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TRANSLATIONAL SCIENCE

Metabolic rewiring controlled by c-Fos governs cartilage integrity in osteoarthritis

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Handling editor Thomas Pap

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/ard-2023-224002).

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Received 9 February 2023
Accepted 11 May 2023
Published Online First 21 June 2023

ABSTRACT

Objectives The activator protein-1 (AP-1) transcription factor component c-Fos regulates chondrocyte proliferation and differentiation, but its involvement in osteoarthritis (OA) has not been functionally assessed.

Methods c-Fos expression was evaluated by immunohistochemistry on articular cartilage sections from patients with OA and mice subjected to the destabilisation of the medial meniscus (DMM) model of OA. Cartilage-specific c-Fos knockout (c-Fos$^{\Delta Ch}$) mice were generated by crossing *c-fos*$^{fl/fl}$ to *Col2a1-CreERT* mice. Articular cartilage was evaluated by histology, immunohistochemistry, RNA sequencing (RNA-seq), quantitative reverse transcription PCR (qRT-PCR) and *in situ* metabolic enzyme assays. The effect of dichloroacetic acid (DCA), an inhibitor of pyruvate dehydrogenase kinase (Pdk), was assessed in c-Fos$^{\Delta Ch}$ mice subjected to DMM.

Results FOS-positive chondrocytes were increased in human and murine OA cartilage during disease progression. Compared with c-Fos^{WT} mice, c-Fos$^{\Delta Ch}$ mice exhibited exacerbated DMM-induced cartilage destruction. Chondrocytes lacking c-Fos proliferate less, have shorter collagen fibres and reduced cartilage matrix. Comparative RNA-seq revealed a prominent anaerobic glycolysis gene expression signature. Consistently decreased pyruvate dehydrogenase (Pdh) and elevated lactate dehydrogenase (Ldh) enzymatic activities were measured *in situ*, which are likely due to higher expression of hypoxia-inducible factor-1α, *Ldha*, and Pdk1 in chondrocytes. *In vivo* treatment of c-Fos$^{\Delta Ch}$ mice with DCA restored Pdh/Ldh activity, chondrocyte proliferation, collagen biosynthesis and decreased cartilage damage after DMM, thereby reverting the deleterious effects of c-Fos inactivation.

Conclusions c-Fos modulates cellular bioenergetics in chondrocytes by balancing pyruvate flux between anaerobic glycolysis and the tricarboxylic acid cycle in response to OA signals. We identify a novel metabolic adaptation of chondrocytes controlled by c-Fos-containing AP-1 dimers that could be therapeutically relevant.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Anaerobic glycolysis is the primary energy source in healthy articular cartilage chondrocytes, but the early metabolic response of chondrocytes at the onset of osteoarthritis (OA) and how this could affect disease outcome is not known.

WHAT THIS STUDY ADDS

⇒ First time *in vivo* investigation of the role of c-Fos/AP-1 (activator protein-1) in chondrocyte metabolism using genetically modified mouse models, *in situ* metabolic enzyme assays and comparative OMIC analyses, providing insights into the early events of OA pathogenesis.

⇒ Demonstration that c-Fos, highly expressed in human and mouse OA samples, is essential for the cartilage-protective response of chondrocytes to stress.

⇒ Discovering that c-Fos regulates cellular bioenergetics and cartilage integrity in OA chondrocytes by modulating the activity of enzyme complexes that control pyruvate usage between anaerobic glycolysis and the tricarboxylic acid (TCA)-cycle/oxidative phosphorylation (OXPHOS).

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides new insights into how articular chondrocytes adapt to OA-associated signals and demonstrates a crucial role of cellular bioenergetics in cartilage integrity. Testing whether therapeutic intervention aimed at boosting the early metabolic stress response of chondrocytes to OA signals in larger and appropriately designed studies is crucial to improve disease outcomes, prevent disability and reduce healthcare and societal costs.

To cite: Matsuoka K, Bakiri L, Bilban M, *et al*. *Ann Rheum Dis* 2023;**82**:1227–1239.

INTRODUCTION

Osteoarthritis (OA) is the most common joint disease and its prevalence is growing in developed countries due to population ageing, more frequent biomechanical trauma and obesity.[1 2] OA has long been considered the consequence of a 'wear and tear' process leading to loss of articular cartilage. However, accumulating clinical and experimental data have changed this perception. Synovial inflammation is frequently present in clinical OA and in animal models[3 4], and metabolic mediators have been implicated in disease onset and/or progression.[5–8] These modulate metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families that breakdown articular

cartilage during OA progression.[9 10] Obesity and metabolic syndrome are recognized as strong risk factors for hand OA, but also knee OA, where low-grade chronic inflammation and systemic metabolic alterations disrupt joint tissue homoeostasis.[2 5 11] Several lines of evidence suggest that cells in the joint are subjected to metabolic alterations and shift from a resting state to a metabolically active state in order to meet the energy requirements of extracellular matrix (ECM) biosynthesis, cell proliferation and survival.[7 12] How articular chondrocytes, that primarily rely on glycolysis in low oxygen homoeostatic conditions, respond to joint damage and adapt to microenvironmental changes in articular cartilage is an important yet poorly understood question.

A large fraction (40%) of knee OA is heritable and genome-wide association studies indicated that most OA risk variants are located in non-coding sequences, and enriched close to genes involved in bone and cartilage development.[13 14] This suggests an important role for chondrocyte regulatory elements together with non-genetic factors in OA onset and/or development. Importantly, mutations in cartilage matrix genes such as *COL2A1*, *COL9A3*, *COL11A2* and cartilage oligomeric matrix protein *(COMP)*, which are produced by articular chondrocytes, cause chondrodysplasia with early-onset OA.[15] Recent advances in imaging technologies revealed that morphological changes in the collagen network and biomechanical changes in the articular cartilage are already present in early-stage OA.[16] This implies that chondrocytes maintain the structural and functional integrity of cartilage and sense microenvironmental changes during OA onset, although the underlying cartilage protective mechanisms remain elusive.

The Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra1, Fra2) proteins are components of the dimeric activator protein-1 (AP-1) transcription factor complex.[17] While Jun proteins can form homodimers or heterodimers, Fos proteins can only form heterodimers. AP-1 is activated by various signals, such as growth factors, inflammatory cytokines, mechanical and oxidative stress,[18–20] and has an essential role in cartilage and bone physiology.[17 21 22] For example, c-Jun is required for joint cell specification and intervertebral disc formation, while Fra2 regulates cartilage development and chondrocyte differentiation.[23 24] Importantly, ectopic expression of c-Fos in H2-*c-fos*LTR transgenic mice drives osteoblast and chondrocyte proliferation resulting in osteosarcoma and chondrogenic sarcoma.[25 26] In humans two single nucleotide polymorphisms in the FOS promoter were found to be associated with knee-OA susceptibility.[27] While the functional role of AP-1 in OA has not yet been thoroughly assessed *in vivo*, these data suggest that AP-1 dimers are likely important players in joint and cartilage biology, which led us to hypothesize that c-Fos expression in chondrocytes is functionally relevant in OA.

Here we show that FOS protein expression is increased in OA cartilage. To investigate the role of c-Fos containing AP-1 dimers (c-Fos/AP-1) in cartilage integrity, c-Fos was genetically inactivated in chondrocytes in the context of a well-established experimental OA model. We demonstrate that c-Fos controls chondrocyte response to microenvironmental stress caused by biomechanical and inflammatory cues. Furthermore, we identify a switch in energy metabolism from aerobic glycolysis to pyruvate oxidation and tricarboxylic acid (TCA) cycle in chondrocytes as a central mechanism in the early stages of experimental OA, with pyruvate and lactate dehydrogenases as critical metabolic enzymes downstream of c-Fos.

RESULTS

Damaged human and murine knee cartilage expresses high c-Fos/AP-1

First, FOS protein expression was evaluated in articular cartilage from 20 patients with knee OA. Cartilage destruction was graded using the Mankin score (MS)[28] and for each patient, two regions with high (MS≥8) or low (MS≤5) scores were selected and subjected to FOS immunohistochemistry (IHC). Regardless of the overall MS of the section, chondrocytes in the damaged regions close to the cartilage surface displayed more intense FOS staining, compared with cells in the middle and/or deeper areas (figure 1A). Quantitative analysis further revealed that FOS-positive articular chondrocytes were more abundant in severely damaged cartilage areas (MS 8–14) compared with non-damaged and/or mildly-damaged (MS 0–5) regions (figure 1B). Thus FOS expression is most elevated in OA-affected cartilage, compared with less damaged regions.

Next, adult wild-type mice were subjected to a well-established, surgery-induced OA model: destabilisation of the medial meniscus (DMM).[29] Articular cartilage damage was observed 8 weeks post DMM (figure 1C and D). IHC revealed increased expression of c-Fos and phosphorylated (activated) c-Fos in articular chondrocytes at 2 and 8 weeks post DMM (figure 1E and F and online supplemental figure 1A,B). c-Jun expression was also significantly elevated, although phosphorylated c-Jun was unchanged (online supplemental figure 1C,D). Sox9, a master regulator of chondrogenesis often cooperating with AP-1 was also increased (online supplemental figure 1E,F).[22] Importantly, the number of c-Fos-positive chondrocytes was already increased 2 weeks post DMM (figure 1E and F), before any damage was observed (figure 1D) suggesting that c-Fos/AP-1 could be implicated in the response of chondrocytes to OA-inducing signals.

Articular cartilage is protected by c-Fos in the DMM mouse model

Cartilage-specific tamoxifen (TAM)-inducible c-Fos loss-of-function (LOF) mice were generated combining *c-fos*$^{flox/flox}$ and *Col2a1-CreERT* by genetic crosses (c-Fos$^{\Delta Ch}$ mice).[30 31] In c-Fos$^{\Delta Ch}$ mice, a nuclear localisation signal-enhanced green fluorescent protein (nls-EGFP) sequence is inserted in the *c-fos* locus to place nuclear EGFP expression under the control of the *c-fos* promoter and regulatory elements on CRE/LoxP deletion (figure 2A). Immunofluorescence (IF) of EGFP in intact knee joints (10 weeks of age) revealed that approximately 70% (62–90%) of articular chondrocytes expressed EGFP 1 week after TAM injection (figure 2B) and a number of c-Fos-deficient (EGFP positive) chondrocytes were still detected in the articular cartilage 1 year later (online supplemental figure 2A,B). While no obvious growth abnormalities were observed in c-Fos$^{\Delta Ch}$ mice, safranin O/fast green staining of knee joint sections revealed lower glycosaminoglycans (GAGs) content, but comparable cartilage area in 1-year-old c-Fos$^{\Delta Ch}$ compared with c-Fos^{WT} mice (online supplemental figure 2B–D).

Next, 10-week-old c-Fos^{WT} and c-Fos$^{\Delta Ch}$ mice were subjected to DMM. While osteophyte (OP)-like bone/cartilage formations were observed in the medial tibial plateau and OP maturity increased over time, no significant differences in OP size and maturity were noted between operated groups (online supplemental figure 2F–G) and synovial thickening was comparable 2 weeks post DMM (online supplemental figure 2H,I). However, OARSI scoring revealed more severe DMM-induced cartilage damage in c-Fos$^{\Delta Ch}$ mice 8 weeks post surgery than those from c-Fos^{WT} mice (figure 2C and D). Consistently, the cartilage area

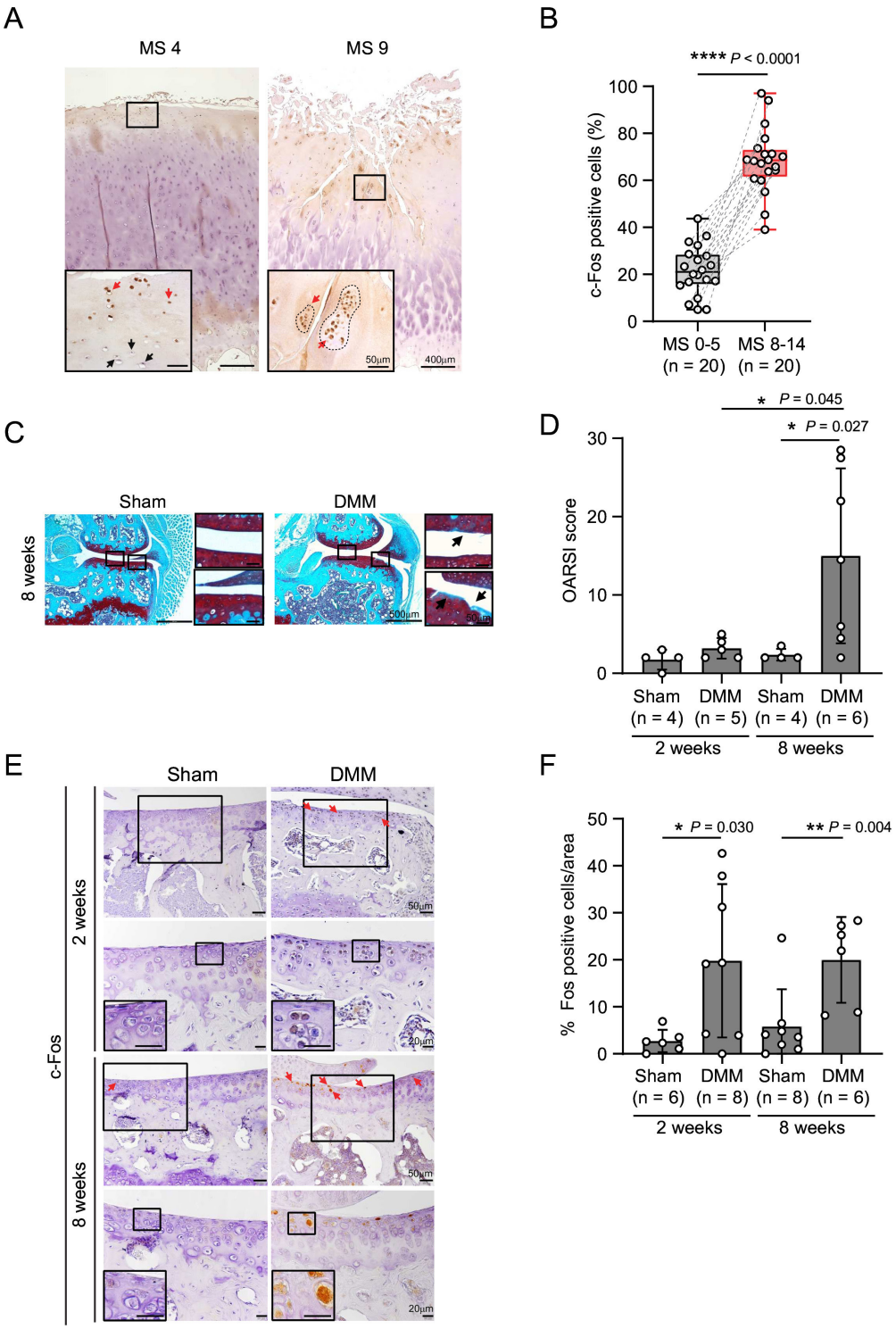

Figure 1 c-Fos is activated in articular chondrocytes in patients with OA and a mouse OA. (A and B) Femoral condyles of 20 patients undergoing knee arthroplasty were collected and histopathologically graded using the Mankin score (MS; 0: most intact; 14: most degenerated). From each patient, tissue regions with MS 0–5 and MS 8–14 were selected and histological sections were stained immunohistochemically (IHC) with antibodies against c-Fos. (A) IHC images of c-Fos in representative cartilage regions with MS 4 (left) and MS 9 (right). (B) Quantification of c-Fos-positive in MS 0–5 and MS 8–14 regions. The dotted lines connect the sample pairs from each patient. Statistical differences between groups were analysed by Mann-Whitney test. (C and D) 10 weeks-old wild-type mice were subjected to DMM (n=7)/sham (n=4) and cartilage damage was evaluated by Osteoarthritis Research Society International (OARSI) system 2 and 8 weeks post surgery. Red and black indicate c-Fos positive and negative cells, respectively. (C) Representative images of safranin O/fast green staining of the joint. (D) Quantification of cartilage damage. Black arrows indicate the damaged area. Statistical differences between groups were analysed by Mann-Whitney test. (E) Representative IHC images of c-Fos at 2 and 8 weeks post surgery. (F) Quantification of c-Fos positive cells. Red arrows indicate positive cells. Bar graphs and plots represent or include mean±SD, respectively. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001. Statistical differences between groups were analysed by non-parametric Mann-Whitney test in B and by two-way ANOVA with Bonferroni post hoc analysis in D and F. ANOVA, analysis of variance; DMM, destabilisation of the medial meniscus; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International.

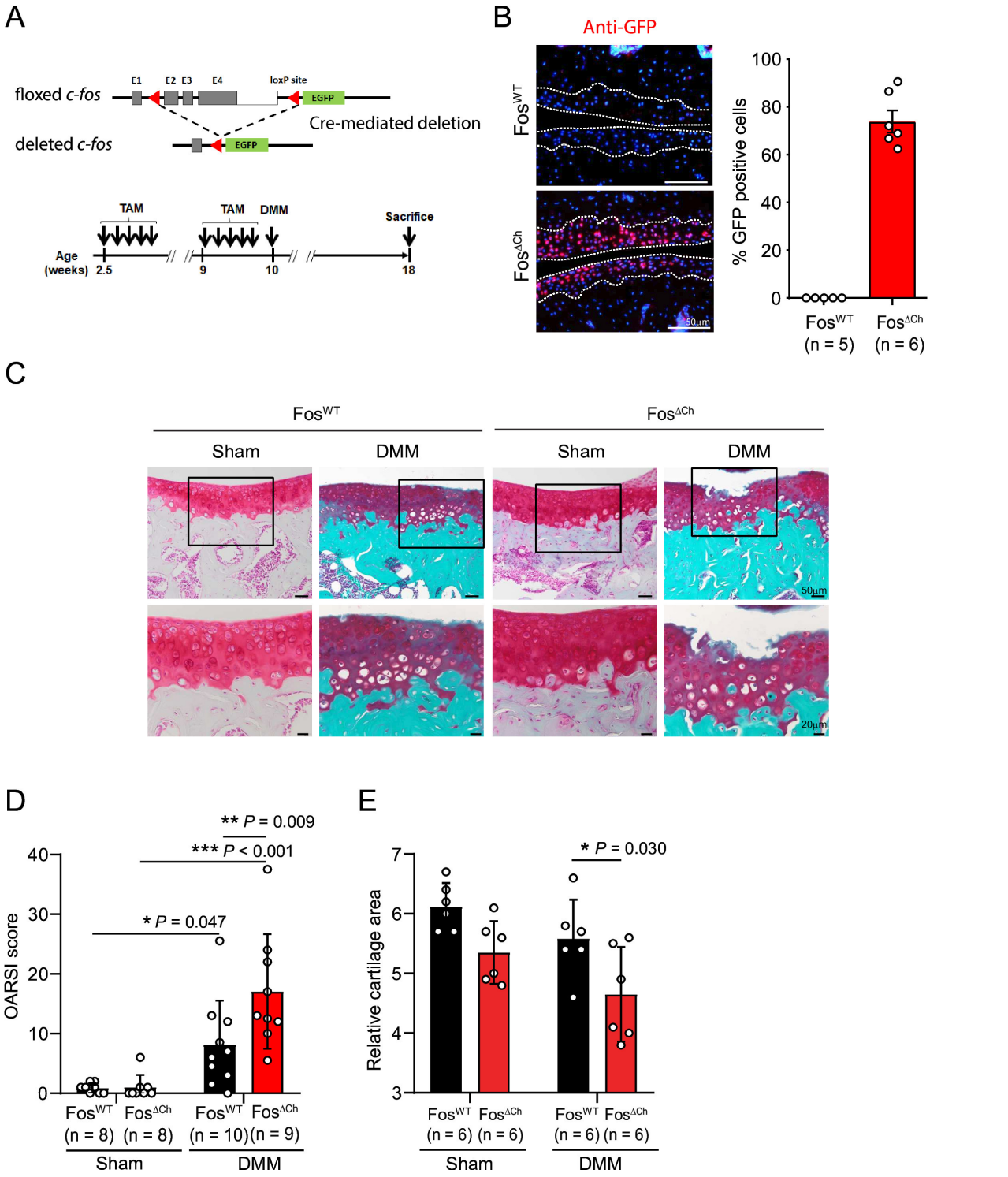

Figure 2 c-Fos protects knee cartilage in experimental OA. (A) Targeting strategy and structure of the floxed/deleted c-Fos allele. Cre expression results in deletion of exons 2–4 and expression of nuclear EGFP under the control of the c-fos promoter. Coding areas (exon 1–4) are depicted in grey boxes. Experimental procedure and timeline to delete *c-fos* in chondrocytes and experimentally induced cartilage damage (DMM). Tamoxifen was injected intraperitoneally at two time points (2.5 and 9 weeks of age, 2 mg/mouse/day, 5 consecutive days) and mice were subjected to DMM/sham at 10 weeks of age and knee joints analysed 8 weeks post surgery. (B) Analysis of c-Fos deletion by anti-GFP immunofluorescence (red) in c-Fos^{WT} and c-Fos$^{\Delta Ch}$ mice articular cartilage at 10 weeks of age. The left panels are representative images of GFP-positive articular chondrocytes before surgery. (nuclei counterstained with DAPI) while % GFP-positive articular chondrocytes are plotted on the right. Articular cartilage is depicted with white dashed lines. (C) Representative images of safranin O/fast green staining of knee joints from c-Fos^{WT} mice and c-Fos$^{\Delta Ch}$ mice 8 weeks post surgery. (D) Quantification of cartilage damage on the medial side. (E) Relative cartilage area quantified by ImageJ analysis. Bar graphs and plots represent or include mean±SD, respectively. *p<0.05, **p<0.01, and ***p<0.001. Statistical differences between groups were analysed by non-parametric Mann-Whitney test in B and by two-way ANOVA with Bonferroni post hoc analysis in D and E. ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; DMM, destabilisation of the medial meniscus; EGFP, enhanced green fluorescent protein; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; TAM, Tamoxifen.

after DMM was significantly smaller in c-Fos$^{\Delta Ch}$ mice than in c-Fos^{WT} mice (figure 2E). These data suggest that c-Fos expression in articular chondrocytes has a protective role during DMM-induced cartilage damage.

Chondrocyte proliferation and collagen production during cartilage degeneration rely on c-Fos expression

DMM-induced articular chondrocyte proliferation was lower in c-Fos$^{\Delta Ch}$ compared with c-Fos^{WT} mice as shown by Ki67 (figure 3A and B) and PCNA IHC (online supplemental figure 3A,B) and this was consistent with decreased chondrocyte density in Fos mutants (figure 3C). Collagen was next assessed by picrosirius red staining and polarised light visualisation as well as by IF for the Col2a1 subunit of collagen type II, the major cartilage extracellular matrix (ECM) component. Eight weeks post surgery, articular cartilage in DMM/sham-treated c-Fos^{WT} mice and sham-treated c-Fos$^{\Delta Ch}$ mice was composed of collagen bundles (yellow/green), while collagen fibres in DMM-treated c-Fos$^{\Delta Ch}$ appeared less stained with picrosirius red (figure 3D). Digital image analyses (online supplemental figure 3C) further documented decreased collagen area (figure 3E) and reduced fibre length in c-Fos$^{\Delta Ch}$ mice after DMM (figure 3F). Consistently, while Col2a1-immunopositive area was increased by DMM in c-Fos^{WT} mice, particularly in the calcified cartilage zone, it was lower in DMM-treated c-Fos$^{\Delta Ch}$ mice (figure 3G and H). MMP-13 and Adamts-5 are key degrading enzymes for collagen and GAGs, respectively, and their gene inactivation in mice leads to resistance to cartilage damage.[32 33] While c-Fos deletion had no notable effect on MMP-13 expression, Adamts-5 was found decreased in knee sections from sham-treated and DMM-treated c-Fos$^{\Delta Ch}$ mice (online supplemental figure 3D–G, online supplemental figure 9A and C). Taken together, increased cartilage damage in c-Fos$^{\Delta Ch}$ mice is likely due to reduced chondrocyte proliferation and decreased collagen/ECM anabolic reactions rather than increased collagen catabolic processes.

Energy metabolism during OA is dependent on c-Fos

To unravel the early molecular events contributing to the c-Fos protective function, articular cartilage was isolated from c-Fos^{WT} and c-Fos$^{\Delta Ch}$ mice 4 weeks after surgery, when cartilage damage was minimal and similar between the two genotypes (online supplemental figure 4A), and subjected to RNA sequencing (RNA-seq). Two data sets were generated: data set 1 consists of differentially expressed genes (DEGs) between DMM-treated and the non-operated contralateral knee in c-FosWT mice, while data set 2 contains DEGs between knee cartilage samples isolated from DMM-treated c-Fos^{WT} and DMM-treated c-Fos$^{\Delta Ch}$ mice (online supplemental figure 4B). A total of 801 DEGs were detected in data set 1 and 5167 in data set 2, with 725 DEGs shared between two data sets (online supplemental figure 4B, online supplemental table 3). Heat map representation of the shared upregulated and downregulated genes along the three analysis groups revealed a striking inverse regulation between DMM-treated c-Fos^{WT} and c-Fos$^{\Delta Ch}$ samples with the expression profile of DMM-treated c-Fos$^{\Delta Ch}$ largely comparable to the profile of c-Fos^{WT} contralateral knee (online supplemental figure 4C).

Pathway analysis was next conducted using gene set enrichment analysis.[34] Overall, 52 pathways classified in six biological process groups emerged in the two data sets (figure 4A, online supplemental table 4). Cell cycle progression/DNA repair (group 1) and inflammatory response/immune system (group 2) were activated by DMM in c-Fos^{WT} (data set 1), while pathways belonging to ECM synthesis/remodelling (group 5) were mostly suppressed (figure 4A, online supplemental figure 4D). In cellular metabolism (group 3), tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS) and heme metabolism, three interconnected pathways often increased in cells on high energy demand, were upregulated in data set 1, while glycolysis and hypoxia appeared downregulated (figure 4A and B). Activation of mammalian target of rapamycin (mTOR) and suppression of transforming growth factor-β (TGF-β) signalling pathways (group 4), was also apparent in data set 1 (figure 4A), consistent with their role in cartilage homoeostasis and OA.[35 36] Importantly, c-Fos deletion (data set 2) completely inverted the picture of DMM-induced genes and pathways (figure 4A), with a marked suppression of OXPHOS, heme metabolism and TCA cycle, while glycolysis and hypoxia were restored to wild-type contralateral levels (figure 4A and B, online supplemental figure 4D). Finally, when correlating Ingenuity Pathway Analysis (IPA) Z-scores with Log2Fc mRNA expression, several transcription factors and secreted proteins relevant to Fos-dependent chondrocyte response to DMM, were found connected to biological processes in group 3, that is, cellular metabolism pathways. For example, Sox9, Smad3 and Hif-1α and their upstream regulator Tgfβ1/2/3 and Bmp2/4 were also activated/increased in data set 2 (figure 4B and C and (online supplemental figure 4E), while these were decreased in data set 1 (figure 4B, online supplemental figure 4F), consistent with the opposite glycolysis/hypoxia signatures between the two data sets (figure 4B). These data reveal that metabolic responses of chondrocytes during cartilage damage are characterised by a shift from aerobic glycolysis to TCA cycle and these responses are strikingly affected by c-Fos expression.

Pyruvate dehydrogenase and lactate dehydrogenase activities are modulated by c-Fos in chondrocytes

We next examined the molecular events mediating the effect of c-Fos on the metabolic pathways affected by DMM. Pyruvate, a cellular metabolite located at the intersection of multiple metabolic pathways, can be either converted to lactate by lactate dehydrogenase (Ldh) to supply glycolysis with NAD+, or to acetyl-coenzyme A by the pyruvate dehydrogenase (Pdh) complex and subsequently oxidized by the TCA cycle. Suppression of Pdh activity by pyruvate dehydrogenase kinases (Pdks) balances carbon flux between the two metabolic circuits. In sharp contrast to the situation observed in the Fos-proficient data set 1 (online supplemental figure 5A), expression of glycolytic enzymes increased in Fos-deficient cartilage after DMM (data set 2), while the majority of TCA cycle enzymes decreased (figure 5A). Increased expression of *ldha*, encoding for a major subunit of Ldh, in c-Fos$^{\Delta Ch}$ samples was confirmed by quantitative PCR (qPCR) (online supplemental figure 5B). RNA-seq and qPCR analyses revealed that *pdk1*, but not other *pdk* isoforms, was upregulated in c-Fos$^{\Delta Ch}$ mice, while Pdh complex subunits, such as *pdha*, *pdhx* and *dld* were largely unaffected (figure 5A and online supplemental figure 5C). Increased Pdk1 in chondrocytes of DMM-treated c-Fos$^{\Delta Ch}$ mice was also apparent by IHC (figure 5B and C). Hif-1α is an important upstream transcriptional regulator of Ldh[37–39] and Pdk1,[37 40] and a suppressor of chondrocyte proliferation in the hypoxic growth plate.[39 41] Consistent with increased Ldh and Pdk1 protein expression but also the IPA prediction, increased Hif-1α protein was apparent in the cartilage of DMM-treated c-Fos$^{\Delta Ch}$ mice compared with c-Fos^{WT} mice (figure 4C, figure 5D and E and online supplemental table 3). Although following the same direction as the

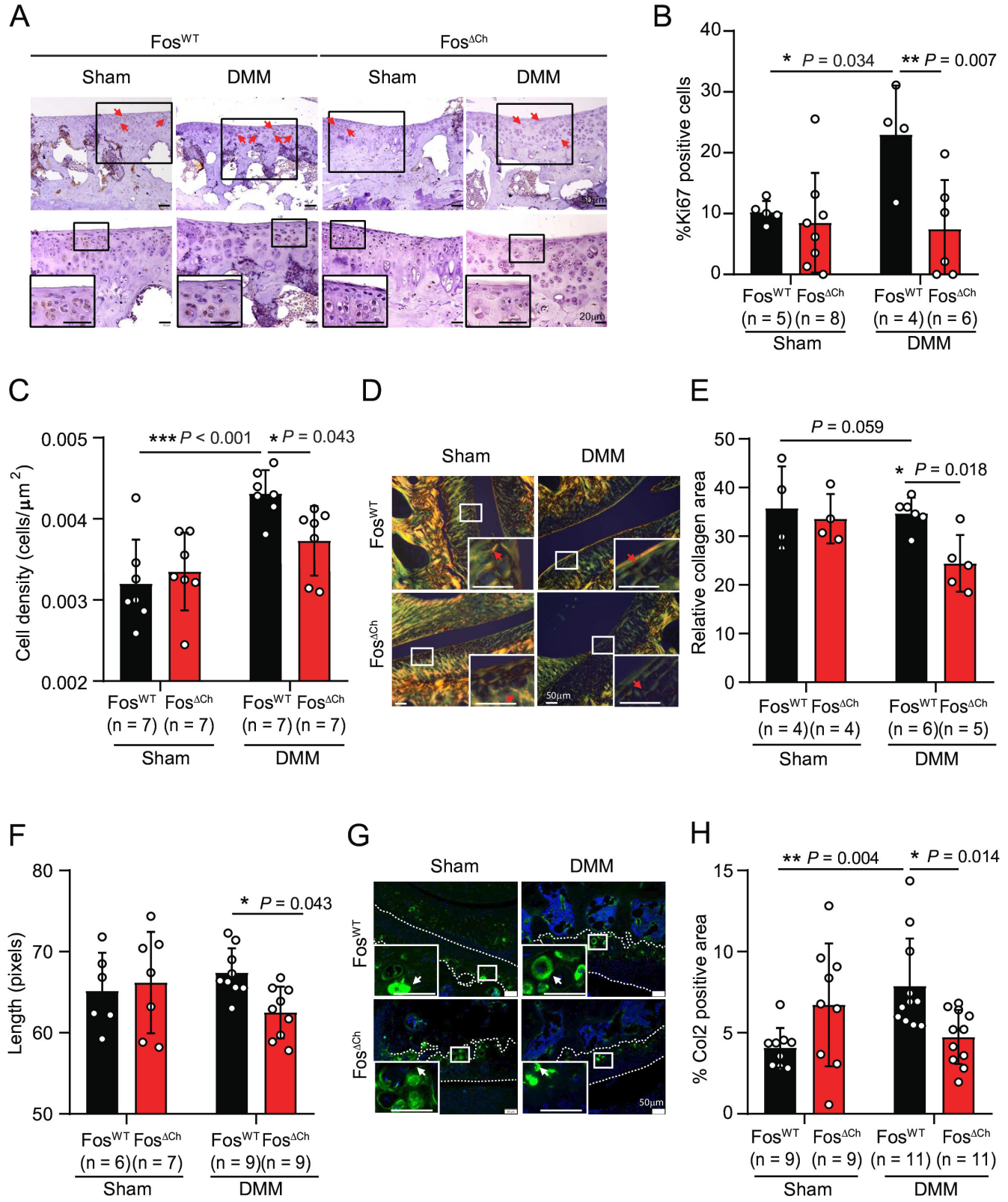

Figure 3 c-Fos affects chondrocyte proliferation and collagen organisation during cartilage damage progression. (A) Representative images of Ki67 and (B) quantification of Ki67-positive cells. (C) Chondrocyte density in articular cartilage from c-Fos^{WT} mice and c-Fos$^{\Delta Ch}$ mice at 8 weeks post surgery. (D) Representative images of picrosirius red staining of knee joints from c-Fos^{WT} mice and c-Fos$^{\Delta Ch}$ mice 8 weeks post surgery. Pictures are taken under the polarised light. (E) Quantification of collagen area in articular cartilage based on picrosirius red staining. (F) Quantification of collagen fibre length. Picrosirius red staining images were analysed using CurveAlign. (G) IF of collagen type 2 (green) in articular cartilage from c-Fos^{WT} mice and c-Fos$^{\Delta Ch}$ mice 8 weeks post surgery. Col2 positive areas are indicated by white arrows. (H) Quantification of Col2-positive area. Bar graphs and plots represent or include mean±SD, respectively. *p<0.05, **p<0.01, and ***p<0.001. In all panels, statistical differences between groups were analysed by two-way ANOVA with Bonferroni post hoc analysis. ANOVA, analysis of variance; DMM, destabilisation of the medial meniscus; IF, immunofluorescence

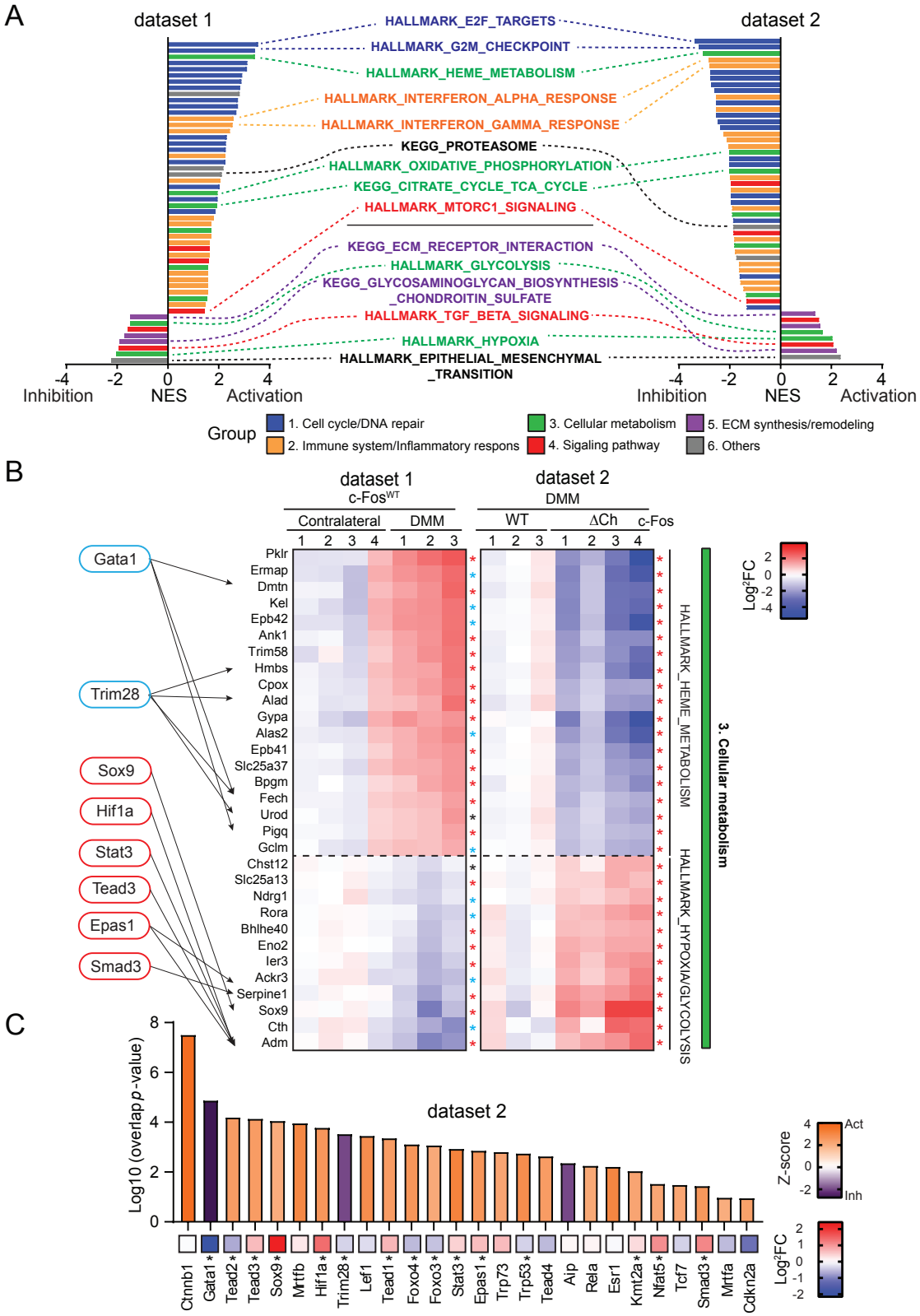

Figure 4 c-Fos transcriptionally controls cellular metabolic pathways. Bulk RNA-sequencing of articular cartilage from DMM-treated mice. (A) GSEA analysis indicating common core biological pathways either enriched or downregulated between data set 1 (c-Fos^{WT} mice: DMM-treated vs contralateral articular cartilage) and data set 2 (DMM-treated articular cartilage: c-Fos^{WT} vs c-Fos$^{\Delta Ch}$ mice). NES, normalised enrichment score. Contralateral c-Fos^{WT}, n=4, DMM c-Fos^{WT}, n=3. DMM c-Fos$^{\Delta Ch}$, n=4. (B) Log2FC-based relative mRNA expression heat map of top-ranked factors enriched in the cellular metabolism (group 3) in figure 4C and online supplemental figure 4A (green) and IPA-predicted transcription factors (TFs) downstream of c-Fos in the DMM-treated side. Target genes of TFs detected by IPA are indicated with arrows. Asterisk indicates p<0.05 (black), p<0.01 (blue) and p<0.001 (red). (C) IPA-predicted upstream TFs from DMM-treated c-Fos$^{\Delta Ch}$ vs c-Fos^{WT} mice, showing activation Z-score (bars) and Log2FC (asterisk indicates p value<0.05). Statistical evaluation of RNA-seq data was performed as indicated in the methods. DMM, destabilisation of the medial meniscus; GSEA, gene set enrichment analysis; IPA, Ingenuity Pathway Analysis; RNA-seq, RNA sequencing; WT, wild-type.

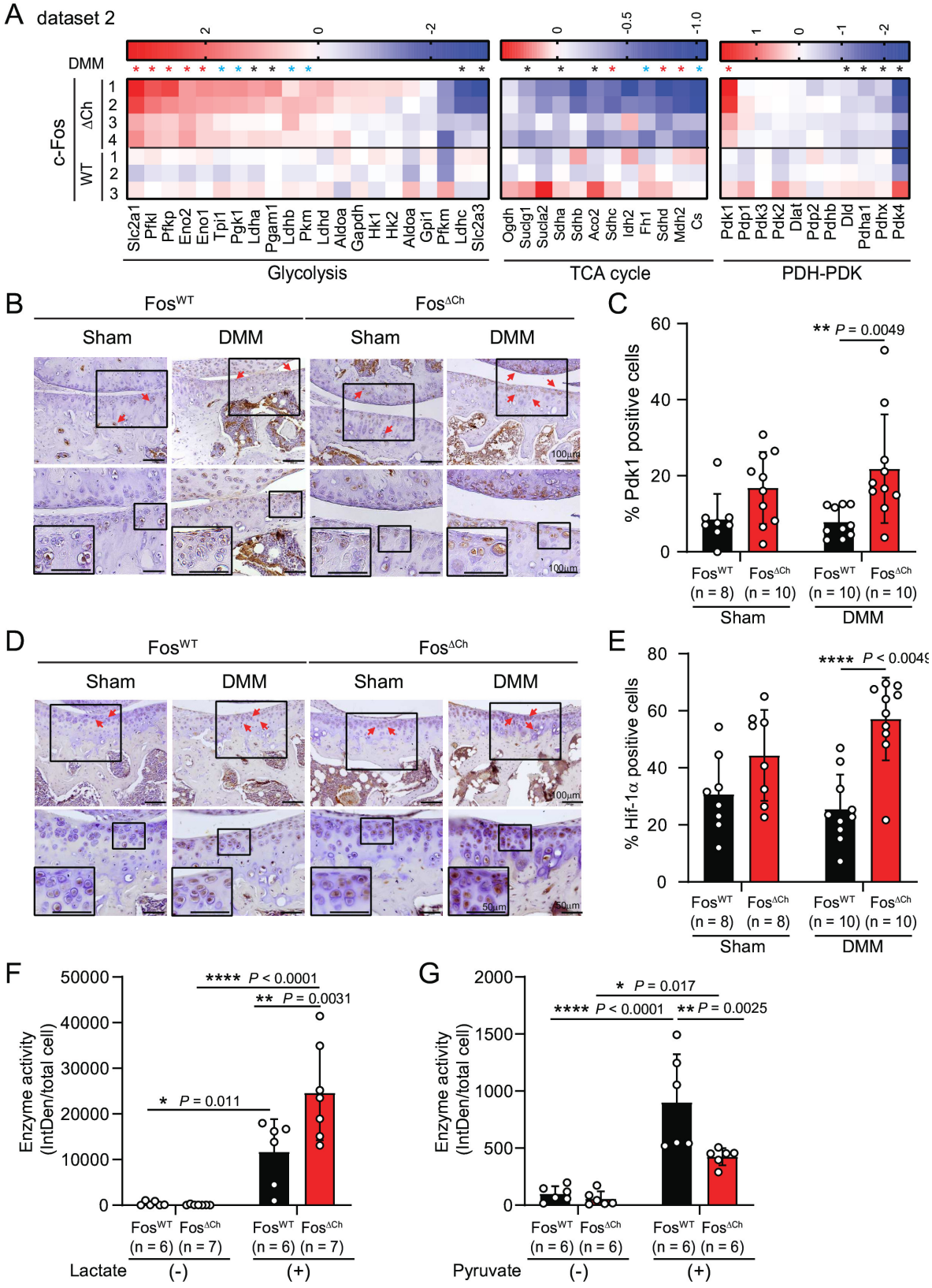

Figure 5 c-Fos maintains pyruvate dehydrogenase activity in experimental OA. (A) Relative mRNA expression heat map of factors in glycolysis, PDH-PDK pathway and TCA cycle based on Log2FC in the DMM-treated side compared with those from the contralateral side in c-Fos^{WT} mice. Asterisk indicates p<0.05 (black), p<0.01 (blue) and p<0.001 (red). (B) Representative images of Pdk1 and (C) quantification of positive cells. (D) Representative images of HIF-1α and (E) quantification of positive cells. Red arrows indicate positive cells. (F and G) *In situ* enzyme activity assay by formazan formation for Ldh (F) and Pdh (G). Bar graphs and plots represent or include mean±SD. *p<0.05, **p<0.01 and ***p<0.001. In all panels, statistical differences between groups were analysed by two-way ANOVA with Bonferroni post hoc analysis. ANOVA, analysis of variance; DMM, destabilisation of the medial meniscus; Ldh, lactate dehydrogenase; OA, osteoarthritis; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; TCA, tricarboxylic acid; WT, wild-type.

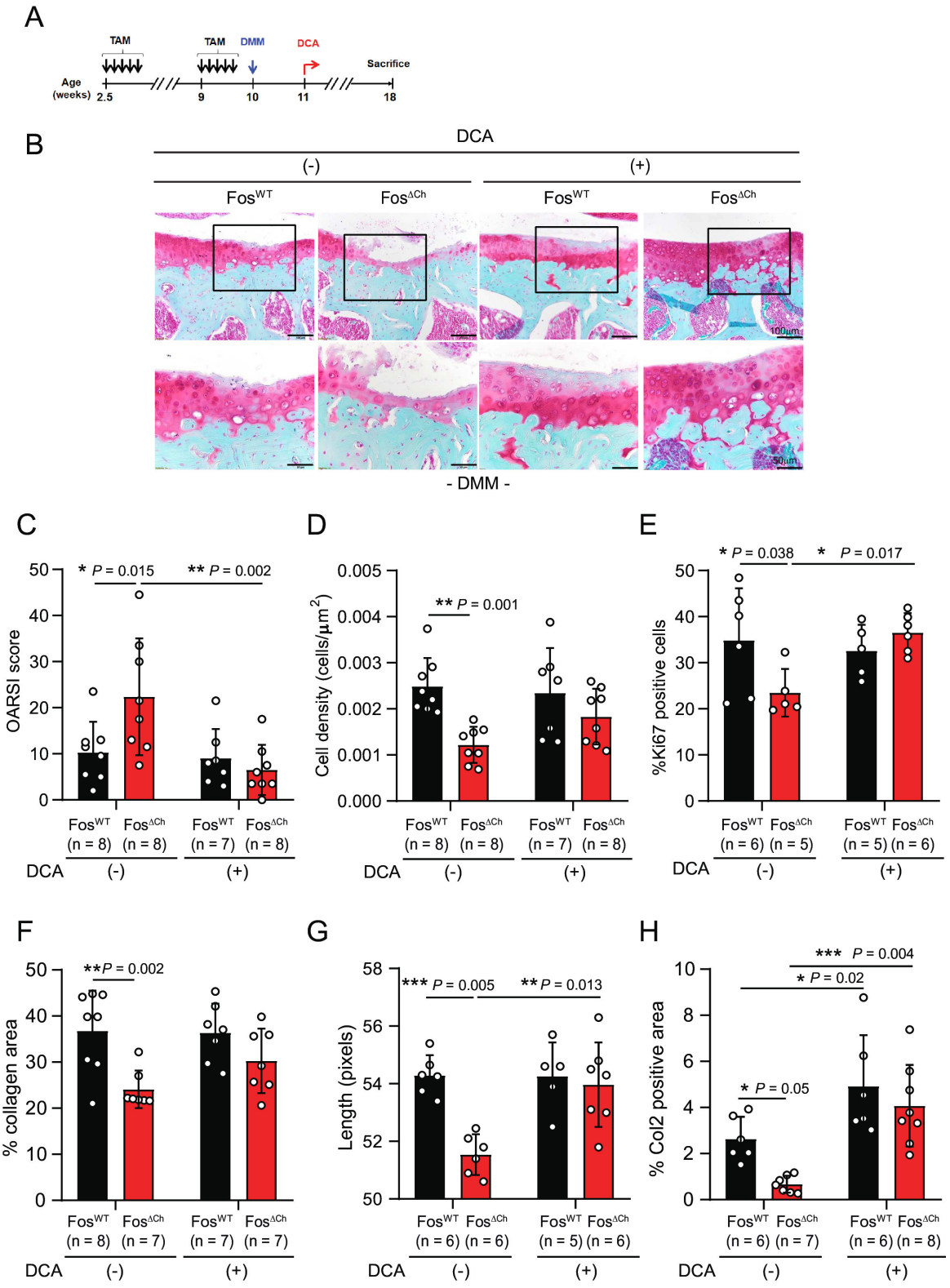

Figure 6 DCA treatment reduces DMM-induced cartilage damage in c-Fos deficient mice. (A) Experimental procedure. Tamoxifen was injected into mice at two time points (2.5 and 9 weeks of age, 2 mg/mouse/day, 5 consecutive days). Mice were subjected to DMM/sham at 10 weeks of age and treated with DCA from 11 weeks of age for 7 weeks. Knee joints were analysed 8 weeks post surgery. (B) Representative images of safranin O/fast green staining of knee joints from c-Fos^{WT} mice and c-Fos$^{\Delta Ch}$ mice treated with or without DCA 8 weeks post DMM. (C) Quantification of cartilage damage. (D) Chondrocyte density in articular cartilage from c-Fos^{WT} mice and c-Fos$^{\Delta Ch}$ mice 8 weeks post surgery. (E) Quantification of Ki67 positive cells. (F) Quantification of collagen area based on picrosirius red staining in the articular cartilage. (G) Quantification of collagen fibre length. The images of collagen fibre from picrosirius red staining were analysed by CurveAlign. (H) Quantification of IF images of collagen type 2 positive area. Bar graphs and plots represent or include mean±SD. *p<0.05, **p<0.01 and ***p<0.001. In all panels, statistical differences between groups were analysed by two-way ANOVA with Bonferroni post hoc analysis. ANOVA, analysis of variance; DCA, dichloroacetic acid; DMM, destabilisation of the medial meniscus; IF, immunofluorescence; TAM, Tamoxifen; WT, wild-type.

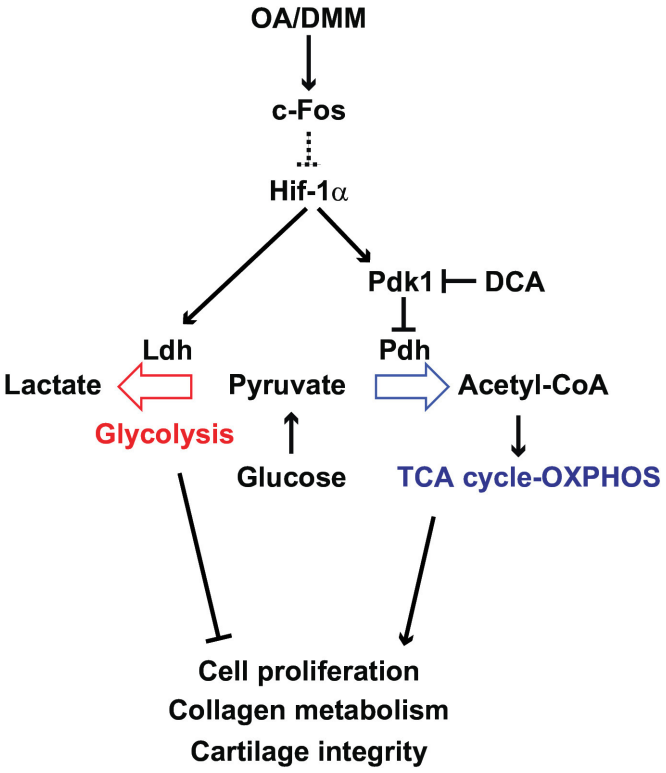

Figure 7 Scheme depicting chondrocyte pyruvate usage pathways modulated by c-Fos/AP-1 in experimental OA. In early OA, the Hif-1α/Pdk1/Pdh and/or Hif-1α/Ldh pathways are suppressed by c-Fos, and pyruvate—acetyl-CoA conversion is predominant, leading to increased TCA cycle/OXPHOS and decreased lactate production. In chondrocytes subjected to DMM, c-Fos/AP-1 modulates pyruvate metabolism through Hif-1α/Pdk1/Pdh and/or Hif-1α/Ldh, thereby controlling cell proliferation and collagen biosynthesis, improving cartilage integrity and counteracting OA progression. The dotted lines between c-Fos and Hif-1α, two nuclear proteins forming heterodimeric transcription factors, indicate yet-to-be-defined pathways, such as the Tgfβ/Smad/Bmp and mTORC1, which are more likely than a direct transcriptional regulation. In Fos-deficient cells, chondrocytes execute these events in the opposite manner, whereby c-Fos-induced protection is lost and the energy deficit leads to decreased proliferation, collagen synthesis and increased OA. Elevated Pdk and Ldh activity can be therapeutically targeted in Fos-deficient cells using DCA to promote TCA cycle/OXPHOS and suppress glycolysis, rescuing the above-mentioned defects in proliferation and cartilage integrity. DCA, dichloroacetic acid; DMM, destabilisation of the medial meniscus; Ldh, lactate dehydrogenase; OA, osteoarthritis; OXPHOS, oxidative phosphorylation; Pdh, pyruvate dehydrogenase; Pdk, pyruvate dehydrogenase kinase; TCA, tricarboxylic acid.

DMM-treated pairs, most of the observed differences between c-Fos^{WT} and c-Fos$^{\Delta Ch}$ samples were not statistically significant between sham-treated (figure 5C and E) or contralateral groups (online supplemental figure 5B,C). mRNA expression of *ldha*, *pdk1* and *hif1a* were not affected by *in vitro* AdenoCre-mediated *c-fos* deletion in primary articular chondrocytes (online supplemental figure 5D), in line with the rather healthy cartilage displayed by un/sham-operated c-Fos$^{\Delta Ch}$ mice. These data collectively indicate that the molecular and cellular events downstream of c-Fos are likely part of the response of chondrocytes to OA-related signals such as biomechanical stress or cartilage damage caused by DMM. Interestingly, while transcripts of Hif-1α modulating factors such as *egln1* and *vhl*, encoding the HIF-prolyl hydroxylase Phd2 and the E3-ubiquitin ligase pVHL respectively, were either increased or unaffected in Fos-deficient cartilage after DMM, proteasome genes and their master transcriptional regulator *nrf1* were decreased (online supplemental figure 5E, data set 2) and the reverse expression signature were observed in the Fos-proficient data set 1 (figure 4A, online supplemental figure 5E). These data suggest that the cellular and metabolic changes observed in DMM-treated Fos-deficient mice could be caused, at least in part, by Hif-1α activation, potentially due to decreased proteasome activity.

Ldh and Pdh activities were next assessed in articular cartilage tissue sections by *in situ* enzyme histochemistry.[42–45] A strong lactate-dependent activity was detected in articular chondrocytes and quantitative analysis revealed higher amounts of active Ldh in sections from DMM-treated c-Fos$^{\Delta Ch}$ mice compared with c-Fos^{WT} (figure 5F and online supplemental figure 6A). Conversely, pyruvate-dependent Pdh activity was lower in articular chondrocytes from DMM-treated c-Fos$^{\Delta Ch}$ mice (figure 5G and online supplemental figure 6B). Finally, while Pdh activity in murine primary articular chondrocytes was not affected by *in vitro* c-fos deletion, treatment with dichloroacetic acid (DCA), a PDK inhibitor, led to a more robust increase of Pdh activity in Fos-deficient chondrocytes (online supplemental figure 6C). These results suggest that the Hif-1α/Ldh/Pdk/Pdh complex axis downstream of c-Fos could be a functional determinant for the switch in energy metabolism occurring in chondrocytes in response to OA signals.

DCA treatment reverts the deleterious effects of c-Fos inactivation in experimental OA

Hif-1α and Pdk1 protein expression and glycolytic flux are increased in chondrocytes in mice lacking prolyl hydroxylase 2 (Phd2), the main negative regulator of Hif-1α, leading to various cartilage dysplasias.[46] Importantly, *in vivo* DCA treatment increased collagen synthesis in cartilage by restoring glucose oxidation, oxygen consumption and cell proliferation in Phd2 mutants, while sparing the wild-type littermates.[46] Cohorts of c-Fos^{WT} and c-Fos$^{\Delta Ch}$ mice were therefore subjected to DMM and treated with DCA in a therapeutic setting (figure 6A).

After 3 weeks of DCA treatment, *in situ* Pdh activity was still lower in most c-Fos$^{\Delta Ch}$ mutants (online supplemental figure 7A,B), while Ldh activity was restored to the levels measured in c-Fos^{WT} samples (online supplemental figure 7C,D). End-point histological analyses 1 month later, revealed that while DCA had no effect on c-Fos^{WT} mice, cartilage damage was reduced in DCA-treated c-Fos$^{\Delta Ch}$ mice to levels similar to c-Fos^{WT} (figure 6B,C). Consistent with a possible implication of Hif-1α downstream of c-Fos, chondrocyte density and Ki67 positivity were increased in DCA-treated c-Fos$^{\Delta Ch}$ mice (figure 6D and E, online supplemental figure 8A). Finally, DCA treatment restored collagen area (figure 6F, online supplemental figure 8B), collagen fibre length (figure 6G) and Col2-positive articular chondrocytes with less cartilage damage (figure 6H and online supplemental figure 8C) in c-Fos$^{\Delta Ch}$ mice, again reaching values comparable to c-Fos^{WT}. Taken together, pyruvate metabolism by Ldh and Pdh is a critical node downstream of c-Fos involved in the response of chondrocytes during OA.

DISCUSSION

Despite a large number of studies, there is no disease-modifying drug or preventive strategy for OA due to limited mechanistic knowledge of how this heterogeneous disease develops.[7] Combining mouse models and patient samples, the present study provides new insights into how articular chondrocytes adapt to

OA-associated signals and demonstrates a crucial role of the AP-1-forming protein c-Fos in modulating cellular pyruvate usage and cartilage integrity (figure 7).

Glycolysis rather than the TCA cycle and subsequent oxidative phosphorylation is the primary energy source for chondrocytes *in vivo*, likely owing to their hypoxic environment.[6 7 47] Our findings provide evidence that the fine-tuning between glycolysis and TCA cycle—OXPHOS that occurs in articular chondrocytes in response to excessive biological/mechanical stress is an important determinant of OA pathogenesis. In the early stages of experimental OA, the shift in cellular energy metabolism in chondrocytes towards increased utilisation of pyruvate in the TCA cycle—OXPHOS is likely necessary to satisfy the high energy demand of early response processes, such as proliferation and collagen/matrix synthesis. Genetic inactivation c-Fos in chondrocytes leads to major impairment of their metabolic response to OA signals, through changes in expression and/or activity of two pyruvate-metabolising enzymes: pyruvate and lactate dehydrogenase. This balance in the activity of these two enzymes determines the usage of rapid and low-energy producing lactic fermentation (glycolysis) or slower but high-energy producing TCA cycle—OXPHOS (figure 7). Decreased Pdh and increased Ldh activities, measured *in situ* in DMM-treated Fos-deficient articular chondrocytes, are in line with a decreased influx of acetyl-CoA to the TCA cycle and a consequent energy deficit in these mutant cells. Fos-deficient articular chondrocytes subjected to experimental OA proliferate less frequently, and produce less and shorter collagen fibres, resulting in increased overall cartilage damage. Chondrocyte-specific gene inactivation of *Ldha* is beneficial in experimental OA[48] and we demonstrate that restoring Ldh/Pdh activities to levels similar to wild-type by treatment with DCA is beneficial in Fos-deficient mutants, rescuing all above-mentioned phenotypes. While DCA had no therapeutic benefit in wild-type mice, strategies mimicking the effect of DCA might still be of interest to potentiate other therapies. Nevertheless, this striking result indicates that in OA chondrocytes, c-Fos is more essential to modulate pyruvate usage than to control the expression of its classical target genes, such as matrix-degrading enzymes and cell cycle/proliferation proteins. Whether c-Fos, a bona fide oncogene, might also modulate pyruvate usage in highly glycolytic solid tumours is worth exploring, for example, using Fos-dependent experimental models of chondrosarcoma and/or osteosarcoma.

Two studies have documented that T-5224, a broad-spectrum AP-1 dimer inhibitor, ameliorates DMM-induced experimental OA.[49 50] While these results might seem in disagreement with our genetic experiment selectively deleting c-Fos in chondrocytes, rather we infer that the role of AP-1 in OA pathogenesis is not limited to the function of Fos-containing AP-1 heterodimers, nor limited to a single cell type in the joint. Broadly inhibiting the binding of AP-1 dimers to DNA can limit the transcription of AP-1 target genes essential to OA pathogenesis in chondrocytes, but also in joint immune and/or synovial cells. Consistently, mice broadly deficient for Batf, a bZIP-containing protein that forms AP-1 dimers only with Jun proteins are resistant to experimental OA.[50] We envisage that the disease resistance reflects changes in AP-1 dimer composition and the relevance of AP-1 activity in other cell types beyond chondrocytes during OA. In this regard, it is striking that Fos inactivation in chondrocytes had little effect on DMM-induced osteophyte formation or synovial thickening, two OA-related pathological events that involve other mesenchymal cells. Examining the effects of c-Fos inactivation in other cell types of the joint and identifying among the Jun, ATF and MAF proteins the essential c-Fos dimerising partner(s) in chondrocytes might provide further mechanistic clues to OA pathogenesis. As c-Jun and JunB are both increased during human[50] and mouse OA, these are the most likely Fos partners to genetically assess in future *in vivo* experiments.

Decreased Pdh and increased Ldh activities in DMM-treated, Fos-deficient chondrocytes are the net outcome of increased Hif-1α and Pdk1 mRNA and protein expression. The essential contribution of HIF-1α to the metabolic changes in the absence of c-Fos is not formally demonstrated by *in vivo* loss and gain of function experiments, but is very likely given the reported role of HIF in chondrocyte metabolism. Beyond its direct control of both Ldh[37–39] and Pdk1[37–40] expression, Hif-1α and HIF signalling, is a crucial determinant of important biological processes in chondrocytes, such as survival and proliferation,[39 41] collagen synthesis and matrix quality.[46] It is therefore not surprising that genetic or pharmacological manipulation of Hif-1α or its upstream regulator Phd2, lead to pleiotropic effects in mouse cartilage. For example, inappropriate HIF-1α signalling in mice lacking Phd2 in chondrocytes resulted in skeletal dysplasia with increased bone mass[46] and reduced articular cartilage thickness.[51] HIF-1α stabilisation in Phd2-deficient chondrocytes also resulted in metabolic reprogramming with enhanced glutamine flux and decreased glucose oxidation leading to collagen overmodifications and the formation of a cartilaginous matrix more resistant to protease-mediated degradation, despite decreased collagen synthesis.[46] As cartilage stiffness, matrix composition and matrix catabolism are also important determinants of OA, the deleterious effects of Hif-1α gene inactivation in experimental OA[52 53] are likely due to the additive effects of imbalanced energy and cartilage matrix metabolism, dysregulated gene expression of matrix-degrading enzymes and acute cell death/autophagy responses.

Several Hif-1α target genes that were reported to increase in Phd-2 deficient chondrocytes are increased by DMM in c-Fos$^{\Delta Ch}$ mice: *pdk1*, *ldha*, the glucose transporter Glut1 encoded by *slc2a1*, the glutamine sensor glutaminase 1 (*gls1*) and the collagen-modifying enzymes *p4ha*, *p4hb*, *lox* and *plod2*, consistent with increased Hif-1α expression and with the cartilage and collagen defects observed in c-Fos$^{\Delta Ch}$ mutants. RNA-seq experiments also revealed increased *Phd2* and *Vhl* mRNA expression in c-Fos$^{\Delta Ch}$ DMM samples (data set 2), consistent with *Phd2* being a transcriptional Hif-1α target. Increased Hif-1α in c-Fos-deficient chondrocytes is likely not due to decreased prolyl and asparaginyl hydroxylase activity, or direct binding of c-Fos/AP-1 to the *hif1a* promoter, but could be a result of reduced proteasome activity. Altered signalling activity from the Tgfβ/Smad/Bmp and/or mTORC1 pathway, which have been documented to interact with and modulate both AP-1 and HIF signalling are also attractive candidates to explore functionally, with the prospect to substantiate the envisaged link between c-Fos/AP-1 and Hif-1α.

Overall, this work highlights the function of Fos/AP-1 in OA pathogenesis and provides convincing evidence that early modulation of the balance in pyruvate usage between lactate production and TCA-cycle-OXPHOS in chondrocytes determines OA outcome. Whether boosting this early metabolic stress response of chondrocytes can facilitate the cartilage regenerating or repair capacity at early stages of the disease or whether therapeutic intervention can trigger this response even at later stages of the disease certainly warrants further experimentation.

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Acknowledgements We thank Sarah Sinclair and Christina Bauer for assisting with tissue sections staining and mouse experiments and Dragana Kubatovic for helping with mouse colony management. We are very grateful to Drs Doug Hanahan, Georg Schett, Nabil Djouder and Rudolf Zechner and to the members of the Wagner Laboratory for helpful comments and discussions. Elisabeth Ponweiser at KILM/MUW for help with *in situ* Pdh/Ldh activity assays; Alexander Stögner and Ruth Grübl-Barabas from the Department of Orthopedics and Trauma Surgery/MUW for help with preparation and IHC of human OA cartilage, Christian Beyer from the University Erlangen for help in establishing the DMM procedure. We thank S Derdak for the help with bulk RNA-seq analysis. RNA-seq was performed at the Core Facilities of the MUW, a member of the Vienna Life-Science Instruments initiative.

Contributors KM designed and performed experiments and wrote the manuscript. LB contributed to mouse colony management, experimental design and manuscript writing. AH contributed to in situ activity assays and MB contributed to bulk RNA-seq analyses of mouse cartilage and primary osteoarthritis (OA) chondrocytes. MK and HY contributed to data mining of single-cell RNA-seq data sets from human OA chondrocytes. ST and RW provided human articular cartilage from patients with OA and analyses of c-Fos and Pdk1 expression and the associated clinical data. EFW directed the study, approved the data and edited the manuscript with input from all the authors. Guarantor: EFW.

Funding ST gratefully acknowledges support from the AFOR Foundation and from a fellowship by Johnson & Johnson Medical Products GmbH. HY is an H2020 – MSCA fellow (ITN 2019-859860-CANCERPREV). The Wagner Laboratory is supported by the ERC (AdG 2016-741888-CSI-Fun), an H2020 – MSCA grant (ITN 2019-859860-CANCERPREV) and the MUW.

Competing interests None declared.

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.

Patient consent for publication Not applicable.

Ethics approval Human articular cartilage was obtained from patients with osteoarthritis during total knee arthroplasty with written informed consent and following the terms of the ethics committee of the Medical University of Vienna (EK-Nr.: 1822/2017, 2166/2020). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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