Chondroitin synthase-3 regulates nucleus pulposus degeneration through actin-induced YAP signaling

Leixin Wei1,2 | Peng Cao1 | Chen Xu1 | Huajian Zhong1 | Xiukun Wang2 | Meizhu Bai2 | Bo Hu1 | Ruizhe Wang1 | Ning Liu1 | Ye Tian1 | Huajiang Chen1 | Jinsong Li2 | Wen Yuan1

1Department of Orthopaedic Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, China 2State Key Laboratory of Cell Biology, Shanghai Key Laboratory of Molecular Andrology, CAS Center for Excellence in Molecular Cell Science, Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China

Correspondence
Jinsong Li, State Key Laboratory of Cell Biology, Shanghai Key Laboratory of Molecular Andrology, CAS Center for Excellence in Molecular Cell Science, Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, 200031, China. Email: jsl@sibcb.ac.cn

Wen Yuan, Department of Orthopaedic Surgery, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China. Email: yuanwenspine@smmu.edu.cn

Funding information
National Nature Science Foundation of China, Grant/Award Number: 82002335, 81972088, 81871802, 81772376, 81601928, 81702149, 31971109 and 31471390; Shanghai Municipal Commission of Health and Family Planning, Grant/Award Number: 17140901500, 18ZK1438900, 20QA1409200 and 20184Y0181; Shanghai Education Development Foundation; Shanghai Municipal Education Commission “Chen Guang” Program, Grant/Award Number: 17CG36; Shanghai “Rising Stars of Medical Talent” Youth Development Program

Abstract
Loss of chondroitin sulfate (CS) has been reported to play a key role during intervertebral disc degeneration (IDD). However, the detailed mechanism of CS and its synthases has not been elucidated. Since CS is mainly synthesized by chondroitin synthases 3 (Chsy3), here, the Chsy3 knockout mice are generated by using CRISPR-Cas9 and semi-cloning technology to study its mechanism during IDD. We find that CS and Chsy3 expression are decreased during IDD both in human and mouse nucleus pulposus (NP) tissue, and knockout of Chsy3 shows that spontaneous IDD phenotype resembles that of human samples in the Chsy3−/− mice. Taking advantage of RNA-Seq data, we confirm increased catabolic and decreased anabolic changes in Chsy3−/− NP cells. By using bioinformatic analysis and validation, we find that Hippo signaling pathway is significantly downregulated, and the activation of Yap1 is mainly affected in Chsy3−/− NP cells. Furthermore, functional analyses have shown that Chsy3 could regulate NP cell degeneration by Actin tension mediated activation of Yap1, which is independent of Hippo/Lats signaling. In summary, our findings reveal a novel mechanism that depletion of CS-related Chsy3 can cause spontaneous intervertebral disc degeneration by mediating Yap activation through CS-related actin-tension in NP cells.

KEYWORDS
chondroitin sulfate, intervertebral disc degeneration, nucleus pulposus, Yap

Abbreviations: ADAMTS, a disintegrin and metalloprotease with thrombospondin motifs; AF, annulus fibrosus; cDNA, complementary DNA; Chsy, chondroitin synthase; CS, chondroitin sulfate; DNA, deoxyribonucleic acid; ECM, extracellular matrix; GAG, glycosaminoglycan; GO, gene ontology; IDD, intervertebral disc degeneration; IVD, intervertebral disc; MMP, matrix metalloproteinases; mRNA, messenger RNA; NP, nucleus pulposus; RNA, ribonucleic acid.

Leixin Wei, Peng Cao, Chen Xu and Huajian Zhong contributed equally to the work.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2020. The Authors. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology.
1 | INTRODUCTION

Low back pain (LBP) is a common symptom, and also a major cause of job disability.1,2 According to estimation, the treatment of LBP has exceeded $100 billion per year in just United States,3 which has been a global matter that need to be solved. The intervertebral disc degeneration (IDD) has been considered as a leading contributor to LBP, but the pathological mechanism is poorly understood.4 The IDD is characterized by imbalanced metabolism of extracellular matrix (ECM), increased inflammatory cytokines, cell apoptosis, and neurovascular ingrowth.5 During IDD, the expression of matrix metalloproteinases (MMPs) and a disintegrin and metallopeptase with thrombospondin motifs (ADAMTSs) is increased, further leading the increasing breakdown of ECM. Meanwhile, the synthesis of important ECM components, such as aggrecan and type II collagen (Collagen II), is decreased.6 Loss of aggrecan is responsible for the disc tissue dehydrating, further resulting in less ability to maintain disc height and absorb compressive loads. Another important function of aggrecan is to inhibit the nerve ingrowth and thus, reduce pain-related symptoms.7 Both the tissue dehydrating and nerve ingrowth during IDD are largely due to loss of the chondroitin sulfate (CS) content inside. The CS, a sulfated glycosaminoglycan (GAG) component of aggrecan, is consisting of repeating disaccharide units.8 This CS consisted GAG chains are highly sulfated, providing aggrecan with normal osmotic properties.9 Our previous studies revealed that CS content was significantly reduced and its synthetases were dysregulated during IDD.10,11 However, it is still unknown that how CS content affects nucleus pulposus degeneration.

To date, six glycosyltransferases have been identified that are involved in the CS biosynthesis, chondroitin synthase 1 (CHSY1),12 CHSY2 (or chondroitin sulfate synthase 3, CSS3),13 CHSY3 (or chondroitin sulfate glucuronyltransferase, CSGlCAT or chondroitin polymerizing factor 2, CHPF2),14,15 CHPF (or CSS2),16,17 chondroitin sulfate N-acetylgalactosaminyltransferase (CSGalNAcT-1)18 and CSGalNAcT-2.19 CHSY1, CHSY2 and CHSY3 possess two glycosyltransferase activities of N-acetylgalactosaminyltransferase-II (GalNAcT-II) and glucuronyltransferase-II (GlcAT-II), while CHPF exhibits little glycosyltransferase activity. A large-scale functional screen for secreted and transmembrane proteins indicated that CHSY1 and CHSY3 were related to skeletal tissue metabolism.20 And researches showed that CHSY1 and CHSY3 were both highly expressed in cartilage and intervertebral discs.11,21,22 CHSY1-deficient mice display skeletal abnormalities containing chondrodysplasia and decreased bone density,23 and our previous study found that the expression of CHSY1 in the nucleus pulposus (NP) was upregulated by TGF-β through activation of MAPK signaling.24 However, evidence showed that overexpression of CHSY3 in HeLa cells significantly increased the amount of CS, which is higher than that of CHSY1.15 Hence, CHSY3 may play a more important role in CS biosynthesis, so as in normal disc function maintenance. However, it is unclear how the CHSY3 functions in the intervertebral disc and IDD. In this study, we use Chsy3 knock-out mice to systemically investigate the molecular function and mechanisms of CHSY3 in IDD.

2 | MATERIALS AND METHODS

2.1 | Chsy3 knockout mice

All animal experiments were approved by the Second Military Medical University Animal Care and Use Committee. The Chsy3+/− female mice were generated by combining CRISPR-Cas9 technology with semi-cloning technology.25-27 We first used the CRISPR-Cas9 technology to generate Chsy3 knock-out artificial spermatids and then injected it into the mature oocytes to obtain Chsy3+/− female mice (F0). To generate Chsy3−/− mice, the Chsy3+/− females were backcrossed with wild-type (WT) males (C57BL/6) to obtain Chsy3+/− male and female mice (F1), which were then crossed to obtain Chsy3−/− mice (F2). The wild-type littermates were used as controls.

2.2 | Sample collection and cell culture

The H19-DMR and JG-DMR DKO-AG-haESCs were used for gene editing as described previously.26 The DKO-AG-haESCs were cultured in ESC medium supplemented with 1500 U/mL LIF (Millipore, Billerica, MA, USA), 3 μM CHIR99021 (Selleck, Houston, Texas, USA), and 1 μM PD0325901 (Selleck, Houston, Texas, USA).

To obtain mice primary disc cells, we harvested the intervertebral discs (from T10-L3) from 8-week-old mice, and then dissected under microscopy to isolate the NP tissue. NP tissue specimens were washed twice with PBS, then, minced and digested with 0.25 mg/mL type II collagenase (Gibco, Grand Island, NY, USA) in DMEM medium (Gibco, Grand Island, NY, USA) for 4 hours at 37°C. NP cell suspension was transferred into a 40 μm cell strainer (BD Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 800 g for 5 minutes. The NP cells were resuspended in DMEM containing 10% FBS (Gibco, Grand Island, NY, USA), 100 U/mL penicillin (Gibco, Grand Island, NY, USA), 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA), and 1% L-glutamine (Gibco, Grand Island, NY, USA). Cells were incubated at 37°C in 5% CO2 and the medium was changed every 3 days. The osmotic pressure of culture medium was adjusted to 450 mOsm/kg H2O by adding proper amount of...
sucrose and NaCl to mimic the in situ osmotic pressure of the NP tissue. Cells at passage three and on were used for all subsequent experimental procedures.

For human NP cell culture, the tissue specimens were treated according to the mouse NP cell culture method, and the isolated NP cells (mainly from L4/5 of degenerated patients) were resuspended in DMEM/F12 containing 10% FBS (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% L-glutamine. The viability of the suspended cells was over 90% when assessed using cell counting kit-8 (Dojindo, Tokyo, Japan). Cells were incubated at 37°C in 5% CO₂ and the medium was changed every 3 days. Cells at the second passage were used for subsequent experimental procedures.

2.3 | In vitro NP cell degeneration model

For in vitro NP cell degeneration, we treated NP cells with recombinant human TNF-α (PEPROTECH, Cranbury, NJ, USA) (50 ng/mL) or recombinant human IL-1β (PEPROTECH, Cranbury, NJ, USA) (25 ng/mL) to establish the degeneration model if not indicated.

2.4 | Human disc tissues information

Informed consent was provided by the patients or their relatives to obtain human intervertebral tissue at surgery. The experiment was authorized by the ethics committee of Second Military Medical University (The approval number was SHCZ0000423). Patients' disc tissues were obtained from 63 patients undergoing lumbar interbody fusion surgery. IDD was confirmed in their MRI images of the lumbar spine and patients diagnosed with classical sciatica, infection or other spinal diseases were excluded from the study. About 51 degenerated nucleus pulposus samples with different Pfirrmann grade (II-V) of disc degeneration were obtained from patients who underwent disc resection surgery or spinal fusion to relieve low back pain. About 12 normal disc tissues were obtained from trauma patients with no previous history of IDD. In this study, samples of grade-I (G-I) were classified as the normal group, samples of grade-II (G-II) and grade-III (G-III) were classified as the mild IDD group, grade-IV (G-IV) and grade-V (G-V) samples were classified as the severe IDD group. The details of patient information can be found in Table S2.

2.5 | Construction of plasmid

To construct the CRISPR-Cas9 plasmid for Chsy3 gene, sgRNA targeted in the exon 1 of Chsy3 gene was designed, synthesized, annealed, and ligated to the pX330-mCherry plasmid which was digested with Bbs I (New England Biolabs, Ipswich, MA, USA).

For Chsy3 or Yap1 overexpression, we used Chsy3 or Yap1 overexpression lentivirus provided by OBiO Technology Corp., Ltd (Shanghai, China). For Chsy3 knockdown, Yap1 knockdown and Lats1 knockdown, we use synthesized chemically modified small interfering RNA mediated knockdown provided by Shanghai GenePharma Co., Ltd (Shanghai, China).

For detection of Yap/Taz activities, we use 8xG-TIIC-luciferase as a gift from Stefano Piccolo (Addgene plasmid # 34615; http://n2t.net/landingpage; RRID: Addgene_34615). The detection method was according to previously reported. For detection of TGF-β/Smad activities, we use SMAD luciferase reporter plasmid provided by Yeasen Biotech Co., Ltd. (Shanghai, China). The detection methods are as previously described.

2.6 | Transfection and FACS

Approximately 1 × 10⁶ DKO-AG-haESCs were transfected with 2 μg of pX330-mCherry plasmid including sgRNA targeting Chsy3 gene by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instruction manual. 24 hours after transfection, the DKO-AG-haESCs expressing red fluorescence protein were sorted with flow cytometry (Aria II cell sorter, BD Biosciences, Franklin Lake, NJ, USA) and plated at a low density. After 5-6 days of cell expansion, single colonies were picked for derivation and genotype using PCR and sequencing. siRNA transfections were done with Lipofectamine RNAi-MAX (Invitrogen, Carlsbad, CA, USA) in antibiotics-free medium according to manufacturer's instructions. Sequences of siRNAs are provided in Supplementary Data S1.

2.7 | Intracytoplasmic AG-haESCs injection (ICAHCI) and embryo transfer

To generate semi-cloned (SC) embryos, the ICAHCI technique was used as described before. The DKO-AG-haESCs containing intended mutation of Chsy3 gene were arrested at M phase by culturing in medium supplemented with 0.05 μg demecolcine for 8 hours before ICAHCI. The DKO-AG-haESCs were trypsinized, washed with HEPES-CZB medium three times, and suspended in HEPES-CZB medium containing 3% (w/v) of polyvinylpyrrolidone. And then each nucleus from M-phase haploid cells was injected into MI-arrested oocyte by using a Piezo-drill micro-manipulator. The reconstructed oocytes were cultured in CZB medium for 1 hour and activated in activation medium without CB for 5-6 hours.
And then, the activated reconstructed embryos were cultured in KSOM medium with amino acid at 37°C under 5% CO₂ in air. After culturing in KSOM medium for 24 hours, the SC embryos reached to the two-cell stage. 15-20 embryos at two-cell stage were then transferred into each oviduct of pseudo-pregnant ICR female mice at 0.5 days post-coitum (dpc).

2.8 | Body length and weight

Under anesthesia, the body length and weight of mice were measured at and after 4 weeks and recorded every two weeks. The body length was defined as the length from dorsal tip of nose to the dorsal base of tail as previously described.³⁰

2.9 | MRI and micro-CT analysis

For MRI analysis, knockout mice were scanned at 4 weeks and 8 weeks old under anesthesia (n = 6 for each group). Imaging was acquired using quadrature extremity coil that were integrated into a clinical 3 Tesla Siemens Trio MRI (Siemens Medical Solutions, Erlangen, Germany). The MRI images of mice or human lumbar disc were used for further analysis. According to the modified Thompson classification, the severity of disc degeneration was divided into four grades: Grade 1, normal; Grade 2, minimal decrease of signal intensity but obvious narrowing of high signal area; Grade 3, moderate decrease of signal intensity; and Grade 4, severe decrease of signal intensity.³¹ The mean grey scale of the disc was measured by ImageJ software.

For micro-CT scanning, knockout mice were scanned at 4 weeks and 8 weeks old under anesthesia (n = 6 for each group). Scanning was performed at 49 kV, a current of 179 μA (Skyscan 1172, Bruker, Kontich, Belgium), an exposure time of 475 ms and a filter of Al 0.5 mm. Hydroxyapatite phantoms (0.25 and 0.75 mg/cm³) were equally scanned for mineral density calibration. After scanning, μCT projections were reconstructed into cross-sectional images using a modified back-projection algorithm (N-Recon, V1.01; Bruker). Reconstructed μCT images were digitally aligned (Dataviewer V1.01; Bruker) to a common global coordinate system to allow for accurate and consistent measurements of anisotropy. Regions of interest for trabecular bone and cortical bone were hand-drawn (Ctan, V1.01.0; Bruker). Analyses of bone densitometry (bone quantity), microarchitecture (bone quality), and anisotropy (bone quality) were performed (Ctan, V1.01.0; Bruker). Individual 2D cross-sections of cortical bone were analyzed for tissue mineral density, BMD, bone surface area, cross sectional thickness, and porosity. Each measure was then averaged providing a mean value. Disc height index (DHI) was calculated by dividing the average disc height by the average height of adjacent vertebral bodies.

2.10 | Histological staining, immunohistochemistry (IHC), and immunofluorescence (IF)

Mice or human lumbar disc and NP tissues were isolated and fixed in 4% (w/v) buffered paraformaldehyde for 24 hours, then embedded in paraffin. A 6 μm disc tissue sections were processed for H&E, Masson and safranin O-fast green staining performed by Shanghai Yubio Co., Ltd (Shanghai, China) according to standard protocols. IHC was also performed according to standard protocols. In brief, antigen retrieval was conducted by trypsin for 30 minutes at 37°C. Then, the sections were blocked with 1% BSA for 15 minutes at room temperature. The sections were incubated at 4°C overnight with rabbit anti-Chsy3 (PL0306862, PLLABS, Richmond, BC, Canada) or rabbit anti-Yap1 (13584-1-AP, Proteintech, Rosemont, IL, USA). After washing three times, the sections were incubated with the secondary antibody peroxidase-conjugated affinipure goat anti-rabbit IgG (1:1000 dilution, SA00001-2, Proteintech, Rosemont, IL, USA). Finally, the sections were counterstained with hematoxylin.

For IF analysis, cells were prepared as previously described,²⁹ and cells were fixed with 4% of paraformaldehyde in PBS pH 7.4 for 10 minutes at room temperature, then washed three times with ice-cold PBS. Permeabilization was performed by incubating the samples for 10 minutes with PBS containing 0.15% Triton X-100, then washed three times with ice-cold PBS. The antigens were blocked using 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) for 30 minutes and then incubated with following antibodies: rabbit anti-Actin (ab179467, Abcam, Cambridge, MA, USA), rabbit anti-CS (BS5178R, Bios, Beijing, China), rabbit anti-Chsy3 (PL0306862, PLLABS, Richmond, BC, Canada), rabbit anti-Yap1 (13584-1-AP, Proteintech, Rosemont, IL, USA), mouse anti-CS (ab11570, Abcam, for human samples, Cambridge, MA, USA). After washing three times, the sections were incubated with the secondary antibody R-PE-conjugated Goat Anti-Rabbit IgG (1:1000 dilution, SA00008-2, Proteintech, Rosemont, IL, USA), CoraLite488-conjugated Affinipure Goat Anti-Mouse IgG (1:1000 dilution, SA00013-1, Proteintech, Rosemont, IL, USA) or CoraLite488-conjugated Affinipure Goat Anti-Rabbit IgG (1:1000 dilution, SA00013-2, Proteintech, Rosemont, IL, USA), and then counterstained with DAPI (Invitrogen, Carlsbad, CA, USA). Samples were imaged using a ZEISS microscope (ZEISS Axio Imager A2, Carl Zeiss microscopy GmbH, Jena, Germany). Immunostained slides were identified independently by three pathologists who were blinded to patients’ data and outcomes. When there were different evaluation results, a consensus result should be achieved after re-examination. Within each sample, 200 cells were counted and the number of immune-positive cells expressed as a proportion of this sample.
2.11 | DMMB (1,9-dimethylmethylene blue) assay

The DMMB assay was performed by using a Blyscan sGAG assay kit (Biocolor, B1000, Biocolor, Carrickfergus, UK). Firstly, harvested tissues or NP cells were washed with PBS, and then, digested with 1 mL papain extraction reagent for 24 hours at 65°C. CS contents were measured by the reaction with DMMB, and staining was quantified by measuring absorbance at 656 nm. Chondroitin-4-sulfate was used as the standard.

2.12 | Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from disc tissues or NP cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Concentration of total RNAs are measured at 260 nm with a spectrophotometer (DU-800; Beckman Coulter, Brea, CA, USA). The PrimeScript RT reagent Kit with gDNA Eraser (TAKARA, Kyoto, Japan) are used to conduct reverse transcription with a 20-µL final reaction mixture according to the manufacturer’s instructions. Real-time PCR was performed by using TB Green Premix Ex Taq (TAKARA, Kyoto, Japan) with a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH was used to normalize the gene expression of other mRNAs. The relative amount of each transcript was calculated according to the comparative Ct method. Each experiment was repeated at least three times independently. Primers used in this article are listed in Supplementary Data S1.

2.13 | Western blot analysis

Cellular protein was extracted with RIPA lysis buffer and the protein concentration was measured by Bicinchoninic Acid Protein assay kit (Pierce, Rockford, IL, USA). Cellular protein was loaded on SDS-PAGE-denaturing gels and transferred to PVDF membranes. Immunolabeling was detected using Pierce ECL Western Blotting Substrate (Pierce Rockford, IL, USA). Following antibodies were used: anti-CHSY3 (ab75050, Abcam, Cambridge, MA, USA), anti-GAPDH (60004-1-Ig, Proteintech, Rosemont, IL, USA), anti-Yap1 (13584-1-AP, ProteinTech, Rosemont, IL, USA), anti-pYap1 (ab76252, Abcam, Cambridge, MA, USA), anti-Ctgf (23936-1-AP, ProteinTech, Rosemont, IL, USA), and Anti-Ankrd1 (11427-1-AP, ProteinTech, Rosemont, IL, USA). Horseradish peroxidase-linked goat anti-mouse (32230, Thermo, Waltham, MA, USA) antibody and goat anti-rabbit (32260, Thermo, Waltham, MA, USA) antibody were used as secondary antibody.

2.14 | RNA-Seq analysis

For transcriptome sequencing, RNA samples of primary disc cells were prepared from 8-weeks-old WT, Chsy3+/− and Chsy3−/− mice. Then, the RNA samples were extracted using the TRIZol solution (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocols. Further library construction, RNA quality control check, purification, quantification, and validation steps are conducted by Shanghai NovelBio Bio-Pharm Technology Co., Ltd. (Shanghai, China). For analysis of transcriptome sequencing data, we aligned approximately 100 bp long reads to the mouse genome (mm9) using Tophat2/Bowtie2. We identified mapped data to gene structures derived from RefSeq using the summarize overlaps function with mode Intersect Strict (Genomic Ranges, Bioconductor). Using the initial raw counts data, we calculated reads per kilobase per million reads mapped (RPKM) values for the same gene set using Cufflinks. The differential analysis was carried out using edgeR, applying TMM (trimmed Mean of M-values) library normalization and a .05 false discovery rate (FDR) to select expressed transcripts. All processed and raw data can be found in GEO database under the Accession No. GSE145649.

Gene Ontology (GO) and KEGG pathway analyses were performed as previously described. In brief, we calculated the P-value of each GO term using right-sided hypergeometric tests, and Benjamini-Hochberg adjustment was used for multiple test correction. An adjusted P-value that is lower than .05 indicated a statistically significant deviation from the expected distribution, and thus, the corresponding GO terms and pathways were enriched in target genes. We analyzed all the differentially expressed mRNAs using GO and KEGG pathway analyses.

2.15 | Statistical analysis

The SPSS 20.0 Software (IBM, San Diego, CA, USA) was used for statistical analysis. Quantitative data were described as means ± SDs. Unpaired two-tailed Student’s t test was used to compare the difference between the two experimental groups. One-way analysis of variance (ANOVA) and Tukey’s post hoc was used for comparing multiple groups. P values <.05 were considered as statistically significant. No statistical method was used to predetermine sample size for these experiments. Data collection was conducted randomly.

2.16 | Data and materials availability

All processed and raw data of the RNA sequencing can be found in GEO database under the Accession No. GSE145649.
Figure 1  Chsy3 is downregulated in human IDD. A, Human disc tissues were collected and classified by Pfirrmann grading system. B, Compared with human normal discs (normal group), CS content was significantly decreased in degenerative discs (IDD group). n = 12 in normal group, n = 51 in IDD group, t test. C, Immunofluorescence CS staining of normal (n = 12) and degenerated (n = 51) human NP tissue also confirmed CS expression was significantly decreased in degenerated NP tissues, t test. qRT-PCR analysis showing expression changes of CS-related synthetases (D) and CS-related transferases (E) between human degenerated (n = 51) and normal samples (n = 12), t test. Degenerated samples were a combination of mild and severe degenerated samples indicated in A. The detailed CHSY3 expression between each degeneration grade were indicated in (F), ANOVA. G, Immunohistochemistry staining confirmed the expression of CHSY3 was significantly decreased in degenerate discs. n = 6, t test. The qRT-PCR experiments were repeated three times independently, and GAPDH is used as an internal control. All data are shown as mean ± SD. *P < .05; **P < .01
FIGURE 2  The generation of IDD-related Chsy3 knockout mice. A, Schematic diagram of Chsy3 sgRNA design. The red bases represent the PAM sequence, while the blue bases represent sequence of Chsy3 sgRNA. B, Schematic diagram of generating DKO-AG-haESCs with −1 bp frameshift mutation of Chsy3. FACS, fluorescence-activated cell sorter. C, Schematic diagram of Chsy3+/− F0 mice generated by ICAHCl using DKO-AG-haESCs carrying −1 bp frameshift mutation of Chsy3. PB, the second polar body from the oocyte; PPB, the pseudo-polar body from metaphase haploid cell nucleus; PPN, pseudopronucleus derived from injected haESCs. D, Schematic diagram of mating of Chsy3+/− F0 mouse to generate Chsy3 F2 mice. E, Genotyping of Chsy3 F2 mice. F, qRT-PCR analysis confirmed the RNA level of Chsys among WT, heterozygous and homozygous F2 mice. t test, WT was served as control. G, Western blot analysis confirmed the protein level of Chsy3 among WT, heterozygous, and homozygous F2 mice. H, Genotyping of H19-DMR and IG-DMR in Chsy3+/− F2 mice. The qRT-PCR experiments were repeated three times independently, and GAPDH is used as an internal control. All data are shown as mean ± SD. *P < .05; **P < .01
More figures and related data can be found in the online supplementary figures and data.

3 | RESULTS

3.1 | CHSY3 expression is decreased during human IDD

The severity of human IDD has been classified into five grades according to the Pfirrmann grading system. By analyzing 62 human NP samples, we found the CS amount was decreased significantly in NP tissue samples of IDD group (IDD group contains both mild and severe degeneration samples, Figure 1A-C). The CS-related synthetases and transferases were also significantly decreased (Figure 1D,E). Because CS content is largely dependent on synthetases than CS-related transferases, here, we chose Chsy3 (NM_019015, human) for further assessments. The expression of CHSY3 was gradually decreased with the increasing degenerative Pfirrmann grade (Figure 1F), and its downregulation was also confirmed by IHC (Immunohistochemistry) analysis in human NP samples (Figure 1G). Thus, combining these human sample data, and concerning the roles of Chsy3 in CS biosynthesis and NP degeneration were not clarified, we constructed Chsy3 (NM_133913, Mus musculus) knockout mice models to further investigate it. We used the CRISPR-Cas9 gene editing technique and semi-cloning technique to generate Chsy3 knock-out mice. We designed the sgRNA targeting the exon 1 of mouse Chsy3. (Figure 2A). The pX330-mCherry-Chsy3 plasmid was transfected into the androgenetic haploid embryonic stem cells carrying both H19-DMR and IG-DMR deletions (DKO-AG-haESCs) that can be used as sperm replacement to “fertilize” mouse oocytes to produce animals (Figure 2B). After 12 hours, we found red fluorescence in the experimental group under the fluorescence microscope (Figure S1A). The ratio of mCherry positive haploid cell was 8.5% in the experimental group (Figure S1B) and we established a haploid cell line with −1 bp frameshift mutation in Chsy3 (Figure S1C,D). Then, we injected the Chsy3−1 bp DKO-AG-haESCs into the MII oocytes, which developed into the two-cell stage in vitro. And we transplanted the embryos into the uterus to gain the F0 Chsy3+/− semi-cloned mice that mated into the F2 mice (Figure 2C,D). The genotypes of Chsy3 in F2 mice were WT/WT, −1 bp/WT, and −1 bp/−1 bp, respectively (Figure 2E). The mRNA and protein levels of CHSY3 decreased significantly in NP of heterozygous and homozygous F2 mice when compared to that of WT (Figure 2F,G). Meanwhile, the H19-DMR and IG-DMR deletions were crossed out in Chsy3−/− F2 mice (Figure 2H). Off-target analysis in Chsy3−/− F2 mice indicated no indel mutation in top 20 predicted sites (Table S1).

3.2 | Chsy3 depletion directly induces IDD in Chsy3−/− mice

The body length was similar among WT, Chsy3+/−, and Chsy3−/− mice at birth (Figure 3A), while Chsy3−/− mice showed the shorter length than those of WT and Chsy3+/− mice at and after 4 weeks (Figure 3B,D). The body weight, however, was similar among the mice with different genotypes (Figure 3C). Interestingly, Chsy3−/− mice exhibited typical IDD phenotypes. The sagittal CT analysis indicated that disc height was narrowed significantly in Chsy3−/− mice at 4 and 8 weeks (Figure 3E-G). The sagittal MRI analysis showed disc radiological degeneration traits in Chsy3−/− mice at 4 and 8 weeks (Figure 3H-M). To further confirm the presence of IDD in Chsy3−/− mice, we performed Safranin O/Fast green staining and Mason's trichrome staining of mouse intervertebral discs. The results revealed that extracellular matrix decreased, NP cell loss and unclear demarcation between NP and AF tissues with the deletion of Chsy3 (Figure 4A). The expression of Chsy3 was also confirmed using IHC in disc tissues (Figure 4B,C), and the CS content of the disc was also significantly decreased in disc tissues (Figure 4D). Combining these data, we confirmed that accelerated degeneration took place when Chsy3 was depleted in intervertebral discs.

To investigate whether the Chsy3 depletion influences the bone development, we performed bone analysis in 4-week mice using micro-CT data. We analyzed the trabecular and cortical bone quality of lumbar vertebrae and femur bone, and found that the cortical bone mineral density (Ct.BMD), cortical thickness (Ct.Th), trabecular bone mineral density (Tb.BMD), trabecular bone volume fraction (Tb.BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) of the vertebrae had no difference between Chsy3+/− mice and WT mice (Figure S2A,B). Meanwhile, the Ct.Th, BMD, Tb.BV/TV, Tb.Th and Tb. N of the femur had also no differences between Chsy3−/− mice and WT mice (Figure S2C,D). Also, IHC and histological analysis also confirmed no significant differences between Chsy3−/− mice and WT mice in knee joint and other organ tissues (Figure S3A-C). These data suggest that Chsy3 deletion has no obvious effect on bone development, implying that Chsy3+/− mouse is a suitable IDD model.

3.3 | Loss of Chsy3 induces NP cell degeneration in vitro

To further explore the function of Chsy3 in NP cells, we dissociated and cultured Chsy3−/− primary NP cells in vitro. Immunofluorescence analysis confirmed the decreased expression of Chsy3 and CS content in Chsy3−/− primary NP cells (Figure 4E-G). The expressions of anabolic genes...
FIGURE 3 Accelerated IDD is found in Chsy3<sup>−/+</sup> mice. Representative images of the body length of WT, Chsy3<sup>−/+</sup>, and Chsy3<sup>−/−</sup> mice at postnatal (A) and at 4 weeks (B). Dynamic change of body weight (C) and body length (D) among WT, Chsy3<sup>−/+</sup> and Chsy3<sup>−/−</sup> mice. n = 6 per group, ANOVA. E. The sagittal reconstructed CT images of WT, Chsy3<sup>−/+</sup>, and Chsy3<sup>−/−</sup> mice at 8 weeks. The disc height index (DHI) of WT and Chsy3<sup>−/−</sup> mice at 4 weeks (F) and 8 (G) weeks are shown. n = 6 per group, t test. The sagittal MRI T2-weighted images of the intervertebral disc of WT, Chsy3<sup>−/+</sup>, and Chsy3<sup>−/−</sup> mice at 4 weeks (H) and 8 (K) weeks are shown respectively. The modified Thompson grade of the disc of WT, Chsy3<sup>−/+</sup>, and Chsy3<sup>−/−</sup> mice at 4 weeks (I) and 8 (L) weeks were compared. n = 6 per group, t test. The mean grayscale of the intervertebral disc between WT, Chsy3<sup>−/+</sup>, and Chsy3<sup>−/−</sup> mice at 4 weeks (J) and 8 weeks (M) are also compared. n = 6 per group, t test. All data are shown as mean ± SD. *P < .05; **P < .01, ****P < .0001.
(Acan, Col2, and Chsy1) were significantly downregulated and catabolic genes (ADAMTS 4/5 and MMP2/13) were significantly upregulated in Chsy3−/− primary NP cells detected by qRT-PCR and Western blot (Figure 4H), and these events aggravated in the NP cells under degeneration environment induced by IL-1β (Figure 4I) or TNF-α (Figure S3F), which resembles the NP cells in the degeneration state. Similar results were observed in human primary NP cells when Chsy3 was knockdown using Chsy3 targeting siRNA (Figure S3G), and were also treated with cell degeneration environment induced by IL-1β (Figure 4J) or TNF-α (Figure S3H). Together, these data imply that Chsy3 plays the degeneration protective role in NP cells, and loss of Chsy3 would aggravate the degeneration in vitro.
FIGURE 4  Chsy3 protects NP degeneration both in vivo and in vitro. A, Safranin O/Fast Green and Masson's trichrome staining of mice intervertebral discs. The bars represent 500 µm. B, Immunohistochemistry analysis of Chsy3 expression in mice disc tissues. The bars represent 500 µm, n = 6. C, Quantifications of Chsy3 positive intensity was measured. n = 6. D, The CS content of the NP tissue was measured between mice. n = 6. E, Representative images of immunofluorescence stained Chsy3 in mice primary NP cells. Cells were counterstained with DAPI and Actin. The bars represent 100 µm. F, Quantifications of Chsy3 positive intensity in different mice primary NP cells were performed. n = 6. G, Representative images of CS content immunofluorescence staining and the quantifications of CS intensity were measured in the right panel. n = 6, the bars represent 100 µm. H, qRT-PCR analysis (left panel) and Western blot analysis (right panel) showing the expression level of anabolic and catabolic genes by different NP cells. qRT-PCR analysis showing the expression level of anabolic and catabolic genes between different NP cells under IL-1β (25 ng/ml) induced cell degeneration (I), and in human NP cells after different treatment (J). WT: wild type, NC: negative control, siCHSY3: CHSY3 knockdown using siRNA. The qRT-PCR experiments were repeated three times independently, and Gapdh is used as an internal control. All data are shown as mean ± SD. *P < .05; **P < .01. All statistical differences were assessed with Student’s t test

3.4  Global transcriptome analysis reveals downregulation of Hippo pathway in Chsy3−/− NP cells

In order to investigate the underlying mechanisms, we performed RNA sequencing of NP cells from WT, Chsy3+/−, and Chsy3−/− mice. Quality control of the sequencing data showed a strong correlation between samples (Figure S4A-C). The hierarchical clustering and PCA analyses indicated that Chsy3−/− NP cells exhibited different gene expression patterns compared with WT and Chsy3+/− ones (Figure 5A,B). The Volcano plot and MA plot showed more than 800 differentially expressed genes among the samples (Figure 5C, Figure S4D-F). 860 for Chsy3−/− vs. WT and 944 for Chsy3+/− vs. WT). Gene ontology (GO) analysis showed dysregulated extracellular matrix organization (GO:0030198) and metalloproteinase activity (GO:0008237) in the Chsy3−/− samples (Figure 5D, Figure S4G,H). And the pathway analysis showed multiple signaling pathways including Wnt, Hippo, Hedgehog, mTOR and PI3K-AKT signaling pathways are involved (Figure 5E,F, Figure S4I,J). To further narrow down the targeted pathway of Chsy3, we performed GSEA (Gene set enrichment analysis) and found that only Hippo pathway was downregulated in either Chsy3−/− versus WT or Chsy3+/− versus WT data (Figure 4G and Figure S6A), implying that Hippo pathway may be affected by Chsy3 knockout. It is noted that during the analysis, we found that GO terms and GSEA categories correlated with bone and skeletal development are significantly modulated (Figures S4G and S5B). And by testing some of the key genes, we found that osteogenesis or ossification-related genes are also upregulated during Chsy3 knockdown (Figure S5C,D), which also implies that osteogenesis or ossification-related genes may also participate in the Chsy3 knockdown-mediated NP cell degeneration phenotype.

3.5  Yap1 is directly regulated by Chsy3

In order to confirm which part of Hippo pathway is targeted by Chsy3, we examined the expression of key genes and downstream targets of the pathway. By combining the qRT-PCR results of Chsy3+/− (Figure 6A,B) and Chsy3 rescue experiments (Figure 6C,D) in mouse NP cells, we found that Yap1 is vividly regulated by Chsy3 expression. The results were further confirmed in the protein level using western blots (Figure 6E) and in human NP cells (Figure S6A-C). However, Chsy3 overexpression in wild type NP cells showed less significant expression change of IDD-related genes (Figure S3I). To show how Yap1 expression changes during IDD, we performed IHC analysis. Results showed that Yap1 was highly expressed in the normal NP cells in the intervertebral disc, and gradually, downregulated after Chsy3 knockout (Figure 6F) and degeneration (Figure 6G). Further immunofluorescence confirmed the downregulation as well as decreased nuclear Yap1 expression after Chsy3 knockout (Figure 6H) and degeneration (Figure 6I). These results gave the idea that Yap1 expression, especially Yap1 activation may be related to Chsy3 knockdown in NP cells.

To confirm the hypothesis, we first examined whether Yap1 activation affects NP cell degeneration. By using Yap1 targeting siRNA or inhibitor (Verteporfin), we found that Yap1 inhibition significantly reduced the expression of anabolic genes and increased the expression of catabolic genes both in normal NP cells and under degeneration environment (Figure 7A, Figure S6D). Yap1 inhibition can significantly reverse the degeneration protective effect brought by Chsy3 overexpression (Figure 7B), and its overexpression in normal NP cells showed similar protective effect to that of Chsy3 (Figure 7C). Interestingly, we found that when overexpression in Chsy3-depleted NP cells, its effect was less significant than that of in normal NP cells (Figure 7D), suggesting that Chsy3 expression may be a prerequisite of Yap1’s degeneration protective function.

3.6  Actin tension is involved in Chsy3 mediated Yap1 activation

We next explored how Chsy3 controls the function of Yap1. Considering that the function of Chsy3 is to generate chondroitin-related extracellular matrix and due to that a previous report showing that Yap1 activation is directly controlled by
FIGURE 5 RNA sequencing and analysis reveals that Hippo signaling pathway is affected in Chsy3−/− NP cells. A, Heatmap of hierarchical cluster of the sequencing data showing differentially expressed genes in WT, Chsy3+/−, and Chsy3−/− NP cells. B, Principal Component Analysis (PCA) among the six samples. C, Volcano plot showing the differentially expressed genes between Chsy3−/− and WT NP cells. D, Gene Ontology (GO) and (E) Pathway analysis of Chsy3−/− versus WT NP cells showing the significantly enriched GO or Pathway terms (left panel) and the overlapping status between samples (right panel). F, The Pathway interacting network constructed using the sequencing data. The red color represents the pathway category is upregulated when comparing to WT NP cells, the green color represents downregulated pathway category, while the yellow color represents pathway category has bilateral expression trends. Note that the Hippo, Hedgehog, mTOR, and Wnt signaling pathways are all significantly downregulated after Chsy3 knockout. G, The Gene Set Enrichment Analysis (GSEA) showed that only Hippo pathway is significantly affected comparing Chsy3−/− and WT NP cell data.
FIGURE 6  Yap1 activation is controlled by Chsy3. qRT-PCR analysis showing the expression changes of Hippo signaling pathway genes (A) and Yap1 downstream targets Ctgf and Ankrd1 (B) between WT, Chsy3+/−, and Chsy3−/− NP cells. qRT-PCR analysis showing the expression changes of Hippo signaling pathway genes (C) and Yap1 downstream targets Ctgf and Ankrd1 (D) after Chsy3 overexpression by transfecting Chsy3 plasmid in Chsy3−/− NP cells. Only Yap1 was significantly affected in Chsy3 overexpression group. E, Western blot analysis confirmed the expression changes of Yap1 and its downstream targets Ctgf and Ankrd1 in mice Chsy3−/− NP cells and after Chsy3 overexpression (oeChsy3) in normal mouse NP cells. F, IHC staining of Yap1 in WT, Chsy3+/−, and Chsy3−/− NP tissues. The bars represent 500 µm, n = 6. The Quantifications of positively stained Yap1 was performed by comparing the intensity in the images. G, IHC staining of Yap1 in human normal, mild degenerated (Pfirrmann Grade II-III) and severe degenerated (Pfirrmann Grade IV-V) NP tissues. The bars represent 500 µm, n = 6. The Quantifications of positively stained Yap1 was shown in the right panel. H, Representative images of WT, Chsy3+/−, and Chsy3−/− NP cells immunofluorescence stained for Yap1 and actin were shown in the left panels and quantifications of nuclear/cytoplasmic Yap1 ratio was measured in the right panels. The bars represent 100 µm, n = 6. I, Representative images of inflammation factors-treated (TNF-α 50 ng/mL, IL-1β 25 ng/mL) NP cells stained for Yap1 and actin were shown in the left panels and quantifications of nuclear/cytoplasmic Yap1 ratio was measured in the right panels. The bars represent 100 µm, n = 6. The qRT-PCR experiments were repeated three times independently, and GAPDH is used as an internal control. All data are shown as mean ± SD. *P < .05; **P < .01. All statistical differences were assessed with Student’s t test.
actin assembly, we thus hypothesized that extracellular CS mediated actin assembly of the NP cells may be responsible for the activation of Yap1 in NP cells. To test this, we used Latrunculin A (Lat.A, 100 nM) to inhibit actin assembly in NP cells and found more severe degeneration phenotype (Figure 7E). In normal NP cell degeneration, actin disassembly could reverse the effect of both Chsy3 and Yap1 overexpression (Figure 8A,B). However, in Chsy3 depleted NP cells, Lat.A treatment showed less effective (Figure 7F), suggesting that Chsy3 expression controls Yap1 activation probably through its CS product-mediated actin tension state in the NP cells. To provide further evidence, we performed Yap1 activity assay using Yap1/Taz luciferase reporter. By detecting the luciferase activities of Yap1/Taz reporter, we

**FIGURE 7** Yap1 is necessary for Chsy3 to protect NP cell degeneration. A, qRT-PCR (left panel) and Western blot (right panel) analysis showing the expression changes of anabolic and catabolic genes related to IDD in Yap1 knockdown (siYap1) or Yap1 inhibited (Verteporfin, 50 nM) NP cell degeneration model induced by IL-1β (25 ng/mL). B, qRT-PCR (left panel) and Western blot (right panel) analysis showing the expression changes of anabolic and catabolic genes related to IDD in Chsy3−/− NP cells after Chsy3 overexpression or/and Yap1 inhibition (Verteporfin, 50 nM) under degeneration environment (IL-1β 25 ng/mL). C, qRT-PCR (left panel) and Western blot (right panel) analysis showing that the anabolic promoting effect of Yap1 overexpression in NP cell degeneration can be reversed by Chsy3 knockdown. D, qRT-PCR (left panel) and Western blot (right panel) analysis showing that in Chsy3 knockout NP cells, Yap1 overexpression did not promote the expression of anabolic genes, and further inhibition of Yap1 also showed no effect, which indicated that Chsy3 is a prerequisite of the anabolic promoting effect of Yap1 during NP cell degeneration. E, qRT-PCR analysis confirmed the expression changes of anabolic and catabolic genes related to IDD in WT NP cells after Latrunculin A (Lat.A) treatment. F, qRT-PCR analysis showing that inhibitor of actin polymerization did not affect the expression changes of anabolic and catabolic genes related to IDD in Chsy3−/− NP cells. The qRT-PCR experiments were repeated three times independently, and GAPDH is used as an internal control. All data are shown as mean ± SD. *P < .05; **P < .01. All statistical differences were assessed with Student's t test.
**Figure 8** Chsy3 modulates Yap1 activation through Actin tension in NP cells. A, qRT-PCR analysis showing the expression changes of anabolic and catabolic genes in Chsy3 overexpressed or combined with actin depolymerization-treated (Latrunculin A, Lat.A, 100 nM) NP cells under degeneration environment (IL-1β 25 ng/mL). B, qRT-PCR analysis showing the expression changes of anabolic and catabolic genes in Yap1 overexpressed or combined with actin depolymerization-treated (Latrunculin A, Lat.A, 100 nM) NP cells under degeneration environment. C, Yap1 activities were determined by luciferase reporter assay under different treatments (oeChsy3: overexpression of Chsy3 by plasmids, Lat.A: Latrunculin A at 100 nM). n = 6. D, Immunofluorescence analysis showing the expression of Yap1 and actin in mice NP cells under different treatments. The bars represent 100 µm, n = 6. Quantifications of nuclear Yap1 ratio was measured in mice NP cells with different treatments were shown in the right panel. E, Western blot analysis confirmed the change of Yap1 and phosphorylated Yap1 under the same treatment as in (C). F, qRT-PCR analysis showing that Lats1 knockdown did not affect the expressions of anabolic and catabolic genes in NP cell degeneration, while inhibition of actin polymerization showed similar effects to that of Chsy3 knockdown, indicated that Lats1 is not a regulator in Chsy3-mediated effects. The qRT-PCR experiments were repeated three times independently, and GAPDH is used as an internal control. All data are shown as mean ± SD. *P < .05; **P < .01. All statistical differences were assessed with Student’s t test.
found that Chsy3 overexpression could significantly increase the Yap1/Taz activities, while actin disassembly completely reversed the effect (Figure 8C). This result was further supported by immunofluorescence analysis (Figure 8D) and western blot (Figure 8E) in NP cells. To further identify Yap1 activities is modulated through Chsy3 mediated CS production, we conducted a timeline analysis of CS content and Yap1 expression (Figure S6E). We found that Yap1 expression is not decreased until CS content dropped significantly, and treatment with Lat.A did not significantly affect the amount of CS produced (Figure S6E,F).

Besides, we performed Lats1 knockdown experiments to investigate whether the canonical Hippo signaling is involved in the effect of Chsy3-Yap1 (Figure S7A). qRT-PCR analysis showed that although Lats1 knockdown reduced the expression of Yap1 downstream targets to some extent, the expression of anabolic genes and catabolic genes were not significantly affected (Figure 8F, Figure S7B). Taken together, these data indicated that Chsy3 activates Yap1 through CS mediated actin tension, which is not related to the canonical Hippo pathway.

3.7 Chsy3 modulate IDD through Yap1/Smad signaling

Our previous reports have shown that Chsyls are directly regulated by TGFβ and inflammatory factors, we investigated the correlation between Chsy3 and TGFβ/inflammatory factors. To this end, we treated NP cells with gradient IL-1β and TNF-α and found the gradual downregulation of Chsyls at RNA level (Figure 9A). Furthermore, the addition of TGFβ to IL-1β-treated NP cells could increase the expression of anabolic genes and Chsy3 while downregulate the expression of catabolic genes, which could be reversed to some extent with Yap1 knockdown (Figure 9B). In IL-1β-treated Chsy3−/− NP cells, although TGFβ treatment could increase the expression of anabolic genes except for Chsy3 while downregulate the expression of catabolic genes, the modulation of Yap1 did not further influence their expression (Figure 9C). Furthermore, we employed Smad inhibitor (SIS3 5 µM) in NP cell degeneration and found that Smad inhibition completely abolished the effect of Yap1 or Chsy3 overexpression (Figure 9D), and is further validated using a Smad luciferase reporter (Figure 9E, Figure S7C) and Western blot analysis (Figure 9F). To show that Chsy3-mediated effect was dependent on CS content, we provided evidence that Chsy1 knockdown could also downregulate the expression of Yap1 downstream targets, while Chsy2 knockdown showed little effect (Figure S7D), which is consistent with previous findings that Chsy3 and Chsy1 were mainly involved in CS production. Taken together, our findings showed that Chsy3-mediated Yap1 activation is dependent on CS/Actin tension mechanism to protect NP cell degeneration by promoting the expression of anabolic genes and reduce the expression of catabolic genes (Figure 9G).

4 DISCUSSION

It has been presumed that loss of CS content plays a key role in human IDD. As previously described, six glycosyltransferases are involved in the CS biosynthesis. A combination of Chsy1 and Chsy3 exhibits the highest activity of CS biosynthesis. Chsy3 plays a unique role in the synthesis of chondroitin sulfate. A 50% knockdown of Chsy3 mRNA in HeLa cells caused reduction amount of CS nearly to 46%, which showed the highest effect among the synthetases. Since CS is considered vital to disc function maintenance, previous reports indicated a vital role of Chsy3 in disc degeneration. However, the molecular function and mechanism of CS or Chsy3 during IDD is not clearly identified. In this article, we try to pursue the function and mechanism of Chsy3 using both in vitro and in vivo methods. Here, we found that Chsy3−/− mice displayed similar features of human IDD, including loss of disc height and water content, narrowing disc space, loss of aggrecan and normal collagen structure, increased expression of matrix degrading enzymes. Our investigation showed that the potential mechanism of Chsy3 is related to the extracellular CS mediated mechanical stress Yap activation manner, which is the first time described the detailed mechanism of how CS regulate IDD in molecular level.

A common feature of human IDD is loss of water content in the disc. In our study, we found that loss of CS can directly induce loss of disc height and water content in Chsy3−/− mice in vivo, which was similar with previous IDD models. The expression of MMP 2/13 and ADAMTS 4/5 was increased, while the expression of Col2 and aggrecan was decreased in Chsy3−/− disc cells, indicating that abnormal ECM turnover and composition existed in Chsy3−/− mice. Furthermore, ossification-related genes are also upregulated during Chsy3 knockdown. Choi et al also found that degenerative NP cells promoted dystrophic mineralization.

To further investigate the potential mechanism of IDD induced by Chsy3 ablation, we used the RNA-seq data to find the involved signal pathways. RNA-seq data showed that the Hippo signal pathway was both downregulated in Chsy3−/− and Chsy3−/− disc cells when compared with WT disc cells. Hippo pathway plays key roles in regulating diverse cellular process, such as cell proliferation and apoptosis. In mammals, the core elements of Hippo pathway contain a Set/Thr kinase cascade, including Mst1/2 and its downstream Lats1/2. Lats1/2 phosphorylates the transcriptional activator Yap, causing it to be excluded from the nucleus to cytoplasm. Nuclear Yap interacts with its downstream Tead to turn on...
FIGURE 9 Chsy3 is mediated by TGFβ and Smad signaling is required for its function in NP cells. A, qRT-PCR analysis showing the expression of CS synthases are controlled by inflammatory factors. qRT-PCR analysis showing expression changes of anabolic and catabolic genes under different treatment in WT NP cells (B) or Chsy3−/− NP cells (C) during IL-1β induced degeneration. TGFβ was used at 20 ng/mL. D, qRT-PCR analysis showing that the effect of Chsy3 and Yap1 overexpression in IL-1β and SIS3 (5 µM, Smad3 inhibitor)-treated NP cells. E, SMAD activities were determined by luciferase reporter assay in WT mice NP cells following different treatments. SIS3, Smad3 inhibitor used at 5 µM, n = 6. F, Western blot analysis showing the expression change of pan-Smad (Smad), phosphorylated Smad (p-Smad), and other IDD-related genes. G, Schematic diagram of the mechanism of Chsy3 regulating IDD. The qRT-PCR experiments were repeated three times independently, and GAPDH is used as an internal control. All data are shown as mean ± SD. *P < .05; **P < .01. All statistical differences were assessed with Student’s t test.
the expression of growth-promoting and apoptosis-inhibiting genes. However, the role of Hippo pathway in the IDD is unclear. In our study, we found that Yap1 and its downstream Tead were both downregulated in Chsy3−/− and Chsy3−/− disc cells. Previous study also reported that Yap expression was decreased with age in healthy intervertebral discs and in the process of IDD, but regulation of Yap during IDD is still unknown. Furthermore, our results showed that Chsy3 modulation greatly affected the expression and activation of Yap1, and functional analysis showed that Lats1 is not involved in Chsy3-mediated Yap1 activation. These findings were also consistent with other reports that showed Yap1 activation in some circumstances is not liable on canonical Hippo signaling pathway.

Some cells could maintain its function through physical and mechanical cues, such as ECM stiffness. Previous study found that Yap was controlled by mechanical signals, such as ECM stiffness, shear stress, and stretching. Nuclear Yap increased in cells with stiff ECM, while nuclear YAP decreased in cells with soft ECM. The change of nuclear YAP will further regulate diverse cell process. This mechanical control depends on RHO GTPase function and actomyosin-driven contractility but is largely independent of kinase regulation. The state of the actin cytoskeleton plays a key role in mediating the effects of shape and tension of nuclear YAP/TAZ. Disturbances of the actin cytoskeleton have strong effects on YAP localization. In our study, we also found YAP activation was largely depended on mechanical stress related to actin tension. And by using actin assembly inhibitor, the active nuclear Yap1 was significantly decreased, further leading more severe phenotype of IDD. In sum, we confirm the Chsy3-actin-Yap1 activation axis, and provide evidence that ECM mediated Yap1 activation is also critical in intervertebral disc degeneration. We assume that Chsy3 ablation causes degradation of disc ECM and less synthesis of CS, changing the anchorage site of NP cells in the watery NP region, transform a relatively stiff state to a soft state. And this ECM change will in turn causes less activated nuclear Yap and further potentiates the NP cells to a degeneration-sensitive state or make the NP cells more vulnerable to inflammatory stimulation, finally inducing the IDD process.

On the other hand, altered CS amount may also affect the osmotic pressure in the NP region, for it mainly maintains the hydrostatic pressure through its hydrophilic property. However, due to lack of proper equipment and method, we did not test the in vivo or in vitro osmotic pressure routinely to investigate the effect of Chsy3 knockout or reduced CS on osmotic pressure. Another limitation of our study is that we only used inflammatory factors induced degeneration in vitro model for cellular mechanism study. Although the inflammatory model is the most studied model for in vitro experiments of NP cells, it is true that multiple factors including osmotic pressure change may also induce degeneration of NP. Whether the proposed mechanism works in other model needs further validation.

In conclusion, our results showed that Chsy3−/− mice displayed typical characteristics of human IDD. Chsy3-mediated extracellular CS content controls IDD through YAP signaling in a mechanical dependent manner. Chsy3−/− mice may be useful for further studying the pathology and treatment of human IDD.

ACKNOWLEDGMENTS
We thank Wei Li and Yongqiong Yu for support with cell sorting. We thank Shanghai NovelBio Bio-Pharm Technology Co., Ltd for performing the RNA-sequencing and analyzing the data. This work was supported by National Nature Science Foundation of China (82002335, 81972088, 82072471, 81672211, 81871802, 81772376, 81601928, 81702149, 31971109, and 31471390), Shanghai Municipal Commission of Health and Family Planning (17140901500, 18ZR1438900, 20QA1409200, and 2018Y0181), Shanghai Education Development Foundation and Shanghai Municipal Education Commission "Chen Guang" Program (17CG36), Shanghai "Rising Stars of Medical Talent" Youth Development Program.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
L. Wei, C. Xu, J. Li, and P. Cao conceived and designed the experiments. L. Wei, C. Xu, HZ, P. Cao, and X. Wang performed the experiments. M. Bai, B. Hu, R. Wang, N. Liu, Y. Tian, J. Li, P. Cao, W. Yuan, and L. Wei collected the samples. L. Wei, C. Xu, H. Zhong, X. Wang, M. Bai, P. Cao, and Y. Tian performed the animal experiment. L. Wei, C. Xu, W. Yuan, and J. Li analyzed the data. L. Wei, C. Xu, and H. Zhong drafted the manuscript. J. Li, W. Yuan, C. Xu, and P. Cao reviewed and edited the manuscript. All authors read and approved the final manuscript.

REFERENCES
1. Buchbinder R, van Tulder M, Öberg B, et al. Low back pain: a call for action. Lancet. 2018;391:2384-2388.
2. Hartvigsen J, Hancock MJ, Kongsted A, et al. What low back pain is and why we need to pay attention. Lancet. 2018;391:2356-2367.
3. Katz JN. Lumbar disc disorders and low-back pain: socioeconomic factors and consequences. J Bone Joint Surg Am. 2006;88 Suppl 2:21-24.
4. Wang SZ, Rui YF, Lu J, Wang C. Cell and molecular biology of intervertebral disc degeneration: current understanding and implications for potential therapeutic strategies. Cell Prolif. 2014;47:381-390.
5. Kepler CK, Ponnappan RK, Tannoury CA, Risbud MV, Anderson DG. The molecular basis of intervertebral disc degeneration. Spine J. 2013;13:318-330.
6. Wang WJ, Yu XH, Wang C, et al. MMPs and ADAMTSs in intervertebral disc degeneration. Clin Chim Acta. 2015;448:238-246.
7. Johnson WE, Caterson B, Eisenstein SM, Hynds DL, Snow DM, Roberts S. Human intervertebral disc aggrecan inhibits nerve growth in vitro. *Arthritis Rheum.* 2002;46:2658-2664.

8. Hutton WC, Elmer WA, Boden SD, Horton WC, Carr K. Analysis of chondroitin sulfate in lumbar intervertebral disks at two different stages of degeneration as assessed by discogram. *J Spinal Disord.* 1997;10:47-54.

9. Sivan SS, Wachtel E, Roughley P. Structure, function, aging and turnover of aggrecan in the intervertebral disc. *Biochim Biophys Acta.* 2014;1840:3181-3189.

10. Hu B, Xu C, Tian Y, et al. Inflammatory microRNA-194 and -515 attenuate the biosynthesis of chondroitin sulfate during human intervertebral disc degeneration. *Oncotarget.* 2017;8:49303-49317.

11. Hu B, Xu C, Cao P, et al. TGF-beta stimulates expression of chondroitin sulfate synthase-1 (Chsy1) is required for bone development and digit patterning. *Dev Biol.* 2012;363:413-425.

12. Kitagawa H, Uyama T, Sugahara K. Molecular cloning and expression of a human chondroitin synthase. *J Biol Chem.* 2001;276:38721-38726.

13. Izumikawa T, Uyama T, Okuura Y, Sugahara K, Kitagawa H. Involvement of chondroitin sulfate synthase-3 (chondroitin synthase-2) in chondroitin polymerization through its interaction with chondroitin synthase-1 or chondroitin-polymerizing factor. *Biochem J.* 2007;403:545-552.

14. Gotoh M, Yada T, Sato T, et al. Molecular cloning and characterization of a novel human chondroitin sulfate glucuronyltransferase that transfers glucuronic acid to N-acetylgalactosamine. *J Biol Chem.* 2002;277:38179-38188.

15. Izumikawa T, Koike T, Shiozawa S, Sugahara K, Tamura J, Kitagawa H. Identification of chondroitin sulfate glucuronyltrans-ferase as chondroitin synthase-3 involved in chondroitin polymerization: chondroitin polymerization is achieved by multiple enzyme complexes consisting of chondroitin synthase family members. *J Biol Chem.* 2008;283:11396-11406.

16. Yada T, Gotoh M, Sato T, et al. Chondroitin sulfate synthase-2. Molecular cloning and characterization of a novel human glycosyltransferase homologous to chondroitin sulfate glucuronyltransferase, which has dual enzymatic activities. *J Biol Chem.* 2003;278:30235-30247.

17. Kitagawa H, Izumikawa T, Uyama T, Sugahara K. Molecular cloning of a chondroitin polymerizing factor that cooperates with chondroitin synthase for chondroitin polymerization. *J Biol Chem.* 2003;278:23666-23671.

18. Uyama T, Kitagawa H, Tamura Ji J, Sugahara K. Molecular cloning and expression of human chondroitin N-acetylgalactosaminyltransferase: the key enzyme for chain initiation and elongation of chondroitin/dermatan sulfate on the protein linkage region tetrasaccharide shared by heparin/heparan sulfate. *J Biol Chem.* 2002;277:8841-8846.

19. Uyama T, Kitagawa H, Tanaka J, Tamura J, Ogawa T, Sugahara K. Molecular cloning and expression of a second chondroitin N-acetylgalactosaminyltransferase involved in the initiation and elongation of chondroitin/dermatan sulfate. *J Biol Chem.* 2003;278:3072-3078.

20. Tang T, Li L, Tang J, et al. A mouse knockout library for secreted and transmembrane proteins. *Nat Biotechnol.* 2010;28:749-755.
40. Zhang C, Wang F, Xie Z, et al. AMOT130 linking F-actin to YAP is involved in intervertebral disc degeneration. *Cell Prolif*. 2018;51:e12492.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

*How to cite this article*: Wei L, Cao P, Xu C, et al. Chondroitin synthase-3 regulates nucleus pulposus degeneration through actin-induced YAP signaling. *The FASEB Journal*. 2020;34:16581–16600. [https://doi.org/10.1096/fj.202001021R](https://doi.org/10.1096/fj.202001021R)