Supplemental Methods

Animals

All animal experiments were conducted under strict governmental and European guidelines and were approved by the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences, under license number AVD80100 2018 7144. Adult pathogen-free male C57BL/6N mice (7–9 weeks old) were obtained from Charles River Laboratories and group-housed at a density of 2–4 individuals in static individually ventilated cages (IVC) (Tecniplast, #GM500) with corncob granules (Rehofix, #MK1500). They were given autoclaved water and high nutrient food chow (RM3 pellets, Special diet) ad libitum. Adult pathogen-free male spiny mice (Acomys; 7–9 weeks old) were bred and maintained in-house. They were group-housed at a density of 2–8 individuals in static IVCs (Tecniplast, #GR1800DD) with powdered cellulose pellets (ARBOCEL) and given autoclaved water and low nutrient and low protein food chow (RM1 pellets, Special diet) ad libitum. Adult pathogen-free male Crl:MON(Tum) Mongolian Gerbil (7–9 weeks old) were obtained from Charles River Laboratories and maintained in-house. Only males were used for all experiments, except for the incision experiment. For the incision experiments only females were used. They were group-housed at a density of 2–8 individuals in static IVCs (Tecniplast, #GR1800DD) with powdered cellulose pellets (ARBOCEL) and given autoclaved water and high nutrient food chow (RM3 pellets, Special diet) ad libitum. On occasion, and always after injury, they additionally received black-oil sunflower seeds and a combination of dried vegetables. All cages were kept in climate-controlled quarters with a 12 h/12 h light/dark cycle.

Tomo-sequencing

After RNA extraction, pellets were resuspended with barcoded primers (containing an anchored poly(T), 6 bp unique barcode, 6 bp unique molecular identifier (UMI), 5' Illumina adapter (used in the Illumina small RNA kit) and a T7 promoter). Barcode design was such

that each pair differed by at least two nucleotides, so that a single sequencing error will not produce the wrong barcode. RNA samples were reverse transcribed to generate cDNA, pooled, and in vitro transcribed for linear amplification using the MessageAmp™ II aRNA Amplification Kit (Invitrogen, #AM1751) according to the CEL-Seq protocol48. Illumina sequencing libraries were prepared using the (Illumina, #RS-200), followed by PCR amplification for 14 rounds (as previously described in detail (Kruse et al., 2016; Junker et al., 2014)). Afterwards, libraries were sequenced paired-end at 26 bp read length for read 1 and 62 bp for read 2, using Illumina NextSeq 500. Read 1 contained barcode information, whereas read 2 was used for alignment. Reads 2 with a valid section barcode were selected, trimmed using TrimGalore (v.0.4.3) with default parameters, and mapped using STAR (v.2.5.3a) with default parameters to the mouse genome (GRCm38), the gerbil genome (MunDraft-v1.0), the Acomys genome (AcoCah_v1_BIUU). Only reads mapping to gene bodies (exons or introns) were used for downstream analysis. Reads mapping simultaneously to an exon and to an intron were assigned to the exon. For section, the number of transcripts was obtained as previously described. We refer to transcripts as unique molecules based on unique molecular identifier correction. Mappabilities for the tomo-seq experiments range from 45% to 80%. Ribosomal and mitochondrial genes were removed from downstream analysis.

Processing of tomo-seq data

To obtain a comparable number of reads per section in one sample, a cut off of either 1×10^3, 5×10^3, or 1×10^4 (reads per section) was used, resulting in the retention of ±75% sections for the analysis. To test for the reproducibility of the results between the biological replicates, Pearson's correlation was applied. One uninjured sample was excluded in this process as the correlation was below <0.9. For the spatial gene expression analysis, read counts were normalized to the total counts per section. Next, the data was renormalized to the mean of total reads across sections to ensure that count numbers roughly corresponded to the number of mapped reads. Afterwards the data was transformed to *Z*-score values and averaged for the three biological replicates (https://github.com/anna-alemany). The cell type markers and

information are displayed in Table S2. For the expression level analysis, the raw read counts from the -5 and +5 sections surrounding the wound edge were summed (Figure S5c). The section containing the wound edge was marked during sectioning. A comparable area was taken in the uninjured area (Figure 3c). After obtaining the raw read count expression matrix, samples were normalized and further processed in R using the DESeq2 Bioconductor package (version 3.12)(Love et al., 2014). Values were transformed using regularized-logarithm transformation (Rld), and log2-transformed fold changes were shrunk using the apeglm method. After differential expression analyses between relevant samples, the DESeq2 results table was exported for analysis. The significant genes were clustered on the basis of *Z*-score expression pattern using self-organizing maps with an initial number of clusters set to about $5\sqrt{n}$, in which n is the total number of genes. Average *Z*-score expression patterns for each cluster were then hierarchically clustered using Euclidean distances and the Wart.D method. Line plots representing gene expression patterns for a gene of interest were smoothed using the package SciPy.interpolate, function interp1d.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Immunohistochemistry

Upon tissue collection, ear samples were fixed overnight in 4% formaldehyde at 4°C, washed in PBS, followed by dehydration and paraffin embedding. 5-10um-thick sections were cut and hydrated. Heat mediated antigen retrieval was performed before staining. Afterwards, sections were permeabilized in 0.5% Tween in PBS for approximately 30 min at RT, and then washed with PBS. Sections were then blocked for non-specific binding with 1% BSA in PBS-T for 60 min at RT, and then incubated with primary antibody in 1% BSA in PBS or PBS-T, O/N at 4 °C in the dark TUBB3-488 (1:500, Biolegend, cat#801203), Anti-alpha smooth muscle Actin antibody (1:100, Abcam, #ab5694), COL1A1 Monoclonal Antibody (1:400, Elabscience, #E-AB-22152), Anti-F4/80/ADGRE1 antibody (1:100, Abcam, #ab6640), CLEC4G Polyclonal

Antibody (1:50, ThermoFisher Scientific, #PA5-53116), Anti-Ki67 antibody (1:200, Abcam, #ab15580), Anti-Fibronectin antibody (1:200, Sigma, #F3648). The next day, following washing, the sections were incubated with Hoechst 33342 nucleic acid stain (Invitrogen, #H3570)(1:10000) and/or DAPI Staining Solution (Abcam, #ab228549)(1:10000) and/or Isolectin GS-IB4-568 (1:200, Invitrogen, #I21412) and/or CD209 (DC-SIGN) Antibody, anti-human, REAdye_lease™ (1:50, Miltenyi, #130-125-076) for 15 min, washed in PBS, mounted with ProLong™ Gold Antifade (Thermo Scientific, #P36934), and stored at RT in the dark until solidified. Samples were then imaged immediately or stored until further analysis at 4 °C in the dark. Samples were imaged using Leica SP8 microscope and Olympus VS200 slide scanner. The images were analyzed with ImageJ 1.53t. The area is expressed as percentage of the area of positive signal over the total area of interest. The quantification from the different micrographs within the sections was averaged to give the final value per sample. When quantifying the positive signal, only the injury area was used.

In situ hybridization probe synthesis

Ear tissue was snap-frozen in liquid nitrogen, and then grinded using a mortar and pestle. Total RNA was isolated from the tissue using a TRIzol Reagent (Invitrogen, #15596018) according to manufacturer's instructions, and eluted in nuclease-free water (Invitrogen, #AM9937). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit with random hexamer primers, according to the manufacturer's instructions (Thermo Scientific, #K1622). The cDNA was diluted 40× with nuclease-free water.

Probes were generated using PCR OneTaq® Quick Load® (New England BioLabs® inc.) on cDNA of intact ear and various timepoints after injury of *Mus, Meriones* or *Acomys* with gene-specific primers (Table S7). After purification of the PCR product with a PCR purification kit (Macherey- Nagel, #12303368), a quality (Gel) and quantity (Thermo Scientific NanoDrop One) check of the PCR product was performed. RNA probe synthesis was carried out overnight using a T7 High Yield kit (New England Biolabs®, #E2040S) and a UTP DIG-RNA labelling mix (Sigma-Aldrich®, #11277073910). The RNA probe was purified with the RNA clean-up kit

(Macherey-Nagel, #15880958) and analyzed with a Thermo Scientific Nanodrop One and gel electrophoresis. A negative control probe was generated using a T3 High Yield kit (Promega, # 9PIP208).

In situ hybridization

Slides were deparaffinised and tissue was stepwise hydrated. After fixation with paraformaldehyde 4% + 0.25% glutaraldehyde for 30-45 min, slides were washed and incubated in Proteinase K 20 µg/mL (Ambion®, #AM2546) for 15 min for antigen retrieval and permeabilization. Proteinase K was blocked with Glycine 0.2% in PBS0, followed by washes in PBS0. The tissue was post-fixed in 4% paraformaldehyde and washed for 2 times in 2× Saline Sodium Citrate buffer (2×SSC). Pre-hybridisation was done for 1 hour at 65 °C to block non- specific signal and was followed by hybridization with a probe concentration of 500 ng/mL at 55 °C. After 24-72 hrs the tissue was incubated 3 times for 15 min with 2×SSC/50% formamide at 55 °C. Thereafter, the tissue was rinsed several times with Tris buffered saline + 0.1% Tween (TBS-T) followed by Maleic Acid Buffer + 0.1% Tween (MABT). This was followed by blocking for 1 hour at room temperature. After blocking, the tissue is incubated with anti-Digoxigenin antibody (AP) (Roche, #11093274910) in MABT overnight at 4 °C. After washing multiple times with MABT, TBS-T and alkaline phosphatase buffer (NTM) the tissue was stained with NBT/BCIP (Roche, #11681451001) and kept in the dark onwards. Staining time was usually 3-5 hrs at 37 °C sometimes followed by staining overnight at 4 °C. After staining was detected well, the slides were washed with NTM followed by PBS0. Slides were mounted with ProLong™ Gold Antifade (Thermo Scientific, #P36934) and stored at RT in the dark until solidified. Samples were imaged immediately using an Olympus VS200 Slide scanner.

Picrosirius red staining and image analysis

Tissue sections were deparaffinized and hydrated through a series of washes in: two in xylene, two in EtOH 100%, one in EtOH 96%, EtOH 70% and two in distilled water. Nuclei were

counterstained in Weigert's hematoxylin solution (Sigma-Aldrich, HT1079) for seven minutes. The tissue slides were washed under running tap water for two minutes and incubated for 60 minutes in a picrosirius red solution, prepared by dissolving 500 mg of Direct Red 80 (Sigma-Aldrich, 36-554-8) in 500 mL of saturated picric acid in distilled water, for a final concentration of 0.1% (w/v) Sirius red. The tissue sections were washed twice in 0.5% acetic acid solution (30 seconds each) and dehydrated through sequential incubations in increasing alcohol concentrations from distilled water to EtOH 70%, EtOH 96% and EtOH 100%, followed by washes in two consecutive xylene solutions. The slides were mounted with Roti-Histokitt (Carl Roth, 6638) and the collagen birefringence was acquired through polarized light using the Olympus SLIDEVIEW VS200 slide scanner. To determine the proportion of the different colors of the collagen fibers based on birefringence (Liu et al., 2021), the images with black background set, were assessed for their hue, saturation and brightness values. Red to orange and yellow signals, indicating thick packaged fibers, were defined by hue values between 1 and 62. Conversely, the green signal, indicating thin, immature fibers, were defined by hue values between 63 and 110. Saturation and brightness were set at intervals of 10-255 and 20-255, respectively for both quantifications of thin and thick fibers. The same settings were used for all the images and the pixel area of thin and thick fibers was quantified. The images were analyzed with ImageJ 1.53t.

Liu, J., Xu, M., Wu, J., Zhang, H., Yang, L., Lun, D., Hu, Y., & Liu, B. (2021). Picrosirius-Polarization Method for Collagen Fiber Detection in Tendons: A Mini-Review. *Orthopaedic Surgery*, *13*(3), 701–707. https://doi.org/10.1111/os.12627

Reagent or resource	Source	Catalog number
Antibodies		
Alexa Fluor® 488 anti-Tubulin β 3	Biolegend	#801203
Isolectin GS-IB4-568	Invitrogen	#I21412
Anti-alpha smooth muscle Actin antibody	Abcam	ab5694
COL1A1 Monoclonal Antibody	Elabscience	E-AB-22152
Anti-F4/80 antibody [CI:A3-1]	Abcam	ab6640
CD209 (DC-SIGN) Antibody, anti-human, REAdye_lease™	Miltenyi	130-125-076
CLEC4G Polyclonal Antibody	ThermoFisher Scientific	# PA5-53116
Anti-Ki67 antibody	Abcam	ab15580
Anti-Fibronectin antibody produced in rabbit	Sigma	F3648
Chemicals, peptides, and recombinant proteins		
TRIzol Reagent	Invitrogen	#15596018
ProLong™ Gold Antifade	Thermo Scientific	#P36934
EdU	Invitrogen	#A10044
Proteinase K	Ambion	#AM2546
NBT/BCIP	Roche	#11681451001
anti-Digoxigenin antibody	Roche	#11093274910
Critical commercial assays		
MessageAmp™ II aRNA Amplification Kit	Invitrogen	#AM1751
TruSeq Small RNA Library Prep Kit	Illumina	#RS-200
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific	#K1622
Click-iT™ EdU Alexa Fluor™ 647 imaging kit	Invitrogen	#C10340
PCR purification kit	Macherey- Nagel	#12303368
T7 High Yield kit	New England Biolabs®	#E2040S
UTP DIG-RNA labelling mix	Sigma-Aldrich	#11277073910
RNA clean-up kit	Macherey-Nagel	#15880958
T3 High Yield kit	Promega	# 9PIP208
PCR OneTaq® Quick Load®	New England BioLabs® inc	

Deposited data		
Raw and analyzed data	This paper	PRJNA898313
single-cell RNAseq data	(Tomasso et al., 2023)	GSE224879 and GSE224433
Mouse reference genome, Build 38, GRCm38	Genome Reference Consortium Mouse	https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_000001635.20/
Gerbil reference genome, MunDraft-v1.0	Reference genome MunDraft-v1.0	https://www.ncbi.nlm.nih.gov/labs/data-hub/taxonomy/10047/
Acomys reference genome, AcoCah_v1_BIUU	Broad institute	https://www.ncbi.nlm.nih.gov/data-hub/genome/GCA_004027535.1/
Oligonucleotides		
Primers for ISH, see table S7	This paper	N/A
Software and algorithms		
Graphpad Prism 9	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
DESeq2 version 3.12	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
R 3.6.0	R Core Team, 2022	https://www.r-project.org/

TrimGalore version 0.4.3	N/A	https://github.com/FelixKrueger/TrimGalore
STAR version 2.5.3a	Dobin et al., 2013	https://github.com/alexdobin/STAR
Python 3.7	van Rossum & Drake, 2009	https://www.python.org/downloads/
Scripts	N/A	https://github.com/anna-alemany
Other		
Benchling	https://www.benchling.com	