

NEDD4 enhances bone-tendon healing in rotator cuff tears by reducing fatty infiltration

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Abstract. Rotator cuff tears (RCT) can cause shoulder pain, weakness and stiffness, significantly affecting daily life. Analysis of the GSE103266 dataset revealed significant changes in the mTOR/PI3K/Akt signaling pathway and lipid metabolism-related pathways, suggesting that fatty infiltration may affect RCT. The analysis indicated that the ubiquitin ligase NEDD4 plays a critical role in RCT. NEDD4 was found to be highly associated with the mTOR/PI3K/Akt signaling pathway. An RCT model in Sprague-Dawley (SD) rats was established to study the role of NEDD4 in regulating the mTOR pathway and investigate its effects on fatty infiltration. SD rats were divided into NEDD4 overexpression and knockout groups. Tissue recovery, apoptosis and fat deposition were measured through histological staining, reverse transcription-quantitative PCR and western blotting. Additionally, cell culture of fibro-adipogenic progenitors and lentiviral transfection were conducted to investigate the effect of NEDD4 on adipocyte differentiation. NEDD4 overexpression significantly reduced lipid accumulation, whereas NEDD4 knockdown enhanced lipid accumulation. NEDD4 was found to regulate the mTOR pathway and the expression of adipogenesis-related genes, promoting fat metabolism and inhibiting adipocyte differentiation. Histological analysis indicated that NEDD4 overexpression improved tissue recovery and reduced apoptosis. Targeting NEDD4 offers a potential therapeutic strategy

to improve the clinical outcomes of patients with RCT by modulating the mTOR pathway and fat metabolism.

Introduction

Rotator cuff tears (RCT) are highly prevalent, especially among individuals over 40 years old and athletes (1). Beyond tendon tears, the development of fatty infiltration is a critical factor complicating RCT (2). Fatty infiltration refers to the abnormal deposition of fat in the muscle tissue surrounding the injured tendon, leading to progressive muscular degeneration (3). This phenomenon is particularly problematic because it hampers the success of rotator cuff repair surgeries by increasing the risk of re-tears, reducing tendon-to-bone healing capacity and impairing shoulder function (4).

Previous studies have indicated that rotator cuff injuries often lead to pathological changes, such as fatty infiltration, severe inflammation, muscular atrophy and fibrosis, leading to irreversible degeneration and persistent muscle waste (5). Fatty infiltration is a primary factor significantly affecting the success of rotator cuff repair surgery (6). Fatty infiltration originates from fibro-adipogenic progenitors (FAPs), which differentiate into adipocytes under certain pathological conditions (7). When chronic inflammation and mechanical stress persist after RCT, FAPs contribute to the accumulation of adipocytes in the muscle, impairing muscle function (8).

FAPs are multipotent mesenchymal cells residing in the muscle tissue, where they play a pivotal role in the repair and regeneration after injury. Under normal conditions, FAPs contribute to tissue homeostasis and muscle repair by differentiating into fibroblasts. However, in the context of chronic injuries, such as RCT, FAPs can aberrantly differentiate into adipocytes, promoting fatty infiltration (9). This adipogenic differentiation is driven by signaling pathways, such as the PI3K/Akt, mTOR and Wnt signaling pathways, which regulate the fate of FAPs in response to injury (10). The pathological transformation of FAPs into adipocytes is a major contributor to muscle dysfunction and a key factor in the failure of rotator cuff repairs (11). Inhibiting pre-adipocyte-related genes [FABP4 and peroxisome proliferator-activated receptor γ (PPAR γ)] can inhibit fatty infiltration.

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RCT significantly modulates fat metabolism, particularly by dysregulating adipogenesis and lipid accumulation (12). The injury promotes an inflammatory response that activates FAPs, driving their differentiation into adipocytes. This not only disrupts normal muscle function but also recruits immune cells to the site of injury (13). Increased infiltration of immune cells exacerbates muscle degeneration by sustaining chronic inflammation, further hindering the healing process (14).

Studies have shown that mTOR, a conserved serine/threonine kinase, can regulate cell proliferation and metabolism by integrating various intracellular and extracellular signals (15). mTOR and its complexes affect protein translation, lipid synthesis and metabolism, mitochondrial energy metabolism and autophagy (16). Understanding the role of the mTOR signaling pathway in fatty infiltration after rotator cuff injuries may provide new insights into the pathogenesis of rotator cuff injury.

Through ubiquitination, NEDD4 tags specific substrate proteins for proteasomal degradation, thereby regulating their intracellular abundance and activity (17). In the context of RCT, NEDD4 modulates fat metabolism and cell signaling by ubiquitinating key regulators of adipogenesis and inflammation. This ubiquitination process modulates the cellular responses to injuries, including the differentiation of FAPs into adipocytes and the activation of the mTOR pathway, which is pivotal for tissue repair (18).

Previous studies have highlighted the key roles of NEDD4 and the mTOR signaling pathway in protein modification and fat metabolism (19), respectively. It was hypothesized that NEDD4 regulates the mTOR pathway through its ubiquitin ligase activity, thereby affecting adipose infiltration. The present study aimed to investigate the interaction between NEDD4 and mTOR by constructing NEDD4 overexpression and interference vectors in a rat model of rotator cuff injury and investigating their mechanisms in regulating fat infiltration. Understanding these interactions may reveal new therapeutic strategies, provide a theoretical basis for treating and preventing fat infiltration after rotator cuff injuries, guide clinical decision-making, and improve patients' outcomes.

Materials and methods

Vector construction and screening. The overexpression vector pcDNA3.1-NEDD4-3xHA was purchased from HonorGene (Changsha Abiwei Biotechnology Co., Ltd.). The plasmid pLVshRNA-EGFP (2A) Puro was used to generate the knockdown interference vectors. A total of 3 different short hairpin (sh) RNA sequences were designed to target NEDD4: sh-NEDD4-1 (5'-GCAGCTCGCAAACCTGTA TCT-3'; concentration, 443.6 ng/ μ l), sh-NEDD4-2 (5'-GGG CTTGTGTAATGAAGATCA-3'; concentration, 560.4 ng/ μ l) and sh-NEDD4-3 (5'-GCAAACATTCTGGAGGATTCT-3'; concentration, 682.3 ng/ μ l). The shRNA negative control (NC) sequence is shRNA-NC (5'-TTCTCCGAACGTGTC ACGT; concentration, 519.4 ng/ μ l). The overexpression plasmid pcDNA3.1-NEDD4-3xHA (687.98 ng/ μ l) and its NC (754.55 ng/ μ l) were used for transfection. For transfection, the PEI transfection reagent (Shanghai Yeasen Biotechnology Co., Ltd.) was used. Cells were incubated at 37°C for 48 h during transfection. Subsequent experiments were performed

24 h after transfection. The expression levels of NEDD4 were detected using RT-qPCR, and vectors were screened based on knockdown efficiency, following the previously described methods (20).

Animal model construction. A total of 18 male SD rats (8-week-old; mean weight, 280 $\pm$ 1.03 g) were purchased from SPF (Beijing) Biotechnology Co., Ltd [Production license number: SCXK (Jing) 2019-0010]. The rats had *ad libitum* access to water and a standard laboratory diet. Rats were housed in a specific pathogen-free (SPF) environment at 22 $\pm$ 2°C, 40-80% humidity, and a 12/12-h light/dark cycle. Rats were acclimatized for 5 days before the experiment. Feed and water were checked daily to ensure they were fresh, clean and adequate. The body weight of all rats was recorded weekly to assess their overall health condition. The present study was approved (approval no. KY20230718-16) by the Experimental Animal Welfare and Ethics Committee of Beijing Jishuitan Hospital Guizhou Hospital (Guiyang, China).

The rats undergoing surgery were divided into the modeling and treatment groups, while the negative control group received no treatment. A total of three SD rats were used in each experimental group. The NEDD4 intervention groups were divided into empty vector groups (sh-NC and OE-NC), overexpression treatment group (Model + OE-NEDD4) and knockdown treatment group (Model + sh-NEDD4). The 'model' refers to the rotator cuff tear (RCT) injury model used in the experiment. The experimental design involves five distinct groups to investigate the role of NEDD4 in RCT. The negative control group establishes baseline changes without any intervention. The empty vector groups (sh-NC and OE-NC) serve as controls to ensure that observed effects are attributable to NEDD4 expression rather than the viral vector itself. The NEDD4 overexpression group is used to assess the impact of increased NEDD4 levels on tissue repair and fatty infiltration. Conversely, the NEDD4 knockdown group investigates the effects of reduced NEDD4 expression on disease progression.

The rats were anesthetized using the anesthetic isoflurane (RWD Life Science Co., Ltd.). Anesthesia was induced with an isoflurane concentration of 2.0-5.0% in a closed system until the rats lost their reflex response. During the maintenance of anesthesia, the isoflurane concentration was reduced to 1.0-2.5%.

Based on previous studies, a method was adopted to establish the rat model of rotator cuff injury. Before conducting the surgery to establish the rat model of rotator cuff injury, animals underwent general anesthesia and received an intramuscular injection of 100,000 units of penicillin to prevent infection (21). The surgical procedure included the following steps: after skin preparation, the surgical area was disinfected with 2.5% iodine. The shoulder joint was externally rotated and abducted. Then, an oblique incision was made along the long axis of the scapula to expose the rotator cuff insertion site. The supraspinatus muscle (SSP) was dissected at the bony attachment point, and a 4-0T muscle suture was passed through the muscle to create a circumferential marker. Thereafter, the deltoid muscle was closed, and the skin incision was sutured, followed by a second disinfection of the incision site. For the first 3 days after the surgery, penicillin was administered at a

dose of 100,000 units/rat via intramuscular injection. Shoulder cuff repair surgery was conducted after 4 weeks. After opening the deltoid muscle, the ruptured SSP was identified using the marker line. The surrounding adhesive tissue was bluntly dissected to reduce tension, and the SSP was appropriately pulled until the muscle end was easily relocated to the greater tuberosity. The synovium and cartilage at the greater tuberosity insertion point were scraped away until the bone bed was visible. Two parallel bone tunnels were established using a sterile 0.5-mm drill. Finally, the SSP tendon was sutured to the insertion point using a 4-0T muscle suture (Fig. S1).

The treatment started on the second day after modeling, with all rats in all groups receiving a 200- μ l tail vein injection of NEDD4 lentivirus (concentration: 1×10^7 TU/ml) once every 2 weeks for 4 weeks.

The humane endpoint criteria were as follows: the experimental rats becoming debilitated due to immune suppression, unable to eat or drink, and showing signs of depression accompanied by hypothermia (body temperature $<37^\circ\text{C}$) without anesthesia or sedation. None of the experimental animals reached these criteria.

Euthanasia was carried out using isoflurane (RWD Life Science Co., Ltd.) anesthesia. At the end of the experiment (at the conclusion of week 6 post-modeling/eighteen SD rats), cardiac blood collection was performed under isoflurane anesthesia following blood sampling.

The rat was anesthetized and positioned supine on a board. The left index finger was used to palpate the strongest heart-beat location, typically located at the left 4th or 5th intercostal space, just below the triangle formed by the front limbs and xiphoid process. Gentle pressure was applied with the left thumb and index finger on the right chest area to stabilize the heart. With the right hand, the needle was inserted vertically at the point of the strongest heartbeat. Once blood appeared in the syringe, the left hand was released and the syringe was carefully supported to slowly draw the blood.

After blood collection, a high concentration (5%) of the anesthetic was administered in a closed system to ensure the animals were euthanized in a state of deep anesthesia. Death was verified by confirming the absence of both a heartbeat and respiratory movement. After these signs of death were observed, gas perfusion was continued for an additional 3 min. The animals were only removed from the euthanasia chamber after death was confirmed. If death was not confirmed, alternative euthanasia methods, such as cervical dislocation, were immediately applied.

This experimental design helped to clarify the specific role of NEDD4 in the pathogenesis of RCT. By comparing the results between different groups, the role of NEDD4 in improving or exacerbating the disease process was investigated.

Histological and pathological staining. A total of 6 weeks after modeling, all rats were euthanized. Researchers harvested the distal end of the supraspinatus tendon along with the connected humeral bone block from each rat to obtain a complete bone-tendon junction (BTJ) sample. Some samples were processed for decalcification and stained using H&E and oil red O staining and terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL)

assay following the established protocols for observation and analysis.

For H&E staining, paraffin-embedded tissue sections were baked at 64°C for 1 h, followed by deparaffinization in xylene and rehydration through graded ethanol solutions. Hematoxylin staining was performed at room temperature for 4 min and eosin for 6–10 sec.

For Oil Red O staining, fresh frozen tissue sections were prepared at a 0.5–10 μm thickness, fixed in 10% formaldehyde for 5–10 min at room temperature, and stained with 0.5% Oil Red O solution for 10–15 min at room temperature. Nuclei were counterstained with hematoxylin for 1–2 min at room temperature, followed by rinsing and sealing with glycerol for observation.

For TUNEL staining, tissue sections were first equilibrated by adding 50 μl of Equilibration Buffer per sample, incubating at room temperature for 10 min. TMR-5-dUTP Labeling Mix and Equilibration Buffer were thawed on ice, and the TdT incubation buffer was prepared using a ratio of 1 μl Recombinant TdT enzyme: 5 μl TMR-5-dUTP Labeling Mix: 50 μl Equilibration Buffer. This mixture was adjusted based on slide size. Negative controls were prepared using ddH₂O instead of the enzyme. For labeling, 56 μl of the TdT incubation buffer was added to each sample, followed by a 1-h incubation at 37°C in the dark, ensuring the slides remained moist. Post-incubation, samples were washed four times with PBS for 5 min each. For nuclear staining, slides were immersed in a freshly prepared DAPI solution at room temperature for 8 min in darkness. After washing the samples with PBS (three times for 5 min each), excess liquid was removed and anti-fade mounting medium was used for sealing. Fluorescence microscopy was employed for observation, with immediate analysis under dark conditions to detect TUNEL-positive cells (red fluorescence) and DAPI-stained nuclei (blue fluorescence), examining at least five fields of view per slide.

FAPs' cell culture. The FAPs used were derived from primary isolation and culture in the laboratory. The FAPs were isolated and cultured from the primary skeletal muscles of normal rats, not from the rats used in the animal model construction. Rat FAPs were used in the study (isolation and culturing of FAPs using a single rat), and FAPs were cultured following previously described methods (22). Frozen FAPs were thawed from liquid nitrogen and quickly dissolved in a 37°C water bath. Subsequently, the cells were transferred to a 15-ml centrifuge tube containing 10 ml of complete culture medium (Shanghai Zhong Qiao Xin Zhou Biotechnology, Co., Ltd.). After centrifugation (room temperature, $20\text{--}25^\circ\text{C}$) at $200 \times g$ for 5 min, the supernatant was removed, and the cells were resuspended in fresh complete culture medium before being transferred to T-25 culture flasks for further cultivation (37°C with 5% CO₂). Euthanasia was performed on the rats used in the aforementioned experiments.

Lentiviral transfection. Before lentiviral infection, adherent FAPs (primary isolation and cultivation in the laboratory) were seeded at 3×10^5 cells per well in a 6-well plate and cultured until reaching $\sim 70\%$ confluence within 18–24 h. Lentiviral transduction was performed using lentivirus produced from 293T cells (iCell Bioscience). The lentiviral vector plasmid (5 μg), along

with packaging plasmid pH1 (3.75 μ g) and envelope plasmid pH2 (1.25 μ g), was transfected into 293T cells using 20 μ l PEI transfection reagent. Transfection was carried out at 37°C for 24 h. After transfection, lentiviral particles were collected. The culture medium was replaced with 10 ml fresh medium at 24 h post-transfection, and supernatants containing lentivirus were harvested at 48 h. The collected supernatant was centrifuged at 500 x g for 10 min to remove cellular debris. The virus-containing supernatant was either directly used for infection or stored at -80°C. The multiplicity of infection for lentiviral transduction was 300. For lentiviral infection, target cells were exposed to lentiviral suspension for 24 h at 37°C, followed by medium replacement. Cells were subsequently selected using 2.5 μ g/ml puromycin for stable expression. Images were captured using a fluorescence microscope to confirm transduction efficiency. Experiments were performed 24 h after transduction. The lentivirus used for transduction was provided in a concentrated format (100X) and includes a fluorescent tag. Images were captured using a fluorescence microscope.

Western blotting. Cellular proteins were lysed in a buffer containing 9M urea and a mixture of HALT protease inhibitor (cat. no. 78430; Thermo Fisher Scientific, Inc.) and HALT phosphatase inhibitor (cat. no. 78428; Thermo Fisher Scientific, Inc.). Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (cat. no. 78835; Thermo Fisher Scientific, Inc.) and kept on ice during processing. The lysates were centrifuged at 16,000 x g for 15 min at 4°C to collect the supernatant. The protein concentration of the lysates was determined using the Pierce BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). The assay was conducted by preparing a standard curve with concentrations of 0, 0.25, 0.5, 1, 2, 3 and 5 mg/ml. Each standard and sample (diluted 50x) was measured in triplicate with 20 μ l in a 96-well plate, followed by the addition of 200 μ l BCA working reagent. Plates were incubated at 37°C for 30 min before measuring absorbance at 562 nm.

Equal amounts of denatured proteins (20 μ g per lane) were dissolved on 4-20% or 12% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Inc.) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in TBST (0.1% Tween-20) for 30 min at room temperature. For phosphoprotein analysis, 5% BSA in TBST was used instead. The membranes were then incubated with the following primary antibodies: BACH1 (1:1,000; cat. no. sc-271211; Santa Cruz Biotechnology, Inc.), HO-1 (1:1,000; cat. no. MA1-112; Thermo Fisher Scientific, Inc.), HK2 (1:2,000; cat. no. PA5-29326; Thermo Fisher Scientific, Inc.), β -actin (cat. no. A228; MilliporeSigma), GAPDH (1:1,000; cat. no. G9295; MilliporeSigma), Histone 3 (1:5,000; cat. no. ab1791; Abcam), NQO1 (1:2,000; cat. no. HPA007308; MilliporeSigma), KEAP1 (1:1,000; cat. no. 8047S; Cell Signaling Technology, Inc.) and NRF2 (1:3,000; cat. no. 12721; Cell Signaling Technology, Inc.). The membranes were incubated with the primary antibodies overnight at 4°C. The next day, the membranes were brought to room temperature for 1 h prior to washing with TBST three times (5 min each).

The membranes were then incubated with secondary antibodies at room temperature for 1 h. The secondary antibodies used were HRP-conjugated Goat Anti-Rabbit IgG (H+L)

(1:3,000; cat. no. GB23303; Wuhan Servicebio Technology Co., Ltd.) and HRP-conjugated Goat Anti-Mouse IgG (H+L) (1:5,000; cat. no. GB23301; Wuhan Servicebio Technology Co., Ltd.). Detection was conducted using Clarity Western ECL Substrate (cat. no. 1705061; Bio-Rad Laboratories, Inc.) and ChemiDoc Touch Imaging System (cat. no. 1708370; Bio-Rad Laboratories, Inc.). The densitometric analysis of bands was performed using ImageJ software (version 1.53k; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was conducted using the $2^{-\Delta\Delta C_q}$ method for relative quantification. RNA was extracted using the RNAsimple Total RNA Kit (Shanghai Yihui Biological Technology Co., Ltd.). mRNA was reverse transcribed using the SureScript First-strand cDNA Synthesis Kit (Guangzhou Saivell Biotechnology Co., Ltd.). Reverse transcription was conducted under the following conditions: 25°C for 5 min, 50°C for 15 min, 85°C for 5 sec, and held at 4°C. qPCR reactions were performed using SYBR Green as the fluorophore (Wuhan Servicebio Technology Co., Ltd) on a CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: Initial denaturation: 95°C for 1 min; denaturation: 95°C for 20 sec; annealing: 55°C for 20 sec; extension: 72°C for 30 sec (for a total of 40 cycles). The $2^{-\Delta\Delta C_q}$ method was used for quantification (23). The primer information used is included in Table SI. β -actin was used as the reference gene.

Bioinformatics analysis. The procured dataset GSE103266 was obtained from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) of the National Center for Biotechnology Information. This dataset included data from bilateral supraspinatus tears and suprascapular nerve resection surgeries conducted on rodent models. Samples were collected at 0, 10, 30 and 60 days after injury, with a sample size of n=4 per group. Untreated specimens were utilized as controls. RNA sequencing of muscle tissue was conducted to acquire the needed data. After data acquisition, the DESeq2 package (v1.38.3) of R was employed for differential analysis. The GSVA package (v1.46.0) was utilized for immune infiltration and single-sample Gene Set Enrichment Analysis (ssGSEA), while the ggpmlsc package (v0.5.5) was utilized for correlation analysis. Enrichment analysis was conducted utilizing the KOBAS (<http://bioinfo.org/kobas/>).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8 (Dotmatics). Data are presented as the mean $\pm$ standard error of the mean (SEM). Measurement data were compared between two groups using unpaired t-tests, and one-way ANOVA was used for multiple-group comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Rotator cuff injury significantly activates the PI3K/Akt, mTOR and lipid metabolism-related pathways. The effect of rotator cuff injury was analyzed using dataset GSE103266. Principal component analysis (PCA) revealed distinct separation among control and treated groups at 10, 30 and 60 days after injury

(Fig. 1A). Compared with controls, differential gene expression analysis identified 767, 539 and 368 upregulated and 883, 960 and 582 downregulated genes after 10, 30 and 60 days, respectively (Fig. 1B). Differential gene expression in response to RCT revealed significant alterations in multiple pathways. Specifically, the PI3K-Akt, mTOR and lipid metabolism pathways showed the most pronounced changes, with upregulation of adipogenesis-related genes (FABP4 and PPAR γ) and downregulation of lipid degradation pathways. Enrichment analysis using the KOBAS platform illuminated the significant role of fatty acid metabolism, oxidative phosphorylation and immune response pathways, underscoring the complex interplay between muscle degeneration, inflammation and fat accumulation after RCT (Fig. 1C) (24).

Rotator cuff injury suppresses fat metabolism and enhances immune activation. ssGSEA scoring of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with fat and other metabolisms was conducted based on differential gene expression to investigate the effect of rotator cuff injury on physiological functions and metabolic pathways. ANOVA revealed significant enrichment of 55 and 56 pathways, respectively (Fig. 2A and B). Fat metabolism-related pathways, such as Reactome glycerophospholipid catabolism and Reactome peroxisomal lipid metabolism, were downregulated, while lipid biosynthesis pathways were upregulated. Additionally, rotator cuff injury modulated cellular functions, signaling pathways and disease-related pathways (Fig. 2A). KEGG enrichment analysis showed the suppression of metabolic pathways, such as fatty acid degradation and glycolysis/gluconeogenesis, and only a few metabolic pathways were upregulated (Fig. 2B). Immune infiltration analysis identified 28 immune-related cells under different treatments (Fig. 2C), and scoring analysis demonstrated the activation of 25 immune-related cells subsequent to rotator cuff injury (Fig. 2D). These findings indicated that rotator cuff injury strongly modulated fat metabolism, primarily affecting physiological responses at the site of injury by inhibiting fat metabolism pathways and promoting immune activation.

NEDD4 is a key regulator of fat metabolism and immune activation after rotator cuff injury. Functional predictions on differential genes across different groups were performed to elucidate the role of key genes in abnormal fat metabolism induced by rotator cuff injury. The results revealed a significant presence of fat metabolism-related genes in the group treated for 10 days. Gene regulatory network analysis identified the key roles of genes SCD, CACNA1H, GNB3, FASN, LPIN1, TUBA1A, CACNA1S, KCNC1, CAV3, SCN5A, KCNH2 and NEDD4 (Fig. 3A). Next, PCA analysis was conducted on all genes within the network across the four groups. Thereafter, the top 20 genes that contributed the most to the two principal components (PC1 and PC2) were selected. By integrating each gene's contribution to both principal components, CACNA1S, EEF1A1 and NEDD4 were identified (Fig. 3B and C). NEDD4, an important E3 ubiquitin ligase, exhibited significantly increased expression after 10 and 30 days of treatment compared with controls (Fig. 3D). Correlation analysis indicated the close association of NEDD4 with fat-related signaling pathways, KEGG signaling pathway and

immune infiltration scores (Fig. 3E and F; Figs. S2-S4). It was hypothesized that NEDD4 plays a crucial role in rotator cuff injury by regulating fat metabolism and immune activation.

NEDD4 is a critical regulator of mTOR, PI3K-Akt and fatty acid metabolic pathways in rotator cuff injury. To unravel the regulatory mechanism of NEDD4 in rotator cuff injury, its associations with relevant pathways were investigated. Gene expression analysis revealed that rotator cuff injury led to the differential expression of genes related to the mTOR and PI3K/Akt signaling pathways (Fig. 4A), fatty acid biosynthesis, unsaturated fatty acid biosynthesis and fatty acid elongation pathways (Fig. 4B). In the present study, mTOR activation was assessed as a key component in accelerating bone-tendon healing in the context of rotator cuff injury. Correlation analysis showed that NEDD4 was significantly and positively correlated with genes in the mTOR pathway (ATP6V1G2, SLC7A5, FZD3 and IGF1R) and the PI3K/Akt pathway (LAMC2, BCL2L11, FGF21 and CCNE1) (Fig. 4C and D, respectively). In addition to the listed genes, numerous other genes were highly correlated with NEDD4 in these pathways (Figs. S5 and S6). Furthermore, NEDD4 was significantly and positively correlated with genes in fatty acid biosynthesis, unsaturated fatty acid biosynthesis and fatty acid elongation (TECR, ACACB, MCAT, PPT1, SCD, ACOX1, HSD17B12, ACSL3 and ACSL4) (Fig. 4E and G). These findings suggested that NEDD4 regulated multiple metabolic pathways, regulating fat metabolism and other physiological functions at the site of rotator cuff injury in rats.

Successful establishment of NEDD4 interference and overexpression models in rats to assess the role of NEDD4 in rotator cuff injury. Interference and overexpression vectors were constructed to investigate the role of NEDD4 in rat rotator cuff injury. Interference vectors included sh-NC (empty vector), sh-NEDD4-1, sh-NEDD4-2 and sh-NEDD4-3, while overexpression vectors included OE-NC and OE-NEDD4. NEDD4 expression levels were assessed using RT-qPCR, with the lowest expression observed in rats treated with sh-NEDD4-1 (Fig. S7A). Fluorescence staining confirmed successful vector packaging, with over 90% of vectors exhibiting high fluorescence intensity (Fig. S7C).

Rats underwent NEDD4 interference and overexpression treatments to evaluate the effects of vector on rotator cuff injury. Monitoring body weight over 49 days revealed significant differences between groups ($P < 0.05$) but there were no overall differences (Fig. S7B), suggesting the successful establishment of the rat model. Blood and tendon attachment tissues were collected from each group for further analysis.

NEDD4 promotes tissue recovery and reduces apoptosis in rat rotator cuff injury. Histological examination of rat rotator cuff injuries using H&E staining revealed distinct differences among the treatment groups. In the control group, chondrocytes were well-organized in the growth plate, and inflammatory bodies were minimal within the ligament, indicating healthy tissue structure. In the model group, there was a marked increase in the abundance of inflammatory bodies in the ligament, accompanied by disorganized chondrocytes in the growth plate, suggesting significant tissue damage. Compared

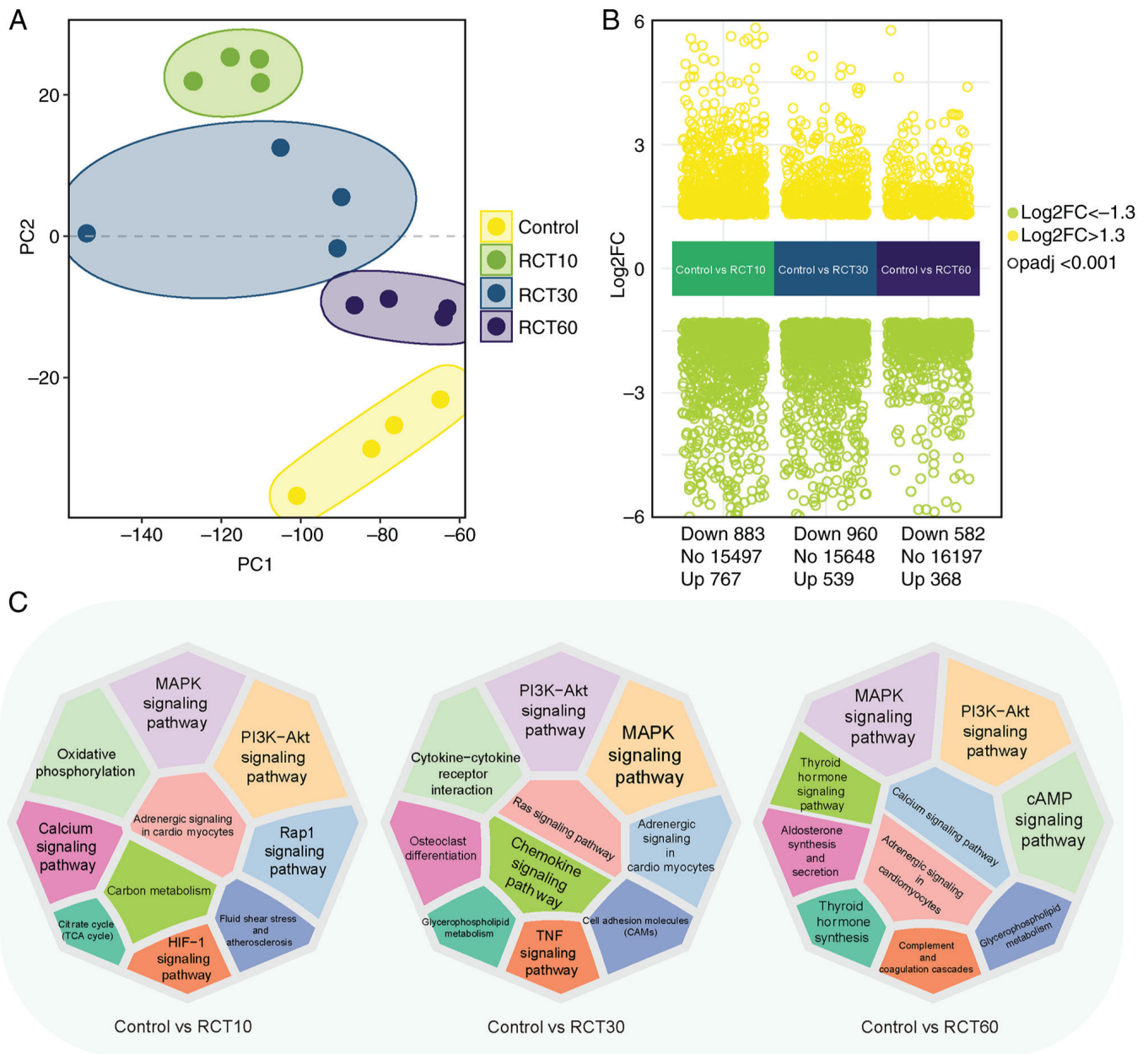


Figure 1. Differential Gene Expression and Pathway Enrichment Analysis Across Treatment Groups. (A) Principal component analysis demonstrates distinct separation among control, RCT10, RCT30 and RCT60 groups. (B) Differential expression analysis reveals the number of upregulated and downregulated genes ($\log_2FC > 1.3$ or < -1.3) between control and treated groups (RCT10, RCT30 and RCT60). (C) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis highlights significantly enriched pathways ($P < 0.05$) related to cellular growth, proliferation and metabolism in RCT10, RCT30 and RCT60 compared with controls. RCT, rotator cuff tear.

with the empty vector group, the NEDD4 overexpression group displayed fewer inflammatory bodies in the ligament and better-organized chondrocytes, indicating enhanced tissue recovery. Conversely, the NEDD4 knockdown group showed a higher number of inflammatory bodies and more disorganized cells, suggesting severe tissue damage (Fig. 5A).

To further elucidate the role of NEDD4, TUNEL staining was conducted on rotator cuff cells. The model group exhibited a significant increase in cell apoptosis compared with the control group, highlighting the detrimental effect of rotator cuff injury on cell viability (Fig. S8). The apoptotic rate was significantly lower in the NEDD4 overexpression group than in the empty vector group, suggesting that NEDD4 promoted

normal cell proliferation and mitigated cell death (Fig. 5B). On the other hand, the NEDD4 knockdown group showed a slightly higher rate of apoptosis compared with the empty vector group, indicating that low NEDD4 expression impairs normal cell proliferation and exacerbates tissue damage.

These detailed observations underscore the critical role of NEDD4 in regulating cell apoptosis and promoting tissue repair after rotator cuff injuries.

NEDD4 regulates fat metabolism and reduces lipid accumulation in rotator cuff injury. Oil Red O staining was applied to tissue samples from each treatment group to investigate the effect of fat infiltration on rotator cuff injuries. The results

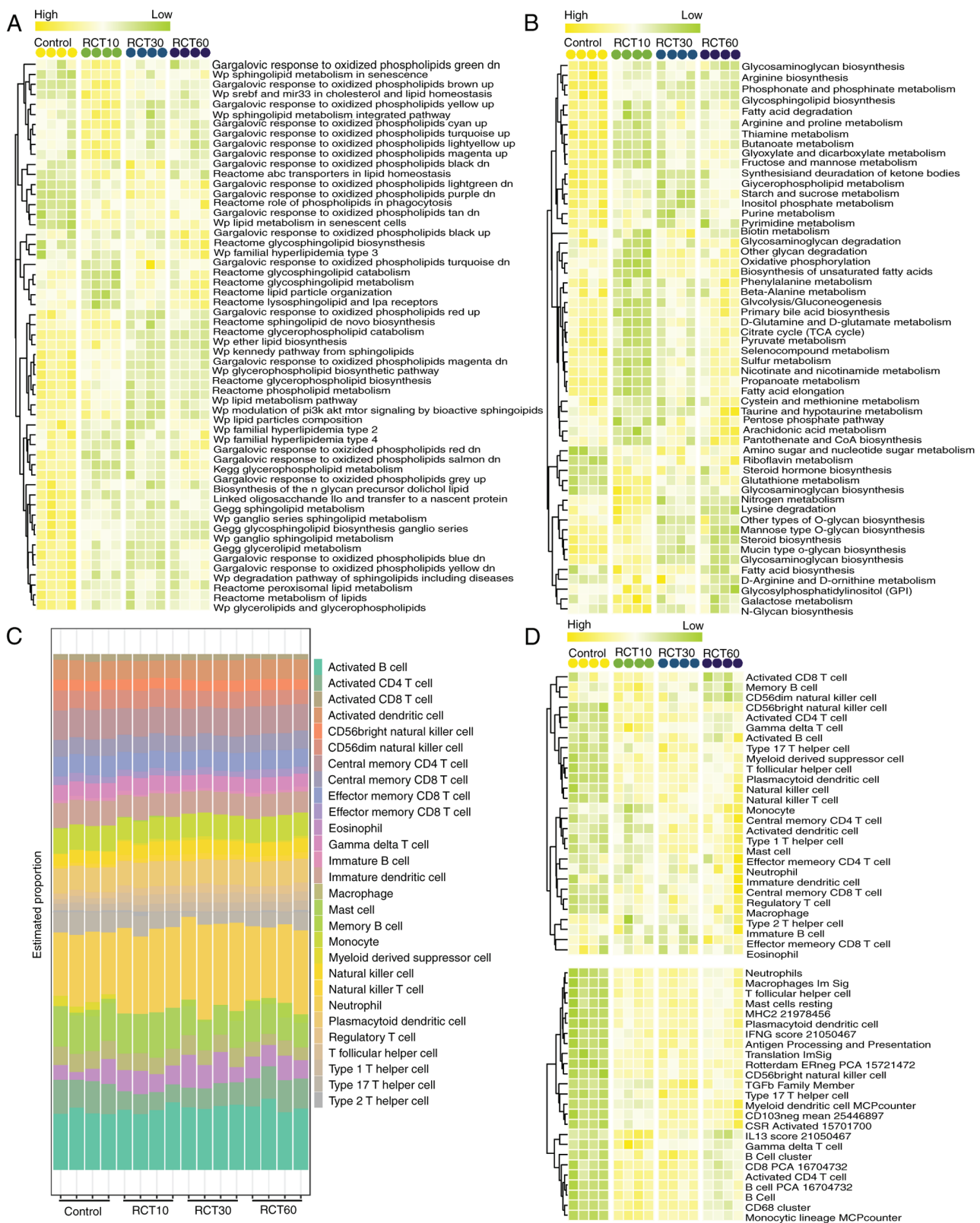


Figure 2. Analysis of fat metabolism pathways and immune function in rotator cuff injury. (A) Heatmap showing ssGSEA scores for fat metabolism-related pathways. Top 55 pathways with significant enrichment ($P < 0.05$) are displayed, with yellow indicating high scores and green indicating low scores. (B) Heatmap showing KEGG pathway ssGSEA scores related to metabolism. Top 56 pathways with significant enrichment ($P < 0.05$) are displayed. (C) Proportion of 28 types of immune-related cells in control, RCT10, RCT30 and RCT60 groups as determined by immuno-infiltration analysis. (D) Heatmap showing the distribution of 28 types of immune-related cells and the scores of related cells and cytokines. Top 25 results with significant P-values ($P < 0.05$) are displayed. ssGSEA, single-sample Gene Set Enrichment Analysis; RCT, rotator cuff tear.

revealed an increase in the abundance of lipid droplets in the model group compared with the control group, underscoring

the significance of fat deposition in rotator cuff injury (Fig. 6A and B). Notably, NEDD4 overexpression reduced the

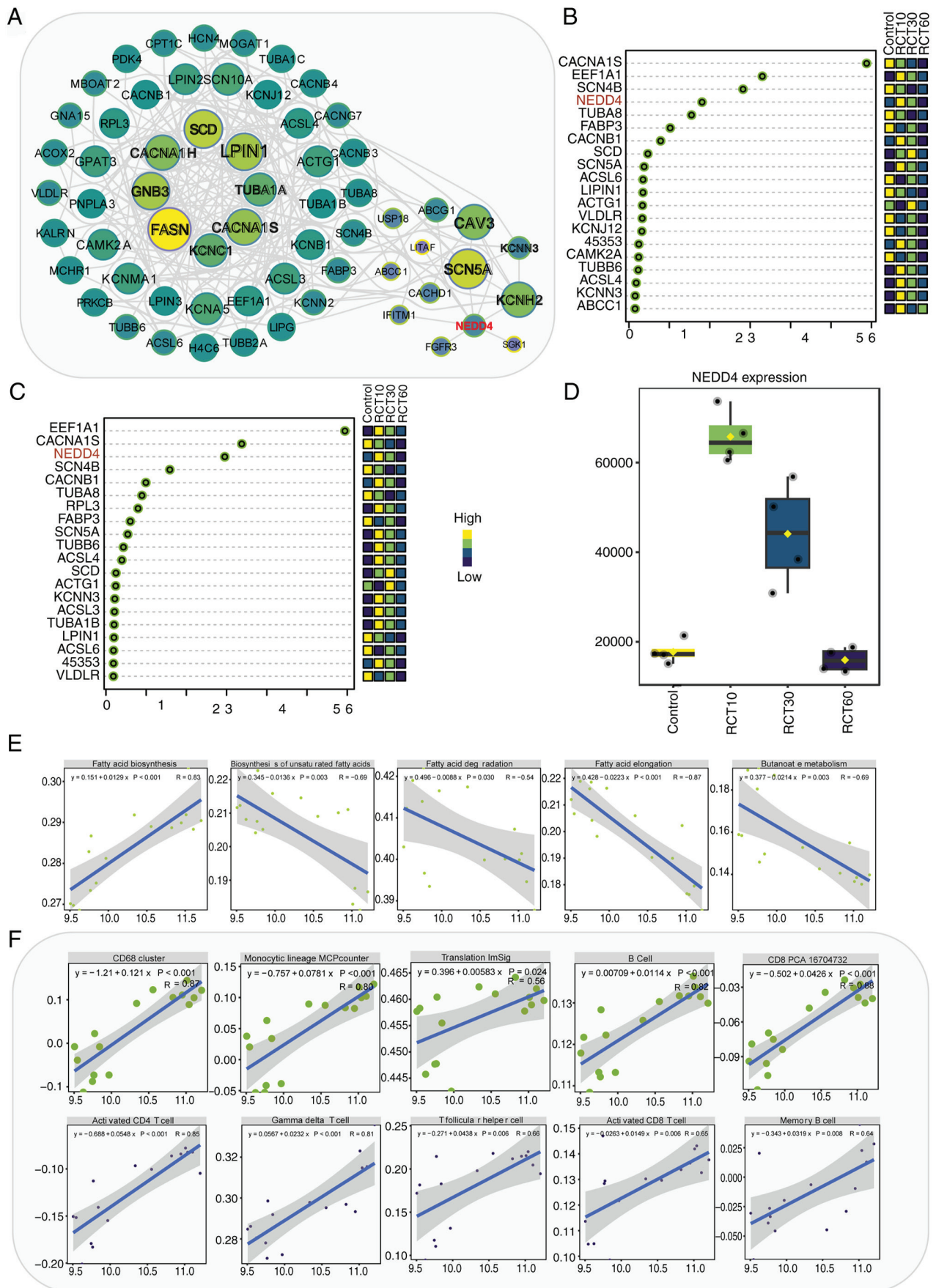


Figure 3. Identification and functional analysis of the key gene NEDD4. (A) Differential gene network interaction of control vs. RCT10. Genes are arranged according to degree value. The larger the circle, the higher the degree of connection between genes. (B and C) Principal Component Analysis showing the contribution of the top 20 genes to PC1 and PC2. (D) Expression levels of NEDD4 across the four groups, with significant differences noted. (E) Correlation analysis of NEDD4 with fat-related signaling pathways. (F) Correlation analysis of NEDD4 with Kyoto Encyclopedia of Genes and Genomes signaling pathways and immune-related factors. RCT, rotator cuff tear.

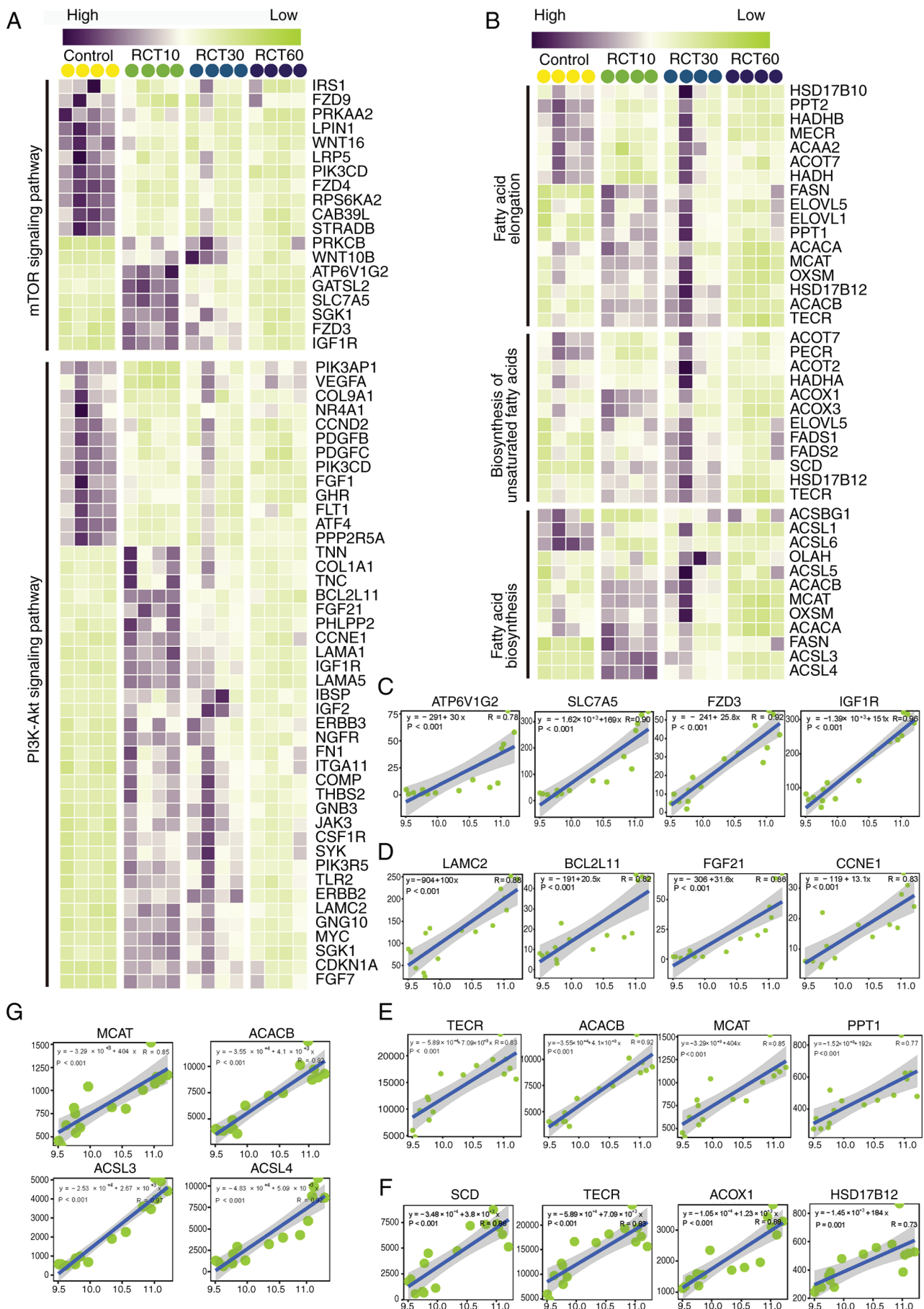


Figure 4. Expression of key genes and correlation with NEDD4. (A) Heatmap of gene expression in mTOR and PI3K-Akt pathways across 4 groups (purple=high, green=low). (B) Heatmap of gene expression in fatty acid pathways across 4 groups. (C-G) Correlation analysis of NEDD4 with mTOR pathway genes, PI3K-Akt pathway genes, fatty acid elongation genes, unsaturated fatty acids biosynthesis genes and fatty acid biosynthesis genes (All correlations, $P < 0.001$).

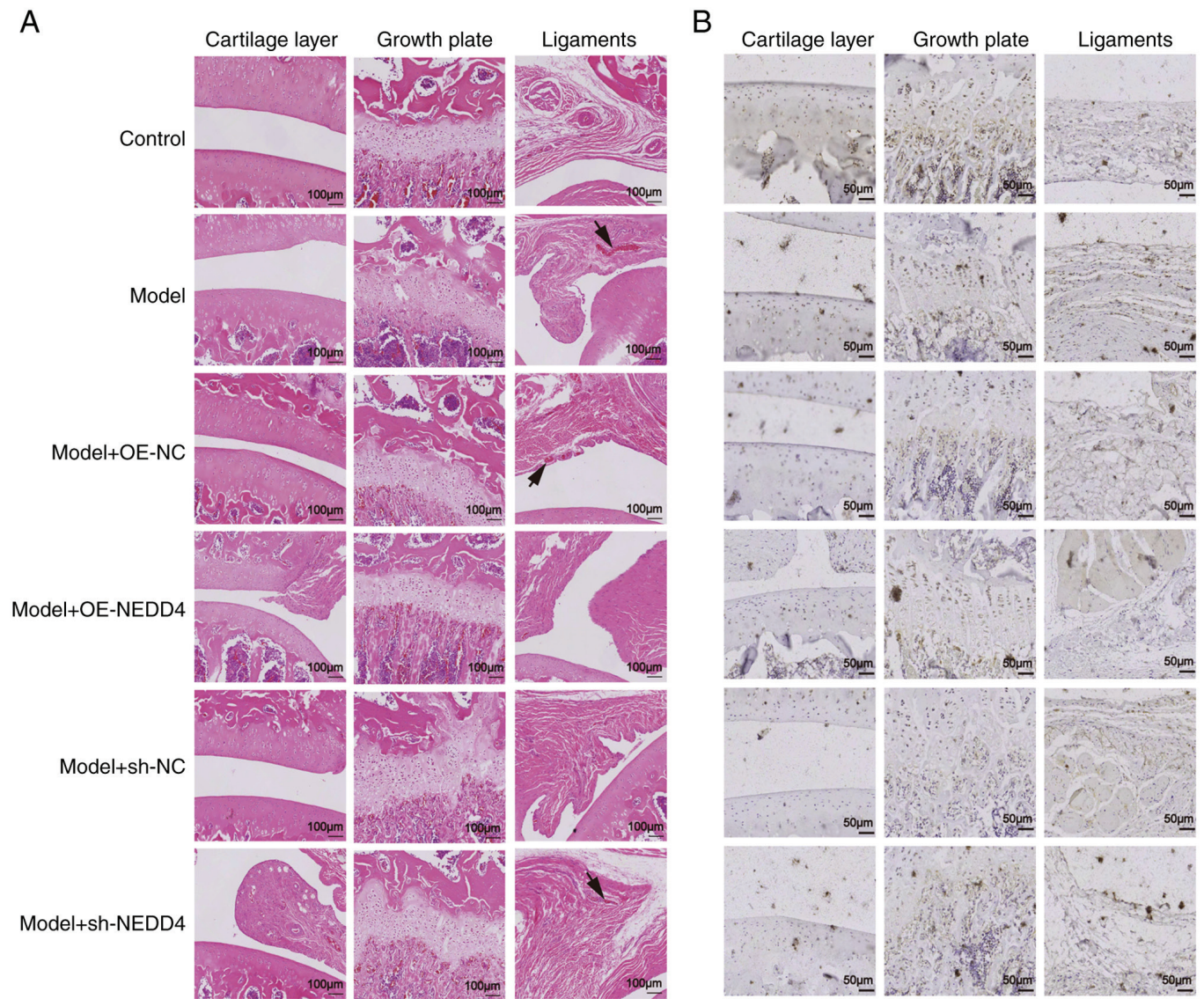


Figure 5. Tissue staining analysis results of rotator cuff tendon junction. (A) H&E staining results of the rotator cuff bone-tendon junction across different treatment groups. Sections show the cartilage layer, growth plate and ligaments. (B) TUNEL staining results of the rotator cuff muscle-tendon junction, highlighting differences in cell apoptosis across different treatment groups.

abundance of lipid droplets, suggesting its role in mitigating fat deposition. By contrast, NEDD4 knockdown resulted in increased lipid accumulation compared with the empty vector control, highlighting the potential of NEDD4 as a therapeutic target (Fig. 6B). Further molecular experiments were conducted to elucidate the relationship between NEDD4 activity and the expression of adipogenic genes. RT-qPCR revealed that the model group exhibited elevated levels of adipogenic markers *C/ebpa*, *PPAR γ* and *Fabp4*, with corresponding reductions in *PI3k*, *Akt* and *mTOR*, suggesting impaired fat metabolism pathways (Fig. 6C). Conversely, NEDD4 overexpression normalized their expression levels, reducing *C/ebpa*, *PPAR γ* and *Fabp4* expression levels and enhancing *PI3k*, *Akt* and *mTOR* expression levels. These findings demonstrated the regulatory effects of NEDD4 on these pathways. The knockdown group mirrored the model group's trends, further validating the regulatory role of NEDD4 in fat metabolism (Fig. 6C). Western blotting confirmed the transcriptional trends observed in RT-qPCR, with protein expression patterns

aligning closely across groups. The model group showed increased levels of adipogenic proteins, which were moderated in the NEDD4 overexpression group, illustrating the function of these genes in controlling adipocyte differentiation and fat deposition (Fig. 6D and E). These results collectively suggested that NEDD4 not only affects adipocyte differentiation but also plays a crucial role in regulating fat metabolism, potentially offering a novel intervention for managing rotator cuff injuries by targeting fat infiltration.

NEDD4 inhibits FAP differentiation into adipocytes. *In vitro* experiments were performed to investigate the effect of NEDD4 on the differentiation of FAPs. FAP cells underwent NEDD4 knockdown and overexpression, and lentiviral infection status was monitored by fluorescence at 0, 4 and 8 days after infection (Fig. 7A). These results suggested that NEDD4 may regulate the differentiation of FAP cells, with a greater effect after prolonged lentiviral infection. Fluorescence intensity increased over time in both groups, indicating that

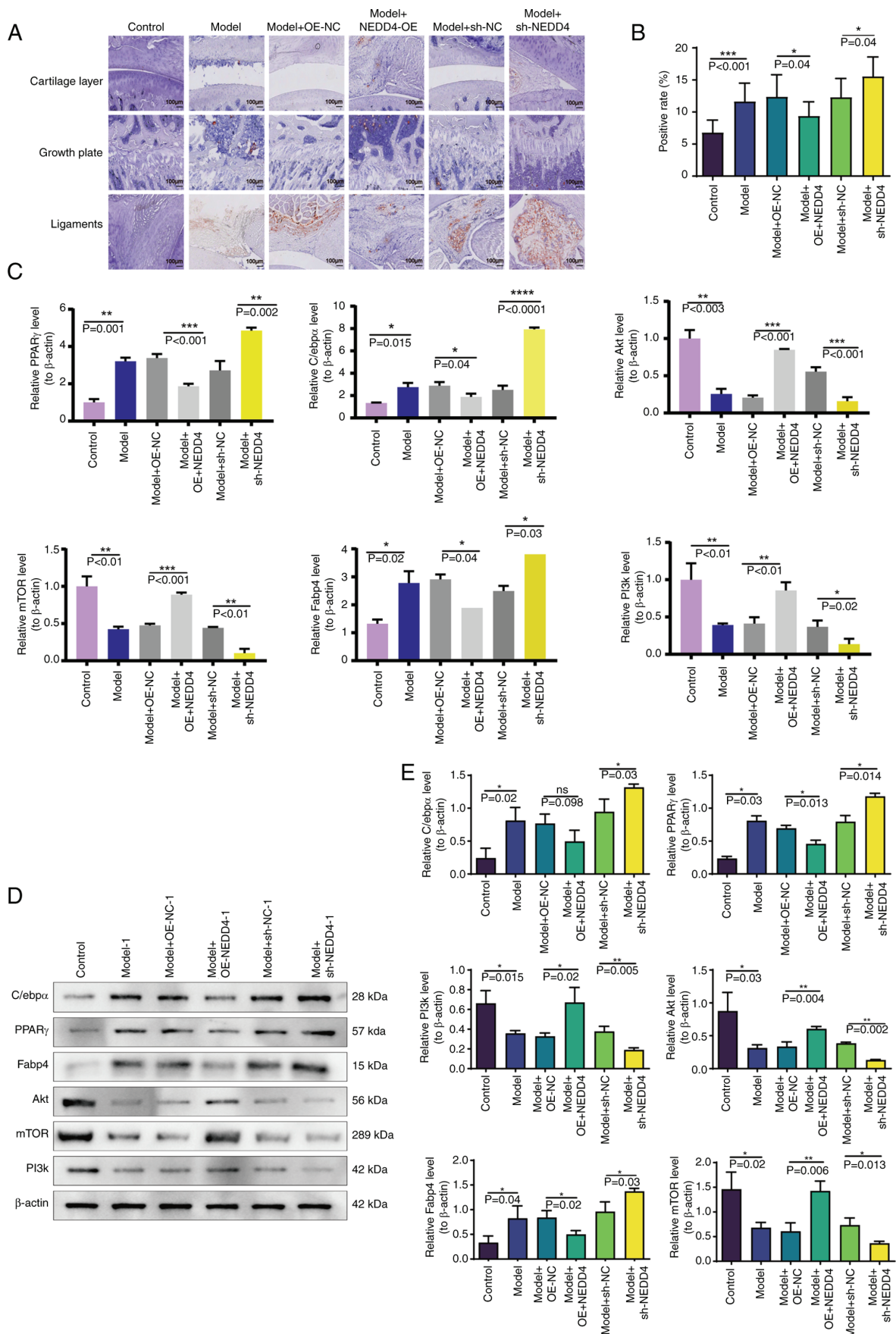


Figure 6. Research results on NEDD4 and fat formation and depletion in rotator cuff injuries. (A) Oil Red O staining results of rotator cuff tissue showing lipid droplets. (B) Histogram demonstrating lipid droplet positivity in rotator cuff tissue across different treatment groups. (C) Reverse transcription-quantitative PCR results for *C/ebpα*, *PPARγ*, *Fapb4*, *PI3k*, *Akt* and *mTOR* expression levels. (D) Western blotting results displaying protein levels of *C/ebpα*, *PPARγ*, *Fapb4*, *PI3k*, *Akt* and *mTOR*. (E) Histogram showing protein levels of *C/ebpα*, *PPARγ*, *Fapb4*, *PI3k*, *Akt* and *mTOR* across different treatment groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$. *PPARγ*, peroxisome proliferator-activated receptor γ ; OE, overexpression; sh- short hairpin; NC, negative control.

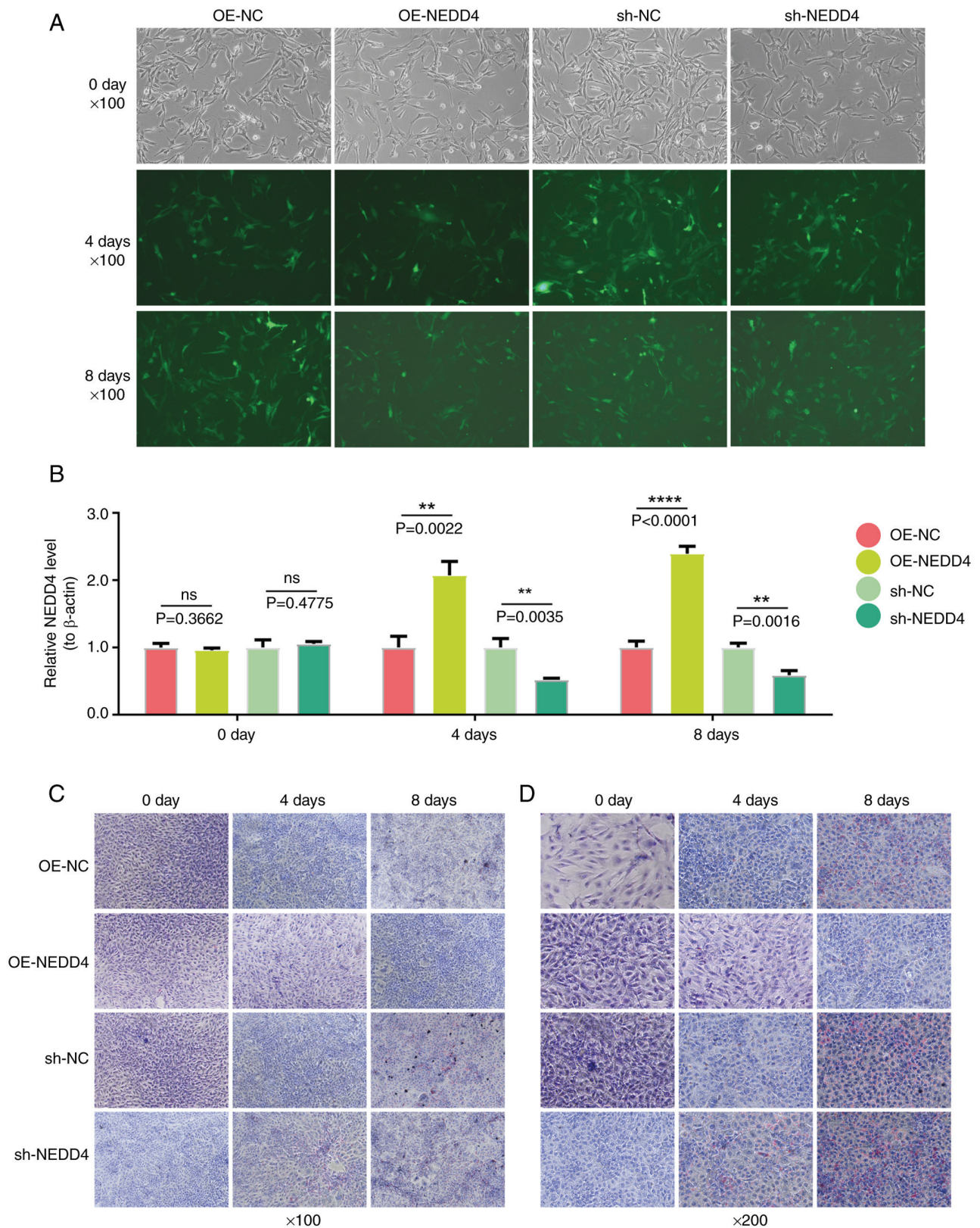


Figure 7. Analysis of NEDD4's regulation of FAPs' cell differentiation. (A) Fluorescence results showing lentiviral transfection of FAP cells at 0, 4- and 8-days post-infection for OE-NC, OE-NEDD4, sh-NC and sh-NEDD4. (B) qPCR detection results of NEDD4 levels in FAP cells at 0, 4 and 8 days post-infection for 4group. (C) Oil Red O staining results of FAP cells at 100x magnification showing lipid droplets at 0, 4- and 8-days post-infection for 4group. (D) Oil Red O staining results of FAP cells at x200 magnification showing lipid droplets at 0, 4- and 8-days post-infection for 4 group. ** $P<0.01$ and **** $P<0.0001$. FAP, fibro-adipogenic progenitor; OE, overexpression; sh- short hairpin; NC, negative control; ns, no significance.

the role of NEDD4 in regulating FAP differentiation may be greater after longer lentiviral infection. After knockdown

and overexpression interventions, RT-qPCR was conducted to measure NEDD4 levels in FAP cells to verify vector

effectiveness. NEDD4 knockdown in FAP cells decreased NEDD4 levels on days 4 and 8, while NEDD4 overexpression increased NEDD4 levels on those days. Both treatments showed significant differences compared with the control group. This suggests that the lentiviral vector affected gene expression, modulated the transcription or translation of target genes, and altered NEDD4 expression. Over time, NEDD4 levels significantly increased in the NEDD4 overexpression group (Fig. 7B), suggesting a stronger regulatory effect on the differentiation of FAPs with time. Oil Red O staining was performed on FAP cells after NEDD4 overexpression and knockdown to confirm the role of NEDD4 in regulating differentiation into adipocytes. Over time, the lipid droplet rate increased in both control groups and the NEDD4 knockdown group, while decreasing in the NEDD4 overexpression group (Fig. 7C and D). These findings suggested that NEDD4 overexpression can inhibit lipid droplet accumulation, indicating that NEDD4 suppresses FAP cell differentiation into adipocytes. Therefore, in rotator cuff injuries with fat infiltration, NEDD4 may be a key factor inhibiting the differentiation of FAP cells into adipocytes.

Discussion

The present study explored the therapeutic mechanisms of rotator cuff injury using a rat model and *in vitro* experiments with FAP cells. The present findings revealed that NEDD4 protects against fat infiltration and promotes the recovery of rotator cuff injury by modulating the mTOR signaling pathway.

Consistent with previous findings, H&E staining showed that NEDD4 overexpression positively affects tissue recovery (25). Furthermore, TUNEL staining indicated that NEDD4 negatively regulates apoptosis, thereby decreasing the severity of rotator cuff injuries, promoting cell proliferation, and facilitating tissue repair (26). As a member of the HECT E3 ubiquitin ligase family, NEDD4 plays a key role in the ubiquitination process. It tags specific substrate proteins for proteasomal degradation, thus regulating their abundance and activity (27). In the context of rotator cuff injuries, NEDD4 regulates fat metabolism and cell signaling by ubiquitinating key regulators of adipogenesis and inflammation. This process affects the cellular response to harmful stimuli, including the differentiation of FAP cells into adipocytes and the activation of the mTOR pathway, both of which are critical for tissue repair (28,29).

Rotator cuff injuries involve complex molecular signaling, in which protein ubiquitination is essential for regulating protein function, stability and cellular localization (30,31). This process can affect muscle inflammation, apoptosis and autophagy, thereby controlling the viability and function of myocytes (32,33). Therefore, NEDD4 may play a pivotal role in modulating fat metabolism and tissue repair by stabilizing key proteins in critical signaling pathways (34). By ubiquitinating specific proteins, NEDD4 ensures their degradation in the proteasome, thereby precisely controlling their abundance and activity (35). In the context of rotator cuff injuries, this mechanism is crucial for regulating fat infiltration and promoting tissue repair (36,37).

The present study underscored the importance of NEDD4 in regulating fat deposition after rotator cuff injury. Specifically,

a significant and positive correlation was found between NEDD4 and several lipid metabolism-related genes, such as ATP6V1G2, SLC7A5 and FZD3 (38). IGF1R and SLC7A5 are pivotal for adipocyte growth and nutrient uptake (39), while FGF21, ACOX1 and ACSL3/4 play central roles in fatty acid oxidation and lipid catabolism (40-43). NEDD4 may exert its effects by ubiquitinating these targets, thus regulating lipid synthesis, fatty acid oxidation and energy homeostasis (44). This correlation highlights the potential role of NEDD4 in metabolic disorders, such as obesity and metabolic syndrome whose hallmark is dysregulated lipid metabolism (45).

Moreover, the present study demonstrated that NEDD4 overexpression significantly reduced fat deposition in rotator cuff tissues, evidenced by decreased expression of adipogenic markers, such as C/EBP α , PPAR γ and Fabp4. This suggests that NEDD4 inhibits adipocyte differentiation and fat accumulation, providing a more favorable environment for tissue repair. Conversely, NEDD4 knockdown leads to increased fat deposition, which is associated with impaired tissue repair. These findings indicate that NEDD4 may mitigate fat infiltration by regulating the expression of genes involved in adipogenesis and lipid metabolism, thereby promoting tissue repair.

Consistent with previous studies, Oil Red O staining also confirmed the pathological role of fat deposition in rotator cuff injury (2). Fat cell infiltration may hinder skeletal muscle function and contribute to the development of injuries (46,47). The current experimental results revealed that NEDD4 overexpression reduces fat deposition in rotator cuff injuries, while its inhibition exacerbates this condition. Thus, NEDD4 may promote tissue recovery by preventing fat deposition. *In vitro* experiments showed that NEDD4 significantly inhibited the differentiation of FAP cells into adipocytes. NEDD4 overexpression prevented lipid accumulation in FAPs, whereas NEDD4 knockdown exhibited the opposite effect. These results suggest that NEDD4 is a potential therapeutic target for preventing fat infiltration in rotator cuff injuries (48).

Mechanistically, the effects of NEDD4 may be mediated through the ubiquitination and degradation of key adipogenic regulators, such as PPAR γ and C/EBP α , which promote adipogenesis (49,50). By facilitating the ubiquitination of these factors, NEDD4 reduces their stability, thereby inhibiting adipogenesis. The direct regulatory effects of adipogenesis-related transcription factors further underscore the potential of NEDD4 in controlling fat infiltration in pathological conditions, where abnormal FAP differentiation leads to muscular dysfunction and impaired tendon healing (51,52).

Additionally, NEDD4 regulates the PI3K/Akt/mTOR axis, affecting lipid metabolism and cell viability (53). NEDD4 overexpression activates the mTOR pathway, which is associated with the inhibition of adipogenesis, enhanced myocyte regeneration and accelerated tissue repair (54). By contrast, NEDD4 knockdown suppresses the mTOR pathway, leading to the differentiation of FAP into adipocytes, thereby exacerbating fat deposition in injured rotator cuff tissues. This mechanism explains the correlation between fat infiltration and impaired healing in rotator cuff injuries.

Overall, the present study elucidated the critical role of NEDD4 in the recovery of rotator cuff injury, by regulating protein ubiquitination and fat metabolism. Through protein

ubiquitination, NEDD4 exerts a dual role in lipid metabolism. On one hand, it inhibits adipocyte differentiation and reduces fat deposition by regulating adipogenesis-related genes. On the other hand, it affects lipid metabolism and cell viability through key signaling pathways, such as the PI3K/Akt/mTOR axis, thereby promoting tissue repair. The mechanism of NEDD4 provides a deeper insight into the relationship between fat infiltration and impaired healing of rotator cuff injuries.

Although NEDD4 overexpression shows beneficial effects in rotator cuff injuries, further studies are needed to uncover additional mechanisms (35). Moreover, NEDD4 may interact with other proteins to form complexes that indirectly regulate lipid synthesis by affecting the function or stability of these proteins (55). NEDD4 may also regulate autophagy in adipocytes by promoting fatty acid degradation and enhancing energy consumption, thus reducing fat deposition (45,56). Additionally, NEDD4 may modulate the insulin signaling pathway, weakening the effect of insulin on lipid synthesis and deposition (56,57).

In conclusion, NEDD4 reduced fat infiltration in rotator cuff injury by targeting the mTOR signaling pathway and regulating protein ubiquitination, thereby enhancing protein stability in key signaling pathways involved in fat metabolism and tissue repair. The present study highlighted the significance of NEDD4 in modulating the mTOR pathway, reducing fat infiltration, and promoting tissue repair in rotator cuff injuries, suggesting its potential as a therapeutic target for improving the outcomes of rotator cuff injury.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JL conceptualized the study, developed methodology, validated and curated data, wrote the original draft, and wrote, reviewed and edited the manuscript. YP performed validation and formal analysis. DZ conducted software analysis. CG conceptualized the study, performed formal analysis, data curation and project administration and acquired funding. WP conceptualized the study, developed methodology curated data, and performed project administration. JL and CG

confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. KY20230718-16) by the Experimental Animal Welfare and Ethics Committee of Beijing Jishuitan Hospital Guizhou Hospital (Guiyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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