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4-Methylcatechol attenuates osteoarthritis progression by preserving GPX4 stability and limiting LAMP2A-associated lysosomal degradation

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Abstract

Background: Loss of glutathione peroxidase 4 (GPX4) and ferroptotic chondrocyte injury have been implicated in osteoarthritis (OA), but the mechanisms

regulating GPX4 protein stability remain unclear. We investigated whether lysosome-associated membrane protein type 2A (LAMP2A)-dependent chaperone-mediated autophagy (CMA) contributes to GPX4 degradation and whether 4-methylcatechol (4MC) modulates this process.

Methods: Primary murine and human OA chondrocytes, paired weight-bearing and non-weight-bearing human OA cartilage, and an anterior cruciate ligament transection (ACLT) model in female C57BL/6J mice were used. Ferroptosis was induced with erastin or L-buthionine-sulfoximine. GPX4 stability and CMA involvement were examined using lysosomal and proteasomal inhibitors, RNA interference, cycloheximide chase, lysate-based thermal shift assay, co-immunoprecipitation, and proximity ligation assay. Mice received weekly intra-articular 4MC, and joints were assessed at 4 and 8 weeks.

Results: Erastin reduced GPX4 protein without altering Gpx4 mRNA. Chloroquine and bafilomycin A1, but not MG132, preserved GPX4, while LAMP2A knockdown attenuated GPX4 loss, supporting the involvement of a LAMP2A-dependent lysosomal process. Ferroptotic stress increased GPX4-LAMP2A association and proximity. 4MC altered GPX4 thermal stability in cell lysates and slowed GPX4 loss, reduced GPX4-LAMP2A association, lipid peroxidation, and cell injury, and improved matrix anabolic-catabolic balance. GPX4 knockdown diminished the protective effect of 4MC, whereas 4MC did not further increase GPX4 after LAMP2A knockdown. In ACLT mice, 4MC reduced cartilage degeneration, restored GPX4, decreased 4-hydroxynonenal and MMP13, and increased COL2A1. Similar protective

effects were observed in primary human OA chondrocytes, and LAMP2A staining was higher in weight-bearing than in non-weight-bearing human OA cartilage.

Conclusions: These findings support the involvement of LAMP2A-dependent CMA-associated lysosomal degradation of GPX4 in chondrocyte ferroptosis. 4MC may alleviate OA-related ferroptotic injury, at least partly, by preserving GPX4 stability and reducing stress-induced GPX4–LAMP2A association.

Keywords: Osteoarthritis, Ferroptosis, GPX4, Chaperone-mediated autophagy, 4-Methylcatechol

Background

Osteoarthritis (OA) is a chronic whole-joint disorder characterized by progressive articular cartilage loss, subchondral bone remodeling, synovial inflammation, pain, and functional impairment [1]. It affects more than 500 million people worldwide and imposes a substantial and increasing socioeconomic burden [2]. Current pharmacological treatments, including analgesics and nonsteroidal anti-inflammatory drugs, primarily relieve symptoms but do not reliably prevent structural disease progression [1,3]. Accordingly, defining cellular mechanisms that drive cartilage degeneration remains essential for the development of disease-modifying interventions.

Articular cartilage homeostasis depends on the survival and metabolic activity of chondrocytes[4], the only resident cell type responsible for maintaining the extracellular matrix (ECM). Because cartilage is avascular and relatively hypoxic, chondrocytes are particularly vulnerable to disturbances in redox balance[5,6].

Persistent oxidative stress can impair mitochondrial function, suppress anabolic matrix synthesis, and promote the expression of catabolic enzymes, thereby contributing to cartilage deterioration [7]. Ferroptosis, an iron-dependent form of regulated cell death driven by phospholipid peroxidation, has emerged as a potential link between oxidative stress and OA-related chondrocyte loss [8]. Increased lipid peroxidation has been reported in human OA cartilage, and pharmacological inhibition of ferroptosis can attenuate experimental cartilage degeneration, supporting a contributory role for ferroptotic injury in OA [9].

Glutathione peroxidase 4 (GPX4) is a central defense against ferroptosis because it reduces membrane phospholipid hydroperoxides and limits the propagation of lipid peroxidation [10]. Genetic or pharmacological loss of GPX4 sensitizes cells to ferroptosis, whereas preservation of GPX4 activity is protective [11]. Reduced GPX4 abundance and activity have been observed in OA cartilage and experimental OA models [8,9]. Although altered transcription, glutathione depletion, and other metabolic changes may contribute to impaired GPX4 function, the post-translational mechanisms that regulate GPX4 protein stability in chondrocytes remain insufficiently understood. Identifying how GPX4 is lost under ferroptotic stress may therefore reveal a therapeutically relevant point of control upstream of lipid peroxidation and cell death.

The autophagy-lysosomal system maintains cellular proteostasis by removing damaged organelles and proteins. Chaperone-mediated autophagy (CMA) is a selective lysosomal degradation pathway in which cytosolic proteins containing

KFERQ-like recognition motifs are identified by heat shock cognate 70 (HSC70) and delivered to lysosome-associated membrane protein type 2A (LAMP2A) for lysosomal translocation and degradation [12–14]. CMA can be enhanced during oxidative stress and nutrient deprivation and can influence cell fate by regulating the abundance of metabolic enzymes and redox-related proteins [15,16]. GPX4 has recently been reported to undergo CMA-associated degradation in a model of neuronal ferroptosis [17,18]. However, whether LAMP2A-dependent CMA-associated lysosomal degradation contributes to GPX4 loss in chondrocytes, and whether this process is relevant to OA, has not been established.

Pharmacological preservation of GPX4 stability may provide a strategy for limiting ferroptotic cartilage injury. 4-Methylcatechol (4MC) is a small catechol derivative with reported antioxidant and anti-inflammatory activities. Our previous study showed that 4MC reduced experimental inflammatory arthritis and modulated macrophage polarization and pyroptosis through pathways involving Nrf2/HO-1 and NF- κ B/NLRP3 [19]. Nevertheless, its effects on chondrocyte ferroptosis and GPX4 protein turnover have not been investigated. Moreover, whether 4MC influences the LAMP2A-associated lysosomal GPX4 loss remains unknown.

In the present study, we investigated whether LAMP2A-dependent CMA-associated degradation participates in GPX4 loss during ferroptotic stress in chondrocytes and whether 4MC modulates this process. We combined experiments in primary murine chondrocytes, primary human OA chondrocytes, paired regions of human OA cartilage, and an anterior cruciate ligament transection-induced post-

traumatic OA mouse model. We examined GPX4 stability, lysosomal and proteasomal degradation, GPX4-LAMP2A association, ferroptosis-related oxidative injury, cartilage matrix homeostasis, and the effects of intra-articular 4MC treatment. This design was intended to clarify the relationship between lysosomal protein degradation and chondrocyte ferroptosis and to evaluate 4MC as a pharmacological modulator of this pathway in experimental OA.

Materials and Methods

Reagents and materials

All small-molecule reagents, including erastin, ferrostatin-1, L-buthionine-sulfoximine (BSO), and 4-methylcatechol (4MC), were purchased from MedChemExpress. Cell culture reagents, including DMEM/F12 medium, penicillin-streptomycin, and 0.25% trypsin-EDTA, were obtained from Gibco. Millipore supplied the PVDF membranes, while the decalcification solution (EDTA-based), safranin O/fast green staining kits, DAPI, and H&E staining kits were sourced from Solarbio. Beyotime Biotechnology provided all other experimental reagents, including bovine serum albumin (BSA), hydrogen peroxide, JC-1 mitochondrial membrane potential assay kit, Annexin V-FITC/PI apoptosis detection kit, BCA protein assay, DCFH-DA probe, and DAB substrate. RIPA cell lysis buffer, ECL chemiluminescent reagent, and the CCK-8 were sourced from Epizyme. Thermo Fisher provided C11-BODIPY™ 581/591 fluorescent dye, while MDA detection kits were acquired from APEXBIO. Protein A/G magnetic beads were purchased through MedChemExpress. ChamQ Universal SYBR qPCR Master Mix was obtained from Vazyme Biotech, and

Lipofectamine 3000 along with TRIzol reagent were supplied by Invitrogen. Duolink® PLA kits were sourced from Sigma-Aldrich. Secondary antibodies labeled with Cy5, Alexa Fluor 488, or Alexa Fluor 647 fluorophores were also obtained from APEXBIO. Gene-specific small interfering RNAs (siRNAs) were synthesized by GenePharma. Primary antibodies targeting GPX4, LAMP2A, HSC70, COL2A1, MMP13, ADAMTS5, Aggrecan, β -actin, and 4-HNE were obtained from respective commercial suppliers.

Ethical approval and sample acquisition

Cartilage specimens from human subjects included in this study were collected from patients diagnosed with advanced knee osteoarthritis undergoing total knee replacement at the First Affiliated Hospital of Shandong First Medical University. The study obtained ethics clearance from the Institutional Review Board (IRB Approval No. [2025S915]), and informed consent was willingly provided by all participants in writing. Animal experiments were performed in accordance with established institutional protocols and approved by the Animal Ethics Committee (Approval No. [2025121101]).

Mouse model of post-traumatic OA and pharmacological intervention

Eight-week-old female C57BL/6J mice were purchased from Jinan Pengyue Laboratory Animal Breeding Company and housed under specific pathogen-free (SPF) conditions, with a standardized 12-hour cycle of light and darkness. Animals were randomly assigned to four groups (n = 5 mice/group/time point): (1) sham control; (2) ACLT + vehicle, in which mice received intra-articular injection of sterile

saline in a final volume of 10 μ L; (3) ACLT + low-dose 4-methylcatechol (4MC), in which mice received intra-articular injection of 4MC at 10 μ g/knee in a final volume of 10 μ L; and (4) ACLT + high-dose 4MC, in which mice received intra-articular injection of 4MC at 20 μ g/knee in a final volume of 10 μ L. For surgical induction of osteoarthritis, mice were anesthetized intraperitoneally with sodium pentobarbital (100 mg/kg). The right knee joint was exposed surgically, and complete transection of the anterior cruciate ligament (ACL) was performed to induce joint instability. Sham-operated mice underwent an identical surgical procedure without ACL disruption. Starting one week post-operation, mice in the treatment cohorts received intra-articular injections of either 4MC solution (10 μ L per injection) or vehicle weekly. At the predetermined experimental endpoints (4 and 8 weeks after surgery), animals were euthanized humanely, and their knee joints harvested for micro-CT imaging, histopathological assessment, and molecular analyses.

Micro-computed tomography (micro-CT) imaging and analysis

Isolated knee joint samples were immersed in 4% paraformaldehyde for fixation lasting 48 hours. Following fixation, samples were scanned using a Quantum GX2 micro-CT imaging system (PerkinElmer) operated at a voltage of 70 kV and current of 142 μ A, combined with a 0.5-mm aluminum filter. The resulting images featured isotropic voxels measuring approximately 9 μ m. Projection data underwent three-dimensional (3D) reconstruction with NRecon software (Bruker), and images were manually adjusted to standard anatomical orientations. Quantitative evaluation of subchondral bone microstructure involved manually defining a cubic region of

interest (ROI) beneath the calcified cartilage interface within the medial tibial plateau. Bone segmentation was executed through global intensity thresholding, followed by the calculation of morphometric indices such as bone volume fraction (BV/TV), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th) using CTAn software (Bruker) [20]. Osteophyte occurrence was determined through visual inspection and enumeration based on full-joint 3D reconstructions. To ensure unbiased results, all image analyses and measurements were performed by investigators blinded to experimental group assignments.

Histological and immunohistochemical (IHC) analyses

Human articular cartilage and murine knee joint samples were subjected to decalcification at room temperature over four weeks in a 10% EDTA solution (pH 7.4). Following this step, tissues underwent standard dehydration, clearing, and paraffin embedding. Subsequently, sections (5 μ m thick) were cut coronally using a microtome. Histopathological examinations involved H&E staining to visualize tissue morphology, and safranin O/fast green staining for evaluating cartilage matrix integrity and proteoglycan distribution. The severity of cartilage damage was independently assessed by two blinded pathologists with specific training, utilizing the Osteoarthritis Research Society International (OARSI) scoring method ranging from 0 to 6 [21].

For immunohistochemistry, paraffin-embedded sections were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen

peroxide, followed by blocking with 5% BSA. Sections were incubated overnight at 4°C with antibodies against GPX4 (1:200), LAMP2A (1:200), or 4-HNE (1:150), followed by HRP-conjugated secondary antibodies, DAB visualization, and hematoxylin counterstaining. Staining was quantified by investigators blinded to group allocation. Chondrocytes with brown DAB staining above background were considered positive, and the percentage of positive chondrocytes was calculated relative to the total number of hematoxylin-stained chondrocytes within the cartilage region of interest. For each human donor and cartilage region, three nonoverlapping fields were analyzed and averaged to generate a single value. Each donor was considered one independent biological replicate. For murine samples, measurements from multiple fields were averaged to obtain one value for each animal.

Isolation and in vitro expansion of primary chondrocytes

Primary chondrocytes were enzymatically extracted from the articular cartilage of neonatal (5-day-old) C57BL/6J mice. Cartilage specimens were carefully minced and then enzymatically digested in DMEM/F12 medium containing collagenase type II (0.2%, Sigma-Aldrich, Catalog No. C6885) for approximately 4-6 hours at 37°C with gentle agitation. After enzymatic digestion, the resulting cell suspension was filtered through a cell strainer (70-µm mesh), centrifuged, and then resuspended in complete growth medium. Cells were plated into appropriate culture dishes and incubated under standard culture conditions (37°C, 5% CO₂, humidified atmosphere). All functional assays were conducted using first-passage (P1) chondrocytes to maintain phenotypic fidelity and minimize dedifferentiation. Human primary

chondrocytes were similarly isolated from osteoarthritic cartilage specimens obtained during total knee arthroplasty using identical enzymatic digestion and culture procedures.

Assessment of chondrocyte viability and cell death

Primary chondrocyte viability was determined through the Cell Counting Kit-8 (CCK-8) assay. Cells were initially seeded into 96-well plates at 5×10^3 cells per well and incubated overnight for adherence. Following various experimental treatments, each well received 10 μ L of the CCK-8 solution, and incubation proceeded for 2 additional hours at 37°C under 5% CO₂ atmosphere. Absorbance was then measured at a wavelength of 450 nm using a multimode microplate reader, with readings adjusted by subtracting the corresponding background values.

Cell death induced by experimental conditions was evaluated using an apoptosis detection kit employing Annexin V-FITC and propidium iodide (PI). After treatments, cells were harvested, rinsed twice using chilled PBS, and resuspended in the provided binding buffer. Cells underwent staining with Annexin V-FITC and PI according to the manufacturer's guidelines, and stained samples were promptly examined (within half an hour) using a BD FACSCanto II flow cytometer. Flow cytometry data were processed via FlowJo software (version 10.8), and the percentages of Annexin V- and/or PI-positive cells were calculated as indicators of cell death.

Quantification of lipid peroxidation and oxidative stress (OS) markers

To evaluate lipid peroxidation, cells were incubated with the fluorescent probe C11 BODIPY™ 581/591 at a concentration of 5 μ M in warmed culture medium for

30 min at 37°C following treatments. Excess staining was eliminated by two gentle PBS washes, after which images were immediately captured via fluorescence microscopy using appropriate optical filters. Lipid peroxidation levels were quantified as the ratio of the oxidized/reduced fluorescence intensity analyzed with ImageJ software.

Malondialdehyde (MDA), frequently employed as a lipid peroxidation marker, was measured through a spectrophotometric assay utilizing a TBA-based detection kit. The absorbance was recorded at 532 nm wavelength, and obtained MDA values were normalized relative to total protein levels measured via the BCA assay.

Reactive oxygen species (ROS) levels within cells were assessed using the fluorescent indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, cells underwent incubation with 10 μ M DCFH-DA in serum-free medium at 37°C for 20 minutes. Following washing, the fluorescence intensity was rapidly analyzed by flow cytometry within one hour. ROS concentrations were expressed as geometric mean fluorescence intensities (gMFI), normalized against untreated controls.

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were assessed by applying the fluorescent dye JC-1. Following exposure to treatments, cells were stained at 37°C for 20 minutes using JC-1 at a concentration of 5 μ g/mL, washed extensively with PBS, and immediately visualized under fluorescence microscopy. Intact mitochondria were identified by red-orange JC-1 aggregates, while mitochondrial membrane depolarization was indicated by green fluorescence from JC-1 monomers. Quantitative evaluation was carried out by determining the red-to-green fluorescence

intensity ratio from microscopy images using ImageJ software.

RNA interference and quantitative real-time PCR (qRT-PCR) analysis

Small interfering RNAs (siRNAs) specifically targeting mouse Gpx4 and LAMP2A genes, along with a scrambled negative control (siNC), were synthesized commercially (GenePharma). Specific siRNA sequences utilized for gene knockdown experiments are detailed in Supplementary Table 1. Transfection of primary chondrocytes with siRNAs at 50 nM concentration was accomplished using Lipofectamine 3000 (Invitrogen). Cells were exposed to siRNA-liposome complexes for 6 hours, after which the medium was refreshed. Subsequent experimental assays took place between 24 to 48 hours post-transfection to achieve maximal gene suppression. Knockdown efficiency was rigorously assessed at the mRNA and protein levels via quantitative real-time PCR (qRT-PCR), Western blotting, or immunofluorescence prior to further testing.

For murine cartilage samples, articular cartilage was carefully dissected from the femoral condyles and tibial plateaus under a stereomicroscope, immediately snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Chondrocytes or cartilage tissues were subjected to RNA isolation with TRIzol reagent (Vazyme), and the integrity and purity of extracted RNA were verified by NanoDrop spectrophotometer, confirming absorbance ratios (A_{260}/A_{280}) of ≥ 1.9 . Subsequently, 1 μg RNA from each sample was reverse transcribed using a commercially obtained reverse transcription reagent kit. Quantitative PCR reactions were conducted in triplicate using ChamQ Universal SYBR qPCR Master Mix (Vazyme) on a

LightCycler 480 II system (Roche). Cycling conditions comprised an initial denaturation at 95°C for 30 seconds, succeeded by 40 amplification cycles consisting of 10 seconds at 95°C and 30 seconds at 60°C. Primer sequences used in the qPCR experiments are provided in Supplementary Table 2. Expression levels of target genes were normalized against Gapdh expression and analyzed using the $2^{-\Delta\Delta Ct}$ comparative method [22].

Molecular docking analysis

Molecular docking was performed to predict the potential binding interaction between 4-methylcatechol (4MC) and GPX4 using an AutoDock Vina-based protocol. The crystal structure of GPX4 was obtained from the Protein Data Bank (PDB ID: 6HKQ), and the three-dimensional structure of 4MC was downloaded from the PubChem database. The ligand structure was energy-minimized using the MMFF94 force field. Prior to docking, the GPX4 structure was prepared by adding hydrogen atoms using PyMOL 2.5.5, and both the receptor and ligand were converted into PDBQT format using ADFRsuite 1.0. Molecular docking was then carried out using AutoDock Vina 1.2.3 [23]. The docking grid box was set to cover the whole GPX4 protein to allow global ligand searching, and the exhaustiveness value was set to 32. The top-ranked binding pose with the lowest docking energy was selected for subsequent analysis. The predicted binding mode and ligand–residue interactions were visualized using PyMOL 2.5.5 and Discovery Studio 2025.

Lysate-based thermal shift assay

A lysate-based CETSA [24] was performed to evaluate whether 4MC alters the thermal stability of GPX4. Chondrocyte lysates were incubated with DMSO or 4MC (40 μ M), divided into aliquots, and heated at 37, 42, 47, 52, 57, or 62°C. After centrifugation, the soluble protein fractions were collected and subjected to Western blotting for GPX4 detection. Band intensities were normalized to the 37°C group, and thermal stability curves were used to calculate the apparent T_{m50} values. After the CETSA temperature-gradient assay, the concentration-dependent effect of 4MC on GPX4 protein abundance was further examined. Chondrocyte lysates were incubated with different concentrations of 4MC (0, 20, 40, and 80 μ M) and heated at 52°C, which was selected based on the temperature-gradient CETSA results. After centrifugation, the soluble protein fractions were collected and analyzed by Western blotting using an anti-GPX4 antibody. GPX4 band intensities were quantified by densitometric analysis and expressed as relative band intensity.

Protein extraction and immunoblotting analysis

To isolate proteins, cultured chondrocytes underwent two washes with chilled PBS, after which they were disrupted in RIPA lysis buffer containing protease and phosphatase inhibitors. Cell lysates remained on ice for 30 minutes, with occasional vortex mixing, and then were centrifuged. The clarified supernatants were transferred to fresh tubes, and protein concentrations were quantified using the bicinchoninic acid (BCA) protein quantification method.

Protein samples (20 μ g each) underwent SDS-PAGE separation and subsequent

transfer onto PVDF membranes. Membranes were blocked at room temperature for 1 h using 5% skim milk in TBST, then incubated overnight at 4°C with primary antibodies diluted in blocking buffer, including anti-GPX4, anti-LAMP2A, anti-HSC70, anti-COL2A1, anti-MMP13, anti-ADAMTS5, anti-Aggrecan (all diluted 1:1000), and anti- β -actin (1:5000 dilution). After multiple washes, membranes underwent incubation with horseradish peroxidase (HRP)-linked secondary antibodies at a 1:5000 dilution for 1 hour at room temperature. Protein bands were detected by applying enhanced chemiluminescence (ECL) detection reagents. Subsequently, band intensities were assessed using Image Lab software (Bio-Rad), normalized to β -actin, and reported as fold changes relative to control samples.

Co-immunoprecipitation (Co-IP) assay

For co-immunoprecipitation experiments to assess biochemical association, chondrocytes were rinsed twice using ice-cold PBS and lysed with IP buffer. Lysates were centrifuged to eliminate insoluble materials, and supernatants containing equal amounts of proteins were incubated overnight at 4°C with primary antibodies specific to LAMP2A or GPX4, or corresponding normal rabbit or mouse IgG antibodies as negative controls. Immunocomplexes were then captured using protein A/G magnetic beads previously equilibrated with IP lysis buffer, followed by an additional 2-hour incubation at 4°C. After five washing steps with chilled IP buffer, bound complexes were eluted in 1× SDS-PAGE sample buffer by boiling for 5 minutes. Protein eluates underwent SDS-PAGE separation and immunoblotting analyses using antibodies recognizing GPX4, HSC70, and LAMP2A. To ensure equal loading, aliquots (5%) of

input lysates were concurrently probed with an anti- β -actin antibody.

Proximity ligation assay (PLA) for in situ detection of GPX4-LAMP2A interactions

The Duolink® In Situ Proximity Ligation Assay (PLA) Kit from Sigma-Aldrich was employed to examine GPX4–LAMP2A proximity in chondrocytes. Positive PLA signals indicate that the antibody-recognized targets are located within approximately 40 nm [25]. Cultured chondrocytes seeded onto sterile glass coverslips were initially fixed using 4% paraformaldehyde, permeabilized with PBS containing 0.3% Triton X-100, and blocked utilizing the Duolink blocking solution provided. Cells were then incubated overnight at 4°C with primary antibodies targeting the proteins of interest. After thorough washing, PLA probes corresponding to the species specificity of the primary antibodies were applied. Subsequent ligation and rolling-circle amplification reactions were performed according to the recommended manufacturer procedures, resulting in fluorescently tagged nucleic acid sequences detectable as distinct, bright dots at protein interaction sites. Following DAPI counterstaining of cell nuclei, fluorescence microscopy imaging was conducted using uniform acquisition settings on a Leica confocal microscope. The number and fluorescence intensity of PLA dots were quantitatively analyzed per cell using ImageJ software.

Toluidine blue staining for proteoglycan visualization and semiquantitative assessment

Mouse primary chondrocytes were plated in 12-well dishes, subjected to the indicated treatments, and subsequently rinsed twice with ice-cold PBS. Cells were

fixed in a 4% paraformaldehyde (PFA) solution for 15 min at ambient temperature and extensively rinsed in distilled water to remove residual fixative. Toluidine blue staining (0.1%) was applied for 20 min in darkness, after which residual stain was carefully removed through repeated washing steps with distilled water until the background staining diminished. Stained monolayers were imaged using bright-field microscopy with a digital camera. For quantitative analysis, images were processed in ImageJ. Toluidine blue-positive regions were identified by intensity thresholding, and integrated optical density (IOD) per field was calculated. Values were normalized to total segmented regions, enabling comparative assessment of proteoglycan content across experimental groups.

Immunofluorescence (IF)

For IF assays, cultured cells on glass coverslips or tissue sections were fixed for 15 minutes at room temperature in 4% paraformaldehyde, then permeabilized for 10 minutes with PBS containing 0.1% Triton X-100. Blocking of nonspecific interactions was performed using 5% normal goat serum in PBST solution (PBS containing 0.05% Tween-20) for 1 hour. Samples were subsequently incubated overnight at 4°C with primary antibodies diluted appropriately: anti-GPX4 (1:200), anti-LAMP2A (1:200), anti-SOX9 (1:200), anti-COL2A1 (1:200), or anti-MMP13 (1:200). After three washes in PBST (5 min each), specimens were treated for 1 hour in darkness with fluorescently labeled secondary antibodies. Following nuclear staining with DAPI for 5 minutes, samples were mounted in antifade mounting medium and examined using a ZEISS fluorescence microscope. A fixed threshold was applied across all groups;

marker-positive cells were expressed as a percentage of DAPI-positive cells, whereas COL2A1 staining was quantified as the percentage of positive area within the cartilage region of interest. Values from multiple fields were averaged for each biological replicate.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Comparisons between two independent groups were performed using an unpaired two-tailed Student's *t*-test, whereas paired human cartilage samples were analyzed using a paired two-tailed Student's *t*-test. Comparisons among multiple groups were performed using one-way or two-way ANOVA followed by Tukey's multiple-comparisons test. OARSI scores were analyzed using the Kruskal–Wallis test followed by Dunn's multiple-comparisons test. Dose–response and thermal denaturation curves were fitted using nonlinear regression. Statistical analyses were performed using GraphPad Prism 8.4, and $P < 0.05$ was considered statistically significant.

Results

1. 4MC protects chondrocytes from erastin-induced injury and restores anabolic-catabolic homeostasis in vitro

To examine the potential of 4-methylcatechol (4MC) (Fig. 1A) to prevent ferroptosis, chondrocytes underwent treatment with 5 μ M erastin (Fig. S1) alongside the established ferroptosis inhibitor ferrostatin-1 (Fer-1, 1 μ M), which served as the positive control. Exposure to erastin significantly impaired chondrocyte viability, whereas 4MC treatment reversed these negative effects in a concentration-dependent

manner (1-100 μ M). At higher doses, 4MC provided protection comparable to Fer-1 (Fig. 1B). These data suggest that 4MC effectively counteracts erastin-induced cytotoxicity. Annexin V/PI staining and flow cytometry confirmed that erastin markedly increased cell death, which was dose-dependently attenuated by 4MC (10 and 40 μ M), with efficacy similar to Fer-1 (Fig. 1C, D). These findings further indicate that 4MC has a cytoprotective effect against erastin-induced cell death.

Next, toluidine blue staining was employed to determine if 4MC preserves matrix homeostasis under ferroptotic stress. Erastin markedly reduced sulfated proteoglycan content, whereas 4MC restored matrix staining intensity, with the higher dose showing protection comparable to Fer-1 (Fig. 1E, F). At the transcriptional level, erastin downregulated anabolic markers (Acan, Col2a1) and upregulated catabolic genes (Mmp13, Adamts5). Treatment with 4MC reversed these changes by restoring anabolic gene expression and suppressing catabolic gene induction (Fig. 1G). Immunoblot analysis corroborated these findings: erastin decreased Aggrecan and COL2A1 proteins and increased MMP13 and ADAMTS5 expression, while 4MC significantly restored anabolic-catabolic balance. Notably, 40 μ M 4MC exerted effects comparable to Fer-1 (Fig. 1H, I). Collectively, these data indicate that 4MC mitigates erastin-induced chondrocyte injury, preserves the proteoglycan-rich matrix, and maintains chondrogenic phenotype by promoting anabolic activity and suppressing catabolic effectors.

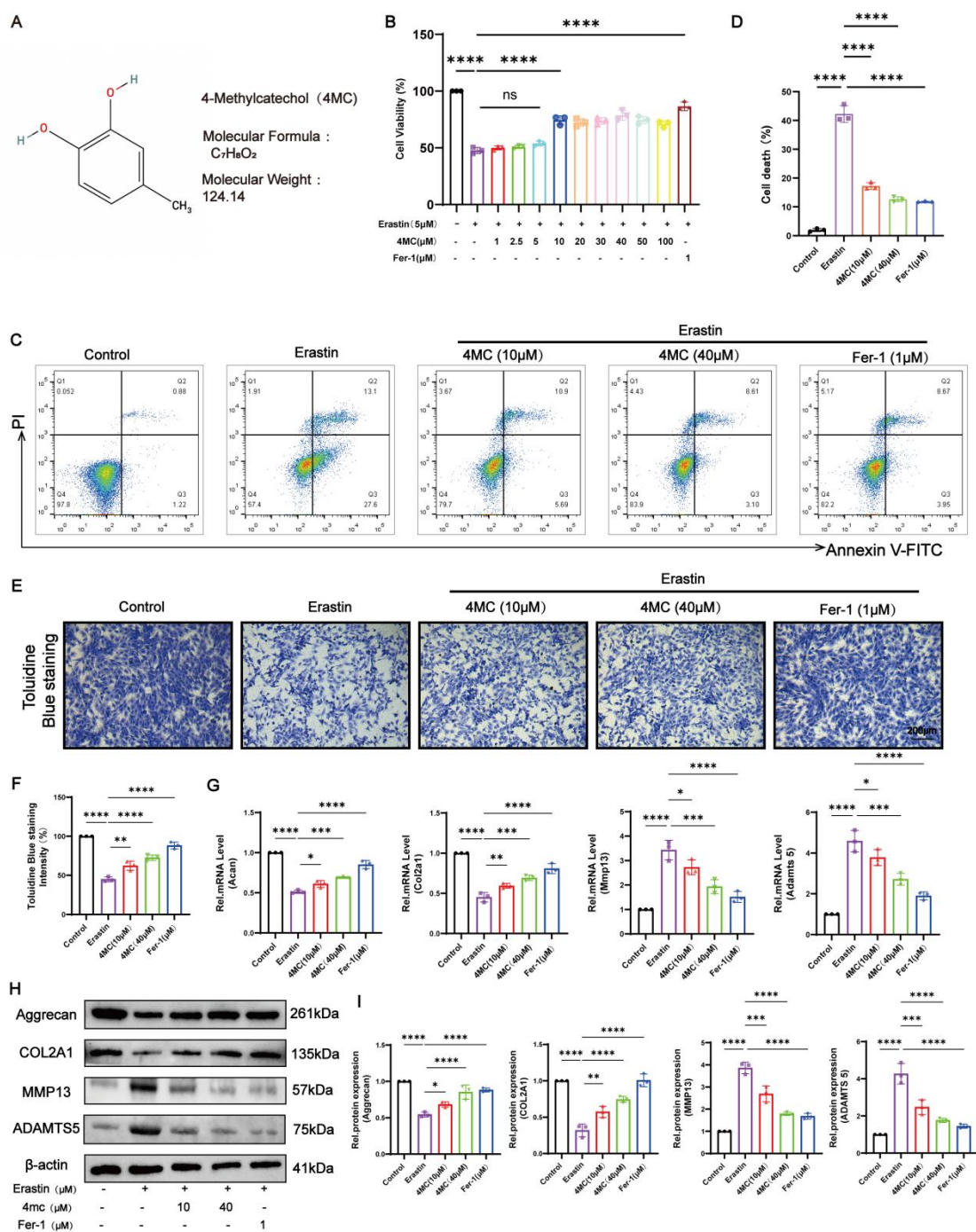


Fig 1. 4MC protects chondrocytes from ferroptosis and maintains cartilage

matrix homeostasis in vitro.

(A) Chemical structure of 4-methylcatechol (4MC). (B) Viability of chondrocytes

treated with erastin (5 μM) and increasing concentrations of 4MC (1-100 μM) for 24

h; ferrostatin-1 (Fer-1, 1 μ M) served as a positive control. (C) Representative flow cytometry plots (Annexin V-FITC/PI staining) showing cell death distribution across treatment groups. (D) Quantification of cell death percentages from (C). (E) Representative images of toluidine blue staining showing proteoglycan content under each condition. Scale bar: 200 μ m. (F) Quantification of toluidine blue staining intensity corresponding to (E). (G) mRNA expression of anabolic markers (Acan and Col2a1) and catabolic genes (Mmp13 and Adamts5) post-treatment. (H) Western blot (WB) analysis of Aggrecan, COL2A1, MMP13, and ADAMTS5 proteins following treatment. (I) Densitometric quantification of protein levels from (H). Statistical significance: Data are presented as mean $\pm$ SD from three independent primary murine chondrocyte isolations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. ns = not significant.

2. 4MC suppresses ferroptosis by preserving GPX4 protein levels and antioxidant function

To clarify the protective mechanism of 4MC, we first examined its effects on GPX4 abundance and ferroptosis-related oxidative injury. Immunoblot analysis revealed that erastin markedly decreased GPX4 protein expression, whereas co-treatment with 4MC restored GPX4 levels in a dose-dependent manner, comparable to the ferroptosis inhibitor Fer-1 (Fig. 2A, B). Consistent with restored GPX4 expression, 4MC significantly reduced intracellular ROS accumulation induced by erastin (Fig. 2C, D). Additionally, 4MC alleviated mitochondrial depolarization (Fig. 2E, G) and suppressed lipid peroxidation, as shown by decreased oxidized/reduced

ratios of the C11-BODIPY probe (Fig. 2F, H) and reduced malondialdehyde (MDA) production (Fig. 2I). Collectively, these findings suggest that 4MC limits ferroptosis-associated oxidative damage by maintaining cellular GPX4 antioxidant activity.

We next investigated whether 4MC confers protection under alternative ferroptotic conditions. In chondrocytes treated with buthionine sulfoximine (BSO), a glutathione synthesis inhibitor, 4MC improved cell viability and partially sustained GPX4 protein levels over time (Fig. 2J-L). However, 4MC did not rescue cell death caused by the GPX4 covalent inhibitor RSL3 (Fig. 2M). Although 4MC restored GPX4 abundance even in the presence of RSL3 (Fig. S2), this was insufficient to prevent ferroptosis, indicating that 4MC protection requires GPX4 catalytic activity rather than mere restoration of protein abundance. Supporting this conclusion, GPX4 knockdown abolished the cytoprotective effect of 4MC, while Fer-1 still enhanced viability (Fig. 2N). Because 4MC did not alter Gpx4 mRNA levels (Fig. S3), we evaluated its influence on GPX4 protein stability. Cycloheximide (CHX) chase assays demonstrated accelerated GPX4 degradation under erastin exposure, whereas co-treatment with 4MC significantly prolonged GPX4 stability (Fig. 2O, P). Taken together, these results indicate that 4MC inhibits ferroptosis by stabilizing GPX4 protein, thereby preserving its antioxidant function.

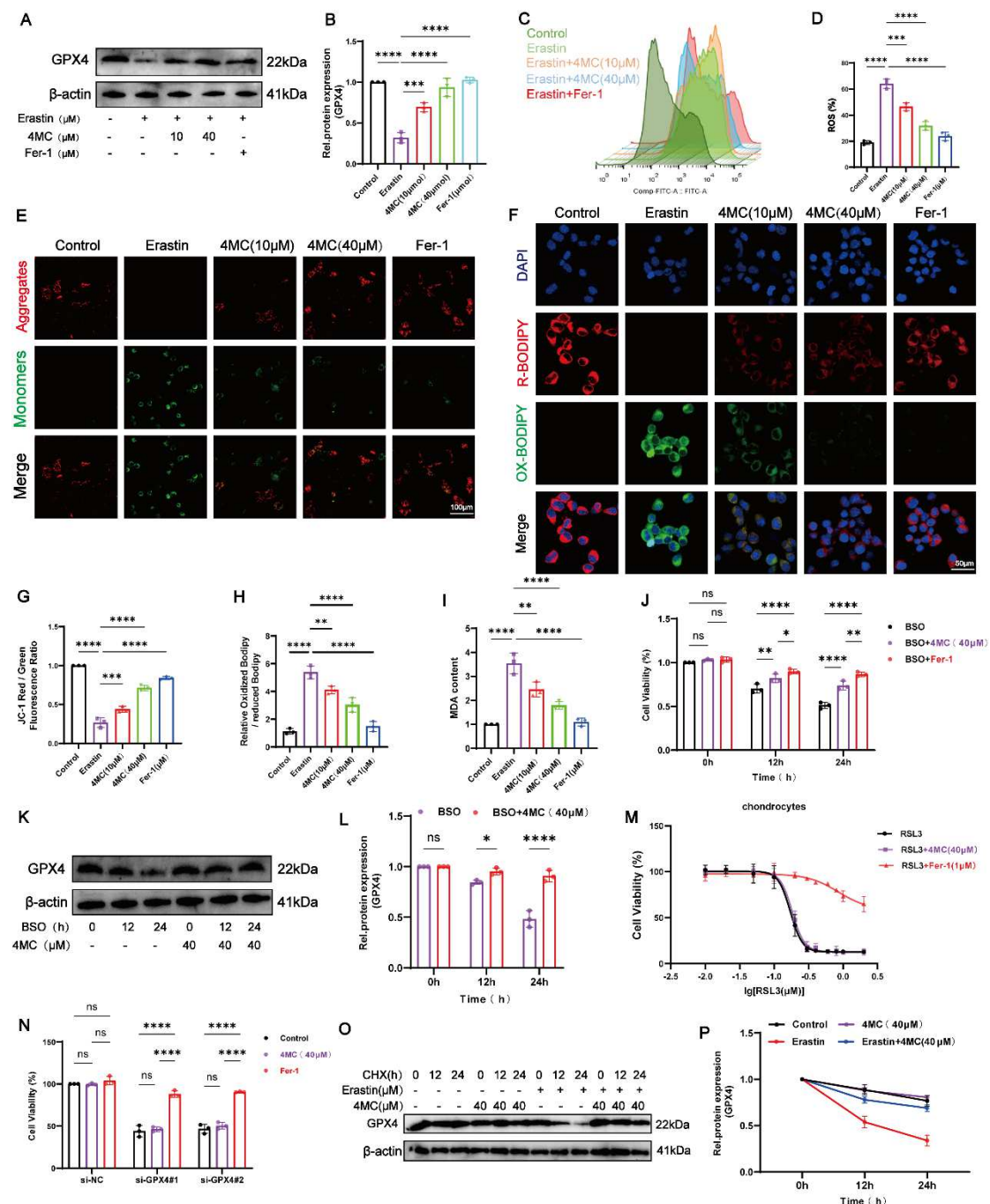


Fig 2. 4MC attenuates chondrocyte ferroptosis by preserving GPX4 stability and redox homeostasis.

(A) Immunoblot analysis of GPX4 in chondrocytes treated with erastin (5 μ M) for 24 h, with or without 4MC (10 or 40 μ M) or Fer-1 (1 μ M). (B) Quantification of GPX4 protein levels normalized to β -actin from panel (A). (C) Representative flow

cytometry histograms of intracellular ROS levels measured by DCFH-DA
fluorescence across indicated groups. (D) MFI quantification from (C).
(E) Representative fluorescence images of mitochondrial membrane potential
assessed by JC-1 staining (red/green fluorescence). Scale bar: 100 μ m. (F)
Representative images of C11-BODIPY staining showing reduced (red) and oxidized
(green) lipid species. Scale bar: 50 μ m. (G) Quantitative analysis of JC-1 red/green
fluorescence ratio. (H) Quantification of oxidized C11-BODIPY fluorescence
intensity. (I) MDA levels in treated cells. (J) Chondrocyte viability following
treatment with BSO (800 μ M), with or without 4MC (40 μ M) or Fer-1 (1 μ M). (K)
Immunoblot analysis of GPX4 after BSO treatment for indicated durations, with or
without 4MC (40 μ M). (L) Quantification of GPX4 protein levels from panel (K). (M)
Dose–response curve of chondrocyte viability to RSL3 with or without 4MC (40 μ M)
or Fer-1 (1 μ M). (N) Cell viability after siRNA-mediated GPX4 knockdown (siGPX4)
or negative control (siNC), followed by erastin treatment with or without 4MC
(40 μ M) or Fer-1 (1 μ M). (O) Cycloheximide (CHX, 10 μ g/mL) chase assay assessing
GPX4 degradation after erastin exposure, with or without 4MC (40 μ M). (P)
Quantification of GPX4 protein levels from panel (O). Statistical significance: Data
are presented as mean $\pm$ SD from three independent primary murine chondrocyte
isolations (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns = not
significant.

3. CMA drives GPX4 degradation during chondrocyte ferroptosis

IHC analysis of paired human OA cartilage specimens showed higher LAMP2A staining in weight-bearing regions than in matched non-weight-bearing regions (Fig. S4). This observation prompted us to investigate whether lysosome-dependent protein degradation contributes to GPX4 loss under ferroptotic stress. Immunoblotting showed that the erastin-induced reduction in GPX4 protein was attenuated by the lysosomal inhibitors chloroquine (CQ) and bafilomycin A1 (BafA1), whereas the proteasome inhibitor MG132 had little effect (Fig. 3B, C). Consistent with these findings, CQ and BafA1, but not MG132, partially protected chondrocytes from erastin-induced loss of viability (Fig. 3A). These results supported the involvement of a lysosome-dependent pathway in erastin-induced GPX4 loss.

We next examined the involvement of LAMP2A, the lysosomal receptor required for CMA substrate translocation. Knockdown of LAMP2A using three independent siRNAs substantially attenuated erastin-induced GPX4 depletion (Fig. 3D–F), supporting the involvement of LAMP2A in this process. Time-course analysis further showed that erastin progressively increased LAMP2A protein abundance while decreasing GPX4 levels, whereas total HSC70 abundance remained relatively stable (Fig. 3G, H). Collectively, these findings support a LAMP2A-dependent lysosomal mechanism consistent with CMA involvement in GPX4 degradation during erastin-induced chondrocyte ferroptosis.

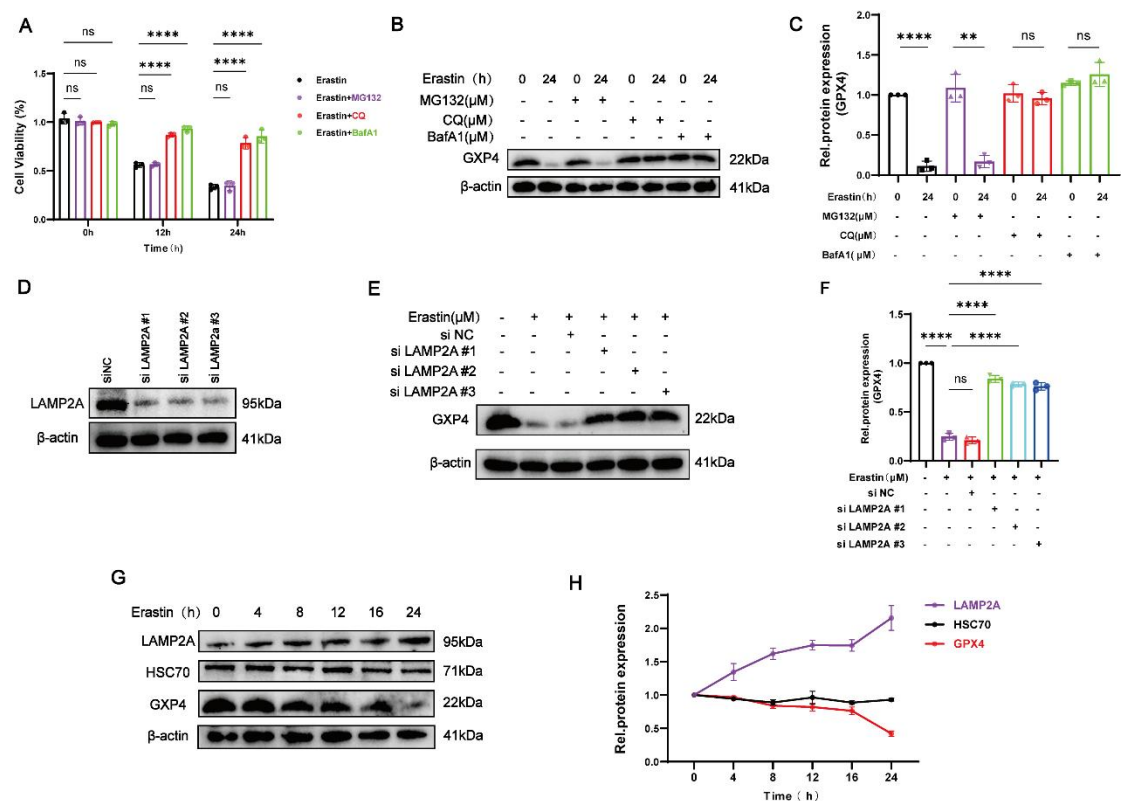


Fig 3. CMA drives GPX4 degradation during chondrocyte ferroptosis.

(A) Viability of chondrocytes treated with erastin (5 μ M) in the absence or presence of proteasome inhibitor MG132 (5 μ M), or lysosomal inhibitors chloroquine (CQ, 20 μ M) and bafilomycin A1 (BafA1, 100 nM). (B) Immunoblot analysis of GPX4 in cells treated with erastin for 24 h, with or without MG132, CQ, or BafA1. (C) Quantification of GPX4 protein levels from (B). (D) Immunoblot validation of LAMP2A knockdown efficiency using three independent siRNAs (siLAMP2A #1-#3). (E) GPX4 immunoblot following transfection with control (siNC) or LAMP2A siRNAs (#1-#3) and subsequent erastin treatment. (F) Quantification of GPX4 protein levels from (E). (G) Time-course immunoblot analysis of LAMP2A, HSC70, and GPX4 following erastin treatment (0–24 h). (H) Densitometric quantification of protein levels from (G). Statistical significance: Data are presented as mean $\pm$ SD

from three independent primary murine chondrocyte isolations (n = 3). *p < 0.05;
p < 0.01; *p < 0.001; ****p < 0.0001. ns = not significant.

4. 4MC preserves GPX4 stability and attenuates stress-induced GPX4–LAMP2A association

We next investigated whether 4MC affects GPX4 protein stability and its association with the CMA receptor LAMP2A. Blind docking yielded a top-ranked predicted pose near a putative CMA-recognition motif, providing a hypothesis for experimental follow-up. The predicted pose was located near one of the putative CMA-recognition motifs of GPX4, providing a structural rationale for further examining the effect of 4MC on GPX4 stability (Fig. 4A). A lysate-based cellular thermal shift assay was subsequently performed. Compared with DMSO, 4MC shifted the GPX4 thermal denaturation curve and increased the apparent T_m50 by 3.92 °C (Fig. 4B). At 52 °C, increasing concentrations of 4MC also resulted in a concentration-dependent increase in soluble GPX4 (Fig. 4C). These findings indicate that 4MC alters the thermal stability of GPX4 in cell lysates.

We next examined whether 4MC affects the association of GPX4 with LAMP2A under ferroptotic stress. Co-immunoprecipitation using an anti-GPX4 antibody showed increased co-precipitation of LAMP2A following erastin treatment, whereas this increase was attenuated by 4MC (Fig. 4D). Total HSC70 abundance remained relatively stable across the treatment groups. Reciprocal co-immunoprecipitation using an anti-LAMP2A antibody showed a similar pattern, with increased GPX4 association after erastin treatment and reduced association following 4MC treatment

(Fig. 4E). These results support the conclusion that 4MC attenuates the erastin-induced association between GPX4 and LAMP2A.

To assess GPX4–LAMP2A proximity in situ, a proximity ligation assay was performed. Erastin markedly increased the number of GPX4–LAMP2A PLA signals, whereas 4MC significantly reduced this increase (Fig. 4F, G). Treatment with 4MC alone did not significantly alter basal GPX4–LAMP2A proximity compared with the control group.

Finally, we examined whether LAMP2A is involved in 4MC-mediated preservation of GPX4 protein. LAMP2A knockdown attenuated the erastin-induced reduction in GPX4, and the addition of 4MC did not further increase GPX4 abundance compared with LAMP2A knockdown alone (Fig. 4H, I). Similarly, bafilomycin A1 restored GPX4 protein levels, while combined treatment with bafilomycin A1 and 4MC produced no further increase (Fig. S5). These non-additive effects are consistent with 4MC acting, at least partly, through an overlapping LAMP2A- and lysosome-dependent process. Collectively, these findings show that 4MC alters GPX4 thermal stability and attenuates the erastin-induced association and proximity between GPX4 and LAMP2A, supporting a mechanism in which 4MC preserves GPX4 protein partly by limiting LAMP2A-associated lysosomal degradation.

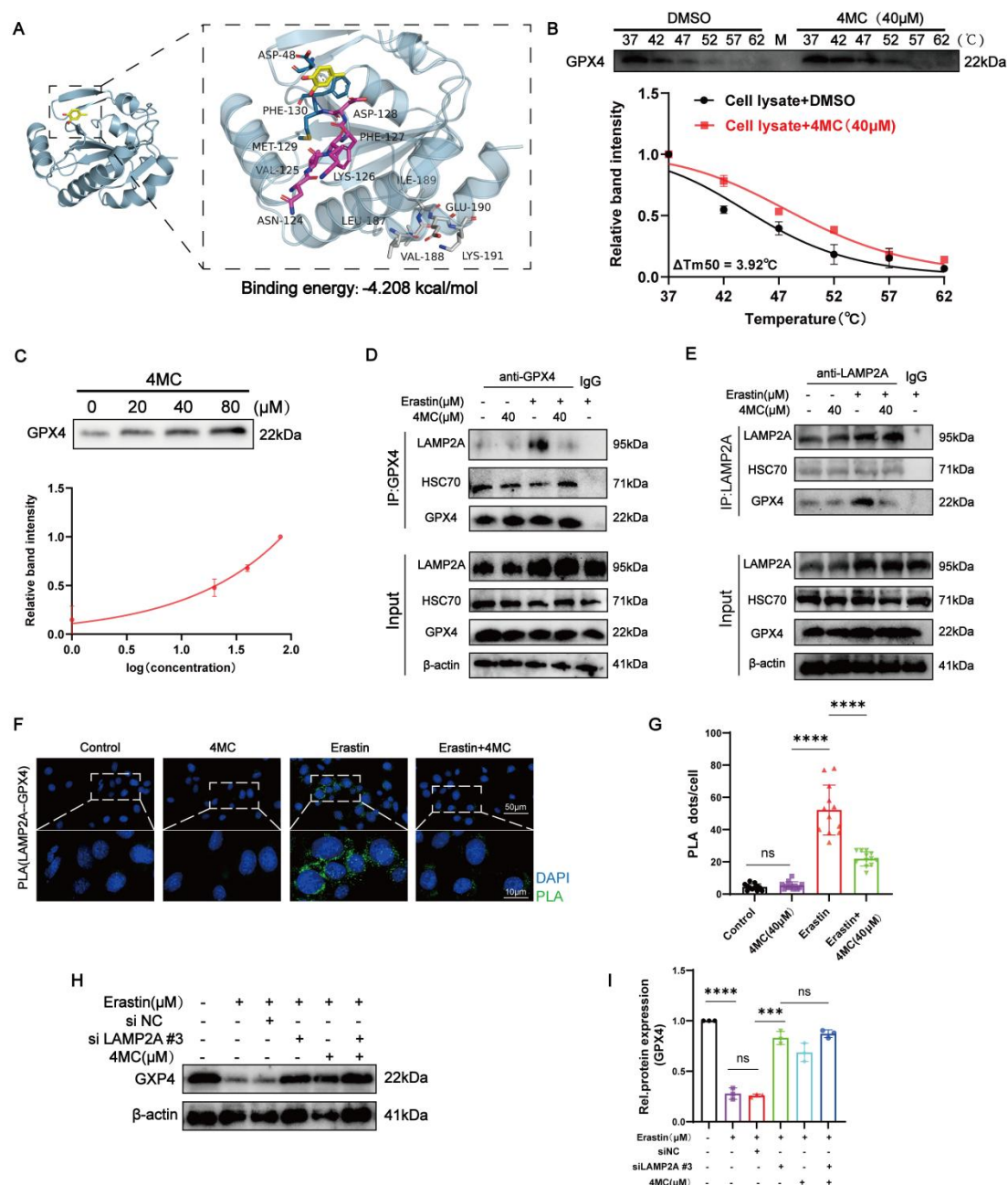


Fig 4. 4MC preserves GPX4 stability and attenuates erastin-induced GPX4–LAMP2A association.

(A) Predicted docking mode of 4MC with GPX4. Binding energy: -4.208 kcal/mol.

(B) CETSA temperature-gradient assay showing that 4MC increased GPX4 thermal

stability, with a ΔTm_{50} of 3.92°C. (C) CETSA concentration-response assay showing

dose-dependent stabilization of soluble GPX4 by 4MC. (D) Co-IP using anti-GPX4 antibodies (or IgG control) demonstrates interactions between GPX4 and LAMP2A or HSC70 in chondrocytes treated with erastin (5 μ M) with or without 4MC (40 μ M). Lysate inputs were probed for indicated proteins. (E) Reciprocal co-IP using anti-LAMP2A antibodies (or IgG control) confirming LAMP2A associations with GPX4 and HSC70 under identical treatment conditions. (F) Representative PLA images visualizing in situ GPX4-LAMP2A interactions in control, 4MC (40 μ M), erastin (5 μ M), and erastin + 4MC groups. Nuclei stained with DAPI. Scale bars: 50 μ m (overview); 10 μ m (magnified images). (G) Quantification of PLA dots per cell corresponding to (F). Data are presented as mean $\pm$ SD from 12 independent primary murine chondrocyte isolations (n = 12). (H) Immunoblot analysis of GPX4 levels in cells transfected with control siRNA (siNC) or LAMP2A-targeting siRNA (siLAMP2A #3), followed by erastin (5 μ M) treatment with or without 4MC (40 μ M). (I) Densitometric quantification of GPX4 protein levels from (H). Statistical significance: Data are presented as mean $\pm$ SD from three independent primary murine chondrocyte isolations (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns = not significant.

5. Intra-articular administration of 4MC attenuates structural deterioration in a murine post-traumatic OA model

To evaluate the in vivo chondroprotective effects of 4MC, we utilized an established anterior cruciate ligament transection (ACLT)-induced OA model in mice. Following surgery, mice received weekly intra-articular injections of vehicle (saline),

low-dose 4MC (4MC-L, 10 µg/knee), or high-dose 4MC (4MC-H, 20 µg/knee) into the affected knee joint. Joint tissues were collected and analyzed at two critical time points, 4 and 8 weeks post-ACLT, to examine early and progressive pathological changes (Fig. 5A).

High-resolution micro-computed tomography (µCT) analysis demonstrated extensive osteophyte formation and disrupted subchondral bone architecture, characterized by increased trabecular spacing and reduced bone mass, in ACLT knees compared to sham-operated controls. Both doses of 4MC significantly mitigated these structural alterations, with dose-dependent efficacy. Compared to the low-dose group, the high-dose 4MC group showed more pronounced suppression of osteophyte formation and improvement in subchondral bone integrity (Fig. 5B). Quantitative µCT measurements at 4 weeks confirmed that ACLT significantly increased osteophyte number and trabecular separation (Tb.Sp), while decreasing bone volume fraction (BV/TV) and trabecular thickness (Tb.Th) (Fig. 5C-F). These protective effects persisted through 8 weeks post-surgery, with fewer osteophytes and improved bone morphology parameters compared with the untreated ACLT group (Fig. 5G-K).

Histological analysis using Safranin O/Fast Green staining revealed significant proteoglycan loss and cartilage surface fibrillation and erosion in ACLT-induced joints compared to sham controls. Conversely, 4MC treatment markedly preserved proteoglycan staining and maintained cartilage structural integrity in a dose-responsive manner (Fig. 5L). Semiquantitative scoring using the OA Research Society International (OARSI) system further supported these observations: both low- and

635 high-dose 4MC groups exhibited significantly lower OARSI scores compared to
636 untreated ACLT animals ($p < 0.01$), with the high-dose group demonstrating the
637 greatest preservation of cartilage structure (Fig. 5M, N). Collectively, these in vivo
638 findings indicate that intra-articular administration of 4MC effectively reduces
639 structural joint degeneration in post-traumatic OA, attenuating osteophyte formation,
640 stabilizing subchondral bone architecture, and preserving ECM integrity.

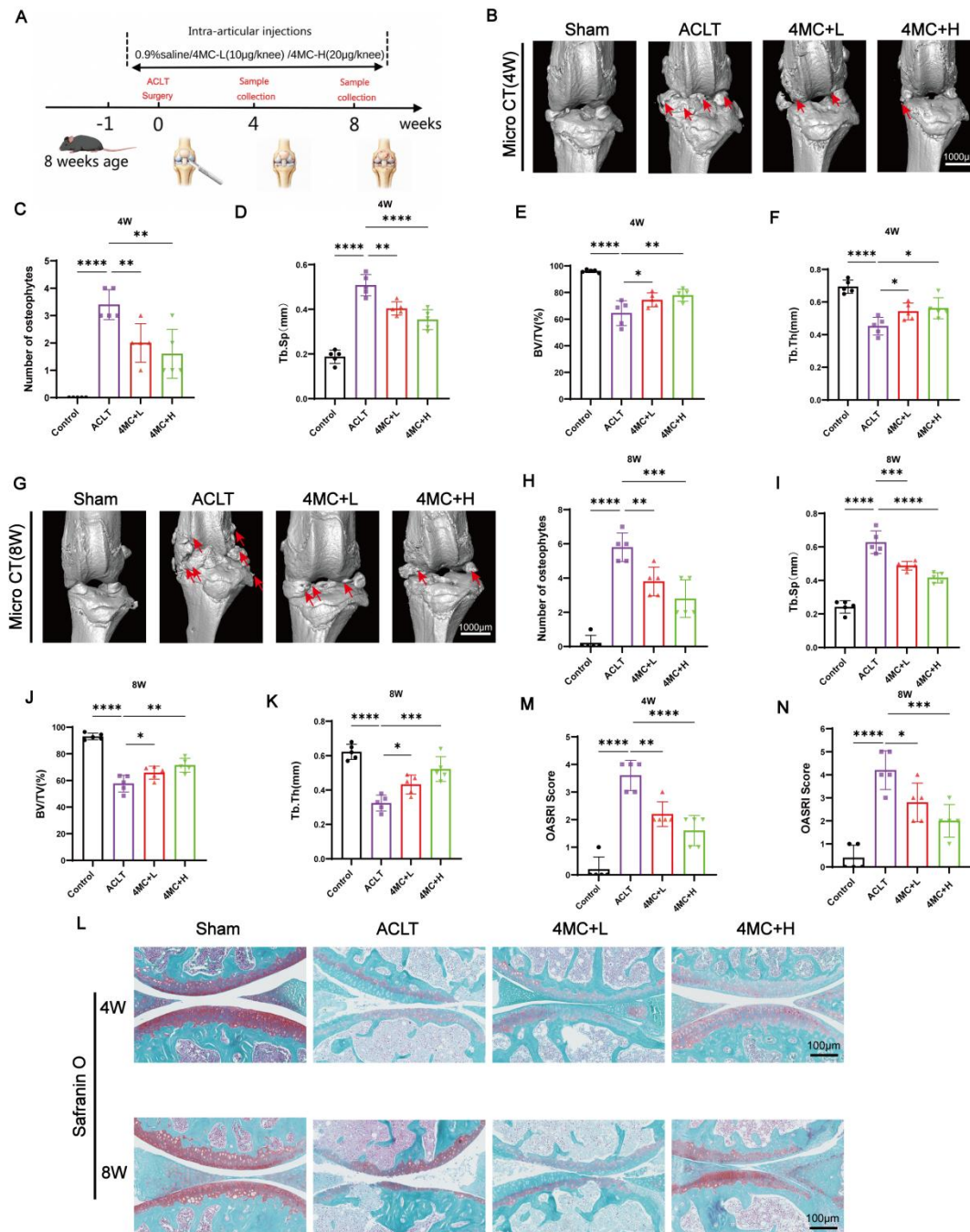


Fig 5. Intra-articular 4MC attenuates structural degeneration in a post-traumatic OA model.

(A) Experimental timeline schematic. Mice underwent sham or ACLT surgery at week 0, followed by weekly intra-articular injections of saline, low-dose 4MC (4MC-L, 10 μg/knee), or high-dose 4MC (4MC-H, 20 μg/knee). Knees were collected at 4 and 8 weeks post-surgery for analysis. (B) Representative 3D μCT reconstructions of

knee joints at 4 weeks post-surgery. Osteophytes indicated by red arrows. Scale bars: 1000 μ m. (C-F) Quantitative μ CT analysis at 4 weeks post-surgery: osteophyte number (C), trabecular separation (Tb.Sp) (D), bone volume fraction (BV/TV) (E), and trabecular thickness (Tb.Th) (F). (G) Representative μ CT reconstructions at 8 weeks post-surgery. Osteophytes indicated by red arrows. Scale bars: 1000 μ m. (H-K) Quantitative μ CT analysis at 8 weeks: osteophyte number (H), Tb.Sp (I), BV/TV (J), and Tb.Th (K). (L) Representative Safranin O/Fast Green-stained sections at 4 and 8 weeks post-surgery, illustrating proteoglycan content and cartilage structural integrity. Scale bar: 100 μ m. (M, N) OARSI histological scores at 4 weeks (M) and 8 weeks (N). Statistical significance: Data are presented as mean $\pm$ SD (n = 5 mice per group). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns = not significant.

6. 4MC attenuates GPX4 loss and lipid peroxidation while restoring cartilage matrix homeostasis in vivo

To determine whether 4MC alleviates ferroptosis-related cartilage injury in vivo, we analyzed articular cartilage after ACLT. IHC showed strong GPX4 expression in sham-operated cartilage, which markedly declined following ACLT at 4 and 8 weeks. Treatment with 4MC partially restored GPX4 immunoreactivity in a dose-dependent manner, with the high-dose (4MC-H) group exhibiting the greatest recovery (Fig. 6A, C, D). Concurrently, staining for the lipid peroxidation marker 4-hydroxynonenal (4-HNE) was elevated in ACLT cartilage. 4MC markedly reduced 4-HNE accumulation, again more effectively in the 4MC-H group (Fig. 6B, E, F). These results indicate that 4MC maintains redox homeostasis in vivo by preserving GPX4 abundance and

669 limiting lipid peroxidation. Improved redox balance correlated with enhanced matrix
670 homeostasis: IF staining demonstrated significant loss of COL2A1 in ACLT cartilage,
671 which 4MC treatment dose-dependently prevented (Fig. 6G-I). In contrast, ACLT
672 increased the expression of matrix-degrading enzyme MMP13, which 4MC
673 significantly suppressed (Fig. 6J-L). RT-qPCR analysis of cartilage tissue further
674 confirmed these observations: ACLT decreased expression of anabolic markers
675 Col2a1 and Acan and increased catabolic markers Mmp13 and Mmp3. 4MC treatment
676 reversed these changes, with high-dose 4MC generally achieving more pronounced
677 normalization (Fig. 6M). Collectively, these findings indicate that intra-articular 4MC
678 administration mitigates OA degeneration by restoring GPX4 protein levels, reducing
679 lipid peroxidation, and rebalancing cartilage matrix turnover toward an anabolic state.

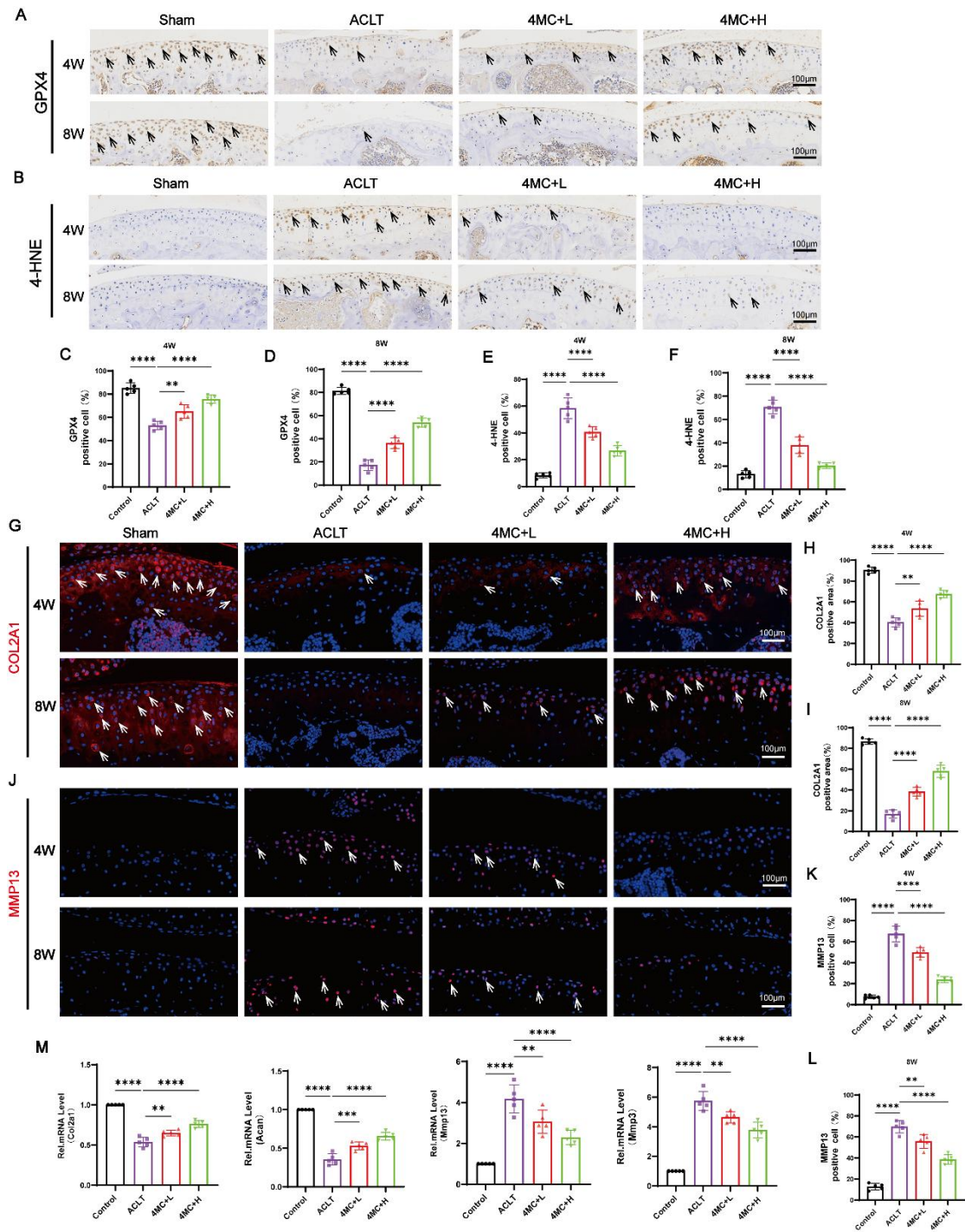


Fig6. 4MC restores GPX4-associated redox balance and cartilage matrix

homeostasis in ACLT-induced osteoarthritis.

(A) Representative IHC staining of GPX4 in articular cartilage from sham-operated,

ACLT, and 4MC-treated mice (4MC-L, 10 µg/knee; 4MC-H, 20 µg/knee) at 4 and

8 weeks post-surgery. Arrowheads indicate GPX4-positive chondrocytes. Scale bars: 100 μ m. (B) Representative IHC staining of 4-HNE in cartilage sections at 4 and 8 weeks. Arrowheads indicate 4-HNE-positive cells. Scale bars: 100 μ m. (C, D) Quantification of GPX4-positive cells (%) at 4 weeks (C) and 8 weeks (D). (E, F) Quantification of 4-HNE-positive cells (%) at 4 weeks (E) and 8 weeks (F). (G) Representative IF staining of COL2A1 (red) with DAPI nuclear counterstaining (blue) in articular cartilage at 4 and 8 weeks. Arrowheads indicate COL2A1-positive regions. Scale bars: 100 μ m. (H, I) Quantification of COL2A1-positive cells (%) at 4 weeks (H) and 8 weeks (I). (J) Representative IF staining of MMP13 (magenta) with DAPI (blue) at 4 and 8 weeks. Arrowheads indicate MMP13-positive cells. Scale bars: 100 μ m. (K, L) Quantification of MMP13-positive cells (%) at 4 weeks (K) and 8 weeks (L). (M) RT-qPCR quantification of Col2a1, Acan, Mmp13, and Mmp3 mRNA in cartilage tissue at 8 weeks post-surgery. Statistical significance: Data are presented as mean $\pm$ SD (n = 5 mice per group). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns = not significant.

Fig7. 4MC protects primary human OA chondrocytes from erastin-induced injury and attenuates GPX4–LAMP2A proximity

To assess the translational relevance of our findings, we evaluated the effects of 4MC in primary human OA chondrocytes. Chondrocytes were isolated from human OA cartilage (Fig. 7A) and exhibited typical adherent morphology in monolayer cultures (Fig. 7B). IF confirmed expression of chondrocyte markers SOX9 and COL2A1 (Fig. 7C). Upon challenge with erastin (1 μ M) (Fig. S6), 4MC dose-

706 dependently improved cell viability, with 40 μ M 4MC exhibiting protection
707 comparable to ferrostatin-1 (Fer-1) (Fig. 7D). Phase-contrast microscopy confirmed
708 that 4MC preserved cellular integrity against erastin-induced damage (Fig. 7E).
709 Mechanistically, erastin enhanced colocalization of GPX4 with LAMP2A, which was
710 effectively suppressed by 4MC treatment (Fig. 7F). PLA provided in situ evidence of
711 increased GPX4-LAMP2A proximity following erastin treatment, and 4MC
712 significantly reduced these proximity signals (Fig. 7G, H). Together, these findings
713 show that 4MC improves cell viability and reduces stress-induced GPX4–LAMP2A
714 proximity in primary human OA chondrocytes, providing supportive human-cell
715 evidence for the observations made in murine chondrocytes.

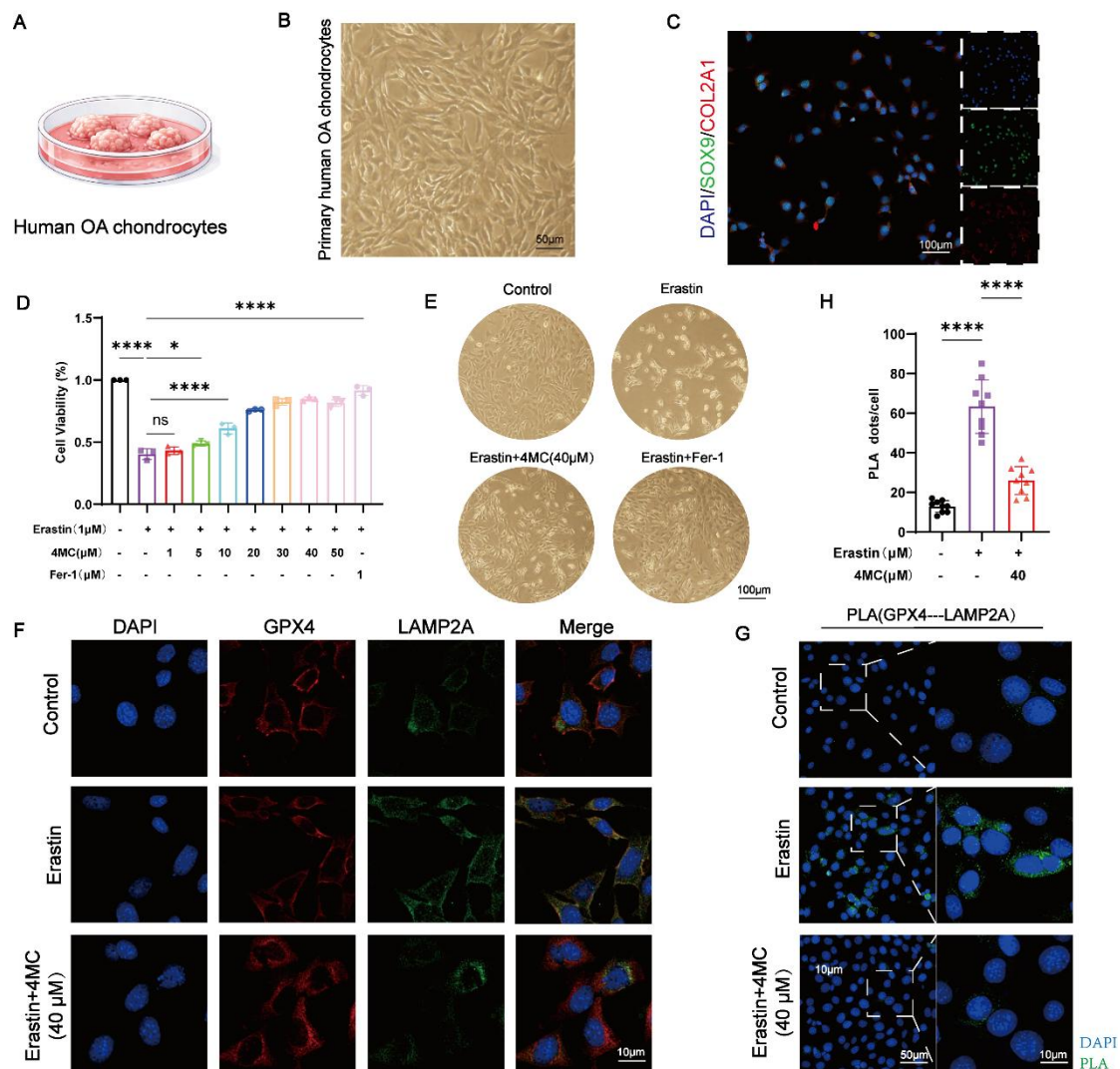


Fig 7. 4MC protects primary human OA chondrocytes from erastin-induced injury and attenuates GPX4-LAMP2A proximity

(A) Schematic illustration of primary human OA chondrocyte isolation from osteoarthritic cartilage specimens. (B) Representative phase-contrast image illustrating morphology of isolated human OA chondrocytes. Scale bars: 50 μm. (C) IF staining confirming chondrocyte identity via expression of SOX9 (green) and COL2A1 (red), with DAPI nuclear staining (blue). Scale bar: 100 μm. (D) Chondrocyte viability assessed by CCK-8 assay after treatment with erastin (1 μM) and increasing concentrations of 4MC (1-50 μM) or Fer-1 (1 μM) for 24 h. Data are presented as mean ± SD from three independent experiments (n = 3). (E)

Representative phase-contrast images of chondrocytes under indicated treatments.

Scale bars: 100 μ m. (F) IF staining of GPX4 (red) and LAMP2A (green) in indicated groups. Nuclei stained with DAPI (blue). Scale bars: 10 μ m. (G) Representative PLA images illustrating GPX4-LAMP2A proximity signals (green dots) in control, erastin-treated, and erastin + 4MC-treated chondrocytes. Scale bars: 50 μ m (overview); 10 μ m (magnified images). (H) Quantification of PLA dots per cell from (G). Data are presented as mean $\pm$ SD; n = 9 independent human OA donors. Statistical significance: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ns = not significant.

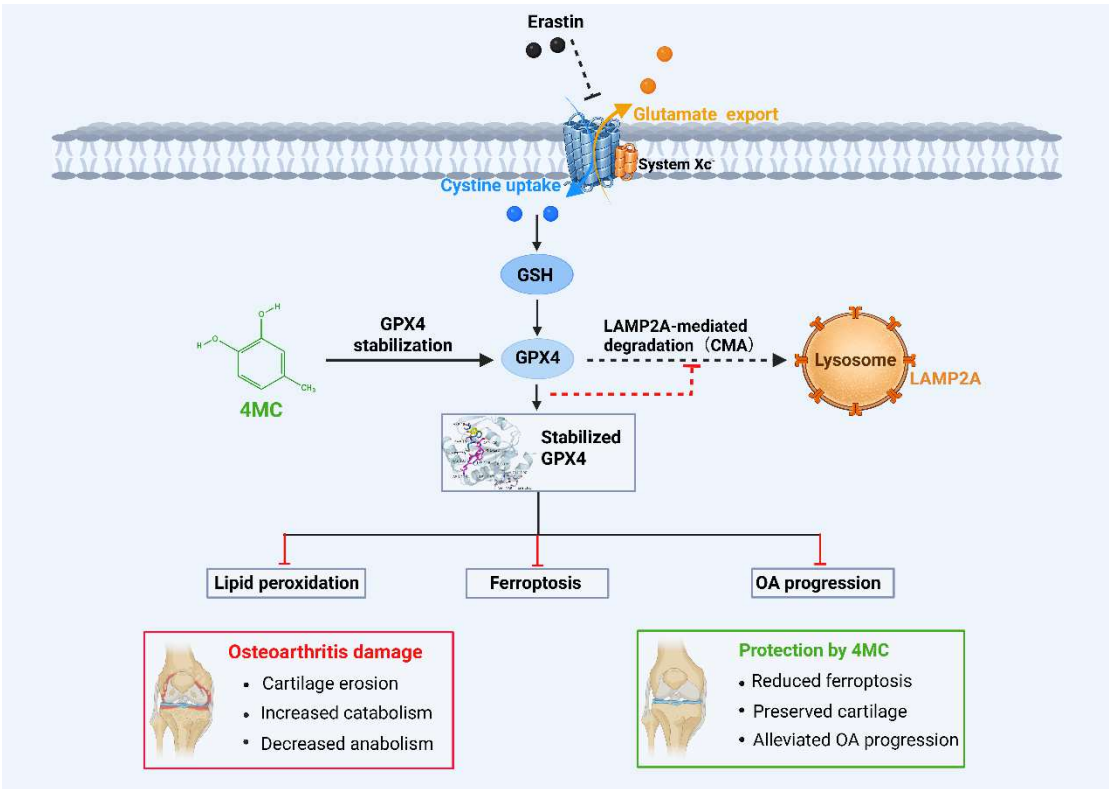


Fig 8. A proposed schematic model illustrating how 4MC preserves GPX4 stability and attenuates ferroptosis-associated cartilage degeneration by reducing stress-induced GPX4-LAMP2A association.

Discussion

OA is a severe and disabling disease characterized by multifactorial pathogenesis

[1,2]. Although mechanical overload is known to induce GPX4 depletion and chondrocyte ferroptosis, thereby contributing to cartilage degradation and OA progression [9], the mechanisms driving GPX4 loss remain poorly understood. In the present study, our data support the involvement of a lysosome- and LAMP2A-dependent process, consistent with chaperone-mediated autophagy (CMA), in GPX4 loss under ferroptotic stress. This process was accompanied by lipid peroxidation, chondrocyte injury, and disruption of extracellular matrix (ECM) homeostasis. In paired human OA cartilage samples, LAMP2A staining was higher in weight-bearing regions than in matched non-weight-bearing regions, indicating an association between LAMP2A abundance and regional cartilage degeneration, although this observation does not by itself establish increased CMA activity. Interestingly, a previous study reported reduced LAMP2A-positive chondrocytes and impaired CMA-related homeostasis in OA cartilage [26]. The apparent difference from our regional comparison may reflect differences in disease stage, cartilage region, mechanical environment, or the distinction between LAMP2A abundance and functional CMA activity. We further found that 4-methylcatechol (4MC) preserved GPX4 abundance, reduced ferroptosis-associated oxidative injury, and improved matrix-related outcomes in cellular and experimental OA models. Together, these findings support a potential link between altered protein homeostasis and ferroptotic injury in OA and suggest that regulation of the LAMP2A-GPX4 axis warrants further investigation.

Articular cartilage is an avascular, specialized connective tissue characterized by chondrocytes embedded within an ECM abundant in collagen and proteoglycans [27].

The biomechanical stability of cartilage depends critically on a delicate equilibrium between anabolic components such as Aggrecan and COL2A1, and catabolic enzymes including MMP13 and ADAMTS5 [28,29]. Disruption of this balance is a central feature of OA pathogenesis and contributes to progressive cartilage degeneration [30]. In our experiments, pharmacological induction of ferroptotic stress reduced chondrocyte viability, increased lipid peroxidation, decreased anabolic matrix markers, and increased catabolic mediators. These changes are consistent with an active contribution of ferroptotic injury to cartilage matrix imbalance, rather than ferroptosis being solely a secondary consequence of oxidative stress [31]. Treatment with 4MC partially corrected these changes and produced effects that, for several readouts, approached those observed with ferrostatin-1. These findings support a chondroprotective effect of 4MC under ferroptotic conditions, but they do not establish 4MC as a disease-modifying OA drug at the present stage.

GPX4 is a central regulator of ferroptosis because it reduces phospholipid hydroperoxides and limits membrane lipid peroxidation[8,9]. Previous OA studies have largely focused on GPX4 expression, enzymatic activity, glutathione availability, or upstream transcriptional regulation[32–34]. Our data suggest that post-translational protein loss also contributes to reduced GPX4 abundance in stressed chondrocytes. The lysosomal inhibitors chloroquine and bafilomycin A1 attenuated erastin-induced GPX4 loss, whereas the proteasome inhibitor MG132 had little effect. Moreover, knockdown of LAMP2A using three independent siRNAs reduced GPX4 depletion. Together with the increased GPX4-LAMP2A association and proximity detected by

co-immunoprecipitation and proximity ligation assays, these findings support the involvement of a LAMP2A-dependent lysosomal process consistent with CMA-mediated GPX4 degradation. This interpretation is biologically plausible because CMA substrates containing KFERQ-like motifs are recognized by cytosolic HSC70 and delivered to the lysosomal membrane, where they bind to LAMP2A for translocation and degradation[35]. Nevertheless, the present study did not directly measure CMA flux, lysosomal uptake of GPX4, or the functional requirement of the predicted CMA-recognition motifs. Accordingly, the data support CMA involvement but do not fully define each step of GPX4 substrate recognition and translocation. Previous studies have implicated CMA-associated GPX4 degradation in other disease contexts [32,33]; our results extend this concept to OA-related chondrocyte injury and suggest that CMA may exert context-dependent effects on redox homeostasis in cartilage.

The present findings also provide insight into how 4MC may influence GPX4 stability. 4MC is a catechol derivative with reported antioxidant and anti-inflammatory properties, and our previous work showed that it ameliorated experimental inflammatory arthritis through effects on macrophage-associated pathways[19]. In the current study, molecular docking predicted a potential 4MC-binding pocket within GPX4 near a putative CMA-recognition motif. This computational result was hypothesis-generating rather than proof of a specific binding interface [36]. A lysate-based cellular thermal shift assay showed that 4MC altered GPX4 thermal stability and increased the soluble GPX4 fraction in a concentration-

related manner. Co-immunoprecipitation and proximity ligation assays further showed that 4MC attenuated the erastin-induced increase in GPX4-LAMP2A association and proximity. In addition, LAMP2A knockdown preserved GPX4 under ferroptotic stress, and the addition of 4MC did not produce a clear further increase. A similar non-additive pattern was observed with bafilomycin A1. These observations are consistent with 4MC and LAMP2A or lysosomal inhibition acting through an overlapping process. However, they do not establish direct binding of 4MC to GPX4, blockade of a specific CMA-recognition motif, selective inhibition of GPX4 translocation, or absence of effects on other CMA substrates. A cautious interpretation is that 4MC preserves GPX4 partly by altering its stability and reducing its stress-induced association with LAMP2A, thereby attenuating LAMP2A-associated lysosomal degradation.

The effects of 4MC were reproduced across primary murine chondrocytes, an ACLT-induced post-traumatic OA model, and primary human OA chondrocytes. In vivo, intra-articular 4MC reduced osteophyte formation, improved subchondral bone parameters, preserved cartilage proteoglycan staining, and lowered OARSI scores. These structural improvements were accompanied by restored GPX4 staining, reduced 4-HNE accumulation, increased COL2A1, and reduced MMP13 expression. The in vivo findings therefore support an association between 4MC treatment, improved redox-related markers, and preservation of cartilage matrix homeostasis. They do not, however, prove that the therapeutic effects of 4MC in the joint are mediated exclusively through the chondrocyte LAMP2A-GPX4 pathway. Given the

830 previously reported anti-inflammatory actions of 4MC [19], effects on synovial cells,
831 macrophages, or other joint tissues may also contribute. In primary human OA
832 chondrocytes, 4MC improved erastin-impaired viability and reduced the erastin-
833 associated increase in GPX4-LAMP2A proximity. These results provide supportive
834 human-cell evidence, although confirmation in a larger, well-characterized donor
835 cohort will be required to establish generalizability and determine whether patient-
836 related factors influence responsiveness.

837 Several limitations should be considered. First, erastin was used as a controlled
838 inducer of ferroptotic stress [37], but the physiological signals that promote LAMP2A-
839 associated GPX4 loss in OA remain unclear. Mechanical overload, inflammatory
840 mediators, metabolic stress, and aging-related changes may act alone or in combination,
841 and the higher LAMP2A staining observed in weight-bearing cartilage provides only
842 indirect support for a relationship with mechanical stress. Second, direct assessment of
843 CMA flux, lysosomal GPX4 uptake, LAMP2A multimerization [17], and mutational
844 analysis of the predicted KFERQ-like motifs will be required to define GPX4 as a CMA
845 substrate more conclusively in chondrocytes. Third, docking and lysate-based CETSA
846 do not establish direct target binding or the precise 4MC-GPX4 binding site [24,38].
847 Orthogonal target-engagement approaches, such as surface plasmon resonance,
848 microscale thermophoresis, drug-affinity responsive target stability, or chemical-probe
849 pull-down, would strengthen this aspect of the mechanism. Fourth, the in vivo
850 dependence of 4MC efficacy on LAMP2A or GPX4 was not directly tested. Cartilage-
851 specific genetic or pharmacological manipulation of this pathway would help

distinguish causal pathway dependence from parallel anti-inflammatory or antioxidant effects. Fifth, only female mice and a single post-traumatic OA model were used; validation in both sexes and in additional OA models would improve generalizability. Finally, pharmacokinetic behavior, joint retention, local and systemic safety, functional outcomes, and long-term efficacy after intra-articular administration remain to be determined before the translational potential of 4MC can be evaluated.

Taken together, our findings support a model in which ferroptotic stress is accompanied by LAMP2A-dependent, lysosome-associated GPX4 loss in chondrocytes, thereby contributing to lipid peroxidation and cartilage matrix imbalance. 4MC preserved GPX4 abundance, attenuated GPX4-LAMP2A association, and improved ferroptosis-associated and structural outcomes in experimental OA. These data identify the LAMP2A-GPX4 interface as a potential regulatory component of OA-related ferroptotic injury and provide a rationale for further investigation of 4MC as a pharmacological modulator of this process. Additional mechanistic and preclinical studies are required before this pathway or compound can be considered a disease-modifying therapeutic strategy.

Declarations

Consent for publication

All authors have seen the manuscript and approved to submit to your journal.

Ethical Approval

All animal research procedures were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Shandong First Medical University. Animal experiments were approved under protocol no. 2025121101, and human sample collection was approved under protocol no. 2025S915.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

Zhendong Ying and **Shufeng Li** contributed equally to this work. **Zhendong Ying**: Writing – original draft, Methodology, Investigation, Data curation, Formal analysis, Visualization. **Shufeng Li**: Writing – original draft, Methodology, Investigation, Data curation, Formal analysis. **Tiantian Tang**: Methodology, Investigation, Data curation, Visualization. **Wenqing Li**: Methodology, Investigation, Data curation, Formal analysis. **Linson Teng**: Methodology, Investigation, Data curation. **Dailin Chen**: Methodology, Investigation, Resources. **Kuocheng Xu**: Methodology, Investigation, Resources. **Lei Zhang**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Informed consent

Informed consent was obtained from all individual participants included in the study.

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