Modulation of matrix metabolism by ATP-citrate lyase in articular chondrocytes

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Certain dysregulated chondrocyte metabolic adaptive responses such as decreased activity of the master regulator of energy metabolism AMP-activated protein kinase (AMPK) promote osteoarthritis (OA). Metabolism intersects with epigenetic and transcriptional responses. Hence, we studied chondrocyte ATP-citrate lyase (ACLY), which generates acetyl-CoA from mitochondrial-derived citrate, and modulates acetylation of histones and transcription factors. We assessed ACLY in normal and OA human knee chondrocytes and cartilages by Western blotting and immunohistochemistry, and quantified acetyl-CoA fluorometrically. We examined histone and transcription factor lysine acetylation by Western blotting, and assessed histone H3K9 and H3K27 occupancy of iNOS, MMP3, and MMP13 promoters by chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR). We analyzed iNOS, MMP3, MMP13, aggrecan (ACAN), and Col2a1 gene expression by RT-qPCR. Glucose availability regulated ACLY expression and function, nucleocytosolic acetyl-CoA, and histone acetylation. Human knee OA chondrocytes exhibited increased ACLY activation (assessed by Ser-455 phosphorylation), associated with increased H3K9 and H3K27 acetylation. Inhibition of ACLY attenuated IL-1β-induced transcription of iNOS, MMP3, and MMP13 by suppressing acetylation of p65 NF-κB, H3K9, and H3K27, blunted release of NO, MMP3, and MMP13, and also reduced SOX9 acetylation that promoted SOX9 nuclear translocation, leading to increased aggrecan and Col2a1 mRNA expression. ACLY is a novel player involved in regulation of cartilage matrix metabolism. Increased ACLY activity in OA chondrocytes increased nucleocytosolic acetyl-CoA, leading to increased matrix catabolism via dysregulated histone and transcription factor acetylation. Pharmacologic ACLY inhibition in OA chondrocytes globally reverses these changes and stimulates matrix gene expression and AMPK activation, supporting translational investigation in OA.

Osteoarthritis (OA)2 is a major and growing public health problem (1). The disease is promoted by factors including aging, biomechanical joint trauma, and changes in systemic metabolism, including obesity and insulin resistance (1–4). As OA progresses, failure of the synovial joint organ frequently develops, with degeneration of articular cartilage as a core disease feature (1). Chondrocytes, the sole cells in articular hyaline cartilage, are responsible for maintaining the homeostatic balance between extracellular matrix anabolism and catabolism (1). Dysfunction of chondrocytes in OA, amplified by local inflammatory and biomechanical injury processes, leads to an excess of matrix catabolic activity, mediated by factors including MMPs and aggrecanases (1, 3).

The avascular, hypoxic environment that articular chondrocytes reside in renders cartilage homeostasis more challenging due to cellular nutritional and metabolic demands (3). In OA, chondrocyte responses to changes in glucose and other available nutrients include transition from resting to a highly metabolically active state (3), associated with development of excess glycolysis, and decreased mitochondrial function with less oxidative phosphorylation, and consequent pro-inflammatory effects (3, 4). As OA advances, altered chondrocyte energy balance is compounded by changes in physiologic chondrocyte nutritional biosensing responses (3, 4). A prime example is decrease in aging, biomechanically injured, and OA chondrocytes in vitro and in vivo of chondrocyte activity of AMPK (5, 6), a master regulator of energy balance (7). AMPK activation inhibits inflammatory and matrix catabolic responses in chondrocytes, partly mediated by inhibition of the master inflammation transcription factor NF-κB (5). Inducible chondrocyte-specific AMPKα knockout (KO) mice develop accelerated mechanical injury–induced and age–related spontaneous knee OA (8).

Metabolism intersects with certain epigenetic and transcription factor modifications mediating cellular re-programming (9). In this light, epigenetic changes that promote OA include not only altered chondrocyte gene methylation (10), but also modification of certain transcriptional responses by histone

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2 The abbreviations used are: OA, osteoarthritis; ACLY, ATP-citrate lyase; AMPK, AMP-activated protein kinase; acetyl-CoA, acetyl-coenzyme A; Ac-H3K9, acetylation of lysine residue 9 of histone 3; ACAN, aggrecan; HCA, hydroxycitric acid; iNOS, inducible nitric-oxide synthase; MMP, matrix metalloproteinase; NO, nitric oxide; PKM2, pyruvate kinase isoenzyme 2; RT-qPCR, reverse transcription quantitative PCR; IHC, immunohistochemistry; IL, interleukin; DMEM, Dulbecco’s modified Eagle’s; ANOVA, analysis of variance.

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ATP-citrate lyase function in articular chondrocytes

A. Acetyl-CoA

B. Protein Expression

C. Acetyl-CoA

D. Protein Expression

Figure 1. Glucose availability in articular chondrocytes influenced nucleocytosolic acetyl-CoA and histone acetylation in an ACLY-dependent manner. Cultured normal primary human knee chondrocytes in DMEM containing 25 mM glucose were subjected to glucose starvation in DMEM without glucose for 5 h, and then placed back in the DMEM containing 25 mM glucose for 1 and 5 h (A and B) or the DMEM containing 10 mM glucose for 5 h in the presence or absence of HCA (20 mM) (C and D). The amounts of total and compartmental (mitochondrial and nucleocytosolic) acetyl-CoA were measured (A and C). Whole cell lysates and nuclear proteins from these cells were studied by Western blotting for expression of PKM2, ACLY, phosphor-ACLY (Ser-455), Ac-H3K9, and Ac-H3K27. Semi-quantitative densitometry analysis of each Western blotting was performed over 3 individual experiments with 3 different donors (B and D). Two-way ANOVA (A and C) and one-way ANOVA (B and D) followed by Bonferroni’s post hoc test were used for statistical data analysis. p values represent comparisons of the mean ± S.D.

decetylases (10). Deacetylation of nuclear histones and transcription factors that impacts gene expression (11, 12), as well as cytosolic protein deacetylation, are countered by acetylation utilizing donor acetyl-CoA, a key metabolic intermediate (11, 12). Mitochondria generate acetyl-CoA via glucose, fatty acid, and amino acid catabolism (11, 12). Acetyl-CoA conveys carbon atoms into the tricarboxylic acid (TCA) cycle for ATP production (11, 12). The TCA cycle intermediate citrate can be exported from mitochondria into the cytosol and nucleus, where ATP citrate lyase (ACLY) converts citrate back to acetyl-CoA (11–13).

Acetyl-CoA abundance in distinct subcellular compartments reflects, in part, the bioenergetic state of the cell (11, 12), rising in nucleus and cytosol when glucose and other carbon sources are abundant (11, 12). Conversely, nutrient deprivation imposes increased requirement for acetyl-CoA residence in the mitochondria for ATP synthesis (11, 12), and nucleocytosolic acetyl-CoA levels decrease (11, 12). ACLY is shown to be responsible for generation of the majority of nucleocytosolic acetyl-CoA in mammalian cells (11–13). Because histone acetylation alters accessibility of chromatin and allows DNA-binding proteins to interact with exposed sites to activate gene transcription and downstream cellular functions (13, 14), ACLY plays a critical role in integrating the cell metabolic state to gene transcription (14). ACLY is essential for growth and development (15). However, ACLY expression and activity are increased in tumor cells and also can be induced by inflammatory mediators in immune cells (16, 17). Moreover, inhibition of ACLY prevents tumor growth (18) and suppresses inflammatory responses (17). As such, overactive ACLY may have a detrimental effect after growth and development.

Altered ACLY activity has also been observed in some metabolic disorders, including dyslipidemia and impaired glucose tolerance (13, 19). Several core features of metabolic syndrome...
(dyslipidemia, obesity, hyperglycemia, and insulin resistance) have been linked to OA (2–4). Because OA chondrocytes exhibit altered metabolism (3, 4), we studied expression, activity, and the role of ACLY in human knee OA articular chondrocytes. We included translationally pertinent studies of pharmacologic ACLY inhibition in OA chondrocytes in vitro, using the naturally occurring and potent ACLY inhibitor (−)-hydroxy-citric acid (HCA), an orally bioavailable citrate analog and caloric restriction mimetic (20). We identified ACLY as a potentially novel metabolic target for OA, as our results suggest a potential role of increased ACLY activity in chondrocytes in OA pathogenesis, and demonstrate that pharmacologically targeting ACLY reverses global changes in chondrocyte from human knee OA cartilages.

Results

Glucose availability in articular chondrocytes influenced the amount of nucleocytosolic acetyl-CoA and histone acetylation in an ACLY-dependent manner

Because ACLY plays an important role linking glucose metabolism to chromatin modification (14), we examined how ACLY expression and function, and epigenetic changes, intersected with glucose availability in articular chondrocytes. To do so, we first glucose-deprived and then glucose-replenished cultured normal primary human knee chondrocytes. Glucose starvation induced a marked decrease in total cellular acetyl-CoA, driven mostly by loss of nucleocytosolic acetyl-CoA. No significant reduction in mitochondrial acetyl-CoA level was seen (Fig. 1A); similar to previous findings that the mitochondrial pool of acetyl-CoA is less susceptible to variations than its nucleocytosolic counterpart (20, 21). Glucose re-feeding rapidly drove an increase of total cellular acetyl-CoA (Fig. 1A). Again, this was mostly due to an increase of nucleocytosolic acetyl-CoA (Fig. 1A). Under the conditions of glucose deprivation, glycolysis was inhibited, indicated by blunted expression of PKM2 (pyruvate kinase isoenzyme M2), which catalyzes the pyruvate-generating final step in glycolysis (Fig. 1B). In parallel, glucose deprivation decreased ACLY expression (Fig. 1B) and lowered acetylation of specific lysine residues 9 and 27 of histone 3 (Ac-H3K9 and Ac-H3K27) (Fig. 1B). Each of these responses was reversed after glucose re-feeding (Fig. 1B). Because acetylation of H3K9 and H3K27 are generally associated with increased gene transcription (22, 23), changes in levels of acetylation of H3K9 and H3K27 in chondrocytes could have an impact on gene expression. We also observed that refeeding the glucose-starved chondrocytes with glucose at 10 mM, but not at 1 mM, sufficiently rescued total acetyl-CoA (Fig. S1). Similarly, this was mostly due to recovery of nucleocytosolic acetyl-CoA (Fig. S1). Pharmacological ACLY inhibitor HCA at 20 mM concentration has been shown to inhibit ACLY in other cell types (20, 21). We found that HCA at the same concentration, which was confirmed to have no effect on chondrocyte cytotoxicity (data not shown), significantly blocked glucose re-feeding–induced recovery of nucleocytosolic and total acetyl-CoA (Fig. 1C), and Ac-H3K9 and Ac-H3K27 levels in glucose-starved chondrocytes (Fig. 1D). This was associated with increased ACLY phosphorylation at Ser-455, which enhances ACLY catalytic activity (13), and ACLY protein expression (Fig. 1D), suggesting ACLY-dependent histone acetylation in chondrocytes.

ACLY is up-regulated in human knee OA chondrocytes/cartilage

Because human OA chondrocytes have an increased capacity of glycolysis (24), we next examined ACLY phosphorylation and expression in either human knee cartilage sections or primary human knee chondrocytes from both normal (grade 0–1) and OA donors (grade 4). Immunohistochemistry (IHC) analysis of cartilage sections demonstrated that ACLY phosphorylation (Ser-455) and expression were low in normal donors, but increased in OA donors, evidenced by in situ positive staining in almost all chondrocytes, particularly prominent in chondrocyte clusters (Fig. 2). Notably, more cells stained positively for both phosphorylated ACLY (Ser-455) and ACLY in donor 2 (age 45), compared with donor 1 (age 24) in normal cartilage sections (Fig. 2). Western blot analysis of primary human knee chondrocytes also showed markedly increased phosphorylation of ACLY (Ser-455) in all 5 OA donors, when compared with 5 normal donors (Fig. 3, top panel). This was correlated with increased levels of total acetyl-CoA (data not shown) and acetylation of H3K9 and H3K27 in OA chondrocytes, compared with normal chondrocytes (Fig. 3A). Semi-quantitative
densitometry analysis of Western blots of 28 normal donors (20 male and 8 female, age 42.3 ± 10.6) and 28 OA donors (10 male and 18 female, age 67.8 ± 7.9) of primary human knee chondrocytes further confirmed that ACLY phosphorylation (Ser-455) and acetylation of H3K9 and H3K27 were significantly increased in OA chondrocytes (Fig. 3B). Levels of ACLY phosphorylation (Ser-455) and expression were not much different between male and female in either normal or OA donors studied, but seemed to increase slightly in normal donors with age. This was in agreement with the IHC results, suggesting a potential age-related effect on ACLY.

Inhibition of ACLY, mimicking glucose starvation, reduced nucleocytosolic acetyl-CoA and promoted phosphorylation of AMPK (Thr-172) in OA chondrocytes

AMPK is activated by glucose starvation in mammalian cells (25). We confirmed that phosphorylation of AMPKα at Thr-172, which is known to be critical for AMPK activity, was greatly increased in chondrocytes (Fig. 4A) under the glucose starvation condition shown in Fig. 1. However, phosphorylation of AMPKα (Thr-172) was reduced after glucose re-feeding (Fig. 4A), correlated with increased ACLY expression and function (Fig. 1), suggesting an inverse relationship between ACLY and AMPK activities. Treatment of OA chondrocytes with HCA resulted in a significant decrease in the amount of nucleocytosolic acetyl-CoA (Fig. 4B), which mimicked glucose starvation. A similar result was observed in OA chondrocytes in which ACLY was knockdown (Fig. 4B). Inhibition of ACLY by HCA or siRNA knockdown also led to a marked increase in phosphorylation of AMPKα (Thr-172) (Fig. 4C). Because activation of AMPK in chondrocytes inhibits catabolic responses to inflammatory cytokines (5), we examined the effect of inhibition of ACLY by HCA on IL-1β–induced nitric oxide (NO) release in AMPKα1 knockout (KO) and wildtype (WT) mouse chondrocytes. As expected, IL-1β–induced NO release was greatly enhanced in AMPKα1KO, compared with WT chondrocytes (Fig. 4D). HCA significantly inhibited IL-1β–induced NO release in not only WT but also AMPKα1KO chondrocytes

Figure 3. Concomitantly increased phosphorylation of ACLY (Ser-455) and acetylation of H3K9 and H3K27 in primary human knee OA chondrocytes. Whole cell lysates isolated from primary human knee chondrocytes from normal (grade 0-I) and OA (grade IV) donors were studied by Western blotting for ACLY expression and phosphorylation (Ser-455), and H3K9 and H3K27 acetylation. β-Actin and histone H3 served as loading controls. Data in A represent 5 normal and 5 OA donors. Age and gender for normal donors were 26 male, 28 male, 34 male, 44 male, 58 male, and age and gender for OA donors were 58 male, 68 female, 71 female, 75 female, 76 male. Semi-quantitative densitometry analyses of Western blots of primary human knee chondrocytes (B) from 28 normal donors (20 male and 8 female, age 42.3 ± 10.6) and 28 OA donors (10 male and 18 female, age 67.8 ± 7.9) were performed. Student’s t test was used for statistical data analysis. p values represent comparison of the mean ± S.E. between normal and OA donors.
IL-1β treatment increased nucleocytosolic acetyl-CoA levels (by 31.5%) in OA chondrocytes (Fig. 5A). HCA treatment decreased basal and IL-1β–induced levels of nucleocytosolic acetyl-CoA by 40 and 45%, respectively (Fig. 5A). In addition, IL-1β increased phosphorylation of ACLY at Ser-455 (Fig. 5B) and H3K9 and K3K27 acetylation (Fig. 5B), effects were also attenuated by HCA (Fig. 5B). Moreover, ChIP-quantitative PCR (qPCR) analysis demonstrated that HCA diminished the IL-1β–induced acetylation of p65 NF-κB and Ac-H3K9 and Ac-H3K27 occupancy of genes that promote loss of chondrocyte extracellular matrix and whose expression is promoted by NF-κB activity, specifically iNOS, MMP3, and MMP13 (Fig. 5C). Furthermore, HCA inhibited IL-1β–induced acetylation of p65 NF-κB at Lys-310, a post-translational modification required for full transcriptional activity of NF-κB (26) (Fig. 5D). HCA also inhibited Ser-536 phosphorylation of p65 NF-κB, a change that enhances NF-κB transcriptional activity (26) (Fig. 5D). Quantitative RT-PCR confirmed that HCA attenuated IL-1β–induced iNOS, MMP3,
**ATP-citrate lyase function in articular chondrocytes**

**Figure 5. Inhibition of ACLY by HCA attenuated IL-1β-induced augmentation of acetylation H3K9 and H3K27 both globally and specifically in the iNOS, MMP3, and MMP13 promoters.** Cultured primary human knee chondrocytes were treated with IL-1β (2 ng/ml) with or without HCA (20 mM) for 5 h. Nucleocytosolic acetyl-CoA was measured (A). Phosphorylation (Ser-455) and expression of ACLY and acetylation of H3K9 and H3K27 (B), and acetylation of p65 NF-κB (Ser-536) (D) were examined by Western blotting, with β-actin and histone H3 as loading controls. Semi-quantitative densitometry analysis of each Western blotting was performed over 3 individual experiments with 3 different donors (B and D). ChIP-qPCR assessed Ac-H3K9 and Ac-H3K27 occupancy of iNOS, MMP3, and MMP13 promoters, and were presented as percentage of input of sonicated genomic DNA (C). Data in C represent the mean ± S.D. of 3 individual experiments with 3 different donors. Two-way ANOVA followed by Bonferroni’s post hoc test (A and C) and Student’s t test (B and D) were used for statistical data analysis. p values represent comparisons of the mean ± S.D.

and MMP13 mRNA expression (Fig. 6A). Moreover, HCA mitigated IL-1β–induced NO, MMP3, and MMP13 release in OA chondrocytes (Fig. 6B). Comparable results were seen in OA chondrocytes subjected to ACLY siRNA knockdown (Fig. 7). Furthermore, cartilage explant studies also demonstrated that HCA suppressed IL-1β–induced GAG, NO, MMP3, and MMP13 release (Fig. 6C).

**HCA increased matrix gene expression in OA chondrocytes by reducing SOX9 acetylation**

Treatment of OA chondrocytes with HCA significantly increased basal levels of *aggrecan* (*ACAN*) and *Col2a1* mRNA expression (Fig. 8, A and B). However, this was unlikely due to acetylation of histones, because HCA markedly reduced basal levels of Ac-H3K9 and Ac-H3K27 in OA chondrocytes (Fig. 5B). Because acetylation of SOX9, the chondrogenic master transcription factor, is recently shown to increase in human knee OA chondrocytes (27), and acetylation of SOX9 reduces its nuclear entry and subsequent ACAN gene expression (27), we examined the effect of HCA on SOX9 acetylation. HCA treatment blunted acetylation of SOX9 (Fig. 8C) in OA chondrocytes. In addition, HCA promoted SOX9 translocation from the cytosol to nucleus, indicated by decreased SOX9 expression in the cytosol and increased SOX9 expression in nucleus (Fig. 8D). These data indicate that ACLY regulated matrix anabolism in OA chondrocytes through modulation of acetylation of SOX9.

**Discussion**

The OA chondrocyte ACLY findings presented here are aligned with growing evidence that histone acetylation is tightly linked to the cellular metabolic state (11–14). The results place ACLY potentially at the hub of metabolic changes in OA chondrocytes that could promote disease progression. Glucose is required as metabolic fuel for chondrocytes, and as a structural precursor in extracellular matrix synthesis (3). However, heightened glycolysis can promote tissue inflammation (28). OA chondrocytes exposed to high glucose have increased glu-
Figure 6. Inhibition of ACLY by HCA attenuated catabolic responses to IL-1β in OA chondrocytes. Cultured primary human knee OA chondrocytes (A and B) or human knee cartilage explants (C) were treated with IL-1β (2 ng/ml) in the presence or absence of HCA (20 mM) for 5, 18, and 24 h, respectively. Total RNA was extracted from 5-h treated samples and subjected to quantitative RT-PCR analysis for mRNA expression of iNOS, MMP3, and MMP13 (A). Conditioned media from the remaining samples were analyzed for release of NO, MMP3, and MMP13 by Griess reaction and ELISA, respectively (B and C). Glycosaminoglycan (GAG) release was determined by dimethylmethylene blue assay (C). Data in A and B represent 3 individual experiments in OA chondrocytes from 3 different donors with replicates for each donor. Data in C represent 18 cartilage explants. Two-way ANOVA followed by Bonferroni’s post hoc test was used for statistical data analysis. p values represent comparisons of the mean ± S.D.

Our findings revealed a regulatory role of ACLY activity in chondrocyte matrix homeostasis by modulation of the nucleocytosolic pool of acetyl-CoA, which impacted on catabolic and anabolic responses via post-translational and epigenetic modifications. Glucose availability in articular chondrocytes influenced nucleocytosolic acetyl-CoA generation and histone acetylation, which were largely dependent on ACLY. As illustrated in our putative model (Fig. 9), ACLY may become overactive in chondrocytes as a result of increased glycolysis and/or inflammatory response, causing an increase in the nucleocytosolic pool of acetyl-CoA. Subsequently, chondrocyte capacity to active AMPK is reduced. In addition, matrix gene expression (ACAN, Col2a1) is inhibited and genes that promote degradation of extracellular matrix (iNOS, MMP3, and MMP13) are activated. These effects are mediated by specific post-translational acetylation modifications of histones and transcription factors (increased acetylation of H3K9, H3K27, p65, NF-κB, and SOX9). Consequently, cartilage homeostasis was impaired due to imbalance between matrix anabolic and catabolic activities in chondrocytes, which could lead to cartilage degradation and OA development.
We do not yet know how ACLY is up-regulated in human knee OA chondrocytes. In cells other than chondrocytes, ACLY mRNA is regulated by the transcription factor sterol regulatory element-binding protein-1 (SREBP-1), via Akt signaling (13). However, ACLY protein levels are independent of SREBP-1 (13). The PI3K/Akt pathway stimulates ACLY catalytic activity predominantly through phosphorylation of ACLY at Ser-455, which also contributes to stabilization of ACLY protein (13).

We speculate that IGF1 and other chondrocyte growth factors have the potential to modulate ACLY expression in OA cartilage chondrocytes. Specifically, growth factor IGF1 can induce phosphorylation of ACLY at Ser-455 via Akt signaling (31). In addition, IGF1 signaling is altered in OA cartilage (32), and OA chondrocytes exhibit decreased anabolic response to IGF-1 (32).

Depletion of the nucleocytosolic pool of acetyl-CoA was recently revealed to activate AMPK (11, 20). We observed the capacity of inhibition of ACLY in OA chondrocytes (by either siRNA knockdown or pharmacologic inhibition) to drop nucleocytosolic acetyl-CoA and promote AMPK activation. Thus, decreased AMPK activity in human and mouse knee OA cartilages (5, 6) may be partly due to increased ACLY activity in OA chondrocytes. Similarly, age-related loss of AMPK activity in knee cartilage (6) may potentially result in part from increased ACLY activity. Because chondrocyte-specific AMPKα KO mice develop accelerated mechanical injury–induced and age–related spontaneous knee OA (8), maintaining AMPK activity by preventing ACLY to become overactive in chondrocytes may potentially limit OA development and progression.

Selective depletion of nucleocytosolic acetyl-CoA is also shown to induce autophagy through inhibition of mTOR via activation of AMPK (11, 33, 34). In addition, acetyl-CoA modulates transcription of core autophagy genes (11, 12). Future study would be of interest to determine whether inhibition of ACLY in OA chondrocytes activates autophagy, which is chondroprotective in vivo (35–37).

Putative translational significance of the work is the potential intersections of ACLY modulation with diet, nutrition, and metabolic syndrome, and respective impact of each of these factors on pathogenesis and progression of OA (1–4). Given that ACLY is emerging as a drug target for long-term use in metabolic disorders (19), it could provide an early opportunity...
to study potential impact of ACLY inhibition on OA onset, symptoms, and progression. Thus, further translational studies in OA, by testing the role of pharmacologic inhibition of ACLY on disease progression, are warranted.

**Experimental procedures**

**Reagents**

Chemical reagents, including HCA, tripotassium salt, were obtained from Sigma. Antibodies to phospho-AMPKα (Thr-172), AMPKα, phospho-p65 NF-κB (Ser-536), acetyl-p65 NF-κB, ACLY, pyruvate kinase isoenzyme M2 (PKM2), H3 (acetyl-K9), H3 (acetyl-K27), and histone H3 were from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies to SOX9, α-tubulin, and lamin B were from Abcam (Cambridge, MA). Antibody to phospho-ACLY (Ser-455) was from Aviva Systems Biology (San Diego, CA). Human ACLY siRNA and control siRNA were from Thermo Fisher Scientific (Waltham, MA).

**Studies of human and mouse articular chondrocytes**

Studies with human knee articular chondrocytes were performed in compliance with institutional IRB reviewed and approved human subjects protocols at the Scripps Research Institute and San Diego VA Medical Center, and abide by the Declaration of Helsinki principles. Studies with mouse knee articular chondrocytes were carried out in compliance with institutional IACUC reviewed animal protocols at the San Diego Veterans Affairs Medical Center. Human knee chondrocytes were isolated from autopsy donors that were graded macroscopically according to a modified Outerbridge scale (38) and cultured in Dulbecco’s modified Eagle’s (DMEM) high glucose medium with 10% fetal calf serum, 100 μg/ml of streptomycin, and 100 IU/ml of penicillin at 37 °C. Once the cells reached confluence (cultured primary chondrocytes), they were either collected and stored in −80 °C for later analyses, or were replated at 3 × 10^5 cells/well in 12-well plates or 7 × 10^5 cells/well in 6-well plates for all other experiments. Mouse articular chondrocytes were isolated from knee joints of 7–8–day-old pups.
ATP-citrate lyase function in articular chondrocytes

Figure 9. Putative model of ACLY in cartilage matrix metabolism and OA. Shown is a schematic model that summarizes our results, in which pharmacologically targetable ACLY was increased in OA chondrocytes in situ and in response to increased glycolysis and IL-1β in vitro, with associated changes that could disrupt cartilage matrix homeostatic balance, leading to cartilage degradation, as discussed in detail in the text.

Measurement of acetyl-CoA
Acetyl-CoA concentrations were determined using a PicoProbe™ Acetyl-CoA Fluorometric Assay Kit (Biovision). To measure cell compartmental acetyl-CoA concentrations, the Thermo Scientific Mitochondria Isolation Kit for Cultured Cells was used to separate mitochondria from the rest of the cytosolic and nuclear components according to the manufacturer’s protocol. Isolated mitochondria and the remaining nucleus and cytosol fraction were then used to measure mitochondrial and nucleocytosolic acetyl-CoA, respectively.

SDS-PAGE/Western blotting
Total cell lysates were prepared using RIPA buffer with 2 mM sodium vanadate and protease inhibitor cocktails (Roche Applied Science). Cytosolic and nuclear proteins were prepared by lysing the cytosolic and nuclear fractions of cells extracted using a Cell Fractionation kit (Abcam, Cambridge, MA). Proteins (10–15 μg) were separated by gradient 4–20% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad), probed with antibodies, exposed to SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), and visualized by autoradiography.

Immunohistochemistry
Slides of human knee cartilage sections from both normal and advanced OA donors were treated with 3% H2O2 for 10 min, then blocked with 10% goat serum for 2 h at room temperature. After washing with Tris-buffered saline, rabbit antibodies to phospho-ACLY (Ser-455), ACLY, and the negative control rabbit IgG (all in 1:100 dilution) were applied to the sections and incubated overnight at 4 °C. Next, sections were washed with Tris-buffered saline, incubated with biotinylated goat anti-rabbit IgG secondary antibody for 30 min, and then incubated for 30 min using the Histostain Plus kit (Invitrogen). Finally, sections were washed and incubated with 3,3′-diaminobenzidine substrate for 2–5 min.

Chromatin immunoprecipitation (Chip) assay followed by quantitative PCR (Chip-qPCR)
Chip assay employed a commercial kit (EMD Millipore, Temecula, CA), according to the manufacturer’s protocol. In brief, cells that were fixed with formaldehyde to cross-link histone and nonhistone proteins to DNA were lysed, and harvested chromatin was fragmented by sonication. Chromatin was immunoprecipitated with antibodies to Ac-H3K9 or Ac-H3K27. The nonimmune IgG was included as a control. The protein-DNA cross-links were then reversed and the DNA was purified. Enriched DNA sequences occupied by H3K9 and H3K27 in promoter regions of iNOS, MMP3, and MMP13 were detected by quantitative PCR using the primers listed in Table S1. The sonicated genomic DNA was included in qPCR as the input control. The PCR was carried out at 95 °C for 5 min for initial denaturation, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 10 s.

Knockdown of ACLY expression in human knee articular chondrocytes
Cultured human knee articular chondrocytes were transfected with ACLY siRNA, and nontarget control using the X-tremeGene siRNA transfection reagent (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s protocol.

of AMPKα1 KO and WT mice using previously described methods (39), and were cultured in the same media aforementioned in 6-well plates. They were then re-plated at 3 × 10⁵ cells/well in 12-well plates for experiments.
Statistical analyses

Most of data were expressed as mean \( \pm S.D \). Some data were expressed as mean \( \pm S.E \). Statistical analyses were performed by either Student’s \( t \) test, one-way ANOVA, or two-way ANOVA with Bonferroni’s post hoc test using GraphPad PRISM 6. \( p \) values less than 0.05 were considered significant.

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ATP-citrate lyase function in articular chondrocytes

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