

Aberrant mechanical stress promotes intervertebral disc degeneration through HUWE1-mediated K48-linked polyubiquitination of CRYAB

Zhicheng Liu

Qilu Hospital of Shandong University

Qunbo Meng

Qilu Hospital of Shandong University

Jiankang Cao

Qilu Hospital of Shandong University

Yifeng Shang

Qilu Hospital of Shandong University

Kaiwen Liu

Qilu Hospital of Shandong University

Haoxin Zhai

Qilu Hospital of Shandong University

Yunpeng Zhao

Qilu Hospital of Shandong University

Yuhua Li

Qilu Hospital of Shandong University

Zexin Wang

Qilu Hospital of Shandong University

Yiming Zhang

Qilu Hospital of Shandong University

Zuyang Wang

Qilu Hospital of Shandong University

Lian Liu

Qilu Hospital of Shandong University

Zhijian Wei

Qilu Hospital of Shandong University

Lianlei Wang

Qilu Hospital of Shandong University

Yuanqiang Zhang

Qilu Hospital of Shandong University

Lei Cheng

Qilu Hospital of Shandong University

Yuan Gao

ygao@email.sdu.edu.cn

Qilu Hospital of Shandong University

Research Article

Keywords: IVDD, CRYAB, Ubiquitination, HUWE1, Mechanical stress

Posted Date: October 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10868611/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

**Aberrant mechanical stress promotes intervertebral disc
degeneration through HUWE1-mediated K48-linked
polyubiquitination of CRYAB**

**Zhicheng Liu^{1,2†}, Qunbo Meng^{1†}, Jiankang Cao^{3†}, Yifeng Shang^{1†}, Kaiwen Liu¹, Haoxin
Zhai¹, Yunpeng Zhao¹, Yuhua Li¹, Zexin Wang¹, Yiming Zhang¹, Zuyang Wang¹, Lian Liu¹,
Zhijian Wei¹, Lianlei Wang^{1*}, Yuanqiang Zhang^{1*}, Lei Cheng^{1*}, Yuan Gao^{1*}**

¹Department of Orthopedic, Qilu Hospital of Shandong University, Jinan 250012, China.

² Articular Orthopaedics, Third Affiliated Hospital of Soochow University, Changzhou, People's
Republic of China.

³Department of Pain, Qilu Hospital of Shandong University, Jinan 250012, China.

† These authors contributed equally to this work.

* Correspondence Author:

Lianlei Wang, Email: wllspine@163.com

Yuanqiang Zhang, Email: drzhyq@126.com

Lei Cheng, Email: chenglei@email.sdu.edu.cn

Yuan Gao, Email: ygao@email.sdu.edu.cn

18 Abstract

19 Background

20 Aberrant mechanical stress is a critical initiator of intervertebral disc degeneration (IVDD), yet
21 the molecular mechanisms driving this process remain incompletely understood. The small heat
22 shock protein CRYAB has been implicated in cellular stress responses, but its role and regulation
23 in mechanically induced IVDD have not been defined. This study aims to investigate the role and
24 regulatory mechanism of CRYAB in mechanically induced IVDD, focusing on post-translational
25 modifications and protein stability control.

26 Methods

27 Integrative single-cell RNA-seq (scRNA-seq) was performed on human nucleus pulposus (NP)
28 tissues across different stages of degeneration. The role of CRYAB under mechanical stress was
29 assessed using gain- and loss-of-function approaches in vitro and *Cryab* conditional knockout mice.
30 Mechanistic studies examined ubiquitination, HUWE1 E3 ligase activity, and the interaction with
31 14-3-3 ϵ . A dual screening strategy was used to identify small molecules modulating CRYAB
32 expression and stability, with lead compound validation in vivo.

33 Results

34 scRNA-seq revealed a progressive loss of CRYAB-expressing NP cells with increasing disease
35 severity, correlating with activation of proteasome-mediated ubiquitin-dependent proteolysis and
36 inflammatory pathways. Mechanical stress downregulated CRYAB, and CRYAB deficiency
37 accelerated IVDD by promoting NP cell apoptosis via NF- κ B signaling. Mechanistically, HUWE1
38 mediated K48-linked polyubiquitination and proteasomal degradation of CRYAB, whereas 14-3-3 ϵ
39 stabilized CRYAB by competitively inhibiting its interaction with HUWE1. Finally, Ginkgolide B
40 was identified as a small molecule that enhances CRYAB expression and stability, effectively
41 ameliorating IVDD in vivo.

42 Conclusions

43 We uncover a 14-3-3 ϵ –HUWE1–CRYAB axis that governs NP cell fate under aberrant
44 mechanical stress. Mechanical overload shifts CRYAB from a 14-3-3 ϵ -stabilized state to
45 HUWE1-mediated degradation, leading to NF- κ B-driven catabolism and apoptosis and accelerating
46 IVDD. Ginkgolide B, a dual-action stabilizer of CRYAB, counteracts this pathogenic mechanism
47 and represents a promising therapeutic strategy.

48 Key Words: IVDD; CRYAB; Ubiquitination; HUWE1; Mechanical stress

49

50 Introduction

51 Low back pain is the leading cause of disability worldwide, imposing a substantial
52 socioeconomic burden and severely compromising patients' quality of life[1]. Intervertebral disc
53 degeneration (IVDD) is recognized as a primary pathological basis for this condition[2]. The
54 intervertebral disc, particularly the nucleus pulposus (NP), physiologically withstands and
55 dissipates the complex mechanical loads of the spine[3, 4]. However, prolonged or abnormal
56 mechanical stress serves as a critical environmental risk factor driving IVDD[5, 6]. Such aberrant
57 stress triggers a cascade of pathological responses in NP cells, including inflammatory activation,
58 extracellular matrix (ECM) degradation, and cellular senescence or death, ultimately leading to
59 structural disruption and functional loss of the disc[7-9]. Although initial mechanotransduction
60 pathways—such as those involving integrins and ion channels—have been explored, the deeper
61 molecular mechanisms by which abnormal stress precisely regulates the stability of key effector
62 proteins to determine cell fate and tissue homeostasis remain unclear[10-12].

63 The small heat shock protein α B-crystallin (CRYAB) plays a central role in cellular stress
64 protection[13]. It functions not only as a molecular chaperone, facilitating the refolding of
65 misfolded proteins, but also modulates various signaling pathways, including NF- κ B and MAPK,
66 to suppress apoptosis and inflammation[14-18]. Recent studies have demonstrated altered CRYAB
67 expression and its protective effects in several degenerative diseases, such as osteoarthritis and
68 atherosclerosis[19, 20]. Our preliminary observations further revealed its downregulation in NP
69 tissues from degenerated discs; however, its specific function in response to mechanical stress, its
70 regulatory mechanisms, and its precise role in IVDD have not been systematically elucidated. This
71 suggests that CRYAB may serve as a critical node linking mechanical stress to disc cellular
72 homeostasis.

73 Intracellular protein homeostasis is meticulously regulated by the ubiquitin-proteasome system
74 (UPS)[21]. Within this system, E3 ubiquitin ligases act as key rate-limiting factors that determine
75 protein fate by specifically recognizing and catalyzing ubiquitination of their substrates, making
76 them attractive therapeutic targets[22]. HUWE1, an important HECT-type E3 ligase, regulates
77 numerous substrates including tumor suppressors and metabolic enzymes, thereby profoundly
78 influencing cell proliferation, apoptosis, and stress responses[23, 24]. Nevertheless, whether
79 HUWE1 targets CRYAB for ubiquitination in the context of disc degeneration remains unknown.
80 Conversely, the 14-3-3 protein family comprises highly conserved adaptor molecules that modulate
81 the subcellular localization, stability, and activity of client proteins by recognizing
82 phosphoserine/threonine motifs[25, 26]. Notably, emerging evidence suggests that 14-3-3 isoforms
83 can stabilize proteins by competitively binding to the same substrate as an E3 ligase[27-29]. This
84 leads to a pivotal hypothesis: within NP cells responding to abnormal mechanical stress, a dynamic
85 regulatory switch composed of HUWE1 and 14-3-3 ϵ exists, which antagonistically controls the
86 ubiquitination and stability of CRYAB, thereby determining the fate of disc cells and tissue
87 homeostasis.

88 To investigate this hypothesis, we first performed integrative single-cell RNA sequencing
89 (scRNA-seq) analysis of human NP tissues across different stages of degeneration. Our analysis
90 revealed a progressive loss of CRYAB-expressing NP cells with increasing disease severity, which
91 correlated with activation of proteasome-mediated ubiquitin-dependent proteolysis and
92 inflammatory pathways. Through a combination of in vitro and in vivo experiments, we uncovered
93 that mechanical stress downregulates CRYAB, and that CRYAB deficiency accelerates IVDD by
94 promoting NP cell apoptosis via NF- κ B signaling. Mechanistically, we identified HUWE1 as the
95 E3 ligase mediating K48-linked polyubiquitination and subsequent proteasomal degradation of
96 CRYAB, and found that 14-3-3 ϵ stabilizes CRYAB by competitively inhibiting its interaction with

97 HUWE1. Furthermore, through a dual screening strategy, we identified Ginkgolide B as a small
98 molecule that enhances CRYAB expression and stability, effectively ameliorating IVDD in vivo.

99 Collectively, this study is the first to uncover the central role of a novel “14-3-3 ϵ -HUWE1-
100 CRYAB” proteostatic axis in stress-induced IVDD. It elucidates the molecular logic by which
101 mechanical stress accelerates degeneration by disrupting this fine-tuned regulatory network. These
102 findings not only provide a fresh perspective for understanding the pathogenesis of IVDD but also
103 lay a solid theoretical foundation for developing disease-modifying therapies centered on
104 stabilizing CRYAB.

105 **Materials and Methods**

106 **Clinical Nucleus Pulposus Specimen Collection and Processing**

107 Human intervertebral disc samples were obtained from patients who underwent lumbar spine
108 surgery at Qilu Hospital of Shandong University. Relatively normal human lumbar disc tissues
109 were collected from five patients with acute lumbar disc herniation or adolescent idiopathic
110 scoliosis without intervertebral disc degeneration (age range: 16–36 years, Pfirrmann grade II).
111 Degenerated nucleus pulposus samples were obtained from ten patients with intervertebral disc
112 degenerative degeneration (age range: 45–70 years, Pfirrmann grade IV). The degree of disc
113 degeneration was assessed using the Pfirrmann grading system. All procedures in this study were
114 approved by the Medical Ethics Committee of Qilu Hospital of Shandong University. All patients
115 participating in the study provided informed consent and voluntarily agreed to participate.

116 Nucleus pulposus tissue samples were obtained from six patients with grade II intervertebral disc
117 degeneration and six patients with grade IV intervertebral disc degeneration. The samples were
118 initially fixed with 10% formaldehyde for 48 hours. Subsequently, paraffin-embedded sections
119 were cut into 6 μ m thick slices for subsequent histological staining.

120 **In vitro experiments**

121 **Antibodies and Reagents**

122 Fetal bovine serum (FBS), and 1% penicillin-streptomycin were sourced from Procell (China).
123 High-glucose Dulbecco’s modified Eagle medium (DMEM; 4.5 g/L glucose) was purchased from
124 Gibco (China). Reverse transcription kit and SYBR Green PCR Master Mix were purchased from
125 Toyobo (Japan). RIPA Lysis Buffer was purchased from Servicebio Corporation (China). BCA
126 Protein Assay Kit, Nuclear and Cytoplasmic Protein Extraction Kit, blocking buffer, Triton X-100,
127 DAPI, TUNEL apoptosis detection kit and Coomassie Blue were purchased from Beyotime (China).
128 Cell Counting Kit-8 (CCK-8) was purchased from Elabscience (USA). Polyvinylidene fluoride
129 membrane and enhanced chemiluminescence were purchased from Millipore (Germany). Lipo8000
130 Transfection Reagent was purchased from Beyotime (China). M-PER™ Mammalian Protein
131 Extraction Reagent was purchased from the Thermo Fisher Scientific (USA). Hematoxylin and
132 eosin staining kit (G1120) was purchased from Solarbio (China). Phalloidin was purchased from
133 Solarbio (China). Protein A&G magnetic beads and MG-132 were purchased from Selleck
134 (Germany). Cycloheximide was purchased from Sigma-Aldrich (USA). All other reagents were
135 sourced from standard commercial suppliers.

136 **Cell Resources and Cell Culture**

137 Human embryonic kidney 293T (HEK293T) cells were purchased from Zhongqiao Xinzhou
138 Biotechnology Co., Ltd. (Shanghai, China; Cat. No. ZQ0098). Cells were cultured in High-glucose

DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was replaced every 2 days. Cells were subcultured at 80–90% confluence using 0.25% trypsin-EDTA and seeded at appropriate densities for subsequent experiments. NP cells were isolated from tissue samples obtained from patients diagnosed with Pfirrmann grade II intervertebral disc herniation. The surgically harvested NP tissues were transported to a clean laboratory in 0.9% sodium chloride solution. Upon arrival, the tissues were washed three times with sterile phosphate-buffered saline (PBS; G0002, CytoBio, China). Subsequently, the samples were digested with 0.25% trypsin-EDTA (G4001, CytoBio, China) for 30 minutes. Following this, the tissues were further digested with 0.2% type II collagenase (Invitrogen, USA) in DMEM/F-12 medium for 1 hour at 37°C with gentle agitation at 75 rpm. Following digestion, the cell suspension was passed through a 70-µm cell strainer (Corning, USA) to remove undigested debris and centrifuged at 1200 rpm for 5 minutes, and the resulting cell pellet was resuspended in complete culture medium (DMEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin). The isolated NP cells were counted and subsequently seeded into T25 culture flasks, maintained in a humidified incubator at 37°C with 5% CO₂.

In vitro mechanical stress culture model

NP Cells were pre-seeded onto 14 mm or 24 mm cell slides, which were placed on a holder. The cell slides and holder were then placed together in a sealed chamber filled with complete culture medium. The top of the sealed chamber was covered with a deformable rubber gasket to apply pressure to the liquid inside. A graded mechanical stress was applied to the cells for 1 hour at a frequency of 1 Hz using a pneumatic component (FESTO, Germany). For the load-response experiments, magnitudes of 0.5, 1.0, 1.5, and 2.0 MPa were applied. In specific mechanistic assays, the Piezo1 agonist Yoda1 (0–20 µM; Sigma, USA) was utilized to chemically mimic mechanical activation. After pressurization, the cells were transferred into freshly prepared complete culture medium.

Plasmid Construction, Virus Packaging, siRNA and Cell Transfection/Infection

Myc-CRYAB, HA-Ub, Flag-HUWE1, and Flag-14-3-3ε expression plasmids were constructed by subcloning the corresponding cDNAs into pcDNA3.1 vector (Invitrogen, USA). The CRYAB K150R/K161R mutant was generated using site-directed mutagenesis (Vigene Biosciences, China). All constructs were verified by DNA sequencing. Cells seeded on the plate overnight were transfected with plasmids using Lipo8000™ Transfection Reagent (C0533-0.5ml; Beyotime, China) according to the manufacturer's instructions. Cells were harvested at 24–72h post transfection for future experiments.

Primary NP cells were seeded in 6-well plates at a density of 2.5×10^5 cells per well and transfected with 1 µg of CRYAB small interfering RNA (CRYAB-siRNA), HUWE1 small interfering RNA (HUWE1-siRNA), 14-3-3ε small interfering RNA (14-3-3ε-siRNA), or a non-specific scrambled control siRNA (NC-siRNA). Transfection was performed using Lipo8000™ Transfection Reagent (C0533-0.5ml; Beyotime, China) according to the manufacturer's instructions, followed by incubation for the indicated durations. Efficiency was validated 48–72h post-transfection by RT-qPCR and immunoblotting.

For overexpression of CRYAB in vitro, adenovirus (ADV-CRYAB) was synthesized under the control of the CMV promoter (Vigene Biosciences, China). The successful overexpression of exogenous genes was measured by immunoblotting.

183 **RNA Extraction and RT-qPCR**

184 After various treatments, total RNA was extracted from the cells of each group using TRIzol
185 reagent (Invitrogen, USA) according to the manufacturer's instructions. Subsequently, RNA
186 samples were reverse transcribed into complementary DNA (cDNA) using a reverse transcription
187 kit (Toyobo, Japan). The obtained cDNA was stored at -20°C. Quantitative real-time PCR (RT-
188 qPCR) was then performed using SYBR Green PCR Master Mix on a Bio-Rad CFX 96 system
189 (Bio-Rad, Hercules, CA, USA). The expression levels of target genes were determined using the
190 $\Delta\Delta C_t$ method, and the results were subjected to statistical analysis. Specifically, the C_t values of
191 target genes were first normalized to the endogenous control gene GAPDH using the ΔC_t method
192 to correct for differences in RNA input and reverse transcription efficiency. Specific primers for
193 the nucleotide sequences of COL2, ACAN, COX-2, iNOS, MMP13, ADAMTs-5, P21 and P16 are
194 listed in Supplementary table 1.

195 **RNA-seq and Bioinformatic Analysis**

196 RNA sequencing was performed by Lianchuan Biotechnology Co., Ltd (China). Differential
197 expression gene (DEG) analysis was conducted using the DEGSeq method, with a threshold set at
198 $P\text{-value} < 0.05$ and $|\text{Fold Change}| > 2$. Enrichment analysis of DEGs was performed using Gene
199 Ontology (GO, <http://www.geneontology.org/>) and the Kyoto Encyclopedia of Genes and Genomes
200 (KEGG, <https://www.kegg.jp/>) databases to explore their potential roles in phenotypic changes.
201 Gene Set Enrichment Analysis (GSEA) was employed to identify significantly enriched biological
202 pathways among the DEGs. All bioinformatics analyses were completed using the OmicStudio
203 platform (<https://www.omicstudio.cn>).

204 **scRNA-seq Data Analysis**

205 Open-access scRNA-seq profiles of human NP tissues were retrieved from the Gene Expression
206 Omnibus. The analytical cohort integrated three healthy (Pfirrmann grade I; GSE160756) and eight
207 degenerated (Pfirrmann grades II–V; GSE165722) specimens. Computational processing of the raw
208 count matrices was conducted in the R environment (version 4.4.1) utilizing the Seurat package
209 (v5). Following the application of standard quality control metrics to exclude artifactual and low-
210 viability cells, cross-study technical variations were mitigated through batch-effect correction
211 algorithms, enabling unbiased dataset integration. Unsupervised clustering and cell-type annotation
212 were performed based on canonical marker expression profiles. The transcriptomic landscape of
213 IVDD progression was further resolved through differential gene expression analysis, paired with
214 comprehensive functional evaluations including GO, KEGG pathway mapping, and GSEA.

215 **Immunoblotting**

216 After cell culture and treatment, cells were lysed in RIPA lysis buffer (G2002, Servicebio, China)
217 supplemented with a protease inhibitor cocktail (78429, Thermo Fisher Scientific, USA) for 30
218 minutes. The lysates were then centrifuged at 13,000 rpm for 30 minutes at 4°C, and the
219 supernatants were collected. Protein concentrations were determined using a BCA Protein Assay
220 Kit (P0010, Beyotime).

221 Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel
222 electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (Millipore,
223 USA). The membranes were blocked with 5% non-fat milk or Protein Free Rapid Blocking Buffer
224 (PS108P, Epizyme, China) for 1 hour at room temperature.

Subsequently, the membranes were incubated overnight at 4°C with primary antibodies targeting the following proteins: β -actin (1:10000, 10494-1-AP, Proteintech), GAPDH (1:10000, 10494-1-AP, Proteintech), CRYAB (1:2000, 15808-1-AP, Proteintech), COL2 (1:500, A19308, Abclonal), ACAN (1:1000, 13880-1-AP, Proteintech), MMP13 (1:1000, 83188-4-RR, Proteintech), ADAMTs-5 (1:1000, A2836, Abclonal), iNOS (1:1000, 18985-1-AP, Proteintech), COX-2 (1:1000, 12375-1-AP, Proteintech), P16 (1:1000, 10883-1-AP, Proteintech), P21 (1:1000, a5682, Abclonal, China), 14-3-3 ϵ (1:1000, 11648-2-AP, Proteintech), Myc (1:5000, 60003-2-Ig, Proteintech), FLAG (1:1000, AE121, Abclonal), HA (1:5000, 51064-2-AP, Proteintech), p65 (1:1000, A19653, Abclonal), p-p65 (1:1000, #3033S, Cell Signaling Technology), HUWE1 (1:500, 19430-1-AP, Proteintech), I κ B α (1:1000, 10268-1-AP, Proteintech), p-I κ B α (1:1000, 82349-1-RR, Proteintech), Bcl-2 (1:500, A0208, Abclonal), lamin B1 (1:1000, 12987-1-AP, Proteintech), Bax (1:1000, 50599-2-Ig, Proteintech), Cleaved Caspase 3 (1:1000, 68773-1-Ig, Proteintech) and ubiquitin (1:1000, 10201-2-AP, Proteintech). After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000, AS014, Abclonal) for 1 hour at room temperature. Protein bands were visualized using an ECL Plus Kit (Thermo Fisher Scientific, USA), and chemiluminescence signals were subsequently captured on Tanon 4800 Imaging System (USA). Densitometric analysis of the bands was performed using Image J software from three independent experiments, and protein expression levels were normalized to GAPDH or β -actin.

For Nuclear Translocation of NF- κ B, The localization of p65 in NP cells was assessed by immunofluorescence staining. The expression levels of p65, Lamin B1, and GAPDH were detected by Western blot analysis.

Co-Immunoprecipitation (Co-IP) Assay

For Co-IP, total cellular proteins were first extracted using lysis buffer. The lysates were then incubated overnight at 4°C with antibody, or control IgG. Subsequently, the mixture was incubated overnight at 4°C with Protein A&G magnetic beads (Cat# B23201, Selleck, Germany). The immunoprecipitates were washed four times with lysis buffer and then subjected to immunoblotting analysis using the indicated antibodies.

Protein-Protein Interaction Profiling by LC-MS/MS

To identify proteins interacting with CRYAB, NP cells overexpressing CRYAB via ADV infection were lysed and subjected to immunoprecipitation using an anti-CRYAB antibody. The immunoprecipitated complexes were resolved by SDS-PAGE, and gel lanes were excised into fractions. Proteins were in-gel digested with trypsin, and the resulting peptides were extracted and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA) coupled with an EASY-nLC 1000 system. The MS data were searched against the SwissProt human protein database using MaxQuant software. Proteins identified in at least two out of three independent immunoprecipitation-mass spectrometry (IP-MS) experiments with a peptide count ≥ 2 were considered as candidate CRYAB-interacting partners.

CHX Chase Assay

To assess protein stability, cells were treated with 50 μ g/mL cycloheximide (CHX, Sigma-Aldrich, Cat# C4859) for the indicated time periods (0, 2, 4, 8 h). In some experiments, cells were pre-treated with 10 μ M MG-132 (Selleck, Cat# S2619) for 2 h before CHX addition to inhibit proteasomal degradation. At each time point, cells were harvested and lysed, and protein levels were analyzed by immunoblotting with indicated antibodies. Band intensities were quantified using

ImageJ software and normalized to the loading control. Target protein half-lives were mathematically derived via non-linear regression analysis of normalized densitometric data utilizing GraphPad Prism software.

Microscale Thermophoresis (MST) Assay

MST assays were performed using a Monolith instrument (NanoTemper Technologies, Germany) to quantitatively evaluate direct molecular interactions. Protein-protein and small molecule-protein binding systems were established according to the specific research objectives[49].

MST Analysis of Protein-Protein Interactions

A purification-free MST strategy utilizing cell lysates was employed to assess the direct interaction of HUWE1 or 14-3-3ε with CRYAB. cDNAs encoding human CRYAB, HUWE1, and 14-3-3ε were cloned into the pcDNA3.1 vector with an N-terminal His tag. Plasmids were transiently transfected into HEK293T cells. After 48 h, cells were harvested and lysed using a non-denaturing lysis buffer containing protease inhibitors to obtain crude cell extracts. His-CRYAB was fluorescently labeled using the Monolith His-Tag Labeling Kit RED (MO-L018, NanoTemper) following the manufacturer's protocol. After labeling, the fluorescence intensity was measured and adjusted to an appropriate range (200–1500 fluorescence units, FU). To minimize non-specific interference, all samples were prepared in the same lysis buffer background, and lysates from empty vector-transfected cells were used as negative controls. Ligand proteins (HUWE1 or 14-3-3ε lysates) were subjected to 16 two-fold serial dilutions in MST buffer and mixed with an equal volume of labeled CRYAB, ensuring a constant final concentration of the labeled protein. The mixtures were incubated at room temperature for 5 min and subsequently loaded into premium coated capillaries (NanoTemper) for measurement. MST parameters were set as follows: LED power at 20%, and MST laser power at 40%. Each concentration point was measured in at least three technical replicates, and the experiment was independently repeated three times. Binding curves were fitted, and apparent dissociation constants (K_d) were calculated using MO.Affinity Analysis software.

MST Analysis of Small Molecule-Protein Interactions

A purified recombinant protein system was used to validate the direct binding of ginkgolide B (GB) to CRYAB. Recombinant CRYAB protein was expressed, purified, and dialyzed into MST buffer (PBS supplemented with 0.05% Tween-20). Protein purity was confirmed by SDS-PAGE. CRYAB was fluorescently labeled using the Monolith Protein Labeling Kit RED-NHS (MO-L011, NanoTemper). Free dye was removed using a desalting column, and the labeled protein concentration was adjusted to the optimal level for detection. GB was dissolved in DMSO and serially diluted in 16 steps using MST buffer, ensuring a consistent final DMSO concentration (≤1%, v/v) across all samples. To exclude potential interference from compound autofluorescence or aggregation, a control group containing GB alone was included for baseline measurement. Each concentration of GB was mixed with an equal volume of labeled CRYAB and incubated at room temperature in the dark for 10 min before being loaded into standard capillaries for measurement. MST parameters were set as follows: LED power at 20%, and MST laser power at 40%. Each concentration point was measured in three technical replicates, and the experiment was independently repeated three times. Data fitting to obtain K_d values was performed using MO.Affinity Analysis software. Final binding curves were generated using GraphPad Prism 10.1.2 (GraphPad Software, USA).

TUNEL Staining

313 The TUNEL assay was conducted with a one-step TUNEL apoptosis detection kit (c1088,
314 Beyotime, China) following the manufacturer's protocol. Following the specific enzymatic labeling,
315 cellular nuclei were counterstained utilizing DAPI. Fluorescent signals were acquired utilizing an
316 Olympus IX73 inverted microscope (Olympus, Japan).

317 **Flow Cytometric Analysis of Cell Apoptosis**

318 Apoptosis was evaluated using an Annexin V-FITC Apoptosis Detection Kit. NP cells from
319 different treatment groups were collected and centrifuged at 1000 rpm for 5 minutes, followed by
320 resuspension in PBS buffer. After discarding the supernatant, the cells were resuspended in 1×
321 Annexin V Binding Buffer. Annexin V/FITC was added to the cell suspension, followed by the
322 addition of PI. The mixture was incubated at room temperature in the dark for 15 minutes, and
323 apoptosis was subsequently detected using a FACS Calibur flow cytometer (Becton Dickinson,
324 USA).

325 **Immunofluorescence Staining**

326 Cells were seeded on cell culture slides in 6-well plates and treated under the indicated conditions
327 for the specified durations. After treatment, the culture medium was removed, and the cells were
328 washed three times with PBS. For fixation, 500 µL of 4% paraformaldehyde solution was added to
329 each well and incubated overnight at 4°C. Subsequently, cells were permeabilized with 500 µL of
330 0.2% Triton X-100 (Sigma, USA) per well for 10 minutes. Following permeabilization, cells were
331 blocked with 1 mL of 5% BSA (Sigma, USA) per well for 1 hour. The cells were then incubated
332 overnight at 4°C in the dark with primary antibodies diluted in blocking buffer: CRYAB (1:100,
333 15808-1-AP, Proteintech), COL2 (1:100, A19308, Abclonal), MMP-13 (1:100, A1606, Abclonal,
334 China), iNOS (1:100, 18985-1-AP, Proteintech, China), ACAN (1:200, 13880-1-AP, Proteintech),
335 p65 (1:100, A19653, Abclonal) and P16 (1:200, 10883-1-AP, Proteintech). After washing, the cells
336 were incubated with goat anti-rabbit secondary antibody (1:100) for 1 hour at room temperature in
337 the dark.

338 **For F-actin cytoskeleton staining:** the Cells were rinsed three times with PBS, followed by
339 staining of the cytoskeleton with rhodamine-labeled phalloidin (1:200, Solarbio, China) for 1 hour.
340 After three additional washes with PBS, the cell-laden coverslips were mounted onto glass slides
341 using an anti-fade mounting medium containing DAPI (S2110, Solarbio, China). Imaging was
342 performed using a fluorescence microscope (Nikon Ti2-U, Japan) or a confocal laser scanning
343 microscope (Olympus SpinSR10, Olympus, Japan).

344 **For NF-κB p65 nuclear translocation detection:** The immunofluorescence staining was
345 performed on NP cells to detect the expression and localization of p65. Cytoplasmic and nuclear
346 protein fractions were isolated using a Nuclear and Cytoplasmic Protein Extraction Kit (P0028;
347 Beyotime, China) according to the manufacturer's instructions, and the expression of p65, lamin
348 B1, and GAPDH was analyzed by immunoblotting.

349 **In vivo experiment**

350 **Experimental Animals**

351 Animal studies utilized male C57BL/6J wild-type mice (Vital River, China), *Cryab-flox/flox*
352 mice (Shanghai Model Organisms Center, China), and *Acan-P2A-CreERT2* mice (GemPharmatech,
353 China). Genotypic confirmation was achieved via PCR amplification of genomic DNA extracted
354 from tail biopsies utilizing specific primer sets (detailed in the Supplementary table 1). To induce
355 conditional knockout, animals received intraperitoneal tamoxifen injections (MCE, no. HY-

13757A, USA; 1 mg daily for five consecutive days), generating Cryab conditional knockout mice. Mice were housed under standard specific pathogen-free conditions (20 ± 2°C, 50%–65% humidity, 12-hour light/dark cycle) with *ad libitum* access to food and water.

Animal IVDD Model Establishment and In vivo Intervention

Lumbar instability model: The lumbar instability model was established in male C57BL/6J mice to simulate IVDD. The mice were anesthetized via intraperitoneal injection of 1.25% Tribromoethanol (Aibei Biotechnology, China). A fine incision was made along the dorsal skin, followed by blunt dissection of the lumbar ligamentous tissues, tendons, and muscles. Bilateral transection of the supraspinous ligaments, interspinous ligaments, and facet joints was performed at the L4/L5 level to induce instability, while the sham group underwent only the surgical exposure procedure. Successful establishment of the model was confirmed by radiographic and histological analyses at 8 weeks post-surgery.

Lumbar Intervertebral Disc Puncture Model: To induce localized lumbar disc degeneration, a microsurgical transabdominal approach was employed. Following the induction of general anesthesia, animals were secured in a supine position. A midline laparotomy was performed to access the retroperitoneal space, and the abdominal viscera were gently retracted utilizing sterile saline-soaked cotton swabs to expose the anterior aspect of the lumbar spine. Utilizing a surgical stereomicroscope for precise visualization, the L4/L5 intervertebral disc was identified. The annulus fibrosus was percutaneously punctured utilizing a sterile 29-gauge needle. The needle was advanced parallel to the vertebral endplates to a predefined depth (approximately 1.0 mm) to avoid spinal cord penetration, rotated 180 degrees, and maintained in situ for 10 seconds to ensure standardized structural disruption. Following needle withdrawal and hemostasis verification, the abdominal cavity was lavaged, and the peritoneum and skin were anatomically closed utilizing absorbable sutures. Sham-operated control animals underwent identical surgical exposure, including laparotomy and visceral retraction, strictly without annular penetration.

Caudal intervertebral disc mechanical overloading model: Pathological axial mechanical loading of the coccygeal intervertebral disc (Co7-8) was simulated utilizing a customized Ilizarov-type compression apparatus, strictly adhering to previously established protocols. Sham-operated control animals underwent identical apparatus installation strictly devoid of spring-induced mechanical loading. **Adeno-associated virus (AAV) in situ injection:** To enhance CRYAB expression in vivo, an AAV vector carrying the rat CRYAB gene under the control of the CMV promoter (AAV-CRYAB, GeneChem, China) was employed. One week following the surgical induction of the IVDD models, the viral suspension was stereotactically microinjected into the punctured intervertebral discs. For murine models, a 0.5 µL volume encompassing 5×10¹⁰ viral genomes was administered. Correspondingly, rat models received a 2.5 µL intra-discal injection comprising 2×10¹¹ viral genomes. The viral construct was delivered via a controlled, slow injection protocol. To preclude fluid reflux and ensure optimal viral retention, the microsyringe needle was maintained *in situ* for 10 minutes post-delivery. The needle was cautiously and slowly withdrawn only after confirming the strict absence of leakage or extravasation. Animal allocation, therapeutic administration, and downstream phenotypic assessments were executed under strictly double-blinded conditions.

Histology staining

For immunohistochemistry (IHC) staining and score: Paraffin-embedded spinal sections were deparaffinized, rehydrated, and subjected to pepsin-based antigen retrieval solution (X1035, Solarbio, China). Subsequently, sections were blocked with 5% BSA. Following blocking, sections

were incubated overnight at 4°C with various primary antibodies. After washing with PBS, the sections were incubated for 1 hour at room temperature with a secondary antibody from an immunohistochemistry kit (PV-9000, ZSGB-BIO). Immunoreactivity was visualized using a DAB chromogenic detection kit (ZLI-9018, ZSGB-BIO), and nuclei were counterstained with hematoxylin. Finally, images were acquired using a light microscope (VS120-S6-W, Olympus, Japan), and quantitative analysis of immunohistochemical staining was performed using ImageJ software.

For Hematoxylin & Eosin (H&E) staining and Safranin O-Fast Green (SO&FG) staining: Tissue sections were deparaffinized in xylene and rehydrated through a graded ethanol series. After deparaffinization and rehydration, H&E staining was performed using an H&E staining kit according to the manufacturer's instructions. Additionally, SO&FG staining was conducted using a Safranin O staining kit (G1371, Solarbio, China) following the manufacturer's protocol. Subsequently, they were subsequently dehydrated, cleared, and mounted with neutral balsam prior to microscopic evaluation.

Drug Target Validation Experiments

High-throughput dual-luciferase reporter assay for drug screening

To seeking small molecule modulators of CRYAB, a small molecule library comprising 500 natural compounds was subjected to a high-throughput drug screening campaign utilising a dual-luciferase reporter system. HEK293T cells were seeded in 96-well plates and co-transfected utilising Lipo8000™ Transfection Reagent with a *CRYAB* transcriptional reporter plasmid (firefly luciferase; pGL4.20-CRYAB) and a pRL-TK control plasmid (Renilla luciferase). Following transfection, compounds were administered at same concentration (10µM) for an additional 24 hours. Luciferase activities were quantitatively evaluated using the Dual-Luciferase Reporter Assay System (Gene&Bio, China) according to the manufacturer's instructions. Compound-induced transcriptional activation was defined by the increase in the firefly-to-renilla luciferase ratio. Candidates demonstrating significant upregulation were identified from the volcano plot of relative luciferase activities and prioritized for downstream validation.

Drug Affinity Responsive Target Stability (DARTS) assay

Cell lysates were prepared in non-denaturing RIPA lysis buffer (Servicebio, China) supplemented with a protease inhibitor cocktail (Thermo Fisher Scientific, USA). Lysates were incubated with small molecule candidates or DMSO control at indicated concentrations (10µM) for 1 hour at room temperature. Enzymatic digestion was performed utilising a serial dilution of pronase (HY-114158; MCE; China) for 15 minutes. Proteolysis was terminated by the addition of 5× SDS sample buffer and heating at 95°C for 5 minutes. The proteolytic stability of CRYAB was resolved utilising Western blot analysis, with GAPDH serving as a non-binding control[50].

Cellular Thermal Shift Assay (CETSA)

For temperature-dependent CETSA (CETSA melting curve), NP cells were treated with 10 µM GB or DMSO control for 1 hour. Cells were harvested into PCR tubes, and subjected to heating across a temperature gradient (50–85°C) for 3 minutes, followed by rapid cooling on ice. For isothermal dose-response CETSA (ITDR-CETSA), cells were treated with a serial dilution of GB (0.001 to 100 µM) for 1 hour, followed by heating at a fixed temperature derived from T_m analysis. The remaining soluble protein fraction was separated from precipitated material utilising centrifugation and subsequently resolved utilising immunoblotting analysis. Apparent T_m (T_m50)

444 and dose-response curves (LogIC50) were mathematically derived utilise non-linear regression
445 algorithms within GraphPad Prism software.

446 **Statistics and Reproducibility**

447 Biological replicate quantities (denoted as n) were established a priori utilizing power estimations
448 informed by preliminary datasets, maintaining minimum group sizes of three for in vitro cellular
449 assays and six animals per experimental group for murine models. To mitigate observational bias,
450 subject allocation and subsequent phenotypic evaluations were executed under randomized, double-
451 blind conditions. Quantitative analytical workflows were processed within GraphPad Prism
452 (v10.1.2) and IBM SPSS (v20.0) software environments. Prior to parametric evaluation, all
453 continuous variables were subjected to Shapiro-Wilk and Levene's testing to confirm Gaussian
454 distribution and homoscedasticity, respectively. Datasets satisfying these prerequisites are
455 expressed as the mean $\pm$ standard deviation (SD). Differences between two independent groups
456 were resolved utilizing unpaired, two-tailed Student's t-tests. For experimental designs
457 encompassing multiple comparators, variance was delineated via one- or two-way ANOVA,
458 complemented by Tukey's post-hoc correction. Conversely, observations violating parametric
459 assumptions were interrogated utilizing the Kruskal-Wallis rank-sum test, integrated with a Holm-
460 adjusted Dunn's post-hoc evaluation. The alpha threshold denoting statistical significance was
461 predefined at $P < 0.05$. Precise P values, exact replicate numbers, and specific analytical
462 frameworks are comprehensively documented within the corresponding figure legends.

463 **Results**

464 **Single-cell transcriptomics links CRYAB downregulation in nucleus pulposus cells to** 465 **intervertebral disc degeneration severity**

466 To map the cellular microenvironmental alterations underlying IVDD at single-cell resolution,
467 we integrated open-access scRNA-seq datasets from human NP specimens (Fig. 1A). The
468 consolidated group comprised 3 healthy (GSE160756, Pfirrmann grade I) and 8 degenerated
469 (GSE165722, Pfirrmann grades II–V) samples, which were stratified into early/mild (Pfirrmann I–
470 II) and advanced/severe (Pfirrmann III–V) degeneration groups. Following stringent quality control,
471 doublet exclusion, and batch-effect correction, unsupervised clustering partitioned the cellular
472 milieu into five distinct lineages: endothelial cells, T cells, myeloid cells, neutrophils, and NP cells
473 (Fig. 1C), with consistent representation across patient origins (Fig. 1D). Cellular identities were
474 robustly annotated using canonical markers, including *PECAMI* and *VWF* for endothelium, *CD2*
475 and *CD8A* for T cells, *CD163* and *HLA-DRA* for myeloid populations, *CSF3R* and *CXCR1* for
476 neutrophils, and the chondrogenic transcripts *COL2A1*, *ACAN*, *COMP*, and *SOX9* for NP cells (Fig.
477 1B, E).

478 Transcriptomic alterations driving IVDD progression were resolved by specifically interrogating
479 the NPC compartment. Differential gene expression analysis between advanced and early-stage NP
480 cells revealed profound transcriptional reprogramming (Fig. 1F). GO annotation of genes
481 upregulated in severe IVDD highlighted a marked enrichment in networks governing aberrant
482 protein homeostasis, specifically proteasome-mediated, ubiquitin-dependent catabolic processes
483 and K48-linked ubiquitination (Fig. 1G). Concurrently, GSEA mapped activated signatures
484 associated with inflammatory cascades and oxidative stress strictly to the advanced IVDD
485 phenotype.

486 Given the prominence of disrupted protein folding and ubiquitination dynamics, we evaluated
487 the expression profiles of molecular chaperones. Notably, the expression of *CRYAB*, an essential

small heat shock protein, inversely correlated with disease severity. Uniform Manifold Approximation and Projection (UMAP) revealed a dense population of *CRYAB*⁺ NP cells in early-stage discs that was substantially depleted in advanced IVDD (Fig. 1H). Quantitative profiling confirmed this depletion: the proportion of *CRYAB*⁺ NP cells declined from nearly 80% in healthy/mild discs to under 40% in severe degeneration (Fig. 1I). Accordingly, both overall *CRYAB* expression and the *CRYAB*⁺ cell fraction were reduced in advanced-stage NP cells (Fig. 1J, K).

To define the functional implications of this loss, we compared the transcriptomes of *CRYAB*⁻ and *CRYAB*⁺ NP cells. *CRYAB*⁻ cells exhibited upregulation of a suite of destructive mediators—including the E3 ubiquitin ligase *HUWE1*, the pro-apoptotic factor *CASP3*, the matrix-degrading proteases *MMP13* and *ADAMTS5*, and the NF- κ B subunit *RELA*—concomitant with the downregulation of structural elements such as *ACAN* and *COL2A1* (Fig. 1L). Pathway enrichment linked the *CRYAB*⁻ state to ubiquitin-dependent protein catabolism, apoptosis, and TNF signaling (Fig. 1M). Consistent with a protective role, *CRYAB* expression across individual cells displayed a robust inverse correlation with the expression magnitudes of *MMP13*, *ADAMTS5*, *RELA*, and *HUWE1* (Fig. 1N). Together, these in vivo single-cell data implicate the progressive loss of *CRYAB* as a critical enabler of ubiquitin-mediated matrix catabolism and inflammation during IVDD progression.

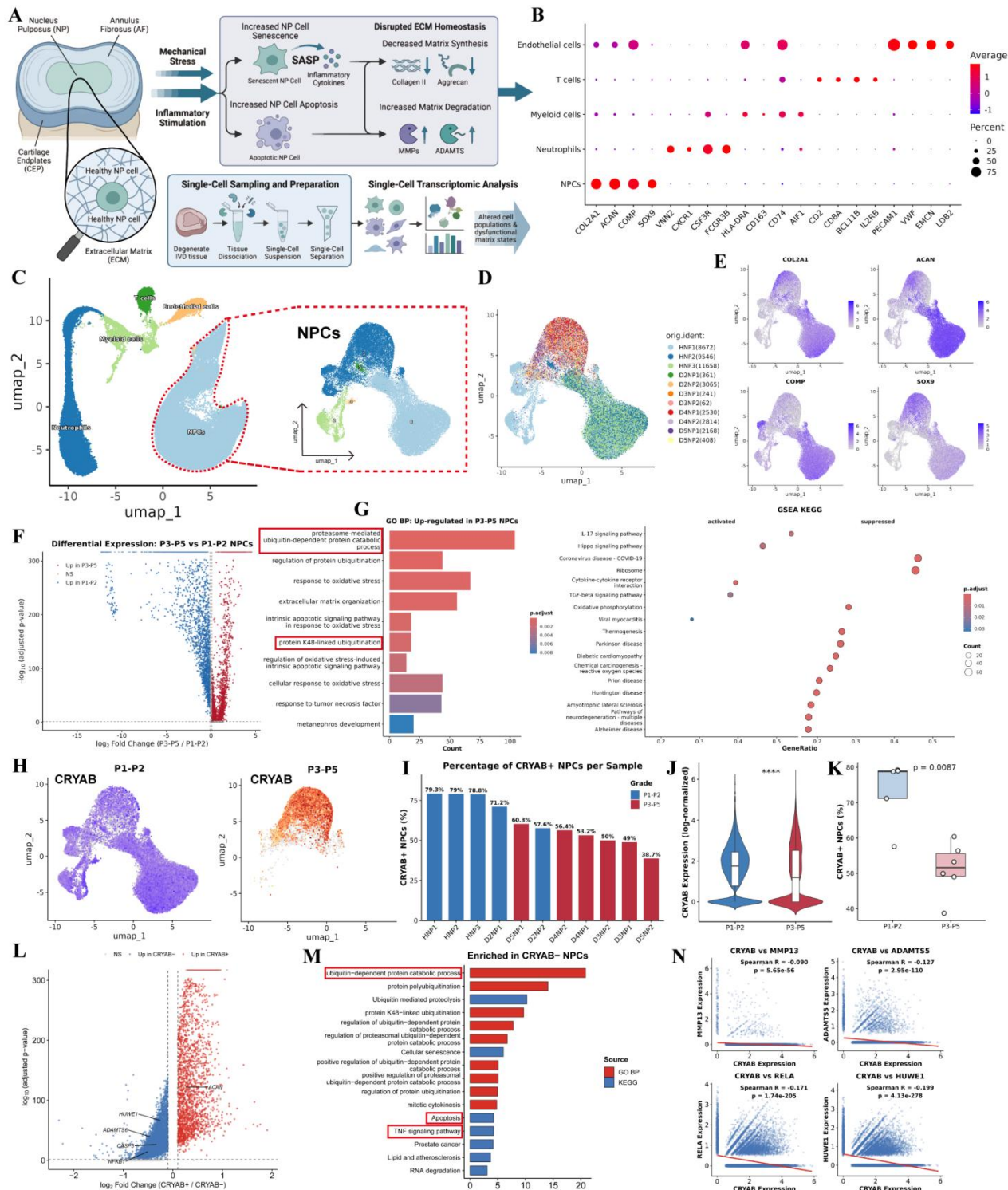


Figure 1. Single-cell transcriptomic profiling reveals *CRYAB* depletion in degenerated human nucleus pulposus cells.

(A) Schematic workflow of scRNA-seq sample preparation and bioinformatic analysis from healthy and degenerated human intervertebral discs. (B) Dot plot illustrating the expression of canonical lineage markers utilized for cell cluster annotation. (C, D) UMAP plots delineating five distinct cellular lineages across the integrated sample cohort. (E) Feature plots visualizing the expression of chondrogenic markers (*COL2A1*, *ACAN*, *COMP*, *SOX9*) specific to . (F) Volcano plot highlighting differentially expressed genes in advanced/severe (P3-P5) versus early/mild (P1-P2)

NP cells. **(G)** GO biological process enrichment analysis of upregulated genes in advanced IVDD, highlighting ubiquitin-dependent protein catabolism. **(H)** UMAP visualization of *CRYAB* expression density between P1-P2 and P3-P5 groups. **(I)** Bar chart quantifying the percentage of *CRYAB*⁺ NP cells across individual clinical grades. **(J, K)** Violin plot and box plot representing overall *CRYAB* expression levels and the *CRYAB*⁺ cell fraction in corresponding groups. **(L)** Volcano plot mapping differentially expressed transcripts between *CRYAB*⁺ and *CRYAB*⁻ NP cells. **(M)** Pathway enrichment analysis of the *CRYAB*⁻ cellular state. **(N)** Scatter plots demonstrating the inverse correlation of *CRYAB* expression with *MMP13*, *ADAMTS5*, *RELA*, and *HUWE1* at single-cell resolution. Scale bars as indicated. Data are represented as mean ± SD. Statistical significance was determined utilizing one-way or two-way ANOVA followed by Tukey's post-hoc test (****P < 0.0001).

CRYAB is down-regulated in abnormal mechanical stress-mediated IVDD

To further elucidate the relationship between *CRYAB* downregulation and IVDD highlighted by our scRNA-seq analysis, we investigated its upstream physiological triggers. Given that aberrant mechanical loading is a primary instigator of disc degeneration, we established an in vitro mechanical stress model. Primary human NP cells (isolated from patients with Pfirrmann grade II degeneration) were subjected to static hydrostatic pressure (2 MPa for 2 h), followed by bulk transcriptomic profiling (RNA-seq). Comparative analysis revealed substantial load-induced transcriptional alterations, comprising the upregulation of 715 genes and the downregulation of 1848 genes (Fig. 2A). Focusing on the mechanosensitive downregulated gene subset, immunoblot screening of the top 10 candidates identified *CRYAB* as the most consistently downregulated target, in concordance with our scRNA-seq findings (Fig. 2B). Furthermore, KEGG and GO enrichment analyses of this mechanosensitive signature primarily mapped to inflammatory response pathways (Fig. 2C, D). Consequently, *CRYAB* was prioritized for downstream mechanistic evaluation.

Clinical relevance of this target was first corroborated at the protein level. Immunohistochemical and immunoblot assessments of human NP tissues confirmed a significant reduction of *CRYAB* protein in severe (grade IV) versus mild (grade II) degenerated specimens (Fig. 2E, F). We subsequently evaluated the magnitude-dependent impact of mechanical stress in vitro. Exposing primary grade II NP cells to escalating magnitudes of hydrostatic pressure (0.5 to 2.0 MPa) provoked a progressive, magnitude-dependent attenuation of *CRYAB* levels, corroborated by quantitative protein assessments across varying pressure gradients. Notably, this mechanically induced *CRYAB* depletion occurred in tandem with the accumulation of catabolic enzymes (*MMP13*, *ADAMTS-5*), inflammatory mediators (*iNOS*, *COX2*), and the senescence marker p16 (Fig. 2G, H; Fig. S1A, D), which phenocopied the advanced IVDD state observed in vivo.

We then investigated whether canonical mechanosensors govern this cascade. Pharmacological engagement of Piezo1, a principal mechanosensitive ion channel in NP cells, using the specific agonist Yoda1 recapitulated the effects of hydrostatic pressure, suppressing *CRYAB* production in a concentration- and time-dependent manner (Fig. 2I, J; Fig. S1B, C).

The mechanosensitive downregulation of *CRYAB* was further confirmed in vivo using established mouse IVDD models subjected to lumbar spine instability (LSI), lumbar needle puncture (LNP), or a combination of both (LSI+LNP). Histological evaluation (H&E and SO&FG staining) at 4 and 8 weeks post-operation revealed progressive disc degeneration across all surgical groups, characterized by continuous cellular disorganization and proteoglycan depletion (Fig. 2K ; Fig. S1E). Concurrently, immunohistochemistry demonstrated a progressive decline in *CRYAB* protein levels that strictly mirrored the severity of structural degeneration (Fig. S1F). Notably, *CRYAB* expression was nearly abolished in the 8-week LSI+LNP group, which exhibited the most severe

mechanical disruption (Fig. 2K). Together, these in vitro and in vivo findings demonstrate that pathological mechanical stress, signaled through mechanosensitive pathways, directly drives the depletion of CRYAB and precipitates the catabolic and inflammatory hallmarks of IVDD.

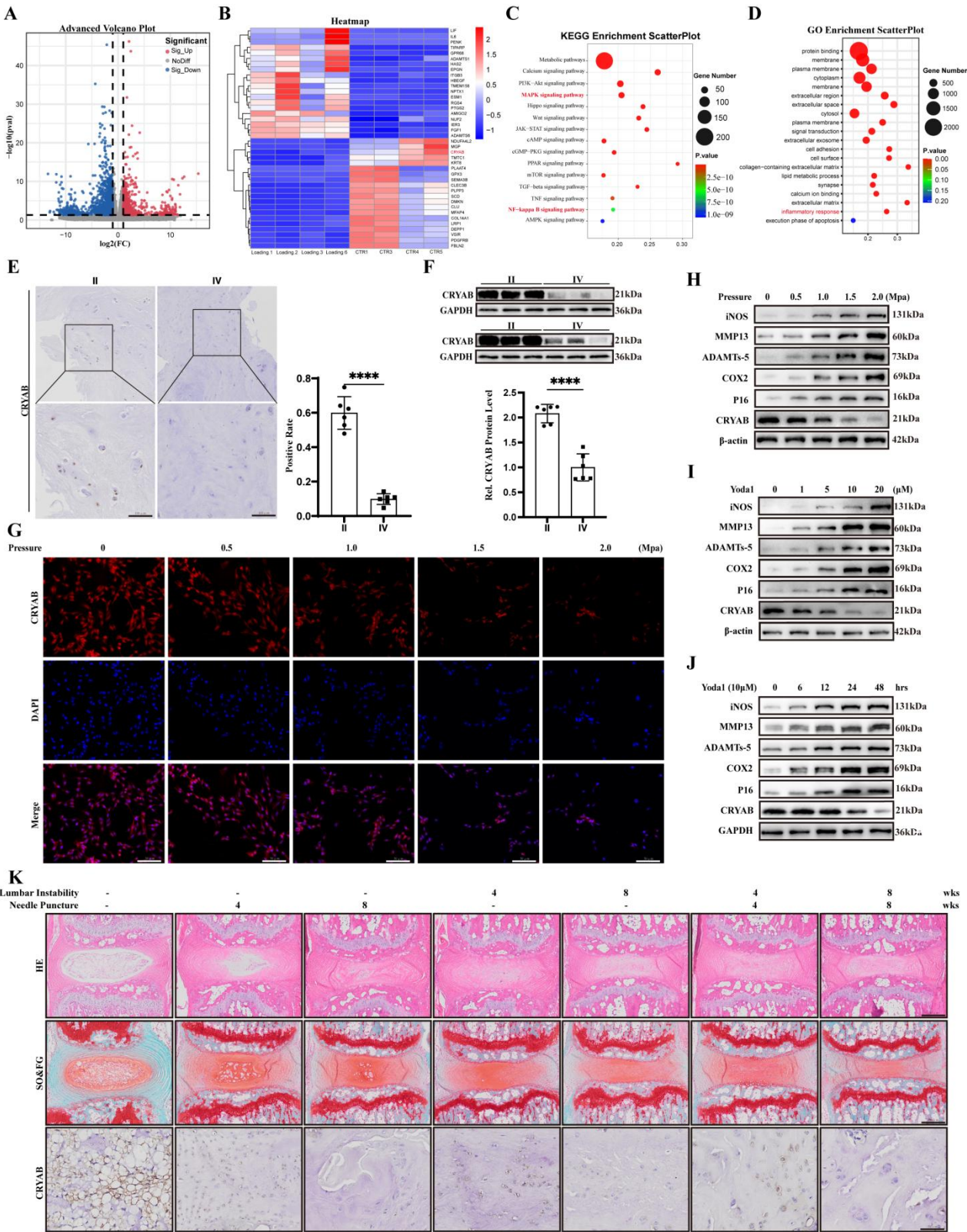


Figure 2. Pathological mechanical stress downregulates CRYAB in vitro and in vivo.

(A) Volcano plot identifying differentially expressed genes in primary human NP cells subjected to 2.0 MPa hydrostatic pressure. (B) Heatmap displaying the top mechanosensitive transcripts, pinpointing *CRYAB* downregulation. (C, D) KEGG and GO enrichment scatter plots of the mechanosensitive gene signature. (E, F) Representative IHC images and immunoblot analysis demonstrating *CRYAB* protein attenuation in grade IV versus grade II clinical NP specimens. (G, H) Immunofluorescence and immunoblot quantification of *CRYAB*, alongside catabolic and senescence markers, in NP cells subjected to escalating magnitudes of hydrostatic pressure (0–2.0 MPa). (I, J) Immunoblot evaluations of *CRYAB* and downstream markers following Piezo1 channel activation via Yoda1 treatment across dose and temporal gradients. (K) Representative H&E, SO&FG, and *CRYAB* IHC staining of mouse intervertebral discs following lumbar instability and needle puncture modeling at 4 and 8 weeks post-operation. Scale bars as indicated. Data are represented as mean $\pm$ SD. Statistical significance was determined utilizing one-way or two-way ANOVA followed by Tukey's post-hoc test (****P < 0.0001).

***CRYAB* deficiency exacerbates mechanical overload-induced catabolism, and senescence in intervertebral discs**

The indispensable role of *CRYAB* in maintaining nucleus pulposus homeostasis was evaluated through targeted gene silencing. Following the verification of knockdown efficiency across three specific siRNA (Fig. 3A), the consequences of *CRYAB* depletion were assessed under both basal and mechanically overloaded conditions. In vitro, *CRYAB* silencing alone provoked a robust upregulation of inflammatory mediators (iNOS, COX2), catabolic enzymes (MMP13, ADAMTS-5), and the senescence marker P16 at both the transcriptional and translational levels, mimicking the pathological alterations induced by 1 MPa hydrostatic pressure (Fig. 3B-D). Crucially, the concurrent application of mechanical stress and *CRYAB* knockdown compounded these effects. Immunofluorescence profiling confirmed that this combined intervention profoundly exacerbated the depletion of ACAN while amplifying COX2, MMP13, and P16 signals (Fig. S2A-I), driving a more severe degeneration than either stimulus alone.

Cellular morphological and phenotypic evaluations further substantiated this functional link. Staining for F-actin (phalloidin) revealed that *CRYAB* deficiency elevated the proportion of senescent cells and triggered cytoskeletal disorganization (Fig. 3E). These degenerative structural deviations were maximally amplified when *CRYAB*-deficient cells were subjected to magnitude-dependent mechanical pressure, highlighting an increased cellular vulnerability.

The physiological protective function of this molecular chaperone was subsequently interrogated in vivo utilizing an *Acan*-driven *Cryab* conditional knockout (*Cryab* cKO) murine model subjected to mechanical overloading. We obtained conditional *Cryab* knockout mice (*Acan*-CreERT2; *Cryab*^{fl/fl}) by crossing *Cryab*^{fl/fl} mice with *Acan*-CreERT2 mice, PCR and Western blot confirmed the specific deletion of *Cryab* in the nucleus pulposus (Fig. S3A–E).

Histological assessments via H&E and SO&FG staining indicated that NP-specific *Cryab* ablation was sufficient to initiate spontaneous disc degeneration even under sham conditions, characterized by minor structural disruption and proteoglycan loss (Fig. 3F, G). Immunohistochemical evaluation confirmed the targeted deletion of *CRYAB* exclusively within the nucleus pulposus compartment of the *Cryab* cKO animals (Fig. 3H, I) and revealed that this localized deficiency profoundly exacerbated the mechanically induced pathological cascade. Relative to mechanically loaded control mice, loaded *Cryab* cKO discs exhibited an accelerated depletion of the structural matrix protein COL2 (Fig. 3J, K), concomitant with extensive accumulation of MMP13, COX2, and P16 across all IVDD animal models (Fig. 3L-Q). These combined functional assessments establish that *CRYAB* operates as a critical protective factor

611 against mechanotransduction-mediated inflammatory and catabolic cascades during IVDD
612 pathogenesis.

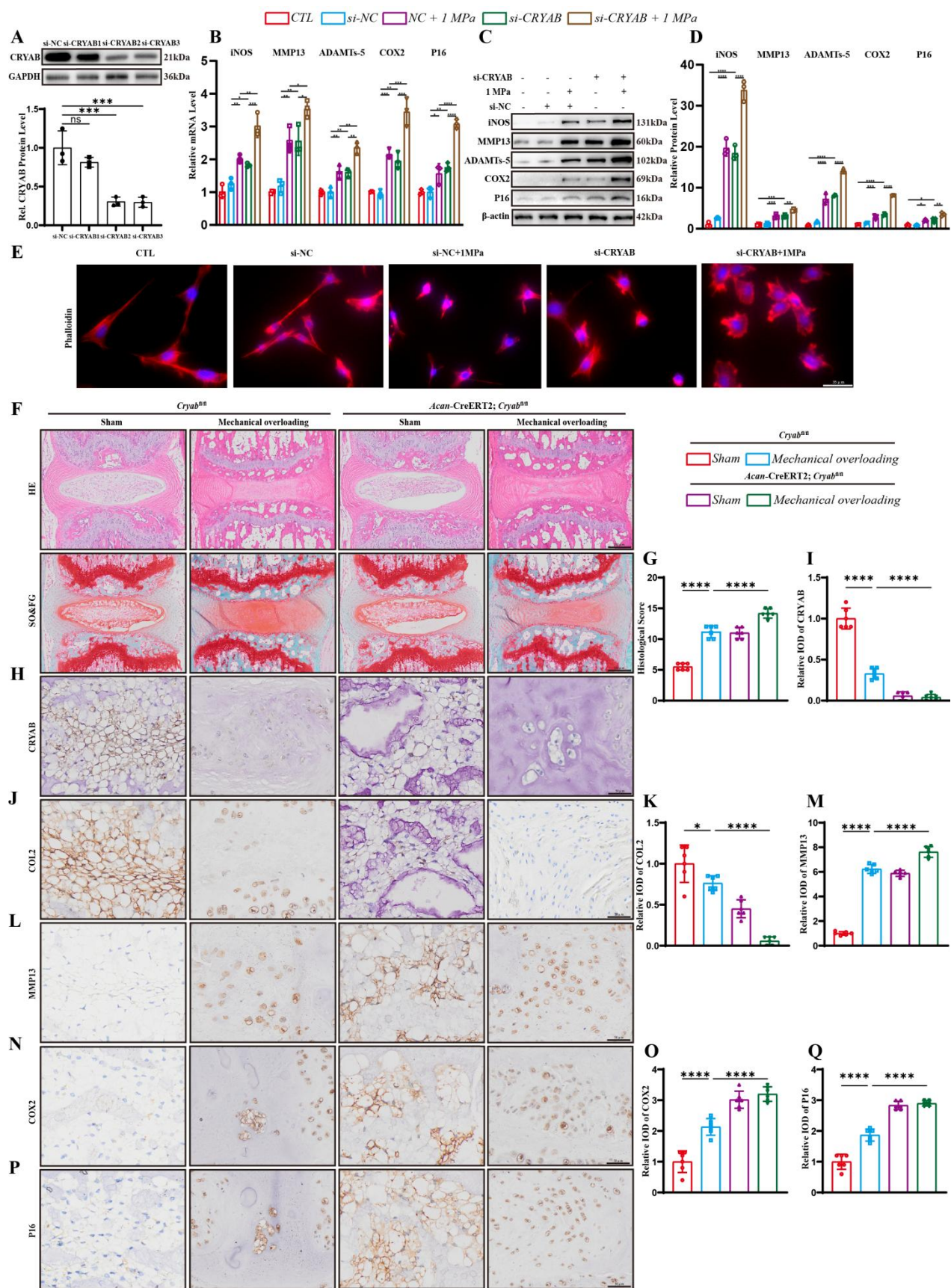


Figure 3. *CRYAB* deficiency exacerbates mechanotransduction-induced matrix catabolism, senescence, and disc degeneration.

(A) Immunoblot validation of *CRYAB* silencing efficiency utilizing three independent siRNAs. (B-D) RT-qPCR and immunoblot assessments of inflammatory, catabolic, and senescence markers in NP cells following *CRYAB* knockdown, with or without concurrent 1.0 MPa mechanical loading. (E) Representative F-actin (phalloidin) staining visualizing cellular senescence and cytoskeletal disorganization under basal and mechanically loaded conditions. (F, G) H&E and SO&FG staining of intervertebral discs from *Cryab*^{fl/fl} and *Acan*-CreERT2; *Cryab*^{fl/fl} (*Cryab* cKO) mice subjected to in vivo mechanical overloading or sham surgery. (H-Q) Representative IHC staining and corresponding quantitative integrated optical density (IOD) analysis of *CRYAB*, *COL2*, *MMP13*, *COX2*, and *P16* within the murine nucleus pulposus compartment across surgical cohorts. Scale bars as indicated. Data are represented as mean $\pm$ SD. Statistical significance was determined utilizing one-way or two-way ANOVA followed by Tukey's post-hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

***CRYAB* overexpression attenuates mechanotransduction-mediated intervertebral disc degeneration in vitro and in vivo**

Given the deleterious effects of *CRYAB* deficiency observed under mechanical stress, the therapeutic potential of its targeted restoration was investigated. Adenovirus-mediated overexpression of *CRYAB* (ADV-*CRYAB*) in primary human NP cells was first confirmed via immunoblotting (Fig. 4A). Following exposure to a 1 MPa mechanical load, control cells harboring the empty vector (ADV-NC) exhibited the anticipated upregulation of catabolic enzymes (*MMP13*, *ADAMTS-5*), inflammatory mediators (*iNOS*, *COX2*), and the senescence marker *P16*. Conversely, *CRYAB* overexpression attenuated these mechanosensitive elevations at both the transcriptional and translational levels, effectively maintaining the molecular baseline (Fig. 4B-D).

Phenotypic preservation was subsequently evaluated utilizing morphological and cellular senescence assays. While unmitigated mechanical stress provoked widespread cytoskeletal disorganization and cellular aging, ADV-*CRYAB* transduction preserved F-actin integrity (Fig. 4E). These in vitro findings indicate that elevated *CRYAB* levels confer robust cellular resistance against load-induced structural and functional deterioration.

The translational capacity of this protective mechanism was assessed in vivo employing an AAV delivery system in the mouse needle puncture model. Histological evaluations (H&E and SO&FG staining) revealed that intra-disc administration of AAV-*CRYAB* visibly ameliorated mechanical injury-induced structural disruption and proteoglycan loss, yielding lower histological scores compared to AAV-NC controls (Fig. 4F, G). Immunohistochemical profiling verified successful *CRYAB* augmentation within the injected tissues (Fig. 4H, I) and demonstrated a corresponding preservation of the *COL2* extracellular matrix (Fig. 4J, K). Furthermore, localized *CRYAB* overexpression suppressed the mechanically triggered accumulation of *MMP13*, *COX2*, and *P16* in vivo (Fig. 4L-Q). To rigorously validate the therapeutic potency of this strategy against *CRYAB* genetic vulnerabilities, AAV-mediated *CRYAB* reconstitution was executed in the *Cryab* cKO murine model. Notably, localized re-introduction of *CRYAB* effectively reversed the exacerbated degenerative phenotype inherent to these deficient mice, restoring structural matrix integrity and significantly buffering the hyperactive catabolic and inflammatory cascades (Fig. S4). Collectively, these findings establish *CRYAB* as a critical intracellular protective factor against mechanical stress-driven intervertebral disc degeneration.

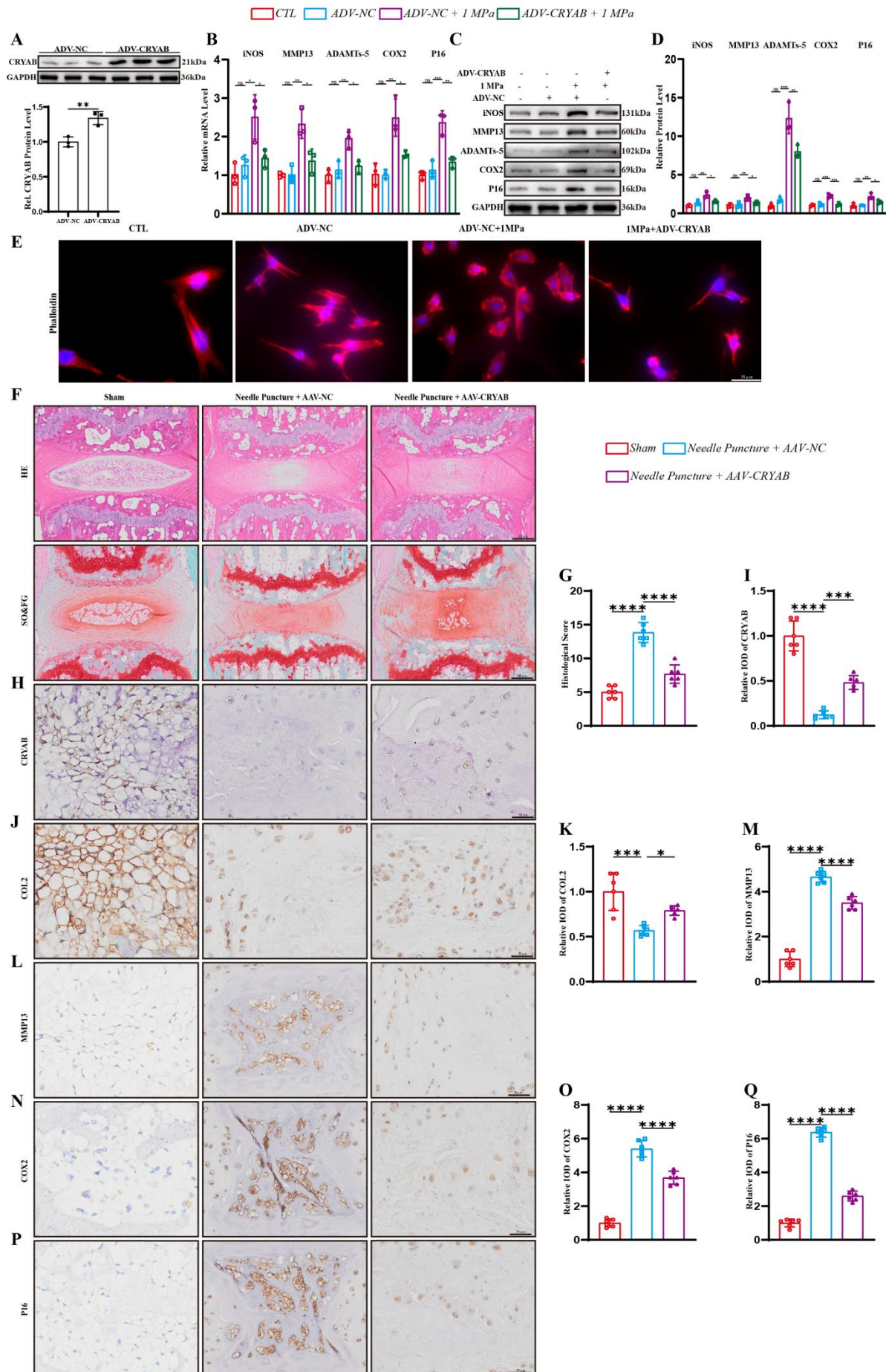


Figure 4. CRYAB restoration ameliorates mechanical overload-induced NP cell deterioration and IVDD progression.

(A) Immunoblot confirming adenovirus-mediated CRYAB overexpression (ADV-CRYAB) in primary NP cells. **(B-D)** Transcriptional (RT-qPCR) and translational (immunoblot) profiling of catabolic and inflammatory markers in ADV-NC and ADV-CRYAB transduced cells exposed to 1.0 MPa hydrostatic pressure. **(E)** phalloidin staining demonstrating preservation of cellular phenotype and cytoskeletal integrity following CRYAB overexpression under mechanical stress. **(F, G)** H&E and SO&FG staining with corresponding histological scoring of mouse intervertebral discs evaluating the in vivo therapeutic efficacy of AAV-CRYAB following needle puncture injury. **(H-Q)** Representative IHC images and quantitative IOD analysis of CRYAB, COL2, MMP13, COX2, and P16, confirming the localized preservation of structural matrix and suppression of catabolism upon AAV-mediated CRYAB augmentation. Scale bars as indicated. Data are represented as mean $\pm$ SD. Statistical significance was determined utilizing one-way or two-way ANOVA followed by Tukey's post-hoc test (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

CRYAB suppresses mechanical stress-induced inflammation and apoptosis by inhibiting canonical NF- κ B signaling

To uncovering the molecular pathways underpinning CRYAB-mediated cellular protection prompted transcriptomic profiling of human NP cells following *CRYAB* knockdown. Differential expression analysis (Fig. 5A, B) coupled with KEGG and GSEA mapped the predominant molecular alterations directly to the activation of the NF- κ B signaling cascade (Fig. 5C, D). Concurrently, GO and GSEA strongly linked *CRYAB* depletion to the profound enrichment of inflammatory responses and intrinsic apoptotic pathways (Fig. 5E-G).

Validation of this regulatory axis at the protein level confirmed that *CRYAB* silencing intrinsically drives NF- κ B signaling activation. Immunoblotting demonstrated enhanced phosphorylation and degradation of I κ B α , accompanied by elevated phosphorylation of the p65 subunit in *CRYAB*-deficient cells (Fig. 5H). Subcellular fractionation and immunofluorescence imaging further verified that the loss of CRYAB facilitated the nuclear translocation of p65, a hallmark of canonical NF- κ B activation (Fig. 5I, J). Downstream of this transcriptional activation, the modified cells exhibited a robust accumulation of inflammatory mediators (iNOS, COX2) and pro-apoptotic executioners (cleaved caspase-3, Bax), paired with a distinct reduction in the anti-apoptotic protein Bcl-2 (Fig. 5K). This molecular shift culminated in exacerbated apoptotic cell death, as quantified by both TUNEL staining and Annexin V/PI flow cytometric profiling (Fig. 5L, M ; Fig. S5A, B).

The specific capacity of CRYAB to counteract mechanosensor-driven intracellular damage was evaluated utilizing the Piezo1 agonist Yoda1 to simulate mechanical overload. While Yoda1 treatment readily provoked canonical NF- κ B activation, inflammatory mediator accumulation, and widespread apoptosis in control cells, adenoviral-mediated *CRYAB* overexpression comprehensively blunted these pathological responses. Elevated CRYAB levels suppressed Yoda1-induced I κ B α degradation, hindered p65 nuclear shuttling (Fig. 5N-P), and effectively maintained the equilibrium of apoptotic and inflammatory regulators (Fig. 5Q). Consequently, this molecular intervention preserved cellular viability, severely restricting Yoda1-induced apoptotic rates in functional assays (Fig. 5R, S; Fig. S5C, D). These mechanistic insights demonstrate that CRYAB safeguards NP cells against mechanical overload-induced injury primarily by restraining the NF- κ B signaling cascade.

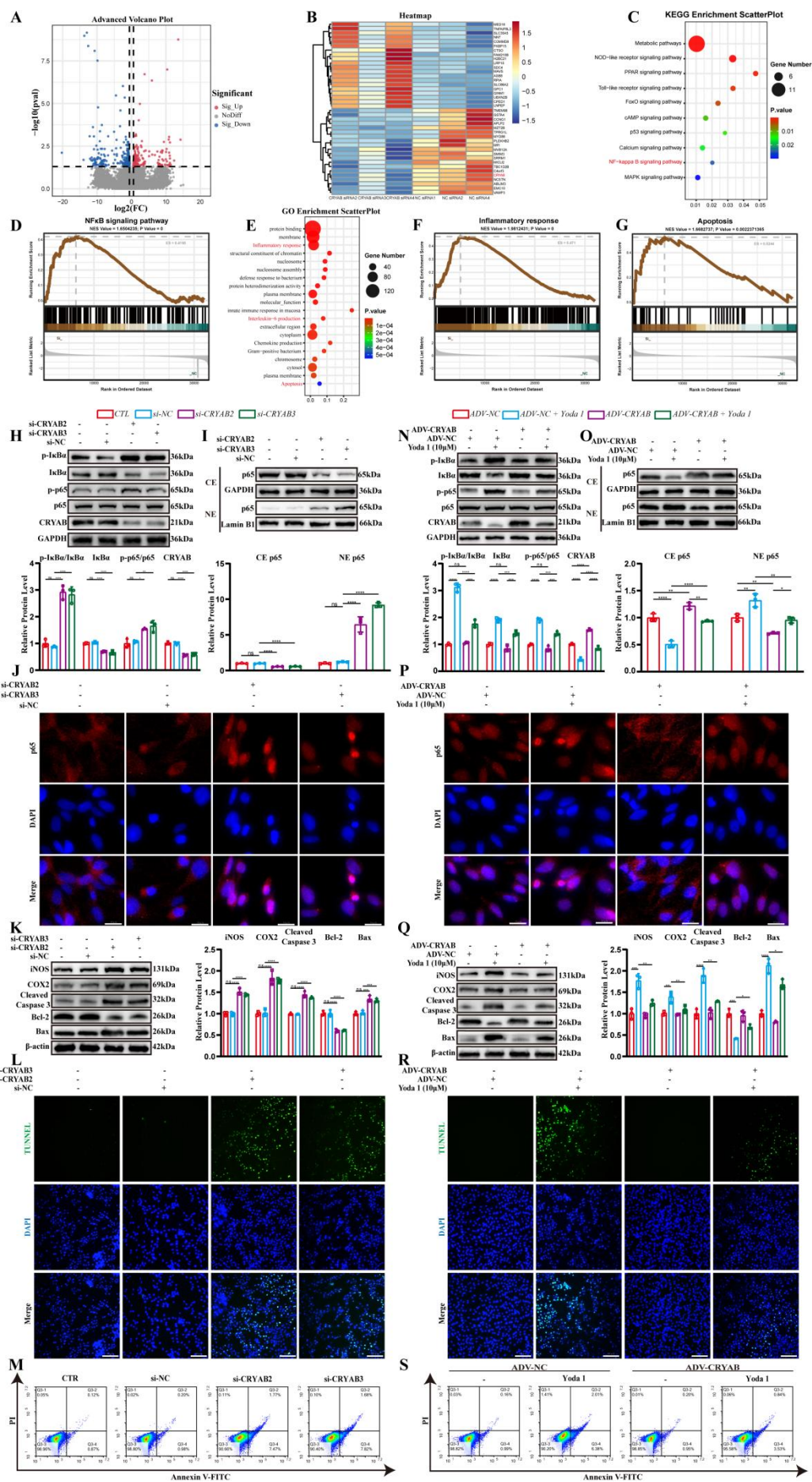


Figure 5. CRYAB protects against mechanosensor-driven inflammation and apoptosis by restricting NF- κ B signaling.

(A, B) Volcano plot and heatmap profiling the transcriptomic alterations induced by *CRYAB* targeted silencing. **(C-G)** KEGG, GO, and GSEA mapping the *CRYAB*-deficient state to hyperactive NF- κ B signaling, inflammatory responses, and intrinsic apoptosis. **(H-K)** Immunoblot and subcellular fractionation analyses validating enhanced I κ B α degradation, p65 nuclear translocation, and accumulation of pro-apoptotic executioners in *CRYAB*-silenced cells. **(L, M)** Representative TUNEL fluorescence imaging and Annexin V-FITC/PI flow cytometric quantification of apoptotic rates. **(N-S)** Immunoblot, TUNEL, and flow cytometric assessments evaluating the capacity of ADV-CRYAB to restrict NF- κ B activation, buffer inflammatory mediator accumulation, and sustain cellular viability against Yoda1-induced mechanical overload simulation. Scale bars as indicated. Data are represented as mean $\pm$ SD. Statistical significance was determined utilizing one-way or two-way ANOVA followed by Tukey's post-hoc test (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

E3 ubiquitin ligase HUWE1 mediates K48-linked polyubiquitination and proteasomal degradation of CRYAB

The profound reduction of CRYAB protein levels under mechanical stress, which exceeded its transcriptional downregulation, strongly implicated post-translational regulation in dictating its cellular abundance. Profiling CRYAB protein stability using the protein synthesis inhibitor CHX revealed a time-dependent decay that was effectively reversed by co-treatment with the proteasome inhibitor MG-132 (Fig. 6A), confirming the reliance of CRYAB turnover on the ubiquitin-proteasome system. Correspondingly, Co-IP assays following exogenous expression of Myc-Ub and HA-CRYAB verified robust baseline ubiquitination of CRYAB within the cellular milieu (Fig. 6B).

Identifying the specific E3 ubiquitin ligase responsible for orchestrating this degradation prompted a proteomics approach. LC-MS/MS analysis of CRYAB immunoprecipitates identified the E3 ligase HUWE1 as a high-confidence interacting partner (Fig. 6C). Histological and immunoblot evaluations of human NP tissues revealed a marked upregulation of HUWE1 in severe (grade IV) compared to mild (grade II) degenerated specimens (Fig. S6A, B), inversely mirroring the CRYAB expression pattern and establishing its clinical association with IVDD. The physical association was rigorously validated through reciprocal co-IP assays in both exogenously transfected primary human NP cells and HEK293T cells (Fig. 6D, E), where endogenous CRYAB-HUWE1 complex formation was detected under basal conditions and visibly enriched following Yoda1-induced mechanical simulation (Fig. 6F). Furthermore, MST analysis utilizing recombinant proteins established a direct, high-affinity interaction between HUWE1 and CRYAB in vitro ($K_d = 2.915E-07$) (Fig. 6G).

The functional capacity of HUWE1 to ubiquitinate CRYAB was subsequently interrogated. Exogenous expression of HUWE1 robustly amplified CRYAB polyubiquitination in the presence of MG-132 (Fig. 6H). Notably, HUWE1 overexpression independently provoked p65 phosphorylation and inflammatory marker accumulation (Fig. S6C, D), verifying its destructive downstream potential. To dissect the linkage specificity of this modification, linkage-restricted ubiquitin mutants were employed. HUWE1 selectively catalyzed the assembly of K48-linked, but not K63-linked, polyubiquitin chains on CRYAB, as evidenced by assays utilizing ubiquitin mutants retaining only specific lysine linkages (HA-K48O or HA-K63O) (Fig. 6I). This linkage specificity was reciprocally confirmed using single lysine-to-arginine ubiquitin mutants; the K48R mutation abrogated HUWE1-driven CRYAB ubiquitination, whereas the K63R mutation yielded

751 no discernible impact (Fig. 6J). Concordant with these biochemical findings, CHX-chase assays
752 demonstrated that HUWE1 knockdown prolonged CRYAB half-life (Fig. 6K, M), whereas
753 HUWE1 overexpression accelerated its degradation in both HEK293T and primary NP cells (Fig.
754 6L, N), further validated by quantitative decay kinetics (Fig. S5E-L).

755 Bioinformatic interrogation of the CRYAB sequence pinpointed distinct lysine residues as
756 putative ubiquitination acceptor sites. Guided by these predictions, a site-directed CRYAB double
757 mutant (K/R) was generated. Co-IP analysis revealed that substituting these specific lysines with
758 arginines substantially attenuated HUWE1-mediated ubiquitination compared to wild-type (WT)
759 CRYAB (Fig. 6O), confirming these residues as the primary targets for HUWE1-directed ubiquitin
760 tagging.

761 The physiological relevance of this targeted degradation was ultimately assessed in the context
762 of mechanical stress. Primary NP cells were co-transduced with adenoviral vectors expressing
763 either WT or mutant CRYAB, alongside HUWE1, and subjected to Yoda1 stimulation. While
764 HUWE1 overexpression effectively neutralized the protective effects of WT CRYAB—
765 exacerbating the accumulation of catabolic (MMP13) and inflammatory (COX2) markers—the
766 ubiquitination-resistant CRYAB K/R mutant sustained its protective efficacy, rendering the cells
767 refractory to HUWE1-mediated molecular deterioration (Fig. 6P; Fig. S5M). These integrated
768 findings establish HUWE1 as the definitive K48-specific E3 ligase for CRYAB, defining a
769 mechanosensitive post-translational axis that drives IVDD pathogenesis.

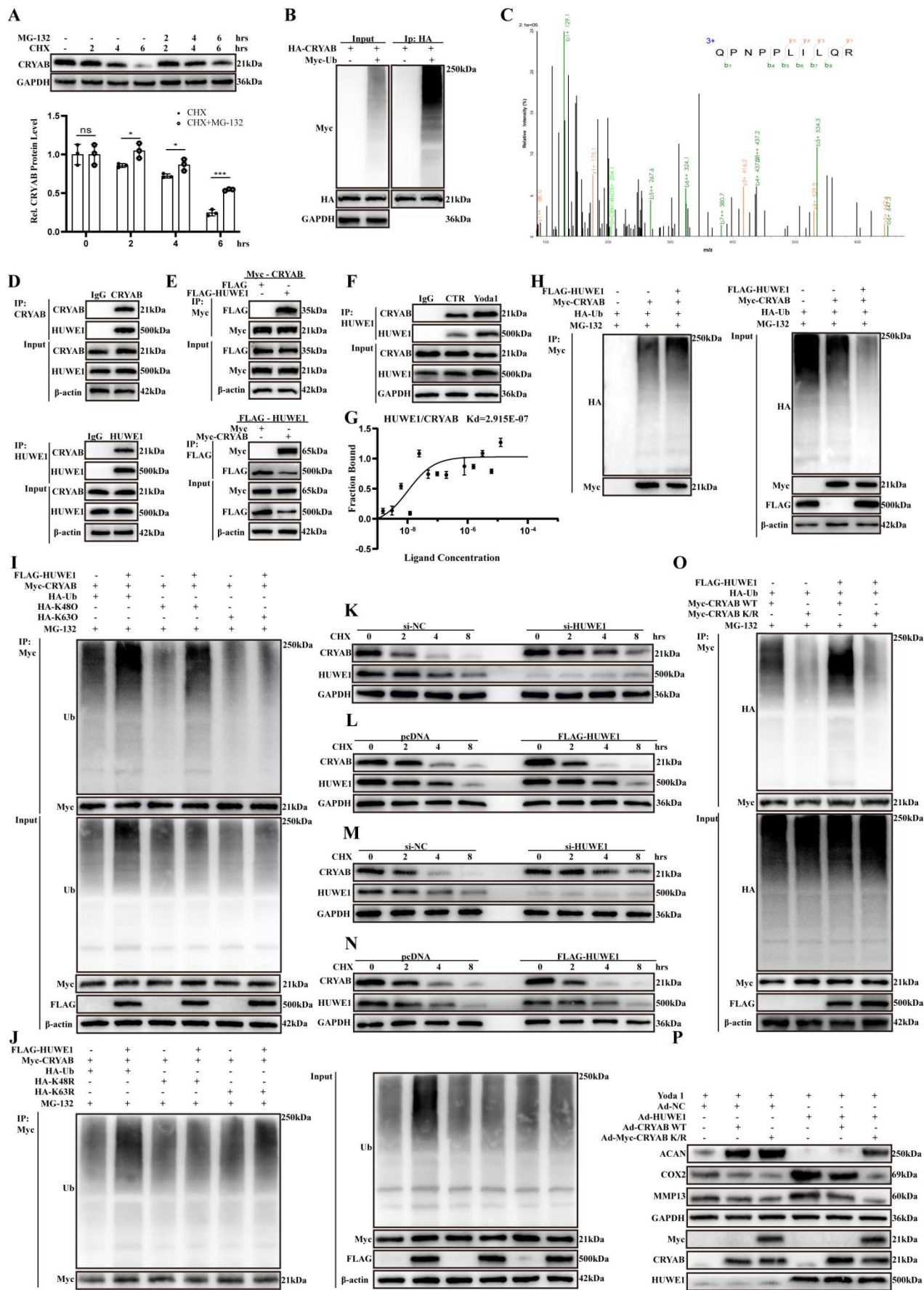


Figure 6. The E3 ligase HUWE1 orchestrates K48-linked polyubiquitination and proteasomal degradation of CRYAB.

(A) Immunoblot demonstrating the temporal decay of CRYAB protein upon CHX treatment and its rescue by the proteasome inhibitor MG-132. (B) Co-IP assay verifying baseline CRYAB polyubiquitination. (C) Mass spectrometry spectra identifying HUWE1 as a CRYAB interacting partner. (D-F) Reciprocal Co-IP analyses confirming the physical association between HUWE1 and CRYAB under basal and Yoda1-stimulated conditions. (G) MST binding curve defining the direct molecular affinity (Kd) between recombinant HUWE1 and CRYAB. (H) Ubiquitination assay demonstrating HUWE1-driven amplification of ubiquitin chain assembly on CRYAB. (I, J) Linkage-specific ubiquitination assays utilizing restricted (K48O/K63O) and mutant (K48R/K63R) ubiquitin constructs, establishing HUWE1 as a K48-specific E3 ligase. (K, L) CHX-chase assays in HEK293T cells detailing CRYAB half-life dynamics following *HUWE1* knockdown or Flag-HUWE1 overexpression. (M, N) CHX-chase assays in primary NP cells detailing CRYAB half-life dynamics following *HUWE1* knockdown or Flag-HUWE1 overexpression. (O) Co-IP assay revealing attenuated ubiquitination upon substitution of critical lysine residues (CRYAB K/R mutant). (P) Immunoblot evaluation assessing the capacity of the ubiquitination-resistant CRYAB mutant to sustain protection against Yoda1 and HUWE1-mediated molecular deterioration. Data are represented as mean $\pm$ SD. Statistical significance was determined utilizing one-way or two-way ANOVA followed by Tukey's post-hoc test (ns $P > 0.05$, * $P < 0.05$).

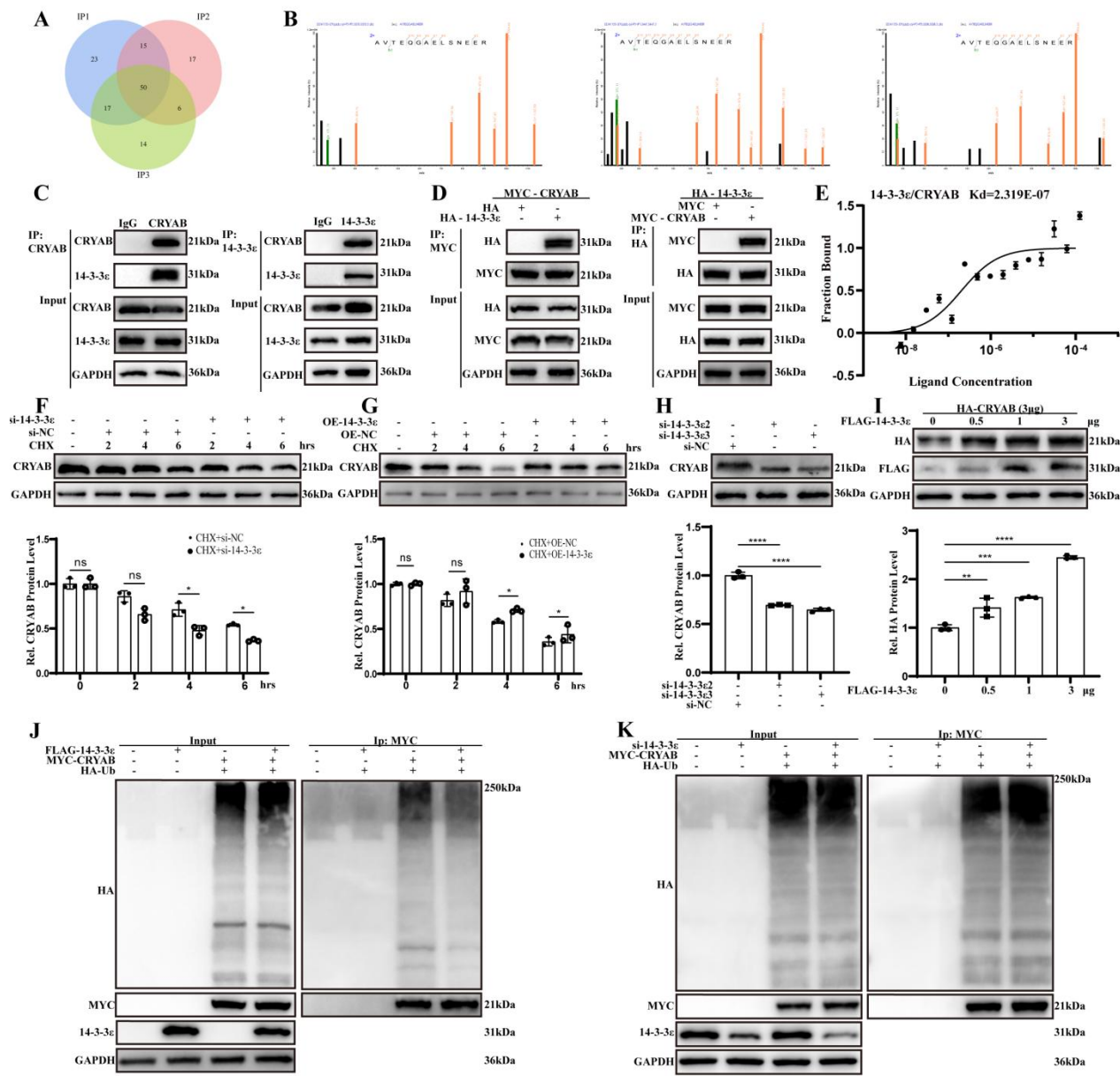
14-3-3 epsilon stabilizes CRYAB by competitively disrupting the HUWE1-CRYAB interaction

Seeking endogenous mechanisms that preserve CRYAB stability against ubiquitin-dependent degradation, we interrogated our triplicate IP-MS datasets. Bioinformatic analysis identified the scaffold protein 14-3-3 ϵ as a high-probability interacting candidate (Fig. 7A, B). Clinical relevance of this target was concurrently established through expression profiling of human NP specimens, revealing a substantial decline in 14-3-3 ϵ protein levels in advanced IVDD tissues compared to mild degeneration (Fig. S6E-H).

The physical association was corroborated endogenously via reciprocal Co-IP in human NP cells (Fig. 7C) and exogenously in HEK293T cells co-expressing Myc-CRYAB and HA-14-3-3 ϵ (Fig. 7D). MST utilizing purified recombinant proteins further established a direct, high-affinity interaction between these two molecules in vitro (Kd=2.319E-07) (Fig. 7E). Mapping the specific interaction interface revealed a structural reliance on the previously identified ubiquitination sites. Co-IP assays and MST assays demonstrated that substituting lysines 150 and 161 with arginines (CRYAB K150R/K161R) impaired the direct binding between CRYAB and 14-3-3 ϵ (Fig. S7A, B). This finding indicates that these specific lysine residues are indispensable for 14-3-3 ϵ recognition.

The physiological consequence of this interaction on CRYAB half-life was evaluated using CHX chase assays. Depletion of 14-3-3 ϵ via small interfering RNA accelerated CRYAB degradation over time (Fig. 7F), whereas 14-3-3 ϵ overexpression prolonged its cellular retention (Fig. 7G). In accordance with these kinetic alterations, steady-state CRYAB protein levels were diminished following 14-3-3 ϵ knockdown (Fig. 7H) and dose-dependently elevated upon its overexpression (Fig. 7I), confirming 14-3-3 ϵ as a positive regulator of CRYAB abundance. To evaluate the functional interdependency between 14-3-3 ϵ and CRYAB, genetic rescue experiments were performed. While 14-3-3 ϵ overexpression effectively blunted Yoda1-induced matrix degradation and inflammatory responses, concurrent silencing of *CRYAB* completely abolished these protective effects, indicating that the protective function of 14-3-3 ϵ is strictly dependent on the presence of CRYAB (Fig. S7C-E).

817 The capacity of 14-3-3 ϵ to shield CRYAB from the ubiquitin-proteasome pathway was evaluated
 818 in the presence of MG-132. Exogenous introduction of 14-3-3 ϵ restricted CRYAB
 819 polyubiquitination (Fig. 7J; Fig. S7F, G), whereas 14-3-3 ϵ silencing exacerbated ubiquitin chain
 820 assembly on CRYAB (Fig. 7K). Mechanistic dissection of this protective effect revealed a
 821 competitive binding dynamic. Co-expression analyses demonstrated that 14-3-3 ϵ overexpression
 822 disrupted the physical association between HUWE1 and CRYAB, thereby restricting HUWE1-
 823 driven ubiquitin tagging. Together, these data establish that 14-3-3 ϵ sterically hinders HUWE1
 824 access to critical lysine residues, suppressing K48-linked ubiquitination and sustaining CRYAB-
 825 mediated cellular protection in the intervertebral disc.



826
 827 **Figure 7. The scaffold protein 14-3-3 ϵ competitively binds CRYAB to antagonize HUWE1-**
 828 **mediated ubiquitination.**

829 (A, B) Venn diagram of triplicate IP-MS datasets and representative MS spectra identifying 14-3-
 830 3 ϵ as a CRYAB interaction candidate. (C, D) Reciprocal Co-IP analyses confirming the endogenous
 831 and exogenous physical association between 14-3-3 ϵ and CRYAB. (E) MST analysis quantifying

the direct in vitro binding affinity (Kd) between purified 14-3-3 ϵ and CRYAB. (F-I) CHX-chase assays and steady-state immunoblotting demonstrating changed CRYAB stability and abundance following 14-3-3 ϵ modulation. (J, K) In vitro ubiquitination assays illustrating the capacity of 14-3-3 ϵ to restrict ubiquitin chain assembly on CRYAB. Data are represented as mean $\pm$ SD. Statistical significance was determined utilizing one-way or two-way ANOVA followed by Tukey's post-hoc test (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Ginkgolide B, a dual CRYAB modulator, ameliorates mechanical stress-induced intervertebral disc degeneration in mice

To further translate these mechanistic insights into therapeutic strategies, it was essential to identify pharmacological agents capable of both elevating and maintaining intracellular CRYAB levels. A high-throughput dual-luciferase reporter screen of a 500-compound natural product library initially identified seven candidates that effectively promoted *CRYAB* transcription (Fig. 8A, B). Beyond this transcriptional activation, the capacity of these small molecules to physically protect the synthesized protein was evaluated. DARTS assays revealed that GB conferred the most robust protection against proteolytic cleavage (Fig. 8C). This direct target engagement was further corroborated utilizing the CETSA, which demonstrated that GB dose-dependently elevated the thermal stability of CRYAB in intact cellular environments (Fig. 8D). Moreover, MST analysis established a direct, high-affinity molecular binding event between purified GB and CRYAB (Fig. 8E).

The functional consequence of this structural engagement on CRYAB turnover was subsequently evaluated. Based on the premise that steric occupation can preserve protein stability, in vitro ubiquitination assays were performed. Under proteasomal inhibition with MG-132, GB administration effectively abrogated HUWE1-driven polyubiquitin chain assembly on CRYAB (Fig. 8F), indicating that pharmacological binding of GB structurally shields the protein from E3 ligase-mediated degradation.

The in vivo therapeutic efficacy of GB and its absolute dependency on the CRYAB target were rigorously interrogated utilizing the *Acan*-driven *Cryab* cKO murine model subjected to mechanical overloading. In mechanically overloaded *Cryab*^{fl/fl} mice, intra-disc administration of GB visibly ameliorated structural disruption, preserving proteoglycan content and the COL2 extracellular matrix while simultaneously suppressing MMP13 accumulation (Fig. 8G, K, M). Correspondingly, GB treatment maintained robust endogenous CRYAB protein levels despite the mechanical stress (Fig. 8I). Crucially, these chondroprotective effects were completely abolished in *Cryab* cKO mice. In the absence of the nucleus pulposus-specific CRYAB target, GB administration failed to halt matrix degradation or restrict catabolic enzyme expression under mechanical overload (Fig. 8G-M). These in vivo genetic validation data definitively establish Ginkgolide B as a targeted, dual-action therapeutic agent that mitigates IVDD progression by concomitantly driving *CRYAB* expression and structurally shielding it from degradation.

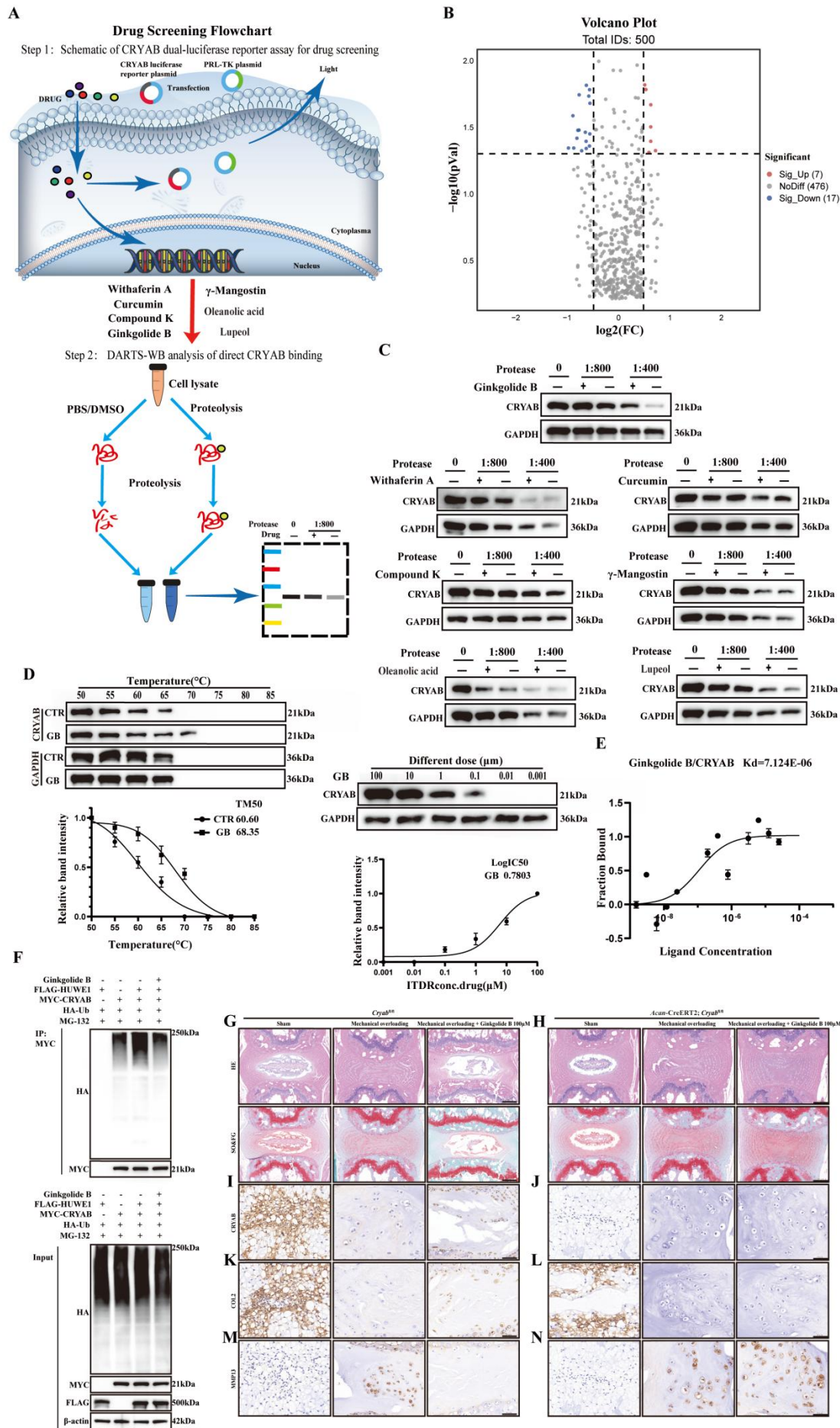


Figure 8. High-throughput screening identifies Ginkgolide B as a targeted dual-action therapeutic that structurally shields CRYAB.

(A) Schematic representation of the dual-luciferase reporter drug screening workflow targeting *CRYAB* transcriptional activation. (B) Volcano plot identifying primary small molecule candidates from a 500-compound natural product library. (C) DARTS assay evaluating proteolytic protection conferred by candidate molecules, pinpointing GB. (D) CETSA establishing the dose- and temperature-dependent elevation of *CRYAB* thermal stability by GB. (E) MST binding curve confirming direct physical engagement between GB and purified *CRYAB*. (F) In vitro ubiquitination assay demonstrating GB-mediated structural shielding against HUWE1-driven ubiquitin tagging. (G-M) In vivo genetic validation utilizing mechanically overloaded *Cryab*^{fl/fl} and *Cryab* cKO mice. Representative H&E, SO&FG, and corresponding IHC analyses demonstrate that intra-discal GB administration mitigates matrix degradation and represses catabolic enzymes strictly in a *CRYAB*-dependent manner. Scale bars as indicated.

Discussion

IVDD is a multifactorial pathological process in which aberrant mechanical stress serves as a critical environmental trigger[30, 31]. Despite this recognition, the precise molecular mechanisms by which mechanical forces disrupt cellular proteostasis to drive disc degeneration have remained elusive. In this study, we identify a previously uncharacterized proteostatic regulatory axis—the 14-3-3ε–HUWE1–CRYAB axis—that acts as a central molecular switch governing mechanically induced IVDD. Our findings demonstrate that pathological mechanical stress shifts the endogenous balance between the E3 ubiquitin ligase HUWE1 and the scaffold protein 14-3-3ε, driving K48-linked polyubiquitination and proteasomal degradation of the small heat shock protein CRYAB. Loss of CRYAB unleashes NF-κB signaling, triggering a cascade of NP cell inflammation, apoptosis, ECM catabolism, and senescence, ultimately precipitating IVDD. Furthermore, we identify Ginkgolide B as a dual-action pharmacological agent that both enhances CRYAB transcription and directly stabilizes the protein to block HUWE1-mediated ubiquitination, exerting robust therapeutic efficacy in vivo. Collectively, this work closes a critical gap in our understanding of mechanical stress-driven IVDD pathogenesis, linking mechanotransduction to post-translational control of protein homeostasis, and provides a promising disease-modifying therapeutic strategy for this prevalent condition.

CRYAB as a mechanosensitive gatekeeper of NP homeostasis

Our study establishes CRYAB as a critical mechanosensitive protective node in NP homeostasis and IVDD progression, with robust clinical and functional validation. Integrative scRNA-seq analysis of human NP tissues provides the first in vivo clinical evidence that CRYAB expression in NP cells declines progressively with worsening IVDD severity. Notably, CRYAB-deficient NP cells from degenerated discs exhibit a pronounced pathological phenotype, with upregulation of catabolic enzymes, inflammatory mediators, and apoptotic effectors, alongside activation of ubiquitin-dependent proteolytic pathways. This single-cell resolution data aligns with the established role of CRYAB as a stress-induced chaperone that preserves cytoskeletal integrity and inhibits apoptosis in other tissues, but its dynamic regulation in response to defined mechanical stimuli within the disc had not been previously characterized[32-34]. Our subsequent in vitro and in vivo experiments using hydrostatic pressure and lumbar instability models definitively link mechanical overload to CRYAB depletion, providing a direct causal line from the biomechanical trigger to the molecular defect. The observation that Piezo1 agonism phenocopies this effect further implicates this mechanosensitive ion channel as a key upstream node. Mechanistically, we demonstrate that CRYAB exerts its protective effects primarily by restraining NF-κB signaling,

blocking p65 nuclear translocation and subsequent transcription of pro-inflammatory and pro-apoptotic genes. These findings position CRYAB as a central molecular link between mechanical stress and NP cell fate, resolving a longstanding gap in understanding how mechanotransduction pathways converge on core effector networks to drive IVDD.

HUWE1 orchestrates CRYAB degradation via K48-linked ubiquitination

A major mechanistic advance of this work is the identification of HUWE1 as the specific E3 ubiquitin ligase that mediates the post-translational degradation of CRYAB in response to mechanical stress. While prior studies have noted CRYAB downregulation in degenerative conditions, most have focused on transcriptional control, leaving the mechanisms governing its protein stability in the intervertebral disc entirely uncharacterized. Our observation that mechanical stress induces a far more pronounced reduction in CRYAB protein than mRNA levels prompted investigation of post-translational regulation, leading to the discovery that CRYAB turnover is governed by the ubiquitin-proteasome system. Through proteomic screening and rigorous biochemical validation, we identify HUWE1, a HECT-type E3 ligase, as a direct, high-affinity binding partner of CRYAB. We further demonstrate that HUWE1 specifically catalyzes K48-linked, rather than K63-linked, polyubiquitination of CRYAB at defined lysine residues (K150/K161), targeting it for proteasomal degradation. Functionally, HUWE1 overexpression accelerates CRYAB turnover and abrogates its protective effects against mechanical stress, whereas HUWE1 knockdown stabilizes CRYAB and blunts load-induced degenerative changes in NP cells. Notably, HUWE1 has previously been implicated in cell proliferation, apoptosis, and stress responses in cancer and metabolic disorders, but its role in IVDD pathogenesis has not been reported[35-37]. Our findings not only define HUWE1 as the critical E3 ligase for CRYAB, but also establish a novel mechanistic link between mechanical stress and dysregulated protein homeostasis in the intervertebral disc.

14-3-3ε competitively blocks HUWE1-mediated degradation to preserve CRYAB stability

Complementing this discovery, we reveal that the scaffold protein 14-3-3ε acts as an endogenous counterbalance to HUWE1-mediated CRYAB degradation, forming a dynamic regulatory switch that maintains CRYAB homeostasis under physiological conditions. The 14-3-3 protein family is classically known to modulate client protein stability, localization, and activity via phosphoserine/threonine motif recognition; emerging evidence suggests that certain isoforms can stabilize targets by competitively disrupting interactions with E3 ubiquitin ligases[38-41]. Here, we demonstrate that 14-3-3ε directly binds CRYAB with high affinity, at the same lysine residues (K150/K161) that serve as HUWE1-mediated ubiquitination acceptor sites. This competitive binding sterically hinders the HUWE1-CRYAB interaction, inhibiting K48-linked polyubiquitination of CRYAB and prolonging its half-life. Functionally, 14-3-3ε overexpression increases steady-state CRYAB levels and protects against its degradation under mechanical stress, while 14-3-3ε depletion accelerates CRYAB turnover and exacerbates degenerative changes in NP cells. This discovery defines a previously unknown endogenous homeostatic mechanism in NP cells: under physiological mechanical conditions, 14-3-3ε dominates the interaction with CRYAB, maintaining its stability; under pathological overload, this balance is disrupted, with enhanced HUWE1 binding driving CRYAB degradation. This competitive binding paradigm represents a sophisticated regulatory logic that may extend to other stress-responsive proteins.

Ginkgolide B as a dual-action therapeutic strategy

From a translational perspective, our identification of GB as a dual-action CRYAB modulator addresses a critical unmet clinical need for IVDD. Current management is limited to symptomatic

relief and surgical intervention, with no approved disease-modifying therapies[42]. Through a dual pharmacological screening strategy, we identified GB as a compound that not only activates CRYAB transcription but also directly binds to CRYAB to enhance its thermal stability and protect it from HUWE1-mediated ubiquitination. Most pharmacological strategies target either transcription or protein stability, but rarely both; GB's dual action likely explains its robust in vivo efficacy. Critically, using NP-specific Cryab conditional knockout mice, we demonstrate that the chondroprotective effects of GB against mechanical overload-induced IVDD are entirely dependent on CRYAB, confirming its on-target mechanism. GB is a well-characterized natural product derived from Ginkgo biloba with documented anti-inflammatory, anti-oxidative, and neuroprotective properties, and a well-established safety profile in clinical use for cerebrovascular disease[43-46]. Our study is the first to report its therapeutic efficacy in IVDD and to define its molecular target as CRYAB, positioning GB as a promising lead compound for disease-modifying therapy.

Strengths of the study

This study has several notable strengths that reinforce the robustness and translational relevance of our findings. First, our work begins with clinical observations from human scRNA-seq data, anchoring mechanistic investigations in the molecular pathology of human IVDD. Second, we employ a comprehensive, multi-modal experimental approach integrating single-cell transcriptomics, biochemical assays, in vitro mechanical stress models, multiple in vivo animal models, and high-throughput pharmacological screening, creating a complete translational pipeline from target discovery to therapeutic validation. Third, we use rigorous genetic models, including NP-specific Cryab conditional knockout mice, to confirm the in vivo function of CRYAB and the target specificity of GB, avoiding off-target effects associated with non-specific gene manipulation. Finally, the discovery of the 14-3-3ε–HUWE1–CRYAB axis provides a multi-layered set of therapeutic targets, from upstream modulation of the HUWE1/14-3-3ε balance to direct stabilization of CRYAB, offering multiple avenues for therapy development.

Limitations

Several limitations should be acknowledged. First, our clinical scRNA-seq and validation cohorts are relatively small; future studies with larger, multi-center cohorts are needed to validate CRYAB as a diagnostic or prognostic biomarker. Second, although we identified Piezo1 as a key upstream mediator, the signaling cascade linking Piezo1 activation to enhanced HUWE1 activity toward CRYAB remains incompletely defined. It is plausible that mechanical stress alters the post-translational modification state of HUWE1, CRYAB, or 14-3-3ε, thereby modulating their interactions; future phosphoproteomic studies are needed to map this upstream network. Third, while our study focuses primarily on NP cells, IVDD is a whole-organ process involving the annulus fibrosus and cartilage endplate[47, 48]; further investigation is required to define the role of this axis in these compartments. Fourth, our in vivo therapeutic studies were conducted in rodent models, which differ from human discs in anatomy, mechanical loading, and cellular composition; future studies in large animal models (e.g., porcine or caprine) are needed to further validate the efficacy and safety of GB for clinical translation. Finally, we employed intradiscal injection in preclinical studies; future work should explore sustained-release delivery systems or the feasibility of systemic administration to improve clinical practicality.

Conclusion

In summary, we have uncovered a previously unrecognized “14-3-3ε–HUWE1–CRYAB” regulatory axis that critically governs NP cell fate in response to aberrant mechanical stress. This

work establishes that mechanical overload disrupts proteostasis by tipping the balance from a CRYAB-stabilizing mode (mediated by 14-3-3 ϵ) to a degradative mode (mediated by HUWE1). The resultant CRYAB loss drives NF- κ B-mediated catabolism and apoptosis, accelerating IVDD. The identification of Ginkgolide B as a dual-action stabilizer of CRYAB provides a compelling therapeutic avenue that directly counteracts this pathogenic mechanism. By integrating biomechanics, molecular biology, and pharmacology, this study offers a cohesive framework for understanding IVDD pathogenesis and lays the groundwork for the development of targeted therapies focused on preserving cellular proteostasis.

Abbreviations

AAV	Adeno-associated virus
ADV	Adenovirus
CCK-8	Cell Counting Kit-8
cDNA	Complementary DNA
CETSA	Cellular Thermal Shift Assay
CHX	Cycloheximide
cKO	Conditional knockout
Co-IP	Co-Immunoprecipitation
CRYAB	α B-crystallin
DARTS	Drug Affinity Responsive Target Stability
DEG	Differential expression gene
DMEM	Dulbecco's modified Eagle medium
ECM	Extracellular matrix
FBS	Fetal bovine serum
GB	Ginkgolide B
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
HEK293T	Human embryonic kidney 293T
IHC	Immunohistochemistry
H&E	Hematoxylin & Eosin
IOD	Integrated optical density

1036	IVDD	Intervertebral disc degeneration
1037	KEGG	Kyoto Encyclopedia of Genes and Genomes
1038	LC-MS/MS	Liquid chromatography-tandem mass spectrometry
1039	LNP	Lumbar needle puncture
1040	LSI	Lumbar spine instability
1041	MST	Microscale Thermophoresis
1042	NP	Nucleus pulposus
1043	PBS	Phosphate-buffered saline
1044	RT-qPCR	Quantitative real-time PCR
1045	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
1046	siRNA	Small interfering RNA
1047	scRNA-seq	Single-cell RNA-sequencing
1048	UMAP	Uniform Manifold Approximation and Projection
1049	UPS	Ubiquitin-proteasome system
1050	SD	Standard deviation
1051	SO&FG	Safranin O-Fast Green
1052	WT	Wild-type
1053	Declarations	
1054	Author contributions	
1055	Conceptualization: Yuan Gao, Kaiwen Liu, Yuanqiang Zhang and Lianlei Wang;	
1056	Methodology: Zhicheng Liu, Qunbo Meng, Jiankang Cao and Yifeng Shang;	
1057	Investigation: Zhicheng Liu, Qunbo Meng, Jiankang Cao and Yifeng Shang;	
1058	Formal analysis: Zexin Wang, Yiming Zhang and Zuyang Wang;	
1059	Visualization: Zhicheng Liu, Qunbo Meng, Jiankang Cao and Yifeng Shang;	
1060	Supervision: Lian Liu, Zhijian Wei, Lianlei Wang, Yuanqiang Zhang , Lei Cheng and Yuan Gao;	
1061	Writing—original draft: Qunbo Meng and Haoxin Zhai;	
1062	Writing—review & editing: Yunpeng Zhao and Yuhua Li;	

1063 Funding acquisition: Yuan Gao and Yuanqiang Zhang.

1064 **Funding**

1065 This work was supported by the National Natural Science Foundation of China (NSFC) Young
1066 Scientists Fund Project (82202752).

1067 **Data availability**

1068 All data associated with this study are available within the paper or the Supplementary Materials.
1069 Data are available from the corresponding author upon reasonable request.

1070 Clinical trial number: not applicable.

1071

1072 **Ethics declarations**

1073 **Ethics approval and consent to participate**

1074 The study protocol was performed in accordance with the Declaration of Helsinki, and written
1075 informed consent was obtained from all participants. The study was approved by the Ethics
1076 Committee of Qilu Hospital of Shandong University (Approval No. KYLL-2024(ZM)-100) on 29
1077 February 2024.

1078 All animal procedures were conducted in accordance with the recommendations of the
1079 International Council for Laboratory Animal Science (ICLAS) guidelines. All experimental
1080 procedures were approved by the Ethics Committee of Qilu Hospital of Shandong University
1081 (Approval No. KYLL-2024(ZM)-100) on 29 February 2024.

1082 **Consent for publication**

1083 All authors gave their consent for publication.

1084 **Competing interests**

1085 The authors declare no competing interests.

1086 **Reference**

1087 1. *Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic*
1088 *analysis for the Global Burden of Disease Study 2019*. Lancet, 2020. **396**(10258): p. 1204-1222.

1089 2. Mohd Isa, I.L., et al., *Discogenic Low Back Pain: Anatomy, Pathophysiology and Treatments of I*
1090 *ntervertebral Disc Degeneration*. International Journal of Molecular Sciences. **24**(1): p. 208.

1091 3. Cachot, U., et al., *A multiscale finite element model of fluid-microstructure interactions in human*
1092 *intervertebral disc compression*. Acta Biomater, 2025. **207**: p. 398-413.

1093 4. Lotz, J.C. and A.H. Hsieh, *The Effects of Mechanical Forces on Nucleus Pulposus and Annulus Fibro sus*
1094 *Cells*. Springer Vienna.

1095 5. Deneuville, J.P., et al., *Dynamic behavior of the nucleus pulposus within the intervertebral disc loading: a*
1096 *systematic review and meta-analysis exploring the concept of dynamic disc model*. Front Bioeng Biotechnol,
1097 **2025**. **13**: p. 1582438.

1098 6. Stokes, I.A. and J.C. Iatridis, *Mechanical conditions that accelerate intervertebral disc degeneration:*
1099 *overload versus immobilization*. Spine (Phila Pa 1976), 2004. **29**(23): p. 2724-32.

1100 7. Yang, Y., et al., *Mechanistic Interactions Driving Nucleus Pulposus Cell Senescence in Intervertebral Disc*
1101 *Degeneration: A Multi-Axial Perspective of Mechanical, Immune, and Metabolic Pathways*. JOR Spine,
1102 **2025**. **8**(3): p. e70089.

1103 8. Wang, S., et al., *High amplitude and low frequency cyclic mechanical strain promotes degeneration of*
1104 *human nucleus pulposus cells via the NF-κB p65 pathway*. J Cell Physiol, 2018. **233**(9): p. 7206-7216.

9. Yurube, T., et al., *Intervertebral disc cell fate during aging and degeneration: apoptosis, senescence, and autophagy*. North American Spine Society Journal (NASSJ), 2023. **14**: p. 100210.
10. Höhfeld, J., et al., *Maintaining proteostasis under mechanical stress*. EMBO Rep, 2021. **22**(8): p. e52507.
11. Dingal, P.C. and D.E. Discher, *Systems mechanobiology: tension-inhibited protein turnover is sufficient to physically control gene circuits*. Biophys J, 2014. **107**(11): p. 2734-43.
12. Elosegui-Artola, A., et al., *Force Triggers YAP Nuclear Entry by Regulating Transport across Nuclear Pores*. Cell, 2017. **171**(6): p. 1397-1410.e14.
13. Manukyan, I., et al., *Cross-talk between calcineurin/NFAT and Jak/STAT signalling induces cardioprotective alphaB-crystallin gene expression in response to hypertrophic stimuli*. J Cell Mol Med, 2010. **14**(6b): p. 1707-16.
14. de Jong, P.R., et al., *Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalized effects*. Cell Stress Chaperones, 2009. **14**(2): p. 117-31.
15. Arya, R., M. Mallik, and S.C. Lakhotia, *Heat shock genes - integrating cell survival and death*. J Biosci, 2007. **32**(3): p. 595-610.
16. Mitzelfelt, K.A., et al., *The Human 343delT HSPB5 Chaperone Associated with Early-onset Skeletal Myopathy Causes Defects in Protein Solubility*. J Biol Chem, 2016. **291**(29): p. 14939-53.
17. Muraleva, N.A., N.G. Kolosova, and N.A. Stefanova, *p38 MAPK-dependent alphaB-crystallin phosphorylation in Alzheimer's disease-like pathology in OXYS rats*. Exp Gerontol, 2019. **119**: p. 45-52.
18. Xie, S. and X. Wang, *CRYAB reduces cigarette smoke-induced inflammation, apoptosis, and oxidative stress by retarding PI3K/Akt and NF-κB signaling pathways in human bronchial epithelial cells*. Allergol Immunopathol (Madr), 2022. **50**(5): p. 23-29.
19. Bao, Q., et al., *αB-crystallin (CRYAB) regulates the proliferation, apoptosis, synthesis and degradation of extracellular matrix of chondrocytes in osteoarthritis*. Exp Cell Res, 2019. **382**(2): p. 111459.
20. Liu, D., J. Kuo, and C.H. Lin, *Computational Investigation of Smooth Muscle Cell Plasticity in Atherosclerosis and Vascular Calcification: Insights from Differential Gene Expression Analysis of Microarray Data*. Bioengineering (Basel), 2025. **12**(11).
21. Yu, P. and Z. Hua, *To Kill or to Be Killed: How Does the Battle between the UPS and Autophagy Maintain the Intracellular Homeostasis in Eukaryotes?* Int J Mol Sci, 2023. **24**(3).
22. Sampson, C., et al., *The roles of E3 ubiquitin ligases in cancer progression and targeted therapy*. Clin Transl Med, 2023. **13**(3): p. e1204.
23. Kao, S.H., H.T. Wu, and K.J. Wu, *Ubiquitination by HUWE1 in tumorigenesis and beyond*. J Biomed Sci, 2018. **25**(1): p. 67.
24. Qi, L., X. Xu, and X. Qi, *The giant E3 ligase HUWE1 is linked to tumorigenesis, spermatogenesis, intellectual disability, and inflammatory diseases*. Front Cell Infect Microbiol, 2022. **12**: p. 905906.
25. Hermeking, H., *The 14-3-3 cancer connection*. Nature Reviews Cancer. **3**(12): p. 931-943.
26. Tzivion, G., Y.H. Shen, and J. Zhu, *14-3-3 proteins; bringing new definitions to scaffolding*. Oncogene, 2001. **20**(44): p. 6331-8.
27. Pennington, K.L., et al., *SGK2, 14-3-3, and HUWE1 Cooperate to Control the Localization, Stability, and Function of the Oncoprotein PTOV1*. Mol Cancer Res, 2022. **20**(2): p. 231-243.
28. Chen, L., et al., *Arabidopsis BPM proteins function as substrate adaptors to a cullin3-based E3 ligase to affect fatty acid metabolism in plants*. Plant Cell, 2013. **25**(6): p. 2253-64.
29. Dar, A., et al., *14-3-3 proteins play a role in the cell cycle by shielding cdt2 from ubiquitin-mediated degradation*. Mol Cell Biol, 2014. **34**(21): p. 4049-61.
30. Fu, F., et al., *Aberrant spinal mechanical loading stress triggers intervertebral disc degeneration by inducing pyroptosis and nerve ingrowth*. Scientific Reports, 2021. **11**(1): p. 772.
31. Ke, W., et al., *An overview of mechanical microenvironment and mechanotransduction in intervertebral disc degeneration*. Experimental & Molecular Medicine, 2025. **57**(10): p. 2157-2168.
32. Wójtowicz, I., et al., *Drosophila small heat shock protein CryAB ensures structural integrity of developing muscles, and proper muscle and heart performance*. Development, 2015. **142**(5): p. 994-1005.
33. Maloyan, A., et al., *Mitochondrial dysfunction and apoptosis underlie the pathogenic process in alpha-B-crystallin desmin-related cardiomyopathy*. Circulation, 2005. **112**(22): p. 3451-61.
34. Wang, C., et al., *Mutation of CRYAB encoding a conserved mitochondrial chaperone and antiapoptotic protein causes hereditary optic atrophy*. JCI Insight, 2024. **10**(1).
35. Jo, S., et al., *O-GlcNAcylation of DDB1 at Ser-764 by OGT promotes cancer cell stemness in colorectal cancer through increased polyubiquitination of p53*. Sci Rep, 2025. **16**(1): p. 1232.
36. Searle, J., et al., *The E3 ubiquitin ligase HUWE1 is required for KRAS-induced lung cancer*. Cell Death Dis, 2026.
37. Jiao, H., et al., *HUWE1 in Skeletal Muscle Prevents Muscle Fatigue via Maintaining Iron and Calcium Homeostasis*. Adv Sci (Weinh), 2026. **13**(4): p. e16719.
38. Barrera, E.E., M. Uhart, and D.M. Bustos, *The 14-3-3 Protein Family, Beyond the Kinases and Phosphatases*. 2025. **3**(4): p. 24.

39. Choi, H.H., et al., *COP9 signalosome subunit 6 stabilizes COP1, which functions as an E3 ubiquitin ligase for 14-3-3 σ* . *Oncogene*, 2011. **30**(48): p. 4791-801.
40. Janosev, M., et al., *Structural basis of ubiquitin ligase Nedd4-2 autoinhibition and regulation by calcium and 14-3-3 proteins*. *Nat Commun*, 2025. **16**(1): p. 4875.
41. Zhang, C., et al., *A phosphorylation-dependent ubiquitination switch orchestrates nuclear immune reprogramming upon chitin perception*. *Nat Commun*, 2026. **17**(1).
42. Gradisnik, L., et al., *Degenerative Disease of Intervertebral Disc: A Narrative Review of Pathogenesis, Clinical Implications and Therapies*. 2026. **13**(1): p. 40.
43. Mohammadi Zonouz, A., M. Ghasemzadeh Rahbardar, and H. Hosseinzadeh, *The molecular mechanisms of ginkgo (Ginkgo biloba) activity in signaling pathways: A comprehensive review*. *Phytomedicine*, 2024. **126**: p. 155352.
44. Masserini, F., et al., *Therapeutic strategies in vascular cognitive impairment: A systematic review and meta-analysis*. *Alzheimers Dement*, 2025. **21**(11): p. e70840.
45. Li, W., et al., *Network meta-analysis of the efficacy of pharmacological treatments for post-stroke cognitive impairment and vascular cognitive impairment*. *Front Neurol*, 2025. **16**: p. 1683496.
46. Hwang, Y.C., et al., *Comparison of Efficacy and Safety of Cilostazol/Extract of Ginkgo biloba vs. Aspirin in Carotid Atherosclerosis in Patients with Diabetes Mellitus*. *Diabetes Metab J*, 2026. **50**(2): p. 357-367.
47. Ashinsky, B., et al., *Intervertebral disc degeneration and regeneration: a motion segment perspective*. *Eur Cell Mater*, 2021. **41**: p. 370-380.
48. Feng, C., et al., *Region-specific mitophagy in nucleus pulposus, annulus fibrosus, and cartilage endplate of intervertebral disc degeneration: mechanisms and therapeutic strategies*. *Front Pharmacol*, 2025. **16**: p. 1579507.
49. Liu, K., et al., *Dapagliflozin regulates chondrocyte homeostasis and protects against osteoarthritis via targets AMPKalpha and SGLT2*. *Cell Death Discov*, 2026.
50. Liu, Z., et al., *Morusin Alleviates Spinal Cord Injury in Rats by Regulating Macrophage Reprogramming Through Targeting RELA and NRF2*. *Phytother Res*, 2026. **40**(2): p. 436-454.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [OriginalWesternBlotImages.pdf](#)
- [Checklist.docx](#)
- [supplementalmaterial.pdf](#)