protease inhibitor and adjusted to a same volume and concentration utilizing NP-40 lysis buffer as previously described[1-3]. In short, cell lysates containing 1 mg proteins were successively mixed with 3 μL 5-TAMRA-azide (20 mM in DMSO), 24 μL TCEP (14.4 mg/mL), and 3.5 μL TBTA (34 mM in DMSO). The click chemistry was initiated by 11.6 μL $CuSO_4$ (50 mM). The reaction was incubated for 1 h at room temperature. The protein pellets were gathered with 6000 g centrifugation and washed using cold methanol. Then, the samples were heated in 1.2 % SDS at 85 °C for 10 min and separated by SDS-PAGE. The fluorescence scanning was conducted with chemiluminescent gel imager scanner (ChemiDoc XRS System, Hercules, CA, USA). For the competitive labelling assay, RAW264.7 cells were incubated with EG (50 and 100 μM) for 1 h before incubating with AD-EG (50 μM).

8. Pull-down assay

For targets identification in situ labelling experiments, AD-EG was incubated with RAW264.7 cells on the basis of stable isotope labelling with amino acids in cell culture (SILAC) proteomics. RAW264.7 cells were cultivated in medium involving either $^{13}C_6{}^{15}N_2$-Lysine/$^{13}C_6{}^{15}N_4$ L-Arginine (Heavy) or $^{12}C_6{}^{14}N_2$-Lysine/$^{12}C_6{}^{14}N_4$ L-Arginine (Light). Cells were grown in heavy or light labelled-medium for at least 6 cell passages to reach 96 % incorporation. RAW264.7 cells (Heavily- or Light-labelled) were stimulated with LPS (1 μg/mL) for 6 h, followed by treatment with AD-EG (50 μM). At the same time, the excess EG (100 μM) for competitive binding was added into light-labelled cells and then further inoculated at 37 °C for 4 h. The click chemistry with biotin-PEG3-azide was performed as above described. The proteins were collected by centrifugation and resuspended in 0.2 % SDS/PBS. Then streptavidin beads were mixed with samples and incubated at room temperature for 3 h. Eventually, the captured proteins on the streptavidin beads were digested by trypsin and determined with LC-MS/MS (NanoLC-LTQ Velos pro MS, Thermo, Waltham, MA, USA). The *p*-value (< 0.01) and protein fold change (> 1.2) were considered to indicate a differentially abundant protein[4].

9. LC-MS analysis

The peptides were separated in C18 reversed-phase column. Elution buffer A and B were 0.1 % and 0.2 % formic acid in water and acetonitrile, respectively. The gradient elution procedure was 2 % - 40 % B for 70 min; 40 % - 95 % B for 5 min; 95 % B for 20 min. The eluent was introduced to the MS at 300 nL/min. The parameters of MS are as below: scan spectra, 350 to 2000 m/z; maximum IT, 50 ms; HCD Collision Energy, 35 %; resolution, 60000; Target FDR for PSMs, 0.01. The analysis of MS data was conducted through Proteome Discoverer (version 1.4) software with SEQUEST (Thermo, Waltham, MA, USA).

10. Drug affinity responsive target stability (DARTS)

DARTS was performed as previously described[5, 6]. RAW264.7 cells were lysed with NP-40 Lysis Buffer (Beyotime, Shanghai, China). Lysates were diluted with 10×TNC buffer (50 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0) and incubated with different concentrations of EG (10, 50 and 100 μM) at room temperature for 1 h. Subsequently, 5 μg/mL of pronase was

added and incubated at room temperature for 20 min in 1 × TNC buffer. After adding the SDS loading buffer, the reactions were terminated, followed by a specific anti-PEBP1 antibody detection via western blot.

11. Cellular thermal shift assay (CETSA)

CETSA was adapted from previous research to identify the ligand-target engagement[7, 8]. For living cells CETSA experiment, RAW264.7 cells were pretreated with EG (50 μM) or DMSO for 3 h at room temperature and harvested in PBS with 1 % protease inhibitor. Then, cells were classified into ten groups and heated at gradient temperature (43 - 61 °C) for two min respectively with T100 Thermal Cycle (Bio-Rad, Hercules, CA, USA). Kinase buffer (CST, Beverly, MA, USA) was added to the samples and repeated freeze-thaw in liquid nitrogen. The lysate was collected and analyzed through western blot with a specific anti-PEBP1 antibody. For the cell lysate CETSA experiments, RAW264.7 cells were gathered and repeatedly frozen and thawed in liquid nitrogen. Subsequently, the cell lysates were classified into two groups, one as the control group (DMSO), and the other group was inoculated using EG (50 μM) for 1 h at 37 °C. The lysates were heated and analyzed as above described in living cells experiment.

12. PEBP1 expression and purification

His-PEBP1, His-PEBP1^{D70A}, His-PEBP1^{Y120F} and His-PEBP1$^{D70A/Y120F}$ plasmids were expressed in *E.coli* BL21 (DE3) cells. The recombinant proteins were induced by isopropyl-β-D-1-thio-galactopyranoside (0.5 mM, IPTG) at 16 °C in 120 rpm for 18 h. The cells were gathered by centrifugation at 4000 rpm for 25 min under a temperature of 4 °C. The cells were lysed and the supernatant was loaded onto Ni-NTA resin (Thermo, Waltham, MA, USA) with HEPES buffer (a gradient of 10-250 mM imidazole, 20 mM HEPES, 250 mM NaCl) to remove impurities. The proteins were concentrated via centrifugal filtration (Amicon Ultra-10, Millipore, Waltham, MA, USA) and dissolved in PBS buffer for confirmation with Coomassie brilliant blue staining.

13. Surface plasmon resonance (SPR) assay

The analysis of interaction between PEBP1 and EG was implemented with Biacore T200 system (GE Healthcare, Uppsala, Sweden). The carboxymethylation 5 (GE Healthcare, Uppsala, Sweden) sensor chip was immobilized with recombinant PEBP1 protein (120 μg/mL) by amine coupling reaction (acetate, pH 4.5). Different concentrations of EG (0.39 to 12.5 μM) in PBS (containing 5 % DMSO) were injected into the sensor chip. The analysis of data was conducted through Biacore evaluation software (GE Healthcare, Uppsala, Sweden).

14. Isothermal titration calorimetry (ITC) analysis

ITC was carried out using a MicroCal PEAQ-ITC200 (Malvern Instruments, Malvern, UK) at 25 °C. PEBP1 protein (100 μM) and EG (1 mM) were prepared in PBS (pH 7.4, 0.1 % DMSO). PEBP1 protein solution (100 μM) was placed in the calorimeter sample cell (280 μL), and EG solution (1 mM) was put into injection syringe. In the process of titration, an initial injection of EG (0.4 μL) was followed by 19 successive injections of 2 μL with a 150 s interval. Ultimately, the binding parameters, including the association constant (K_D), enthalpy value (ΔH), and entropy value (ΔS), were analyzed by the Origin 9.0.

15. Tryptophan fluorescence quenching study

Recombinant PEBP1 protein was titrated utilizing the vehicle or EG (1-500 μM) in a quartz plate (96-well). The fluorescence intensities after continuous addition of EG solution were detected via setting excitation wavelength (280 nm) and emission wavelengths from 300 to 500 nm at 1 nm increment with fluorescence spectrophotometer (PerkinElmer, Waltham, MA, USA). Then, the intensities of fluorescence were corrected by the buffer contribution.

16. Determination of EG-binding site on PEBP1

Recombinant PEBP1 protein was inoculated with DMSO or AD-EG (50 μM) at 37 °C for 2 h, which was exposed to 365 nm UV for 10 min on ice. The SDS loading buffer was added into samples and heated at 98 °C for 10 min. Subsequently, the samples were separated with SDS-PAGE. The proteins were digested with trypsin and analyzed with Q-Exactive HF mass spectrometer coupled with an UltiMate 3000 RSLCnano System (Thermo Fisher Scientific, Waltham, MA, USA). The samples were bound onto a trap column (3 μm) packed with

ReproSil-Pur C18-AQ (100 μm, 12 cm). The peptides were isolated on a C18 column (3 μm) packed with ReproSil-Pur C18-AQ (75 μm,12 cm) and eluted as the below gradient through phase B (0.1 % formic acid in acetonitrile) at the flow rate of 300 nL/min: 0 - 70 min (2 %-40 %), 70 - 75 min (40 % - 95 %), 75 - 95 min (95 %). Mass spectrometers were operated in positive ionization mode with the MS parent scan of a mass range from 350 to 1500 m/z with resolution 60000 to 200 m/s. RAW files were analyzed through Proteome Discoverer (PD) 2.2 (Thermo Fisher Scientific, Waltham, MA, USA) according to the database of ratus UniProt FASTA. The maximum missed cleavage sites is 2. Fragment mass tolerance was 0.02 Da and precursor mass tolerance was 10 ppm. Oxidation at methionine was set as dynamic modification, carbamidomethylation at cysteine was set as static modification, and 318.1098 was set as dynamic modification. The percolator was used to filter the results with FDR < 1 %.

17. Molecular Docking

The entire docking process was performed using Induced Fit Docking module of Schrodinger 2018. From RCSB Protein Data Bank, PEBP1 protein crystal structure (PDB code: 1BD9) was downloaded. The potential receptor binding site was predicted by the Schrödinger SiteMap component. The grid box around the active site was generated using Receptor Grid Generation module, with the coordinate values of center_x = -17.332, center_y = 32.139, center_z = 44.032, and dock ligand with length ≤ 20 Å. EG and PEBP1 were docked with Induced Fit docking module to obtain precise binding conformation. The Glide XP was chosen for docking precision. Finally, Pymol software (Schrödinger, LLC, Palo Alto, CA, USA) was used to show the interaction types of docked PEBP1 protein with EG ligand.

18. Hydrogen-deuterium exchange mass spectrometry (HDXMS) analysis

HDXMS experiment was carried out as previously described[9]. Briefly, deuterium labelling was initiated with a 20-fold dilution into D_2O buffer of a pre-equilibrated aliquot of PEBP1 protein in the presence or absence of EG at 37 °C for 30 min. The labelling reaction was quenched through adding concentrated hydrochloric acid at specified time points (0.08, 0.25, 1, 30, 60, and 240 min). Subsequently, samples were injected and online digested by a Waters ENZYMATE BEH pepsin column (2.1 × 30 mm, 5 μm). The peptides were trapped and desalted on aVanGuard Pre-Column trap (ACQUITY UPLC BEH C18, 1.7 μm) for 3 min,

eluted in the trap by 15 % acetonitrile at 100 μL/min flow rate, and isolated through an ACQUITY UPLC BEH C18 column (1.0 × 100 mm, 1.7 μm). The relative deuterium content of all peptides was calculated by subtracting the mass of the undertreated control sample from that of the deuteron-labelled sample.

19. Co-immunoprecipitation (co-IP) analysis

The cells were transfected with HA-PEBP1 for 48 h, and inoculated with 1 μg/mL LPS and treated in the presence and absence of EG (50 μM) for 1 h. The cells were lysed and collected via centrifugation at 10000 rpm for 15 min. Anti-HA-tag antibody-conjugated magnetic beads were added into cell lysate for 4 h at 4 °C. Then, the immunoprecipitated proteins were separated by SDS-PAGE and determined with western blot.

20. Immunofluorescence assay

RAW264.7 cells were seeded on uncoated confocal dish, and stimulated with LPS (1 μg/mL) and EG (50 μM) for 1 h. The cells were fixed through 4 % (w/v) paraformaldehyde for 20 min, and subsequently permeabilized by 0.5 % Triton X-100 for 0.5 h. The cells were blocked with 5 % BSA for 1 h. Anti-NF-κB p65 antibody was added for incubation overnight under a temperature of 4 °C. Next, the cells were incubated with the secondary antibody labelled by Dylight 594 (1 : 200) for 1 h at 37 °C. After washes with PBS, the cells were incubated with 20 μg/mL DAPI for 20 min at 37 °C. The fluorescence (340 nm/488 nm for DAPI; 594 nm/618 nm for Dylight 594) was visualized via Zeiss LSM880 (Zeiss, Oberkochen, Germany).

21. Transient transfection with PEBP1 siRNA

For siRNA knockdown, cells were transfected by lipofectamine RNAimax (Invitrogen, Carlsbad, CA, USA) with NC or PEBP1 siRNA (Gene Pharma, Shanghai, China) in Opti-MEM medium. After 6 h, the cells were washed with DMEM medium and further cultured for 48 h. The PEBP1-siRNA sequence was shown as listed in Table 1.

Table 1 SiRNA sequences for transfection.

Name	Sequence
PEBP1 siRNA	5'–3' (sense) GAUCCUGGGAAACUCUACATT 5'–3' (antisense) UGUAGAGUUUCCCAGGAUCTT
Negative siRNA	5'–3' (sense) UUCUCCGAACGUGUCACGUTT 5'–3' (antisense) ACGUGACACGUUCGGAGAATT

22. Western blot analysis

RAW264.7 cells were collected after treatment and lysed in RIPA with 1 % protease suppressor. The concentration of protein was examined utilizing BCA protein assay reagent. SDS loading buffer was added into protein samples and subsequently heated under a temperature of 98 °C for 10 min. Next, the proteins were isolated with 10-15 % SDS-PAGE, and transferred to PVDF membrane. Afterwards, the membrane was blocked in 5 % (w/v) skimmed milk at 37 °C for 30 min, and incubated with primary antibodies overnight at 4 °C and HRP-conjugated secondary antibodies for 1 h at 37 °C. Finally, the membranes were visualized by ECL (Thermo, Waltham, MA, USA), and scanned with Tanon 5200 Imaging Analysis System (Tanon, Shanghai, China).

23. Statistical analysis Results

The data were presented as means ± standard error of mean (SEM). Statistical analysis was performed with Student's t-test of variance (ANOVA) using GraphPad Prism 8.0. $P < 0.05$ was considered as statistically significant.

II. Supplementary figures

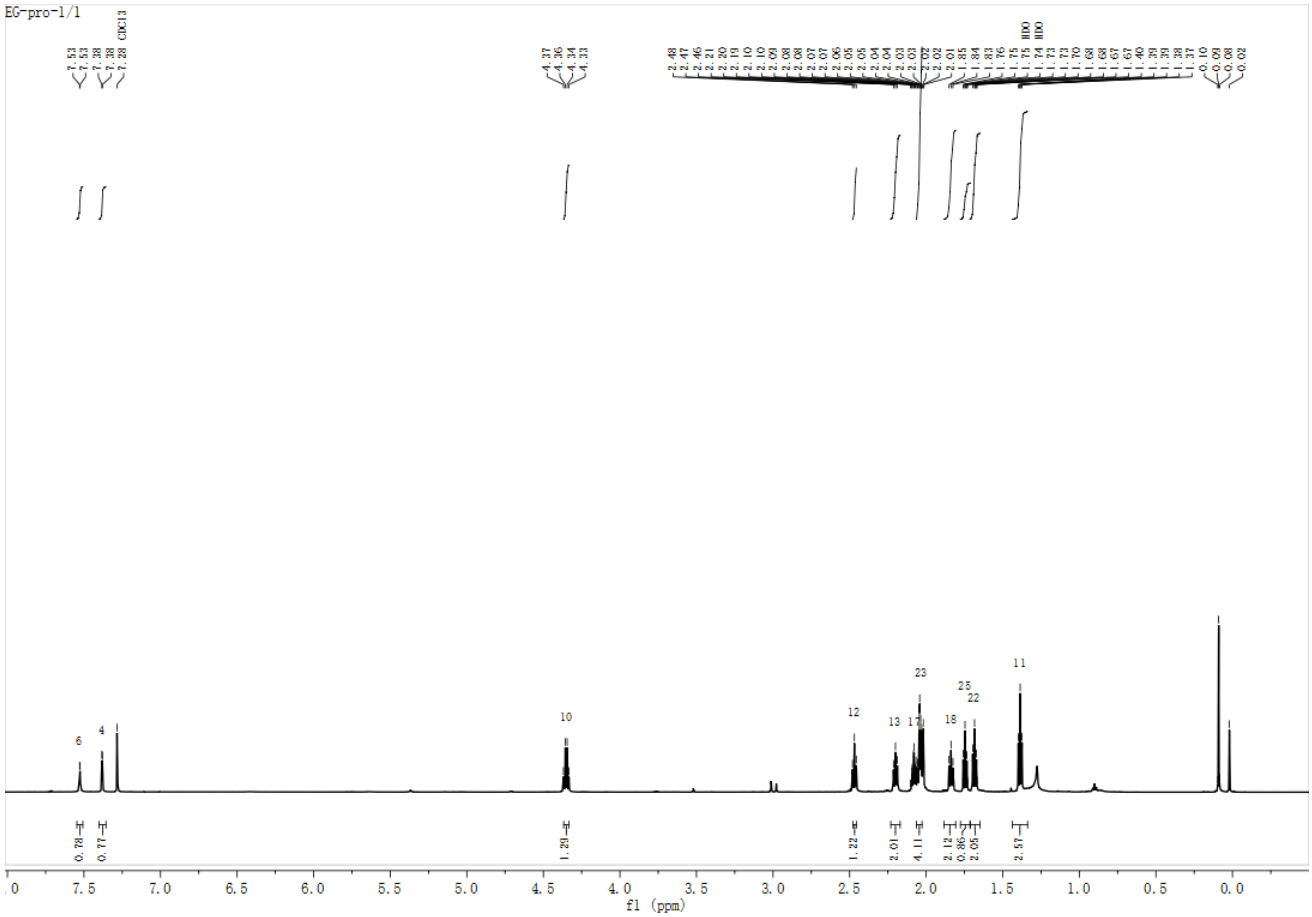

Fig. S1 ^{1}H NMR spectrum of AD-EG.

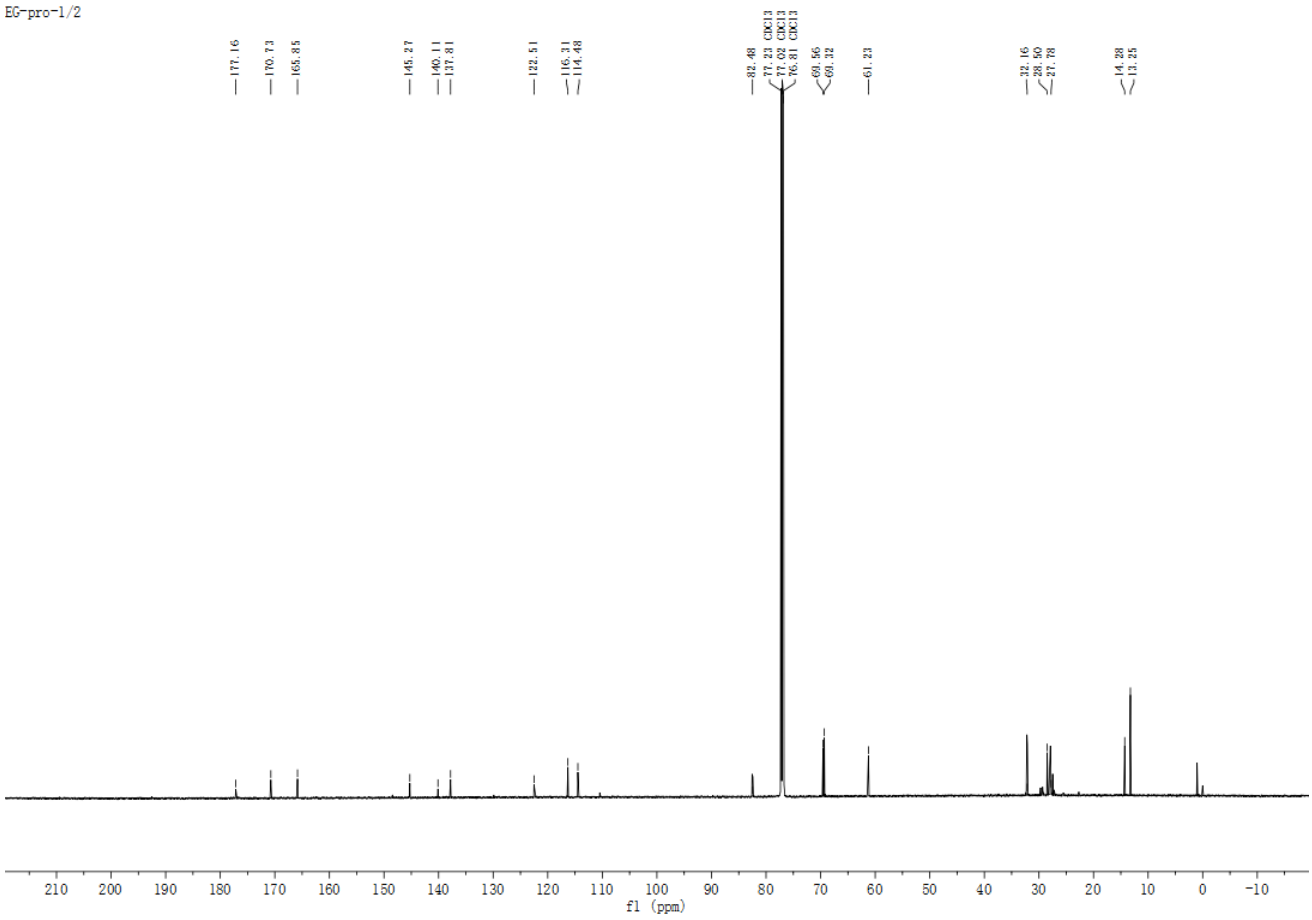

Fig. S2 ^{13}C NMR spectrum of AD-EG.

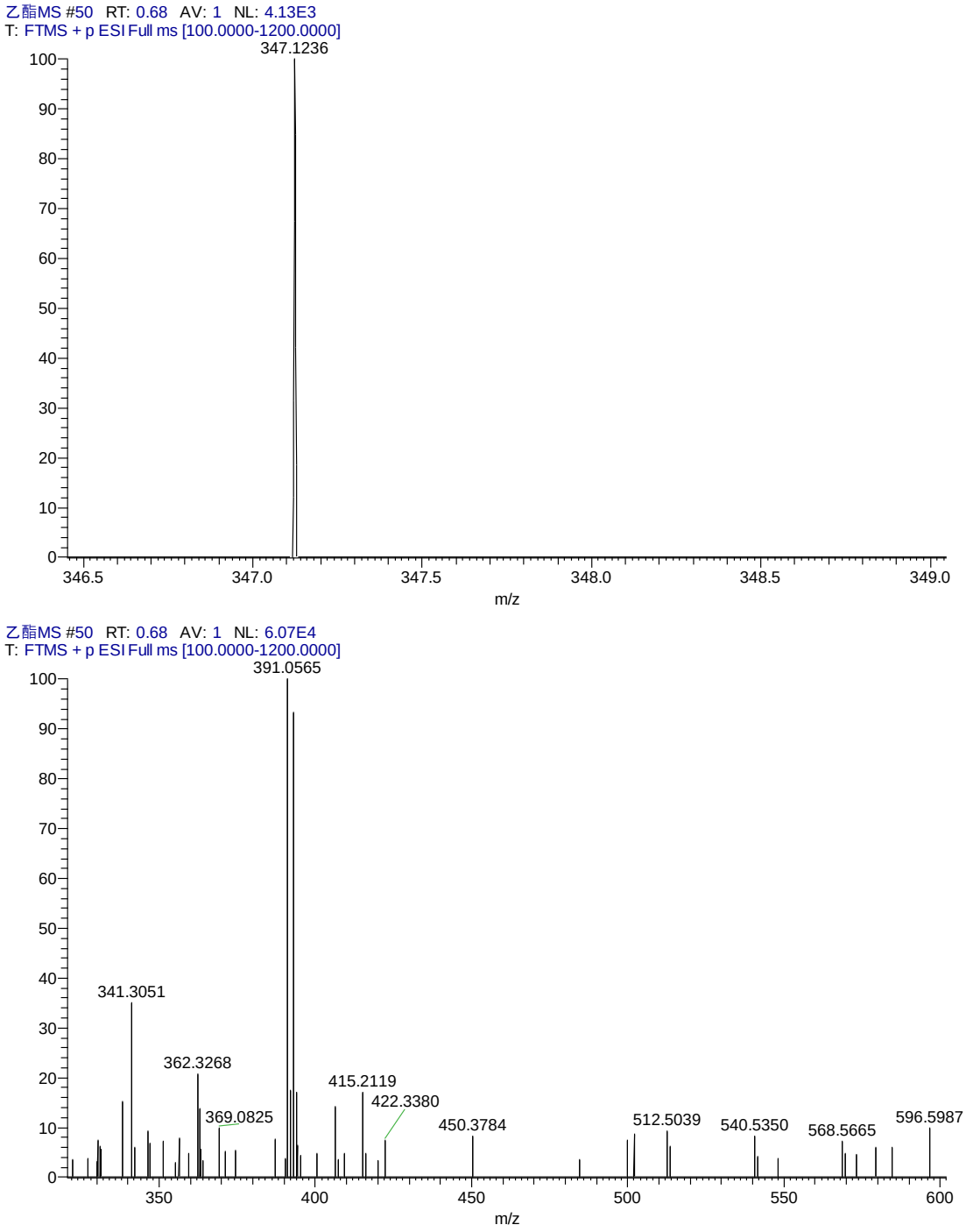

Fig. S3 MS of AD-EG.

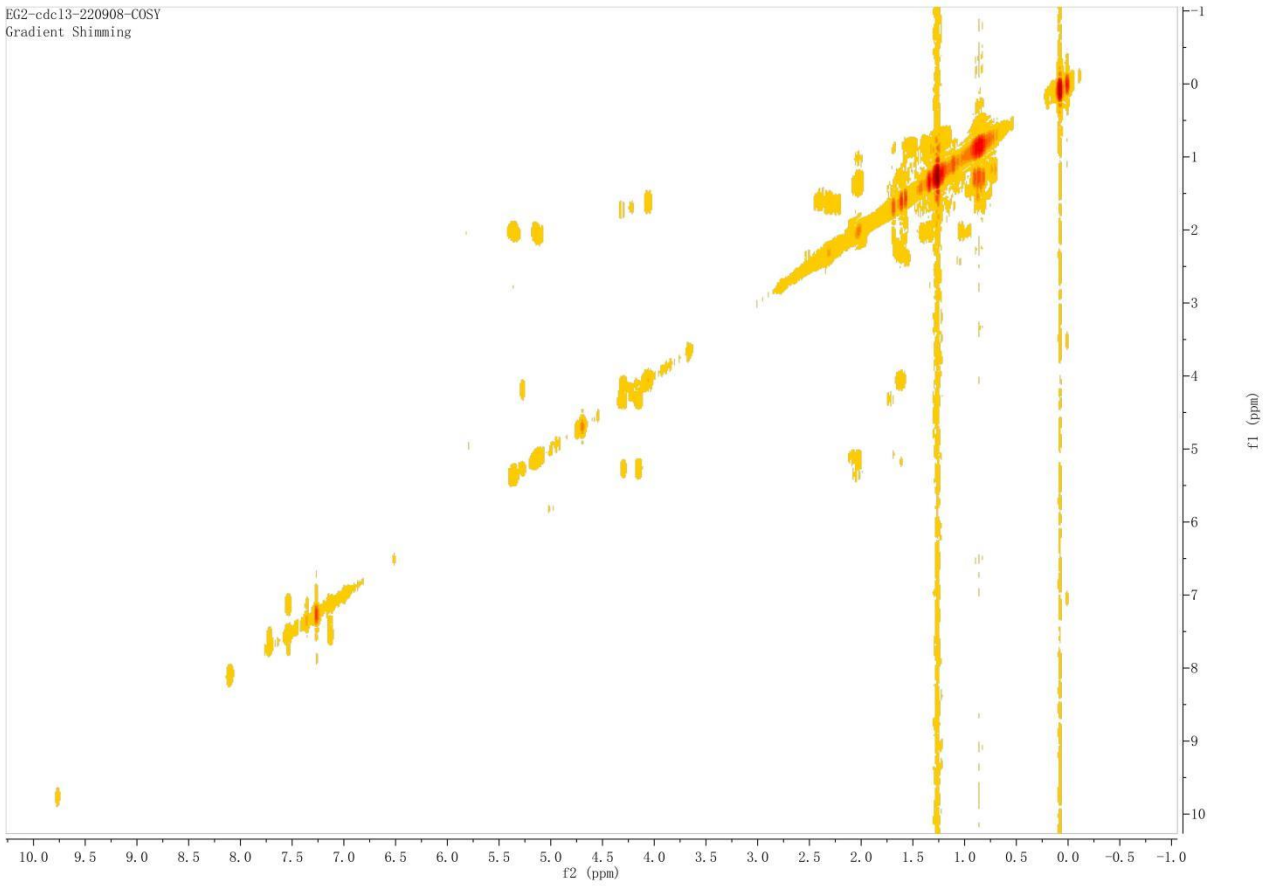

Fig. S4 ^{1}H-^{1}H COSY spectra for AD-EG.

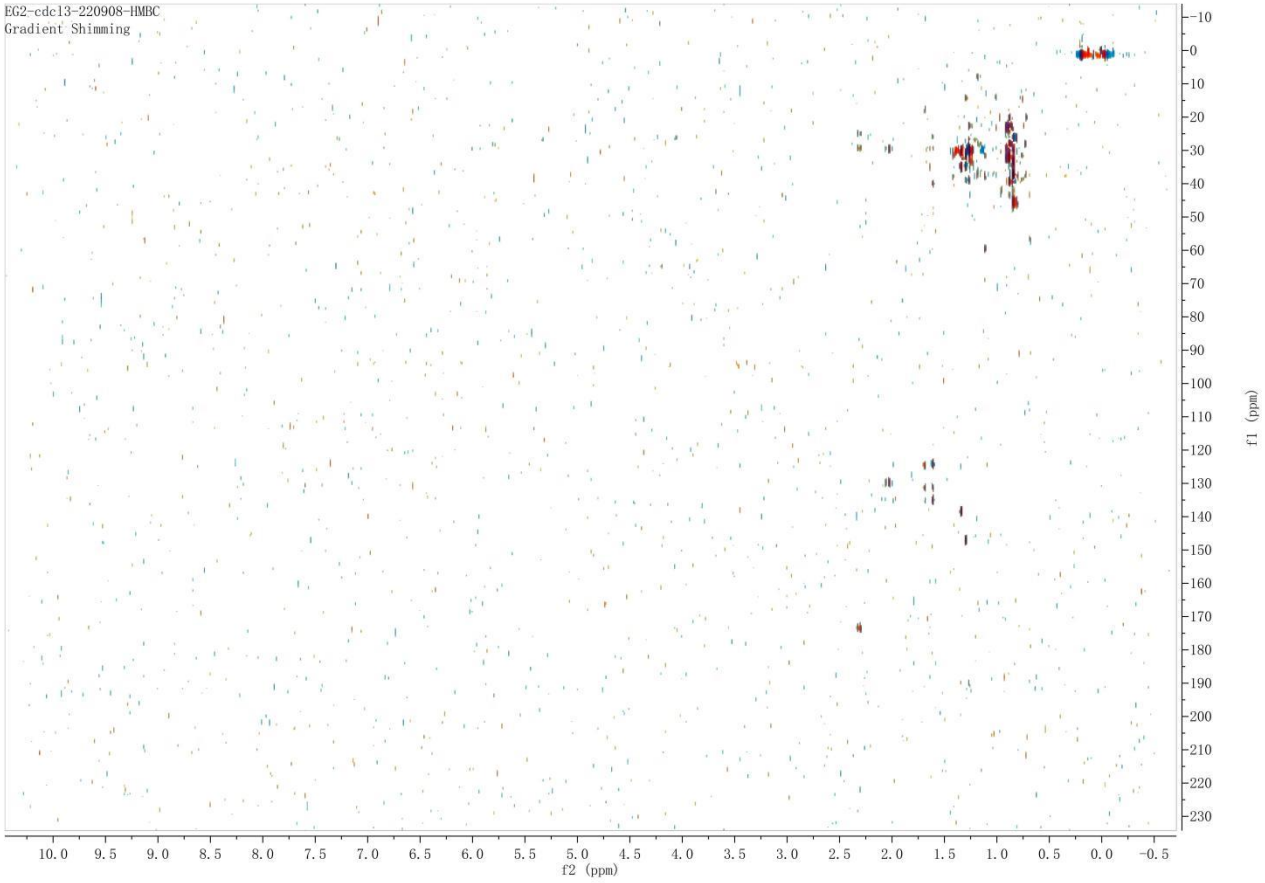

Fig. S5 HMBC spectra for AD-EG.

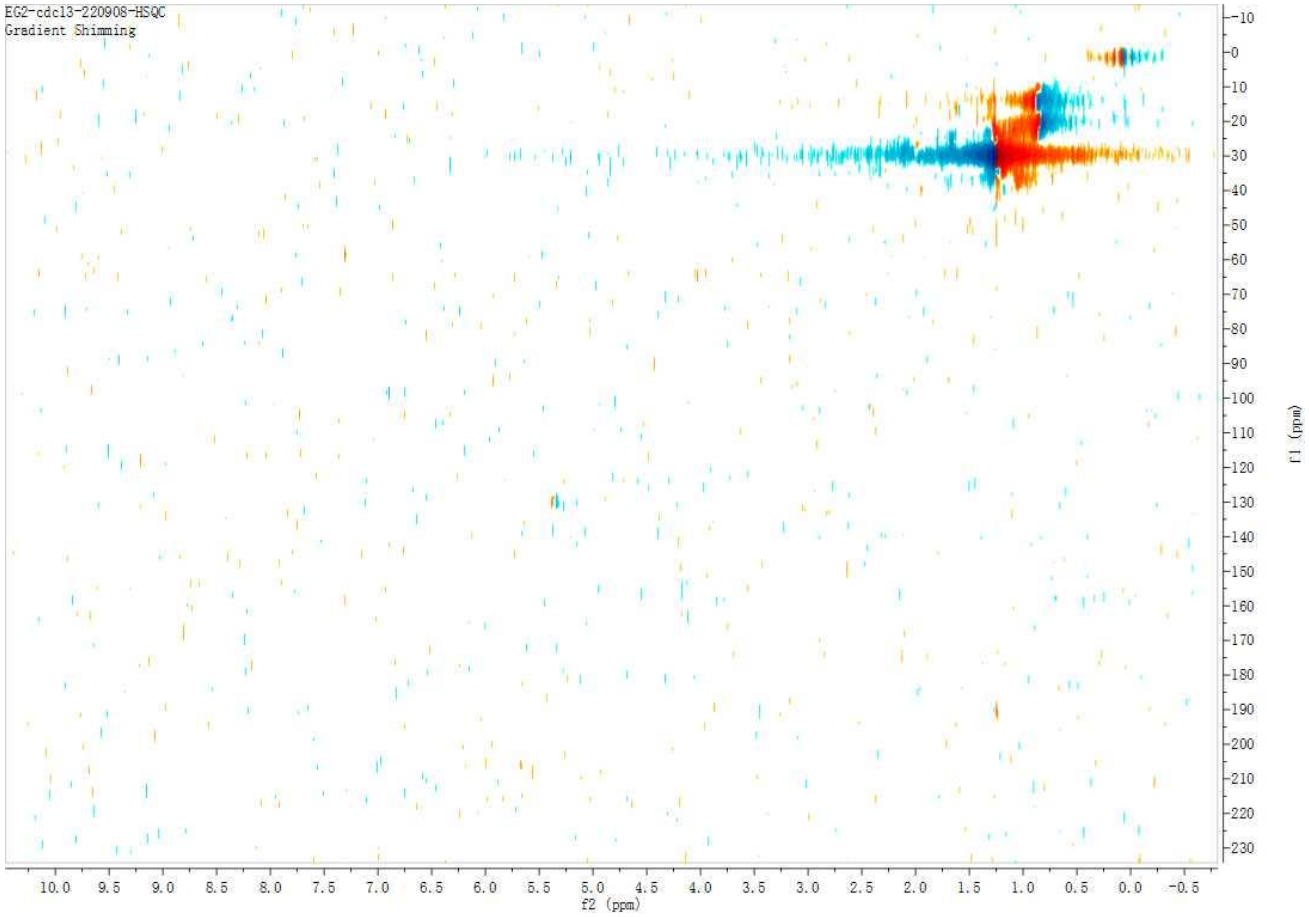

Fig. S6 HSQC spectra for AD-EG.

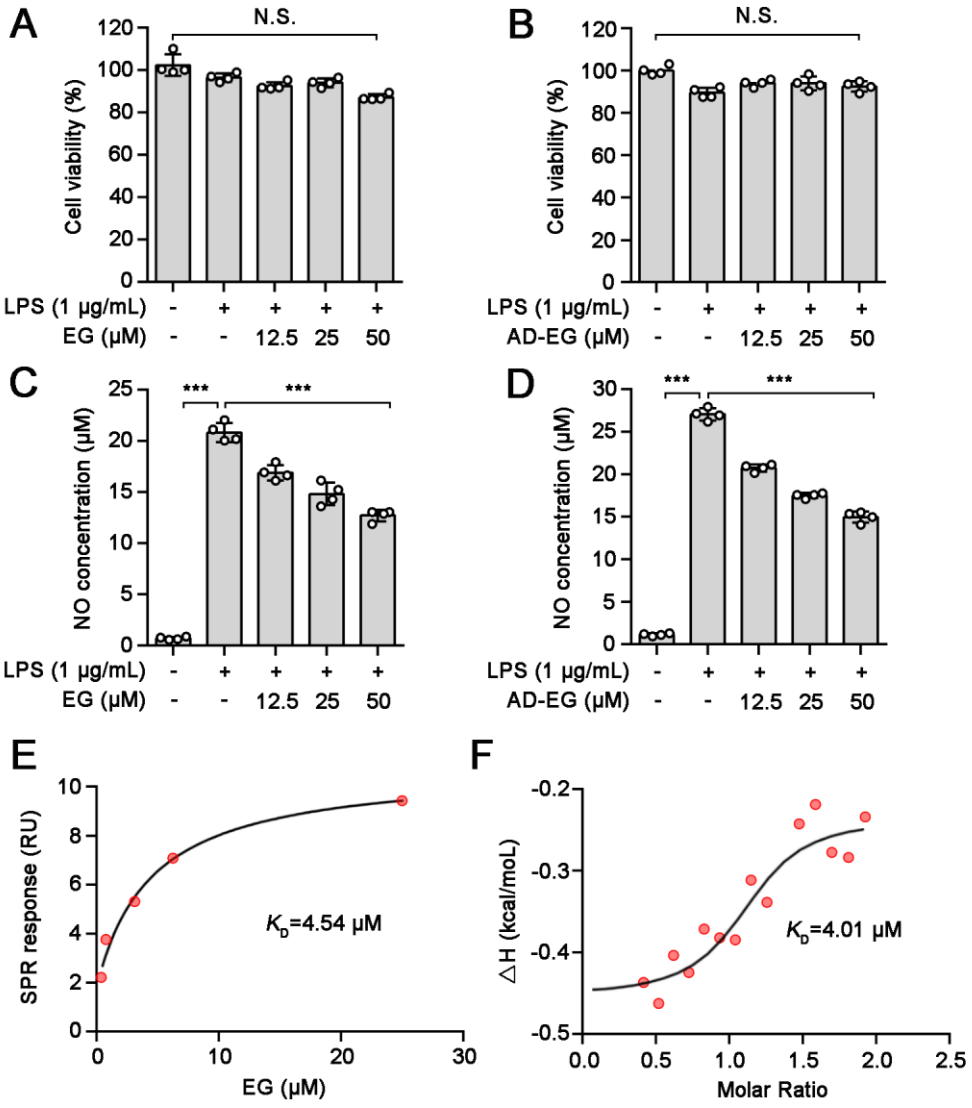

Fig. S7 PEBP1 serves as a cellular target of EG. (A-B) The low cytotoxicity of EG and AD-EG in RAW264.7 cells. (C-D) EG or AD-EG inhibited the level of NO production. (E) Binding affinity analysis of EG with PEBP1 determined by SPR. (F) Typical ITC binding isotherms for the EG interaction of PEBP1. Data are expressed as mean ± SEM for four individual experiments. ***P < 0.001, **P < 0.01, *P < 0.05.

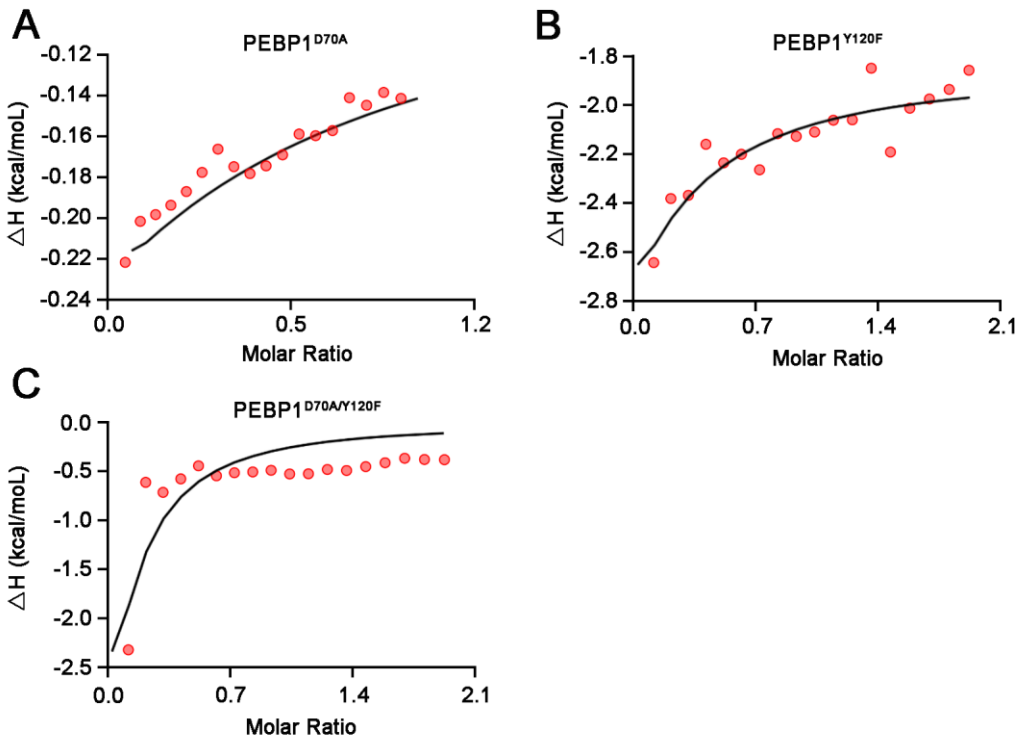

Fig. S8 ITC analysis for the binding of EG with PEBP1^{D70A} (A), PEBP1^{Y120F} (B) and PEBP1$^{D70A/Y120F}$ (C) mutants.

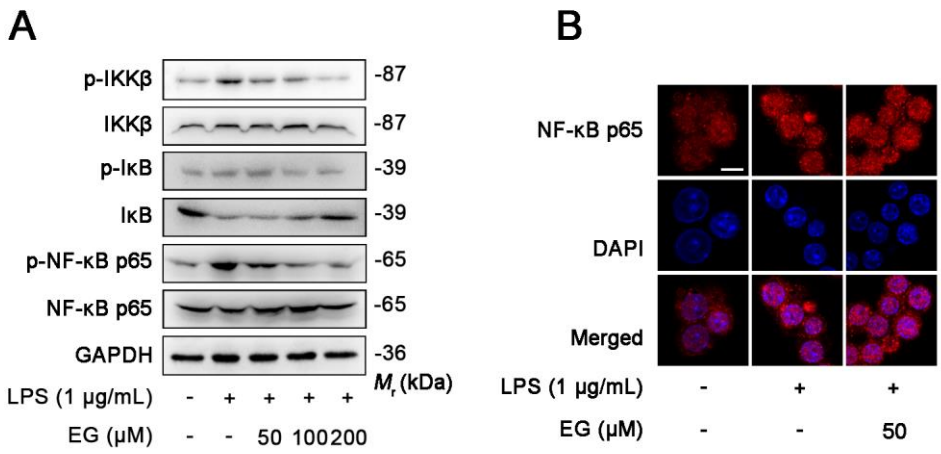

Fig. S9 EG inhibited NF-κB pathway activation. (A) Total and phosphorylation expressions of IKKβ, IκB-α and NF-κB p65 were determined by western blot assay. (B) The nuclear translocation of NF-κB p65 was detected by immunofluorescence assay. Representative images were displayed with NF-κB p65 (red) and nucleus (blue), scale bar = 20 μm.

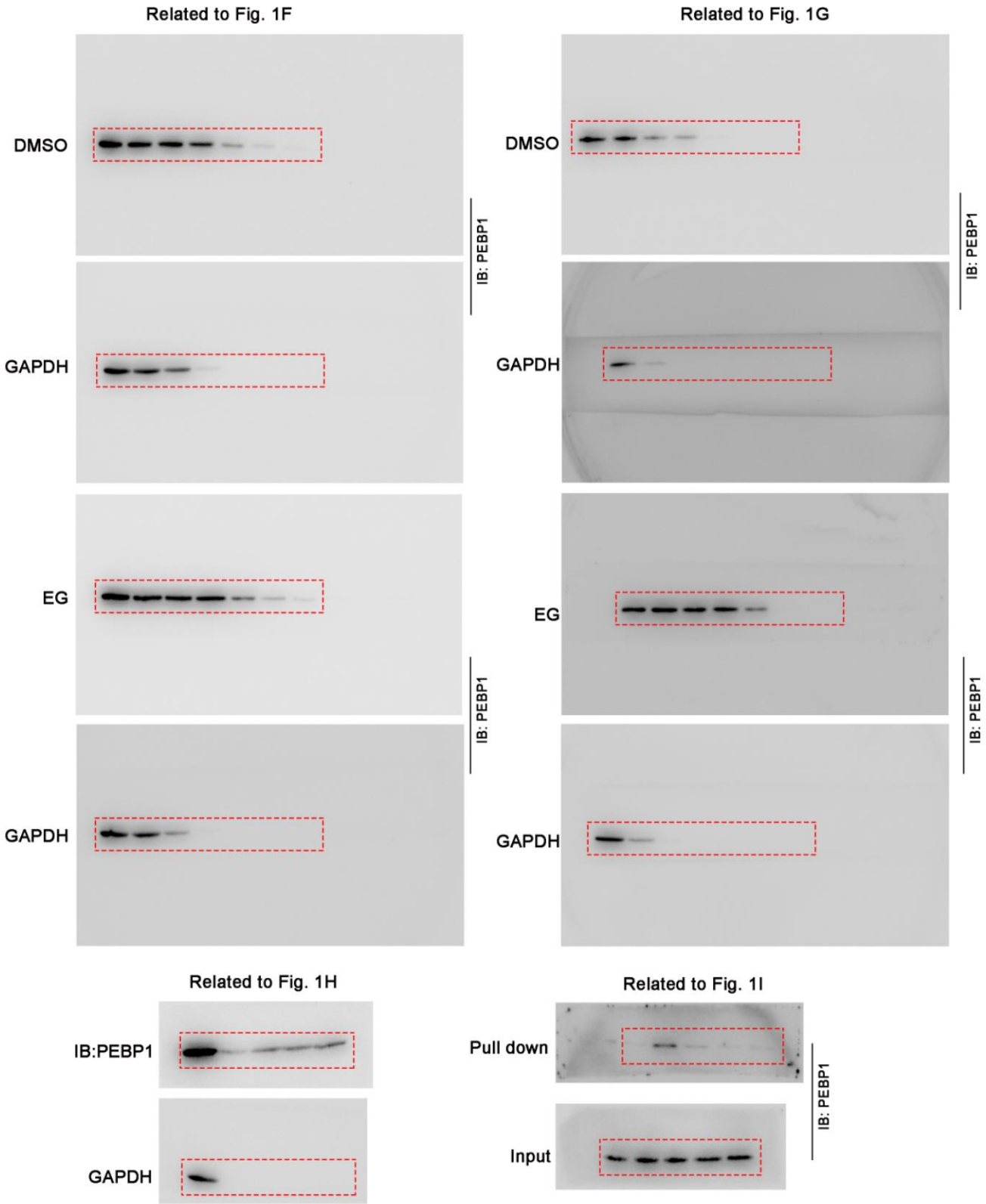

Fig. S10 Whole uncropped images of the original western blots.

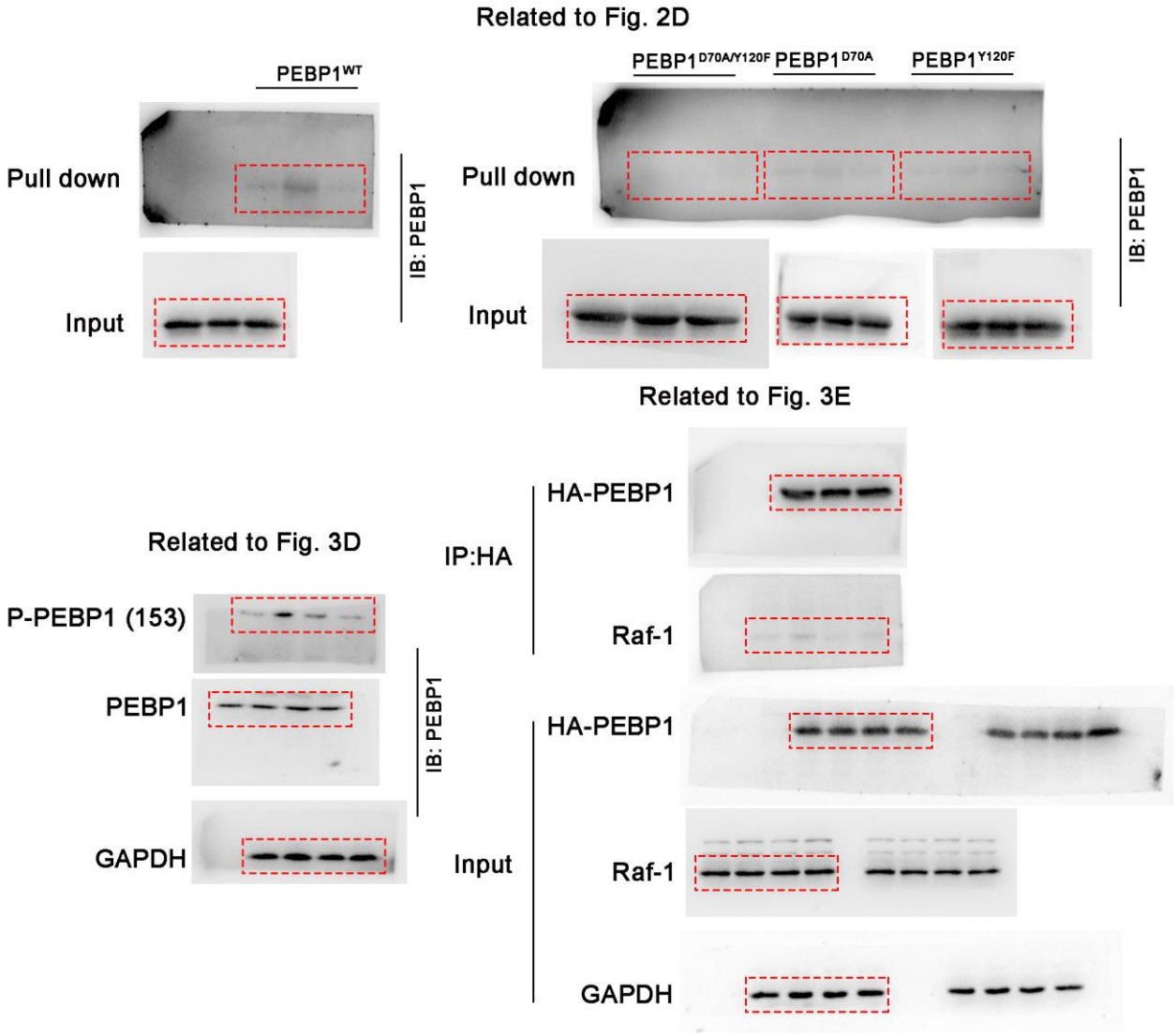

Fig. S11 Whole uncropped images of the original western blots.

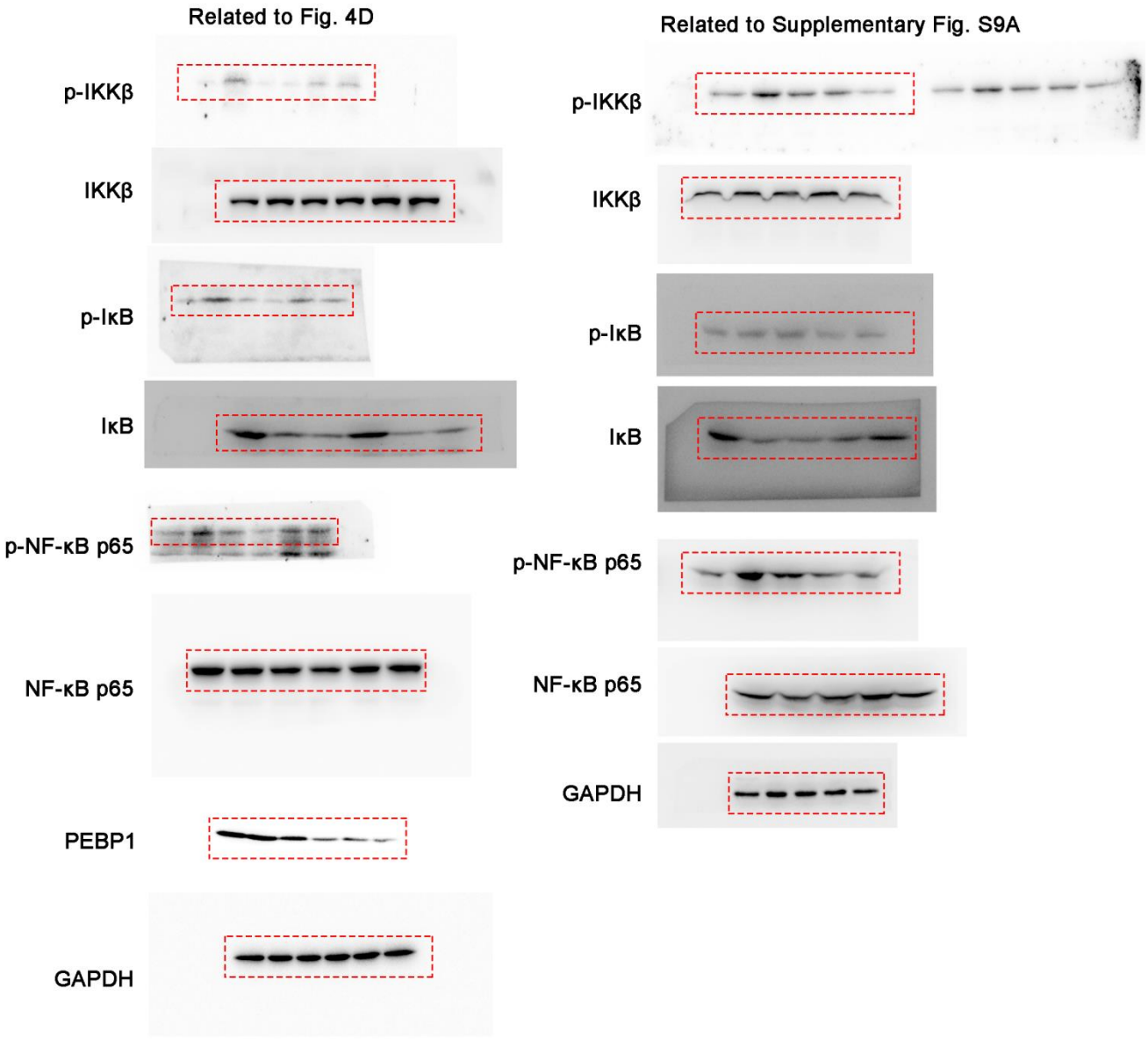

Fig. S12 Whole uncropped images of the original western blots.

III. Supplementary references

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