# Supporting materials

## AMPKα1 Deficiency in Macrophages Impairs Tendon Regeneration and Tendon Stem Cell Function via a TNF-α – FBP2 Signaling

## Methods

*In vivo subcutaneously transplantation*

To evaluate the effect of *Fbp2* knockdown on the tendon regeneration, cells were first treated with siRNA against *Fbp2* or negative control siRNA for 24 hours. Then, TSPCs ($1 \times 10^6$ cells) with treatment were seeded on sterilized scaffolds, and implanted subcutaneously in the dorsum of athymic mice (6 – 8 weeks, BLAB/c nude mice) under anesthesia. Implants from 8 weeks were retrieved and fixed with 4% paraformaldehyde. Paraffin sections at a thickness of 4 μm were harvested, followed by HE, Masson's trichrome, and immunofluorescence staining.

*Construction of a biomimetic parallel scaffold*

Building upon our previously established protocol for collagen self-assembly, the biomimetic parallel scaffold was achieved through the following adapted procedure: A solution of Type I tropocollagen (Corning, 3,88 mg/mL) was injected into a dialysis bag (3500 Da), submerged in a phosphate buffer solution. Simultaneously, axial tensile force was applied to both ends of the dialysis bag using mechanical apparatus, and assembly was conducted at 37°C for 24 hours under constant temperature conditions. The scaffolds underwent overnight freeze-drying after freezing and were subsequently cross-linked for 4 hours under light-avoiding conditions using a cross-linking agent containing 1 wt% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 80% ethanol. Finally, they underwent three alternating washes with distilled water and a 1% glycine solution, followed by freeze-drying and lyophilization for further use.

*Flow cytometry*

The mice were euthanized, and the harvested tendon was minced and digested completely with 3 mg/mL collagenase type I (Thermo Fisher Scientific) and 4 mg/mL dispase (Roche) at 37 ℃ for 1 h. After passing through a 70 μm strainer, the single-cell suspensions were incubated with the appropriate cocktail of antibodies (CD45, CD11b, F4/80, CD206, 1 μL/test) for 30 min at 4℃. Then, cells were washed two times with PBS and resuspended in 500 μL of flow buffer for analysis.

*Macrophage phagocytosis*

Efferocytosis was assessed in macrophages with autologous apoptotic TSPCs and evaluated through immunofluorescence staining. For the in vitro macrophage phagocytosis assays using fluorescence microscopy, apoptotic TSPCs were pre-labeled with PKH26. Subsequently, BMDMs were co-cultured with apoptotic TSPCs at the ratio of 1:10 of BMDMs to apoptotic TSPCs for 1 hour. Macrophages were then collected and washed three times with PBS, and fixed with 4% paraformaldehyde for 15 min to evaluate efferocytosis by microscopy.

*Enzyme-linked immunosorbent assay (ELISA)*

To measure IL-6, TNF-α, IL-4 and IL-10 levels in culture supernatants, BMDM supernatants were collected, centrifuged at 500 × g for 5 min to remove cell debris and then assayed using the IL-6, TNF-α, IL-4 and IL-10 Mouse commercial ELISA Kit (ExCell Bio, Inc., Jiangsu, China) according to the manufacturer's instructions.

*CFU-F assays*

For CFU-F assay, single-cell suspensions of TSPCs (1000 cells/well) for 12 – 14 days in growth medium and fixed with 4% paraformaldehyde. Then, 0.1% crystal violet solution (Solarbio, Beijing, China) was used to stain the cells. Colonies of >50 cells were defined as single colony unit, and the number of clusters was counted using Image J 1.52a software.

*Scratch assay*

A wound healing assay was also employed to assess the migration of TSPCs. TSPCs were seeded in 6-well plates and cultured until confluent. A straight line was scratched using a pipettor spear head in each well. The 6-well plate was washed twice with PBS buffer to remove floating cells. At 0, 24 and 36 hours, scratch wound healing was recorded using an inverted microscope, and the scratch width was measured by Image J software for statistical analysis.

*Macroscopic evaluation of regenerated tendons.*

At 1- and 4-week postoperatively, mice were euthanized and the Achilles tendon was fully exposed. Then the full-length tendon complexes including partial gastrocnemius and calcaneus were harvested, and photographed using a camera (Nikon, Japan).

*ROS assay*

Mitochondrial ROS were measured using Mito SOX following the manufacturer's instructions. First, the cells were washed with PBS, then cells were incubated in 5 μM Mito SOX Red solution (Yeasen, China) at 37 ℃ for 10 min. Finally, the red fluorescence of cells was calculated by a flow cytometer (BD Accuri C6).

Cellular reactive oxygen species were tested by using DCFH-DA. A final working concentration of 10 μM DCFH-DA was added and incubated at 37 ℃ for 20 min. Then the cells were washed by prewarmed PBS. Finally, the cells were checked with a laser-scanning microscope (LSM 510, Zeiss, Germany).

*Assessment of F6P*

The content of F6P was measured by using the fructose-6-phosphate assay kit (Beyotime, China) by examining the fluorescence intensity of Ex/Em (535/587 nm).

## Supplementary Figures

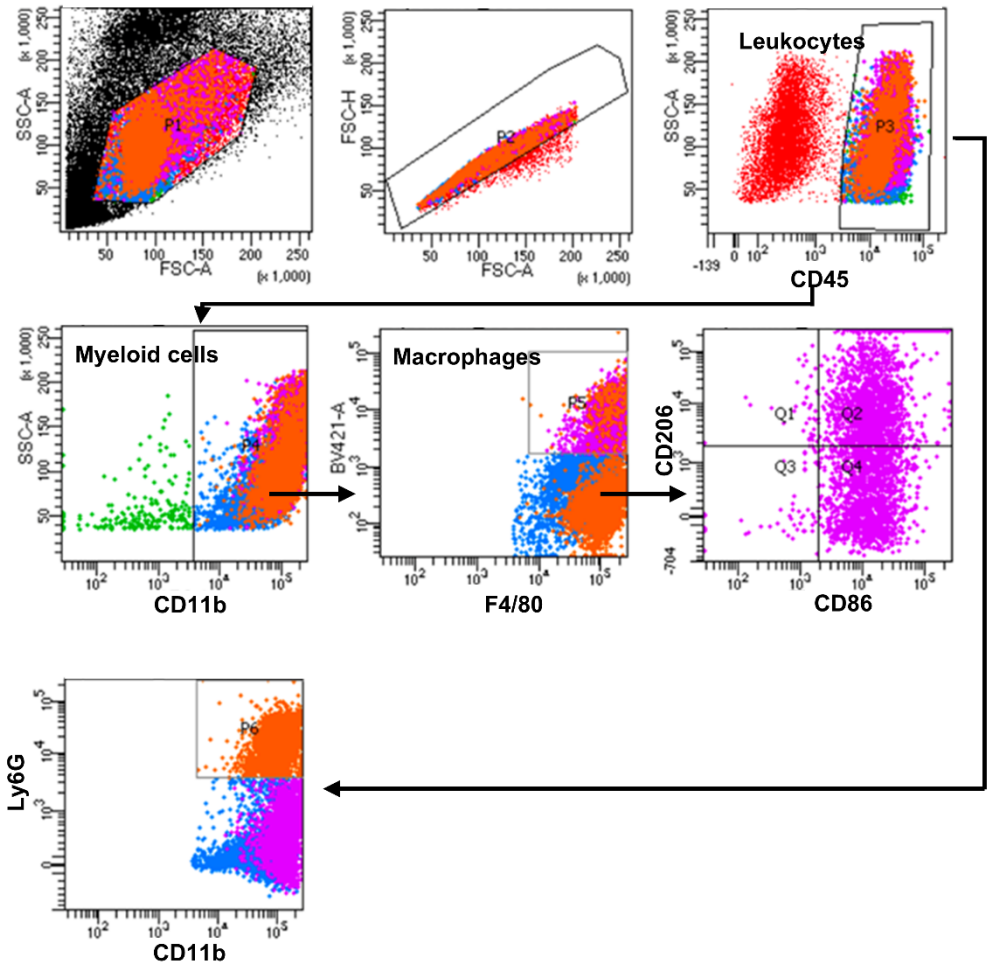



**Fig. S1** Gating strategy for flow cytometric analysis.

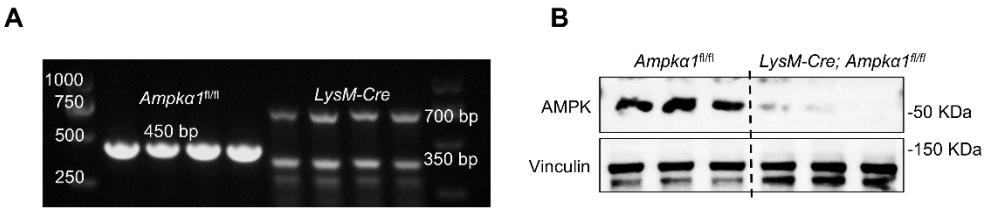



**Fig. S2** Validation and characterization of *LysM-Cre; Ampkα1*$^{fl/fl}$ mice. **(A)** Agarose gel electrophoresis qRT-PCR products of *LysM-Cre; Ampkα1*$^{fl/fl}$ mice. **(B)** Western blotting analysis of AMPKα1 expression in bone marrow-derived macrophages (BMDMs) of control and *LysM-Cre; Ampkα1*$^{fl/fl}$ mice.

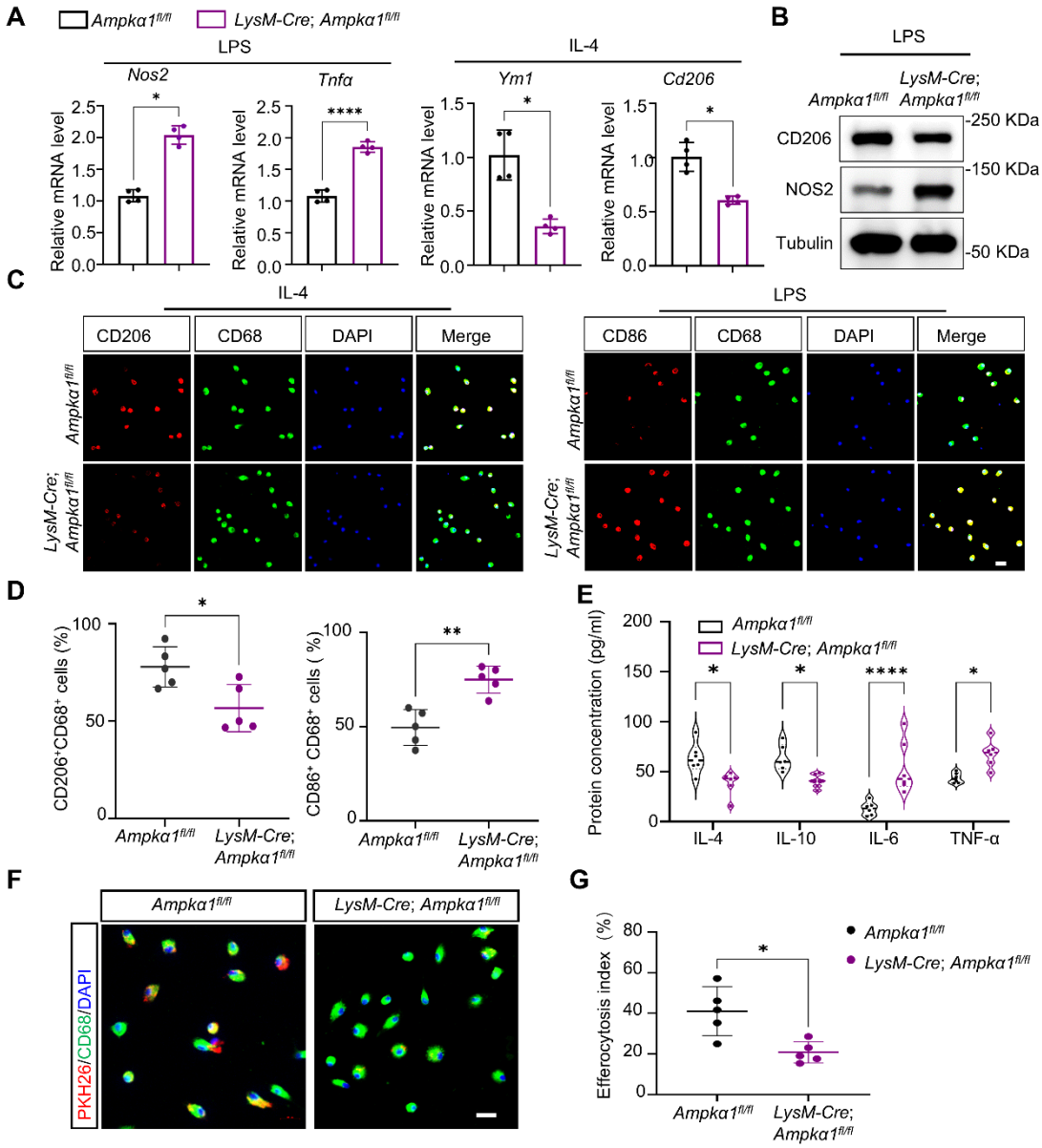



**Fig. S3** Knocking out *Ampkα1* hinders macrophage transition to an anti-inflammatory phenotype and its phagocytic function. **(A)** The expression of *Nos2*, *Tnf-α*, *Ym1* and *Cd206* in the polarization of macrophages under different treatments by qRT-PCR analysis ($n$ = 4). **(B)** Western blotting analysis of CD206 and NOS expression in the macrophages under lipopolysaccharide (LPS) treatments. **(C)** Immunofluorescent co-staining of CD206 or CD86 (red) with CD68 (green) in the polarization of macrophages under different treatments. Scale bars: 20 μm. **(D)** Left: proportion of CD206 and CD68 double-positive cells among CD68-positive cells ($n$ = 5). Right: proportion of NOS2

and CD68 double-positive cells among CD68-positive cells ($n$ = 5). **(E)** Expression levels of the cytokines IL-4, IL-10, IL-6, and TNF-α in macrophage supernatants of control and *LysM-Cre; Ampkα1*$^{fl/fl}$ group were detected by ELISA. **(F)** Immunofluorescent co-staining of PKH26 with CD68 in the macrophages ($n$ = 6). Red: PKH26 labeled apoptotic TSPCs; Green: CD68 labeled BMDMs. Scale bars: 20 μm. **(G)** Percent efferocytosis was quantified as the number of macrophages with engulfed apoptotic cells as a percentage of total macrophages (n = 5). * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

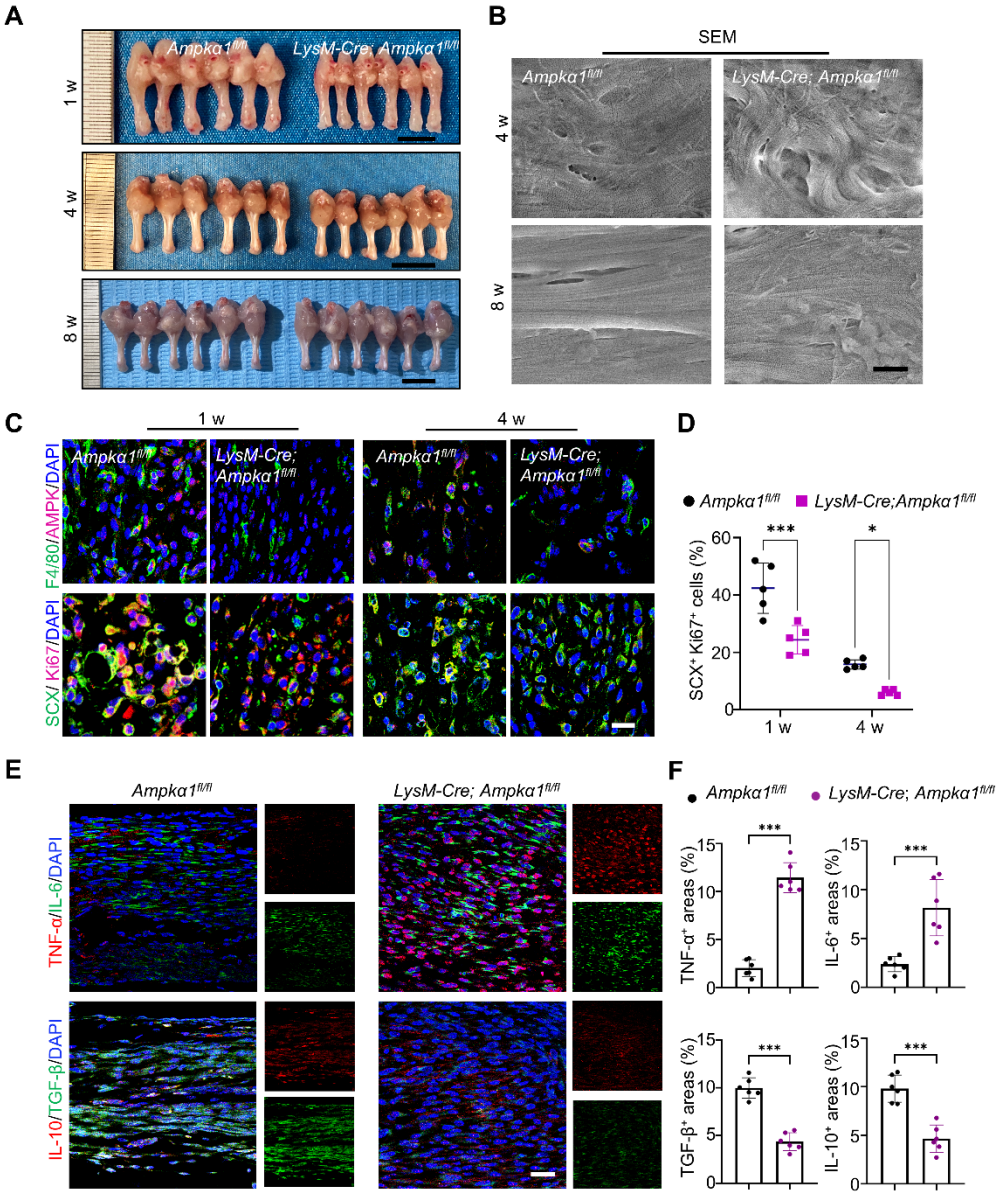



**Fig. S4** Macrophage AMPKα1 deficiency impairs tendon regeneration and repair capacity. **(A)** Gross view of tendons in newborn and adult mice at 1-, 4- and 8-week after injury. Scale bars: 1 cm. **(B)** Representative scanning Electron Microscope (SEM) images illustrating the microstructural characteristic of regenerated collagen fibers in the control and *LysM-Cre; Ampkα1*$^{fl/fl}$ group at 4- and 8-week after injury. Scale bars: 1 μm. **(C)** Immunofluorescent co-staining of AMPK (red) with F4/80 (green), Ki67 (red) with SCX (green) in the control and *LysM-Cre; Ampkα1*$^{fl/fl}$ group at 1- and 4-week after injury. Scale bars: 20 μm. **(D)** Proportion of SCX and Ki67 double-positive cells

among SCX-positive cells ($n = 5$). **(E)** Immunofluorescence staining results showed the protein expression levels of TGF-β, IL-10, IL-6, and TNF-α of *LysM-Cre; Ampkα1*$^{fl/fl}$ group and control group. Scale bars: 50 μm. **(F)** Quantification analysis of p-AKT$^{+}$SCX$^{+}$-positive cells and p-AKT$^{+}$CD146$^{+}$-positive cells ($n = 6$). * $p < 0.05$, and ***$p < 0.001$.

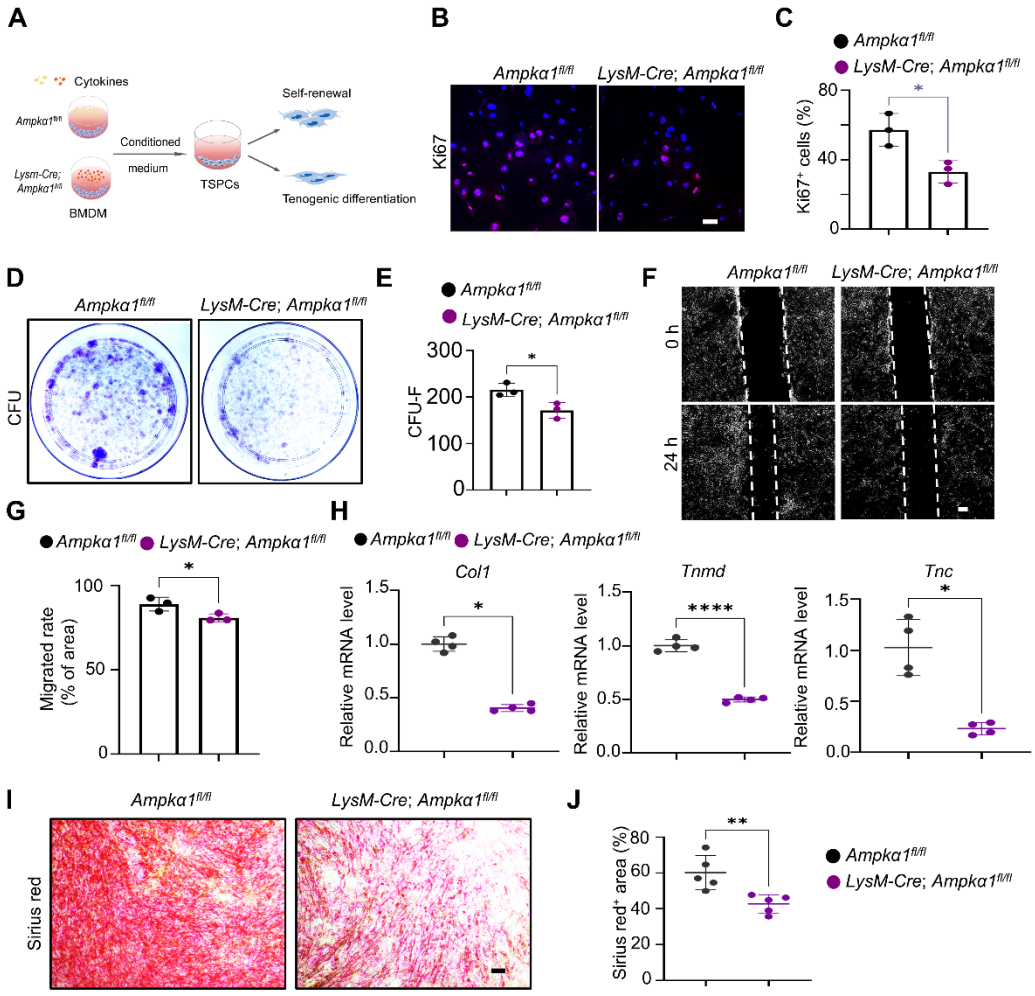
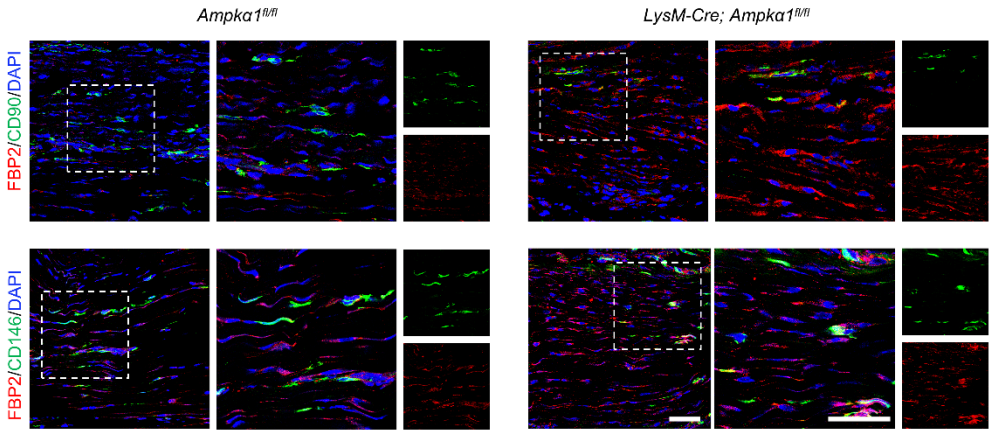



**Fig. S5** Macrophage knockout of *Ampkα1* impairs the proliferation, migration, and differentiation of TSPCs. **(A)** Schematic representation of TSPCs stimulated by supernatant of *LysM-Cre; Ampkα1*$^{fl/fl}$ and control mouse-derived macrophages. **(B)** The protein level of Ki67 in TSPCs stimulated with different culture supernatants was analyzed by immunofluorescent staining. Scale bars: 20 μm. **(C)** Quantitative analysis of Ki67-positive cells ($n$ = 3). **(D)** Photograph of crystal violet staining in both TSPCs groups after 14 days of culture. **(E)** Quantification of crystal violet staining ($n$ = 3). **(F)** Images of cell scratch assay for migration of TSPCs cultured for 0 and 24 h in both supernatant groups. Scale bars: 200 μm. **(G)** The percentage of recovered area in scratch assay ($n$ = 3). **(H)** The expression of *Col1*, *Tnmd*, and *Tnc* in both TSPCs groups was evaluated by qRT-PCR analysis ($n$ = 4). **(I)** Photomicrographs of Sirius red staining of

both TSPCs groups were cultured for two weeks after tenogenic differentiation induction. Scale bars: 100 μm. **(J)** Quantification of Sirius red-positive areas ($n = 5$). *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

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127 **Fig. S6** The colocalization of FBP2 and tendon-specific markers (CD146 and CD90) in

128 tendon tissue. Scale bars: 50 μm.

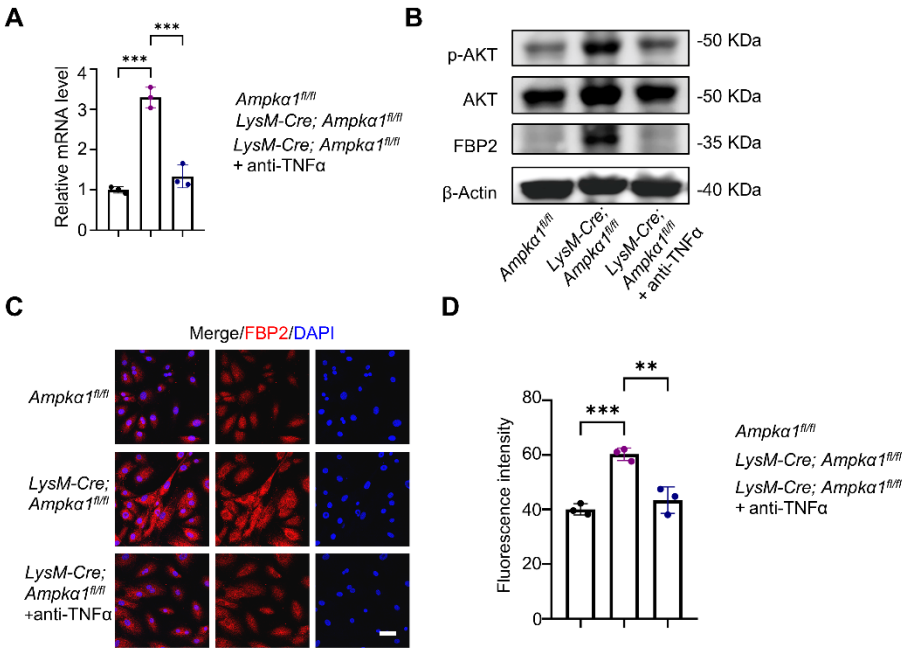
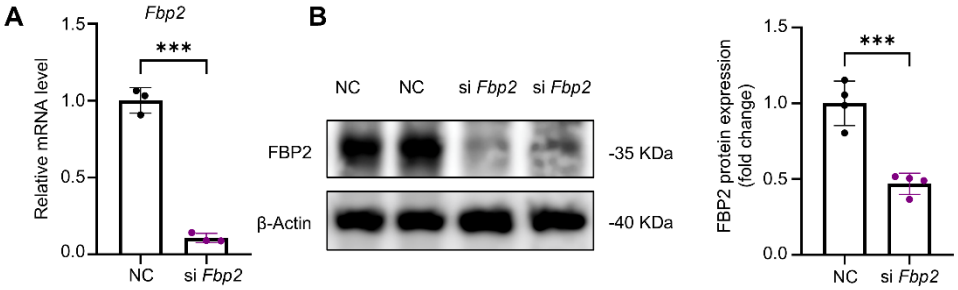



**Fig. S7** TNF-α increased FBP2 levels through PI3K /AKT signaling. **(A)** The expression of *Fbp2* in TSPCs treated with a TNF-α neutralizing antibody was evaluated by RT-PCR analysis ($n$ = 3). **(B)** Western blotting analysis of p-AKT, AKT and FBP2 expression in TSPCs treated with a TNF-α neutralizing antibody. **(C)** Immunofluorescence staining results showed the protein expression levels of FBP2 (red) in TSPCs treated with a TNF-α neutralizing antibody. **(D)** Quantification of FBP2 fluorescent intensity ($n$ = 3). Scale bars:50 μm. $^{**}p < 0.01$, and $^{****}p < 0.0001$.




137

138 **Fig. S8** Verification of the FBP2 knockdown efficiency. (**A)** The expression of *Fbp2*
139 after siRNA treatment was evaluated by qRT-PCR analysis ($n$ = 3). (**B)** Western
140 blotting analysis of FBP2 expression after siRNA treatment ($n$ = 4). ***$p$ < 0.001.

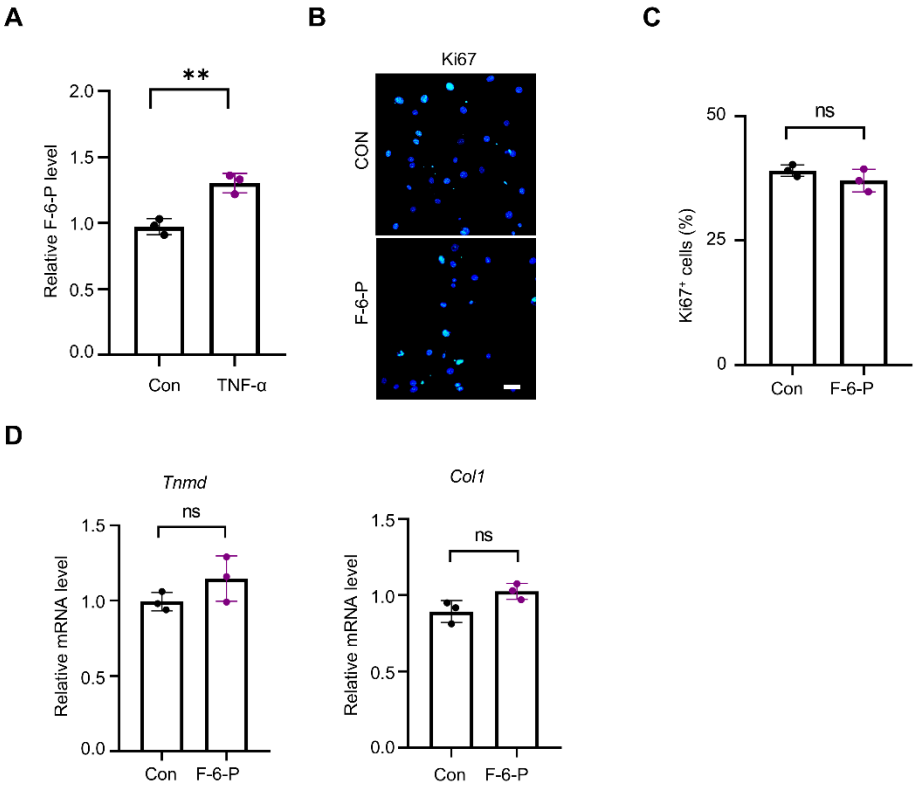



**Fig. S9** Assessment of F6P content and its impact on TSPCs. **(A)** The levels of F6P of TSPCs stimulated by TNF-α was measured by using the fructose-6-phosphate assay kit. **(B)** Immunofluorescence staining results showed the protein expression levels of Ki67 in TSPCs were treated with F-6-P (10mM) for 24 h, compared to control group. Scale bars: 20 μm. **(C)** Quantification of ratio of Ki67 positive cells ($n$ = 3). **(D)** The expression of *Tnmd*, and *Col1* in TSPCs treated with F-6-P for 24 h was evaluated by RT-PCR analysis ($n$ = 3). ns: no significance; **$p < 0.01$.

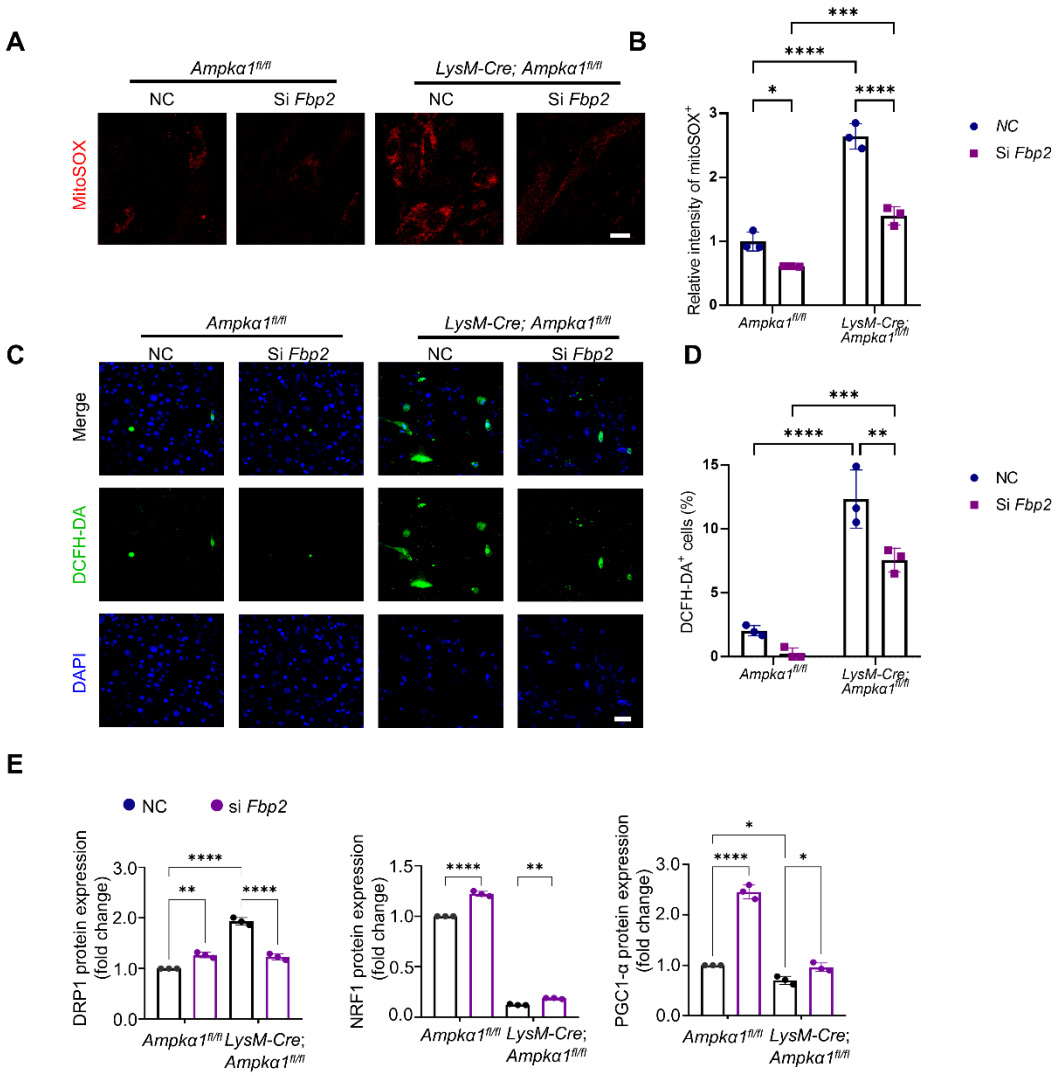
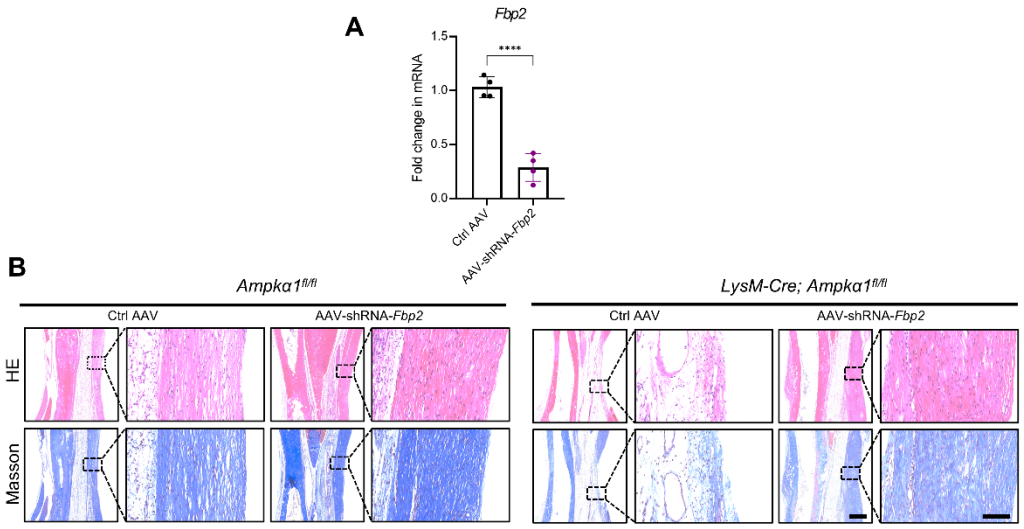



**Fig. S10** The impact of *Fbp2* knockdown on TSPCs mitochondria. (A) Immunofluorescence staining results showed the fluorescence intensity of mitoSOX in *Fbp2*-knockdown TSPCs, compared to NC group. Scale bars: 20 μm. (B) Quantification of mitoSOX fluorescent intensity ($n$ = 3). (C) Immunofluorescence staining results showed the fluorescence intensity of DCFDA in *Fbp2*-knockdown TSPCs, compared to NC group. Scale bars: 50 μm. (D) Quantification of DCFDA fluorescent intensity ($n$ = 3). (E) Quantification of western blotting results in Figure 6J ($n$ = 3). *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001 and ****$p$ < 0.0001.




158

159 **Fig. S11** *Fbp2* knockdown in TSPCs notably enhances tendon regeneration. **(A)** The
160 knockdown efficiency of shRNA targeting Fbp2 in TSPCs were examined with qRT-
161 PCR ($n$ = 3). **(B)** Representative HE staining and Masson trichrome staining of the
162 tendon from *LysM-Cre; Ampkα1*$^{fl/fl}$ mice and *Ampkα1*$^{fl/fl}$ mice injected with *AAV-Fbp2-*
163 *shRNA* and control *AAV-shRNA* at 4 weeks after injury. Low magnification image scale
164 bars: 500 μm; high magnification image scale bars: 100 μm.**** $p < 0.0001$.

## Supplementary Tables

**Table S1. The primers used for qRT-PCR identification.**

| Gene | Primer sequences（5’-3’） |
|---|---|
| *Ampkα1*$^{flox/flox}$ Forward | CCCACCATCACTCCATCTCT |
| *Ampkα1*$^{flox/flox}$ Reverse | AGCCTGCTTGGCACACTTAT |
| *LysM*-Cre Mutant | CCCAGAAATGCCAGATTACG |
| *LysM*-Cre Common | CTTGGGCTGCCAGAATTTCTC |
| *LysM*-Cre Wild type | TTACAGTCGGCCAGGCTGAC |

**Table S2. The commercial reagents.**

| Reagents | Supplier |
|---|---|
| Formaldehyde Fixative Solution | Servicebio, China |
| Glutaraldehyde | Sigma, USA |
| Decalcification Solution | Servicebio, China |
| 0.1% Triton Solution | Solarbio, China |
| 5% Goat Blocking Serum | Zsbio, China |
| Bovine Serum Albumin | Sigma, USA |
| Hematoxylin-Eosin Staining Kit | Solarbio, China |
| MASSON Staining Kit | Beijing Solarbio |
| Picrosirius Red Staining Kit | Solarbio, China |
| Low-glucose DMEM | Hyclone, USA |
| Fetal Bovine Serum | Gibco, USA |
| Penicillin/streptomycin | Hyclone, USA |
| L-glutamine | Hyclone, USA |
| Trypsin | Hyclone, USA |
| Dimethyl Sulfoxide (DMSO) | Solarbio, China |
| Recombinant TGF-β Factor | Peprotech, USA |
| GDF-5 Factor | R&D Systems, USA |
| Recombinant Mouse M-CSF Protein | Novoprotein, China |
| Recombinant Mouse TNF-α Protein | Novoprotein, China |
| lipopolysaccharide | Sigma, USA |
| Recombinant Mouse IL-4 Protein | Peprotech, USA |
| Collagenase I | Solarbio, China |
| Dispase | Roche, Switzerland |
| Lymphoprep™ | Stemcell Technologies, Canada |
| 0.1% Crystal Violet Staining Kit | Solarbio, China |
| Cell Transfection Kit | Polyplus, France |

| Reagents | Supplier |
|---|---|
| PKH26 Red Staining Kit | Solarbio, China |
| Staurosporine | MedChemExpress, USA |
| Mouse ELISA Kit | MeiMian, China |
| Trizol | Thermo Fisher Scientific, USA |
| DEPC Water | Beyotime, China |
| Tris-HCl | Beyotime, China |
| Reverse Transcription Kit | Takara, Japan |
| SYBR Green Real-time Quantitative PCR Kit | Roche, Switzerland |
| RIPA Lysis Buffer | Thermo Fisher Scientific, USA |
| Protease/Phosphatase Inhibitor Cocktail | Thermo Fisher Scientific, USA |
| BCA Protein Quantification Kit | Thermo Fisher Scientific, USA |
| High-purity Heat-resistant DNA Polymerase | Solarbio, China |
| Tricolor Pre-stained Protein Standard | Biotides, China |
| Mouse Tail Collagen Solution | Corning, USA |
| 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide | Tcichemicals, China |
| N-Hydroxysuccinimide | Sigma, USA |
| JC1 staining | Solarbio, China |
| Mito SOX Red | Yeasen, China |
| MitoTracker Red | Beyotime, China |
| HE staning | Solarbio, China |
| Sirius Red staining | Solarbio, China |
| Masson trichrome staining | Solarbio, China |

169

**Table S3. The target sequences for *Fbp2* knockdown and negative control.**

| Gene | Primer sequences（5’-3’） |
|---|---|
| si *Fbp2* Forward | GCUAUUUACAGAAAGACCATT |
| si *Fbp2* Reverse | UGGUCUUUCUGUAAAUAGCTT |
| si NC Forward | UUCUCCGAACGUGUCACGUTT |
| si NC Reverse | ACGUGACACGUUCGGAGAATT |

172 **Table S4. List of RT-qPCR primers.**

| Gene | Primer sequences（5’-3’） |
|---|---|
| *Actin*-F | CATGTTTGAGACCTTCAACACCC |
| *Actin*-R | TGGCATAGAGGTCTTTACGGATG |
| *Gapdh*-F | CATGGCCTTCCGTGTTCCTA |
| *Gapdh*-R | CCTGCTTCACCACCTTCTTGA |
| *Ym1*-F | CCAGTTGGGCTAAGGACAGG |
| *Ym1*-R | CAGGTGAGTACACAGGCAGG |
| *Cd206*-F | GTGGAGTGATGGAACCCCAG |
| *Cd206*-R | CTGTCCGCCCAGTATCCATC |
| *Tnf*-F | CCTGTAGCCCACGTCGTAGC |
| *Tnf*-R | AGCAATGACTCCAAAGTAGACC |
| *Nos2*-F | GTTCTCAGCCCAACAATACAAGA |
| *Nos2*-R | GTGGACGGGTCGATGTCAC |
| *Col1*-F | CCACCCCAGCCGCAAAGAGTC |
| *Col1-R* | GTCATCGCACACAGCCGTGC |
| *Tnmd*-F | GGGCTGTCACATTCTAAATGCAG |
| *Tnmd*-R | TTCTTCTTCTCGCCGTTGCT |
| *Tnc*-F | TTCTTCTTCTCGCCGTTGCT |
| *Tnc*-R | AGAGGGTATGCTATAAGCCAGAA |
| *G6pc*-F | TCCGTGCCTATAATAAAGCAGT |
| *G6pc*-R | TGGCTTTTTCTTTCCTCGAAAG |
| *Pgam1*-F | CAGGTAAAGATCTGGAGACGAT |

| | |
|---|---|
| *Pgam1*-R | CTTGCTGATGTTGCTGTAGAAG |
| *Eno1*-F | TTCGCACCTAACATCCTGGAGAAC |
| *Eno1*-R | GAACTCGGAGGCAGCCACATC |
| *Pfk1*-F | ACGGTATACATCGTGCATGAT |
| *Pfk1*-R | GATGTTGTAGGTGCGGAGATTC |
| *Fbp2*-F | ACAGGACAAGGAGTAGATCTCT |
| *Fbp2*-R | TAGCAGCATCAAAATACTTGGC |
| *Gpi1*-F | ATTGCTCTGCATGTAGGTTTTG |
| *Gpi1*-R | CTTGGTGATGTACTTTCCGTTG |
| *Pgk1*-F | CAAATTCTGCTTGGACAATGGA |
| *Pgk1*-R | CCACACAATCCTTCAAGAACAG |
| *Pkm*-F | TATCATTGCCGTGACTCGAAAT |
| *Pkm-R* | AAGTTTACACGAAGGTCGACAT |
| *Aldoc*-F | ATGGCGCTGTGTACTAAAAATC |
| *Aldoc-R* | TGTAACATACTGGCAACGTTTG |
| *Hk1*-F | ATTAAGAAGCGAGGGGACTATG |
| *Hk1*-R | CTCCCCATTCCGTGTTAATACA |
| *Slc2a3*-F | TGGGATCAATGCTGTGTTCTAT |
| *Slc2a3-R* | CCAGGAACAGAGAAACTACAGT |
| *mt DNA* | GCCAGCCTGACCCATAGCCATAAT |
| *mt DNA* | GCCGGCTGCGTATTCTACGTTA |
| *nu-DNA* | TTGAGACTGTGATTGGCAATGCCT |
| *nu-DNA* | CCAGAAATGCTGGGCGTACT |

174 **Table S5. The list of antibodies.**

| Antibody | Catalog No. | Supplier |
|---|---|---|
| NOS2 | 18985-1-AP | Proteintech, USA |
| CD206 | 18704-1-AP | Proteintech, USA |
| F4/80 | ab90247 | Abcam, USA |
| CD86 | 13395-1-AP | Proteintech, USA |
| CD68 | ab303565 | Abcam, USA |
| p-AMPK | ab90247 | Thermo Fisher Scientific, USA |
| AMPK | #2532 | Cell Signaling Technology, USA |
| SCX | ab58655 | Abcam, USA |
| Ki67 | ab16667 | Abcam, USA |
| TNC | MA5-16086 | Thermo Fisher Scientific, USA |
| COL1 | 14695-1-AP | Proteintech, USA |
| FMOD | 60108-1-Ig | Proteintech, USA |
| FBP2 | PA5-97265 | Thermo Fisher Scientific, USA |
| FBP2 | sc-166097 | Santa Cruz，USA |
| GAPDH | TA-08 | Zsbio, China |
| Vinculin | 66305-1-lg | Proteintech, USA |
| β-Tubulin | 10068-1-AP | Proteintech, USA |
| ACTB | TA-09 | Zsbio, China |
| DRP1 | sc-101270 | Santa Cruz，USA |
| PGC-1α | sc-518025 | Santa Cruz，USA |
| NRF-1 | sc-28379 | Santa Cruz，USA |
| HRP-linked anti-mouse IgG | ZB-2305 | Zsbio, China |
| HRP-linked anti-rabbit IgG | ZB-2301 | Zsbio, China |

| Antibody | Catalog No. | Supplier |
|---|---|---|
| FITC-labeled goat anti-mouse IgG (H + L) | ZF-0312 | Zsbio, China |
| FITC-labeled goat anti-rabbit IgG (H + L) | ZF-0311 | Zsbio, China |
| FITC-labeled goat anti-rat IgG (H + L) | ZF-0315 | Zsbio, China |
| Rhodamine labeled goat anti-mouse IgG (H + L) | ZF-0313 | Zsbio, China |
| Rhodamine labeled goat anti-rabbit IgG (H + L) | ZF-0316 | Zsbio, China |
| Rhodamine labeled goat anti-rat IgG (H + L) | ZF-0318 | Zsbio, China |
| Mounting Medium with DAPI | ZLI-9557 | Zsbio, China |