PHLPP1 deficiency protects against age-related intervertebral disc degeneration

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Funding information
NIH/NIAMS R01, Grant/Award Number: AR078908; NIH/NIAMS R21, Grant/Award Number: AR072222; The department of Orthopaedics at Emory University School of Medicine

Abstract

Background: Intervertebral disc (IVD) degeneration is strongly associated with low back pain and is highly prevalent in the elderly population. Hallmarks of IVD degeneration include cell loss and extracellular matrix degradation. The PH domain leucine-rich-repeats protein phosphatase (PHLPP1) is highly expressed in diseased cartilaginous tissues where it is linked to extracellular matrix degradation. This study explored the ability of PHLPP1 deficiency to protect against age-related spontaneous IVD degeneration.

Methods: Lumbar IVDs of global Phlpp1 knockout (KO) and wildtype (WT) mice were collected at 5 months (young) and 20 months (aged). Picrosirius red–alcan blue staining (PR-AB) was performed to examine IVD structure and histological score. The expression of aggrecan, ADAMTS5, KRT19, FOXO1 and FOXO3 was analyzed through immunohistochemistry. Cell apoptosis was assessed by TUNEL assay. Human nucleus pulposus (NP) samples were obtained from patients diagnosed with IVD degeneration. PHLPP1 knockdown in human degenerated NP cells was conducted using small interfering RNA (siRNA) transfection. The expression of PHLPP1 regulated downstream targets was analyzed via immunoblot and real time quantitative PCR.

Results: Histological analysis showed that Phlpp1 KO decreased the prevalence and severity of age-related IVD degeneration. The deficiency of PHLPP1 promoted the increased expression of NP phenotypic marker KRT19, aggrecan and FOXO1, and decreased levels of ADMATS5 and cell apoptosis in the NP of aged mice. In degenerated human NP cells, PHLPP1 knockdown induced FOXO1 protein levels while FOXO1 inhibition offset the beneficial effects of PHLPP1 knockdown on KRT19 gene and protein expression.

Conclusions: Our findings indicate that Phlpp1 deficiency protected against NP phenotypic changes, extracellular matrix degradation, and cell apoptosis in the process of IVD degeneration, probably through FOXO1 activation, making PHLPP1 a promising therapeutic target for treating IVD degeneration.

Keywords

aging, FOXO1, intervertebral disc degeneration, matrix homeostasis, nucleus pulposus, PHLPP1
1 | INTRODUCTION

Back pain is a leading cause of physical disability worldwide and its socioeconomic impact increases exponentially as the global population ages. Intervertebral disc (IVD) degeneration is the most common cause of back pain in older adults. As the IVD ages, the expression of the NP phenotypic matrix catabolism, chronic inflammation, cell death, and extracellular matrix (ECM) degradation. Notochordal cells are replaced by chondrocyte-like cells and the number of apoptotic cells in the IVD increases. In addition to the cellular and ECM alterations, proinflammatory cytokines are upregulated in degenerated IVDs, contributing to matrix breakdown by enhancing the expression of matrix metalloproteinases (MMPs), a disintegrin and metalloprotease with thrombospondin motif (ADAMTS), and inhibiting the synthesis of matrix molecules.

The Ser/Thr protein phosphatase PH domain Leucine-rich-repeats protein phosphatase 1 (PHLPP/1) regulates several kinases involved in cell survival and apoptosis, autophagy, differentiation, inflammation, and matrix catabolism, including protein kinase B (AKT), mammalian sterile 20-like kinase 1 (MST1), and ribosomal protein S6 kinase (S6K1). Genetic depletion of PHLPP1 in mice protects against osteoarthritis progression, and provides cardio protection via AKT signaling.

However, spontaneous development of IVD degeneration, not injury or trauma, is the main risk factor for the high prevalence of IVD degeneration. The aim of this study was to first determine if PHLPP1 deficiency can delay the progression of spontaneous IVD degeneration by suppressing matrix degradation and cell apoptosis in aged mice and second, if PHLPP1 knockdown can promote NP cell regeneration of human degenerated NP cells from older patients. Lumbar IVDs from global PHLPP1 KO and WT mice were examined through histo-logy, immunohistochemistry and TUNEL assay at 5 and 20 months.

2 | MATERIALS AND METHODS

2.1 | Mouse model

All animal research was conducted in accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (U.S. Department of Health, Education, and Welfare, NIH 78-23, 1996). All animal protocols were approved by the Atlanta Veteran’s Affairs Medical Center’s Institutional Animal Care and Use Committee (Protocol V016-19). C57BL/6j mice and PHLPP1 KO mice were sacrificed at 5 (WT: n = 4, one female and three males; KO: n = 4, two females and two males) and 20 (WT: n = 11, five females and six males; KO: n = 13, eight females and five males) months of age. Lumbar IVDs (L3–L6) were isolated and used for downstream analysis.

2.2 | Histology and immunohistochemistry

IVDs were fixed in 0.1% (Sigma Aldrich, Z2902-3.75L) for 2 days and decalcified in 5% Fornic acid (Millipore Sigma, FX0440-6) for 3 days. Decalcified IVDs were embedded in paraffin and 5 µm thick mid sagittal sections were used for histology and immunohistochemistry. IVD morphology was visualized using Picrosiri used red–alcan blue staining (PR-AB).

For immunohistochemistry, antigen retrieval was performed by applying 0.8% hyalurondase (Sigma Aldrich, H3506) for 1 h at 37°C, followed by a 30-minute incubation in 2.5% normal horse serum for blocking (ImmPress–AP Horse Anti-Rabbit IgG Polymer Reagent, MP-5401-50, Vector Laboratories). Samples were then incubated with primary antibodies against ADAMTS5 (ab41037, ABCAM), aggrecan (BS-1223R, Bios), KRT19 (TROMA-III, DSHB), FOXO1 (2880, cell signaling) and FOXO3a (2497, cell signaling). After overnight incubation at 4°C, the samples were washed and incubated with an alkaline phosphatase conjugated secondary antibody (ImmPress–AP Horse Anti-Rabbit IgG Polymer Reagent, MP-5401-50, Vector Laboratories) for 30 min and visualized using alkaline phosphatase substrate (SK-5105 ImmPACT Vector Red, Vector Laboratories). For KRT19 immunostaining, the samples were treated with peroxidase suppressor (35 000, Thermo Fisher) and visualized using alkaline phosphatase conjugated secondary antibody (ImmPress–AP Horse Anti-Rabbit IgG Polymer Reagent, MP-5401-50, Vector Laboratories) for 30 min after primary antibody incubation, and then incubated with Signal Stain Boost IHC detection reagent (I1205, Thermo Fisher) for 30 min.

2.3 | TUNEL assay

TUNEL assay was performed following the manufacturer’s protocol (C10618, Thermo Fisher). In brief, 5 µm thick mid sagittal sections were deparaffinized, dehydrated, and permeabilized in proteinase K solution for 15 min at room temperature. Samples were then incubated with TUNEL reaction mixture in a humidified atmosphere for 1 h at 37°C. Sections were mounted with antifade mounting medium with DAPI (H1200, Vector laboratories) and visualized using a
Immediately after surgery, the specimens were washed. Human IVD donors were minced into small fragments (Amphotericin B, and 1 mM 50% ethanol, followed by 0.025% collagenase P (Roche Diagnostics, 40 341 623) digestion for 4 h. Isolated cells were filtered through 100-μm mesh and rinsed twice with 1× PBS. NP cells were expanded in low glucose DMEM containing 10% premium fetal bovine serum (FBS), 1% pen/strep, 1% primocin, and 50 μg/ml ascorbate. All cells were grown at 37°C under 5% CO₂, 20% O₂, and 90% humidity. Culture medium was changed every other day. Cells of passage 2–3 were used for experiments.

### Human NP cell extraction and culture

Following informed consent in accordance with the institutional review board approval, four human NP tissues were obtained from patients with discogenic pain and graded by surgeons as Grade IV or Von the Pfirrmann scale (Table 1). Immediately after surgery, the specimens were washed with five dips of 70% Ethanol, 1× PBS with 3% pen/strep and 1.5% Amphotericin B, and 1× PBS to prevent contamination. NP tissues were minced into small fragments (~1 mm³). Cells were released from the tissues by digestion in 0.2% protease (Sigma Aldrich, P5147-1G) for 1 h, followed by 0.025% collagenase P (Roche Diagnostics, 40 341 623) digestion for 4 h. Isolated cells were filtered through 100-μm mesh and rinsed twice with 1× PBS. NP cells were expanded in low glucose DMEM containing 10% premium fetal bovine serum (FBS), 1% pen/strep, 1% primocin, and 50 μg/ml ascorbate. All cells were grown at 37°C under 5% CO₂, 20% O₂, and 90% humidity. Culture medium was changed every other day. Cells of passage 2–3 were used for experiments.

### Inhibitor treatment

The 100 nM FOXO1 inhibitor AS1842856 (Millipore Sigma, 344 355) or 100 nM PI3K/AKT inhibitor Wortmannin (Millipore Sigma, W1628) were dissolved in DMSO. To collect cells for RNA, degenerated human NP cells were transfected for 24 h, followed by FOXO1 inhibitor stimulation for another 24 h. To collect cells for protein, NP cells were transfected for 48 h and then stimulated with FOXO1 inhibitor for 24 h. An equivalent amount of DMSO was used in the untreated group.

### Immunoblot analysis

Whole-cell extracts were isolated from human degenerated NP cells followed by lysis in RIPA buffer (Millipore Sigma, R0278) containing freshly added protease inhibitor cocktail (Thermofisher Scientific, 78 430). Protein concentration was determined by Bicinchoninic Acid assay (Thermofisher Scientific, J23227) using bovine serum albumin as the standard. Equal amounts of protein (20 μg) were loaded onto gradient gels (4%–20%, precast SDS-PAGE gels [BioRad, 4 561 094]), and transferred to PVDF membrane (BioRad, 1 620 177). The membrane was blocked with 5% Milk/TBS-T buffer for 1 h and then incubated with primary antibodies against PHLPP1 (Sigma-Aldrich, 071341), Phospho-AKT Ser473 (Cell Signaling Technology, 9271), AKT (Cell Signaling Technology, 9272), phospho-p44/42 MAPK ERK1/2 (Cell Signaling Technology, 9101), p44/42 MAPK ERK1/2 (Cell Signaling Technology, 9102), FOXO1 (Cell Signaling Technology, 28805), FOXO3a (Cell signaling, 2497), KRT19 (TROMA-III, DSHB) or GAPDH (Cell Signaling Technology, 8845). All antibodies were diluted at 1:1000. After overnight incubation with primary antibodies at 4°C, membranes were washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Cell Signaling Technology, 70745) or mouse HRP (1:5000, Cell Signaling Technology, 70765) for 1 h. Membranes incubated with GAPDH were imaged directly after washing with TBST. Protein bands were visualized using Amersham ECL select western blotting detection reagent (VWR, 89233-310) and iBright FL 1500 imaging system (Thermo Fisher). Band densities were calculated using image J.

### Table 1: Human IVD donors

| Patient | Gender | Age (years) | IDD grade |
|---------|--------|-------------|-----------|
| 1       | M      | 74          | 5         |
| 2       | M      | 61          | 5         |
| 3       | M      | 61          | 5         |
| 4       | F      | 72          | 4         |

RNA was extracted using TRIZol reagent (Thermofisher Scientific, 15 596 026) and chloroform (Thermofisher Scientific, AAJ67241AP), followed by RNEasy micro kit (Qiagen, 70 044) according to manufacturer's instructions. RNA concentration and purity were determined using NanoDrop 2000 spectrophotometers (Thermofisher Scientific, ND-2000). For mRNA, 1 μg of RNA was used for reverse transcription into cDNA using Superscript VILO cDNA synthesis kit (Thermofisher Scientific, 11 754 050). For each quantitative PCR reaction, 5 ng cDNA was mixed with Taqman primers (KRT19: Hs01051611_g1 and GAPDH: Hs02758991_g1), 2× Taqman universal PCR master mix (Thermofisher Scientific, 4 304 437), and RNase-free water to a total volume of 20 μl. Gene expression was measured with the Rotor-Gene Q detection system (Qiagen). GAPDH was used for the endogenous control. Fold change of KRT19 gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.
2.9 | Statistics

Two-way analysis of variance (ANOVA) testing was utilized to assess the effect of aging, genotype, and sex on IVD degeneration (Table A1). Because sex was not identified as a significant variable, sexes were combined and two-way ANOVAs were utilized to analyze differences between genotypes and age, with subsequent Bonferroni post hoc testing. Power analysis was performed using G*Power to determine the sample size (using a significance level of 0.05 and a power of 0.8). Human data were analyzed by t tests (knockdown experiments) or one-way ANOVAs, followed by Bonferroni post hoc testing (inhibitor treatment). The statistical analyses were performed using GraphPad Prism9 (GraphPad Software, Inc., La Jolla, CA). A p value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Phlpp1 deficiency alleviated age-related spontaneous IVD degeneration in IVDs of aged mice

To investigate if Phlpp1 depletion is protective against spontaneous IVD degeneration in aged mice, we analyzed IVDs of lumbar spines from both WT and KO mice at (5-young and 20-months old) of age, corresponding to the ages with low (20–30 years) and high (50–60 years) prevalence of IVD degeneration in humans. Picrosirius red-alcian blue staining (PR-AB) was performed to assess the alterations of IVD compartment organization and matrix composition. We quantified histopathological changes using a previously established histological grading system. The IVD degeneration score for each IVD is listed in Table A2. In young mice, no detectable differences were observed in the IVD structure and proteoglycan accumulation in WT and KO IVDs. All mice displayed a typical healthy IVD structure, consisting of a central notochordal NP cell band embedded in pronounced proteoglycans, which surrounded by the highly organized collagenous matrix of the annulus fibrosus (AF, Figure 1A). Compared to young WT and agedKO IVDs, the occurrence of IVD degeneration in agedWT mice was significantly increased (Figure 1B–D; Table 2). We observed that 75% of agedWT IVDs were degenerated, while only 33% of KO IVDs showed degenerative changes. In addition to the lower incidence of IVD degeneration, agedKO IVDs displayed less severe degenerative features. Specifically, 21% of the degenerated IVDs in agedWT

FIGURE 1  PHLPP1 deficiency decelerated age-related spontaneous IVD degeneration. (A–C) Picro-sirius red/alcian blue (PR-AB) staining of WT and KO IVDs. (A) No obvious differences in IVD structure were detected between young and aged mice. (B) Severely degenerated IVDs of agedWT mice (left) displayed chondrocyte-like cells scattered in fibrocartilaginous matrix with less alcian blue stain in the NP. Only mild degeneration was observed in KO IVDs and proteoglycans were preserved in all KO IVDs (right). Blue boxes indicate regions of interest. (C) Quantification of histological scores. (D) Contribution of NP, AF and NP/AF boundary to histological scorings of aged mouse IVDs. Data are represented as mean ±/− SD. Young: n = 12 IVDs/genotype; AgedWT: n = 23 IVDs; AgedKO: n = 30 IVDs. Scale bar = 200 μm and 50 μm (insert). *p < 0.05. **p < 0.01. ****p < 0.0001.
groups were severely degenerated (Table 3), which is characterized by a complete replacement of notochordal NP cells with hypertrophic chondrocytes, the formation of fibrocartilaginous matrix and clefts, and loss of discernable AF/NP boundaries (Figure 1B, left).

None of these severe degenerative features were detected in IVDs from agedKO mice, which all maintained high NP proteoglycan deposition (Figure 1B, right). The improvement of histological features in agedKO mice was also demonstrated by the distribution of histological scores in each compartment (Figure 1D). Taken together, our findings suggest that Phlpp1 deficiency maintained a higher proteoglycan content and decreased both the prevalence and severity of spontaneous IVD degeneration in aged mouse IVDs.

3.2 | Phlpp1 deficiency protected against aggrecan degradation in aged mice

Aggrecan is the main proteoglycan in the NP and an important component for proper mechanical and structural function of IVDs. We performed immunostaining to determine whether Phlpp1 depletion maintained aggrecan deposition in NPs of agedKO mice. Consistent with the decrease of proteoglycans evidenced from PR-AB staining, aggrecan deposition was reduced in agedWT IVDs, where severely degenerated IVDs rarely expressed any aggrecan (Figure 2A–D). In contrast, aged IVDs from KO mice maintained aggrecan content at levels similar to youngKO mice, which were significantly higher compared to agedWT IVDs (Figure 2A–D).

TABLE 2 Incidence of IVD degeneration in young and aged mice

| Number of IDD (%) | WT | KO |
|-------------------|----|----|
| Young Mice        | 0 (0%) | 0 (0%) |
| IVDs              | 0 (0%) | 0 (0%) |
| Aged Mice         | 11 (100%) | 10 (72%) |
| IVDs              | 18 (75%) | 11 (33%) |

TABLE 3 Incidence of severe IVD degeneration in young and aged mice

| Number of severe IDD (%) | WT | KO |
|--------------------------|----|----|
| Young Mice               | 0 (0%) | 0 (0%) |
| IVDs                     | 0 (0%) | 0 (0%) |
| Aged Mice                | 3 (27%) | 0 (0%) |
| IVDs                     | 5 (21%) | 0 (0%) |

FIGURE 2 PHLPP1 deficiency protected against aggrecan degradation in aged IVDs. Representative images of (A) aggrecan (B) and ADAMTS5 immunostaining of young and aged mice. Positive immunosignal is stained with red. Arrows indicate positive cells. Lower panel shows the magnified area in the black box. (C) Quantification of aggrecan. Young WT and KO IVDs showed comparable aggrecan expression. AgedKO IVDs displayed more aggrecan expression than agedWT IVDs. (D) Quantification of ADAMTS5 immunopositivity. ADAMTS5 expression did not show obvious differences in the NP of young mice, but ADAMTS5 was increased in agedWT compared to agedKO NPs. Red = aggrecan and ADAMTS5, Purple = hematoxylin counterstain; Scale bar = 200 μm and 50 μm (insert). Data are represented as mean ±/− SD. Young: n = 12 IVDs/genotype; AgedWT: n = 23 IVDs; AgedKO: n = 30 IVDs. **p < 0.01. ***p < 0.001
The aggrecanase ADAMTS5 is one of the key mediators of aggrecan degradation in the NP. Only minor ADAMTS5 expression was observed in the NP of young WT and KO mice which was maintained at low levels in aged KO mice (Figure 2B–E). In contrast, its expression was significantly increased in the NP of aged WT mice and was significantly higher compared to KO mice (Figure 2B–E). Collectively, these results indicate that Phlpp1 depletion maintained aggrecan deposition in the NP of aged KO mice, partially by protecting against aggrecan degradation through repression of ADAMTS5.

3.3 | Phlpp1 deficiency increased the expression of NP phenotypic marker KRT19 in aged IVDs

To examine if the enhanced matrix deposition and reduced degenerative changes were accompanied by a healthier NP phenotype, we immunohistochemically assessed the level of the NP phenotypic marker KRT19 (Figure 3). KRT19 expression patterns confirmed the
protective effect of Phlp1 depletion on NP tissues in agedKO mice. Specifically, no changes in the expression of KRT19 were detected in young IVDs between two genotypes, but its expression significantly increased in the NP of agedKO compared to agedWT IVDs, potentially as a response to the worsening microenvironment in the aging NP.

### 3.4 | Phlp1 deficiency protected against cell apoptosis in aged IVDs

Apoptosis is yet another hallmark of IVD degeneration during aging. To investigate the effects of PHLPP1 on cell apoptosis, TUNEL assays were performed on IVDs between two genotypes, but its expression significantly increased in the NP of agedKO compared to agedWT IVDs, potentially as a response to the worsening microenvironment in the aging NP.

### 3.5 | PHLPP1 deficiency promoted the expression of transcription factor FOXO1 in aged IVDs

It has recently been demonstrated that FOXOs are important players in modulating IVD homeostasis and that FOXO deficiency induced degenerative features in mouse IVDs. FOXOs are under indirect control of PHLPP1. To investigate the impact of PHLPP1 on the expression of FOXO1 and FOXO3a in aged mice, we performed immunohistochemistry on young and aged WT and KO mice. Young WT and KO mice did not show significant differences in FOXO1 or FOXO3a expression in the NP (Figure 5A, B). However, a significant increase in the expression of FOXO1 was observed in agedKO compared to WT mice (Figure 5D), while no differences were observed in FOXO3a expression (Figure 5E). These results suggest that Phlp1 deficiency promoted FOXO1 expression and may delay age-related, spontaneous IVD degeneration.

### 3.6 | PHLPP1 knockdown increased protein levels of KRT19 via FOXO1 signaling in human degenerated NP cells

We transiently knocked down PHLPP1 (KD) by siRNA-mediated transfection to determine whether PHLPP1 silencing could induce a healthy NP phenotype in human degenerated NP cells. PHLPP1 protein levels of KD NP cells were significantly reduced compared to the degenerated NP cell control group (Figure 6A). As expected, PHLPP1 knockdown promoted the phosphorylation of AKT, a known PHLPP1 substrate (Figure 6A). In contrast, ERK phosphorylation was not altered by PHLPP1 knockdown (Figure 6A).

Consistent with the findings in mice, protein levels of FOXO1, but not FOXO3, increased significantly after PHLPP1 knockdown (Figure 6A). To investigate if FOXO1 activity was increased and if FOXO1 was the downstream effector of PHLPP1, the KD NP cells were treated with FOXO1 inhibitor AS1842856 which binds to the active form of FOXO1. FOXO1 inhibition offset the beneficial effect of PHLPP1 knockdown on KRT19 gene expression (Figure 6B). Surprisingly, AKT inhibition with the small molecule
PI3K/AKT inhibitor Wortmannin did not abolish KRT19 gene expression (Figure 6B). FOXO1 dependent KRT19 expression was further confirmed by immunoblotting. Human NP cell culture with the FOXO1 inhibitor decreased KRT19 expression in PHLPP1 knockdown cells (Figure 6C). Taken together, these findings suggest that PHLPP1 silencing promoted a healthy NP phenotype through targeting FOXO1 signaling, independent of AKT.

4 | DISCUSSION

This study demonstrated that PHLPP1 played a significant role in age-induced spontaneous IVD degeneration in mice and suggested a role of PHLPP1 in FOXO1 signaling in human NP cells. Our results indicated that Phlpp1 KO maintained a healthy NP phenotype and proteoglycan content and protected against cell apoptosis while suppressing matrix degradation in aged mice. Concomitantly, suppressing the function of PHLPP1 promoted FOXO1 activation in both aged mouse NP tissue and degenerated human NP cells. Blocking the activity of FOXO1 abolished the beneficial effects of PHLPP1 knockdown on promoting a healthy NP cell phenotype.

Spontaneous IVD degeneration during aging is partially caused by a catabolic shift in matrix metabolism, causing progressive IVD matrix breakdown and loss of structural integrity. Our data showed that systemic inhibition of PHLPP1 function improved proteoglycan deposition, specifically aggrecan deposition, reduced the expression of the matrix degradation enzyme ADAMTS5, and prevented the formation of fibrous NP tissues that occurred only in severely degenerated IVDs from aged WT mice. Anabolic effects of PHLPP1 inhibition have also been shown to be beneficial in other tissues where Phlpp1 deficiency promoted bone formation and mitigated the onset of osteoarthritis in a mouse model of osteoarthritis by increasing aggrecan and glycosaminoglycans production.

High incidence of cell apoptosis has been reported in the IVD with age and degeneration, causing the loss of cell number and failure in replenishing the functional extracellular matrix. Targeting pathways that are involved in cell apoptosis have been explored as a therapeutic approach to halt IVD degeneration. Our data demonstrated that, in addition to the protective effects of PHLPP1 deficiency on maintaining proteoglycan content, apoptosis was attenuated in the NP of aged KO
mice. Inhibition of PHLPP1 function has been found to attenuate apoptosis in myocytes, intestinal epithelial cells, and neurons, providing protection against tissue injuries. PHLPP1 is known to directly dephosphorylate and promote apoptotic pathways such as Akt, PKC and p70S6 kinase. One of the key regulators in IVD cell apoptosis is Akt signaling. Our in vitro findings showed that PHLPP1 knockdown increased Akt phosphorylation in human degenerated NP cells while inhibiting Akt activity did not reverse the effects of PHLPP1 knockdown on KRT19 gene expression. It is possible that Akt signaling is not involved in promoting NP phenotype, but regulating cell survival in the IVD. These findings, together with our data on apoptosis, suggest that PHLPP1 is a potential target for preventing cell loss in IVD degeneration.

Recent studies found that deletion of FOXOs caused accelerated IVD degeneration in aged mice which was accompanied by severe cell loss in the NP and endplate. Our data in mice demonstrated that Phlp1 KO promoted FOXO1 expression in mouse NP tissue, and that silencing PHLPP1 in degenerated human NP cells promoted a healthy NP cell phenotype by increasing FOXO1 expression. Inhibiting FOXO1 function abolished the PHLPP1 knockdown induced expression of the NP phenotypic marker KRT19. Liu et al. demonstrated in NP cells that FOXOs expression can suppress matrix degradation induced by oxidative stress. In human meniscus cells, FOXO1 overexpression rescued expression of matrix related genes. In mouse meniscus, FOXOs depletion accelerated age-related damage by suppressing the expression of genes related to autophagy, antioxidant defense, and meniscus phenotype. On the contrary, Bradley et al. demonstrated that Phlp1 deficiency promoted chondrocyte proliferation by inhibiting FOXO1 activity. These contrasting results demonstrate that PHLPP1 and FOXO1 activities are complex and highly tissue dependent. Becerra et al. revealed that FOXO3a regulated PHLPP1 expression in chondrocytes. However, in our study, we did not observe any relationship between FOXO3a and PHLPP1. It should also be noted that the levels of FOXO3a and FOXO1 were determined from the whole NP cell extract. Therefore, it may mask the effect of PHLPP1 on FOXO3a activity in human NP cells. Further investigation on delineating the mechanisms between FOXO1/3a and PHLPP1 during IVD degeneration is warranted.

Our study used mice with global Phlp1 knockout. It cannot be excluded that the surrounding tissues contributed to the delayed age-related spontaneous IVD degeneration. Contrary to observations in other tissues, where systemic PHLPP1 deficiency displayed thicker articular cartilage in young mice, we did not observe any morphological differences between young WT and KOS mouse IVDs. One reason might be that IVD cells are quiescent and therefore only display minor PHLPP1 activity in young and undegenerated IVDs.

In summary, our data demonstrated conclusively that inhibition of PHLPP1 function promoted healthier IVD histological features and prevented severe IVD degeneration in aged mice. Phlp1 depletion suppressed apoptosis and matrix degradation and promoted KRT19 expression, potentially via FOXO1 activation. The improved histological, immunohistochemical, and apoptotic outcomes of its depletion indicate PHLPP1 as a potential therapeutic target for treating IVD degeneration.

**AUTHOR CONTRIBUTIONS**

Changli Zhang—study design, data collection and analysis, manuscript creation and editing. Katherine M. Joseph—HIC processing, image acquisition, data analysis, and manuscript editing. Nazir M. Khan—study design, data interpretation, critical suggestions, and manuscript review. Martha Elena Diaz-Hernandez—human specimen collection, data interpretation, critical suggestions, manuscript review. Hicham Drissi—study design, data interpretation, critical suggestions, manuscript review. Svenja Illien-Junger—study design, data analysis, graphic designs, manuscript review and editing, and fund acquisition. All authors have read and approved the final manuscript.

**ACKNOWLEDGMENTS**

Funded by NIH/NIAMS R21 AR072222, NIH/NIAMS R01 AR078908, and the Department of Orthopedics at School of Medicine, Emory University. The authors thank Mr. Shayan Parvini Najafabadi for his technical assistance. Phlp1 KO mice originated from Dr. Alexandra Newton at UC, San Diego.

**CONFLICT OF INTEREST**

The authors have no conflict of interest associated with this work.

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APPENDIX

### TABLE A1  Respective p-values of assays an age or sex

| Assay     | Age (p-values) | Sex (p-values) |
|-----------|----------------|----------------|
|           | Interaction    | Genotype | Age | Interaction | Genotype | Sex |
| KRT19     | 0.237          | 0.060    | *0.002 | 0.245 | *0.015 | 0.395 |
| Aggrecan  | *0.033         | 0.145    | *<0.0001 | 0.708 | *0.017 | 0.407 |
| ADAMTS5   | 0.158          | *0.018   | 0.100 | 0.783 | *0.001 | 0.525 |
| TUNEL     | *0.027         | 0.053    | *<0.0001 | *0.012 | *0.020 | *0.001 |
| FoxO1     | *0.003         | 0.531    | *0.002 | 0.335 | *0.021 | 0.361 |
| FoxO3     | 0.493          | 0.780    | 0.164 | *0.030 | 0.506 | 0.807 |

Asterisks denote significance.

### TABLE A2  IVD degeneration scores

| WT | Male (6 months) | Female (20 months) | Male (20 months) | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score |
|----|-----------------|--------------------|-------------------|-----------------|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|    | 1               | 3-4                | 0                 | 1               | 3-4   | 0     | 1    | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     |
|    | 4-5             | 0                  | 5-6               | 0               | 5-6   | 0     | 2    | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     |
|    | 2               | 3-4                | 0                 | 4-5             | 0     | 3     | 3-4   | 9.2   | 3     | 3-4   | 1.5   | 4-5   | 0     | 5-6   | 1     | 3     | 3-4   | 9.2   | 3     | 3-4   | 1     | 2     | 3-4   | 9.2   |
|    | 5-6             | 0                  | 5-6               | 0               | 5-6   | 0     | 4-5   | 1     | 4-5   | 1.7   | 5-6   | 0     | 5-6   | 0     | 3     | 3-4   | 1     | 2     | 3-4   | 9.2   | 3     | 3-4   | 1     |
|    | 1               | 3-4                | 0                 | 4-5             | 0     | 5     | 3-4   | 25    | 4     | 3-4   | 4.2   | 4-5   | 0     | 5-6   | 1     | 5     | 3-4   | 2.5   | 4     | 3-4   | 2.5   | 4     | 3-4   | 2.5   |
|    | 4-5             | 0                  | 5-6               | 0               | 5-6   | 0     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 4     | 3-4   | 1.3   | 5     | 3-4   | 1.3   | 5     | 3-4   | 1.3   |
|    | 5-6             | 0                  | 5-6               | 0               | 5-6   | 0     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 4     | 3-4   | 1.3   | 5     | 3-4   | 1.3   | 5     | 3-4   | 1.3   |
|    | 6               | 3-4                | 2.7               | 5-6             | 15.5  | 4-5   | 0     | 5-6   | 1.2   | 4-5   | 1     | 5-6   | 1     | 4-5   | 1     | 5-6   | 1     | 4-5   | 1     | 5-6   | 1     | 4-5   | 1     |
| KO| Male (6 months) | Female (20 months) | Male (20 months) | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score | Mouse | Level | Score |
|    | 1               | 3-4                | 0                 | 1               | 3-4   | 0     | 1    | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     | 3-4   | 1     |
|    | 4-5             | 0                  | 5-6               | 0               | 5-6   | 0     | 2    | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     | 2     | 3-4   | 1     |
|    | 2               | 3-4                | 0                 | 4-5             | 0     | 3     | 3-4   | 9.2   | 3     | 3-4   | 1.5   | 4-5   | 0     | 5-6   | 1     | 3     | 3-4   | 9.2   | 3     | 3-4   | 1     | 2     | 3-4   | 9.2   |
|    | 5-6             | 0                  | 5-6               | 0               | 5-6   | 0     | 4-5   | 1     | 4-5   | 1.7   | 5-6   | 0     | 5-6   | 0     | 3     | 3-4   | 1     | 2     | 3-4   | 9.2   | 3     | 3-4   | 1     |
|    | 1               | 3-4                | 0                 | 4-5             | 0     | 5     | 3-4   | 25    | 4     | 3-4   | 4.2   | 4-5   | 0     | 5-6   | 1     | 5     | 3-4   | 2.5   | 4     | 3-4   | 2.5   | 4     | 3-4   | 2.5   |
|    | 4-5             | 0                  | 5-6               | 0               | 5-6   | 0     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 4     | 3-4   | 1.3   | 5     | 3-4   | 1.3   | 5     | 3-4   | 1.3   |
|    | 5-6             | 0                  | 5-6               | 0               | 5-6   | 0     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 5-6   | 1     | 4     | 3-4   | 1.3   | 5     | 3-4   | 1.3   | 5     | 3-4   | 1.3   |
|    | 6               | 3-4                | 2.7               | 5-6             | 15.5  | 4-5   | 0     | 5-6   | 1.2   | 4-5   | 1     | 5-6   | 1     | 4-5   | 1     | 5-6   | 1     | 4-5   | 1     | 5-6   | 1     | 4-5   | 1     |

6 3-4 2.5
6 3-4 2.5
7 3-4 1
8 3-4 1