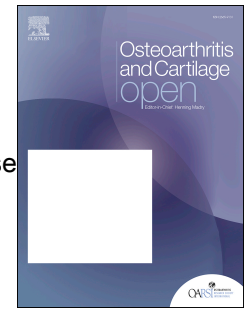

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Interleukin 13 (IL-13)-regulated expression of the chondroprotective metalloproteinase ADAM15 is reduced in aging cartilage.

C. Y. Yang[1], A. Chanalaris[1, a], S. Bonelli[2], O. McClurg[3], G. Lorenzatti Hiles[4], A. L. Cates[4], J. Miotla Zarebska[1], T. L. Vincent[1], M. L. Day[4], S. A. Müller[5,6], S. F. Lichtenthaler[5,6,7], H. Nagase[1], S. D. Scilabra[2, 5,6], L. Troeberg[1,3]*

[1]Centre for OA Pathogenesis Versus Arthritis, Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive, Oxford, OX3 7FY, United Kingdom

[2] Fondazione Ri.MED - ISMETT, Department of Research, via Ernesto Tricomi 5, 90145 Palermo, Italy

[3] Norwich Medical School, University of East Anglia, Bob Champion Research and Education Building, Rosalind Franklin Road, Norwich, NR4 7UQ, United Kingdom

[4] Division of Urologic Oncology, Department of Urology, University of Michigan, Ann Arbor, Michigan, United States of America

[5] German Center for Neurodegenerative Diseases (DZNE), Feodor-Lynen Strasse 17, 81377 Munich, Germany

[6] Neuroproteomics, School of Medicine, Klinikum rechts der Isar, Technical University of Munich, 81675 Munich, Germany

[7] Munich Cluster for Systems Neurology (SyNergy), 81377 Munich, Germany

* Address correspondence and reprint requests to: L. Troeberg, Norwich Medical School, University of East Anglia, Bob Champion Research and Education Building, Rosalind Franklin Road, Norwich, NR4 7UQ, United Kingdom. Tel: 01603-591910

E-mail address: L.Troeberg@uea.ac.uk (L. Troeberg).

[a] Present address: Kromek Group plc, NETPark, Thomas Wright Way, Sedgefield, County Durham, TS21 3FD, UK

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SUMMARY

Objective: The adamalysin metalloproteinase 15 (ADAM15) has been shown to protect against development of osteoarthritis in mice. Here, we have investigated factors that control ADAM15 levels in cartilage.

Design: Secretomes from wild-type and *Adam15*$^{-/-}$ chondrocytes were compared by label-free quantitative mass spectrometry. mRNA was isolated from murine knee joints, either with or without surgical induction of osteoarthritis on male C57BL/6 mice, and the expression of *Adam15* and other related genes quantified by RT-qPCR. ADAM15 in human normal and osteoarthritic cartilage was investigated similarly and by fluorescent immunohistochemistry. Cultured HTB94 chondrosarcoma cells were treated with various anabolic and catabolic stimuli, and ADAM15 mRNA and protein levels evaluated.

Results: There were no significant differences in the secretomes of chondrocytes from WT and *Adam15*$^{-/-}$ cartilage. Expression of ADAM15 was not altered in either human or murine osteoarthritic cartilage relative to disease-free controls. However, expression of ADAM15 was markedly reduced upon aging in both species, to the extent that expression in joints of 18-month-old mice was 45-fold lower than in that 4.5-month-old animals. IL-13 increased expression of ADAM15 in HTB94 cells by 2.5-fold, while modulators of senescence and autophagy pathways had no effect. Expression of *Il13* in the joint was reduced with aging, suggesting this cytokine may control ADAM15 levels in the joint.

Conclusion: Expression of the chondroprotective metalloproteinase ADAM15 is reduced in aging human and murine joints, possibly due to a concomitant reduction in IL-13 expression. We thus propose IL-13 as a novel factor contributing to increased osteoarthritis risk upon aging.

Introduction

Osteoarthritis (OA) remains the most prevalent of the musculoskeletal diseases, affecting 10% of men and 18% of women over the age of 60, with significant socioeconomic and healthcare impacts. Age is the major risk factor for development of OA, followed by obesity and joint injury. There is considerable interest in understanding the molecular mechanisms by which aging increases OA risk, so that strategies can be developed to reduce incidence, halt progression and treat the disease. Molecular factors such as chondrocyte senescence, 'inflamm-aging', and oxidative stress increase with age, while joint biomechanics are altered due to sarcopenia, tendon and ligament damage. These molecular and mechanical risk factors converge to initiate catabolic signalling pathways that lead to adaptive and ultimately damaging remodelling of joint tissues. There are also likely to be additional molecular risk factors that elevate OA susceptibility in aging joints.

Bohm *et al.*[1] showed that adamalysin metalloproteinase 15 (ADAM15) protects against OA in a mouse model of the disease. Although deletion of the enzyme caused no evident phenotype at birth[2], knockout mice developed more severe OA at 12-14 months of age, in both C57BL/6 and 129/SvJ strains[1]. Male *Adam15*$^{-/-}$ mice had 3-fold higher histological scores than wild-type animals, with females showing a similar trend towards increased susceptibility with age[1]. Cartilage fibrillation, fissuring, eburnation and necrosis were all higher in *Adam15*-null mice, along with increased proteoglycan loss, synovial hyperplasia, and osteophyte formation[1].

Metalloproteinases have been widely studied in the context of OA cartilage degradation, with enzymes such as matrix metalloproteinase (MMP) 13 and adamalysin with disintegrin and thrombospondin motifs (ADAMTS)-4 and -5 shown to mediate degradation of type II collagen and aggrecan respectively in murine models of disease.

Pathological roles have similarly been suggested for many other MMPs and ADAMTSs which are thought to degrade cartilage matrix components, contribute to bone remodelling, and participate in cellular signalling pathways.

In contrast with these secreted metalloproteinases, the role of transmembrane ADAM 'sheddases' in adult joints is less well understood. One of the ADAMs, ADAM15, is of particular interest as it is the only member of the metalloproteinase family shown to protect against OA. Its mechanism of action remains unclear. The enzyme is thought to be catalytically active, since as it has an HEXXHXLGXXHD zinc-binding consensus sequence in its catalytic domain, and cleavage of substrates including E- and N-cadherin[3,4], CD44[5], pro-heparin-binding epidermal growth factor[6] and FGF receptor IIIb[7] has been described. However, the *in vivo* relevance of these substrates and of ADAM15's catalytic activity to its chondroprotective role have not been tested. The enzyme has been shown to enhance chondrocyte survival[1], possibly by promoting cell-cell[3,8,9] and cell-matrix[1,3] attachment, and by reducing apoptosis[10–12]. While the detailed molecular mechanism underlying these protective effects is not known, ADAM15 has been shown to interact with integrins such as $\alpha v\beta 3$[13] and $\alpha 5\beta 1$[13], and the cytoplasmic domain of the enzyme interacts with intracellular signalling molecules such as c-src and focal adhesion kinase[1,11,12,14].

The protective role of ADAM15 is so far not known to extend beyond the joint. Its expression is elevated in several cancers, and correlates with worse prognosis in some prostate[15], lung[16] and breast[17] cancers. Loss of *Adam15* reduces tumour growth[4,18] and protects against metastasis in murine melanoma[19] and bladder[4,18] cancer models. ADAM15 expression is also elevated in the synovial membrane[20] and serum[21] of rheumatoid arthritis (RA) patients, and siRNA targeting of ADAM15 reduced arthritis scores and joint damage in a rat collagen-induced model of RA[11]. Of relevance to both

cancer and RA, pathological neovascularization is increased in *Adam15*-null mice[2]. Roles in the vasculature are additionally supported by studies indicating ADAM15 expression in endothelial cells is increased by sheer stress[12] and that it promotes LPS-induced vascular hyperpermeability[22].

Little is known about factors regulating ADAM15 expression. Early studies using *in situ* hybridization[23] and immunohistochemistry[10] indicated that expression of ADAM15 is increased in OA cartilage, although subsequent microarray studies have reported either no change or a slight but statistically insignificant increase in expression in OA (reviewed by Yang et al.[24]).

In the current study, we investigated the regulation of ADAM15 in cartilage with aging and OA, and in response to modulation of pathways postulated to increase OA risk with age. We found that expression of ADAM15 was not altered in OA cartilage, but that expression of the enzyme reduced markedly with age in both human and murine joints. *In vitro* analysis indicated *ADAM15* expression was not regulated downstream of senescence or autophagy, but was increased by IL-13 treatment. mRNA levels of *Il13* in the joint dropped with age, suggesting this cytokine may control ADAM15 expression in the joint and so be a novel factor contributing to increased osteoarthritis risk upon aging.

Materials and methods

Further details are provided in the online Supplementary Materials and Methods file.

Mass spectrometry analysis of murine chondrocyte secretome

Chondrocytes were isolated from 6-day-old wild-type (WT) and *Adam15*$^{-/-}$ mice[2] (n=3 animals per group), and conditioned media analysed by label-free quantitative (LFQ) LC-MS/MS. LFQ was performed only for proteins with at least two ratio counts of unique peptides. LFQ values were log2 transformed and a two-sided Student's t-test was used to evaluate proteins significantly regulated between *Adam15*$^{-/-}$ and WT chondrocytes. P-values were false discovery rate (FDR)-adjusted to less than 5%. Only proteins detected in all 3 *Adam15*$^{-/-}$ and WT samples were statistically analysed.

Cartilage samples

Human OA cartilage (n=8 donors, 58-84 years of age) was obtained from donors with late-stage OA, undergoing unicompartmental knee arthroplasty or total knee arthroplasty for OA. Human normal cartilage (n=16, aged 9-75 years of age) was purchased from Articular Engineering (Northbrook, IL, USA) or obtained from Stanmore Biobank (Royal National Orthopaedic Hospital, Stanmore, UK) from donors undergoing amputation for low limb malignancies with no involvement of the cartilage. All cartilage samples were collected with informed donor consent and in compliance with national and institutional ethical requirements, the United Kingdom Human Tissue Act, and the Declaration of Helsinki (HTA Licence 12217 and Oxford REC C 09/H0606/11).

RNA was isolated from whole knee joints of male C57BL/6 mice (Charles River Laboratories) with or without surgical destabilisation of the medial meniscus (DMM) performed at 10 weeks of age. Sham-operated animals underwent capsulotomy without destabilisation of the meniscus.

Porcine cartilage explants were dissected from metacarpophalangeal joints of 3-9 month old pigs within 24 h of slaughter. Explants were rested for 3 days in serum-free cartilage medium (DMEM supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 2 mg/ml amphotericin B, 10 mM HEPES) and treated with IL-13 (100 ng/ml) for 48 h. Conditioned media were concentrated by addition of 5% trichloroacetic acid and analysed by immunoblotting.

RT-qPCR

RNA was extracted from cultured cells using RNeasy Mini Kits (Qiagen), and from human cartilage and whole murine knee joints using TRIzol followed by RNeasy Mini Kits. After generation of cDNA using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific), qPCR was performed with TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific). Human Taqman primer/probes sets (Thermo Fisher Scientific) were as follows: *ADAM15* Hs00187052_m1, *RPLP0* Hs99999902_m1. Murine Taqman primer/probes sets (Thermo Fisher Scientific, Waltham, MA, USA) were as follows: *Adam15* Mm00477328_m1, *Adam17* Mm00456428_m1, *Cdkn1a* Mm04205640_g1, *Cdkn2a* Mm00494449_m1, *Il6* Mm00446190_m1, *Il13* Mm00434204_m1, *Rplp0* Mm00725448_s1, *Runx2* Mm00501584_m1, and *Sirt1* Mm01168521_m1.

Immunofluorescent staining

Cryosectioned cartilage explants were air dried and fixed in neutral-buffered formalin and ice-cold acetone. After treatment with chondroitinase ABC, sections were incubated in blocking buffer (PBS containing 1% goat serum, 5% BSA) and washed in PBS. Primary antibodies were applied to detect ADAM15 (rabbit anti-ADAM15, Atlas Antibodies, HPA011633, 2 μg/ml) and/or perlecan (rat anti-perlecan, Millipore). After further washing in PBS, sections were incubated with appropriate Alexa Fluor-conjugated secondary antibodies and visualised on an Olympus BX51 fluorescent microscope. Optimal exposure time for each protein was determined as that giving a signal in positively-stained sections and no detectable staining in negatively-stained sections, avoiding over-exposure and signal saturation. Exposure times of individual channels were kept constant. Quantification was done on raw, unaltered images from at least six random regions using ImageJ (NIH, Bethesda, MD).

To validate ADAM15 staining, the anti-ADAM15 antibody was pre-incubated with a 10-fold molar excess of recombinant ADAM15, centrifuged, and the supernatant applied to sections.

Immunoblotting

Proteins were separated by SDS-PAGE and transferred to PVDF. Membranes were incubated (overnight, 4 ℃) with primary antibodies against ADAM15 (rabbit anti-ADAM15, Atlas Antibodies, HPA011633, 0.4 μg/ml) or actin (Abcam, ab3280), washed in PBS containing 0.2% TWEEN 20, and further incubated with appropriate detection antibodies for 1 h. After washing as before, signal was visualised using Clarity Western

ECL Blotting Substrate (Bio-Rad).

Statistical analysis

Statistical tests were performed in GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, CA), with all significant changes annotated in figures.

To evaluate whether there was significant variation in the age or sex of cartilage donors, groups were tested for normality using the D'Agostino and Pearson omnibus test. Two-way analysis of variance (ANOVA) was performed, and the forest plots (Fig. 2) were examined.

RT-qPCR results were analysed using two-tailed Student's t-tests (to compare between 2 groups), or one-way ANOVA (to compare between more than 2 groups). For the latter, the derived P values were corrected for multiplicity using Tukey's test.

For analysis of immunofluorescence images, the background-corrected integrated intensity values per cell were aggregated for the normal and OA groups (n=3-6 per group)[25], and the results analysed by one-way ANOVA.

Results

The secretome of murine Adam15$^{-/-}$ chondrocytes does not differ significantly from WT

To investigate potential substrates of ADAM15 in cartilage, we compared the abundance of proteins secreted into conditioned media of chondrocytes isolated from 6-day-old WT and *Adam15*$^{-/-}$ mice using LFQ LC-MS/MS. The analysis identified 108 ECM proteins (based on Uniprot annotation; Supplemental Table 1), including collagens, aggrecan, other matrix components and regulators of matrix turnover. The abundance of these was not altered in the conditioned media of *Adam15*$^{-/-}$ chondrocytes compared to their WT counterparts (Fig. 1, Supplementary Table 1). Abundance of the other 1297 proteins analysed was also not altered in *Adam15*$^{-/-}$ samples. This supports previous studies indicating *Adam15*$^{-/-}$ mice have no evident developmental cartilage defects or phenotype at birth[2].

Expression of ADAM15 is not altered in human OA cartilage

To evaluate whether expression of ADAM15 was altered in OA cartilage, we analyzed RNA isolated from cultured chondrocytes and also isolated directly from cartilage of age- and sex-matched (Fig. 2A) normal (n=12) and OA (n=8) donors. Expression of *ADAM15* was about 30% lower in OA chondrocytes than in normal chondrocytes [P=0.0129, 95% CI (-0.6904, -0.09374), Fig. 2B]. However, there was no significant difference in *ADAM15* expression in mRNA samples isolated directly from cartilage [P=0.7883, 95% CI (-1.263, 1.64), Fig. 2C]. This indicates that *ADAM15* expression is affected by chondrocyte isolation and *in vitro* culture, so we sought to analyze RNA isolated directly

from joint tissues in subsequent analyses. There was also no difference in expression of *ADAM15* in male and female cartilage [P=0.2187, 95% CI (-2.204, 0.5396), Fig. 2D].

On the other hand, we saw a strong age-dependent variation in *ADAM15* expression in cartilage, with low levels of expression in cartilage from young (<20 years of age) and older (>65 years of age) donors, and highest expression at intermediate ages (Fig. 2E). Since we had observed no significant difference in expression in OA donors, Fig. 2E shows data for both normal (closed circles) and OA (open circles) donors.

To evaluate whether ADAM15 is also reduced at the protein level with aging, immunohistochemistry (IHC) was performed on cryosections of cartilage using a polyclonal rabbit anti-human ADAM15 antibody. The specificity of the antibody was evaluated by pre-incubating it with a ten-fold molar excess of recombinant ADAM15 before IHC was performed. This successfully blocked antibody staining (Supplementary Fig. 1), confirming that the conditions used generated a specific ADAM15 signal. Semi-quantitative evaluation of ADAM15 staining in 6 donors confirmed a trend towards reduced ADAM15 expression in cartilage with age (Fig. 3).

Expression of Adam15 is not altered in murine OA cartilage

We also quantified *Adam15* expression in the DMM surgical model of OA, which recapitulates many of the molecular and pathological features of human OA. There was no change in *Adam15* expression in DMM-operated versus sham-operated knees 6 hours after joint destabilisation [P=0.4944, 95% CI (-0.2273, 0.1152), Fig. 4A], or 8 weeks [P=0.4100, 95% CI (-0.1467, 0.9667), Fig. 4B] and 12 weeks after surgery [P=0.1700, 95% CI (-0.2659, 0.6059), Fig. 4B].

Expression of Adam15 drops markedly in 18-month old mice

Changes in *Adam15* expression with age were analyzed by isolating RNA from knee joints of mice aged 0.2-18 months. Expression of *Adam15* was readily detectable in all samples, with C_t values below 20, but expression of the enzyme was significantly lower in old (18 month) individuals than in younger adults (3, 4.5 and 5.5 months). For example, *Adam15* expression was 45-fold lower at 18 months of age than at 4.5 months [P<0.0001, 95% CI (25.18, 64.48), Fig. 4C].

Age-dependent expression of a range of other genes was also examined in these samples. Expression of *Adam17*, a related metalloproteinase, was also higher at 4.5 months than at 18 months, but only by 2-fold [P=0.0012, 95% CI (0.2944, 1.478), Fig. 4D]. Similarly, expression of *Sirt1* and *Runx2* were 1.3-fold higher [P=0.0851, 95% CI (-0.1153, 0.2604), Fig. 4E] and 0.8-fold higher [P=0.0003, 95% CI (0.3250, 1.263), Fig. 4F], respectively, in 4.5-month-old mice compared with 18-month-old mice. No age-dependent variation in *Cdkn1a* (Fig. 4G) was observed, but expression of and *Cdkn2a* (Fig. 4H) and *Il6* (Fig. 4I) increased with age.

IL-13 stimulates expression of ADAM15 in human HTB94 chondrosarcoma cells

In an attempt to understand what may drive the age-dependent changes in ADAM15 expression observed, we screened a number of candidate regulators suggested to regulate ADAM15 in other cell types, or with possible relevance to OA, aging or inflammation using a human cell culture model.

Dihydrotestosterone (DHT) has been shown to increase *ADAM15* expression in MCF-7 breast cancer cells, while estradiol had no effect[26]. Neither hormone had any effect on

ADAM15 expression in HTB94 chondrosarcoma cells (Fig. 5A). Mirin, a small molecule inducer of senescence[27–29], suppressed *ADAM15* expression after 24 hours of treatment [P=0.0298, 95% CI (-0.6994, -0.06061), Fig. 5A], but this effect was not observed at 48 hours and mirin had no effect on protein levels of ADAM15. No effect was seen with the SIRT1 activator resveratrol or the SIRT1 small molecule inhibitor EX-527. Similarly, 3-methyladenine (3-MA), which inhibits autophagosome formation by inhibiting phosphatidylinositol 3-kinase (PI3K), had no effect on *ADAM15* expression.

Among the cytokines and inflammatory mediators tested, GM-CSF [P=0.0009, 95% CI (3.256, 0.6236), Fig. 5B] and IL-13 [P=0.0047, 95% CI (3.016, 0.3836), Fig. 5B] both stimulated *ADAM15* expression. No effect was seen with the other cytokines (M-CSF, TNF, IFNγ, IL-1, IL-4, IL-6, IL-10), growth factors (activin, BMP-7, CTGF, FGF2, FGF18, TGFβ) or inflammatory mediators (dexamethasone, PMA, retinoic acid, LPS) tested (Fig. 5A, B).

Treatment of HTB94 chondrosarcoma cells with 100 ng/ml IL-13 for 48 h also increased levels of ADAM15 by 2.5-fold at the protein level [P=0.0017, 95% CI (08426, 2.691), Fig. 5C]. IL-13 similarly stimulated expression of ADAM15 in primary porcine cartilage explants [P=0.0105, 95% CI (2.528, 0.6091), Suppl. Fig. 2].

To investigate whether IL-13 may contribute to the age-dependent changes in *Adam15* expression in the joint, we measured *Il13* expression in the samples used in Fig. 4. This showed that *Il13* expression was reduced by 75% in joints from 18-month-old mice compared with 4.5-month old animals [P=0.0062, 95% CI (0.903, 6.953), Fig. 5E]. Expression of *Adam15* in individual animals correlated with their expression of *Il13* (Suppl. Fig. 3, $R^2 = 0.7538$).

Discussion

Metalloproteinases have been extensively studied as mediators of cartilage degradation in OA, with enzymes such as MMP-13 and ADAMTS-5 thought to be of particular importance in degrading the cartilage extracellular matrix and promoting structural failure of joints. As a result, these enzymes have been the target of pharmaceutical and academic OA drug discovery programmes. Metalloproteinases were similarly extensively studied in the context of cancer, but translational development of metalloproteinase inhibitors failed in this field, due in part to high homology between the metalloproteinase catalytic domains, which caused off-target inhibition of metalloproteinases with homeostatic roles. This cross-reactivity is also likely to be important in the development of metalloproteinase-targeted OA therapies, in particular due to the chronic nature of OA and the high incidence of co-morbidities in aging OA patients. It is thus of considerable importance to identify metalloproteinases that have protective or homeostatic functions in the joint, and to establish the timing, location and regulation of their activities.

ADAM15 is the first metalloproteinase shown to protect against OA in adult murine joints, with *Adam15*$^{-/-}$ mice exhibiting higher levels of spontaneous OA at 12-14 months of age[1]. The mice have no evident developmental defects[2], and we observed no difference in secretion of extracellular matrix molecules by chondrocytes isolated from young animals. These data indicate the enzyme serves its protective function primarily in adult cartilage.

Using RNA extracted directly from normal and OA human knee cartilage, we found that expression of ADAM15 was not increased in OA samples relative to age-matched controls. Similarly, we saw no regulation of *Adam15* in the DMM surgical model of murine OA, either in the hours immediately after surgery or at the time of joint damage (8- and 12-weeks after surgery). These findings are in agreement with several microarray

studies, as previously reviewed by Yang *et al*.[24].

In contrast, we saw strong down-regulation of ADAM15 with age. In both human and murine samples, expression of ADAM15 was highest in adult joints, with lower expression in young and old donors. This bell-shaped expression profile further supports the conclusion that ADAM15 functions largely in adult tissues, and is required for cartilage maintenance rather than development. This expression pattern did not reflect global changes in expression with age, since minimal changes were observed in expression of the related metalloproteinase *Adam17* or the transcription factor *Runx2*, for example. Expression of the senescence markers *Il6*[30] and *Cdkn2a*[31,32] but not *Cdkn1a*[31,32] also increased in murine joints with age, in line with previous reports. We did not observe the previously reported aging-dependent decrease in expression of the histone deacetylase *Sirt1*, although this reduction has largely been reported at the protein level[33,34], possibly reflecting post-translational regulation. Based on the increase in *Il6* and *Cdkn2a*, we conclude that the decrease in *Adam15* expression occurs when joints showed cellular and biochemical signs of aging.

Given its apparent protective role in the joint, we were keen to explore what factors could drive expression of ADAM15 and underlie its reduced expression with aging. Mirin, a chemical inducer of cellular senescence, transiently reduced *ADAM15* mRNA levels in HTB94 chondrosarcoma, but had no effect on protein levels or on mRNA expression after 24 hours. Modulators of SIRT1 (resveratrol and EX-527) and autophagy (3-MA) had no effect on ADAM15 expression. Similarly, factors previously shown to stimulate small (2-4-fold) increases in *ADAM15* expression in cancer cells and RA synovial fibroblasts (i.e. dihydrotestosterone[26], LPS[11]) were ineffective in HTB94 chondrosarcoma cells, as were a range of other pro- and anti-inflammatory stimuli.

However, we saw a 3-fold increase in ADAM15 expression upon treatment with GM-

CSF and IL-13. GM-CSF may be of importance in immune contexts, but we considered IL-13 more likely to have a role in the joint, since it has been previously been shown to block collagen release from IL-1/oncostatin M-stimulated bovine nasal cartilage *in vitro*, potentially via suppressing *MMP3* and *MMP13* expression[35]. Broader suppressive effects on inflammatory signalling in OA[36] and RA synovial explants[37,38] and *in vivo* murine RA models[39–41] have also been reported.

IL-13 can bind either to the type I IL-13 receptor (a dimer of the IL-13 Receptor, IL13R, and the IL1-4 Receptor, IL4R) or to the decoy type II IL-13 receptor (a dimer of the IL13R). Both the IL13R and IL4R are expressed in cartilage, with no change in expression reported in OA[42]. IL-4 has been shown to block metalloproteinase expression[42] and matrix breakdown[43] in cytokine-stimulated OA cartilage explants, and polymorphisms in IL4 and IL4R have been associated with OA[44,45], supporting a potential role for this signalling pathway in joint homeostasis. To our knowledge, this is the first report showing a reduction in *Il13* expression in murine joints upon aging. Spadaro *et al*. found that IL-13 levels were higher in the synovial fluid of patients with inflammatory (rheumatoid and psoriatic) arthritis than those with OA[46], but direct comparison between OA and healthy synovial fluid was not reported. Analysis of OA susceptibility in *Il13*$^{-/-}$ mice would shed light on the role of this cytokine in the joint environment.

Recent studies have indicated that ADAM15 expression is increased by sheer stress in endothelial cells[12] and by scratch wounding of cultured glomerular mesangial cells[47]. ADAM15 expression may thus be regulated by mechanical stimuli, which would be of considerable importance in cartilage, although the absence of *Adam15* regulation in the DMM model argues against this possibility. Activity of ADAM17 can be mechanically stimulated through c-Src mediated signalling pathways[48], raising the possibility that joint biomechanics could alter ADAM15 activity as well as expression. ADAM15 has low

sensitivity to tissue inhibitor of metalloproteinases 3 (TIMP-3)[49], the physiological inhibitor of most ADAMs, supporting the possibility that access to its active site may be conformationally regulated. Changes in joint mechanics change with age may synergise with changes in cytokine profile to effect significant changes in ADAM15 expression and activity in the joint.

The molecular mechanism(s) by which ADAM15 protects cartilage remain unclear. Our proteomic analysis did not identify any novel ADAM15 substrates in chondrocytes, in line with previous limited identification of ADAM15 substrates in other cell types. This indicates that the enzyme either has high substrate specificity, requires activation through as yet unknown mechanisms, and/or serves its protective function by mechanism(s) other than proteolytic shedding. Cleavage of soluble and not membrane-bound substrates, for example, would require different proteomic approaches for identification. Alternatively, ADAM15 effects in cartilage may be independent of proteolytic activity, as has been shown for its effects on pathological retinal neovascularisation[50].

The roles of many other ADAM and ADAMTS metalloproteinases in adult cartilage and joints have also not yet been established. Many of these show strong regulation in OA[24], and given their participation in cell signalling and survival pathways, it is likely that at least some of these enzymes contribute to joint homeostasis. Further investigation of their activities will strengthen our ability to target dysregulated matrix turnover in OA.

Authors' contributions

Conception and design: CYY, GLH, TLV, MLD, SAM, SFL, HN, SDS, LT.

Collection and assembly of data: CYY, AC, SB, OM, JMZ, GLH, ALC, JMZ, SDS.

Analysis and interpretation of data: CYY, AC, OM, SAM, SFL, HN, SDS, LT.

Provision of study materials: TLV, MLD.

Drafting and revising the manuscript: CYY, HN, SDS, LT.

All authors approved the final article.

Competing interests

The authors have no competing interests.

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Fig. 1. Secretome of *Adam15*$^{-/-}$ chondrocytes is not significantly different from WT.

(A) Volcano plot showing the −log10 of P-values versus the log2 of protein abundance in media of *Adam15*$^{-/-}$ versus WT chondrocytes (n=3 animals per group). The hyperbolic curves represent a permutation-based FDR correction for multiple hypotheses (P = 0.05, s0 = 0.1). Detected proteins were below the curve after FDR, and thus were not significantly different between the groups. ECM proteins (based on Uniprot annotation) are shown in blue. (B) Abundance of collagens and aggrecan were not altered in the media of *Adam15*$^{-/-}$ chondrocytes compared to WT. (C) Abundance of other selected extracellular matrix components and regulators was also not altered in the medium of *Adam15*$^{-/-}$ chondrocytes.

Fig. 2. Expression of *ADAM15* is not altered in human OA cartilage, but is reduced with age.

(A) Forest plot showing that donors used for (B-D) were age- and sex-matched. Mean age for all samples was calculated and subtracted from the mean age of OA females (n = 5), OA males (n = 3), normal females (n = 6) and normal males (n = 6). Data are depicted as differences in means (ΔMean) with 95% CI. ΔMeans were not significantly different (P = 0.7886). (B) Chondrocytes were isolated from normal (n = 12, 58-75 years of age) and OA (n = 8, 58-84 years of age) cartilage and cells cultured in monolayer for 5 days. RNA was isolated and expression of *ADAM15* quantified by RT-qPCR, relative to *RPLP0* and to mean expression in normal chondrocytes (mean ± SD). (C) RNA was extracted from normal (n = 12, 58-75 years of age) and OA (n = 8, 58-84 years of age) cartilage samples matching those in (B), and expression of *ADAM15* quantified by RT-qPCR, relative to *RPLP0* and to mean expression in normal cartilage (mean ± SD). (D) Data shown in (C) were analysed by gender, irrespective of OA status, with expression normalised to the

mean of expression in female donors (mean ± SD). (E) Data shown in (C), along with data from an additional 4 normal donors (9-16 years of age), were analysed by age. Samples from normal cartilage are shown in closed circles (n = 16) and those from OA cartilage shown in open circles (n = 8). * $P < 0.05$, ***$P < 0.001$.

Fig. 3. Cartilage expression of ADAM15 also decreases with age at the protein level. (A) Cartilage sections from donors aged 47, 54 and 90 were stained with antibodies against ADAM15 (red) and perlecan (green), and counterstained with DAPI. Exposure times for each channel were kept constant for all samples. (B) Expression of ADAM15 was semi-quantitatively evaluated in 3 normal (red) and 3 OA (orange) donors of various ages, with each dot representing integrated fluorescence intensity per single cell, calculated from at least six random fields of view on three sections per donor. Median fluorescence intensity with 95% CIs of the mean are shown.

Fig. 4. Expression of *Adam15* is reduced with age in murine joints. (A) RNA was extracted from joints of male mice 6 hours after DMM or sham surgery and expression of *Adam15* quantified by RT-qPCR, relative to *Rplp0* and average expression in sham-operated joints (mean ± SD, n = 8 animals per group). (B) RNA was extracted from joints of male mice 8 and 12 weeks after DMM or sham surgery or from age-matched naïve controls, and expression of *Adam15* quantified by RT-qPCR, relative to *Rplp0* and average expression in naïve joints (mean ± SD, n = 5-13 animals per group). (C-I) RNA was extracted from joints of male mice aged 0.2 to 18 months as indicated, and expression of *Adam15* (C), *Adam17* (D), *Sirt1* (E), *Runx2* (F), *Cdkn1a* (G), *Cdkn2a* (H) and *Il6* (I) were quantified by RT-qPCR, relative to *Rplp0* (mean ± SD, n = 6-13 animals per group). Data are expressed relative to average expression in 18-month old mice (C) or 0.2 month old

mice (D-I). * $P \leq 0.05$, ** $P \leq 0.01$, ****, $P \leq 0.0001$.

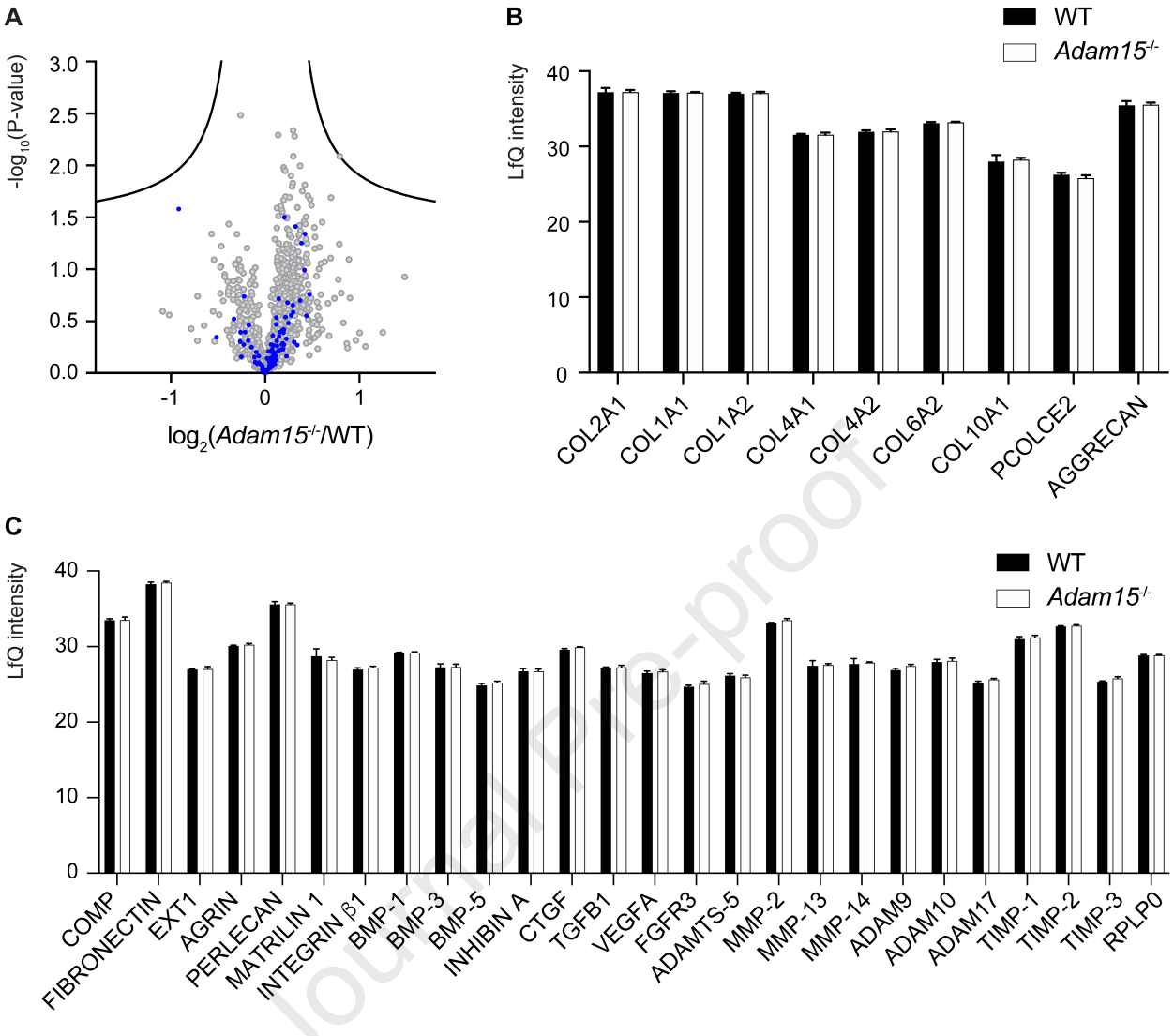
Fig. 5. IL-13 stimulates expression of ADAM15.

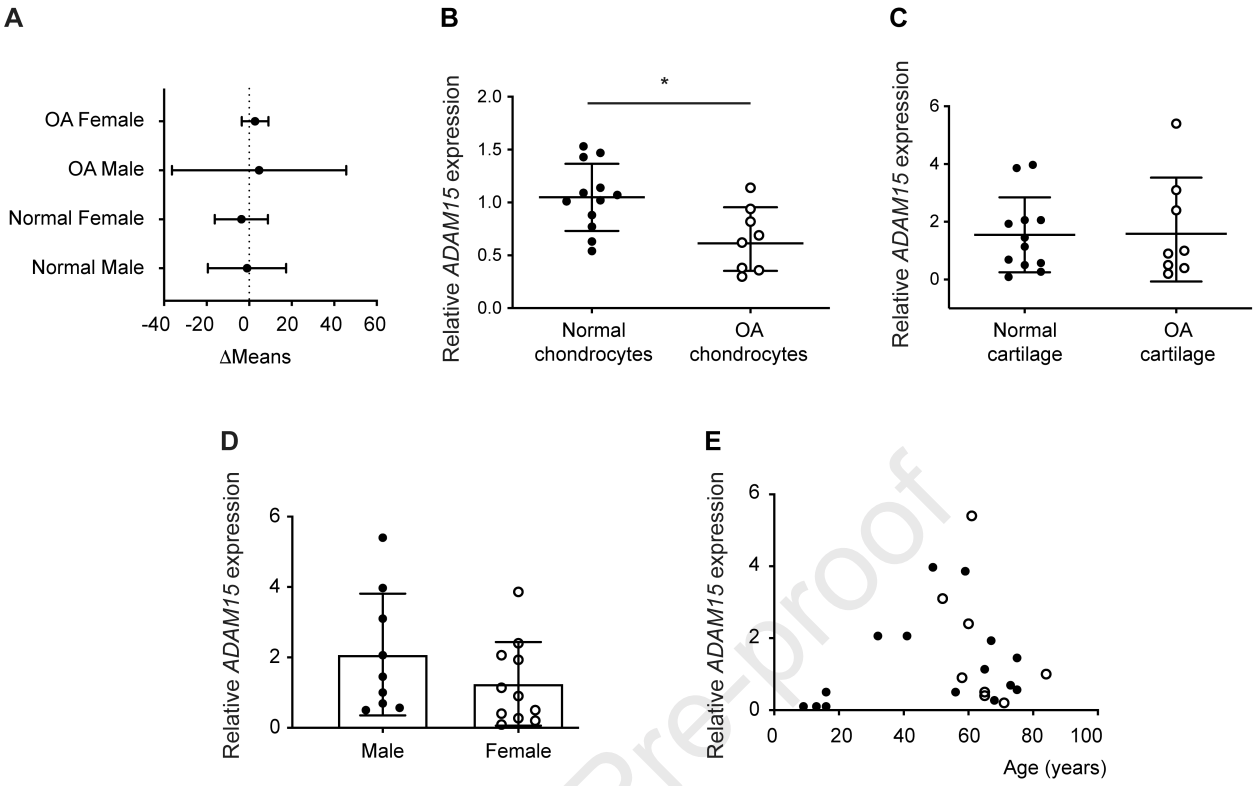
(A) After 24 h of serum starvation, HTB94 cells were treated for a further 24 h with Dexamethasone (1 μM, DMSO control), phorbol 12-myristate 13-acetate (DMSO control), dihydrotestosterone (DHT, 0.1 μM, methanol control), estradiol (0.1 μM, ethanol control), mirin (50 μM, DMSO control), resveratrol (10, 50, 100 μM, ethanol control), EX-527 (1, 10 μM, DMSO control) or 3-MA (5 μM, DMSO control). RNA was extracted and expression of *ADAM15* quantified by RT-qPCR, relative to *RPLP0* and mean expression the respective controls (mean ± SD, n = 3). (B) After 24 h of serum starvation, HTB94 cells were treated for a further 24 h with M-CSF (100 ng/ml), GM-CSF (50 ng/ml), TNF (100 ng/ml), IFNγ (100 ng/ml), IL-4 (20 ng/ml), IL-6 (20 ng/ml), IL-10 (10 ng/ml), IL-13 (20 ng/ml), LPS (100 ng/ml), activin A (50 ng/ml), BMP-7 (100 ng/ml), CTGF (100 ng/ml), FGF2 (100 ng/ml), FGF18 (100 ng/ml) or TGFβ (10, 100 and 1000 pg/ml), for 24 h. RNA was extracted and expression of *ADAM15* quantified by RT-qPCR, relative to *RPLP0* and the average expression in control (left panel) or DMSO-treated (right panel) cells (mean ± SD, n = 3). (C) After 24 h of serum starvation, HTB94 cells were treated with IL-13 (100 ng/ml) for 48 h, cells lysed in SDS sample buffer, and expression of ADAM15 and actin analysed by immunoblotting. (D) Band intensities from (B) were quantified and expression of ADAM15 normalised to actin plotted (mean ± SD, n = 6). Arrow indicates predicted position of active ADAM15. (E) RNA was extracted from joints of male mice aged 0.2 to 18 months as indicated, and expression of *Il13* quantified by RT-qPCR, relative to *Rplp0* and to average expression in 18-month old mice (mean ± SD, n = 6-13). * $P < 0.05$, ** $P < 0.01$.

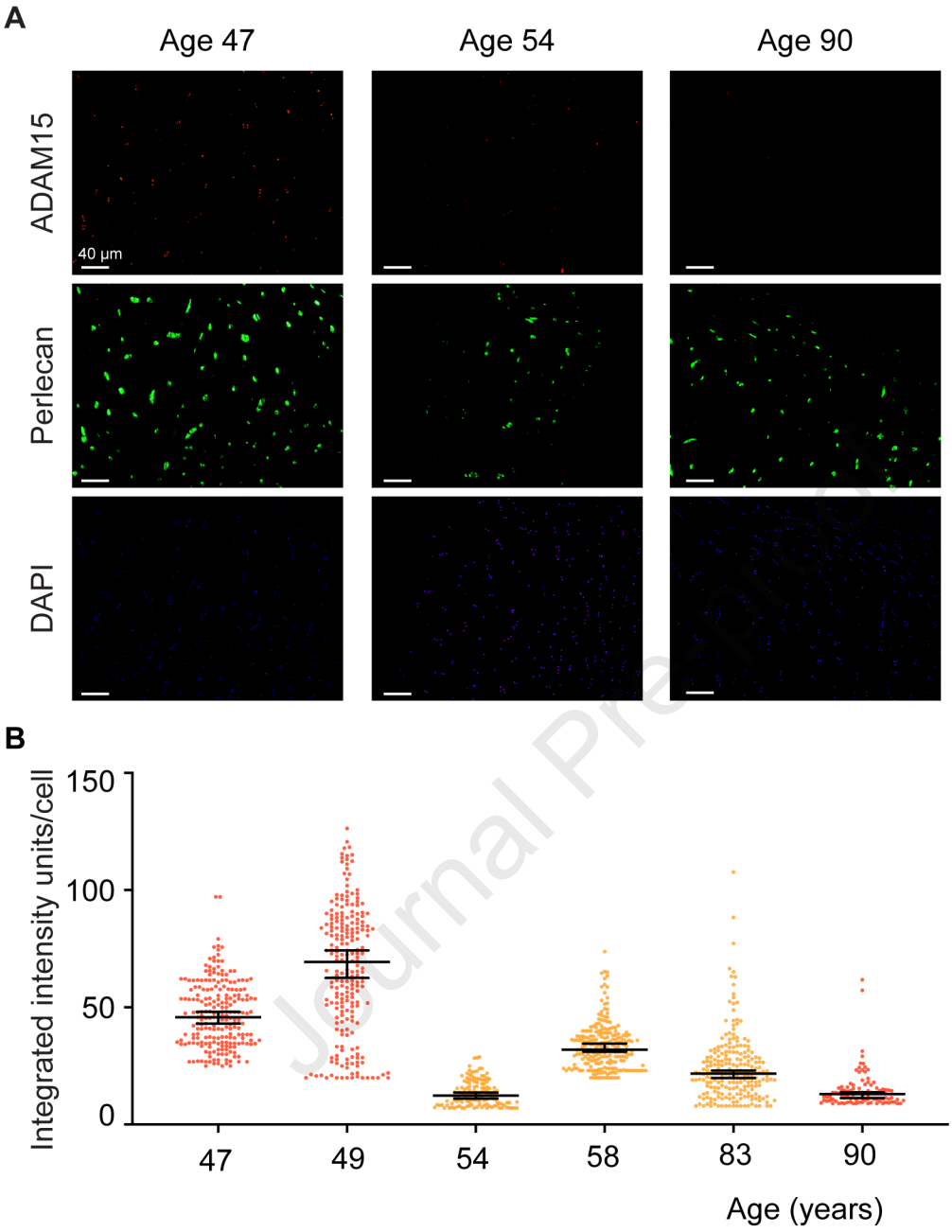
Supplementary Fig. 1. Validation of anti-ADAM15 antibody specificity by antigen blocking. Cryo-sections of OA cartilage were stained with antibodies against ADAM15 (red) and perlecan (green), and counterstained with DAPI. In right hand panels, the anti-ADAM15 antibody was pre-incubated with a 10-fold molar excess of recombinant ADAM15 in PBS for 30 minutes at room temperature. The antigen-antibody mixture was centrifuged (15 min, 17000 x g) and the supernatant applied to tissue sections. Images were taken using the same exposure times.

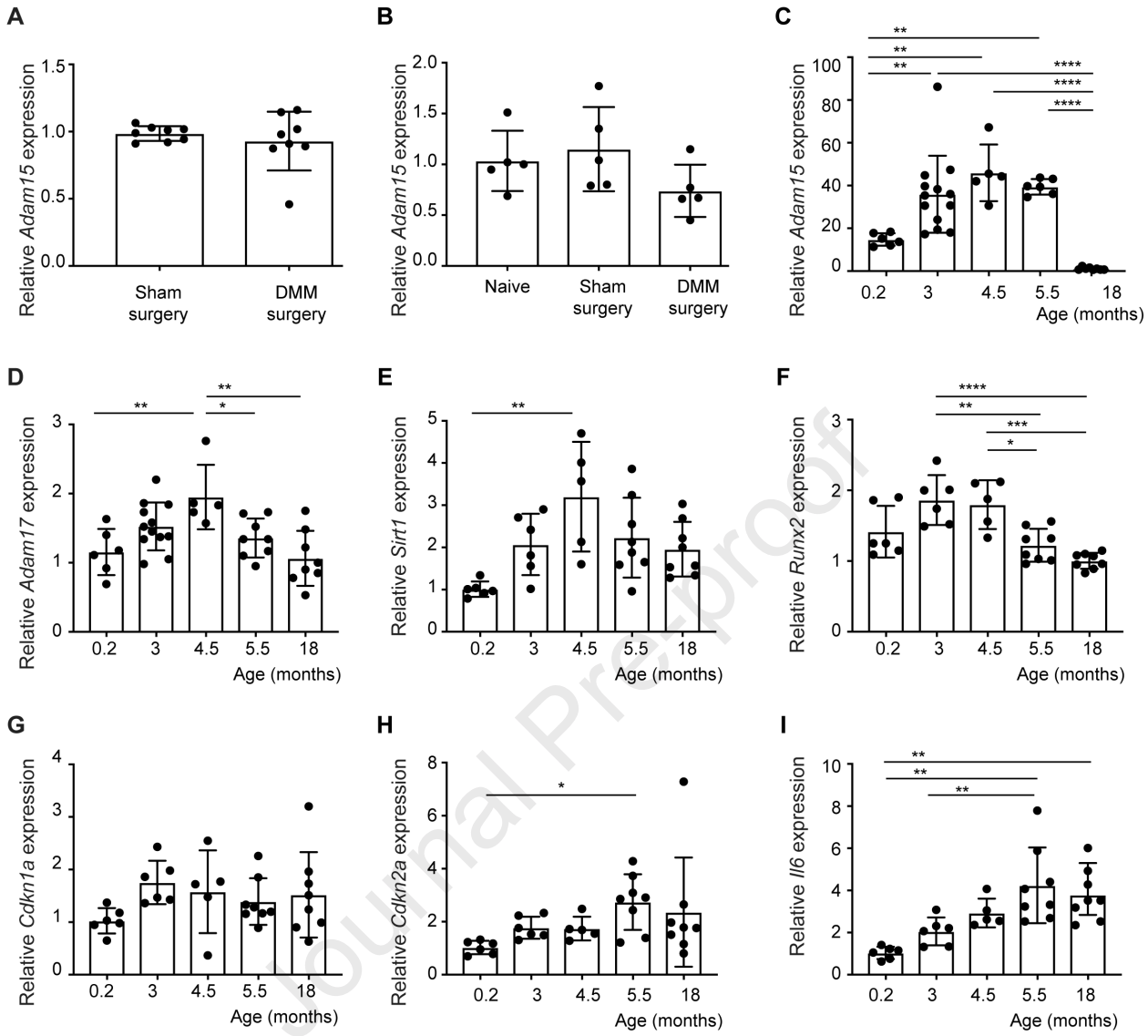
Supplementary Fig. 2. IL-13 stimulates expression of ADAM15 in porcine cartilage explants. Porcine cartilage explants were treated with IL-13 (100 ng/ml) for 48 h in serum-free DMEM, and conditioned media concentrated by addition of 5% trichloroacetic acid. Samples were analysed for ADAM15 expression by immunoblotting, and band intensities quantified (mean ± SD, n=3 technical replicates per group). * $P < 0.05$

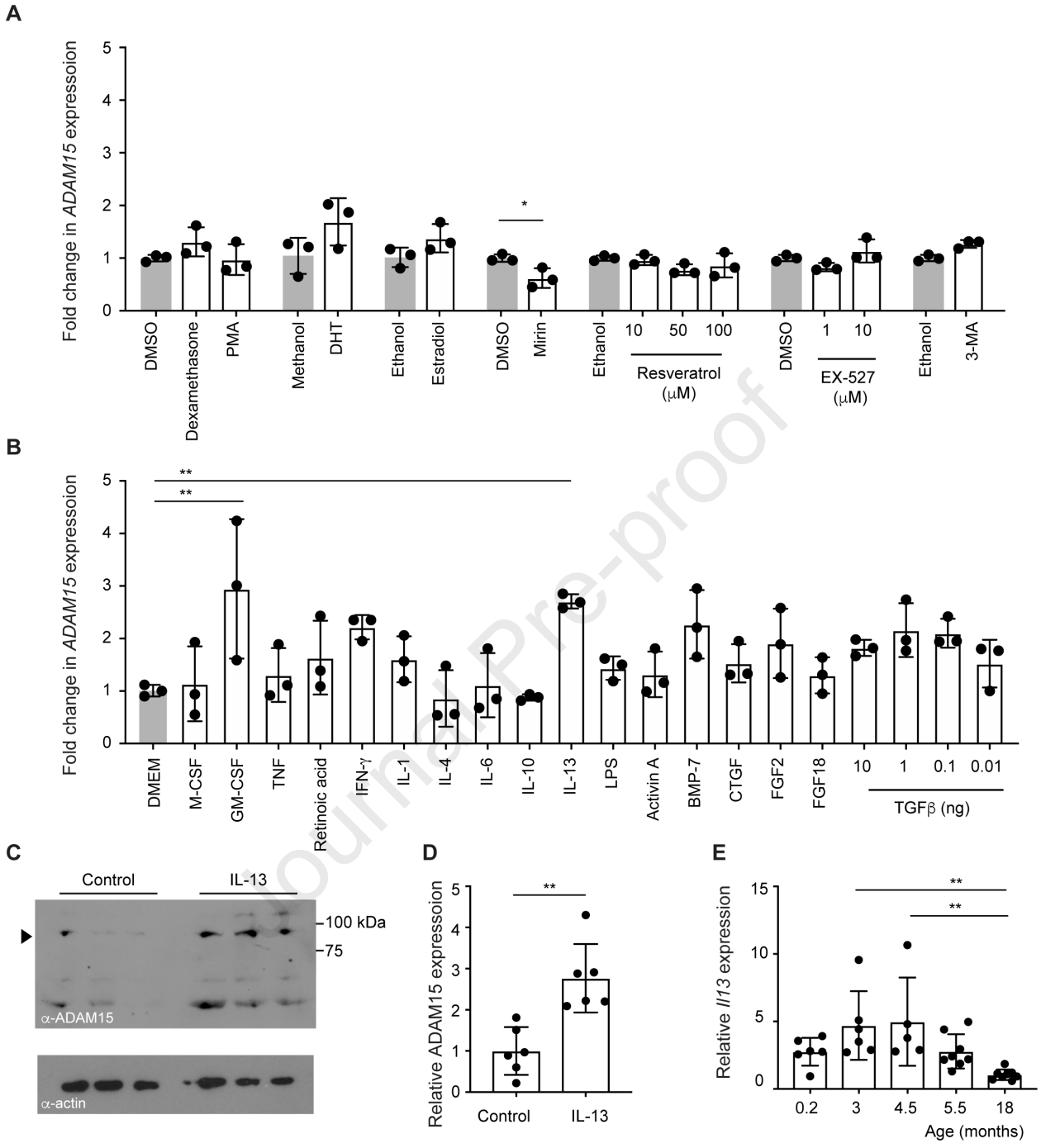
Supplementary Fig. 3. Correlation between expression of *Adam15* and *Il13* in murine joints. RNA was extracted from joints of male mice (aged 0.2, 3, 4.5, 5.5 and 18 months, n = 6-13 animals per age group) and expression of *Adam15* and *Il13* quantified by RT-qPCR, relative to *Rplp0*. ΔCt values for *Adam15* expression in each animal were plotted against their ΔCt values for *Il13*. Data were normally distributed and had a Pearson's correlation coefficient of 0.7538.










A
Fold change in ADAM15 expressoion
DMSO
Dexamethasone
PMA
Methanol
DHT
Ethanol
Estradiol
DMSO
Mirin
Ethanol
10
50
100
Resveratrol (μM)
DMSO
1
10
EX-527 (μM)
Ethanol
3-MA
B
Fold change in ADAM15 expressoion
DMEM
M-CSF
GM-CSF
TNF
Retinoic acid
IFN-γ
IL-1
IL-4
IL-6
IL-10
IL-13
LPS
Activin A
BMP-7
CTGF
FGF2
FGF18
10
1
0.1
0.01
TGFβ (ng)
C
Control
IL-13
100 kDa
75
α-ADAM15
α-actin
D
Relative ADAM15 expressoion
Control
IL-13
E
Relative Il13 expression
0.2
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4.5
5.5
18
Age (months)

OSTEOARTHRITIS AND CARTILAGE

AUTHORS' DISCLOSURE

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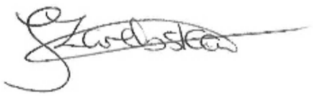
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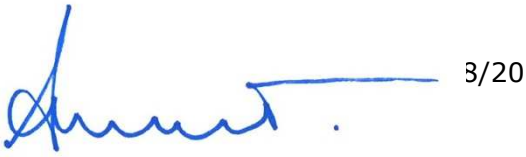
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