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The clock transcription factor BMAL1 is a key regulator of extracellular matrix homeostasis and cell fate in the intervertebral disc

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ABSTRACT

The circadian clock in mammals temporally coordinates physiological and behavioural processes to anticipate daily rhythmic changes in their environment. Chronic disruption to circadian rhythms (e.g., through ageing or shift work) is thought to contribute to a multitude of diseases, including degeneration of the musculoskeletal system. The intervertebral disc (IVD) in the spine contains circadian clocks which control ~6% of the transcriptome in a rhythmic manner, including key genes involved in extracellular matrix (ECM) homeostasis. However, it remains largely unknown to what extent the local IVD molecular clock is required to drive rhythmic gene transcription and IVD physiology. In this work, we identified profound age-related changes of ECM microarchitecture and an endochondral ossification-like phenotype in the annulus fibrosus (AF) region of the IVD in the *Col2a1-Bmal1* knockout mice. Circadian time series RNA-Seq of the whole IVD in *Bmal1* knockout revealed loss of circadian patterns in gene expression, with an unexpected emergence of 12 h ultradian rhythms, including FOXO transcription factors. Further RNA sequencing of the AF tissue identified region-specific changes in gene expression, evidencing a loss of AF phenotype markers and a dysregulation of ECM and FOXO pathways in *Bmal1* knockout mice. Consistent with an up-regulation of FOXO1 mRNA and protein levels in *Bmal1* knockout IVDs, inhibition of FOXO1 in AF cells suppressed their osteogenic differentiation. Collectively, these data highlight the importance of the local molecular clock mechanism in the maintenance of the cell fate and ECM homeostasis of the IVD. Further studies may identify potential new molecular targets for alleviating IVD degeneration.

Introduction

Evolutionarily conserved circadian (~24 h) rhythms persist throughout biology, with almost every aspect of our physiology and behaviour having evolved around the rotation of the earth. Endogenous circadian rhythms define our daily patterns of sleep, eating and exercise, moderate the scale of an immune response we mount, determine how our body responds to medications, and gate daily patterns of metabolism [1]. Evolved as a homeostasis mechanism, this temporal alignment of behaviour and physiology to the external environment ensures optimisation of metabolism and energy allocation to anticipate daily variations in physiological demands [2]. In a tissue context-dependent manner, circadian clocks drive 24 h rhythmicity in approximately 5–10% of the genome [3], 5–20% of the proteome [4–6] and ~25% of the phosphoproteome [7]. Like many other regulatory processes, the circadian clock changes during ageing, losing its precise temporal control with the robustness of circadian rhythms, in terms of oscillatory amplitude and circadian phase, declining with age in both animal models and humans [8]. As such, the functional decline of circadian rhythms has been proposed as a potential mechanism driving an increased risk of various diseases, including metabolic syndromes, cancer and musculoskeletal conditions [8–10]. Indeed, genetic models of circadian disruption

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exhibit age-related musculoskeletal conditions including osteoporosis, osteoarthritis and tendinopathy [11–14]. In humans, prolonged misalignment of internal circadian rhythms with environmental rhythms, such as those seen in chronic shift workers or frequent long-haul travellers, is associated with profound consequences for health and well-being [15,16]. Given that both population ageing and chronic circadian misalignment are increasingly prevalent, understanding the importance of biological timing mechanisms in age-related diseases is becoming more and more relevant.

Intervertebral discs (IVDs) are extracellular matrix (ECM)-rich fibrocartilaginous joints that allow flexion and shock absorption in the spine. They consist of an outer fibrous ring, the annulus fibrosus (AF), which surrounds an inner gelatinous centre, the nucleus pulposus (NP) rich in proteoglycans. The AF consists of several weaved layers (laminae) of tendon-like tissue composed predominantly of collagen. Correct arrangement of the layers as well as the collagen fibres is essential to counteract mechanical forces and contain the NP when the spine is subject to mechanical load. The AF consists of distinct regions - the inner AF, adjacent to the NP and rich in type II collagen, and an outer AF to the peripheral edge of the disc consisting primarily of type I collagen. The inner AF is populated by rounded fibrocartilage cells, while cells in the outer AF show a more fusiform fibroblast-like morphology. These cells are organised in a linear arrangement, with the degree of linear alignment increasing as proximity to the NP decreases. With age, there is progressive degeneration of the IVD, which is reported to affect approximately 90% of individuals over the age of 50 years [17]. This degeneration is characterised by reduced tissue hydration (due to loss of proteoglycan), fibrosis, calcification and altered cell population and morphology [18–23]. These pathological changes form a vicious circle whereby alterations in cell behaviour and matrix composition impair tissue mechanics (and ultimately tissue function), which in turn drive an exaggerated and aberrant cellular response.

Despite the notion of daily variations in spine physiology being recorded as early as 1724, evidence of an intrinsic circadian rhythm within cells of the IVD was only provided recently [24]. The molecular circadian clock within the mouse IVD drives rhythmic IVD transcription and shows strong tissue-specificity, with little overlap even within the musculoskeletal systems. These rhythmic outputs include genes relevant to ECM turnover (e.g., *Adamts1, Timp4, Serpinh1 and Itgb1*) and endoplasmic reticulum (ER) stress (e.g., *Pak1, Atf6 and Chac1*) and are believed to be essential for coping with the daily stresses that the IVD is exposed to within its unique physiological niche [25]. Degeneration of the IVD is a complex and multifactorial process associated with ageing. Significantly, these IVD clocks are disrupted by ageing and inflammation, two known risk factors for IVD degeneration, suggesting the involvement of clock mechanisms in driving a predisposition to the development of accelerated ageing and degeneration in the IVD [25]. However, it remains largely unknown how the IVD clock regulates ECM homeostasis and cell fate in the IVD, and what the underlying molecular links are.

Here, a circadian clock disruption model (*Col2a1-Bmal1* knockout mouse) was analysed using atomic force microscopy and electron microscopy, and time-resolved and tissue-type specific transcriptomic analysis, to investigate the importance of circadian clocks in the homeostasis and degeneration of the ECM-rich intervertebral disc tissue. Our data reveal a profound age-related degeneration characterised by dysregulated ECM microarchitecture in the *Bmal1* KO IVDs, with a major osteogenic component linked to the emergence of a putative ultradian 12 h clock. The circadian transcriptome of the whole disc as well as a comparison of the AF transcriptome indicate an apparent loss of AF phenotype markers and altered ECM homeostasis, with FOXO genes identified as key drivers of the *Bmal1* KO phenotype.

Results

Tissue-specific knockout of Bmal1 predisposes the IVD to age-related extracellular matrix degeneration

Masson's trichrome staining of the *Col2a1-Bmal1* knockout (KO) mouse showed age-related intervertebral disc phenotypes, characterised by thinning of the cartilage end plate and bony protrusions into the growth plate (Fig. S1), consistent with our earlier report using standard histology and X-ray imaging [25]. To gain further insight into changes to the ECM architecture and cell morphology, a newly developed RGB-trichrome staining method [26] was employed (Fig. 1A). This histology analysis allows for clear differentiation not only between tissue types, but also between calcified and uncalcified ECM. Wildtype (WT) IVDs showed clear demarcation between IVD tissue components, with the collagen-rich AF staining in the red spectrum and more proteoglycan-rich tissues such as the NP staining blue. While the lamellae of the inner AF were still evident in the *Bmal1* KO tissue, the resident AF cells appear rounder in morphology when compared with the flattened fibroblast-like cells typical of the healthy AF. The lamellar structure of the outer AF was progressively lost, and large rounded cells appeared. These cells appeared to have migrated outwards towards the periphery of the outgrowth, suggested by the brush stroke-like pattern of dark blue staining that radiated outwards from the lamellar AF (Fig. 1A and B). This blue region then transitions into a distinct yellow-green matrix (Fig. 1B(4)), likely representing calcified ECM [26]. This cartilaginous structure is visible macroscopically upon dissection of the discs (Fig. S2). It appeared that this tissue was derived from rounded hypertrophic chondrocyte-like cells in the KO AF (Fig. 1B). This change of cellular phenotype was reiterated by scanning electron microscopy, showing large, rounded cells with electron dense cytoplasmic compartments in the outer AF of *Bmal1* KO (Fig. 1C). Segmentation of the serial electron microscopy images revealed striking difference in cell morphology. The *Bmal1* KO cells appeared polarised with numerous protrusions on one side of the cell and rounded plasma membrane on the other. Some of the protrusions containing electron dense material became disconnected from the main body as if left behind by a migrating cell (Fig. 1D, Fig. S3 and Supplementary videos 1–4).

Dysregulated collagen organisation and nano-architecture in the IVD of Bmal1 KO mice

Given the disruption to the lamellar arrangement of the collagen-rich AF in *Bmal1* KO mice, AFM and EM were used to examine collagen architecture on the nanoscale. The inner AF of young mice (6 weeks old) showed similar collagen arrangements and typical D period length of 67 nm between WT and *Bmal1* KO (Fig. 2A). However, fibrils showed a significantly thinner mean diameter of 85.12(±19.73) nm in *Bmal1* KO tissue as compared with 90.45(±23.28) nm in wildtype ($P<0.001$, Fig. 2B). Moreover, there was a negative shift in the distribution of fibril diameters, with no fibrils of ≥150 nm identified in *Bmal1* KO tissue (Fig. 2B). Collagen fibrils in the ageing WT inner AF were arranged in a linear fashion, as visualised by AFM at coronal and EM at transverse planes (Fig. 2C). Fibrils in the *Bmal1* KO inner AF appeared slightly disorganised both by AFM and EM imaging (Fig. 2C). Thinning of collagen fibrils was evident in *Bmal1* KO animals, with these fibrils having a significantly smaller mean diameter of 101(±20.62) nm compared with 156.8(±24.2) nm in wildtype ($P<0.0001$, Fig. 2D). Plotting the distribution of fibril diameters showed a leftwards shift in diameter in *Bmal1* KO fibrils. Length of the D period showed no significant change (Fig. 2D). The outer AF of ageing *Bmal1* KO animals showed a more profound change than the inner AF. Linear fibril arrangement was maintained in the outer AF of the wildtype disc. However, visible collagen architecture was almost unidentifiable in *Bmal1* KO (Fig. 2E). The tissue was also pitted by numerous small indentations and mounds, which may have been left by calcification

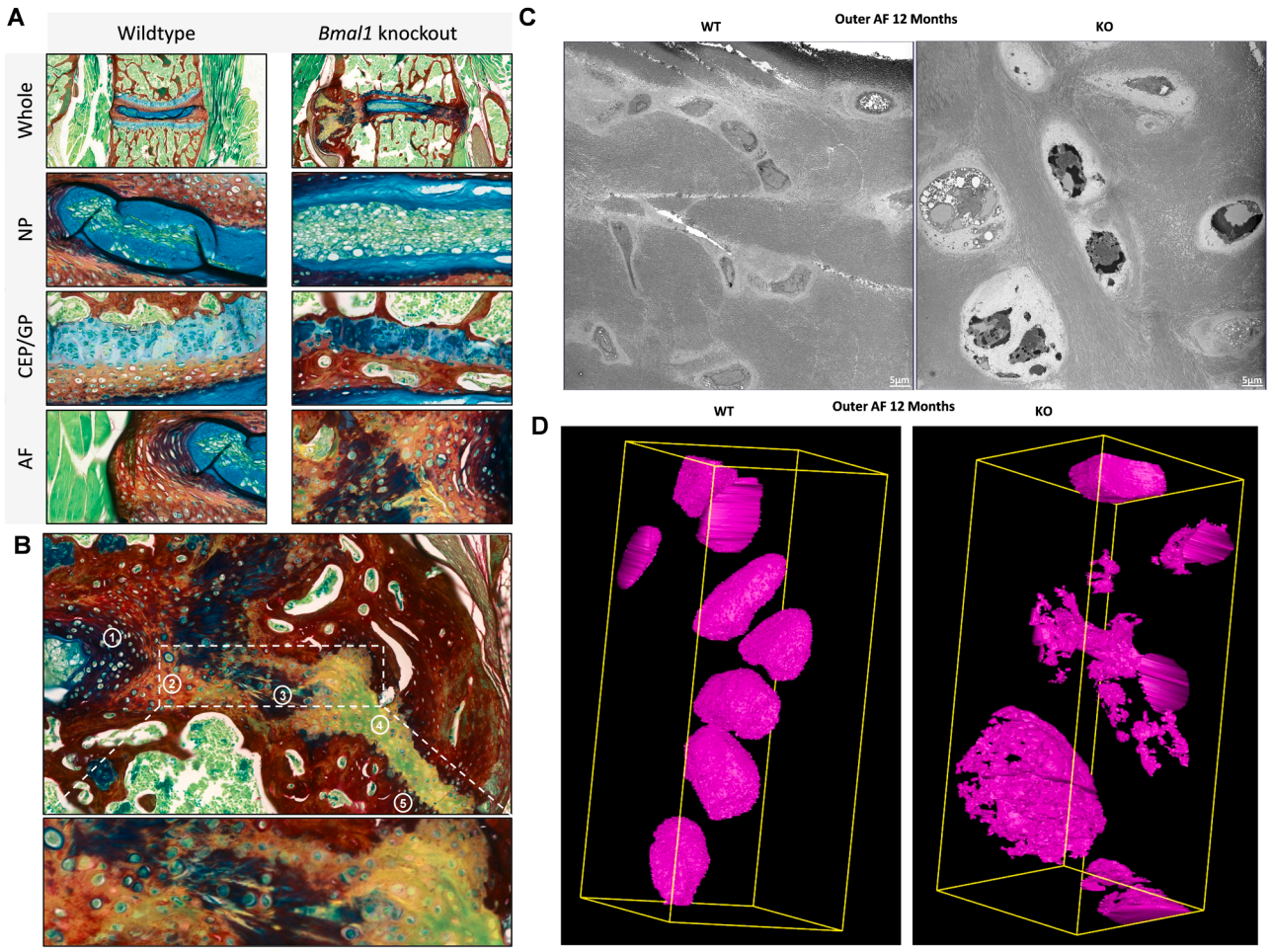

Fig. 1. Col2a1-specific *Bmal1* KO leads to age related IVD degeneration. (A). RGB trichrome staining of 12-month-old wildtype versus Col2a1-*Bmal1* KO lumbar intervertebral disc. **(B).** Representative heterotopic ossification region of the aged Col2a1-*Bmal1* KO IVD. Top, Composite image of 10x magnification snapshots, stitched together to visualise the whole region of heterotopic ossification. Numbers represent a proposed migratory route of cells originating from the inner AF (1), moving to the outer AF (2), the proteoglycan-rich region fanning out from the IVD (3), the non-calcified cartilage region (4) and finally cartilage-bone interface showing pits with resident hypertrophic chondrocyte-like cells (5). Bottom panel, magnification of the dashed rectangle in the top panel. Staining appears to show a brushstroke-like effect from the centre of the disc **(C).** Representative images from scanning electron microscopy (SEM) showing WT and *Bmal1* KO outer AF tissues at 12 months of age. **(D).** 3D rendering of segmented cells from serial block-face SEM from WT and *Bmal1* KO outer AF tissues at 12 months of age.

deposits (Fig. 2E).

Outer AF of the Bmal1 KO IVD undergoes endochondral ossification

Given the apparent deposition of cartilage and bone-like ECM within the outgrowth region of the *Bmal1* KO IVDs, resident cells were examined to gain further insight into their phenotype. Their rounded morphology and cartilaginous territorial matrix led to the hypothesis that these cells resemble hypertrophic chondrocytes. This hypothesis was supported by immunohistochemistry (IHC) staining for chondrogenic makers such as COLX (Type X collagen), SOX9 (SRY-box transcription factor 9) and ALPL (Alkaline phosphatase) (Fig. 3 and Fig. S4). COLX expression was visible in the AF of KO mice already at 3 months of age (before visible degeneration) and remained at high levels at 6 and 12 months. While in WT, only CEP region stained for COLX (Fig. 3). Moreover, primary AF cells isolated from 3 months old KO animals retained high COLX expression in culture (Fig. S5). SOX9 was expressed both in the WT and KO AF at low levels at 3 months of age, but SOX9-positive cells appeared to be more abundant in the *Bmal1* KO IVDs at 6 months, particularly in the NP and inner AF. At 12 months of age elevated SOX9 staining was visible throughout the AF of KO IVDs (Fig. 3, Fig. S4A). ALPL-positive cells were generally absent within the WT AF, however, were abundant in *Bmal1* KO animals. These cells were present mostly within the central cartilaginous region and peripheral edges of the outgrowth and showed a rounded morphology (Fig. S4B).

Comparative analysis of the WT and Bmal1 KO circadian transcriptome revealed emergence of 12 h rhythmic genes in KO

Bmal1 is an essential component of the circadian clock mechanism without which the 24 h oscillations patterns in gene expression will be abolished. To fully assess the effect of IVD-specific *Bmal1* knockout, we performed circadian time series RNAseq in *Bmal1* KO whole IVDs and performed a comparative analysis with our published WT mouse IVD data [25]. This analysis identified 788 genes (approximately 6% of the IVD transcriptome) that cycled with a circadian pattern (Fig. 4A) in WT. GO term analysis of circadian genes showed over-represented terms including ‘fatty acid metabolic process’, ‘circadian regulation of gene expression’, ‘response to endoplasmic reticulum stress’ and ‘proteasomal protein catabolic process’ (Fig. 4B). It is of particular interest to note the circadian expression of genes relating to ECM homeostasis and chondrocyte/AF cell physiology, such as *Tgfβ1*, collagen family genes (*Col2a1, Col6a3, Col10a1 and Col11a1*) and proteases *Adam17* and *23,* and *Adamts3* and *12* (Supplementary Tables). Unsurprisingly, the circadian expression patterns of these transcripts were lost in the *Bmal1* knockout IVDs, supporting the role of BMAL1 as a critical regulator of IVD circadian rhythmicity.

Somewhat surprisingly, a profound ultradian rhythmic pattern in

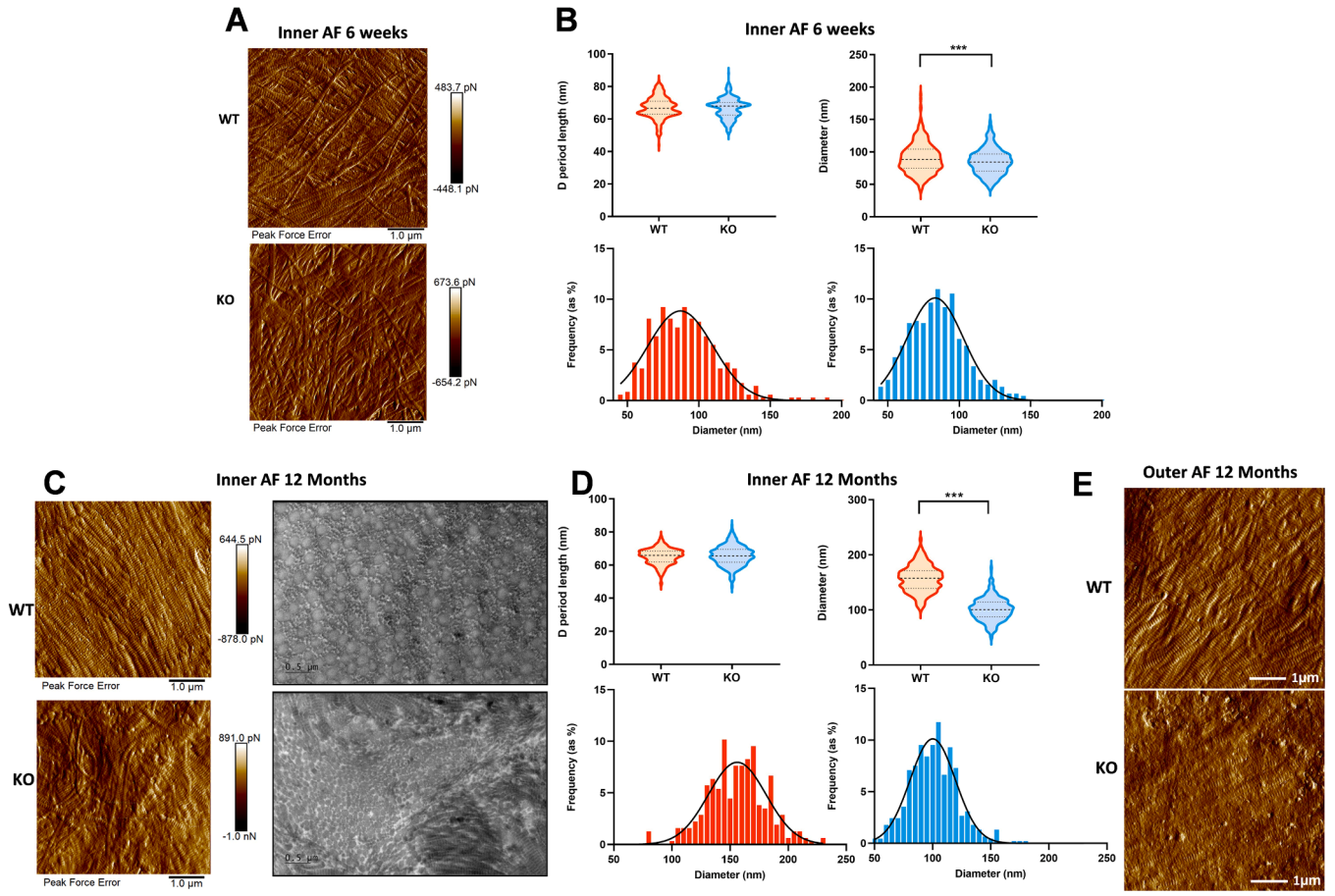

Fig. 2. AFM analysis of the AF in mouse lumbar intervertebral discs reveals thinner and disorganised collagen fibres in the Col2a1-*Bmal1* KO. (A). Representative peak force error AFM images of WT and *Bmal1* KO inner AF at 6 weeks of age showing collagen fibril morphology. **(B).** Quantification of the length of fibril D periods and diameter from AFM images of WT and *Bmal1* KO inner AF at 6 weeks of age. **(C).** Representative peak force error AFM and TEM images of WT and *Bmal1* KO inner AF at 12 months of age showing morphology of collagen fibrils. **(D).** Quantification of the length of fibril D periods and diameter from AFM images of wildtype and *Bmal1* KO inner AF at 12 months of age. **(E).** Representative peak force error AFM images of wildtype and *Bmal1* KO outer AF at 12 months of age showing morphology of collagen fibrils. Tissues from three animals per genotype and per condition were imaged and quantified. Student's t-test, *** - $p < 0.001$.

transcriptional dynamics was identified in the *Bmal1* knockout (Fig. 4C). 3427 genes exhibited a 12 h oscillatory period ($p<0.01$). This represents a substantial 17% of the total transcripts identified in the KO mouse IVD. 12 h gene expression patterns were less apparent within the WT disc (528 genes at $p<0.01$). among these, only 111 genes were common to both genotypes. 3316 genes therefore showed *de novo* ultradian oscillations in *Bmal1* knockout (Fig. 4C). The majority of these ultradian genes did not show 24 h rhythms in WT IVDs (Fig. 4C and Supplementary Tables). This implies a profound switch in temporal gene expression dynamics upon disruption of the molecular circadian clock.

Clustering of the ultradian transcriptome by temporal pattern produced 11 clusters with robust oscillatory patterns (Fig. 4D). Of note, these ultradian genes can be largely segregated into two 'time of peak' groups. Approximately 70% of the ultradian transcriptome showed expression peaks at 5 am and 5 pm, while the remaining 30% of genes showed peaks in their expression at 1am and 1pm. GO term analysis revealed a clear distinction between the two groupings (Fig. 4E). 5 am/5 pm peaking genes showed significant enrichment for processes relating to matrix and cell-cell signalling such as 'glycosaminoglycan metabolic process', 'non-canonical Wnt signalling pathway', 'ossification', 'cartilage development', 'osteoblast differentiation' and 'regulation of cell response to growth factor stimulus', with a list of collagen family genes (Fig. 4E; Fig. S6, Fig. S7). In contrast, 1 am/1 pm peaking genes showed a strong enrichment for terms pertaining to the electron transport chain subunits, mitochondrial translation, cytoplasmic translation, protein transportation and proteasome assembly (Fig. 4E). Of the 88 nuclear-derived genes encoding Complexes I–X of the electron transport chain, a striking 72% were found to be significantly ultradian. None of these genes show a significant ultradian pattern in the WT disc. Electron microscopy imaging of mitochondria from WT and *Bmal1* KO AF showed electron dense inclusion bodies in KO tissues, suggesting that gene expression changes may indeed have effects on the structure or functioning of this organelle (Fig. S8).

Promoter analysis of ultradian genes and AF-specific transcriptomics implicate FOXO transcription factors as effectors for the osteogenic AF phenotype

UPR analysis of genes peaking at 5 am/5 pm showed strong enrichment of forkhead box (FOX) factor motifs (FOXA1, FOXD3, FOXI1, FOXO3, FOXQ1), ARID3A, CEBPA, HOXA5, NFATC2, NOBOX and SRY (Fig. 5A). 11 out of 25 Fox genes expressed in the mouse IVD showed significant ultradian rhythmicity in the *Bmal1* knockout IVDs (Fig. 5B). Genes peaking at 1 am/1 pm were enriched for motifs annotated to ELK4, IRF2, NR1H2:RXRA, NR3C1, SRF and ZNF143 (Fig. S9).

To gain more insight into the AF phenotype, we performed comparative RNAseq on AF tissues dissected at 8 am from WT and *Bmal1* KO mice. Several hundred of genes were differentially expressed between WT and *Bmal1* KO AF (Fig. S10A and Supplementary Tables). Enrichment analysis identified pathways related to ECM organisation, tissue development and FOXO target genes (Fig. S10B and Supplementary Tables). Differentially expressed genes included key regulators of IVD and cartilage homeostasis, such as *Ccn2* (also known as *Ctgf*), *Cnmd,*

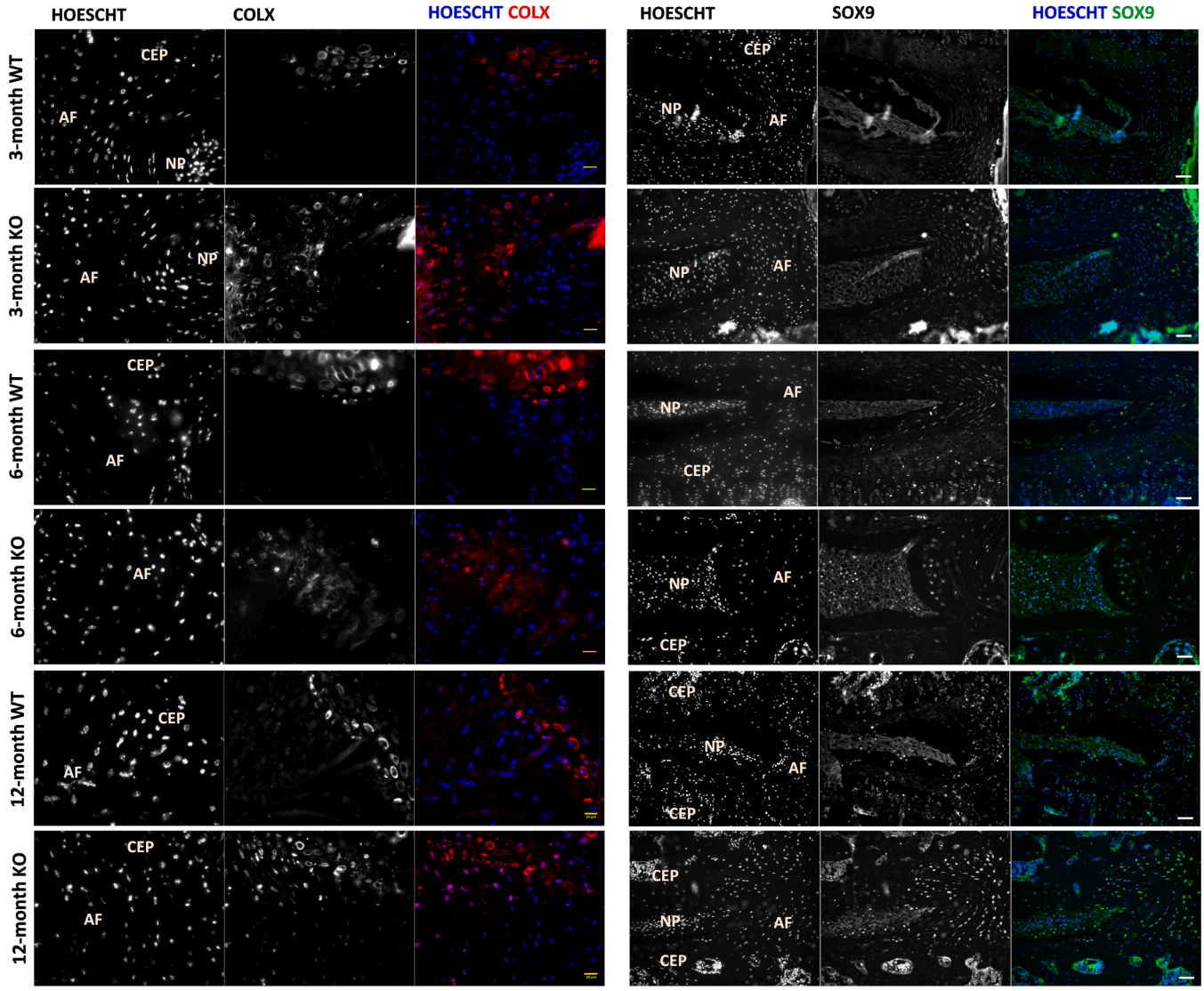

Fig. 3. ***Bmal1*** **KO AF expresses increased levels of hypertrophic chondrocyte markers**. Representative immunofluorescence images of hypertrophic chondrocyte markers COLX and SOX9 in 3, 6 and 12-month-old WT and Col2a1-*Bmal1* KO mice. AF – annulus fibrosus, NP – nucleus pulposus, CEP – cartilaginous end plate. Scale bar: 20 μm in COLX and 50 μm in SOX9 images. $N = 3$.

Foxa2, Gdf6, Grem1, Gli1 and *Ptch1*. Interestingly, many genes described as putative AF marker genes were downregulated in *Bmal1* KO AF tissue, including *Fbln1, Fbln2, Col1a1, Col1a2, Chad* and *Comp*. This is indicative of a loss of AF phenotype in *Bmal1* knockout and supports a transition of AF cells to a more hypertrophic chondrocyte-like phenotype in *Bmal1* knockout.

Among the FOXO factors, FOXO1 has been reported to play a role in age-related IVD degeneration [27,28], making it a key candidate for further investigation. *FOXO1* showed a strong 12 h rhythmic expression pattern in *Bmal1* KO IVDs ($P<0.001$), with much higher expression levels at the 5 pm peaks (Fig. 5B). As such, protein levels of FOXO1 were assessed using IHC. In 6-month-old WT mouse IVDs, FOXO1 signals were detectable. However, in age-matched *Bmal1* KO IVDs, there was a clear increase in expression levels of FOXO1 and number of FOXO1 positive cells, particularly in the outer AF where osteogenesis is observed (Fig. 5C). In an *in vitro* osteogenic differentiation model of human AF cells, *FOXO1* mRNA levels showed an approximately 3-fold induction following osteogenic differentiation, implying *FOXO1's* involvement in osteogenic differentiation of AF cells (Fig. 5D). Consistent with this notion, pharmacological inhibition of FOXO1 with AS1842856 led to an inhibition of osteogenic differentiation in human AF cells (Fig. 5E). Taken together, these data support a significant role of FOXO1 in mediating the osteogenic AF phenotype in *Bmal1* KO IVDs.

Discussion

In this study, using *Col2a1*-specific knockout of the essential circadian clock component *Bmal1*, we investigated the effect of circadian clock disruption on the tissue architecture and gene expression profiles of the intervertebral discs. Profound age-related IVD degeneration was identified in the AF region of the knockout animals; further analysis using electron microscopy and atomic force microscopy identified changes in cell phenotype as well as disorganisation of extracellular matrix structure. Analysis of the IVD circadian transcriptome revealed a loss of the 24 h rhythmic gene patterns and emergence of significant ultradian 12 h oscillation patterns in the *Bmal1* knockout IVDs. Both the circadian transcriptome of the whole disc as well as a comparison of the AF transcriptome pointed to ECM homeostasis as a major *Bmal1* target pathway, revealing FOX genes as key drivers of the *Bmal1* KO phenotype. Importantly, our data demonstrate FOXO1 as a critical *Bmal1*-regulated transcription factor involved in the osteogenic differentiation of human AF cells.

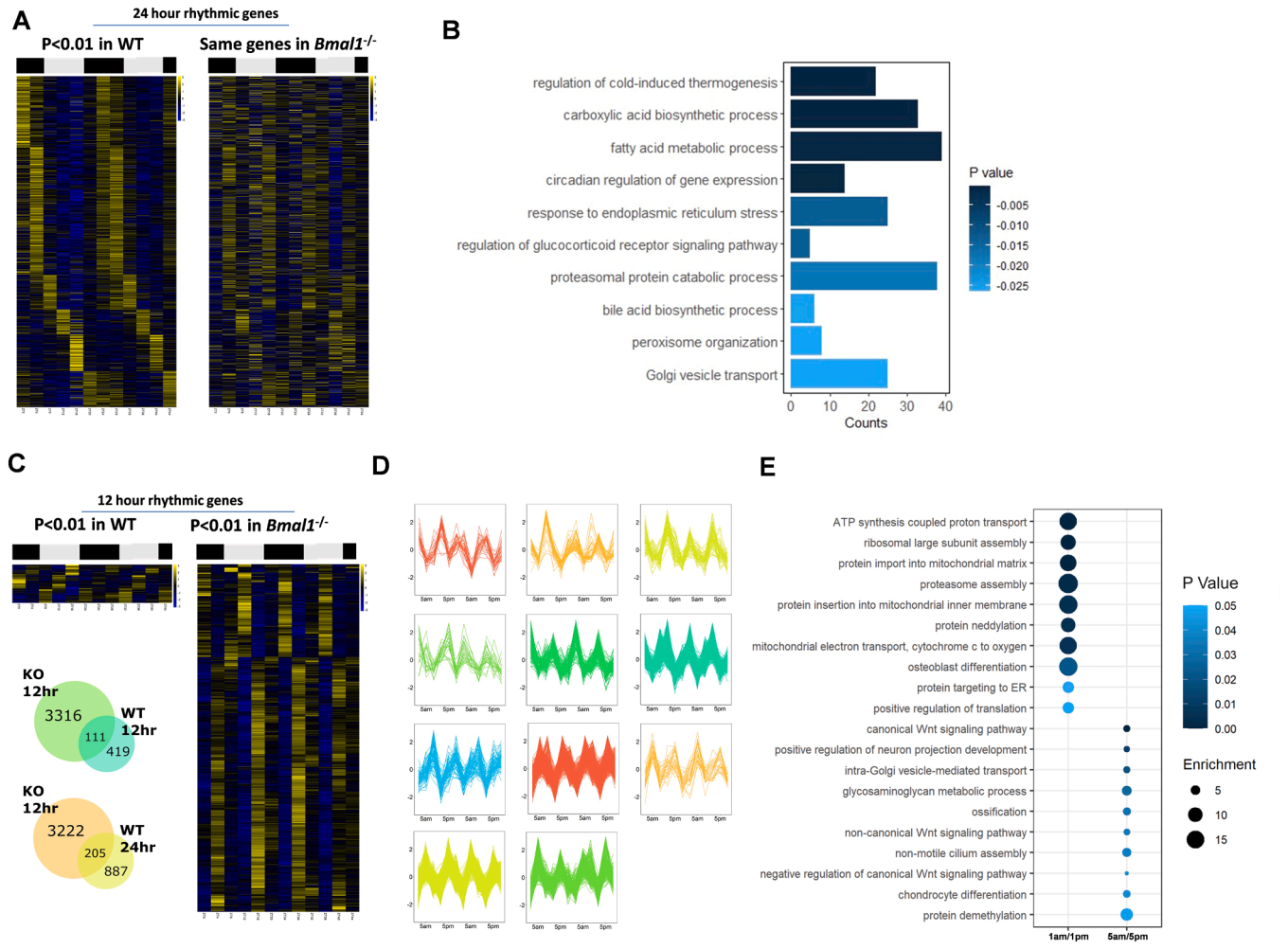

Fig. 4. Emergence of 12 h ultradian genes in *Bmal1* KO IVDs. (A). Heatmaps depicting the expression patterns of the 788 rhythmic genes ($P<0.01$) in WT (left). Equivalent expression patterns of these genes were shown for the *Col2a1-Bmal1* KO IVD (right). Genes were organised according to timing of peak expression. White bars represent the day; black bars represent the night. **(B).** GO term enrichment analysis of the circadian transcriptome, showing the top 10 terms according to P value. **(C).** Heatmaps depicting the expression patterns of the ultradian transcriptome in WT and *Bmal1* KO IVDs and Venn diagram showing overlap between *Bmal1* KO and WT ultradian transcriptome, and *Bmal1* KO ultradian and WT circadian transcriptome. **(D).** Waveform clustering of ultradian transcripts expressed in the *Bmal1* KO mouse intervertebral disc. Y axis values show normalised expression. **(E).** GO enrichment analysis of the ultradian transcriptome in the *Bmal1* KO IVDs, segregated by the time of oscillation peak. The top 10 enriched terms were displayed, ranked by P value.

Despite the highly conserved expression of core clock genes, cross tissue comparisons of circadian functions have revealed an amazing degree of tissue specificity, both in terms of the input pathways to the clock, and output clock-controlled targets [3,29]. As shown previously and in this work, the molecular circadian clock within the WT mice drives rhythmic transcription of ~6% of the IVD transcriptome and shows strong tissue-specificity. These rhythmic outputs are thought to be essential for coping with the daily stresses that the IVD is exposed to within its unique physiological niche [30–32]. The majority of identified circadian genes cycled with a peak expression during waking hours. This 'active phase' group consisted of genes relevant to ECM turnover (e.g., *Adamts1, Timp4, Serpinh1, Itgβ1*) and ER stress (e.g., *Pak1, Atf6, Chac1*), among others.

Age-related degeneration of the intervertebral disc is a multifactorial process, involving a complex interplay between discrete tissue subtypes within the IVD. We have previously shown that ageing disrupts circadian rhythms in IVDs and that the *Bmal1* KO IVD phenotype resembles some of the features seen in aged mice and IVD degeneration [25]. Disruption to the collagen architecture of the ECM is a hallmark of age-related degeneration in cartilaginous tissues. Collagen permits structural flexibility to the disc, necessary for coping with compressive, tensile and shear stresses. In the non-degenerative disc, the AF consists mainly of thick type I collagen fibres with a well-defined linear arrangement. With ageing, the collagen content of the AF declines [33], thinner fibres become more prevalent [34] and linear arrangement is disrupted [35]. These changes are consistent with a shift to a more cartilage-like collagen structure [36]. Our finding that collagen fibrils within the inner AF are thinner in *Bmal1* KO animals as early as 6 weeks (before the onset of observable disc degeneration) suggests that the inner AF could be the primer for later degeneration. We have previously reported that disruption of the circadian clock in tendons leads to malfunctioning of the secretory pathway, and causes abnormal collagen fibrils and collagen accumulation [37]. As a tissue of comparable composition and structure, a similar mechanism could be at play in the AF of intervertebral discs. Thinner collagen fibrils may render the AF less resilient to mechanical strain, leading to accelerated damage or an exaggerated repair response.

An exaggerated cell repair response could explain the cartilaginous protrusion and cell morphology changes observed in the outer AF. Although we have not been able to determine the origin of these cells, studies using single cell RNA-sequencing have identified multiple cell type subsets within the healthy NP and AF, including progenitor-like cells in both tissues [38,39]. Moreover, AF cells have been shown to be capable of differentiating into chondrocytes and osteoblasts *in vitro* as

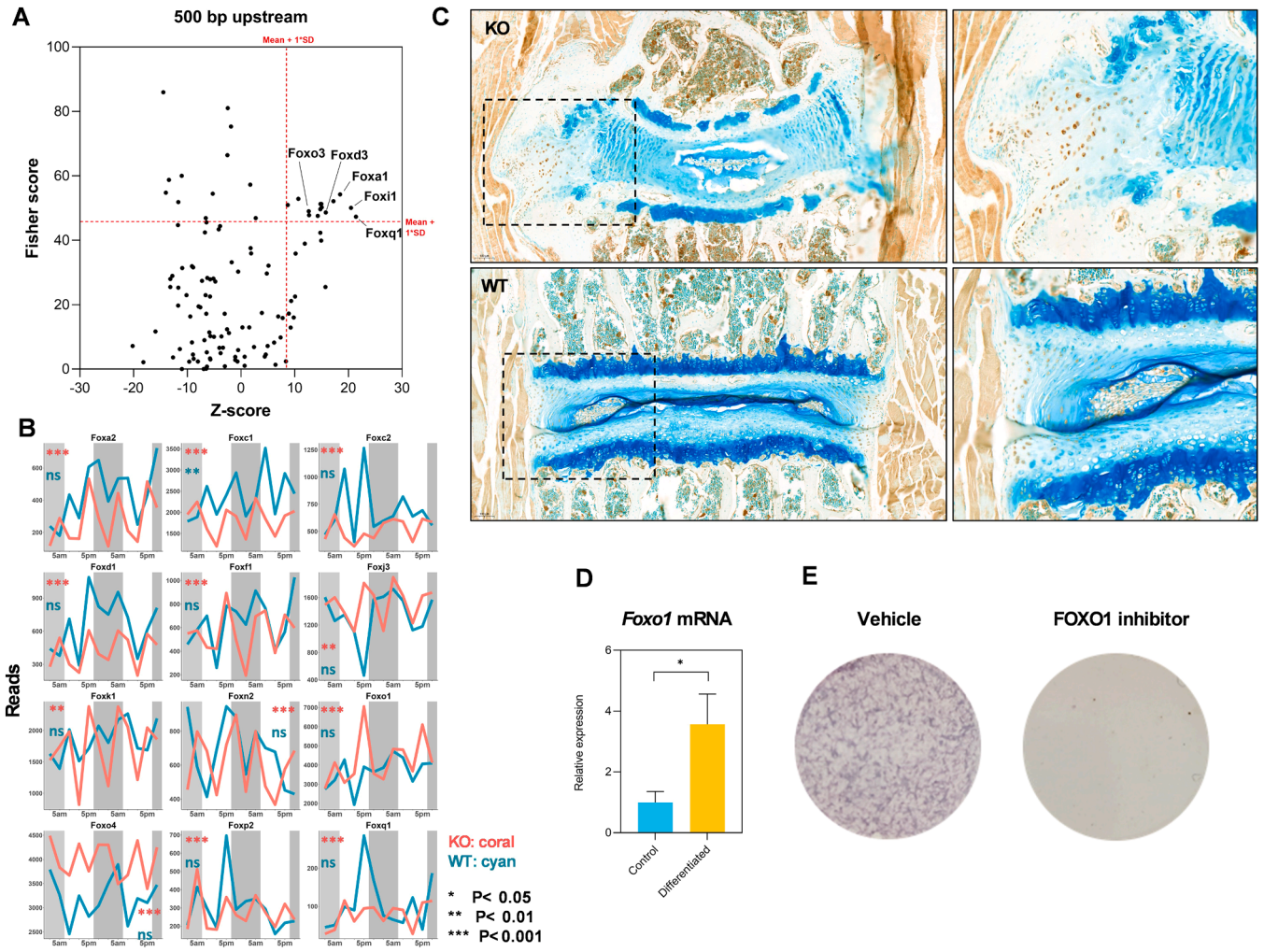

Fig. 5. FOXO1 is involved in the development of the degenerative IVD phenotype in *Bmal1* KO mice. **(A).** Scatter plot of individual transcription factor binding sites (TFBPS) plotted against the Fisher scores and Z-score rankings of their enrichment in the regions 500 bp upstream of the transcription start sites of 5 am/5 pm peaking genes in the ultradian transcriptome. **(B).** Traces of WT (cyan) and *Bmal1* KO (coral) gene expression of FOX transcription factor genes showing significant ultradian rhythmicity in the *Bmal1* KO IVDs. Asterisks denote significance level of 12 h rhythm. Note only *Foxc1* exhibited significant ultradian rhythm in WT IVDs. **(C).** Immunohistochemistry staining of FOXO1 in 6-month-old wildtype and Col2a1-*Bmal1* KO mice. Scale bar: 100 μm. Right panel. Magnification of the dashed rectangle. **(D).** *Foxo1* gene expression in AF-S cells following 3 weeks of culture in osteogenic or control medium ($N = 3$). **(E).** Alkaline phosphatase staining of AF-S cells following 3 weeks of culture in osteogenic medium in the presence or absence of 0.5 μM a FOXO1 inhibitor AS1842856 ($N = 3$).

well as *in vivo,* driving ossification in rat lumbar disc needle puncture model [23]. Migration of progenitor-like cells within the IVD tissue has also been identified previously, such as the migration of cells from a growth plate-adjacent niche through the AF into the centre of the disc [40,41]. Therefore, in the AF of *Bmal1* KO, transition of local cells or invasion by neighbouring regions are equally plausible. What is evident is that these cells express hypertrophic chondrocyte markers such as SOX9, COLX and ALPL.

In line with the AFM and EM findings of changes in collagen matrix composition, the circadian transcriptome of WT IVDs showed 'response to endoplasmic reticulum stress' and 'Golgi vesicle transport' as among the overrepresented circadian regulated processes. Rhythmicity in most of the genes belonging to these categories was lost in the *Bmal1* KO discs. Surprisingly, a new ultradian pattern of expression emerged in the KO IVDs with two antiphase 12 h oscillations. among the 5 am/5 pm peaking genes we revealed overrepresented processes such as 'intra-Golgi vesicle mediated transport', 'glycosaminoglycan metabolic process' and 'ossification', suggesting dysregulation of protein maturation and secretion processes. In WT liver tissues, ultradian rhythms have been referred to as 'harmonics' of the circadian rhythm, i.e., a component frequency of the 24 h circadian oscillation. This implies that ultradian rhythms and circadian rhythms could share a common origin [42]. However, this mechanism is unlikely to be at play in the KO datasets presented here due to the molecular defects of the 24 h circadian clocks. Genes encoding components of the circadian clock for the most part did not show a shift towards a 12 h pattern in *Bmal1* knockout IVDs, with the exception of *Npas2,* a binding partner of BMAL1 to activate E-box containing genes (Fig. S11). Indeed, promoter analysis for over-represented transcription factor binding sites did not identify the E-box motif to be significantly enriched within the ultradian dataset. This suggests that alternative oscillators may be at play in the *Bmal1* knockout IVD to drive ultradian gene transcription.

The FOX family, consisting of 44 transcription factors in humans and mice, regulate diverse cellular functions, from mitochondrial homeostasis and stress response to cell fate and differentiation [43], through binding of the highly conserved forkhead box domain. FOXO1 has been directly implicated in osteogenic differentiation [27,44–46], in lifespan determination and pathogenesis of age-related diseases such as osteoarthritis [47,48]. Recently, FOXO genes have been implicated in age-related IVD degeneration [27,28]. FOXO1/3/4 were shown to be required for IVD homeostasis during aging and their conditional deletion promoted IVD degeneration [27]. FOXO1 was also found to show

decreased levels in aged mouse IVDs and degenerative human IVDs [28]. Although the conditional Bmal1 KO mouse model we used may impact on multiple pathways beyond FOXO factors, it is reassuring to note that dysregulated rhythmic patterns of FOXO1 and its complete KO bring about similar IVD degeneration, highlighting the central importance of the precise temporal and spatial control of this transcription factor in the IVD. Though FOX factors have not yet been explicitly implicated in ultradian rhythmicity, FOXO1 was identified as a predicted upstream regulator of circatidal rhythms in the intertidal mollusc *C. rota* [49]. Given the commonality between these findings, the conservation of ultradian rhythms previously described, enrichment of FOX binding motifs in the ultradian transcriptome as well as ultradian rhythmicity of many FOX family genes, FOX transcription factors may represent a putative regulator of ultradian transcriptional rhythms in the *Bmal1* KO IVDs.

Collectively, our findings implicate circadian rhythms and the core clock transcription factor *Bmal1* as critical regulators of intervertebral disc physiology. Given the increasing expansion of the ageing populations in a modern world which antagonises our innate circadian timing mechanisms, the circadian regulation of ECM homeostasis and IVD cell fate identified here may provide a new molecular mechanism and potential therapeutic targets for age-related IVD degeneration and low back pain.

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Data availability

All the data have been included in supplementary and the raw RNAseq data have been deposited.

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Supplementary materials

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