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Assi, R.; Cherifi, C.; Cornelis, F.M.F.; Zhou, Q.F.; Storms, L.; Pazmino, S.; ... ; Monteagudo, S.

TRANSLATIONAL SCIENCE

Inhibition of KDM7A/B histone demethylases restores H3K79 methylation and protects against osteoarthritis

Reem Assi ,[1] Chahrazad Cherifi,[1,2] Frederique M.F. Cornelis ,[1] Qiongfei Zhou,[1] Lies Storms,[1] Sofia Pazmino ,[3] Rodrigo Coutinho de Almeida ,[4] Ingrid Meulenbelt ,[4,5] Rik J. Lories ,[1,6] Silvia Monteagudo[1]

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For numbered affiliations see end of article.

Correspondence to
Professor Silvia Monteagudo; silvia.monteagudo@kuleuven.be
Dr Chahrazad Cherifi; cherifichahrazad@gmail.com

RA and CC are joint first authors.
RJL and SM are joint senior authors.

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ABSTRACT

Objectives In osteoarthritis, methylation of lysine 79 on histone H3 (H3K79me), a protective epigenetic mechanism, is reduced. Histone methylation levels are dynamically regulated by histone methyltransferases and demethylases. Here, we aimed to identify which histone demethylases regulate H3K79me in cartilage and investigate whether their targeting protects against osteoarthritis.

Methods We determined histone demethylase expression in human non-osteoarthritis and osteoarthritis cartilage using qPCR. The role of histone demethylase families and subfamilies on H3K79me was interrogated by treatment of human C28/I2 chondrocytes with pharmacological inhibitors, followed by western blot and immunofluorescence. We performed C28/I2 micromasses to evaluate effects on glycosaminoglycans by Alcian blue staining. Changes in H3K79me after destabilisation of the medial meniscus (DMM) in mice were determined by immunohistochemistry. Daminozide, a KDM2/7 subfamily inhibitor, was intra-articularly injected in mice upon DMM. Histone demethylases targeted by daminozide were individually silenced in chondrocytes to dissect their role on H3K79me and osteoarthritis.

Results We documented the expression signature of histone demethylases in human non-osteoarthritis and osteoarthritis articular cartilage. Inhibition of Jumonji-C demethylase family increased H3K79me in human chondrocytes. Blockade of KDM2/7 histone demethylases with daminozide increased H3K79me and glycosaminoglycans. In mouse articular cartilage, H3K79me decayed rapidly upon induction of joint injury. Early and sustained intra-articular treatment with daminozide enhanced H3K79me and exerted protective effects in mice upon DMM. Individual silencing of KDM7A/B demethylases in human chondrocytes demonstrated that KDM7A/B mediate protective effects of daminozide on H3K79me and osteoarthritis.

Conclusion Targeting KDM7A/B histone demethylases could be an attractive strategy to protect joints against osteoarthritis.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Osteoarthritis (OA) is the most common chronic joint disease worldwide for which no disease-modifying therapy is yet available. Alterations in histone modifications, which epigenetically regulate gene transcription, contribute to the mechanisms of OA.

⇒ OA is associated with deficient histone methylation of lysine 79 on histone H3 (H3K79me) in articular cartilage.

⇒ Reduction in H3K79me in human articular chondrocytes from non-OA donors via inhibition of DOT1L, the major H3K79 methyltransferase, shifted their molecular signature towards an OA-like profile. Intra-articular injection of a DOT1L inhibitor in mice triggered OA. Similarly, decreased H3K79me in mice on genetic deletion of *Dot1l* in cartilage is associated with increased progression of age-associated and post-traumatic OA. Altogether, these findings show that maintaining or restoring H3K79me is key to preserve joint health and prevent the onset and progression of OA.

WHAT THIS STUDY ADDS

⇒ We identify that KDM7A/B histone demethylases from the Jumonji C family regulate H3K79 methylation status in articular chondrocytes.

⇒ We provide evidence that inhibition of KDM7A/B histone demethylases can counteract H3K79me deficiency in OA cartilage and protect against the disease.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Targeting KDM7A/B histone demethylases provides a potential new disease-modifying strategy in OA.

INTRODUCTION

Osteoarthritis (OA) is the most common chronic joint disease, affecting more than 500 million people globally in 2019.[1] It is characterised by progressive damage to the articular cartilage, often accompanied by subchondral bone thickening, osteophyte formation and synovial inflammation.[2] OA is a leading cause of disability and enduring pain in older adults. Ageing and increasing obesity of the population are likely to compound this. The onset of clinical symptoms is preceded by poorly understood molecular and cellular changes in the joint that lead to loss of homoeostasis. Current management approaches are limited to symptom relief, exercise and rehabilitation strategies, and joint replacement surgery in severe cases. Thus, the development of a disease-modifying therapy is urgently needed.

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Joint homeostasis is highly dependent on epigenetic control mechanisms,[3] largely operated via post-translational histone modifications. Such mechanisms maintain the molecular identity of the articular chondrocyte. This is essential to preserve tissue integrity and cope with challenges such as joint injury or ageing.[4] Losing epigenetic control of the chondrocyte's molecular identity increases the susceptibility to develop OA.[4] Following the identification of a genetic association between histone methyltransferase gene *disruptor of telomeric silencing 1-like* (*DOT1L*) and OA,[5] functional studies revealed a key protective role for DOT1L in this disease.[6 7] DOT1L is the major methyltransferase that methylates Lysine-79 of Histone H3 (H3K79), thereby controlling gene expression programmes.[8 9] Levels of H3K79 methylation (H3K79me) are lower in articular chondrocytes from patients and mice with OA than in chondrocytes from healthy counterparts.[6 7] Reduction of H3K79me in articular chondrocytes from healthy patients shifted their cell identity towards an OA-like profile.[7] Similarly, decreased H3K79me in mice upon genetic deletion of *Dot1l* is associated with increased progression of age-associated and post-traumatic OA.[6] Thus, maintaining or restoring H3K79me levels seems to be critical to preserve joint health and prevent onset and progression of OA. Yet, mechanisms that regulate H3K79me beyond DOT1L remain largely unknown in cartilage biology and beyond.

Histone methylation was regarded irreversible until two families of histone demethylases erasing methyl marks were identified, underscoring the dynamic nature of this chromatin modification.[10] In this study, we hypothesised that blocking specific histone demethylases may increase H3K79me, independent of DOT1L activity. Using a discovery approach, we aimed to identify which histone demethylases regulate H3K79me in the articular chondrocyte and investigate whether their targeting can counteract H3K79me deficiency in OA cartilage and protect against the disease.

RESULTS

Gene expression profile of histone demethylases in human articular cartilage

First, we mapped the expression signature of all known histone demethylases in human articular cartilage. Histone demethylases are divided into two main families: the lysine-specific demethylases (LSD) and Jumonji-C (JmjC) demethylases. The LSD family consists of LSD1 and LSD2, whereas the JmjC demethylases are further classified into six subfamilies: KDM2, KDM3, KDM4, KDM5, KDM6 and KDM7.[10] We determined their baseline mRNA expression in primary human articular chondrocytes (hACs) isolated from patient donors with no history of OA (figure 1A). KDM2A showed the highest expression levels, while KDM2B, KDM4D and KDM4E were almost undetected. The differences observed in basal levels of histone demethylases suggested that articular chondrocytes have a specific expression pattern of these enzymes, which may be altered in OA. Next, to explore the differential role for specific histone demethylases in OA, we evaluated changes in their mRNA expression upon an OA-mimic stimulus in hACs. We treated these cells with interleukin-1β (IL1β), a known catabolic stimulator of chondrocytes (figure 1B).[11] As expected, IL1β reduced expression of healthy marker collagen 2a1 (*COL2A1*) and increased catabolic marker Matrix Metalloproteinase 13 (*MMP13*) (online supplemental figure 1A). Expression of *KDM2A*, *KDM2B*, *KDM3A*, *KDM3B*, *KDM5C*, *KDM6A*, *KDM6B*, *KDM7A* and *KDM7B* was increased upon IL1β treatment, while expression of *KDM4A* was decreased (figure 1B). Moreover, we determined whether there are differences in expression of histone demethylases in damaged areas of articular cartilage from OA patients, compared with their corresponding preserved areas. Expression of *KDM3A* and *KDM7A* was upregulated in damaged areas (figure 1C). These results show that expression of specific histone demethylases fluctuates in OA conditions, suggesting a differential role of these enzymes in this disease.

Inhibition of JmjC histone demethylases increases H3K79me in human chondrocytes

Next, we aimed to screen whether histone demethylases from the LSD or JmjC family are involved in H3K79 demethylation. To this end, human chondrocyte C28/I2 cells[12] were treated with JmjC family pan-inhibitor JIB-04. In parallel, LSD1 was pharmacologically inhibited using S2101 and LSD2 was knocked down by siRNA, as a pharmacological inhibitor for LSD2 is currently unavailable. We assessed levels of H3K79me by western blot analysis. Targeting LSD1 and LSD2 did not change H3K79me levels (figure 2A,B). The methylation mark H3K4me2, used as positive control in LSD1 and LSD2 experiments, was enhanced (online supplemental figure 2A, C). LSD2 knock down showed 80%–90% efficiency (online supplemental figure 2B). In contrast, inhibition of the JmjC demethylase family using JIB-04 resulted in increased H3K79me (figure 2C). H3K36me2 and H3K27me3 were used as positive controls (online supplemental figure 2D). DOT1L protein expression did not increase upon JIB-04 treatment, indicating that the increase in H3K79me occurs in a DOT1L-independent manner (figure 2D). To further confirm our observations, immunofluorescence for H3K79me was performed. Immunofluorescence signal showed an increase in H3K79me when cells were treated with JIB-04 and not upon LSD1 inhibition (figure 2E). The selective DOT1L inhibitor EPZ-5676 was used as a negative control demonstrating specificity of the staining (figure 2E). Collectively, these results show that JmjC histone demethylases are linked to H3K79me mark removal.

Inhibition of KDM2/7 histone demethylases by daminozide increases H3K79me and glycosaminoglycans in human chondrocytes

To dissect the role of the different JmjC demethylase subfamilies in H3K79me regulation, we first used different selective inhibitors, namely daminozide, CP2, C70 and GSK-J4, which respectively target KDM2/7, KDM4, KDM5 and KDM6 subfamilies. Pharmacological blockade of KDM2/7 demethylases with daminozide resulted in increased H3K79me in C28/I2 cells (figure 3A). Inhibition of KDM4, KDM5 and KDM6 demethylases by CP2, C70 or GSK-J4, respectively, did not change H3K79me (figure 3B–D).

Then, we screened whether blockade of the JmjC histone demethylase subfamilies indicated earlier leads to beneficial effects in human chondrocytes in vitro. To this end, we treated three-dimensional micromass cultures of C28/I2 cells with the different JmjC subfamily inhibitors and evaluated changes in glycosaminoglycans by Alcian blue staining (figure 3E–H). We found that treatment of these micromasses with daminozide (figure 3E), and to a lesser extent with GSK-4 (figure 3H), increased glycosaminoglycan content. Treatment with CP2 and C70 did not show a positive effect (figure 3F,G). Altogether, these results indicate that blockade of KDM2/7 histone demethylases by daminozide increases H3K79me and has pro-chondrogenic effects in human chondrocytes. Thus, daminozide exhibited an

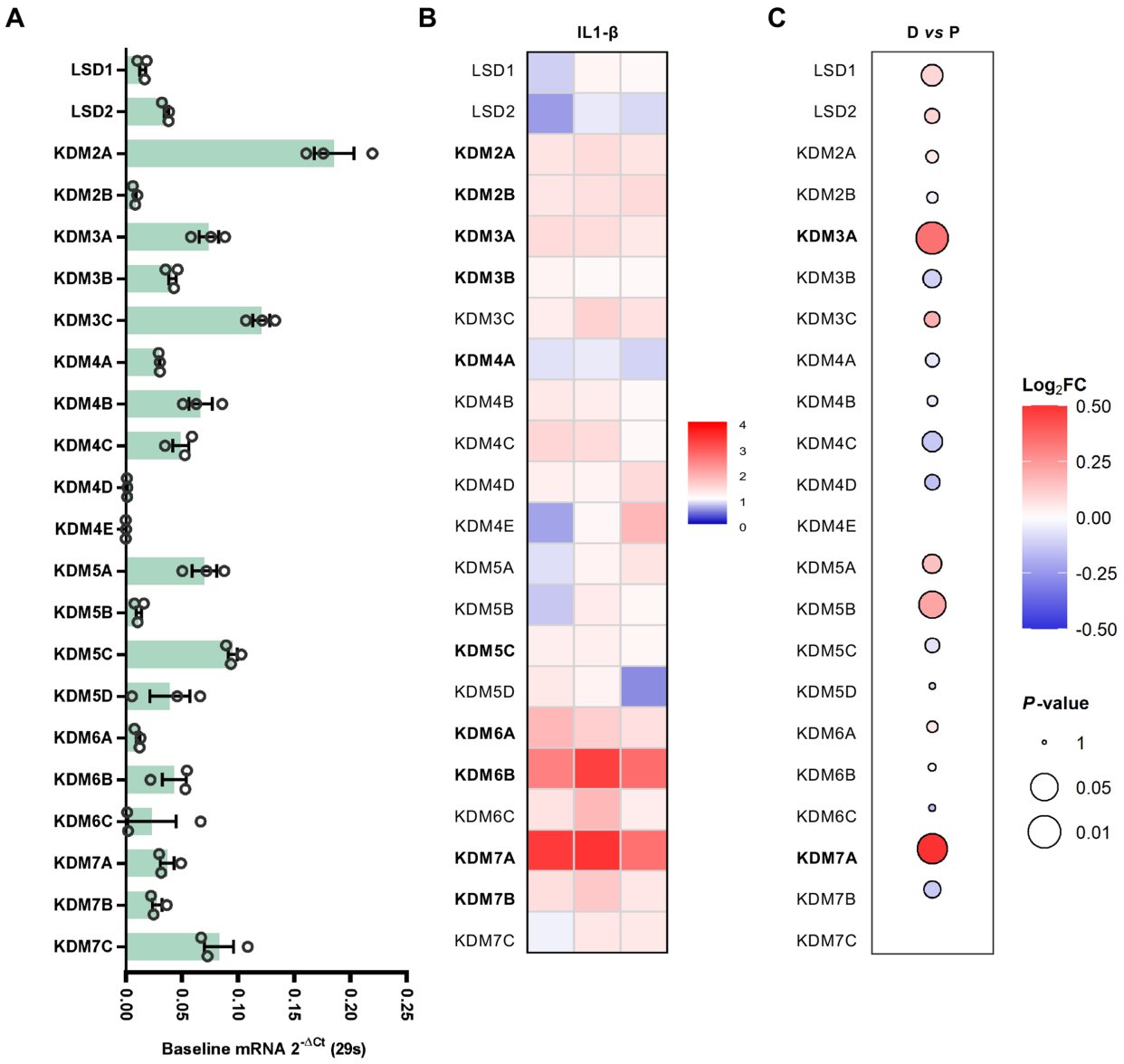

Figure 1 Gene expression profile of histone demethylases in human articular chondrocytes and cartilage. (A) Real-time PCR of histone demethylases gene expression in cultured primary human articular chondrocytes (hACs) obtained from non-OA controls, calculated as relative expression ($2^{-\Delta Ct}$) using S29 as reference gene. Three independent experiments with three technical replicates per condition were performed. Error bars indicate±SEM. (B) Real-time PCR of histone demethylases gene expression in hACs treated with IL1β. Each column represents one of three independent experiments with three technical replicates per condition. Paired t-test with false discovery rate (FDR) adjusted p value (*p<0.05 q<0.1 indicated in bold). (C) RNA sequencing data showing expression of histone demethylases in preserved (P) and damaged (D) articular cartilage from hips and knees of osteoarthritic patients (log2-fold change (Log2FC) of D vs P) (n=21, p values by Benjamini-Hochberg (BH) adjusted paired t-test). OA, osteoarthritis.

interesting potential for in vivo evaluation in an OA preclinical animal model.

Intra-articular treatment with daminozide restores H3K79me and reduces severity of OA in mice upon joint injury

We investigated the effects of KDM2/7 inhibition by daminozide on OA, using a well-established mouse model of the disease. We previously demonstrated that H3K79me is reduced in the articular cartilage of wild-type (WT) mice upon induction of OA by destabilisation of the medial meniscus (DMM) and upon ageing.[6 7] H3K79me kinetics at different time points (3 days, 1 week, 2 weeks, 12 weeks) after DMM surgery showed that H3K79me decays rapidly, with large differences 1 and 2 weeks after injury (difference of means in relative intensity 40.15—(95% CI 25.5 to 54.73)—p<0.0001, 36.22—(95% CI 21.64 to 50.81)—p<0.0001 at 1 and 2 weeks, respectively) compared with sham controls (figure 4A). Low levels of H3K79me were maintained 12 weeks after DMM (figure 4A). Considering these observations, we started intra-articular treatment with daminozide 1 week after DMM and repeated it every 10 days, up to 12 weeks. Knee joints were collected 12 weeks after surgery for histological analysis (figure 4B). Treatment with daminozide increased H3K79me after in vivo injury (difference of means in relative intensity 28.89 (95% CI 0.9947 to 56.79), p=0.0423) (figure 4C). To assess disease severity, cartilage damage and synovial inflammation were scored following OARSI guidelines. Daminozide treatment reduced cartilage damage observed in DMM mice (difference of means in OARSI score, 2.159 (95% CI 0.2320 to 4.086), p=0.0264)

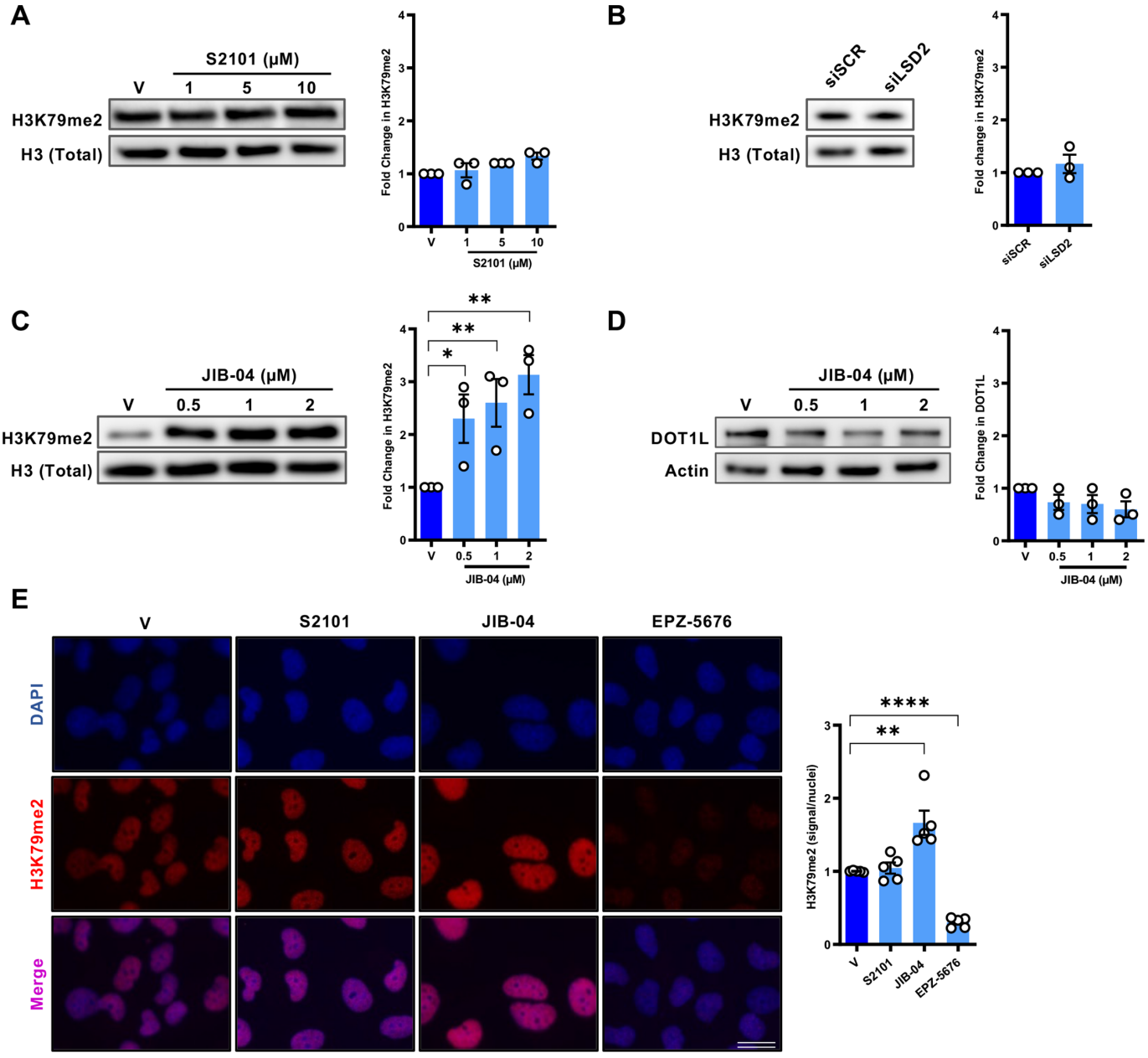

Figure 2 Inhibition of JmjC histone demethylases increases H3K79me in human chondrocytes. (A–D) Immunoblot of H3K79me or DOT1L (with total histone 3 (H3) or Actin as loading control) in C28/I2 cells in monolayer treated with S2101 which targets LSD1 (A), siRNA against LSD2 (siLSD2) or scrambled control (siSCR) (B), JmjC family pan-inhibitor JIB-04 (C–D). Quantification graphs of fold change for H3K79me or DOT1L normalised to H3 or Actin using FIJI software are presented on the right of each corresponding immunoblot. Three independent experiments with three pooled technical replicates per condition were performed. H3K79me/H3 blots were run on two different gels and processed in parallel. Ordinary one-way ANOVA with Dunnett's multiple comparisons test correction and paired t-test **p<0.01, ***p<0.001. (D) Immunofluorescence staining of H3K79me (red) and DAPI (blue) in C28/I2 cells in monolayer following treatment with vehicle (V), S2101, JIB-04 or EPZ-5676 (negative control). Images are representatives of five independent experiments with technical duplicates. Scale bar: 20 µm. Fluorescence intensity relative to V is represented in the graph (n=20 images per condition for each experiment). Ordinary one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test correction **p<0.01, ****p<0.0001. Error bars indicate±SEM.

(figure 4D) but did not affect the mild synovial inflammation caused by DMM injury (online supplemental figure 3). We also evaluated the presence of osteophytes on sections of the knee joint. DMM mice treated with daminozide displayed less osteophyte formation compared with DMM mice treated with vehicle (difference of means in osteophyte score, 0.2700 (95% CI −0.04231 to 0.5823), p=0.0983) (figure 4E). Low H3K79me on DMM surgery was linked to increased ectopic chondrocyte hypertrophy in articular cartilage in vivo.[6] To determine whether daminozide treatment can also reduce the presence of hypertrophic chondrocytes in the articular cartilage of DMM mice, immunohistochemistry for the chondrocyte hypertrophy marker collagen type 10 (COLX) was performed. This analysis indicated that daminozide treatment decreased COLX protein levels in DMM mice (difference of means in relative intensity, 201.8 (95% CI 78.55 to 325.1), p=0.0024) (figure 4F). Furthermore, we detected neoepitopes of aggrecan degradation using NITEGE staining. Daminozide treatment prevented the increase of NITEGE observed in DMM mice

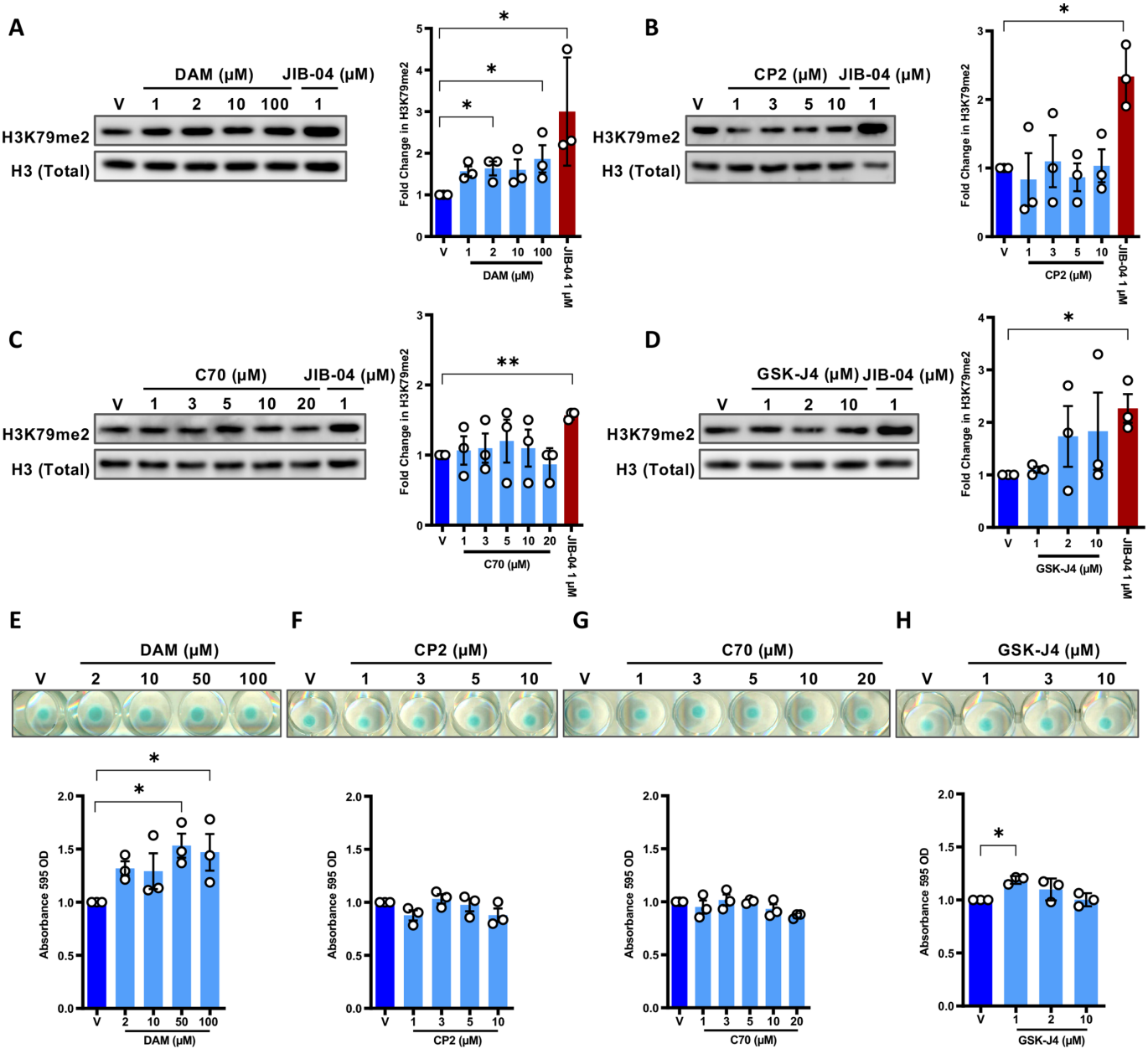

Figure 3 Inhibition of KDM2/7 histone demethylases by daminozide increases H3K79me and glycosaminoglycans in human chondrocytes. (A–D) Immunoblot of H3K79me (with H3 as loading control) in C28/I2 cells in monolayer treated with vehicle (V), daminozide (DAM), CP2, C70, GSK-J4 which, respectively, target KDM2/7, KDM4, KDM5 and KDM6 subfamilies, or with JmjC family pan-inhibitor JIB-04 (positive control). Quantification graphs of fold change for H3K79me normalised to H3 using FIJI software are presented on the right of each corresponding immunoblot. Three independent experiments with three pooled technical replicates per condition were performed. H3K79me/H3 blots were run on two different gels and processed in parallel. Ordinary one-way ANOVA with Dunnett's multiple comparisons test correction *p<0.05, **p<0.01. (E–H) Alcian blue staining of C28/I2 micromasses treated with V, DAM, CP2, C70 or GSK-J4. Images are representative of three independent experiments with three technical replicates per condition. Quantification of staining relative to V was determined by colorimetry at 595 nm and presented in a bar graph below each corresponding treatment. Ordinary one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test correction *p<0.05. Error bars indicate±SEM.

(difference of means in relative intensity 136.4 (95% CI 70.59 to 202.1), p=0.0004) (online supplemental figure 4). All these in vivo data indicate that local inhibition of KDM2/7 histone demethylases by daminozide increases H3K79me and reduces the progression of OA in mice.

Daminozide increases H3K79me and protects against OA via KDM7A/B inhibition

Next, we aimed to find out the mechanism via which daminozide increases H3K79me and protects against OA. Daminozide is reported to be a selective KDM2/7 subfamily inhibitor, with IC50 values of 1.5, 2.1 and 0.55 µM for KDM2A, KDM7A and KDM7B, respectively.[13] First, we interrogated whether KDM2A, KDM7A or KDM7B inhibition mediates effects of daminozide on H3K79me in human chondrocytes using monolayer cultures. Individual silencing of both *KDM7A* and *KDM7B* increased H3K79me, while silencing of *KDM2A* had no effect (figure 5A and online supplemental figure 5A). Then, we investigated whether KDM2A, KDM7A or KDM7B inhibition mediates protective effects of daminozide in OA. We previously reported

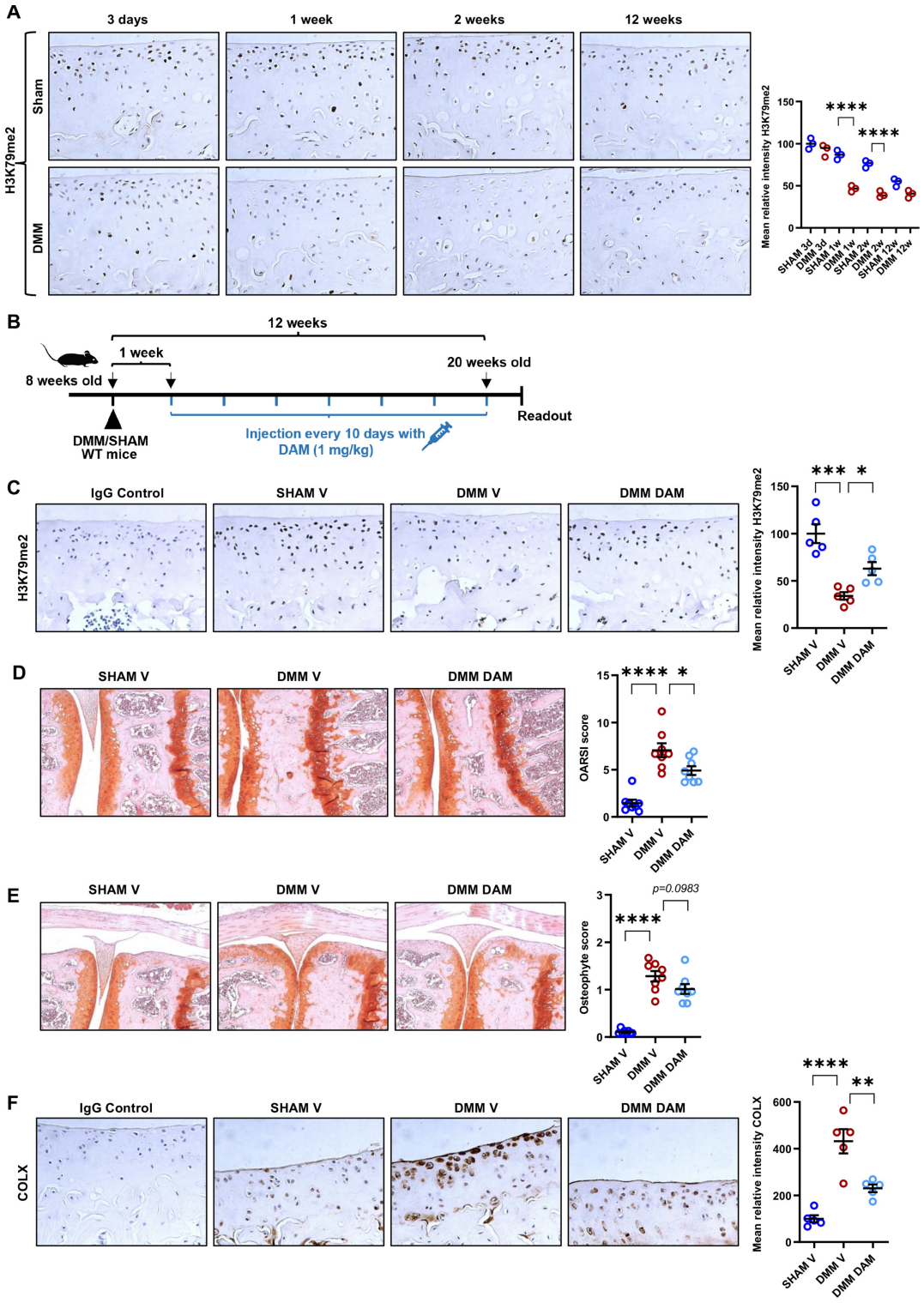

Figure 4 Intra-articular treatment with daminozide restores H3K79me and reduces the severity of osteoarthritis in mice on joint injury. (A) Time course of immunohistochemical detection of H3K79me in articular cartilage of DMM or sham-operated wild-type mice. (B) Scheme of intra-articular injections of daminozide (DAM) or vehicle (V) in DMM or sham-operated wild-type mice. (C) Immunohistochemical detection of H3K79me in articular cartilage of wild-type mice treated with dam or V on oa triggered by DMM surgery compared with sham-operated mice. (D) frontal haematoxylin-safraninO staining of the medial tibia and femur and quantification of articular cartilage damage at the four quadrants, evaluated by OARSI score (see the Methods section). (E) Frontal haematoxylin-safraninO staining of the medial tibia and femur and quantification of osteophytes at the medial compartment of tibia and femur. (F) Immunohistochemical detection of collagen 10 (COLX) in articular cartilage of wild-type mice treated with dam or V on oa triggered by DMM surgery compared with sham-operated mice. In a, the images are representatives of three mice per group. Scale bar: 50 µm. (D and E) images are representatives of 8 mice per group. Scale bar: 200 µm. (C and F) images are representatives of five mice per group. Quantification graphs of DAB staining using image J software are presented on the right of corresponding images. Scale bar: 50 µm. Ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test correction *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars indicate±SEM.

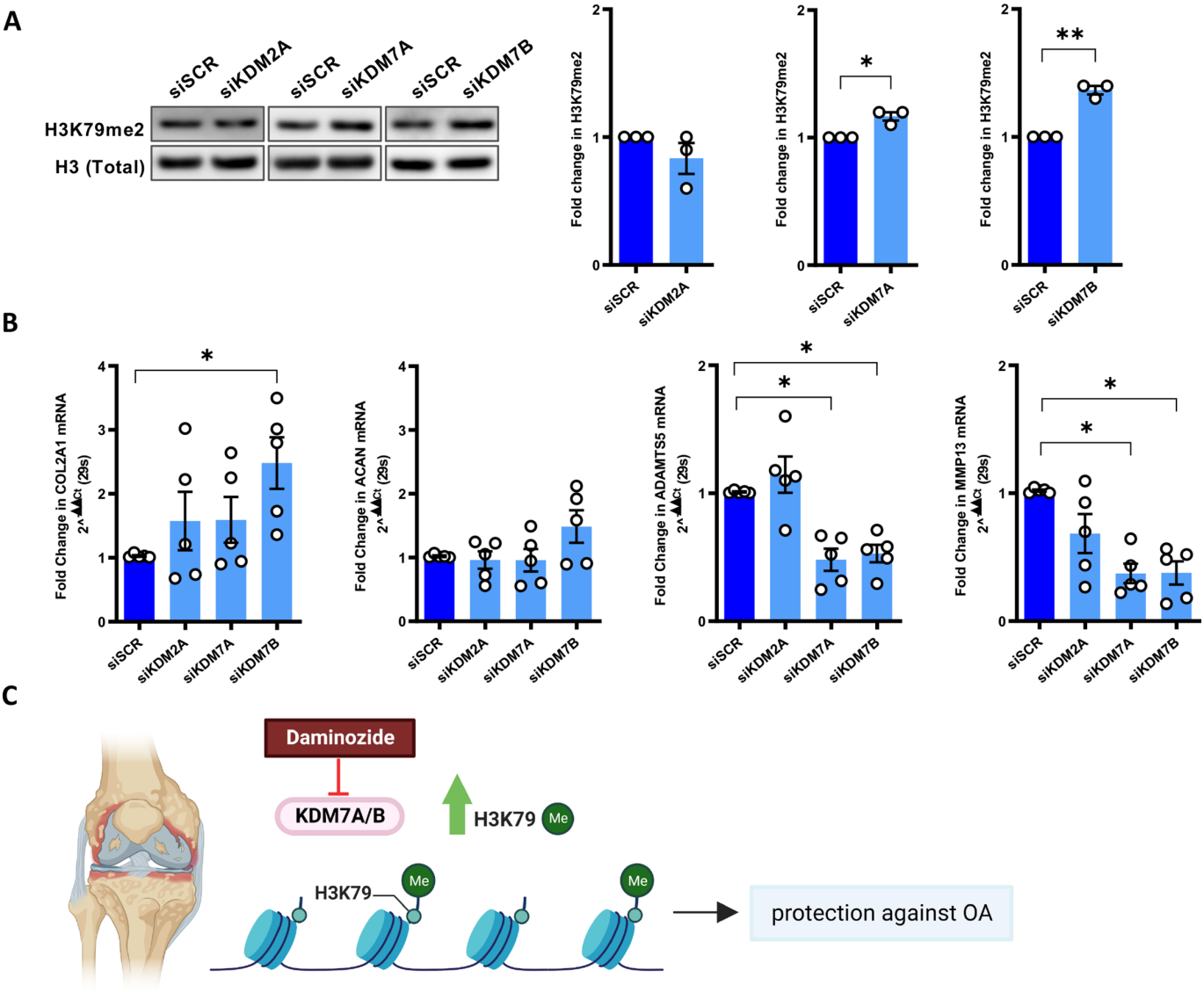

Figure 5 Daminozide increases H3K79me and protects against osteoarthritis via inhibition of KDM7A/B demethylases. (A) immunoblot of H3K79me (with H3 as loading control) following siRNA mediated silencing of *KDM2A, KDM7A*, *KDM7B* or scrambled control (SCR) in C28/I2 cells in monolayer. Quantification graphs of fold change for H3K79me normalised to H3 using Fiji software are presented on the right of immunoblots, respectively. Three independent experiments with three pooled technical replicates per condition were performed. H3K79me/H3 blots were run on two different gels and processed in parallel. Paired t-test *p<0.05, **p<0.01. (B) Real-time PCR of *COL2A1*, *ACAN*, *ADAMTS5* and *MMP13* in monolayer cultures of primary human articular chondrocytes isolated from five OA patients following siRNA mediated silencing of *KDM2A, KDM7A*, *KDM7B* or SCR. Repeated measures one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test correction *p<0.05. Error bars indicate±SEM. (C) Schematic diagram demonstrating the role of targeting KDM7A/B in regulating H3K79me and thereby protecting against OA. Created with BioRender.com. OA, osteoarthritis .

that H3K79me levels are reduced in hACs from OA patients, compared with cells from non-OA donors.[7] In these disease cells, cell identity is lost, and they exhibit a downregulation in healthy cartilage anabolic markers such as *COL2A1* and aggrecan (*ACAN*) while there is often increased expression of catabolic markers such as MMPs, especially MMP13, and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs), especially ADAMTS4 and ADAMTS5.[14 15] We assessed the impact of individual silencing of KDM2A, KDM7A and KDM7B histone demethylases on anabolic and catabolic markers in monolayer cultures of hACs from OA patients. *KDM7A* and *KDM7B* knockdown resulted in increased expression of *COL2A1* together with decreased expression of cartilage degrading enzymes *MMP13* and *ADAMTS5* (figure 5B and online supplemental figure 5B). In contrast, KDM2A silencing had no effects on anabolic or catabolic markers (figure 5B and online supplemental figure 5B). Thus, inhibition of KDM7A/B demethylases mediates the protective effects of daminozide in OA. Globally, our data demonstrate that targeting *KDM7A/B* demethylases increases H3K79me and favours the restoration of the chondrocyte identity, thereby protecting against OA (figure 5C).

DISCUSSION

In this study, we provide novel evidence that specific histone demethylases from the JmjC family control H3K79me in articular cartilage. We show evidence that pharmacological inhibition of KDM2/7 histone demethylases by daminozide enhances

H3K79me and positively affects the chondrogenic potential of human chondrocytes. We demonstrate that H3K79me decays rapidly in articular cartilage after induction of OA in mice by DMM, and early intervention with local daminozide treatment was efficient to prevent loss of H3K79me and protect against OA. Mechanistically, we demonstrate that these beneficial effects of daminozide are mediated by blockade of KDM7A/B histone demethylases, as demonstrated by their individual targeting in hACs from OA patients.

Up to date, different histone demethylases have been explored in OA.[16] Dysregulation in LSD1-mediated demethylation was detected in human OA chondrocytes. Increased LSD1 expression in the superficial and middle zones of OA cartilage contributed to aberrant H3K9 demethylation and LSD1 recruitment to the promoter of microsomal prostaglandin E synthase 1 (mPges-1), and stimulation of gene expression, suggesting potential involvement of this demethylase in OA.[17] In another study, LSD1 negatively regulated the anabolic gene *COL9A1* and potentially disrupted cartilage matrix.[18] Jun *et al* revealed that KDM6B is overexpressed in human OA cartilage, and its intra-articular inhibition by GSK-J4 can reverse cartilage damage in the DMM mouse model of OA. GSK-J4 attenuated the production of pro-inflammatory cytokines and catabolic enzymes in response to IL-1β in mouse chondrogenic cell line ATDC5 cells.[19] These findings were in contrast with other studies showing that KDM6B is essential for cartilage health.[19–21] Knockdown of KDM6B impaired cartilage development and accelerated OA progression in DMM mice.[20] Other histone demethylases reported to be implicated in OA include KDM2A, KDM3A, KDM5C and KDM6A.[22–25] Yet, to date, KDM7A/B demethylases have never been explored in OA.

Here, we demonstrate that KDM7A/B histone demethylases target H3K79me in human chondrocytes. Although extensive studies have been conducted to characterise the role of H3K79me in transcriptional regulation and its physiologic outcome, the identity of H3K79 demethylases has remained unknown. Thus, the scientific community assumed that H3K79 demethylation of certain genes was only due to histone renewal and cell division.[26] However, a recent report pointed out that active H3K79 demethylation occurs by KDM2B in the human kidney HEK293T cell line.[27] Yet, it was still unknown which specific histone demethylases control H3K79me in articular cartilage and whether their blockade can affect OA.

We document H3K79me kinetics in articular cartilage along the course of OA progression on DMM in mice. Our data evidence that H3K79me decays rapidly after OA induction, which pinpoints the importance of an early intervention after joint trauma to rewire the altered genetic landscape. Intra-articular treatment with daminozide increased H3K79me and protected against OA in this translational mouse model of the disease. Daminozide, previously widely employed as a plant growth regulator, has never been tested earlier with a disease-modifying aim in a preclinical animal model. Daminozide is reported to be a highly specific inhibitor of the KDM2/7 family of JmjC histone demethylases.[13] KDM2/7 histone demethylases display a distinctive expression pattern in articular chondrocytes that is altered in OA conditions (figure 1A–C). KDM7A displayed the highest level of upregulation in articular chondrocytes from healthy controls upon stimulation with IL1-β, and in damaged OA cartilage compared with its corresponding preserved cartilage from the same donor. Additionally, we observed an upregulation of both KDM2A and KDM7B upon IL1-β stimulation. Our mechanistic data demonstrated that daminozide exerts its protective effects via inhibition of KDM7A/B histone demethylases, and not via KDM2A inhibition. Daminozide at a much higher concentration (50 mg/kg) in combination with GSK-J4 in C57BL/6 mice reduced TNF-triggered leucocyte adhesion to endothelial cells.[28] In another study, daminozide impaired stemness and angiogenesis of breast cancer cells in vitro via KDM2A inhibition.[29] To our knowledge, our study is the first to investigate the in vivo effects of daminozide in a diseased joint and further explore the individual role of the histone demethylases targeted by the drug.

Our study has some limitations worth mentioning. Although we identify that KDM7A/B demethylases are involved in H3K79 demethylation in chondrocytes, we acknowledge that there might be additional histone demethylases involved in this histone mark's removal that remain unidentified. Yet, intra-articular administration of daminozide was efficient in eliciting protective effects in a translational mouse model of OA. The injection frequency we used for daminozide in vivo seemed to be well tolerated by the mice. However, in terms of practical application, injections every 10 days could be considered as an intensive treatment regimen and may not be feasible for patients. Intra-articular delivery of drugs to cartilage offers many advantages over the systemic exposure by providing better local bioavailability and reducing side effects resulting from systemic exposure.[30] Yet, local approaches will likely not be sufficient for patients with generalised OA. Although we show that daminozide exerts its effects via KDM7A/B demethylases, it is still a wide range pharmacological inhibitor that could induce off-target and undesirable effects. Therefore, further research should focus on the development of more specific inhibitors and reformulate them into molecules with good retention in cartilage and joint to optimise drug-release and disease-modifying effects. Moreover, the in vitro and in vivo experiments shown in this study evaluated the short-term effects of blocking histone demethylases in OA. Experiments with long-term inhibition of histone demethylases are needed to investigate the translational potential of these targets in ageing models of the disease.

In conclusion, this study identifies that KDM7A/B histone demethylases regulate H3K79me levels in the articular chondrocyte and demonstrates that their inhibition protects against OA. Thus, our findings pinpoint that targeting KDM7A/B histone demethylases can be an interesting potential treatment approach worth further investigation for this disabling widespread joint disease.

MATERIALS AND METHODS

Materials

JIB-04, S2102, daminozide, and GSK-J4, were purchased from Sigma. KDM5-C70 (C70) and CP2 were purchased from Xcess Biosciences and Selleckchem. IL-1β was obtained from R&D. EPZ-5676 was purchased from Chemietek. Non-targeting siGENOME siRNA (siSCR) or siGENOME siRNA against KDM2A, KDM7A and KDM7B were purchased from Dharmacon.

Cell culture

Human immortalised chondrogenic cell line, C28/I2,[31] was obtained from Merck Millipore and maintained in monolayer culture containing DMEM/F12 (Gibco), 10% fetal bovine serum (FBS) (Biowest), 1%(vol/vol) antibiotic/antimycotic (Gibco) and 1% l-glutamine (Gibco) at 37°C and 5% CO2 humidified atmosphere. HACs were isolated from patients' hips undergoing hip replacement surgery. They were obtained from OA patients, or from patients with no history of OA for osteoporotic or malignancy-associated fractures. First, cartilage is dissected into fragments using a scalpel and then rinsed in PBS supplemented

with 1% (vol/vol) antibiotic/antimycotic (Gibco). The fragments are cut into small pieces further incubated with 2 mg/mL pronase solution (Roche) in rotating motion at 37 °C for 2 hours and digested overnight at 37 °C in 1.5 mg/mL collagenase B solution (Roche). The preparation was filtered through a 70 µM strainer and cells cultured for in vitro studies in a humidified atmosphere at 37 °C and 5% CO_2. Culture medium consisted of DMEM/F12 (Gibco), 10% FBS (biowest), 1% (vol/vol) antibiotic/antimycotic (Gibco) and 1% L-glutamine (Gibco).[7]

Immunofluorescence

C28/I2 cells were seeded in eight-well chamber slides (Nunc Lab-Tek II —ThermoFisher Scientific). Cells were treated with EPZ-5676 (3 µM) for 96 hours, and with S2101 (1 µM) and JIB-04 (0.5 µM) for 72 hours. Then the cells were fixed with 3.7% Formaldehyde in PBS for 10 min at room temperature. 1% SDS in PBS was used to permeabilise fixed cells for 2 min, and 1% BSA in PBS was applied to block non-specific binding sites for 30 min at room temperature. Then cells were incubated with a primary antibody against H3K79me2 (Abcam, ab3594; dilution 1:2000) for 1 hour. After, cells were incubated with Alexa Fluor 555-conjugated secondary antibody (ThermoFisher, A-31572, 1:1000) for 1 hour at room temperature. Nuclei were stained with DAPI (ThermoFisher, #62249, 1:10 000). Visualisation was done using Olympus IX83 microscope in which images of ×40 magnification were taken per sample. FIJI software was used to quantify fluorescence intensity signals using 20 pictures per condition for each independent experiment.

Micromasses

C28/I2 cells were cultured in 10 µL droplets in 24-well plates at a density of 250 000 cells/micromass. Culture medium consisted of DMEM/F12 (Gibco), 10% FBS (Gibco), 1% (vol/vol) antibiotic/antimycotic (Gibco), 1% L-glutamine (Gibco) and insulin-transferrin-selenium (ITS) (Thermo Fisher). Micromasses were treated with vehicle (DMSO), daminozide (DAM), C70, CP2 and GSK-J4 for 72 hours. The micromasses were washed with PBS and fixed with ice-cold methanol for 1 hour at −20°C. After rinsing with PBS, the micromasses were stained with Alcian Blue (0.1% AB 8GX, Sigma) for 3 hours, washed with water and air dried. Quantification of the staining was performed by dissolving the micromasses with 6M guanidine (Sigma) for 6 hours and measuring the absorbance at 595 nm with a spectrophotometer (BioTek Synergy).

DMM mouse model of OA

WT male C57Bl/6 mice were purchased from Janvier (Le Genest St Isle, France). Post-traumatic OA was induced by the DMM. To this end, a mild instability of the knee was obtained by surgical transection of the medial menisco-tibial ligament of the right knee.[32] Sham-operated mice served as control.

Intra-articular DAM injections

One week after DMM surgery, mice were intra-articularly injected with daminozide (1 mg/kg) or vehicle (30% PEG400 and 5% DMSO in PBS) every 10 days for a total of 7 injections. The dose was determined empirically in a pilot assessment of three different doses (0.5, 1 and 5 mg/kg) and the administration schedule was similar to previous studies.[33 34] Twelve weeks after surgery, the knees were harvested and analysed. For the analysis, we focus on the interaction between surgery and drug intervention with OARSI score (see below) as primary outcome and considering 30% improvement biologically meaningful. Power calculations were based on historical and simulated data using the partial eta-squared value to estimate the effect size using the G*Power suggested 9 per group for 90% power.

Histology

Dissected mouse knees were fixed overnight at 4°C in 2% formaldehyde, decalcified for 3 weeks in 0.5 M EDTA pH 7.5 and embedded in paraffin. Severity of disease was determined by histological scores on haematoxylin safraninO stained sections throughout the knee (6 sections at 100 µm distance). Cartilage damage and synovial inflammation were assessed based on OARSI guidelines.[35] Depth of lesion (0–6) was scored on frontal knee sections. Lesion grades represent the following features: (0) surface and cartilage morphology intact, (1) small fibrillations without loss of cartilage, (2) vertical clefts below superficial layer and some loss of surface lamina, 3–6: vertical clefts/erosions to the calcified cartilage extending, (3) less than 25%, (4) 25%–50%, (5) 50%–75% and (6) more than 75%. The score represents the sum of the means of four quadrants. For synovial inflammation, severity was determined on a 0–3 scale. Osteophytes were scored following an in-house scoring system earlier reported.[6] The scoring of mouse joints was done in a blinded fashion by our team's expert (FMFC).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded EDTA-decalcified knee sections. The sections underwent heat induced epitope retrieval using NA-Citrate buffer (pH 6) for 10 min at 95°C and were treated with 3% H_2O_2/methanol or 3% H_2O_2/PBS for 10 min to inactivate endogenous peroxidase activity. Following that, the sections were blocked in 10% goat serum for 30 min and incubated overnight at 4°C with the primary antibody against H3K79me2 (5 µg/mL, Abcam, ab3594). For incubation with primary antibody against Collagen X (COLX) (1:400, Abcam, Ab58632) and NITEGE (1:50, PA1-1746, ThermoFisher Scientific), epitope retrieval was done using hyaluronidase (10 mg/mL, Sigma, H3884) for 40 min at 37°C and blocked in 1/5 normal goat serum for 1 hour. Rabbit IgG (Santa Cruz, sc-2027) was used as negative control. After incubation with the primary antibody, 1:100 biotin-conjugated-goat anti-rabbit (Vectastain ABC kit, Vector Laboratories) was applied for 30 min. DAB staining was applied, and pictures were captured using an Olympus IX83 microscope. Quantification of DAB staining by digital image analyses after immunohistochemistry was performed in a blinded fashion with colour deconvolution plugin (Jacqui Ross, Auckland University) in ImageJ Software (NIH Image, National Institutes of Health). Quantification was performed using the average of two technical replicates for each mouse per condition, with staining intensity reported relative to the average SHAM+Vehicle mice.

Statistics

Data analysis and graphical presentation were performed with GraphPad Prism V.9 and R-studio. Data are presented as individual data points, representing the mean of technical replicates and SEM as indicated in the figure legends. Gene expression data and ratio data were log-transformed for statistical analysis. All tests performed were two tailed where applicable. For comparisons between two groups, Student's t-test (unpaired and paired as indicated in figure legends) assuming equal variance was used. For comparisons between more than two groups, one-way analysis of variance (ANOVA) was used. Dunnett and Tukey corrections were used for multiple comparisons. For

multiple PCR testing in the screening experiments, correction for multiple testing was performed by applying a false discovery rate (FDR) test with q<0.10. RNA sequencing data were analysed as published before for the RAAK dataset,[36] with differential gene expression being tested pairwise between preserved and damaged samples by paired t-tests (preserved and damaged area per individual patient) followed by Benjamini-Hochberg correction to adjust for multiple testing. Data are reported by p values (significance <0.05).

Study approval

According to Belgian law and UZ Leuven's biobank policies, the joints are considered human biological residual material. Only age and sex of patients were shared between surgeons and investigators involved in this study. The material is fully anonymised without links to medical files. All mouse studies were performed with approval from the Ethics Committee for Animal Research (P114-2008, P198-2012, P159-2016; KU Leuven).

Author affiliations
[1]Development and Regeneration, Skeletal Biology and Engineering Research Center, Laboratory of Tissue Homeostasis and Disease, KU Leuven, Leuven, Belgium
[2]Glycobiology Cell Growth Tissue Repair and Regeneration Research Unit, Gly-CRRET, Univ Paris Est Créteil, Créteil, France
[3]Development and Regeneration, Skeletal Biology and Engineering Research Centre, KU Leuven, Leuven, Belgium
[4]Biomedical Data Sciences, Section of Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands
[5]Integrated research on Developmental determinants of Ageing and Longevity (IDEAL), Leiden University Medical Center, Leiden, The Netherlands
[6]Division of Rheumatology, University Hospitals Leuven, Leuven, Belgium

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

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ORCID iDs
Reem Assi http://orcid.org/0000-0002-3956-5610
Frederique M.F. Cornelis http://orcid.org/0000-0002-4144-5861
Sofia Pazmino http://orcid.org/0000-0001-8579-6914
Rodrigo Coutinho de Almeida http://orcid.org/0000-0001-7966-056X
Ingrid Meulenbelt http://orcid.org/0000-0001-7786-7081
Rik J. Lories http://orcid.org/0000-0002-5986-3092

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