

## Supplementary material

### Supplementary methods

#### Micro-CT

Micro-CT analyses were performed with a SCANCO Medical  $\mu$ CT 40 scanner using the following parameters: voltage, 40kV; X-ray current, 250 $\mu$ A; exposure time, 5,000ms/projection for 720 projections; matrix, 1024 $\times$ 1024; voxel size in reconstructed image, 9 $\mu$ m. Images were analysed with SCANCO evaluation software for parameters at the metaphysis of the proximal tibiae: ratio of bone volume to total volume, trabecular number, and trabecular thickness.

#### Immunodetection and apoptosis detection

Briefly, 5 $\mu$ m thick sections of the joints were deparaffinised, post fixed in PFA and the antigen was retrieved by enzymatic digestion with 15mg/ml of pepsin in 0.02% HCl for 45' at 37°C. The sections were then blocked in 20% foetal bovine serum.

For WNT16 analysis, anti WNT16 antibody (BD-Pharmingen), was diluted 1:200 in 20% FBS or isotype matched non-immune IgG at the same concentration as negative control. After incubation with a biotinylated rabbit anti-mouse IgG (Dako, 1:200), the sections were incubated with StreptABComplex/HRP (Dako). Liquid DAB Substrate Chromogen System (Dako) was used as peroxidase substrate for development.

For Lubricin analysis, sections were incubated with a rabbit anti-Lubricin polyclonal antibody (Abcam); for the detection of MMP- and Aggrecanase-dependent aggrecan neoepitopes, the sections were respectively incubated with the mouse monoclonal Anti-Aggrecan Antibody, MMP cleaved, against the N-terminus FFGVG neoepitope (Novus Biologicals) (10 $\mu$ g/ml), and rabbit-polyclonal Anti-Aggrecan Antibody, aggrecanase cleaved, against the NITEGE neoepitope (Novus Biologicals) (1 $\mu$ g/ml). Incubation with the primary antibodies was followed by incubation with Cy3-conjugated rabbit anti-mouse and goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch). Mouse IgG1 (Sigma-aldrich) and rabbit IgG (Abcam) were used at matching concentrations as negative controls. Nuclei were counterstained using DAPI.

Apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (Roche) according to the manufacturer's instruction.

Images were acquired at 22°C using an Olympus BX61 microscope with a fixed exposure using a 10x/0.4 objective lens. Images were optimised using Adobe Photoshop CS3 for better rendering without altering any relationship between target and control images.

**Quantification of lubricin staining** was performed using ImageJ. Control sections stained using non-immune IgGs were used to set the threshold above which signal was considered positive and which was applied to all images. Each articular cartilage compartment was then outlined using the manual selection tool and the stained area for each compartment above the threshold was measured and was normalised for the total selected area.

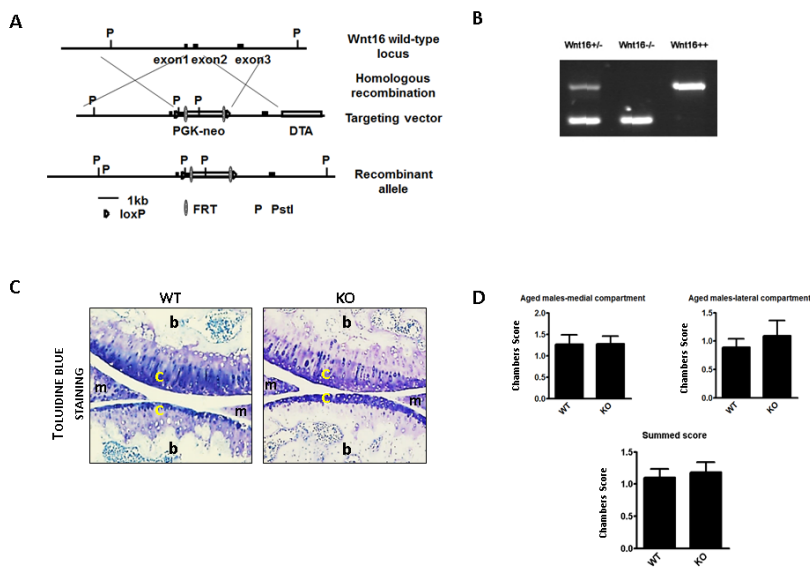
**Supplementary Table 1**

Species	Gene	Sense primer	Antisense primer	Amplicon Length	Annealing temperature(°C)
mouse/ human	β-actin	TGACGGGGTCACCCACACTGTGCCATCTA	CTAGAAGCATTGCGGTGGACGATGGAGG	661	55
mouse	Aggrecan	GAAGAGCCTCGAATCACCTG	ATCCTGGGCACATTATGGAA	132	55
mouse	Col2A1	CATTGTTGGTCTGCCTGGTC	TTCTCTCTGCCCCTAAGCC	750	55
mouse	Sox9	TATGTGGATGTGTGCGTGTG	CCAGCCACAGCAGTGAGTAA	137	55
mouse	Lubricin	TGGAGTGCTGCTGATTCAAGAG	GGTGATTTGGGTGAGCGTTTGTA	256	55
mouse	Matrilin1	CCGTGGCAGACCACTACTTT	ACTCACAAGCACAGGGGTCT	108	68
mouse	Wnt16	GCCACTACCACTCCACCC	GAGCCACCATTCTGCAAGG	188	60
mouse	Axin2	AGCGCCATCGACAGCGAGTT	CAGGCGGTGGGTTCTGGGAA	182	50
mouse	ADAMTS5	TCTCAGAGGCCTTCTGCATT	CTGCCATAATTGCACTCGT	184	55
mouse	ADAMTS4	GATACAGCGATGTGGTCACG	GGCGTAAGAACCGTCAGAAA	119	55
mouse	MMP13	CCAGAACTCCAACCATGT	GTCTCAAACCCCGTGTTCT	200	55
mouse	Col10A1	GCAGCATTACGACCCAAGAT	CCTGAAGCCTGATCCAGGTA	177	55

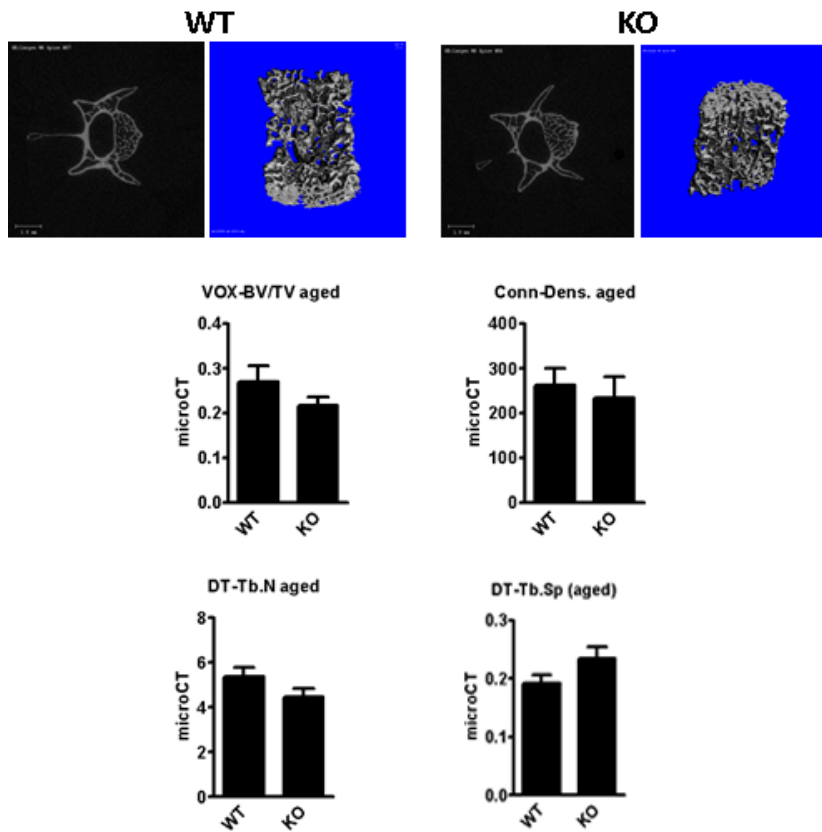
<b>mouse</b>	GDF5	CACAAACCACGCAGTCATTC	TGTTGGCAGAGTCGATGAAG	122	55
<b>human</b>	Lubricin	TCACAGTTGTTATTGTTTACAGACC	GGGTAAGTGAATACACTAGTTTTGA	152	60
<b>human</b>	Axin2	TACCGGAGGATGCTGAAGGCC	CCACTGGCCGATTCTTCCTT	345	55
<b>bovine</b>	Lubricin	GGAGATGTGGGAAGGGTAA	TCTGAGATGCTCCTGGAGGT	288	55
<b>bovine</b>	Axin2	GAAATAGCGGTCCTGGTCCT	GTCTTCCCAAAGCTGTCTGC	119	55

### Supplementary Figure 1. WNT16 deficient mice do not develop spontaneous osteoarthritis. (A)

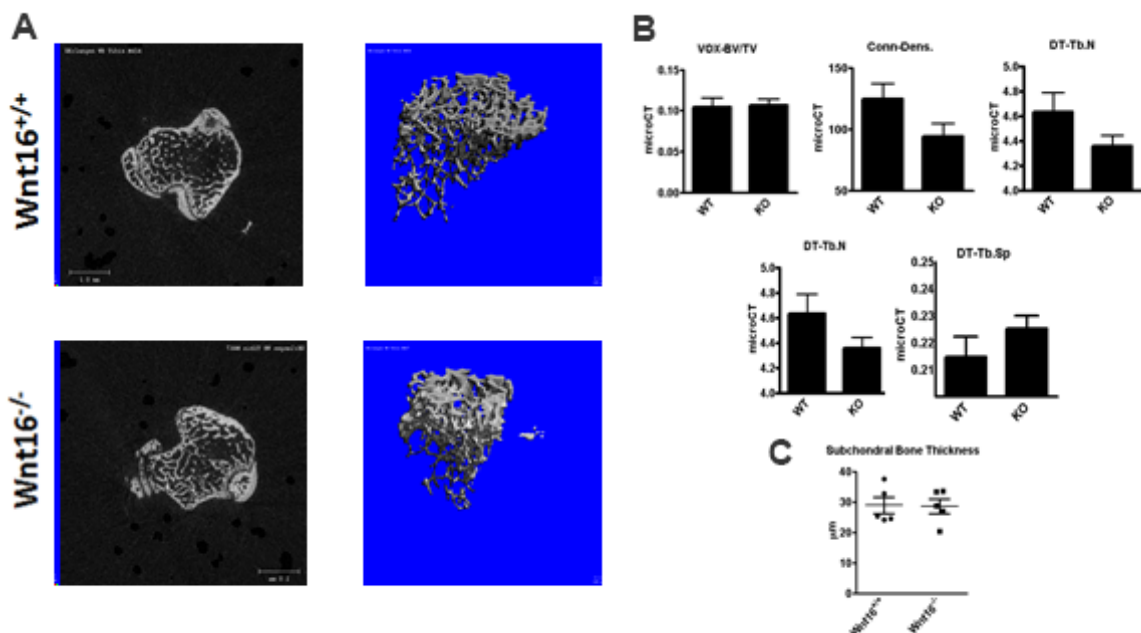
Diagrammatic representation of the cloning strategy. Exon 2 of the WNT16 gene was cloned into the targeting vector downstream of a loxP sequence and upstream of an FRT-flanked PGK-neo selection cassette, which was followed by a second loxP sequence. PGK-DTA in the targeting vector was used for negative selection during ES cell targeting. Following recombination, the LoxP flanked region including exon 2 and PGK-neo cassette were excised by crossing the mice with transgenics expressing cre recombinase under the transcriptional control of the  $\beta$  actin promoter. (B) PCR on genomic DNA from wild-type and mutants showing shift in size following successful recombination. (C) Toluidine blue staining of *wnt16*<sup>-/-</sup> (KO) and control joints (WT). c = cartilage; b = bone; m = meniscus. Modest cartilage surface irregularities were detected in both wild-type and *wnt16*<sup>-/-</sup> mice. (D) Osteoarthritis histological scoring (Chambers' score) revealed no differences between wild-type and *wnt16*<sup>-/-</sup> mice. N=4 per phenotype.



**Supplementary Figure 2. In unchallenged conditions, aged *wnt16*<sup>-/-</sup> mice display no differences in their vertebrae from wild-type (129/Sv background). No significant differences were observed in several bone parameters (including bone density and trabecular thickness) in the vertebrae of 18 months old *wnt16*<sup>-/-</sup> and wild-type mice, as evaluated by micro-CT analysis. (N=4) per phenotype.**

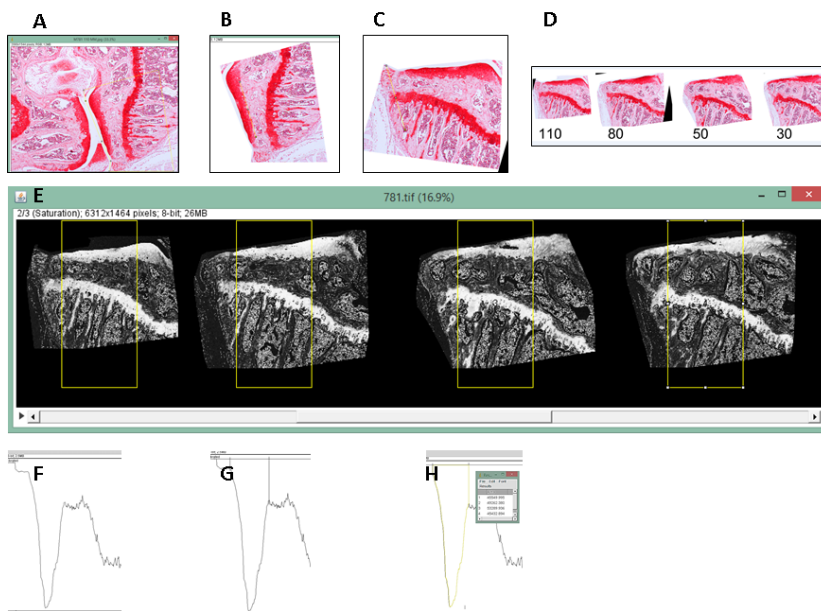


**Supplementary Figure 3. 8 weeks following DMM, *wnt16*<sup>-/-</sup> mice display no significant differences in their long bones from wild-type (129/Sv background).** A&B) No significant differences were observed in several bone parameters (including bone density and trabecular thickness) in the epiphysis of the tibiae of *wnt16*<sup>-/-</sup> and wild-type mice 8wks post DMM, as evaluated by micro-CT analysis. (N=4) per phenotype. C) Histomorphometrical measurement of the subchondral bone. The subchondral bone thickness was measured using Image J software with the Bone J plugin. Briefly, before starting the analysis calibrate the scale bar from pixel to microns. The subchondral bone area below the tibial plateau was selected avoiding the edges near the medial part of the epiphysis and oppositely near the cruciate ligament. Select the “clear outside” followed by “Fill” function from the “Edit” menu and deselect the area. “Invert” the colours and transform the image in an 8 bit picture (greyscale). In the Bone J plugin select the “thickness” function and record the measurement. Five sections per mouse (n=5/phenotype) spanning 200µm in the load bearing area of the joint were used for the analysis. The graph shows the average measurement/mouse ± SEM.



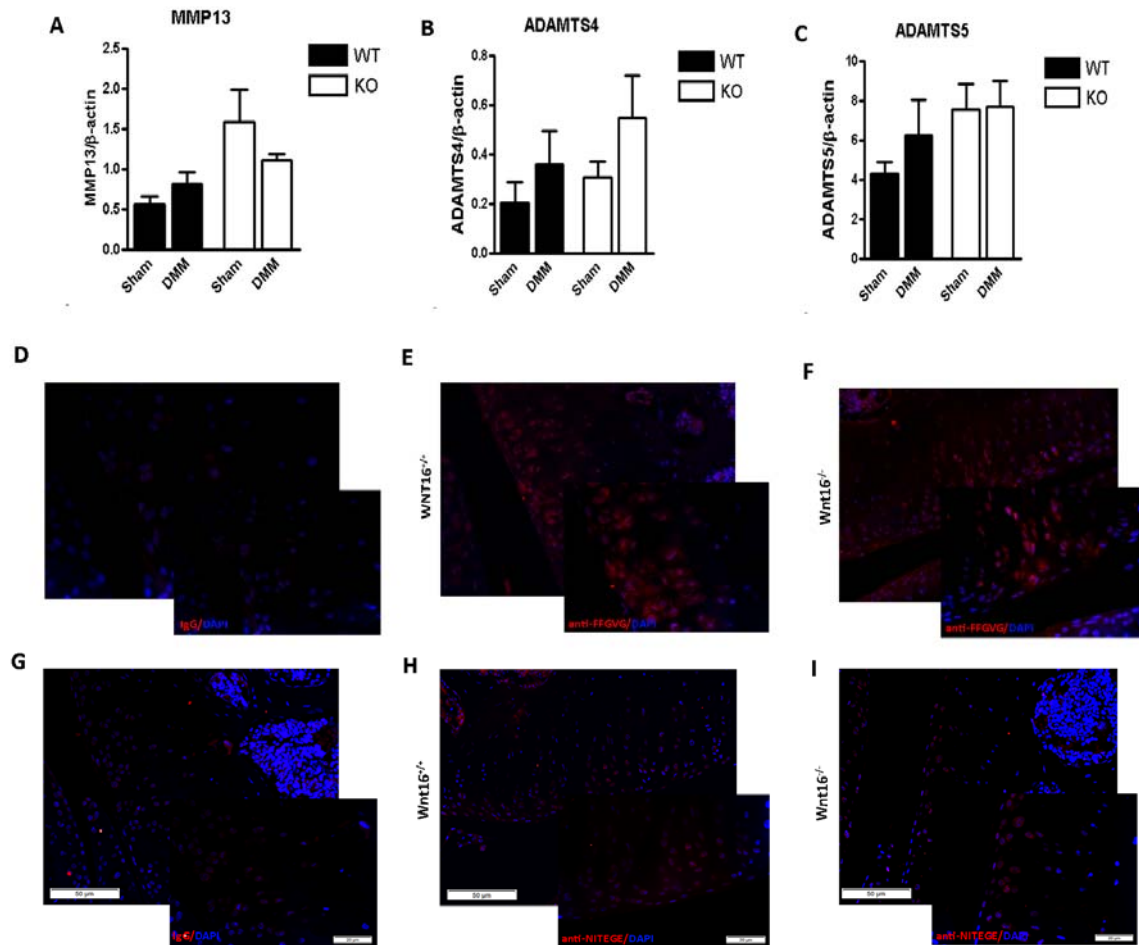
**Supplementary Figure 4. Procedure for histomorphometry.** All stainings must be performed in one single batch. All images must be taken on the same day using the same settings and exposure. Discard sections with cutting or staining artefacts, and do not include knees with less than 3 sections. For optimum consistency there should be 4-6 sections covering 200 microns around the centre of the tibial plateau. The central section is identified as the one with the shortest cross-section of the medial meniscus. Digital images must be taken using identical parameters and turning off all the auto-enhancing features of the camera and of the acquisition program which, by normalizing the total luminosity and contrast, or modifying the colours, might reduce the differences and therefore lead to false-negative results. Each image is opened in ImageJ. The tibial plateau is segmented using a polygon or free-hand tool (A). The region of interest is isolated using Edit>Clear Outside (B) and rotated so that a line drawn along the cartilage-bone junction in the middle of the plateau is perfectly horizontal (C). This is very important so that the non-cartilage staining in the bone marrow spaces can be eliminated (see below). This is accomplished by drawing a line using the line tool and noting the angle as doing so. This measurement will be used to rotate the image. For each knee, all available images (at least 3 parallel images ~50 microns apart) are placed on the same canvas, perfectly aligned horizontally so that all images from the same knee are processed using the same region of interest (D). To isolate the Safranin O metachromatic staining from the background and most of the orthochromatic staining (bone) transform the RGB image in HSB stack (Image>Type>HSB Stack) and press right arrow until selecting the "Saturation" slice. This will abolish background and staining of the bone (E). Setting the region of interest (E): draw a rectangle to include the tibial plateau, making sure to exclude any osteophyte (left side in this figure) and the "bulgy" part near the intercondylar notch. Osteophytes are best identified in the original figure rather than in the "saturation" slice. Press Ctrl+1. Slide the rectangle laterally over the next section and press Ctrl+2 and, when over the last section, press Ctrl+3. This will show the density profile of each section (F). The first negative peak is the articular cartilage. Draw one horizontal line to cut the residual background, if any, and lateral lines to limit the articular cartilage peak (G) and

eliminate the staining of the bone marrow spaces. Repeat this for each of the profiles of the images in the same canvas. Select the “Wand” tool and click inside the area under the curve in each of the density profiles corresponding to each section (G). This will highlight the area under the curve and return the area measurement. Copy the results from the measurements box into a statistics programme to calculate the average. The average of all the sections of each knee is utilized as a score for that individual knee.

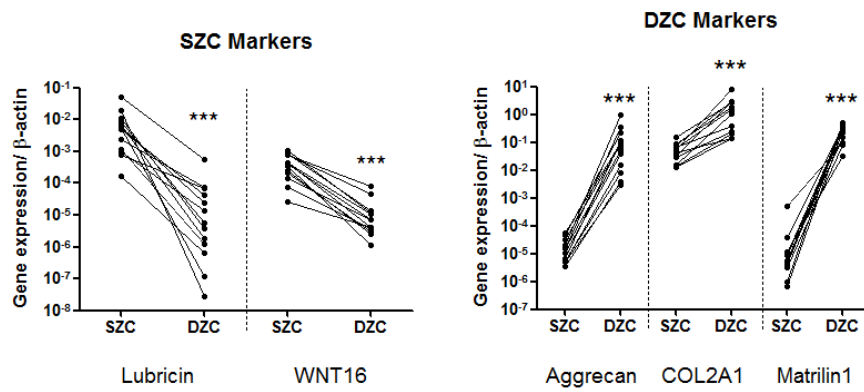




**Supplementary Figure 5. *wnt16*<sup>-/-</sup> mice display no significant differences in the activity of MMPs and Aggrecanases compared to wild type mice.** Gene expression levels of MMP13 (A), ADAMTS4 (B), and ADAMTS5 (C), 7 days following DMM. Immunofluorescence for neo epitopes of MMP cleavage activity (E&F), and Aggrecanase activity (H&I) (N=3). IgG controls shown in D (for MMP cleavage), and G (Aggrecanase cleavage), and G (Aggrecanase cleavage).



**Supplementary Figure 6. Superficial zone cells were successfully isolated separately from the deep zone cells of the mouse neonatal joint (Yasuhara, 2011).** mRNA expression of superficial zone markers Lubricin and WNT16, mature chondrocyte markers COL2A1 and Aggrecan, and epiphyseal marker Matrilin1 was detected in the correct compartment following isolation. Any samples not displaying the correct marker profile were excluded from further analysis (representative data from a single isolation from wild-type mice). (N=13).



### Supplementary Figure 7. WNT16 upregulates Axin2 and Lubricin in Bovine articular chondrocytes.

Stimulation of BAC with WNT16. WNT16 caused a weak activation of the canonical WNT pathway compared to WNT3a, as shown by an increase in canonical target gene, Axin2 (A). Lubricin expression was increased by WNT16 stimulation and was far greater than the upregulation caused by WNT3a (B). (Both N= 2 donors, 3≤ replicates per experiment). Canonical pathway inhibitor, DKK1 (100ng/ml), reduced the WNT16 induced Lubricin upregulation (N=3).

