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## APPENDIX

### Supplementary Methods

#### *Histopathology, histochemistry, and immunohistochemistry (IHC)*

After euthanasia of the experimental animals, spine tissue samples comprising the segments of lumbar 3 to sacral 1 (L3-S1) vertebrae and facet joints were harvested from *Nfat1*<sup>-/-</sup> and WT mice (controls) at the ages of 2, 6, 12, and 18 months for histopathological analyses. We chose to examine L3-S1 lumbar tissue samples because FJOA most commonly occurs at these spinal levels in humans. Harvested lumbar spine samples were fixed in 2% paraformaldehyde, decalcified in 25% formic acid, embedded in paraffin, and sectioned at 5 µm. The tissue sections were stained with safranin-O and fast green to identify cartilage cells and matrices, as well as haematoxylin and eosin (H&E) for structural and cellular analyses of bone and soft tissues. Tissue sections that had been decalcified in 10% EDTA were used for IHC using specific antibodies to identify the location and intensity of the proteins of interest. To observe both immunoreaction and cellular morphology, some tissue sections were stained by the avidin-biotin peroxidase complex methods. DAB chromogen was used for color detection.

The primary antibodies and their dilutions used for IHC in this study are monoclonal IL-1β antibody (Santa Cruz, SC-52012, 1:100), monoclonal MMP-3 antibody (LSBio, LS-C800335, 1:100), monoclonal TNF-α antibody (Santa Cruz, SC-52746, 1:50), polyclonal ADAMTS-5 antibody (Santa Cruz, SC-83186, 1:50), and polyclonal NFAT1 (NFATc2) antibody (Santa Cruz, SC-32993, 1:50).

#### *Development of a novel FJOA scoring system*

To develop a novel semi-quantitative scoring system for comparative assessments of FJOA severity of *Nfat1*<sup>-/-</sup> and WT mice in different age groups, twenty-three representative microscopic images of coronal sections covering all joint tissues and surrounding muscles of the facet joints with safranin-O staining were selected for scoring. The images were scored by an experienced observer (J.W., Observer 1), a well-trained observer (M.J.M., Observer 2), and a novice observer (X.L., Observer 3) with no previous experience in OA histology to assess inter-observer variability as well as to determine the ease of use of the scoring system. The images were also scored twice with a minimum time interval of one week to obtain intra-observer variability from the same observer.

Since L5-6 *Nfat1*<sup>-/-</sup> facets usually displayed the most severe OA among the examined segments and significant differences in averaged group FJOA scores between the paired facet joints (left vs. right) at the same vertebral level were detected in the *Nfat1*<sup>-/-</sup> animals at 12 and 18 months, the score of each animal for statistical analysis was generated from one of the paired facet joints at the level of L5-6. When a paired facet joints at the same segment show different FJOA scores, the joint with a more severe score was used for scoring. FJOA histologic scoring was conducted by 3 independent observers who were blinded regarding genotypes and ages. The

maximum OA score for each facet joint was set at 24, which covered cartilage lesions (score range: 0-6 per facet, 0-12 per joint), chondro-osteophyte formation (score range: 0-2 per facet, 0-4 per joint), subchondral bone changes (score range: 0-2 per facet, 0-4 per joint), and synovitis (score range: 0-2 per proximal or distal end, 0-4 per joint). A detailed semi-quantitative FJOA scoring system is presented in **Table I**. Cumulative or summed scores of a representative facet joint of each animal from all observers at each time point were averaged for statistical analysis to determine the genotype-specific and age-dependent changes in FJOA severity.

### ***Quantitative real-time reverse transcription polymerase chain reaction (qPCR)***

Facet joint cartilage and synovium (containing a thin layer of fibrous capsular tissue due to technical difficulties in obtaining pure synovium from mouse facet joints) were freshly dissected under a microscope. Cartilage and synovium samples were collected separately in RNeasy lysis solution (Qiagen, Crawfordsville, IN, USA) at the age of 2, 6, and 12 months. No tissue samples were collected for qPCR analysis at 18 months due to severe OA changes with articular destruction. Cartilage or synovium samples from L3-S1 facet joints of the same animal were pooled for RNA extraction to obtain enough RNA from each individual mouse. Tissue samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with a DNA Digestion Kit (Ambion) to remove DNA. One microgram of total RNA and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) were used to yield cDNA. Specific primers used in this study are presented in **Table II**. qPCR reactions were performed in triplicate using a 7500 Real-Time qPCR system and SYBR Green reagents (Applied Biosystems). *Gapdh* expression levels were used as internal controls for load normalization of cDNA samples. Gene expression levels were quantified using  $2^{-\Delta\Delta C_t}$  methods using the expression level of age-matched WT samples as controls.

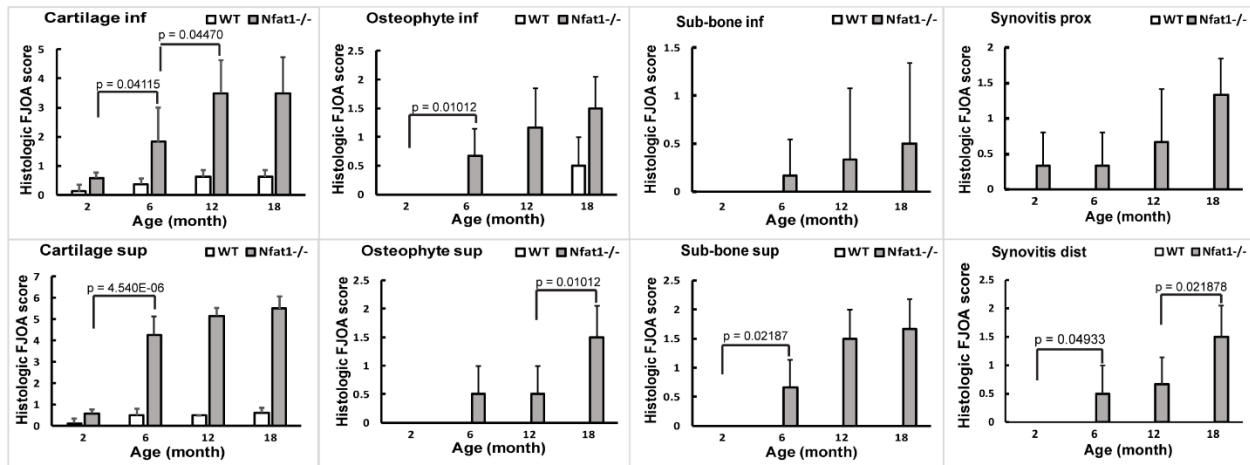
## **Supplementary Results**

### ***Tissue- and facet-specific involvement in initiation and progression of FJOA***

**Facet-specific scoring:** To determine the progression rate of FJOA in specific facets of the joint, we conducted facet-specific assessments using the new FJOA scoring system with statistical analyses. From 2 to 6 months, cartilage lesions were significantly progressed in both the inferior (inf) and the superior (sup) facets, osteophyte formation was significantly progressed in the inferior facet but not the superior facet, subchondral bone changes significantly progressed in the superior facet but not the inferior facet, and synovitis significantly progressed in the distal (dist) end but not in the proximal (prox) end. From 6-12 months, cartilage lesions were significantly progressed in the inferior facet but not the superior facet. From 12 to 18 months, osteophyte formation significantly progressed in the superior facet but not the inferior facet, while synovitis became more severe in the distal end but not in the proximal end (**Supplementary Figure 3**).

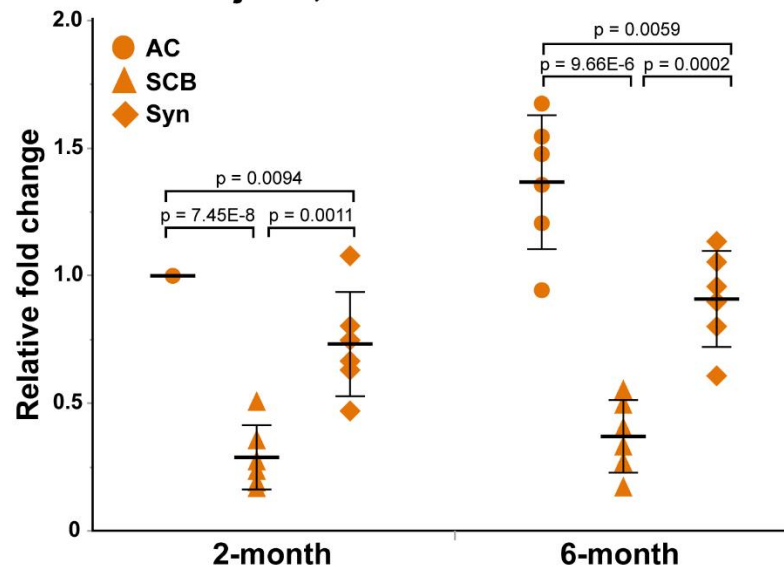
**Supplementary Figure 1.** Comparative analyses of first measurement of area-specific inter-observer variabilities in FJOA scores at 6, 12, and 18 months from three observers (Observer 1, 2, and 3) using the novel FJOA scoring system. Area-specific histologic FJOA scores for 2-month-old *Nfat1*<sup>-/-</sup> mice are not included as they are either zero or too low to display. The histologic FJOA scores for specific areas from the three observers are highly reproducible with no significant differences among the observers, except the severity of osteophyte formation in the superior facets at 18 months and subchondral bone change in the superior facets at 12 months. inf = inferior facet/articular process, sup = superior facet/articular process, Sub-bone = subchondral bone, prox = proximal/cephalic end of the facet joint, dis = distal/caudal end of the facet joint. Student's t-test (unpaired, two-tailed); n = 6; \*p < 0.05.

**Supplementary Figure 2.** Comparative analyses of the second measurement of area-specific inter-observer variabilities in FJOA scores at 6, 12, and 18 months from three observers (Observer 1, 2, and 3) using the novel FJOA scoring system. The histologic FJOA scores for specific areas from the three observers are highly reproducible with no significant differences among the observers, except the severity of synovitis in the proximal end of facet joints at 12 months. inf = inferior facet/articular process, sup = superior facet/articular process, Sub-bone = subchondral bone, prox = proximal/cephalic end of the facet joint, dis = distal/caudal end of the facet joint. Student's t-test (unpaired, two-tailed); n = 6; \*p < 0.05.

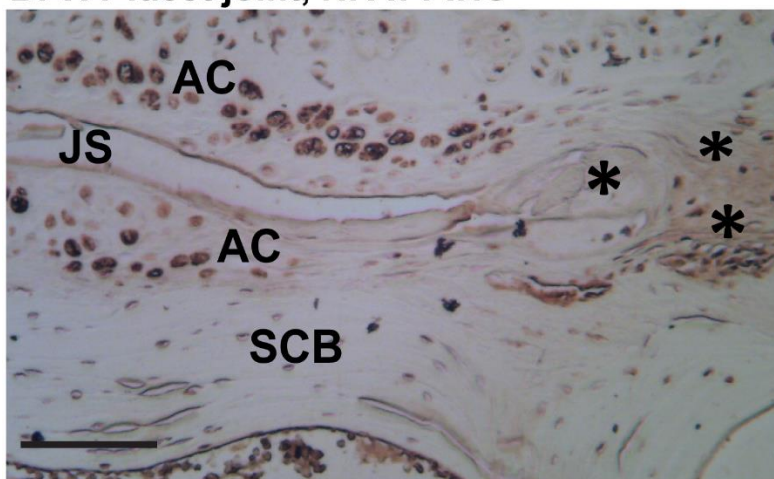


**Supplementary Figure 3.** Age-related progression rates of FJOA in specific areas of *Nfat1*<sup>-/-</sup> mice. The FJOA scores (averaged from all 3 observers) from WT and *Nfat1*<sup>-/-</sup> facet joints were determined by the novel FJOA scoring system. inf = inferior facet/articular process, sup = superior facet/articular process, Sub-bone = subchondral bone, prox = proximal/cephalic end of the facet joint, dis = distal/caudal end of the facet joint. The p values determined by Student's t-test (unpaired, two-tailed) between two age points are indicated in the graphs.

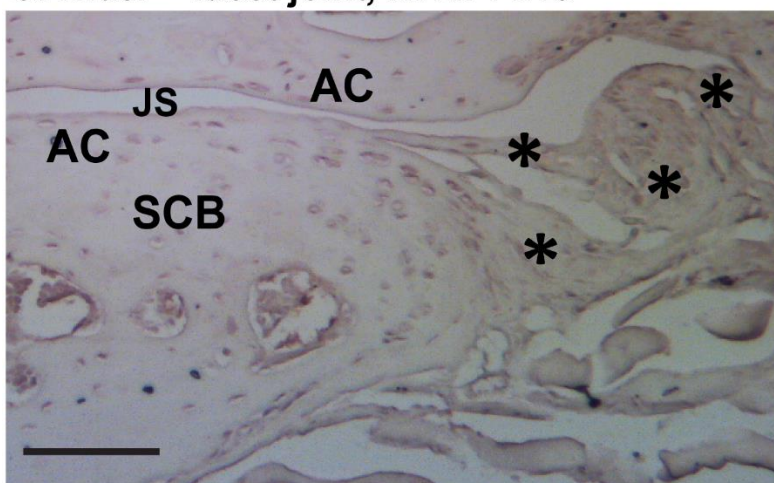
### A: WT facet joint, *Nfat1* mRNA



### B: WT facet joint, NFAT1 IHC



### C: *Nfat1*<sup>-/-</sup> facet joint, NFAT1 IHC



**Supplementary Figure 4.** NFAT1 expression in the facet joint tissues of young adult (2-6 months old) WT and *Nfat1*<sup>-/-</sup> (negative control) mice. (A) qPCR analysis with Student's t-tests (unpaired, two-tailed) showing differential expression of *Nfat1* mRNA in facet joint articular cartilage (AC), subchondral bone (SCB), and synovium (Syn) of 2- and 6-month-old WT mice. The expression level of each 2-month AC sample has been normalized to "1.0". N = 6. (B) A representative photomicrograph of NFAT1 immunohistochemistry (IHC) of WT facet joints showing the cellular localization and staining intensity of NFAT1 protein expression (stained in dark brown) in AC, Syn, and SCB. JS = joint space. \*denotes the area of facet synovium. (C) A representative photomicrograph of NFAT1 IHC of *Nfat1*<sup>-/-</sup> facet joints showing no detectable NFAT1 protein expression in any joint tissues. N = 6 per genotype for (B) and (C). NFAT1 IHC with hematoxylin counterstaining, scale bar = 100 μm.