Supplementary Materials for

Targeting VPS35-ZNT1 Interaction by Engeletin Prevents Zinc Overload and Alleviates Myocardial Ischemia-Reperfusion Injury via Surface ZNT1 Stabilization

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This PDF file includes:

Materials and Methods
Figures. S1 to S5
Tables S1 to S4

Materials and Methods

Animals

Male C57BL/6 mice (8–10 weeks old; weight 21–23 g) were purchased from Beijing Sibeifu Laboratory Animal Center (Beijing, China). The mice were housed under standard specific pathogen free (SPF) conditions with a 12-h light/dark cycle, ambient temperature of 23 ± 3°C, and humidity of 30–70%, with free access to food and water. The experimental protocols were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by Animal Care and Use Committee (Zhongshan Hospital, Fudan University). Only male mice were used in this study to minimize potential hormonal variability associated with the estrous cycle in females. Mice were randomly assigned to experimental groups after facility labeling. All surgical procedures, including myocardial I/R and echocardiography, were performed by a single experienced operator who was blinded to group allocation. Similarly, data acquisition and analysis were conducted by investigators unaware of group assignments. No mice were excluded from the study except in cases of mortality.

Study design and MI/R model

Wild-type C57BL/6J male mice aged 8-10 weeks were subjected to sham-operated or myocardial ischemia reperfusion (I/R) according to the procedures described previously[1]. In brief, mice were anesthetized with isoflurane and underwent transient (45min) ligation of the left anterior descending artery (LAD) using a 6.0 silk suture, followed by reperfusion for 3 or 7 days. Sham-operated mice underwent identical procedures without LAD occlusion. Firstly, the heart was exposed by making a skin incision along the left sternal border and gently separating the intercostal muscles to enter the thoracic cavity between the 4th and 5th ribs. LAD was then ligated with a slipknot, which was released after 45 minutes. Engeletin (25 mg/kg, Cat# HY-N0436, MCE) or saline (vehicle) was administered intraperitoneally daily post-reperfusion.

Echocardiography

Transthoracic echocardiography was performed using a VeVo 2100 Imaging System (VisualSonics, Toronto, ON, Canada) to assess cardiac function in mice. Mice were anesthetized with isoflurane in a box and then positioned on a heated pad in a supine position. Heart rate was maintained at 450 to 600 bpm in all mice. Two-dimensional guided M-mode images were acquired in the left ventricular short and long axis planes. Key left ventricular parameters, including left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were measured from the M-mode tracings. Data are presented as the average of five consecutive cardiac cycles.

Flow Cytometry

The procedure for the flow cytometric analysis of hearts with or without myocardial I/R injury was in accordance with previous research[2]. Heart tissues were fully excised, weighted, minced and enzymatically digested in PBS buffer containing collagenase type II, DNase I, and elastase (all from Worthington Biochemical Corporation) for 30 minutes at 37 °C with gentle agitation according to the manufacturer's protocol. Single-cell suspensions were stained with fluorescently conjugated antibodies for 30 minutes at 4°C in the dark. The complete list of antibodies used is provided in Supplementary Table I. Immunophenotyped cells were then analyzed and sorted on a BD FACSAria II Cell Sorter (BD Biosciences). Data analysis was performed using FlowJo software (v.9.5.2).

The major immune cell populations were identified using the following sequential gating strategy, consistent with established protocols[2, 3]:

Neutrophils $CD45^+$ $CD11b^+$ $Ly6G^+$

Monocytes $CD45^+$ $CD11b^+$ $Ly6G^-$ $F4/80^-$ $Ly6C^+$

Macrophages $CD45^+$ $CD11b^+$ $Ly6G^-$ $F4/80^+$ $Ly6C^-$

M1 Macrophages ($CD45^+CD11b^+F4/80^+$) $CD206^-$

M2 Macrophages ($CD45^+CD11b^+F4/80^+$) $CD206^+$

Infarct Size Measurement

To quantify myocardial infarction following I/R injury, hearts were excised 24 hours after reperfusion. The detailed protocols for 2,3,5-triphenyltetrazolium chloride (TTC, Cat# E2129, Sigma) staining were as described previously with minor revision[1]. The left anterior descending (LAD) coronary artery was re-occluded at the original site, and the heart was retrogradely perfused via the aorta with 1% TTC in phosphate buffer (pH 7.4) at 37°C for 15 minutes. The heart was then frozen in -80℃ and sectioned transversely into 5–7 slices (~2 mm thick) from apex to base. These pieces were incubated with TTC (1%, Cat# T8877, Sigma) at 37℃ for 10-15 minutes Both sides of each slice were photographed with a digital camera. The images were analyzed in a blinded manner using ImageProPlus. The following areas were manually traced and calculated: Area at Risk (AAR): The non-TTC-stained region (pale/white) plus the TTC-stained region (brick-red); Infarct Area (IA): The pale/white.

Masson's Trichrome Staining

Paraffin-embedded heart sections obtained 7 days post-I/R were used to evaluate interstitial fibrosis and scar formation. Sections were deparaffinized, rehydrated, and stained using a standard Masson's trichrome protocol. Stained sections were scanned using a bright-field slide scanner, and the extent of fibrosis (percentage of blue-stained collagen area) within the infarct border zone and remote myocardium was quantified from at least 3 sections per heart using ImageJ software with color deconvolution plugins.

Hematoxylin and Eosin (H&E) Staining

Paraffin sections (5μm) from hearts harvested 3 days post-reperfusion were stained with H&E following standard protocols. Histopathological evaluation was performed by a blinded observer under a light microscope. Myocardial injury was scored based on the degree of myofibrillar loss, vacuolization, and necrosis. Inflammatory cell infiltration was quantified by counting the number of nucleated leukocytes per high-power field (HPF, 400× magnification) in at least 5 random fields within the infarct and peri-infarct regions.

Immunofluorescence

Immunofluorescence (IF) was performed on both murine cardiac tissues and BMDMs. Murine heart sections from sham-operated or myocardial I/R models and BMDMs were paraffin embedded, followed by multiple staining steps. Sections were blocked at room temperature for 1 hour with a solution of 1% bovine serum albumin (BSA) in 1× PBS to minimize non-specific antibody binding. Sections were incubated with specific primary antibodies (see list below) diluted in blocking buffer at 4°C overnight. After three 5-minute washes with 1× PBS, sections were incubated with appropriate fluorophore-conjugated secondary antibodies (Supplementary Table II) for 1 hour at room temperature in the dark. Following PBS washes, nuclei were counterstained with DAPI.

TUNEL (TdT-mediated dUTP nick end labeling) assay

To evaluate apoptosis, paraffin-embedded heart tissue sections were processed for TUNEL staining using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Following the TUNEL reaction, nuclei were counterstained with DAPI (Beyotime, Shanghai, China). TUNEL-positive cells were visualized under a fluorescence microscope (Zeiss, Jena, Germany). The apoptotic index was

quantified as the percentage of TUNEL-positive nuclei relative to the total number of DAPI-positive nuclei.

Isolation and Culture of Primary Adult Mouse Cardiomyocytes

Primary adult ventricular cardiomyocytes were isolated from 6–8-week-old male C57BL/6J mice using a modified Langendorff-free enzymatic perfusion method, as previously described with minor adaptations[1]. Mice were deeply anesthetized via intraperitoneal injection of 1% Pentobarbital sodium. The thoracic cavity was opened under aseptic conditions, and the heart was rapidly exposed. To arrest the heart in diastole and flush blood components, 7 mL of ice-cold EDTA buffer was slowly injected into the inferior vena cava or right ventricle. The heart was retrogradely perfused with 10 mL of EDTA buffer, followed by 3 mL perfusion buffer. The heart was transferred to a digestion apparatus where it was perfused in a recirculating manner with 20–40 mL of enzyme digestion buffer containing 1.5 mg/mL Collagenase Type II and 0.1 mg/mL Protease at 37°C for 8–12 minutes until the tissue became soft and swollen. The ventricles were separated, minced into small pieces with fine forceps in a dish containing stop buffer (perfusion buffer supplemented with 10% fetal bovine serum, FBS) to terminate enzymatic activity. The cell suspension was gently filtered through a 100-μm nylon mesh. Cardiomyocytes were allowed to sediment by gravity, and the calcium concentration was gradually restored to physiological levels by sequential incubation in three intermediate buffers with increasing calcium concentrations each for 5–10 minutes. The final pellet of calcium-tolerant rod-shaped cardiomyocytes was resuspended in primary cardiomyocyte culture medium. Cells were plated on culture dishes pre-coated with laminin and maintained in a humidified incubator at 37°C with 5% CO_2. After 1–2 hours of adhesion, the medium was replaced to remove unattached cells.

Generation and stimulation of bone marrow-derived macrophages (BMDMs)

Bone marrow-derived macrophages (BMDMs) were generated from wild-type (WT) mice according to a previously established protocol[2]. Briefly, after being anesthetized, mice were sterilized and the femurs and tibias were isolated and aseptically flushed from the femurs and tibiae with sterile PBS. A 70-μm filter was used to obtain single cell suspension. The harvested cells were plated in non-tissue culture-treated petri dishes and cultured in BMDM differentiation medium, consisting of RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and 50 ng/mL recombinant murine macrophage colony-stimulating factor (Cat# 50315-02-50UG, PeproTech). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. Additional DMEM containing M-CSF was added at day 3 and 5. After 7 days of differentiation, mature BMDMs were obtained. For polarization experiments, mature BMDMs (day 7) were stimulated with a combination of lipopolysaccharide (LPS, 100 ng/mL, Cat# L2880, Sigma, USA) and interferon-gamma (IFN-γ, 20 ng/mL, Cat# RP01070, Abclonal, China) for 24 h to induce a pro-inflammatory (M1-like) activation state. At the indicated time points post-stimulation, cells were harvested for total RNA extraction and subsequent downstream analyses.

Cell Culture and Transfection

HEK293T and RAW264.7 cell lines were obtained from the Stem Cell Bank, Chinese Academy of Sciences (CAS). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2. The isolation and culture of primary mouse bone marrow-derived macrophages (BMDMs) are described in the previous section.

For transient overexpression, plasmids were transfected into HEK293T with Hieff Trans Liposomal Transfection Reagent (Cat# 40802ES03, Yeason, Shanghai, China) according to the manufacturer's protocol. For gene manipulation in BMDMs and RAW264.7 cells, cells were transduced with siRNAs targeting *Vps35* and *Slc30a1*

by Hieff Trans® LipoBooster 3000 Transfection Reagent Lipo3000 (Cat# 40801ES01, Yeason, Shanghai, China) or electroporation.

Vps35: sense (5'-CUACGUCGAUAAAGUUCUA-3'), antisense (5'- UAGAACUUUAUCGACGUAG-3')

Slc30a1: sense (5'- GGAUUGUUAUGUACUAUAAAU-3'), antisense (5'- UUAUAGUACAUAACAAUCCAU-3')

Isolation of Plasma Membrane and Cytosolic Fractions

To biochemically assess the subcellular distribution of proteins, membrane and cytosolic fractions were separated using Minute™ Plasma Membrane Protein Isolation Kit (Cat# SM-005, Invent Biotechnology) according to the manufacturer's instructions. Cells were harvested and washed once with ice-cold PBS, followed by centrifugation at 500 × g for 5 minutes at 4°C. The cell pellet was then thoroughly resuspended in 200–500 μL of ice-cold Buffer A and incubated on ice for 5–10 minutes to allow hypotonic swelling. After vigorous vortexing for 10–30 seconds, the lysate was immediately transferred to a pre-chilled proprietary filter cartridge and centrifuged at 16,000 × g for 30 seconds at 4°C. The filtrate was collected, vigorously vortexed for 10 seconds, and centrifuged at 700 × g for 1 minute at 4°C to pellet the nuclear fraction. The supernatant was carefully transferred to a new tube and centrifuged at 16,000 × g for 10–30 minutes at 4°C to separate the cytosolic fraction (supernatant) from the crude total membrane fraction (pellet). The cytosolic supernatant was retained for further analysis. The crude membrane pellet was resuspended in 200 μL of Buffer B and centrifuged at 7800 × g for 5 minutes at 4°C. The resulting final pellet, representing the enriched plasma membrane fraction, was solubilized in an appropriate lysis buffer (e.g., RIPA buffer) for downstream applications such as western blot analysis.

Measurement of Intracellular Reactive Oxygen Species (ROS) Generation

Intracellular ROS levels in cultured BMDMs and 293T cells were quantified using the fluorogenic probe 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) via a commercial Intracellular ROS Assay Kit (Cat# S1105S, Beyotime, Shanghai, China), following the manufacturer's protocol. Briefly, following experimental treatments, cells were gently washed once with pre-warmed phosphate-buffered saline (PBS) or serum-free culture medium. They were then incubated with 10 μM DCFH-DA in a dark, humidified incubator at 37°C for 20 minutes. After incubation, cells were washed twice with PBS to remove excess probe. Fluorescence images were immediately acquired using flow cytometry.

Measurement of Intracellular GSSG/GSH

Cells were harvested and lysed in 100–200 μL of ice-cold lysis buffer followed by centrifugation. The supernatant was collected for further experiments. For Total GSH Measurement, working solution was prepared by combining 50 μL of sample/standard, 100 μL of DTNB solution, and 50 μL of NADPH solution in a 96-well plate. Incubate the plate at room temperature (or 25°C) for 5–15 minutes and measure the absorbance at 412 nm (A412) using a microplate reader. For GSSG Measurement, pre-treat an aliquot of the sample supernatant with an appropriate volume of the provided GSH scavenger (e.g., 2 μL per 100 μL sample). Incubate at room temperature for 60 minutes to derivative all free GSH. Follow the same detection steps as for Total GSH using the pre-treated sample. Calculate the total GSH or GSSG concentration in the sample (μM) from the standard curve and determine key parameters according to the following formula: Free GSH = Total GSH – (2 × GSSG), GSH/GSSG Ratio = Free GSH / GSSG.

Detection of Labile Zinc with TSQ Staining

Intracellular labile zinc ions were detected using the membrane-permeant, zinc-specific fluorophore N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ, Cat# HY-119287, MCE, China). Cells were loaded with 10 μM TSQ diluted in serum-free culture medium and incubated in a humidified incubator at 37°C for 30 minutes in the dark. After incubation, cells were washed three times with warm PBS to remove excess dye. Fluorescence imaging was performed immediately using flow cytometry.

Co-immunoprecipitation (Co-IP)

Cells were lysed on ice for 30 minutes in NP-40 lysis buffer supplemented with fresh protease and phosphatase inhibitor cocktails. The lysate was clarified by centrifugation at 12000g for 15 minutes at 4°C. The supernatant was collected, and a small aliquot (typically 5% of the total volume) was saved as the "Input" control. The remaining lysate was pre-cleared by incubation with 20–50 μL of protein A/G magnetic beads for 1 hour at 4°C with gentle rotation to reduce non-specific binding. After brief centrifugation, the pre-cleared supernatant was transferred to a new tube. For each immunoprecipitation (IP) reaction, 20–50 μL of protein A/G bead slurry was washed three times with 1 mL of ice-cold lysis buffer. The washed beads were then incubated with 1–5 μg of the target-specific primary antibody (or an equivalent amount of isotype control IgG) in 500 μL of lysis buffer for 16–18 hours (overnight) at 4°C with rotation to facilitate antibody coupling. Following incubation, the antibody-bound beads were washed three times with lysis buffer to remove unbound antibodies. The pre-cleared cell lysate was then added to the prepared antibody-bead complexes and incubated overnight at 4°C with gentle rotation to allow immunoprecipitation of the target protein and its interacting partners. The next day, the beads were collected by brief centrifugation and subjected to a series of stringent washes (e.g., with lysis buffer, high-salt buffer, and low-detergent buffer) to remove non-specifically bound proteins. After the final wash, the beads were briefly centrifuged, and all supernatant was carefully aspirated. Finally, he beads were briefly centrifuged, and all supernatant was carefully removed. The immunoprecipitated proteins were eluted from the beads for western blot or mass spectrometry (IP-MS) analysis.

Western Blot

Cells or tissue samples were lysed on ice using RIPA lysis buffer supplemented with 1×protease and phosphatase inhibitor cocktail. The lysates were clarified by centrifugation at 12,000 × g for 15 minutes at 4°C. The protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) assay according to the manufacturer's instructions. Equal amounts of protein (typically 20–40 μg) were mixed with 5×SDS-PAGE loading buffer, denatured at 95°C for 10 minutes, and then separated by electrophoresis on 10–15% SDS-polyacrylamide gels. The separated proteins were subsequently transferred onto polyvinylidene difluoride (PVDF) membranes using a wet or semi-dry transfer system. The membranes were blocked with 5% (w/v) bovine serum albumin (BSA) or non-fat milk dissolved in TBST (Tris-buffered saline with 1% Tween-20) for 1 hour at room temperature to prevent non-specific binding. Following blocking, the membranes were incubated with specific primary antibodies diluted in blocking buffer at 4°C overnight. The details of all primary antibodies used (including sources, catalog numbers, and dilutions) are provided in Supplementary Table III. After extensive washing with TBST (3 × 10 minutes), the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Immunoreactive bands were visualized using immobilon western chemiluminescent HRP substrate (Cat# WBLUF0500, Millipore, Billerica, MA).

RNA extraction and quantitative RT-PCR

Total RNA was isolated from cultured BMDMs or RAW264.7 cells using TRIzol (Cat#15596026, Thermo Fisher Scientific). Reverse Transcription Kit (Cat#RK20433, ABclonal) was used for synthesis of cDNA from mRNA, according to manufacturer's instructions. The resulting cDNA fragments were amplified in a real-time quantitative PCR machine (Applied Biosystems) using SYBR Green PCR kit (Cat#RK21203, ABclonal). The

mRNA level of each target gene was normalized to endogenous *18S* expression and calculated as relative mRNA expression or fold change using the ΔΔCT method. Primer sequences for genes of interest are provided in Supplemenrary Table IV.

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-1β, IL-6 and TNF-α in the supernatant were quantified using commercial sandwich ELISA kits according to the manufacturers' protocols. Specifically: Mouse IL-1β ELISA Kit (ABclonal, Cat# **RK00006),** Mouse IL-6 ELISA Kit (ABclonal, Cat# **RK04845),** Mouse TNF-α ELISA Kit (ABclonal, Cat# **RK04595).** Briefly, standards and appropriately diluted cell lysate samples were added to antibody-precoated 96-well plates and incubated. Following a series of incubation and washing steps to capture the target protein and bind a detection antibody, a horseradish peroxidase (HRP)-conjugated streptavidin solution was added. After final washes, tetramethylbenzidine (TMB) substrate was added for color development. The enzymatic reaction was stopped by adding the provided stop solution. The absorbance of each well was immediately measured at 450 nm. A standard curve was generated by plotting the mean absorbance of the standards against their known concentrations. The concentration of RSPO in each sample was interpolated from this curve.

RNA Sequencing

RNA samples were sequenced and analyzed by Illumina. Briefly, total RNA was extracted from BMDMs pretreated with DMSO or ENG (n=3 in each group) and sequencing libraries were constructed. Bioinformatic analysis was performed as follows: Raw reads were quality-trimmed and aligned to the mouse reference genome. Gene-level counts were quantified and differential gene expression analysis was conducted using DEGseq in R, with genes satisfying FoldChange≥1.5 and an p-value < 0.05 defined as significantly differentially expressed. For functional exploration, all expressed genes were subjected to K-means clustering based on Euclidean distance, which partitioned them into 9 distinct co-expression clusters. Pathway and Gene Ontology (GO) enrichment analyses were subsequently performed on each cluster using the clusterProfiler R package.

Metabolomic Profiling

Polar metabolites were extracted from cell pellets using a pre-chilled solution of 80% methanol/water (v/v). After vortexing and centrifugation, the supernatants were dried under vacuum and reconstituted for analysis. Metabolite separation was achieved and the eluted metabolites were detected in both positive and negative electrospray ionization modes. Data analysis for metabolomic projects generated by LC-MS/MS system (Q Exactive series Orbitrap mass spectrometer, Thermo Fisher Scientific, USA) is structured into a three-tiered pipeline to ensure comprehensive biological interpretation. Primary (Foundation) Data Analysis focuses on processing the raw qualitative and quantitative metabolomic results. This stage involves univariate statistical analysis (e.g., Student's t-test, fold-change calculation) and multivariate analysis (MVA), including Principal Component Analysis (PCA) and (Orthogonal) Partial Least Squares Discriminant Analysis [(O)PLS-DA]. The objective is to rigorously identify and filter metabolites that exhibit statistically significant alterations between experimental conditions. Advanced (Biological) Data Analysis builds upon the identified differential metabolites. This phase employs a suite of bioinformatics tools for functional annotation and biological insight extraction. Key activities include pathway enrichment analysis (using databases such as KEGG or MetaboAnalyst), metabolic network visualization, and the integration of metabolite changes into broader biological contexts to generate testable hypotheses. Customized Data Analysis is offered to address specific research objectives. This flexible, client-driven tier may involve integrated multi-omics correlation analyses (e.g., linking metabolomic with transcriptomic data), custom statistical modeling, or the design and generation of publication-ready figures and graphical abstracts tailored to the project's narrative. Identification of significantly altered metabolites (Variable Importance in the Projection, VIP>1; P-value<0.05) was performed according to the criteria: VIP

(Variable Importance in the Projection)>1; P-value<0.05.

Limited Proteolysis Mass Spectrometry (LiP-MS)

Chemical proteomics by LiP-MS approach was in accordance with previous studies[4]. In brief, the cell pellet was subjected to at least three cycles of freeze-thaw by alternating between immersion in liquid nitrogen and rapid thawing in a 37°C water bath. Following lysis, an appropriate volume of ice-cold lysis buffer was added to the sample, which was then vortexed thoroughly. The lysate was centrifuged at 20,000 × g for 10 minutes at 4°C, and the resulting supernatant was carefully collected. Protein concentration was determined using a bicinchoninic acid (BCA) assay according to the manufacturer's protocol. For the limited proteolysis assay, 100 µg of protein was incubated with ENG or DMSO at room temperature for 10 minutes. Subsequently, Proteinase K was added to all samples at a mass ratio of 1:100 (enzyme to substrate). The limited proteolysis was allowed to proceed at room temperature for 5 minutes. For tryptic digestion, the protein solution was first reduced with Tris(2-carboxyethyl)phosphine (TCEP) at a final concentration of 40 mM and alkylated with iodoacetamide (IAA) at 100 mM by incubation in a boiling water bath for 5 minutes. Subsequently, an appropriate volume of hydroxypropyl-β-cyclodextrin (HP-β-CD) solution (250 mM) and 100 µL of 100 mM ammonium bicarbonate were added to each sample. Proteolytic digestion was performed by adding trypsin at an enzyme-to-substrate ratio of 1:50 (w/w) and incubating overnight at 37°C. The digestion reaction was terminated by acidifying the samples with trifluoroacetic acid (TFA) to pH below 2. Each sample was then subjected to nanoflow liquid chromatography separation using Easy nLC 1200 system (Thermo Fisher Scientific). The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 80% acetonitrile). Samples were loaded onto an analytical column (Acclaim PepMap RSLC, 50 µm × 15 cm, nano viper; Thermo Fisher Scientific; P/N 164943) equilibrated with 100% solvent A. The eluted peptides were analyzed online using a Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) operated in positive ion mode with a total analysis time of 120 minutes. Full MS scans (m/z 350–1800) were acquired at a resolution of 60,000 with an AGC target of 1e6 and a maximum injection time (IT) of 50 ms. The top 10 most intense precursor ions from each full scan were selected for higher-energy collisional dissociation (HCD) fragmentation with an isolation window of 2 m/z. MS/MS scans were acquired at a resolution of 15,000 with an AGC target of 2e5, a minimum AGC target of 2e3, a maximum IT of 60 ms, and a normalized collision energy of 28 eV. Only precursors with charge states of 2–5 were selected for fragmentation, and a dynamic exclusion duration of 30 seconds was applied. The significance of inter-group differences and FoldChange for peptides was calculated using the limma package (V3.58.1) in R (V4.3.1). Peptides meeting both of the following criteria were defined as significantly differentially expressed: 1. A fold change ≥ 1.5 (up or down regulated); 2. A p-value < 0.05 as determined by Student's t-test.

Surface Plasmon Resonance (SPR) Binding Analysis

A research-grade CM5 sensor chip was preconditioned according to the manufacturer's protocol. Using standard amine-coupling chemistry, recombinant VPS35 protein was diluted in sodium acetate buffer and immobilized on the surface of a flow cell. A reference flow cell was activated and deactivated without ligand to serve as a blank for background subtraction. Two-fold serial dilutions of ENG in running buffer were prepared. Samples were injected sequentially over the VPS35-coated and reference surfaces at a constant flow rate. The sensor surface was regenerated between cycles with a brief pulse of mild regeneration buffer to remove any residual bound analyte. After each sample cycle, the sensor surface was regenerated by a 60-second pulse of 10 mM glycine-HCl (pH 2.0) to completely remove any bound analyte, restoring the baseline before the next injection. Sensograms were processed by double-referencing (subtracting both the reference flow cell and a buffer blank injection). The resulting binding curves were globally fitted to a 1:1 Langmuir binding model using the Biacore T200 Evaluation Software (v.X.X, Cytiva). The software calculated the kinetic rate constants for association (K_a)

and dissociation (K_d), from which the equilibrium dissociation constant (K_D) was derived.

Cellular Thermal Shift Assay (CETSA)

BMDMs or 293T cells transfected with either wild-type or mutant VPS35 constructs were harvested and resuspended in PBS supplemented with protease and phosphatase inhibitors. Aliquots of the cell suspension were subjected to a gradient of heat challenges (ranging from 37°C to 62°C) for 5 minutes in a thermal cycler, followed by immediate cooling on ice. The heat-treated cells were then lysed by at least three cycles of freeze-thaw in liquid nitrogen, and the lysates were clarified by centrifugation at 20,000 × g for 20 minutes at 4°C to separate the soluble (non-aggregated) protein fraction. The supernatants were collected, and protein concentrations were determined by BCA assay. Equal amounts of protein from each temperature point were resolved by SDS-PAGE and immunoblotted.

Molecular Dynamics (MD) Simulations

All-atom, explicit-solvent molecular dynamics simulations were performed using GROMACS (version 2024.05). The system was constructed with the AMBER99SB-ildn force field and solvated in a TIP3P explicit water model. The simulation temperature was maintained at 310 K using the V-rescale thermostat, and pressure was kept at 1 bar using the Berendsen barostat during equilibration. Prior to production runs, the system underwent energy minimization for 50,000 steps using the steepest descent algorithm to relieve steric clashes. This was followed by system equilibration in two phases: a 100-ps simulation in the NVT ensemble, and a 100-ps simulation in the NPT ensemble (1 bar), each comprising 50,000 steps. The production MD simulation was carried out for 100 nanoseconds with no positional restraints. A time step of 2 fs was used, with the Verlet cutoff scheme updating the neighbor list every 10 steps and a cutoff distance of 1.2 nm. Trajectory data were saved every 10 ps. The binding free energy and per-residue energy decomposition for the protein-ligand complex were calculated using the gmx_MMPBSA tool based on frames extracted from the stable trajectory.

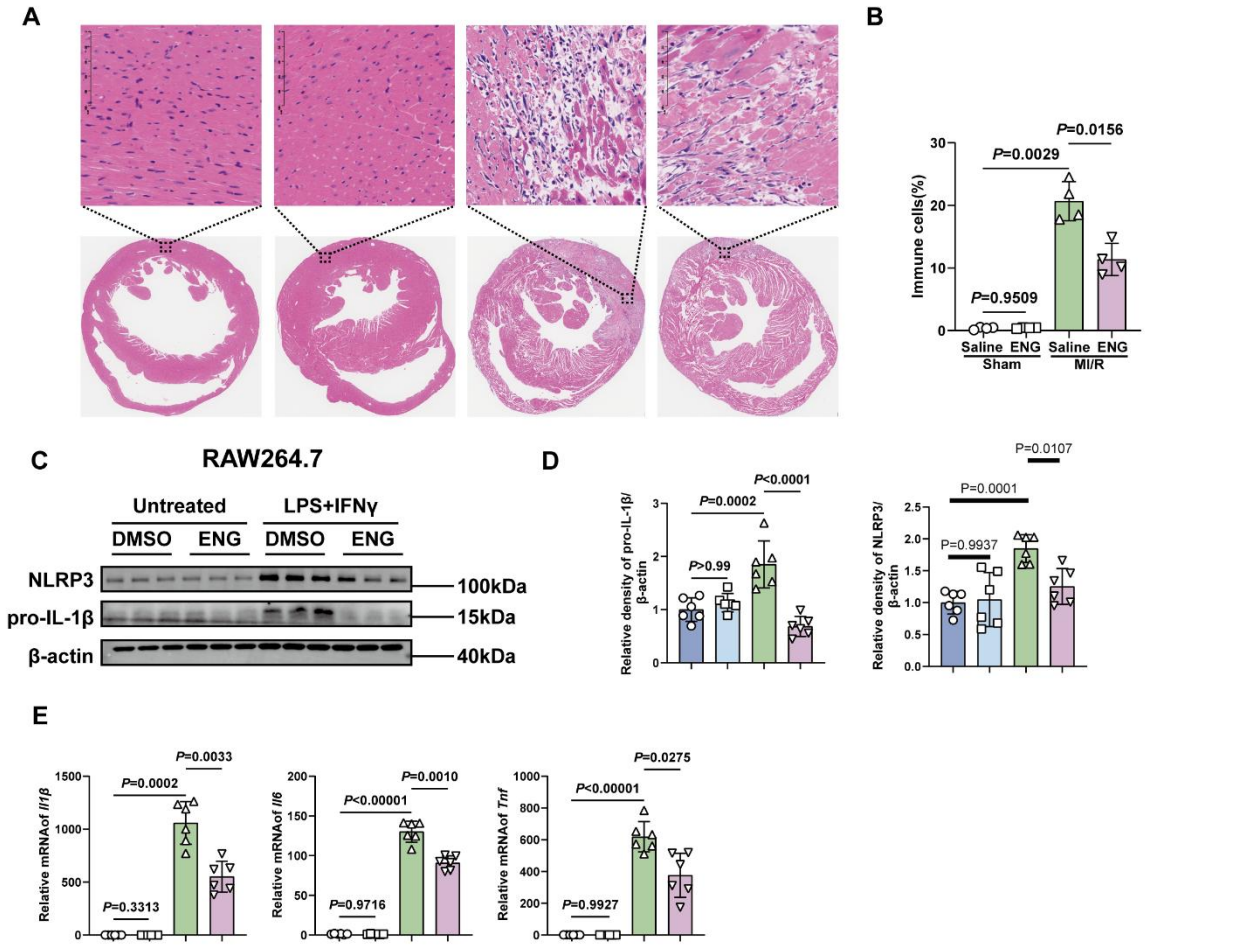
Molecular Docking

The three-dimensional structure of the VPS35 protein was obtained from the Protein Data Bank (PDB ID: 8ttc). The 3D structure of the ligand (Engeletin) was retrieved from the PubChem database (CID: 5281811). Both structures were prepared and optimized for docking (e.g., adding hydrogen atoms, assigning charges). Molecular docking was performed using AutoDock Vina. The search space for docking was defined as a box centered on the specific amino acid region of interest within the VPS35 binding pocket, as requested. The resulting docking poses were visualized and analyzed using Discovery Studio Visualizer.

Statistical Analysis

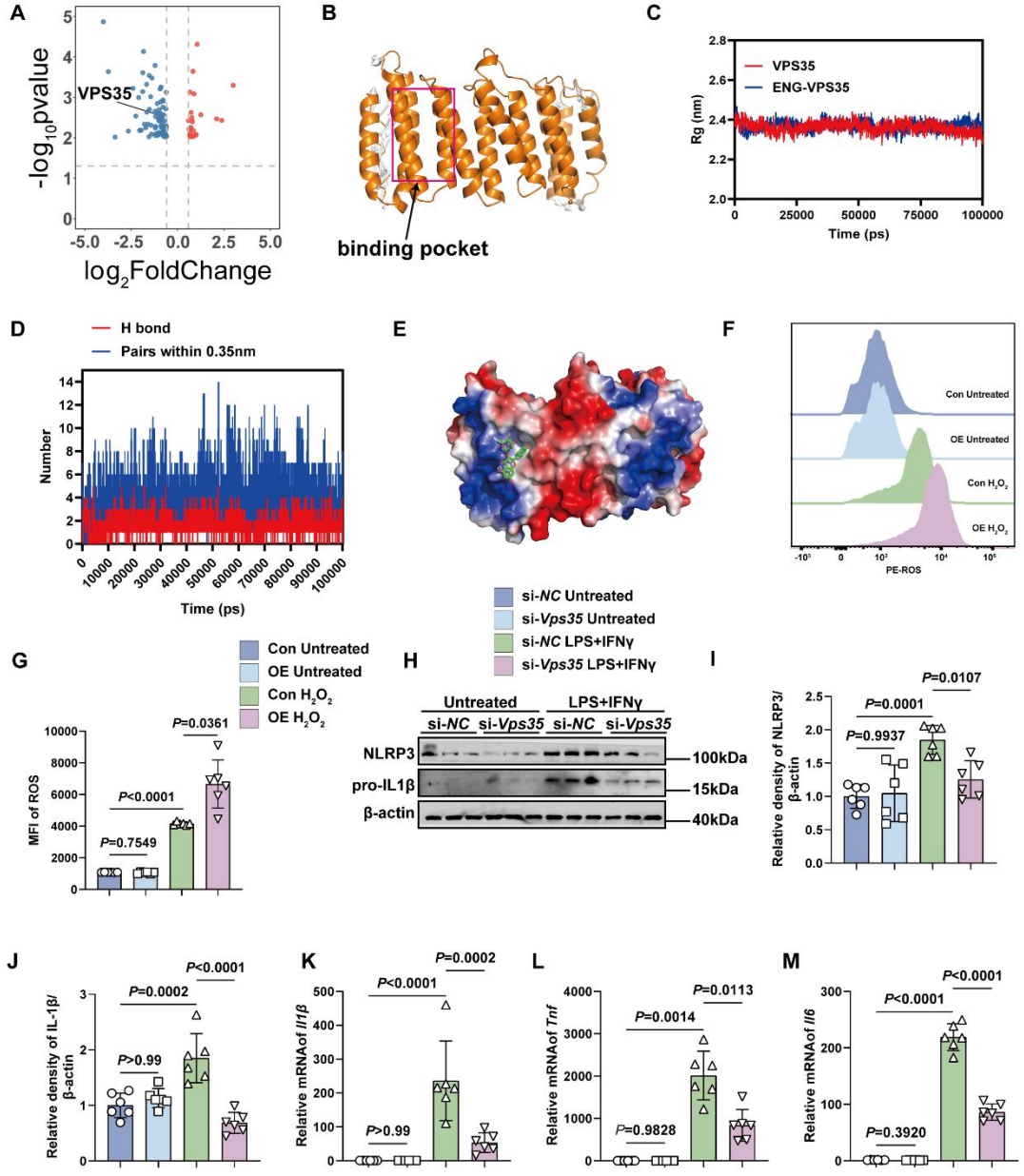
Statistical analyses were performed using GraphPad Prism software (version 8.0.2). Prior to comparative analysis, all datasets were assessed for normality using the Shapiro-Wilk test and for homogeneity of variances using Brown-Forsythe test. Based on these assessments, the appropriate parametric or non-parametric tests were selected. For comparisons between two groups meeting both assumptions, unpaired 2-tailed Student's t-test was used. For comparisons among three or more groups, data conforming to assumptions were analyzed by one-way ANOVA followed by an appropriate post hoc test (e.g., Bonferroni's test). If the assumptions of normality was violated, the non-parametric Kruskal-Wallis test was used, followed by Dunn's multiple comparisons test. If the equal variance was violated, Welch ANOVA test was used, followed by Games-Howell multiple comparison test. Specific tests used for each experiment are detailed in the corresponding figure legends. $p < 0.05$ was considered significant. Quantitative data are presented as the mean ± SD.

Figure. S1.



S1 Engeletin alleviated myocardial inflammation post MI/R. A-B Infiltration of leukocytes was assessed by staining with hematoxylin–eosin (H&E). n=4 per group. C-D Expression of NLRP3, pro-IL1β and β-actin in RAW264.7 cells treated with DMSO or engeletin and quantified data of immunoblotting band intensity in RAW264.7 cells. n=6 per group. E qPCR detection of mRNA expression levels of *Il6*, *Il1β* and *Tnf* in RAW264.7 cells treated with DMSO or engeletin. $n = 6$ per group. Data were analyzed using Welch ANOVA analysis followed by Games-Howell multiple comparison test (B, NLRP3 in D and *Il1β, Tnf* in E) and One-way ANOVA analysis followed by Bonferroni's multiple comparison test (pro-IL1β in D and *Il6* in E).

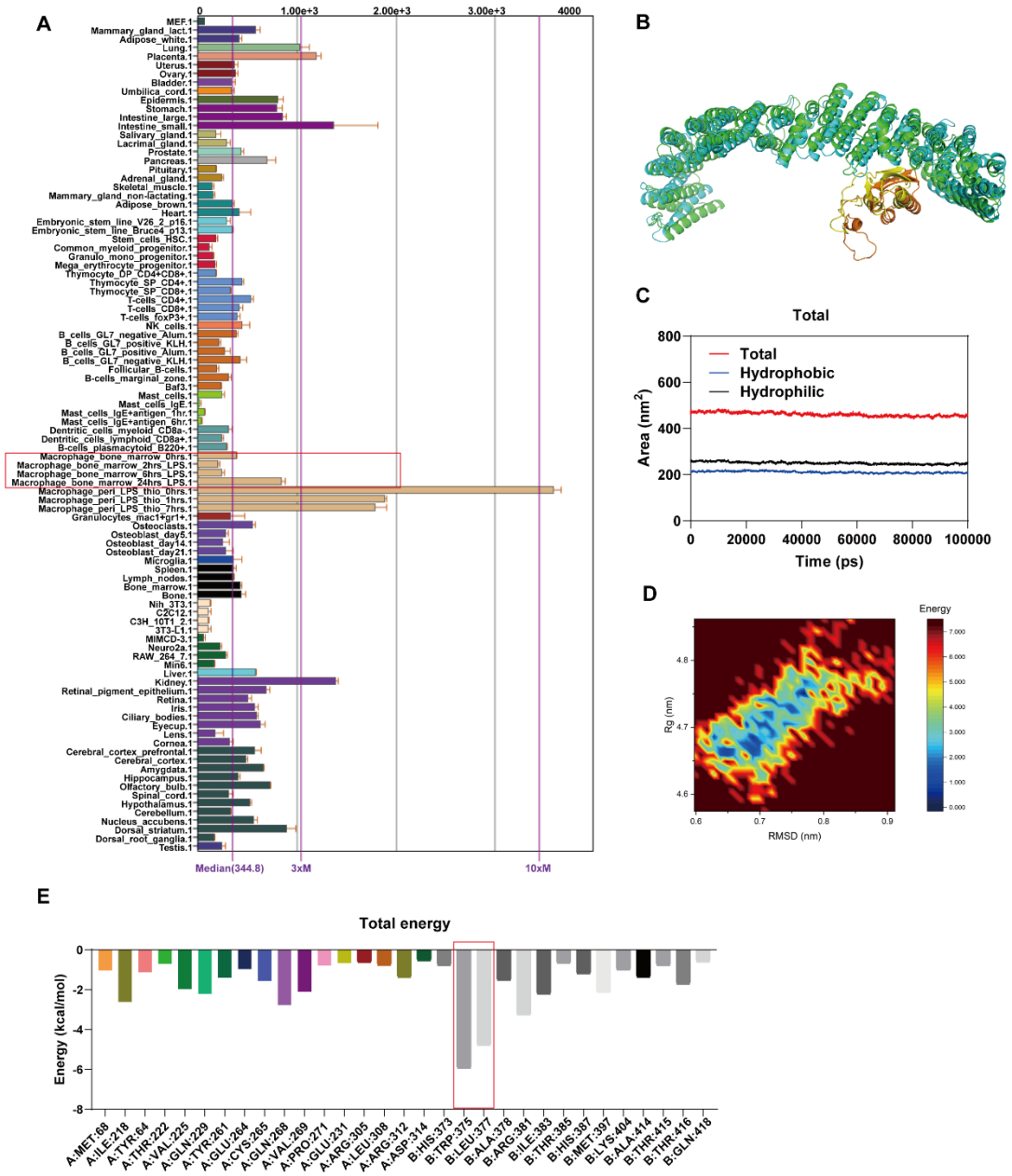
Figure. S2.



S2 VPS35 was identified as the direct target of engeletin. A Volcano plot displaying the differentially expressed proteins between ENG-treated and saline-treated BMDMs in Lip-MS. n=3 per group. B The binding pocket of engeletin. C Time-dependent changes in the radius of gyration (Rg) of the VPS35 and ENG-VPS35 complex during molecular dynamics simulation. D H bond identified in molecular dynamics stimulation. E The binding energy of ENG to VPS35 was analyzed by molecular docking. F DCFH-DA staining of 293T cells transfected with empty vectors or VPS35-OE plasmid and quantified analysis of MFI (mean fluorescent intensity) by flow cytometry. n=6 per group. H-J Expression of NLRP3, pro-IL1β and β-actin in RAW264.7 cells transfected with si-*NC* or si-*Vps35* and quantified data of immunoblotting band intensity in RAW264.7 cells. n=6 per group. K-M qPCR detection of mRNA expression levels of *Il6*, *Il1β* and *Tnf* in RAW264.7 cells transfected with si-*NC* or si-*Vps35*. *n* = 6 per group. Data were analyzed using Welch ANOVA analysis followed

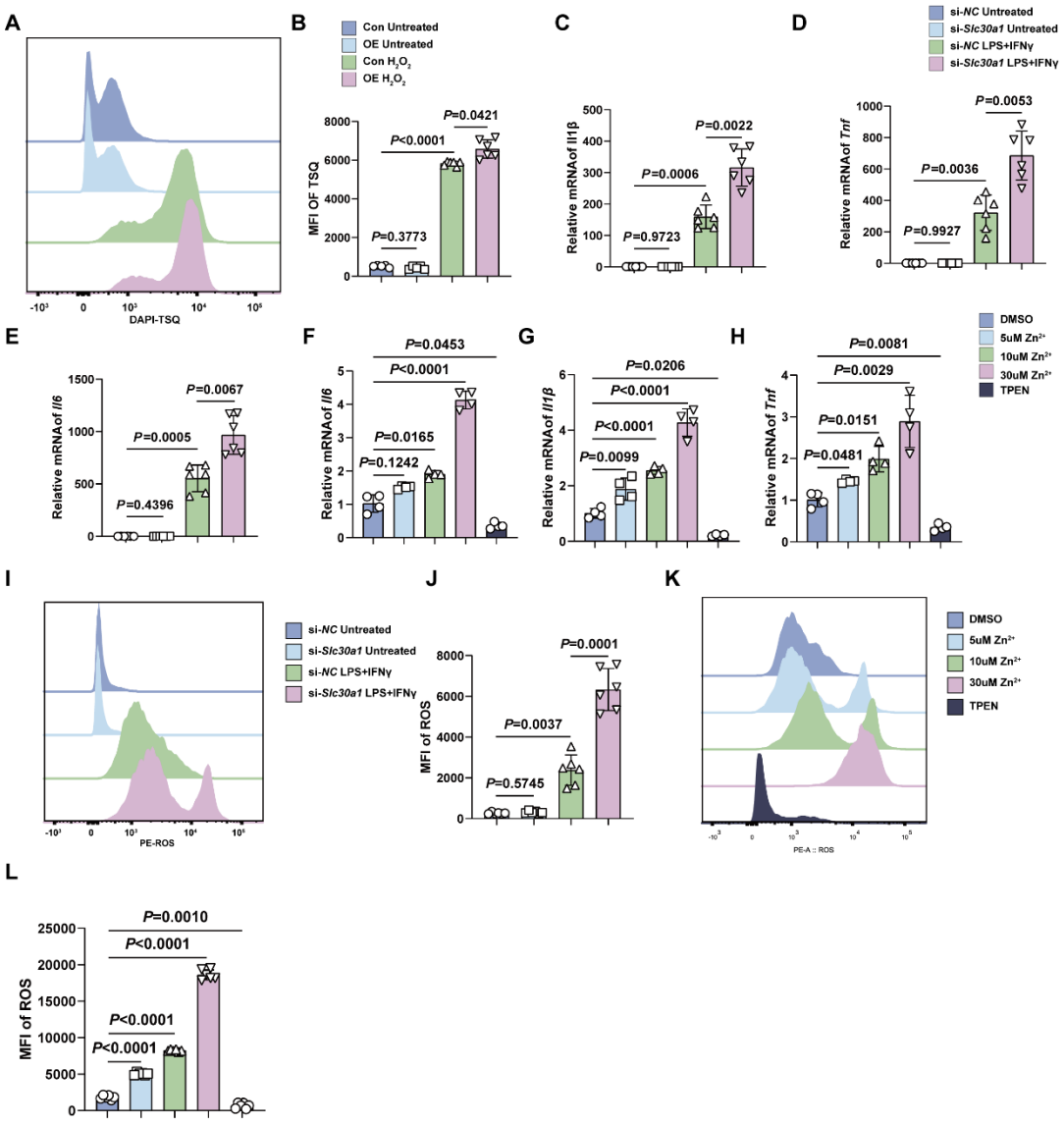
by Games-Howell multiple comparison test (G, I, L and M) and One-way ANOVA analysis followed by Bonferroni's multiple comparison test (J and K).

Figure. S3.



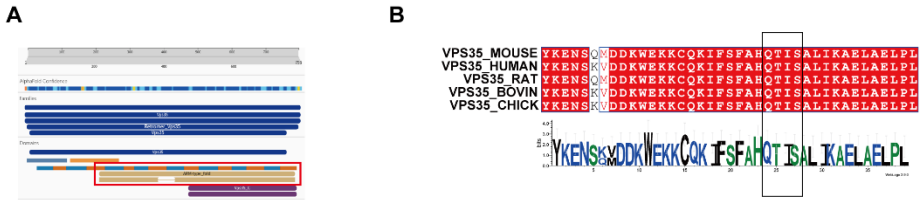
S3 Engeletin disrupted interaction between VPS35 and ZNT1. A Expression of Slc30a1 from Online database BioGPS. B The binding predicted by molecular dynamics stimulation. C Solvent-accessible surface area (SASA) analyzed by molecular dynamics stimulation. D Molecular dynamics trajectories from molecular dynamics stimulation. E Free energy decomposition from molecular dynamics stimulation.

Figure S4



S4 Zinc homeostasis was affected by engeletin. A-B TSQ staining of BMDMs transfected with empty vectors or VPS35-OE plasmid and quantified analysis of MFI (mean fluorescent intensity) by flow cytometry. n=6 per group. C-E qPCR detection of mRNA expression levels of *Il6*, *Il1β* and *Tnf* in BMDMs transfected with si-*NC* or si-*Slc30a1*. *n* = 6 per group. F-H qPCR detection of mRNA expression levels of *Il6*, *Il1β* and *Tnf* in BMDMs treated with DMSO, zinc or TPEN (100uM). *n* = 4 per group. I-J DCFH-DA staining of BMDMs transfected with si-*NC* or si-*Slc30a1* and quantified analysis of MFI (mean fluorescent intensity) by flow cytometry. n=6 per group. K-L DCFH-DA staining of BMDMs treated with DMSO, zinc or TPEN (100uM) and quantified analysis of MFI (mean fluorescent intensity) by flow cytometry. n=6 per group. Data were analyzed using Welch ANOVA analysis followed by Games-Howell multiple comparison test (B, C-E, F, H, J and L) and One-way ANOVA analysis followed by Bonferroni's multiple comparison test (G).

Figure S5



S5 GLN566is essential for the binding between engeletin and VPS35. A The domains of VPS35 obtained from online Interpro database. B Comparative sequence analysis of VPS35.

Table S1.

Supplementary Table I. Antibodies used for flow cytometric analysis.

Antibodies	Manufacturer	Clone	Cat. number
CD45-APC/Cy7	BD Biosciences	30-F11	557659
CD11b-PE/Cy7	BD Biosciences	M1/70	552850
Ly6G-FITC	BioLegend	1A80	127606
Ly6C-BV421	BioLegend	HK1.4	128032
F4/80-PE	BioLegend	BM8	123110
CD206-AF647	BioLegend	C068C2	141712

Table S2.

Supplementary Table II. Antibodies used for immunofluorescent staining.

Antibodies	Concentration	Source	Manufacturer	Cat. number
F4/80	1:200	Rabbit	Abcam	Ab300421
Ly6G	1:200	Rat	Abcam	ab25377
CD68	1:200	Rabbit	Abcam	ab125212
CD11b	1:200	Rabbit	Abcam	ab128797
CD206	1:200	Rabbit	CST	24595
Na, K-ATPase alpha1	1:200	Rabbit	CST	23565
VPS35	1:200	Rabbit	Abcam	ab10099
ZNT1	1:200	Rabbit	Invitrogen	PA5-104383

Table S3.

Supplementary Table III. Antibodies used for western blot.

Antibodies	Concentration	Source	Manufacturer	Cat. number
NLRP3	1:1000	Rabbit	Abcam	ab263899
IL-1β	1:1000	Rabbit	Abcam	ab234437
VPS35	1:200	Rabbit	Abcam	ab10099
ZNT1	1:200	Rabbit	Invitrogen	PA5-104383
Na, K-ATPase alpha1	1:200	Rabbit	CST	23565
BAX	1:1000	Rabbit	CST	2772
Cleaved-Caspse3	1:1000	Rabbit	CST	9661

Table S4.

Supplementary Table IV. Primer list.

Gene	Primer Sequence
Nlrp3	5’- TAGTCCTTCCTACCCCAATTTCC-3’
	5’- TTGGTCCTTAGCCACTCCTTC-3’
Il1b	5’-GAAATGCCACCTTTTGACAGTG-3’
	5’- TGGATGCTCTCATCAGGACAG-3’
Tnf	5’-CCCTCACACTCAGATCATCTTCT-3’
	5’- GCTACGACGTGGGCTACAG-3’
β-actin	5’-GTGACGTTGACATCCGTAAAGA-3’

5’- GCCGGACTCATCGTACTCC-3’

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1. Shi, H., et al., *GSDMD-Mediated Cardiomyocyte Pyroptosis Promotes Myocardial I/R Injury.* Circ Res, 2021. **129**(3): p. 383-396.
2. Huang, C.K., et al., *Lgr4 Governs a Pro-Inflammatory Program in Macrophages to Antagonize Post-Infarction Cardiac Repair.* Circ Res, 2020. **127**(8): p. 953-973.
3. Fan, Q., et al., *Dectin-1 Contributes to Myocardial Ischemia/Reperfusion Injury by Regulating Macrophage Polarization and Neutrophil Infiltration.* Circulation, 2019. **139**(5): p. 663-678.
4. Piazza, I., et al., *A machine learning-based chemoproteomic approach to identify drug targets and binding sites in complex proteomes.* Nat Commun, 2020. **11**(1): p. 4200.