



The intervertebral disc contains intrinsic circadian clocks that are regulated by age and cytokines and linked to degeneration

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**The intervertebral disc contains intrinsic circadian clocks
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3 **The intervertebral disc contains intrinsic circadian clocks that are regulated by age**
4 **and cytokines and linked to degeneration**
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Abstract

Objectives: The circadian clocks are internal timing mechanisms that drive ~24 hr rhythms in a tissue-specific manner. Many aspects of the physiology of the intervertebral disc (IVD) show clear diurnal rhythms. However, it is unknown whether IVD tissue contains functional circadian clocks and if so, how their dysregulation is implicated in IVD degeneration.

Methods: Clock gene dynamics in *ex vivo* IVD explants (from PER2::LUC reporter mice) and human disc cells (transduced with lentivirus containing *Per2::luc* reporters) were monitored in real-time by bioluminescence photon counting and imaging. Temporal gene expression changes were studied by RNAseq and qRT-PCR. IVD pathology was evaluated by histology in a mouse model with tissue-specific deletion of the core clock gene *Bmal1*.

Results: Here we show the existence of the circadian rhythm in mouse IVD tissue and human disc cells. This rhythm is dampened with ageing in mice and can be abolished by treatment with IL-1 β but not TNF α . Time series RNAseq revealed 607 genes with 24 hr patterns of expression representing several essential pathways in IVD physiology. Mice with conditional knockout of *Bmal1* in their disc cells demonstrated age-related degeneration of IVDs.

Conclusions: We have established autonomous circadian clocks in mouse and human IVD cells which respond to age and cytokines, and control key pathways involved in the homeostasis of IVDs. Genetic disruption to the mouse IVD molecular clock predispose to IVD degeneration. These results support the concept that disruptions to circadian rhythms may be a risk factor for degenerative IVD disease and low back pain.

Introduction

The circadian clocks are internal timing mechanisms which drive ~24 hr rhythms in physiology and behaviour. In mammals, the central pacemaker Suprachiasmatic Nuclei (SCN) in the hypothalamus synchronizes peripheral clocks in most major body organs.¹⁻³ Circadian rhythms coordinate tissue-specific physiology with light/darkness, rest/activity feeding cycles and body temperature fluctuations.¹⁴ Disruptions to circadian rhythms (during ageing or in shift workers) have been linked to increased risk of diseases (e.g. obesity, diabetes, cardiovascular disease, and osteoarthritis).^{5,6} At the molecular level, the circadian clock consists of a network of transcriptional activators (*Clock*, *Bmal1*) and repressors (*Per1/2* and *Cry1/2*) organized in a negative feedback loop.⁶ This core oscillator generates 24 hour rhythms in the expression of not only its core components but also a myriad of clock controlled genes (CCGs). Depending on the tissue, expression of 3-16% of the whole transcriptome exhibits a circadian rhythm.⁷

The spine is comprised of bony vertebral bodies alternating with fibro-cartilagenous intervertebral discs (IVD). IVD degeneration is among the most prevalent musculoskeletal disorders affecting one in five people under 60 and more than half of the people above 60 years of age.⁸ Low back pain, which is often associated with IVD degeneration, is the number one cause of Years Lived with Disability in the developed countries.⁹ Existing evidence suggests that the IVD is a highly rhythmic tissue, experiencing a diurnal cycle of higher loading (activity phase),^{10,11} followed by a period of low-load recovery (resting phase). Under high load the pressurized interstitial fluid flows to regions of lower pressure through the outer annulus fibrosus (AF) and the cartilaginous end plate (CEP), resulting in decreased disc height, AF outward bulging and an increase in osmolarity of the central gelatinous nucleus pulposus (NP). During the recovery period, the process is reversed by high osmotic pressure inside the disc causing fluid flow to the NP.¹² Exchange of nutrients/metabolites that occurs with fluid flow during this cycle maintains disc cell homeostasis.¹³

Consistent with the rhythmic nature of IVD tissue, shift work (a factor known to disrupt circadian rhythms) was reported to be associated with higher risk of LBP and IVD degeneration.¹⁴⁻¹⁸ We have previously shown that environmental disruption of circadian rhythm in mice, when combined with high fat diet, leads to degeneration of the lumbar IVD tissue in mice.¹⁹ More recently, changes in the expression of circadian clock genes have been identified in rat IVD tissues following passive smoking (a risk factor for LBP).²⁰ However, no studies have examined whether IVD cells express intrinsic circadian clocks, how these IVD clocks are regulated, what their targets are, and whether genetic disruption to the IVD clock impact on tissue homeostasis and susceptibility to degeneration.

In this study, we systemically characterized the molecular circadian clock mechanisms in mouse and human IVD tissue/cells. Moreover, by generating a tissue-specific *Bmal1* KO mouse model, our study provides the first genetic evidence linking a core clock factor to IVD degeneration.

Results

Intervertebral disc possesses a functional, temperature entrainable circadian clock

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3 To test whether the IVD contains a molecular circadian clock capable of driving circadian
4 rhythm of gene expression we monitored the dynamics of PER2::Luc protein in IVD explant
5 cultures isolated from PER2::Luc reporter mice.²¹ Real-time bioluminescence photon
6 counting demonstrated robust circadian rhythm of PER2::Luc activity which lasted for more
7 than 5 days, with a period of 23.93 +/- 0.10 hrs (mean +/- SEM, n=6, Fig. 1A). As the IVD
8 comprises two distinct cell types, the NP and AF cells, we wanted to know if both regions
9 exhibit circadian rhythms. Live imaging of the mouse IVD explants using high sensitivity EM-
10 CCD camera revealed rhythmic PER2::Luc signals from both AF and NP cells (see
11 Supplementary videos 1-3). To extend these studies to humans, primary human NP cells
12 were transiently transfected with a vector carrying the luciferase gene under the control of
13 the *Per2* promoter. This approach revealed cell autonomous circadian oscillations of
14 *Per2::luc* expression, indicating the operation of a functional clock machinery in these human
15 disc cells (Fig. 1B). IHC staining of human NP tissue sections using antibodies against
16 BMAL1 and CLOCK confirmed presence of these essential circadian clock components in
17 human discs (Fig. 1C).

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21 One of the key properties of a peripheral circadian clock is their ability to respond to time
22 cues that are controlled by the SCN clock, such as hormones or changes in body
23 temperature. Since the IVDs are not vascularised or innervated (except in pathological
24 conditions)²², we hypothesised that daily body temperature oscillations may be a mechanism
25 of clock entrainment for IVDs. To test this, IVD explants from the same mouse were placed
26 in different incubators programmed to have oppositely phased cyclic temperature changes
27 for 4 days (38.5°C for 12 hrs/35.5°C for 12 hrs, or vice versa), before returning to a constant
28 37°C. As a control, another IVD explant from the same mouse was incubated under constant
29 37°C. The PER2::Luc rhythms in IVD explants were all in similar circadian phase for the first
30 3 days before the temperature protocol (Fig. 1D). Once the antiphasic protocol was
31 introduced, the oscillations were driven 180 ° out of phase with each other. Interestingly, the
32 antiphasic oscillations were maintained for at least three more days after the tissues were
33 released to constant temperature. In contrast, the IVD explant that remained at constant
34 temperature gradually lost its ability to oscillate by day 7, mainly due to desynchronization in
35 culture (Fig. 1D). These results clearly indicate that temperature cycles that approximate
36 body temperature changes are capable of not only entraining the circadian phase of the IVD
37 oscillation, but enhancing the oscillation amplitude.

41 **Aging affects the circadian rhythm of IVDs**

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43 Daily systemic time cues in body temperature and hormone release are known to be altered
44 with aging.²³ In addition, intrinsic properties of the clock oscillator could deteriorate with age
45 as well.^{23 24} Indeed, we have previously demonstrated that the amplitude of circadian
46 oscillations in cartilage and tendon tissues dampen with aging.^{25 26} Therefore, we
47 hypothesized that circadian rhythms may change in aging disc, compromising the daily
48 control of IVD physiology. To assess this, we compared the oscillations of PER2::Luc
49 expression in mouse IVD explant cultures from animals aged 2 and 12 months (Fig. 2A,
50 supplementary Video 1). The amplitude of oscillations in IVDs from 12 months old mice was
51 severely reduced (by ~60%) as compared to 2 month old mice. Additionally, the average
52 period of oscillations was significantly lengthened by 1.6 hrs in IVDs from 12 month old mice
53 (Fig. 2A). IHC staining showed decreased expression of the core circadian transcription
54 factors BMAL1 and CLOCK in 12 month (Fig. S1) and 24 month old mice as compared to 2
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3 month old (Fig. 2B). These data demonstrate that the IVD clock becomes dysregulated with
4 ageing.
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8 **The circadian rhythm of IVD is disrupted by IL-1 β in a NF- κ B dependent manner**

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10 Chronic inflammation is a known factor associated with IVD degeneration and lower back
11 pain.²⁷ To investigate the effects of catabolic cytokines on disc circadian clock, we treated
12 IVD explants from the PER2::Luc reporter mice with IL-1 β , LPS and TNF α . Tissues were
13 under continuous bioluminescence recording. Treatment with IL-1 β (or LPS, Fig. S2A)
14 resulted in complete disruption of the PER2::Luc circadian rhythm, associated with
15 significant changes of clock genes (*Bmal1*, *Per2* and *Nr1d1*)(Fig. 3A, Fig.S3). The disrupted
16 rhythm could be reinstated by dexamethasone (an anti-inflammatory glucocorticoid, Fig. 3A)
17 or IL-1RA (an antagonist of IL-1, Fig.S2B), but not by forskolin (a clock synchronising agent
18 without anti-inflammatory properties, Fig. S2C). NF κ B is one of the classic pathways through
19 which IL-1 β can mediate its effects. To evaluate the involvement of NF κ B, we used the
20 IKK1/2 inhibitor BMS-345541 to block the activation of NF κ B. The clock-disrupting effect of
21 IL-1 β was blocked by pre-treating the IVD explant with BMS-345541, supporting a role of
22 NF κ B pathway in the IL-1 β -mediated clock disruption. In contrast to IL-1 β , treatment of IVD
23 explants with TNF α had no effect on their circadian rhythms (Fig. 3B). In contrast, both IL-1 β
24 and TNF α elicited a strong induction of NF κ B signalling in a lung epithelial cell line,
25 suggesting a possible cell-type specific response (Fig.S2D). Next, we took advantage of a
26 transgenic mouse strain expressing the p65-DsRedXP protein fusion construct²⁸ to observe
27 the nuclear translocation of p65, one of the major components of the NF κ B complex. Live
28 imaging showed that treatment of IVD explants with IL-1 β caused rapid nuclear translocation
29 of p65 both in AF and NP cells. However, addition of TNF α (up to 40 ng/mL) had no effect
30 on p65 translocation (Fig.3C).
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35 There are at least two potential mechanisms through which IL-1 β could disrupt the IVD
36 circadian rhythm. Individual cells may still have robust clocks but become desynchronised,
37 with their clocks being in different phases, leading to reduced oscillation amplitude; or
38 individual cells may have lost their pacemaking properties. To distinguish between these
39 two possibilities, we used a high sensitivity EM-CCD camera to visualize the PER2::Luc
40 bioluminescence signals from individual cells in the presence or absence of IL-1 β .
41 Consistent with the lack of effect of forskolin, this imaging approach revealed loss of
42 bioluminescence at single cell level, excluding the desynchronization hypothesis (Fig. 3D
43 and Supplementary video 2). Therefore, disruption to the IVD clock could be a hitherto
44 undiscovered response to pro-inflammatory cytokines.
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49 **Identification of the first IVD circadian transcriptome**

50 Circadian clocks in different tissues exert their local functions through regulating diverse yet
51 highly tissue-specific set of target genes. To reveal the extent of rhythmic genes in IVD
52 tissue under physiological conditions, we performed a time-series RNAseq study using IVD
53 tissues (collected every 4 hours for 48 hours) from mice kept in 12 hr light/12 hr darkness.
54 We used a well-recognized JTKCycle²⁹ algorithm to pick out rhythmic genes. Using
55 $P_{\text{adjust}} < 0.05$ as a cut-off, we identified 607 genes (3.5% of expressed genes in IVD) with
56 rhythmic 24 hr expression patterns (Figure 4A, Supplementary Table 1). Further phase
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3 clustering analysis of these rhythmic genes using R package revealed 4 main clusters (Fig.
4 S4), with more than 70% of these genes peaking at night time points (representing the active
5 phase of mouse). Gene ontology (GO)-term analysis using topGO revealed dozens of
6 overrepresented functional groups with an adjusted $p < 0.01$, including “fatty acid metabolic
7 process”, “circadian rhythm”, “intracellular protein transmembrane transport”, “intrinsic
8 apoptotic signaling pathway”, “carboxylic acid metabolic process”, and “response to
9 endoplasmic reticulum stress”. We next compared the IVD rhythmic gene list to that of the
10 mouse cartilage and tendon we published earlier.^{25 26} There was a very small number of
11 genes (6-16%) overlapping between any two of these skeletal tissues, with only 16 genes
12 common to all three, supporting the tissue-specific function of the peripheral clocks (Figure
13 4B). Of these 16 common genes, 8 were core circadian clock genes. The expression
14 profiles of canonical clock genes (*Bmal1*, *Per2*, *Dbp*) and selected target genes *Follistatin* (a
15 BMP antagonist)³⁰ and *Timp4* (a tissue inhibitor of MMPs)³¹ relevant to IVD physiology and
16 catabolism were validated by temporal qRT-PCR in mouse IVD tissues (Figure 4C, Fig S5).
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20 21 22 Targeted deletion of *Bmal1* causes age-dependent IVD degeneration

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24 *Bmal1* is an essential circadian clock component for the generation of 24 hr rhythms. The
25 global *Bmal1* knock-out mouse shows multi-tissue pathologies, including ectopic calcification
26 of IVDs.³² However, the severe disruption to whole body circadian rhythms confounds
27 interpretation of phenotype. To evaluate the function of local IVD clocks, we produced a
28 conditional KO mouse model (*Col2a1-Bmal1* KO, cKO) with a cell type-specific abolition of
29 the transcription factor *Bmal1* in $\alpha 1(\text{II})$ collagen expressing cells, including NP and AF cells,
30 and chondrocytes.³³ We have previously shown that the central SCN clock and behavioural
31 locomotion rhythms in the cKO mice are not affected.³³ IHC staining of IVDs confirmed loss
32 of BMAL1 expression in the majority of the AF cells and chondrocytes of the cartilaginous
33 end plate in cKO mice (Fig. 5A). The cKO mouse was crossed with the PER2::Luc mouse to
34 enable real-time tracking of clock rhythms. Photon counting of PER2::Luc bioluminescence
35 demonstrated a lack of circadian oscillations in the cKO IVDs, with no response to
36 dexamethasone treatment (Fig. 5B). Bioluminescence imaging of the cKO IVDs confirmed
37 lack of circadian oscillations of PER2::Luc in both AF and NP cells (Fig. 5C and
38 Supplementary video 3).
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42 Histological analysis revealed early signs of degeneration of the lumbar IVDs in cKO mouse
43 at 6 months of age, such as thinning of the growth plate of vertebral body (Fig. 6A), and
44 gradual disappearance of the CEP (Fig. S6). At 12 months, there was widespread
45 degeneration of lumbar IVDs in cKOs. Bone bridges appeared within the growth plate, the
46 CEP was almost completely replaced by bone (Fig. 6A, black arrow), and the height of the
47 disc was significantly reduced in cKO IVDs (Fig. 6A). In addition, staining with Safranin O
48 and picrosirius red revealed disorganisation of the outer annulus structure and signs of
49 fibrosis (with organized collagen bundles) appearing at the periphery of the IVDs (Fig. 6A-C,
50 asterisk). Finally, using X-ray studies, the cKO mice showed clear signs of calcification and
51 narrowing of spaces between vertebrae at 6 months (in tail IVDs, data not shown) and 12
52 months (in lumbar IVDs, Fig. 6C). No signs of degeneration were evident in age-matched
53 WT mice up to the age of 12 months (Fig. 6B). However, similar degenerative changes to
54 the cKO mutants were visible in WT mice at 24 months of age (Fig. S7), suggesting the
55 possibility that loss of *Bmal1* and/or circadian rhythm in IVD cells leads to accelerated
56 ageing of the tissue. TUNEL assay and qPCR were performed to explore the underlying
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3 mechanisms for the observed phenotype. There were no obvious signs of apoptosis,
4 although significant upregulation of catabolism-related genes (*Adamts1*, *Adamts5*, *Adamts15*
5 and *Follistatin*) were observed in cKO IVDs (Fig.S8, S9). Together, these results indicate the
6 essential role of the locally expressed core clock factor BMAL1 in IVD homeostasis, loss of
7 which led to profound tissue degeneration.
8

13 Discussion

15 Low back pain is amongst the most prevalent spinal diseases associated with increasing
16 age, with over 80% of the UK population predicted to experience back pain within their
17 lifetime. Progressive degeneration of the IVD tissue, partly caused by increased catabolism
18 driven by inflammatory/catabolic cytokines, is a major contributing factor in LBP.³⁴ It has long
19 been known that the physiology of IVD is under strong influence by a diurnal rhythm
20 associated with the rest/activity cycles, i.e., daily cycles of loading (activity phase) and low-
21 load recovery (resting phase).¹⁰⁻¹³ Exchange of nutrients/metabolites that occurs with fluid
22 flow during this cycle maintains disc cell homeostasis. Recent epidemiological and
23 experimental studies have linked shift work (in humans) and chronic disruption of circadian
24 rhythms (in mice) to higher risk of IVD degeneration.^{14 15 17-19} However, our study represents
25 the first critical analysis of the molecular and cellular mechanisms of the IVD clock under
26 physiological and pathological conditions. Using the clock gene reporter mouse/cell models,
27 as well as a conditional *Bmal1* KO mouse model that had disrupted IVD clock, we
28 established autonomous circadian clocks in mouse and human IVD cells that respond to
29 temperature cycles, dampen with age and become dysregulated by catabolic cytokines.
30 Genetic disruption to the mouse IVD molecular clock predisposes to IVD degeneration.
31 Global *Bmal1* KO also showed a phenotype in the skeletal system, including the spine.
32 However, our conditional KO model allows us to conclude the essential role of locally
33 expressed BMAL1 or circadian rhythm in maintaining IVD homeostasis. These results
34 support the notion that disruptions to circadian rhythms during ageing or in shift workers may
35 be a contributing factor for the increased susceptibility to degenerative IVD diseases and low
36 back pain.
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41 We also revealed for the first time the circadian transcriptome of the IVD tissue. Of particular
42 interest are the genes and pathways that have been previously implicated in IVD physiology
43 and pathology, such as genes involved in matrix homeostasis/repair (e.g *Follistatin*, *Timp4*,
44 *Adamts1*, *Adamts5*, *Adamts15* and *Adam17*),^{30 31} mitochondria function and fatty acid
45 metabolism (e.g. *Pex1*, *Pex2*, *Pex5*, *Pex15*, *Adipoq*, *Adipor2*, *Fasn*).^{35 36} Although glucose
46 and anaerobic glycolysis represent major metabolic pathways in IVD, there is evidence that
47 mitochondria in the NP are functional and they retain the capacity to metabolise fatty acids
48 through mitochondrial oxidative metabolism.³⁵ Other relevant pathways include ER stress
49 and apoptosis (e.g. *Aifm1*, *Atf6*, *Chac1*, *Bak1*, *Bbc3*, *Opa1* and *Fas*).^{37 38} The diverse clock-
50 controlled pathways identified by this approach implicate circadian rhythm as a critical
51 regulatory mechanism for IVD biology.
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55 Using IVD tissue explants, we have identified the disruption of the circadian clock in IVD as
56 hitherto undiscovered response to pro-inflammatory cytokines. Similar clock disruptions by
57 inflammatory cytokines have been found in other cell types, such as in macrophages,³⁹
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3 synovial fibroblasts,⁴⁰ and chondrocytes.²⁸ The involvement of NFκB pathway in mediating
4 the effects of IL-1 is consistent with our earlier findings in chondrocytes, where NFκB
5 interferes with the core clock complex to disrupt circadian pacemaking.²⁸ Given the diverse
6 pathways controlled by the IVD clock, cytokine-mediated circadian disruption may be
7 involved in driving key aspects of the catabolic response of IVD to chronic inflammation.
8 Therefore, there is the possibility of stabilizing IVD clock rhythm as a novel strategy to
9 combat tissue catabolism. Although the concentration we used for IL-1β (5 ng/mL) in these
10 tissue explant studies was higher than that in degenerative IVD (~50 pg/mL), this dose is in
11 line with most *ex vivo/in vitro* studies. We also identified a lack of response of the IVD clock
12 (and cartilage clock)²⁸ to TNFα, possibly due to the defective NFκB nuclear translocation.
13 These findings suggest IL-1 and TNFα may act on distinct downstream pathways and
14 regulate different target genes within the IVD, as seen in chondrocytes. In SW1353
15 chondrocyte-derived cells, catabolic genes such as IL-6, BMP-2, MMP13 and COX-2 only
16 respond to IL-1, with almost no response to TNFα.^{41 42} Such results are intriguing because
17 we have shown that IL-1β plays a more prominent role in driving disc degeneration than
18 TNFα.^{43 44} Therefore, anti-inflammatory drugs that selectively target IL-1 are more likely to
19 bring therapeutic benefits.
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24 In conclusion, our results provide a firm basis for future studies that aim to elucidate the
25 functional implication and therapeutic potential of the human IVD circadian rhythm in health
26 and disease of the spine.
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Figure legends

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3 **Figure 6. Loss of *Bmal1* leads to degeneration of IVDs and cartilaginous tissues of the**
4 **spine.** (A) Safranin O staining of 12 month old WT and *Bmal1* cKO mouse lumbar IVDs;
5 n=4. Red arrow-loss of CEP; Black arrow- fragmentation of growth plate; *-fibrosis
6 (magnification 2.5x). Analysis of the IVD height and growth plate thickness was shown (two-
7 tailed nonparametric Mann-Whitney test; n=4) *- p<0.05; *** - p<0.001. (B) Picrosirius red
8 staining of lumbar IVDs from 12 month old WT and *Bmal1* cKO mouse showing organisation
9 of collagen (magnification 2.5x left and 5x right panels); n=4. Images were visualized under
10 brightfield or polarized light. (C) X-ray radiography of 12 month old WT and *Bmal1* cKO
11 mouse spines; n=3. Yellow arrows- calcification of IVDs; Red arrows- calcification of tissues
12 surrounding the IVDs.
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Supplementary figure legends

24 **Figure S1. Reduced expression of BMAL1 and CLOCK in ageing IVDs.** IHC of BMAL1
25 and CLOCK on sections of IVDs from 3 and 12 months old mice; n=4. Magnification, 5x left
26 and middle panels, 10x right panels. BMAL1 staining was visible in the AF, but not the CEP,
27 of 12 month old mice. CLOCK staining was largely absent in both AF and CEP in 12 month
28 old mice.
29

30 **Figure S2. Effects of LPS, IL-1RA, forskolin and TNF α on IVD oscillations.**
31 Representative bioluminescence traces of PER2::Luc mouse IVD explants; n=3. (A) LPS
32 treatment (1 μ g/mL, red trace) disrupted the rhythm, which could be rescued by treatment
33 with dexamethasone (100 nM). (B, C) Disrupted circadian rhythm by IL-1 β treatment (5
34 ng/mL, red trace) was not rescued by application of forskolin (10 μ M), but by pre-treatment
35 with IL-1RA (1 μ g/mL). Arrows indicate time of treatment. (D) Both IL-1 β (5 ng/mL) and
36 TNF α (40 ng/mL) induced strong NF κ B signalling in lung epithelial cells stably transfected
37 with NF κ B::luc reporter. Representative, n=3.
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40 **Figure S3. Effects of IL-1 β on endogenous clock gene expression.** qPCR of several
41 clock genes, *Adamts1* and *IL-6* in IVD explants upon IL-1 β treatment (5 ng/mL for 4 hours).
42 *, p<0.05; ***, p<0.001, n=4.
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45 **Figure S4. Phase clustering analysis of rhythmic genes in mouse IVDs.** Clustering
46 analysis was performed using cluster (A) and Rtsne (B) of R package. These analyses
47 revealed 4 main clusters with different peak times (two at night and two during the day).
48 Example genes for each cluster were highlighted. There was a good concordance between
49 these two methods of analysis.
50

51 **Figure S5. Time course qPCR of β -actin in mouse IVDs.** Note the lack of circadian
52 rhythms. Mean and SEM (n = 6).
53

54 **Figure S6. Early onset of IVD degeneration in IVDs from 6 month old cKO mice.**
55 Safranin O/methyl green staining revealed gradual disappearance of CEP in the *Bmal1* cKO
56 mouse (black arrow); n=4.
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3 **Figure S7. Spontaneous degeneration of IVDs from aged WT mice.** Picrosirius red
4 staining and polarised light microscopy were performed on IVDs from 3 and 24 month old
5 wild type mice; n=4. The aged WT mouse IVDs display a phenotype similar to *Bmal1* cKO
6 IVDs, with fibrosis of the outer AF composed of bundles of organised collagen visible under
7 polarised light microscope.
8

9 **Figure S8. TUNEL staining of IVDs from 12 months old WT and *Bmal1* cKO mice.** Note
10 there were no detectable signs of apoptosis in either WT or cKO IVDs. N=4.
11

12 **Figure S9. Time course qPCR of catabolic genes in IVDs from 3 months old WT and**
13 ***Bmal1* cKO mice.** Mean and SEM (n = 4). *, p<0.05; **, p<0.01; ***, p<0.001.
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15 16 17 **Supplementary table and videos**

18 **Supplementary Table 1. List of rhythmic IVD genes with a ~24 hr period.**
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21 **Supplementary Video 1, Live bioluminescence imaging of the PER2::Luc mouse IVD**
22 **explants (2 month on the left and 12 month on the right) using high sensitivity EM-**
23 **CCD camera.**
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26 **Supplementary Video 2, Live bioluminescence imaging of the PER2::Luc mouse IVD**
27 **explants treated with IL-1 β (at 48 hr), followed by Dex (at 96 hr).**
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30 **Supplementary Video 3, Live bioluminescence imaging of the mouse IVD explants**
31 **from a WT mouse (left) and a *Bmal1* cKO mouse (right).**
32

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48 submission of this work.
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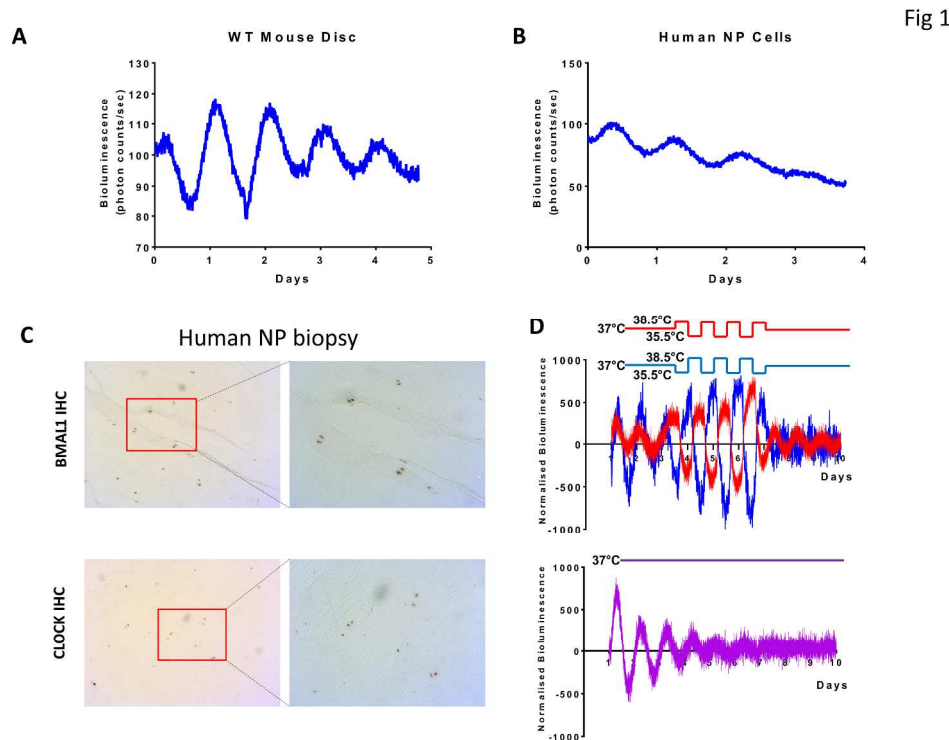


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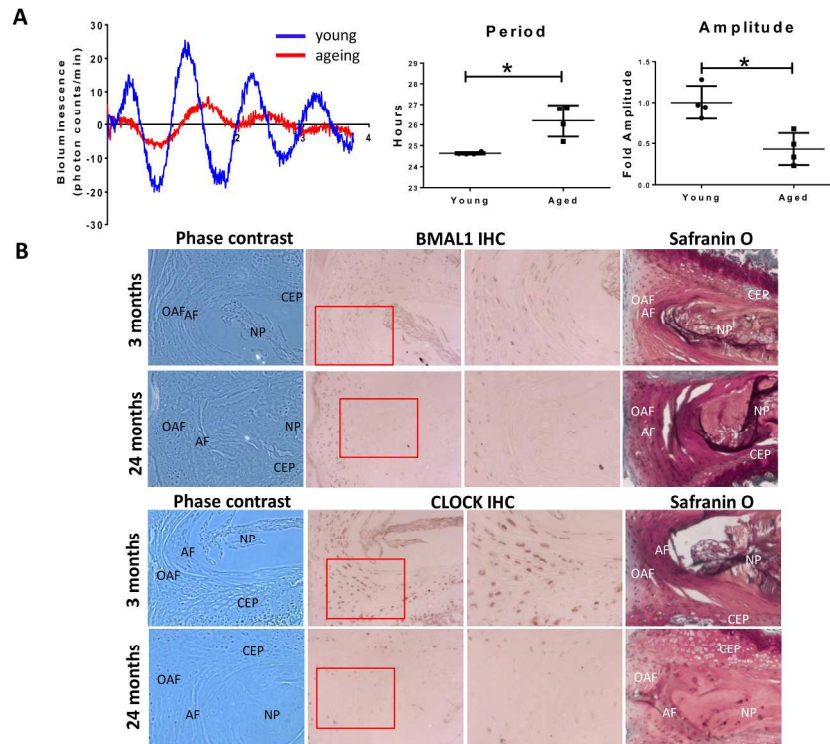


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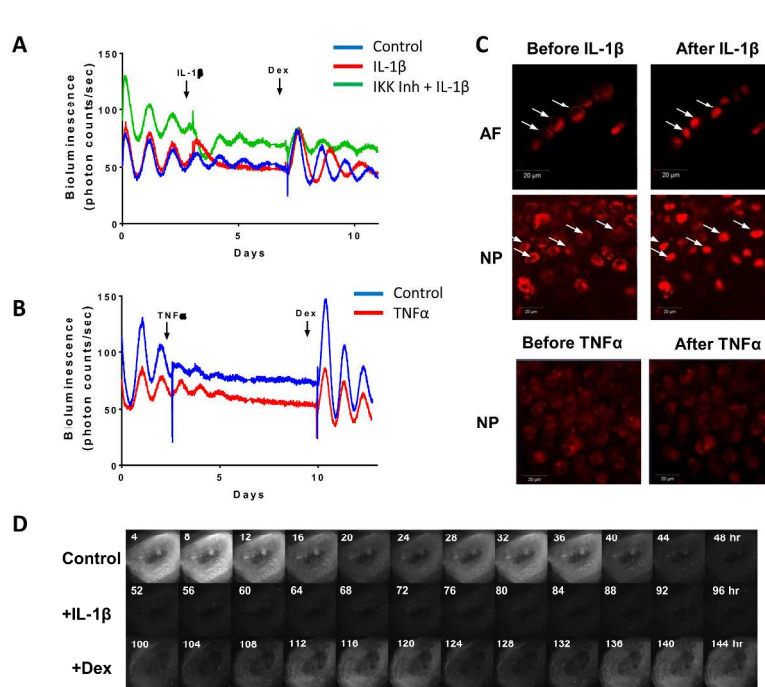


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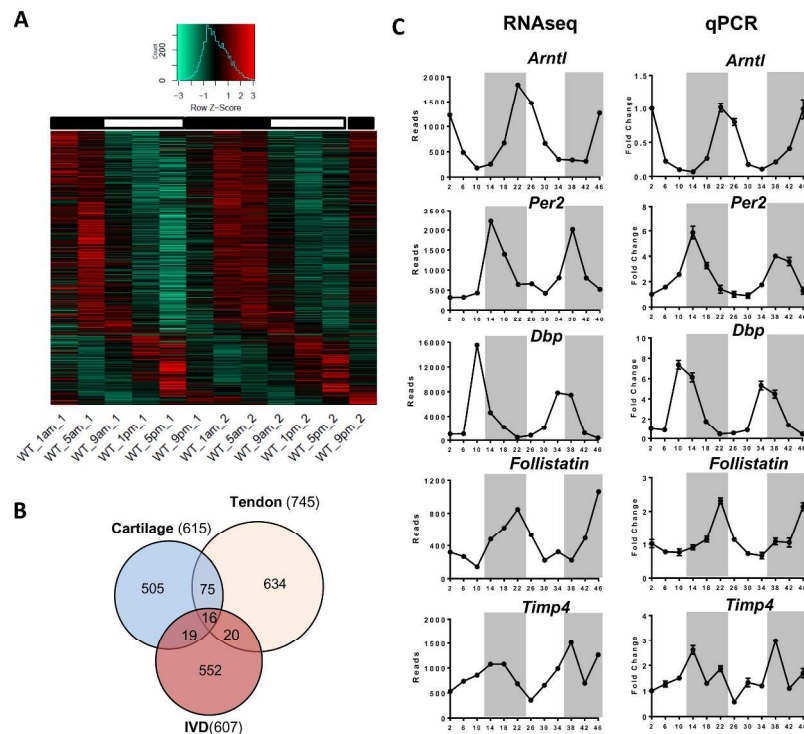


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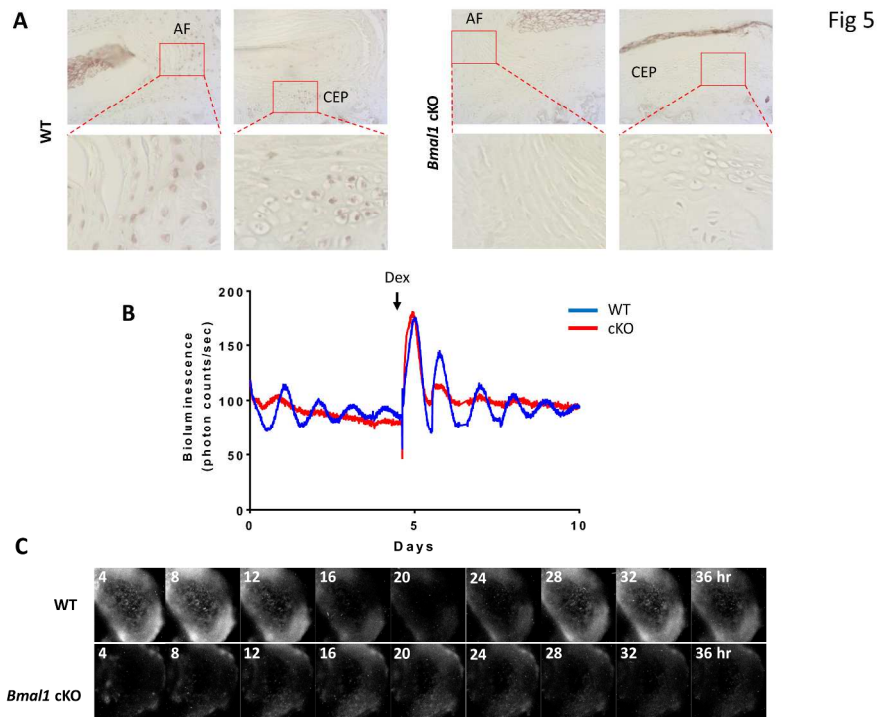


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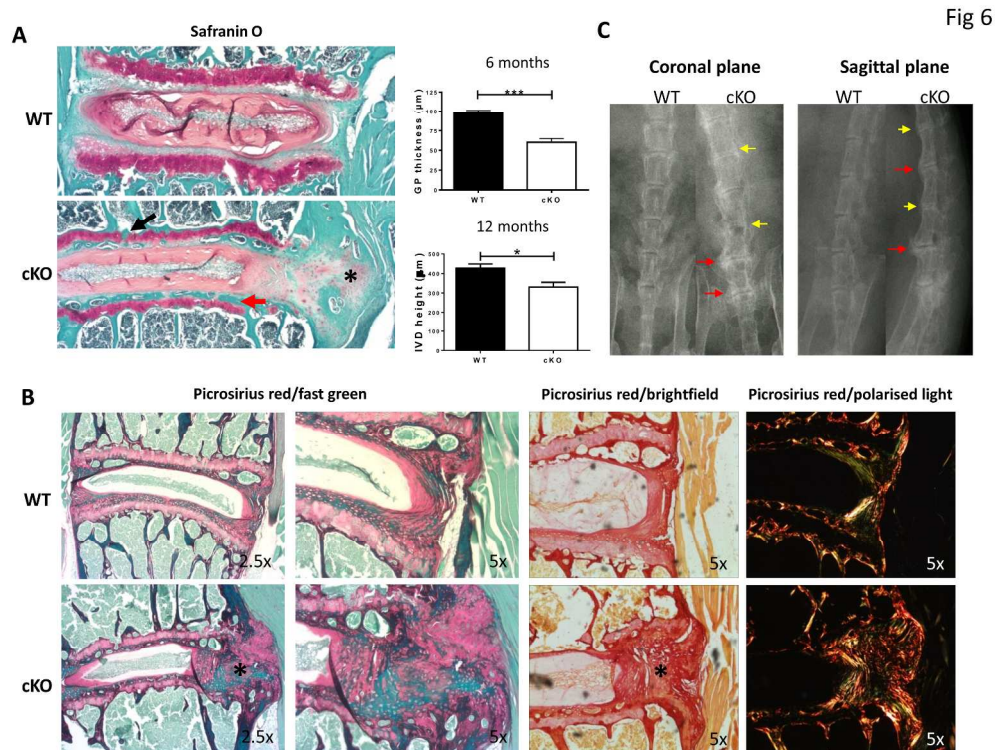
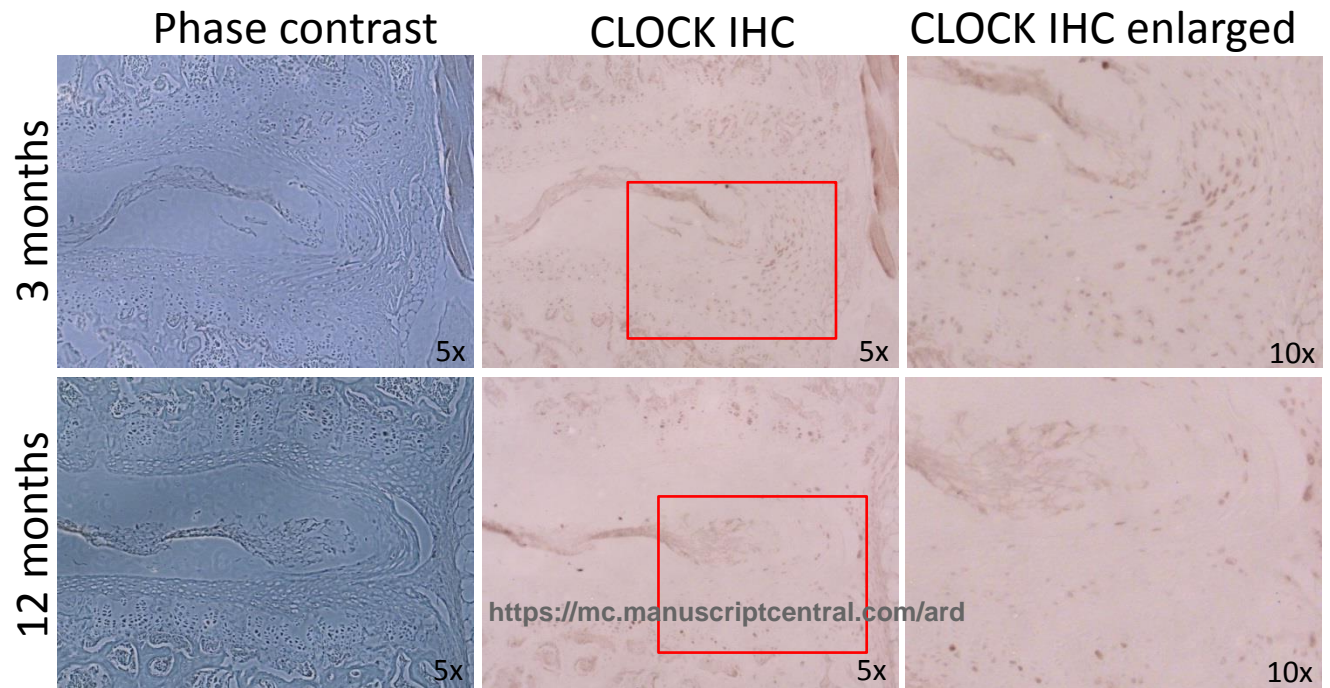
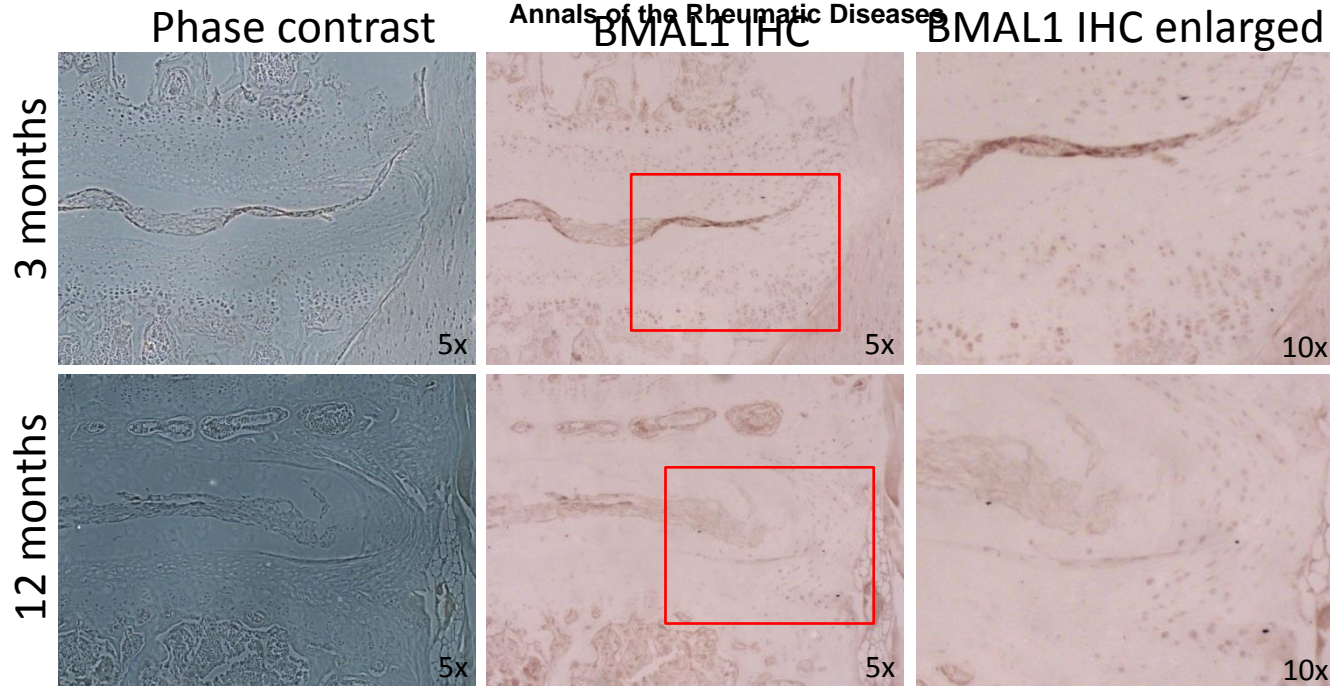
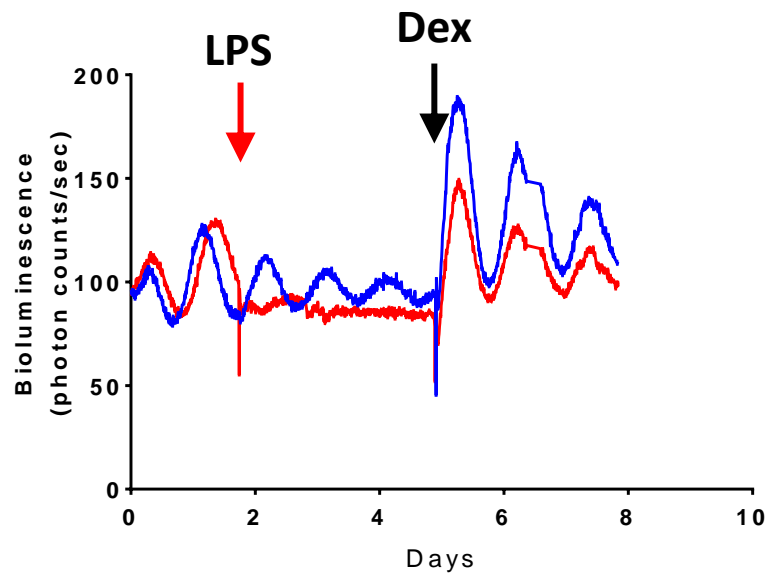


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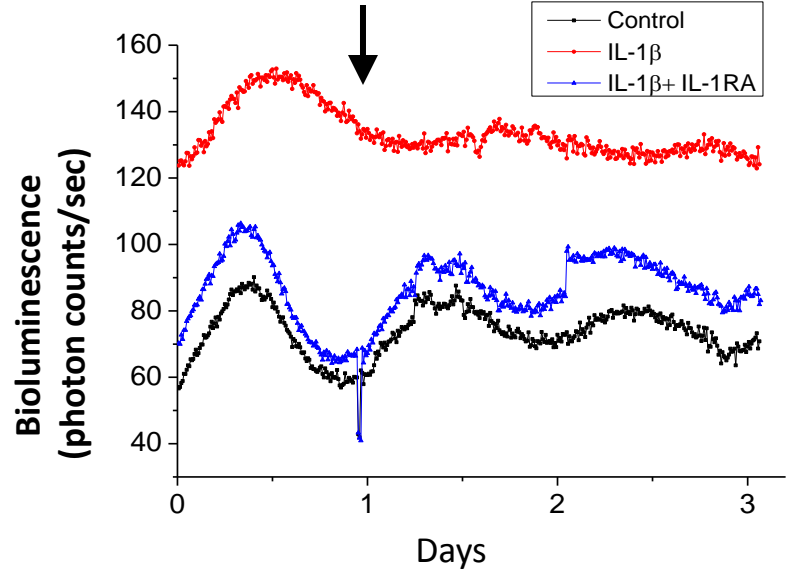
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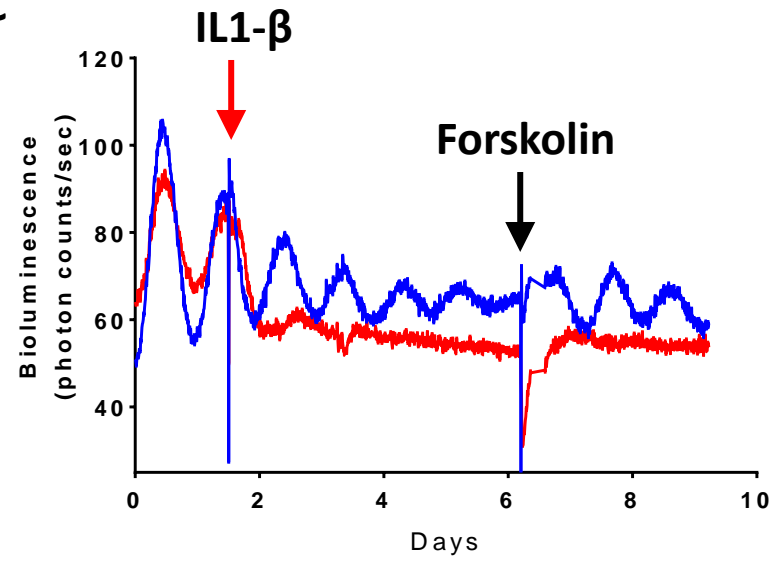
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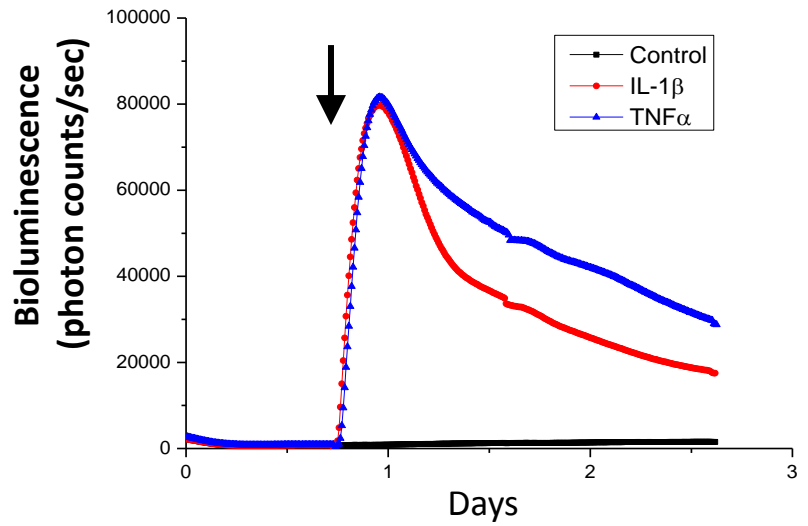


Fig S3

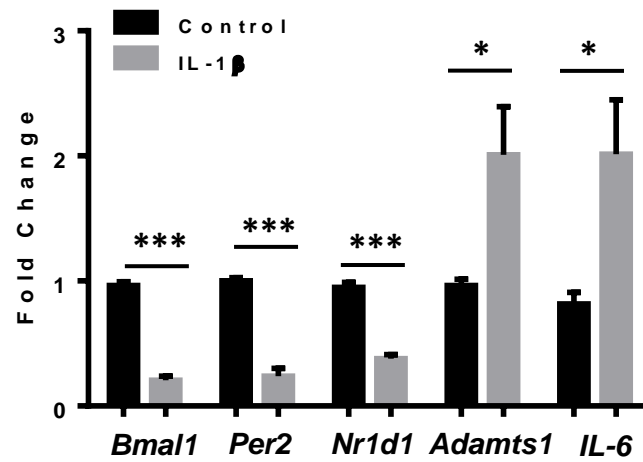


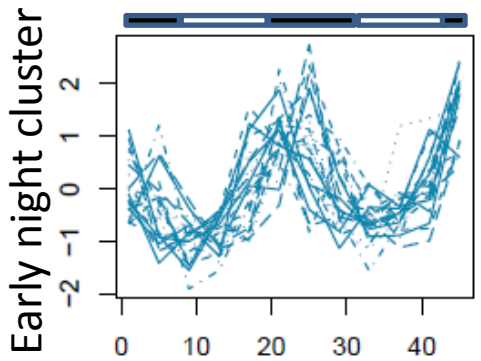
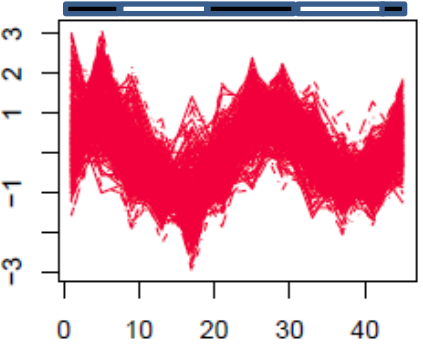
Fig S4

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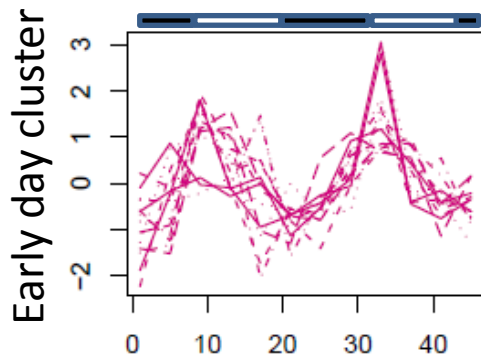
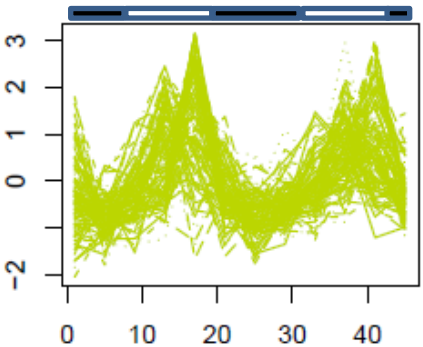
Arntl (Bmal1), Npas2, Cry1, Rorc, Follistatin, Adam17, Adamts1, Adamts15, Atf6

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Dbp, Nr1d1, Bak1, Calm3, Calm4

Mapk6, Sirt6



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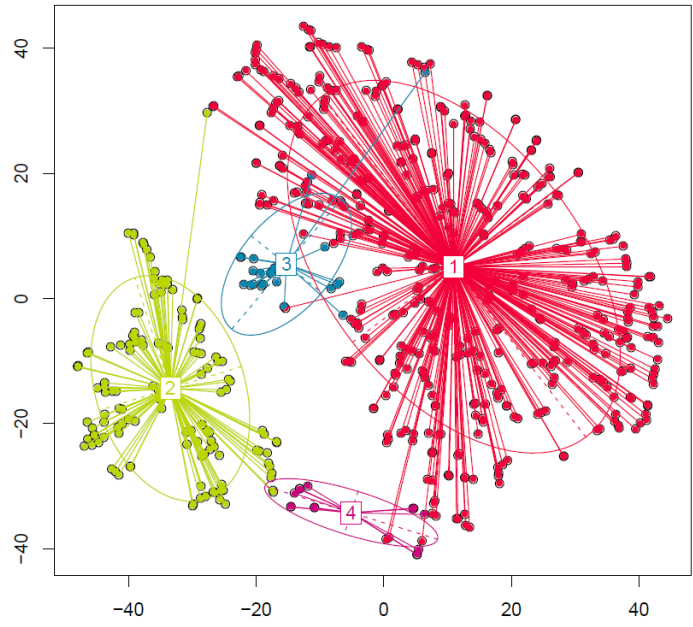


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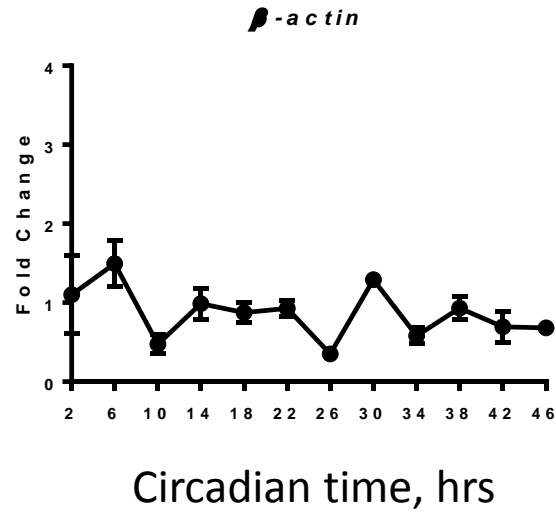
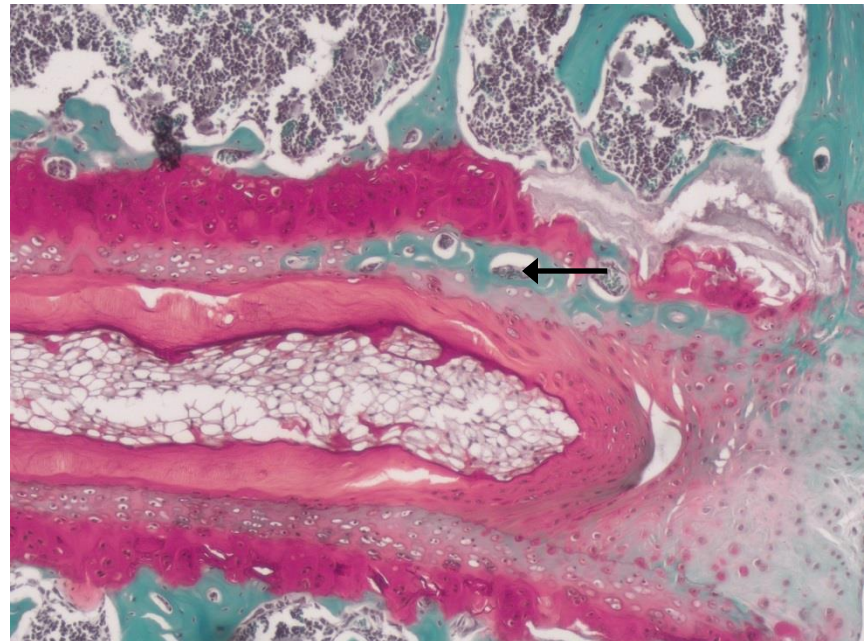
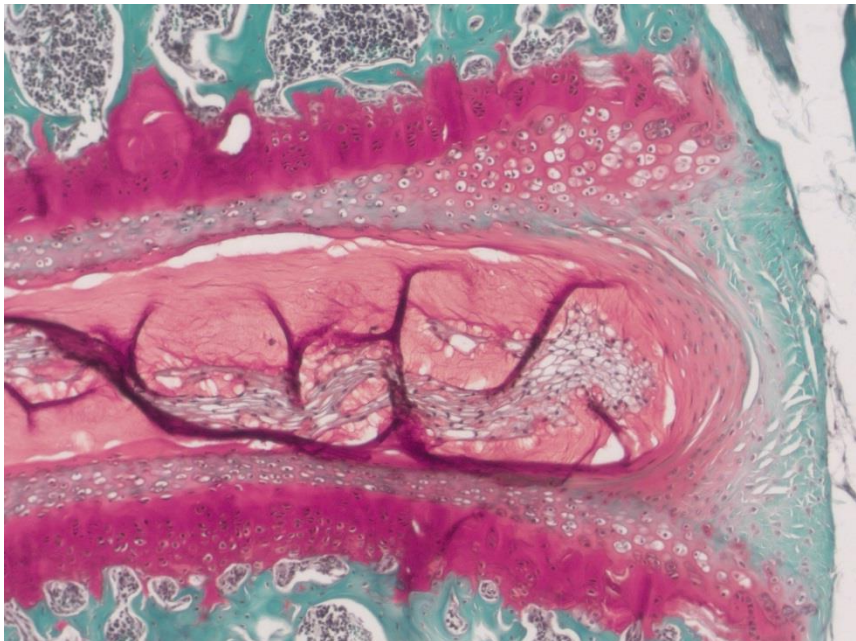


Fig S6

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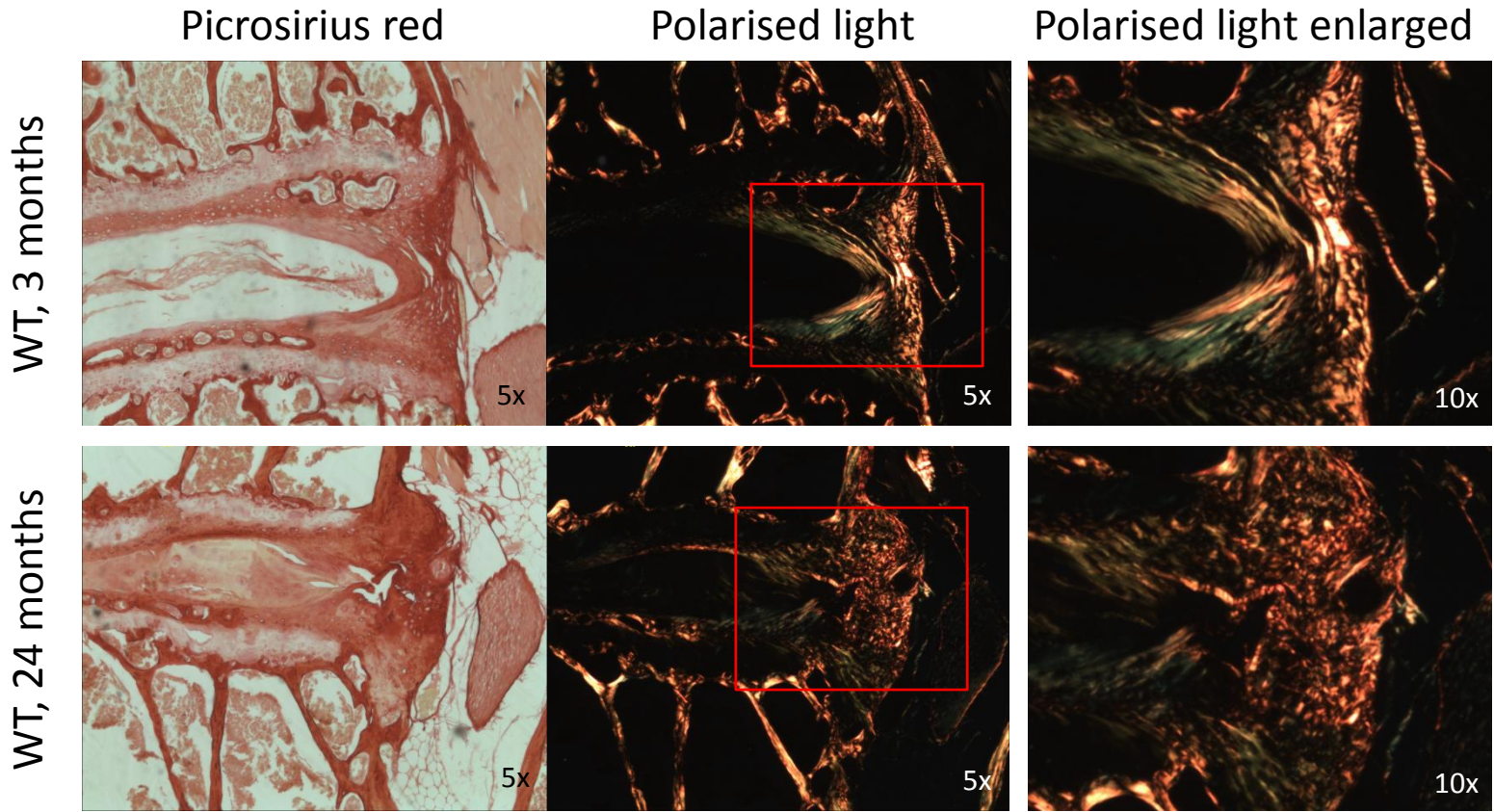
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Fig S7



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Fig S8

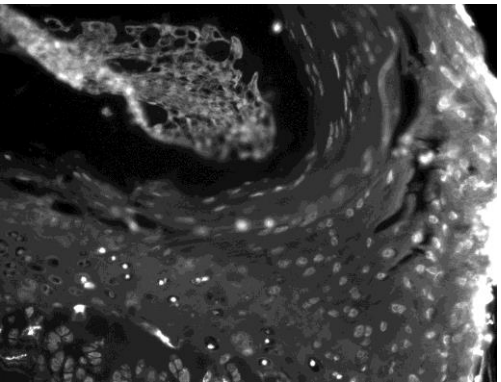
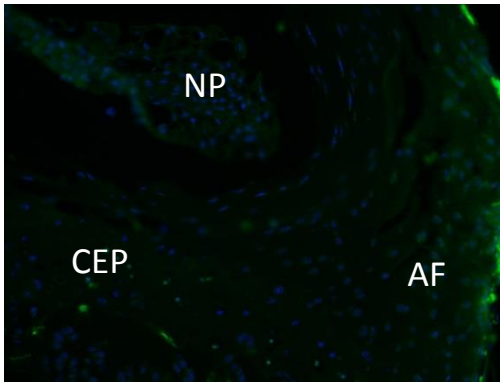
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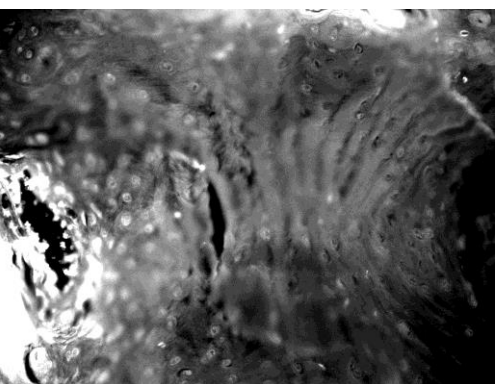
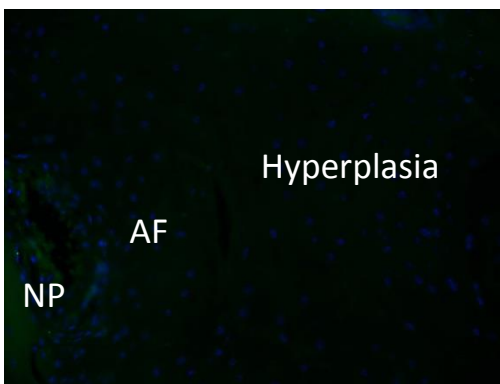
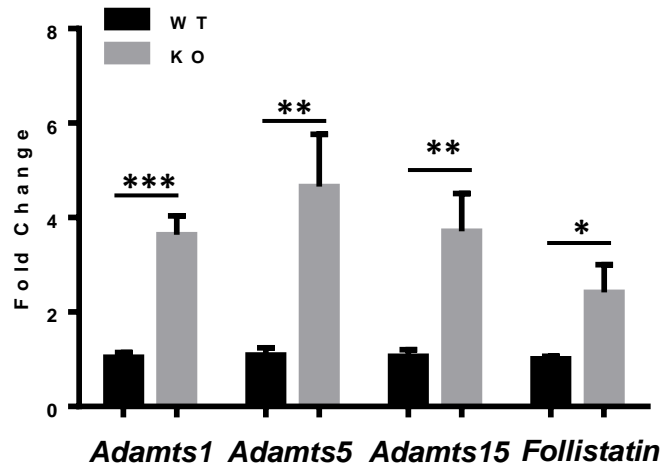


Fig S9



	ID	gene_name	ADJ.P	PERIOD	LAG
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4	ENSMUSG00000038084.10	Opa1	9.81E-05	24	4
5	ENSMUSG00000020283.5	Pex13	9.81E-05	24	4
6	ENSMUSG00000009687.8	Fxyd5	0.000265	24	12
7	ENSMUSG00000027086.10	Fastkd1	0.000265	24	0
8	ENSMUSG00000046417.8	Fam211a	0.000265	24	2
9	ENSMUSG00000022053.7	Ebf2	0.000265	24	5
10	ENSMUSG00000047797.8	Gjb1	0.000265	24	16
11	ENSMUSG00000028989.3	Angptl7	0.000645	24	20
12	ENSMUSG00000025809.9	Itgb1	0.000645	24	4
13	ENSMUSG00000074766.4	Ism1	0.000645	24	3
14	ENSMUSG00000026643.10	Nmt2	0.000645	24	4
15	ENSMUSG00000019883.4	Echdc1	0.000645	24	4
16	ENSMUSG00000095512.1	Gm17222	0.000645	24	4
17	ENSMUSG00000047370.4	Gm7367	0.000645	24	12
18	ENSMUSG00000040605.6	Bace2	0.000645	24	4
19	ENSMUSG00000039956.2	Mrap	0.000645	24	4
20	ENSMUSG00000078234.5	Klhdc7a	0.000645	24	4
21	ENSMUSG00000073176.4	Zfp449	0.000645	24	4
22	ENSMUSG00000090305.1	Gm5459	0.000645	24	16
23	ENSMUSG00000029054.2	Gabrd	0.000645	24	2
24	ENSMUSG00000030972.5	Acsm5	0.001042	24	4
25	ENSMUSG00000021903.5	Galnt15	0.00144	24	20
26	ENSMUSG00000041607.10	Mbp	0.00144	24	18
27	ENSMUSG00000032010.8	Usp2	0.00144	24	22
28	ENSMUSG00000059824.4	Dbp	0.00144	24	18
29	ENSMUSG00000026922.7	Agpat2	0.00144	24	4
30	ENSMUSG00000041653.4	Pnpla3	0.00144	24	4
31	ENSMUSG00000026663.6	Atf6	0.00144	24	4
32	ENSMUSG00000029482.3	Aacs	0.00144	24	4
33	ENSMUSG00000090084.1	Srpx	0.00144	24	4
34	ENSMUSG00000039364.7	Sectm1b	0.00144	24	16
35	ENSMUSG00000051367.8	Six1	0.00144	24	6
36	ENSMUSG00000040370.7	Lym5	0.00144	24	2
37	ENSMUSG00000001700.9	Gramd3	0.00144	24	4
38	ENSMUSG00000031959.8	Wdr59	0.00144	24	2
39	ENSMUSG00000044645.7	Gm7334	0.00144	24	2
40	ENSMUSG00000025940.6	Tmem70	0.00144	24	4
41	ENSMUSG00000036225.8	Kctd1	0.00144	24	4
42	ENSMUSG00000021418.9	Rpp40	0.00144	24	2
43	ENSMUSG00000027663.6	Zmat3	0.00144	24	4
44	ENSMUSG00000031725.8	Ces1f	0.00144	24	4
45	ENSMUSG00000013523.7	Bcas1	0.00144	24	16
46	ENSMUSG00000073233.3	Gm9144	0.00144	24	16
47	ENSMUSG00000037016.7	Frem2	0.00144	24	4
48	ENSMUSG00000047473.8	Zfp30	0.00144	24	2
49	ENSMUSG00000025584.11	Pde8a	0.00144	24	4
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51	ENSMUSG00000079168.3	Cd209g	0.00144	24	4

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3	ENSMUSG00000087235.1	Gm4750	0.00144	24	16
4	ENSMUSG00000044951.7	Mylk4	0.002993	24	22
5	ENSMUSG00000082476.1	Gm12242	0.002993	24	15
6	ENSMUSG00000076432.6	Ywhaq	0.002993	24	4
7	ENSMUSG00000063427.7	Rps10-ps2	0.002993	24	16
8	ENSMUSG00000040181.8	Fmo1	0.002993	24	2
9	ENSMUSG00000008540.5	Mgst1	0.002993	24	4
10	ENSMUSG00000021877.5	Arf4	0.002993	24	4
11	ENSMUSG00000029190.13	D5Ert579e	0.002993	24	2
12	ENSMUSG00000036078.10	Sigmar1	0.002993	24	4
13	ENSMUSG00000022774.6	Ncbp2	0.002993	24	2
14	ENSMUSG00000027309.12	4930402H24Rik	0.002993	24	4
15	ENSMUSG00000022707.10	Gbe1	0.002993	24	4
16	ENSMUSG00000020777.10	Acox1	0.002993	24	2
17	ENSMUSG00000023832.7	Acat2	0.002993	24	3
18	ENSMUSG00000028567.8	Txndc12	0.002993	24	4
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20	ENSMUSG00000039377.6	Hlx	0.002993	24	12
21	ENSMUSG00000041737.7	Tmem45b	0.002993	24	4
22	ENSMUSG00000020932.8	Gfap	0.002993	24	16
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25	ENSMUSG00000026749.5	Nek6	0.002993	24	2
26	ENSMUSG00000038517.9	Tbkbp1	0.002993	24	14
27	ENSMUSG00000005501.8	Usp40	0.002993	24	1
28	ENSMUSG00000020829.9	Slc46a1	0.002993	24	8
29	ENSMUSG00000094686.1	Ccl21a	0.002993	24	6
30	ENSMUSG00000073633.3	Fbxo36	0.002993	24	5
31	ENSMUSG00000027684.10	Mecom	0.002993	24	4
32	ENSMUSG00000052593.9	Adam17	0.002993	24	4
33	ENSMUSG00000040502.5		Mar-09 0.002993	24	2
34	ENSMUSG00000090659.2	Zfp493	0.002993	24	1
35	ENSMUSG00000040717.5	Il17rd	0.002993	24	4
36	ENSMUSG00000027792.5	Bche	0.002993	24	4
37	ENSMUSG00000054000.4	Tusc1	0.002993	24	14
38	ENSMUSG00000033182.6	Kbtbd12	0.002993	24	4
39	ENSMUSG00000037577.6	Ephx3	0.002993	24	4
40	ENSMUSG00000032517.9	Mobp	0.002993	24	16
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42	ENSMUSG00000028572.7	Hook1	0.002993	24	18
43	ENSMUSG00000029503.10	P2rx2	0.002993	24	16
44	ENSMUSG00000019890.3	Nts	0.00442	24	4
45	ENSMUSG00000029195.7	Klb	0.00442	24	4
46	ENSMUSG00000039099.7	Wdr93	0.00442	24	6
47	ENSMUSG00000037071.2	Scd1	0.005847	24	4
48	ENSMUSG00000025153.8	Fasn	0.005847	24	4
49	ENSMUSG00000021775.10	Nr1d2	0.005847	24	19
50	ENSMUSG00000030082.8	Sec61a1	0.005847	24	4
51	ENSMUSG00000021610.7	Clptm1l	0.005847	24	4

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11	ENSMUSG00000060450.7	Rnf14	0.005847	24	2
12	ENSMUSG00000055116.7	Arntl	0.005847	24	6
13	ENSMUSG00000056973.6	Ces1d	0.005847	24	2
14	ENSMUSG00000047539.4	Fbxo28	0.005847	24	2
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16	ENSMUSG00000028496.11	Mllt3	0.005847	24	2
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19	ENSMUSG00000031489.8	Adrb3	0.005847	24	4
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21	ENSMUSG00000032724.5	Abtb2	0.005847	24	4
22	ENSMUSG00000001467.4	Cyp51	0.005847	24	3
23	ENSMUSG00000040564.7	Apoc1	0.005847	24	4
24	ENSMUSG00000003809.8	Gcdh	0.005847	24	2
25	ENSMUSG00000022131.2	Gpr180	0.005847	24	2
26					
27	ENSMUSG00000004394.6	Tmed4	0.005847	24	2
28	ENSMUSG00000021666.10	Gfm2	0.005847	24	0
29	ENSMUSG00000041153.3	Osgin2	0.005847	24	2
30	ENSMUSG00000096795.1	Zfp433	0.005847	24	4
31	ENSMUSG00000015852.7	Fcrls	0.005847	24	4
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33	ENSMUSG00000026181.11	Ppm1f	0.005847	24	4
34	ENSMUSG00000005069.6	Pex5	0.005847	24	0
35	ENSMUSG00000027341.4	Tmem230	0.005847	24	4
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37	ENSMUSG00000030787.3	Lyve1	0.005847	24	2
38	ENSMUSG00000031358.11	Msl3	0.005847	24	4
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40	ENSMUSG00000029348.7	Asphd2	0.005847	24	4
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42	ENSMUSG00000038180.5	Spag4	0.005847	24	5
43	ENSMUSG00000025102.8	3110040N11Rik	0.005847	24	2
44	ENSMUSG00000020038.9	Cry1	0.005847	24	4
45	ENSMUSG00000062480.5	Acat3	0.005847	24	4
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47	ENSMUSG00000078923.4	Ube2v1	0.005847	24	14
48	ENSMUSG00000022074.5	Tnfrsf10b	0.005847	24	6
49	ENSMUSG00000027938.5	Creb3l4	0.005847	24	4
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51	ENSMUSG00000097643.1	A130051J06Rik	0.005847	24	6
52	ENSMUSG00000024334.8	H2-Oa	0.005847	24	14
53	ENSMUSG00000096935.1	1700113A16Rik	0.005847	24	4
54	ENSMUSG00000002578.9	Ikzf4	0.005847	24	8
55	ENSMUSG00000034460.8	Six4	0.005847	24	4
56					
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58	ENSMUSG00000040177.9	2310057M21Rik	0.005847	24	4
59	ENSMUSG00000005268.14	Prlr	0.005847	24	5
60	ENSMUSG00000073427.3	Gm4924	0.005847	24	2
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3	ENSMUSG00000069208.5	Zfp825	0.005847	24	4
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5	ENSMUSG00000053038.7	Gm6180	0.005847	24	16
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7	ENSMUSG00000081497.1	Gm15560	0.005847	24	16
8	ENSMUSG00000041930.7	Fam222a	0.005847	24	4
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11	ENSMUSG00000027762.5	Sucnr1	0.008333	24	4
12	ENSMUSG00000018865.8	Sult4a1	0.008333	24	14
13	ENSMUSG00000070436.5	Serpinh1	0.01082	24	4
14	ENSMUSG00000020585.4	Laptm4a	0.01082	24	4
15	ENSMUSG00000020889.11	Nr1d1	0.01082	24	16
16	ENSMUSG00000033022.7	Cdo1	0.01082	24	4
17	ENSMUSG00000024914.10	Drap1	0.01082	24	16
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19	ENSMUSG00000020532.12	Acaca	0.01082	24	4
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24	ENSMUSG00000066632.2	Pgk1-rs7	0.01082	24	0
25	ENSMUSG00000032193.8	Ldlr	0.01082	24	3
26	ENSMUSG00000027879.3	Sec22b	0.01082	24	2
27	ENSMUSG00000061838.5	Suc1g2	0.01082	24	0
28	ENSMUSG00000037379.4	Spon2	0.01082	24	7
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30	ENSMUSG00000023827.2	Agpat4	0.01082	24	6
31	ENSMUSG00000029231.9	Pdgfra	0.01082	24	4
32	ENSMUSG00000019872.7	Smpd13a	0.01082	24	4
33	ENSMUSG00000027088.4	Phospho2	0.01082	24	2
34	ENSMUSG00000031467.4	Agpat5	0.01082	24	2
35	ENSMUSG00000027742.8	Cog6	0.01082	24	2
36	ENSMUSG00000029009.11	Mthfr	0.01082	24	4
37	ENSMUSG00000063275.9	Ptpla	0.01082	24	4
38	ENSMUSG00000029685.9	Asb15	0.01082	24	22
39	ENSMUSG00000020471.5	Pold2	0.01082	24	13
40	ENSMUSG00000038416.8	Cdc16	0.01082	24	2
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42	ENSMUSG00000029782.12	Tmem209	0.01082	24	16
43	ENSMUSG00000021916.9	Glt8d1	0.01082	24	3
44	ENSMUSG00000028955.3	Vamp3	0.01082	24	2
45	ENSMUSG00000024014.6	Pim1	0.01082	24	14
46	ENSMUSG00000034981.9	Parm1	0.01082	24	4
47	ENSMUSG00000026082.6	Rev1	0.01082	24	22
48	ENSMUSG00000031610.3	Scrg1	0.01082	24	2
49	ENSMUSG00000069631.8	Strada	0.01082	24	12
50	ENSMUSG00000027465.7	Tbc1d20	0.01082	24	4
51	ENSMUSG00000024150.5	Mcf2	0.01082	24	2

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3	ENSMUSG00000024810.10	Il33	0.01082	24	2
4	ENSMUSG00000030545.8	Pex11a	0.01082	24	2
5	ENSMUSG00000031938.8	4931406C07Rik	0.01082	24	2
6	ENSMUSG00000021559.7	Dapk1	0.01082	24	4
7	ENSMUSG00000075486.3	Commd6	0.01082	24	3
8	ENSMUSG00000012422.8	Tmem167	0.01082	24	4
9	ENSMUSG00000074182.4	Znhit6	0.01082	24	22
10	ENSMUSG00000047554.7	Tmem41b	0.01082	24	4
11	ENSMUSG00000028293.8	Slc35a1	0.01082	24	4
12	ENSMUSG00000007777.3	0610009B22Rik	0.01082	24	5
13	ENSMUSG00000028256.10	Odf2l	0.01082	24	0
14	ENSMUSG00000075054.4	Yae1d1	0.01082	24	3
15	ENSMUSG00000039706.7	Ldb2	0.01082	24	5
16	ENSMUSG00000032018.7	Sc5d	0.01082	24	3
17	ENSMUSG00000034413.8	Neurl1b	0.01082	24	4
18	ENSMUSG00000019791.5	Hint3	0.01082	24	2
19	ENSMUSG00000097649.1	Gm10561	0.01082	24	4
20	ENSMUSG00000027555.8	Car13	0.01082	24	4
21	ENSMUSG00000046636.4	Gm7729	0.01082	24	16
22	ENSMUSG00000028990.7	Lzic	0.01082	24	2
23	ENSMUSG00000021567.8	Nkd2	0.01082	24	2
24	ENSMUSG00000070572.6	Trmt112-ps2	0.01082	24	14
25	ENSMUSG00000013646.11	Sh3bp5l	0.01082	24	1
26	ENSMUSG00000063439.6	B9d2	0.01082	24	16
27	ENSMUSG00000055917.8	Zfp277	0.01082	24	4
28	ENSMUSG00000039354.10	Smarcal1	0.01082	24	2
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30	ENSMUSG00000089797.1	Gm16118	0.01082	24	18
31	ENSMUSG00000032346.7	Ooep	0.01082	24	4
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34	ENSMUSG00000020000.7	Moxd1	0.01082	24	4
35	ENSMUSG00000040321.3	Zfp770	0.01082	24	4
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37	ENSMUSG00000096544.1	Gm4617	0.01082	24	15
38	ENSMUSG00000082530.1	Gm12168	0.01082	24	16
39	ENSMUSG00000030336.8	Cd27	0.01082	24	14
40	ENSMUSG00000015437.4	Gzmb	0.01082	24	13
41	ENSMUSG00000065824.1	Gm26315	0.01082	24	9
42	ENSMUSG00000046733.7	Gprc5a	0.01082	24	6
43	ENSMUSG00000041617.4	Ccdc74a	0.01082	24	8
44	ENSMUSG00000016181.4	Diexf	0.01082	24	4
45	ENSMUSG00000091803.1	Cox16	0.01082	24	2
46	ENSMUSG00000020407.7	Upp1	0.01082	24	6
47	ENSMUSG00000055137.6	5033411D12Rik	0.01082	24	4
48	ENSMUSG00000079343.3	Gm5077	0.01082	24	2
49	ENSMUSG00000037940.9	Inpp4b	0.01082	24	4
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51	ENSMUSG00000039278.10	Pcsk1n	0.01082	24	16

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8	ENSMUSG00000086869.2	Gm7809	0.01082	24	0
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10	ENSMUSG00000083567.2	Gm11451	0.01082	24	4
11	ENSMUSG00000041878.3	8430432A02Rik	0.01082	24	4
12	ENSMUSG00000074345.3	Tnfaip8l3	0.01082	24	4
13	ENSMUSG00000033615.9	Cplx1	0.01082	24	16
14	ENSMUSG00000048186.8	Bend7	0.01082	24	4
15	ENSMUSG00000081194.1	Gm8424	0.01082	24	4
16	ENSMUSG00000066438.6	Plekhd1	0.01082	24	16
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18	ENSMUSG00000070577.5	Gm572	0.014955	24	4
19	ENSMUSG00000017300.9	Tnnc2	0.01909	24	18
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21	ENSMUSG00000028618.5	Tmem59	0.01909	24	2
22	ENSMUSG00000035493.9	Tgfb1	0.01909	24	2
23	ENSMUSG00000036309.8	Skp1a	0.01909	24	1
24	ENSMUSG00000026509.10	Capn2	0.01909	24	2
25	ENSMUSG00000022816.5	Fstl1	0.01909	24	3
26	ENSMUSG00000079017.3	Ifi27l2a	0.01909	24	2
27					
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29	ENSMUSG00000030168.7	Adipor2	0.01909	24	4
30	ENSMUSG00000027605.12	Acss2	0.01909	24	4
31	ENSMUSG00000030774.7	Pak1	0.01909	24	20
32	ENSMUSG00000052837.5	Junb	0.01909	24	13
33	ENSMUSG00000019370.10	Calm3	0.01909	24	16
34	ENSMUSG00000022048.8	Dpysl2	0.01909	24	2
35	ENSMUSG00000027668.7	Mfn1	0.01909	24	0
36	ENSMUSG00000020893.11	Per1	0.01909	24	18
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38	ENSMUSG00000030062.6	Rpn1	0.01909	24	0
39	ENSMUSG00000020817.10	Rabep1	0.01909	24	2
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41	ENSMUSG00000022940.10	Pigp	0.01909	24	1
42	ENSMUSG00000020523.8	Fam114a2	0.01909	24	2
43	ENSMUSG00000042688.10	Mapk6	0.01909	24	9
44	ENSMUSG00000029390.7	Tmed2	0.01909	24	4
45	ENSMUSG00000081604.4	Gm11518	0.01909	24	14
46	ENSMUSG00000083899.2	Gm12346	0.01909	24	0
47	ENSMUSG00000052428.5	Tmco1	0.01909	24	4
48	ENSMUSG00000027131.3	Emc4	0.01909	24	3
49	ENSMUSG00000061731.3	Ext1	0.01909	24	4
50	ENSMUSG00000024370.10	Cdc23	0.01909	24	4
51	ENSMUSG00000045294.10	Insig1	0.01909	24	2
52	ENSMUSG00000044221.8	Grsf1	0.01909	24	1
53	ENSMUSG00000032998.10	Foxj3	0.01909	24	2
54	ENSMUSG00000071984.4	Fndc1	0.01909	24	4
55	ENSMUSG00000026585.7	Kifap3	0.01909	24	2
56	ENSMUSG00000025911.8	Adhfe1	0.01909	24	4
57	ENSMUSG00000016494.3	Cd34	0.01909	24	2
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59	ENSMUSG00000030298.4	Sec13	0.01909	24	4
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2	ENSMUSG00000022436.9	Sh3bp1	0.01909	24	14
3	ENSMUSG00000021417.8	Eci2	0.01909	24	4
4	ENSMUSG00000040928.9	S100pbp	0.01909	24	0
5	ENSMUSG00000029864.5	Gstk1	0.01909	24	4
6	ENSMUSG0000001962.8	Fam50a	0.01909	24	21
7	ENSMUSG00000005078.10	Jkamp	0.01909	24	4
8	ENSMUSG00000014402.8	Tsg101	0.01909	24	0
9	ENSMUSG00000031799.9	Tpm4	0.01909	24	4
10	ENSMUSG00000021756.6	Il6st	0.01909	24	2
11	ENSMUSG0000000194.7	Gpr107	0.01909	24	2
12	ENSMUSG00000055866.8	Per2	0.01909	24	22
13	ENSMUSG00000036940.9	Kdm1a	0.01909	24	2
14	ENSMUSG00000036099.10	Vezt	0.01909	24	11
15	ENSMUSG00000034893.7	Cog3	0.01909	24	0
16	ENSMUSG00000029474.6	Rnf34	0.01909	24	4
17	ENSMUSG00000024645.4	Timm21	0.01909	24	1
18	ENSMUSG00000051232.7	Tmem199	0.01909	24	4
19	ENSMUSG00000029554.9	Mad1l1	0.01909	24	16
20	ENSMUSG00000066233.5	Tmem42	0.01909	24	2
21	ENSMUSG00000027217.7	Tspan18	0.01909	24	4
22	ENSMUSG00000057789.7	Bak1	0.01909	24	12
23	ENSMUSG00000023992.8	Trem2	0.01909	24	16
24	ENSMUSG00000036513.9	Commd2	0.01909	24	22
25	ENSMUSG00000032030.10	Cul5	0.01909	24	4
26	ENSMUSG00000038936.7	Sccpdh	0.01909	24	2
27	ENSMUSG00000029759.3	Pon3	0.01909	24	3
28	ENSMUSG00000043635.6	Adamts3	0.01909	24	4
29	ENSMUSG00000040158.6	Tax1bp3	0.01909	24	2
30	ENSMUSG0000001300.9	Efnb2	0.01909	24	4
31	ENSMUSG00000026271.9	Gpr35	0.01909	24	10
32	ENSMUSG00000037001.10	Zfp39	0.01909	24	12
33	ENSMUSG00000049892.7	Rasd1	0.01909	24	4
34	ENSMUSG00000028369.9	Svep1	0.01909	24	2
35	ENSMUSG00000020653.5	Klf11	0.01909	24	14
36	ENSMUSG00000046731.3	Kctd11	0.01909	24	5
37	ENSMUSG00000035890.8	Rnf126	0.01909	24	8
38	ENSMUSG00000058729.7	Lin9	0.01909	24	17
39	ENSMUSG00000015971.4	Actr8	0.01909	24	2
40	ENSMUSG0000004356.7	Utp20	0.01909	24	0
41	ENSMUSG00000027313.3	Chac1	0.01909	24	16
42	ENSMUSG00000026482.7	Rgl1	0.01909	24	2
43	ENSMUSG00000036995.7	Asap3	0.01909	24	4
44	ENSMUSG00000086316.1	2210013O21Rik	0.01909	24	4
45	ENSMUSG00000069378.7	Prdm6	0.01909	24	6
46	ENSMUSG00000037669.8	1110057K04Rik	0.01909	24	2
47	ENSMUSG00000031533.3	Mrps31	0.01909	24	4
48	ENSMUSG00000025507.7	Lrdd	0.01909	24	14
49	ENSMUSG00000069255.6	Dusp22	0.01909	24	4
50	ENSMUSG00000030317.6	Timp4	0.01909	24	22
51	ENSMUSG00000007476.12	Lrrc8a	0.01909	24	4

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2	ENSMUSG00000063889.10	Crem	0.01909	24	2
3	ENSMUSG00000037296.6	Lsm1	0.01909	24	4
4	ENSMUSG00000047182.5	Irs3	0.01909	24	4
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6	ENSMUSG00000036819.8	Jmjd4	0.01909	24	11
7	ENSMUSG00000021339.3	Mrs2	0.01909	24	2
8	ENSMUSG00000020178.5	Adora2a	0.01909	24	6
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10	ENSMUSG00000004500.8	Zfp324	0.01909	24	2
11	ENSMUSG00000034748.10	Sirt6	0.01909	24	10
12	ENSMUSG00000039033.5	Tasp1	0.01909	24	0
13	ENSMUSG00000033581.10	Igf2bp2	0.01909	24	4
14	ENSMUSG00000078897.4	Gm4724	0.01909	24	2
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16	ENSMUSG00000007805.3	Twist2	0.01909	24	6
17	ENSMUSG00000039512.11	Uhrf1bp1	0.01909	24	4
18	ENSMUSG00000020354.9	Sgcd	0.01909	24	3
19	ENSMUSG00000097080.1	1700086O06Rik	0.01909	24	12
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21	ENSMUSG00000030935.9	Acsm3	0.01909	24	4
22	ENSMUSG00000033825.9	Tpsb2	0.01909	24	4
23	ENSMUSG00000078249.4	Hmga1-rs1	0.01909	24	14
24	ENSMUSG00000049916.9	2610318N02Rik	0.01909	24	14
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26	ENSMUSG00000053870.6	Fpgt	0.01909	24	4
27	ENSMUSG00000020151.10	Ptpr	0.01909	24	3
28	ENSMUSG00000081121.1	Gm12791	0.01909	24	17
29	ENSMUSG00000003062.8	Stard3nl	0.01909	24	0
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31	ENSMUSG00000025221.9	Kcnp2	0.01909	24	4
32	ENSMUSG00000022206.6	Npr3	0.01909	24	2
33	ENSMUSG00000041762.10	Gpr155	0.01909	24	1
34	ENSMUSG00000057895.5	Zfp105	0.01909	24	1
35	ENSMUSG00000026048.10	Ercc5	0.01909	24	0
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37	ENSMUSG00000074657.4	Kif5a	0.01909	24	16
38	ENSMUSG00000090215.2	Trim34b	0.01909	24	15
39	ENSMUSG00000043943.8	Naalad2	0.01909	24	4
40	ENSMUSG00000041020.8	Map7d2	0.01909	24	11
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42	ENSMUSG00000050994.13	Adgb	0.01909	24	16
43	ENSMUSG00000030325.10	Klrb1c	0.01909	24	16
44	ENSMUSG00000036634.9	Mag	0.01909	24	16
45	ENSMUSG00000056592.8	Zfp658	0.01909	24	0
46	ENSMUSG00000028145.7	Them4	0.01909	24	4
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48	ENSMUSG00000027001.4	Dusp19	0.01909	24	3
49	ENSMUSG00000086877.1	A230072C01Rik	0.01909	24	4
50	ENSMUSG00000097124.1	A530020G20Rik	0.01909	24	4
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52	ENSMUSG00000078349.2	AW011738	0.01909	24	16
53	ENSMUSG00000031428.5	Zcchc18	0.01909	24	18
54	ENSMUSG00000044033.10	Ccdc141	0.01909	24	4
55	ENSMUSG00000041945.6	Mfsd9	0.01909	24	4
56	ENSMUSG00000042389.7	Tsen2	0.01909	24	4
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58	ENSMUSG00000011751.10	Sptbn4	0.01909	24	17
59	ENSMUSG00000032556.9	Bfsp2	0.01909	24	16
60	ENSMUSG00000012187.7	Mogat1	0.01909	24	4
	ENSMUSG00000092124.1	B930094E09Rik	0.01909	24	4
	ENSMUSG00000024827.9	Gldc	0.01909	24	21

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2	ENSMUSG00000081895.3	Gm10294	0.01909	24	20
3	ENSMUSG00000083859.1	Gm12003	0.01909	24	16
4	ENSMUSG00000059511.3	Gm20563	0.01909	24	4
5	ENSMUSG00000036095.10	Dgkb	0.01909	24	3
6	ENSMUSG00000055188.6	2900002K06Rik	0.01909	24	5
7	ENSMUSG00000087569.1	Gm8464	0.01909	24	16
8	ENSMUSG00000086670.1	Gm13194	0.01909	24	16
9	ENSMUSG00000061988.2	Rpl10a-ps2	0.01909	24	16
10	ENSMUSG00000031297.8	Slc7a3	0.01909	24	1
11	ENSMUSG00000049832.5	Gm9840	0.01909	24	18
12	ENSMUSG00000083668.1	Gm5648	0.01909	24	16
13	ENSMUSG00000035983.4	Gm7008	0.01909	24	16
14	ENSMUSG00000084941.1	Gm11944	0.01909	24	13
15	ENSMUSG00000033765.4	Calm4	0.01909	24	22
16	ENSMUSG00000040035.8	Disp2	0.01909	24	18
17	ENSMUSG00000034739.11	Mfrp	0.025686	24	4
18	ENSMUSG00000044948.10	Wdr96	0.025686	24	6
19	ENSMUSG00000049699.3	Ucn2	0.025686	24	16
20	ENSMUSG00000085893.1	Gm12091	0.032282	24	12
21	ENSMUSG00000022389.8	Tef	0.032282	24	21
22	ENSMUSG00000079037.3	Prnp	0.032282	24	3
23	ENSMUSG00000081992.1	Gm13408	0.032282	24	16
24	ENSMUSG00000021794.9	Glud1	0.032282	24	1
25	ENSMUSG00000030435.9	U2af2	0.032282	24	14
26	ENSMUSG00000031299.10	Pdha1	0.032282	24	1
27	ENSMUSG00000030058.11	Copg1	0.032282	24	2
28	ENSMUSG0000002257.7	Def6	0.032282	24	14
29	ENSMUSG00000049421.7	Zfp260	0.032282	24	2
30	ENSMUSG00000034902.11	Pip5k1c	0.032282	24	12
31	ENSMUSG00000041220.6	Elovl6	0.032282	24	3
32	ENSMUSG00000021748.8	Pdhb	0.032282	24	2
33	ENSMUSG00000030245.10	Golt1b	0.032282	24	4
34	ENSMUSG00000049760.5	2410015M20Rik	0.032282	24	4
35	ENSMUSG00000039100.9		Mar-06 0.032282	24	2
36	ENSMUSG00000014444.10	Piezo1	0.032282	24	8
37	ENSMUSG00000031770.9	Herpud1	0.032282	24	22
38	ENSMUSG00000022893.8	Adamts1	0.032282	24	2
39	ENSMUSG00000032116.11	Stt3a	0.032282	24	2
40	ENSMUSG00000037049.8	Smpd1	0.032282	24	2
41	ENSMUSG00000054690.11	Emcn	0.032282	24	3
42	ENSMUSG00000028150.8	Rorc	0.032282	24	2
43	ENSMUSG00000025511.8	Tspan4	0.032282	24	22
44	ENSMUSG00000029776.10	Hibadh	0.032282	24	1
45	ENSMUSG00000032563.9	Mrpl3	0.032282	24	3
46	ENSMUSG00000090266.4	Mettl23	0.032282	24	3
47	ENSMUSG00000026077.9	Npas2	0.032282	24	8
48	ENSMUSG00000025239.2	Limd1	0.032282	24	0
49	ENSMUSG00000017686.10	Rhot1	0.032282	24	2
50	ENSMUSG00000021395.10	Spin1	0.032282	24	1
51	ENSMUSG00000028149.6	Rap1gds1	0.032282	24	1

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3	ENSMUSG000000091512.1	Lamtor3	0.032282	24	2
4	ENSMUSG00000002017.9	Fam98a	0.032282	24	2
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6	ENSMUSG000000032353.7	Tmed3	0.032282	24	2
7	ENSMUSG000000055319.7	Sec23ip	0.032282	24	0
8	ENSMUSG000000043252.8	Tmem64	0.032282	24	2
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10	ENSMUSG000000063001.8	Rps23-ps	0.032282	24	0
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13	ENSMUSG00000002210.5	Smg9	0.032282	24	12
14	ENSMUSG000000095115.1	Itpripl2	0.032282	24	3
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16	ENSMUSG000000016481.10	Cr1l	0.032282	24	0
17	ENSMUSG000000040374.7	Pex2	0.032282	24	3
18	ENSMUSG000000002949.8	Timm44	0.032282	24	4
19	ENSMUSG000000020114.6	Cand1	0.032282	24	0
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21	ENSMUSG000000024269.5	Tpgs2	0.032282	24	3
22	ENSMUSG000000097971.2	Gm26917	0.032282	24	10
23	ENSMUSG000000020963.8	Tshr	0.032282	24	4
24	ENSMUSG000000036334.7	Igsf10	0.032282	24	4
25	ENSMUSG000000036782.7	Klh13	0.032282	24	4
26					
27	ENSMUSG000000001098.9	Kctd10	0.032282	24	2
28	ENSMUSG000000019874.5	Fabp7	0.032282	24	6
29	ENSMUSG000000030203.11	Dusp16	0.032282	24	0
30	ENSMUSG000000059689.8	Zfp637	0.032282	24	4
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32	ENSMUSG000000037613.9	Tnfrsf23	0.032282	24	8
33	ENSMUSG000000027519.4	Rab22a	0.032282	24	3
34	ENSMUSG000000024778.6	Fas	0.032282	24	4
35	ENSMUSG000000035459.9	Stab2	0.032282	24	16
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37	ENSMUSG000000020623.5	Map2k6	0.032282	24	20
38	ENSMUSG000000032705.8	Exd2	0.032282	24	1
39	ENSMUSG000000040659.3	Efhd2	0.032282	24	14
40	ENSMUSG000000048376.5	F2r	0.032282	24	4
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42	ENSMUSG000000036646.7	Man1b1	0.032282	24	0
43	ENSMUSG000000026608.7	Kctd3	0.032282	24	4
44	ENSMUSG000000025241.9	Fyco1	0.032282	24	2
45	ENSMUSG000000025144.11	Stra13	0.032282	24	12
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47	ENSMUSG000000034263.6	Vwa9	0.032282	24	4
48	ENSMUSG000000034300.10	Fam53c	0.032282	24	1
49	ENSMUSG000000067369.6	Trmt2b	0.032282	24	0
50	ENSMUSG000000066150.6	Slc31a1	0.032282	24	3
51					
52	ENSMUSG000000017724.8	Etv4	0.032282	24	6
53	ENSMUSG000000028184.8	Lphn2	0.032282	24	5
54	ENSMUSG000000096173.1	Gm3150	0.032282	24	4
55	ENSMUSG000000015806.6	Qdpr	0.032282	24	0
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57	ENSMUSG000000029125.8	Stx18	0.032282	24	4
58	ENSMUSG000000047777.9	Phf13	0.032282	24	2
59	ENSMUSG000000046532.7	Ar	0.032282	24	0
60	ENSMUSG000000038764.8	Ptpn3	0.032282	24	0
	ENSMUSG000000039476.7	Prrx2	0.032282	24	5
	ENSMUSG000000036932.8	Aifm1	0.032282	24	4

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2	ENSMUSG00000052504.6	Epha3	0.032282	24	4
3	ENSMUSG00000087635.2	Gm13414	0.032282	24	16
4	ENSMUSG00000037762.6	Slc16a9	0.032282	24	6
5	ENSMUSG00000005907.8	Pex1	0.032282	24	3
6	ENSMUSG00000028024.8	Enpep	0.032282	24	8
7	ENSMUSG00000029815.7	Malsu1	0.032282	24	0
8	ENSMUSG00000064105.6	Cnm2	0.032282	24	4
9	ENSMUSG00000028621.11	Cyb5rl	0.032282	24	4
10	ENSMUSG00000028152.4	Tspan5	0.032282	24	0
11	ENSMUSG00000070000.7	Fcho1	0.032282	24	14
12	ENSMUSG00000038816.8	Ctnnal1	0.032282	24	2
13	ENSMUSG00000039633.6	Lonrf1	0.032282	24	0
14	ENSMUSG00000032401.9	Lctl	0.032282	24	4
15	ENSMUSG00000026810.6	Dpm2	0.032282	24	8
16	ENSMUSG00000024277.8	Mapre2	0.032282	24	2
17	ENSMUSG00000036186.5	Fam69b	0.032282	24	4
18	ENSMUSG00000005225.9	Plekha8	0.032282	24	2
19	ENSMUSG00000029576.11	Radil	0.032282	24	4
20	ENSMUSG00000021646.8	Mccc2	0.032282	24	8
21	ENSMUSG00000024780.6	Cdc37l1	0.032282	24	2
22	ENSMUSG00000000148.11	Brat1	0.032282	24	4
23	ENSMUSG00000041406.8	BC055324	0.032282	24	16
24	ENSMUSG00000042487.5	Leo1	0.032282	24	6
25	ENSMUSG00000030763.6	Lcmt1	0.032282	24	22
26	ENSMUSG00000045410.11	Akr1e1	0.032282	24	2
27	ENSMUSG00000093392.1	Gm6061	0.032282	24	20
28	ENSMUSG00000029536.7	Gatc	0.032282	24	3
29	ENSMUSG00000017400.4	Stac2	0.032282	24	12
30	ENSMUSG00000030722.7	Nfatc2ip	0.032282	24	16
31	ENSMUSG00000044715.6	Gskip	0.032282	24	4
32	ENSMUSG00000093548.1	Gm6407	0.032282	24	15
33	ENSMUSG00000012126.10	Ubxn11	0.032282	24	11
34	ENSMUSG00000041650.9	Pcca	0.032282	24	1
35	ENSMUSG00000080875.2	Gm7332	0.032282	24	14
36	ENSMUSG00000031111.10	Igsf1	0.032282	24	6
37	ENSMUSG00000089764.1	Gm16580	0.032282	24	16
38	ENSMUSG00000071291.4	Zfp58	0.032282	24	6
39	ENSMUSG00000044636.5	Csrnp2	0.032282	24	4
40	ENSMUSG00000043122.6	A530016L24Rik	0.032282	24	2
41	ENSMUSG00000095675.1	Ccl21b	0.032282	24	5
42	ENSMUSG00000040164.3	Kcns1	0.032282	24	5
43	ENSMUSG00000037463.8	Fbxo27	0.032282	24	2
44	ENSMUSG00000022371.9	Col14a1	0.032282	24	2
45	ENSMUSG00000075318.6	Scn2a1	0.032282	24	5
46	ENSMUSG00000044122.8	Proca1	0.032282	24	2
47	ENSMUSG00000042505.6	Acn9	0.032282	24	2
48	ENSMUSG00000014782.9	Plekhg4	0.032282	24	8
49	ENSMUSG00000085738.2	Gm12335	0.032282	24	0
50	ENSMUSG00000046561.8	Arsj	0.032282	24	4
51	ENSMUSG00000028102.9	Pex11b	0.032282	24	2
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2	ENSMUSG00000086679.1	Gm15551	0.032282	24	2
3	ENSMUSG00000037418.5	Best1	0.032282	24	4
4	ENSMUSG00000018500.2	Adora2b	0.032282	24	3
5	ENSMUSG000000091613.1	Gm17046	0.032282	24	4
6	ENSMUSG00000031482.8	Slc25a15	0.032282	24	0
7	ENSMUSG00000002083.6	Bbc3	0.032282	24	10
8	ENSMUSG000000070713.4	Gm10282	0.032282	24	14
9	ENSMUSG00000020396.8	Nefh	0.032282	24	16
10	ENSMUSG00000032593.5	Amigo3	0.032282	24	6
11	ENSMUSG00000047747.9	Rnf150	0.032282	24	20
12	ENSMUSG00000038583.6	Pln	0.032282	24	4
13	ENSMUSG00000042401.6	Crtac1	0.032282	24	14
14	ENSMUSG00000098495.1	RP24-113D21.1	0.032282	24	14
15	ENSMUSG00000047497.9	Adamts12	0.032282	24	0
16	ENSMUSG00000057157.3	Gm6054	0.032282	24	4
17	ENSMUSG00000092454.1	Gm2991	0.032282	24	14
18	ENSMUSG00000015002.10	Efr3a	0.032282	24	4
19	ENSMUSG00000028597.11	Gpx7	0.032282	24	2
20	ENSMUSG00000098198.1	Gm9169	0.032282	24	16
21	ENSMUSG00000079157.3	Fam155a	0.032282	24	0
22	ENSMUSG00000020599.7	Rgs9	0.032282	24	4
23	ENSMUSG00000097554.1	Gm26825	0.032282	24	22
24	ENSMUSG00000074398.5	Gm15441	0.032282	24	15
25	ENSMUSG00000095724.1	Gm21319	0.032282	24	20
26	ENSMUSG00000098985.1	RP24-570C10.6	0.032282	24	19
27	ENSMUSG00000081540.3	Gm12538	0.032282	24	3
28	ENSMUSG00000068165.2	Gm10233	0.032282	24	3
29	ENSMUSG00000005360.8	Slc1a3	0.032282	24	4
30	ENSMUSG00000062794.8	Zfp599	0.032282	24	12
31	ENSMUSG00000066270.2	Gm10157	0.032282	24	22
32	ENSMUSG00000045062.3	Pcdhb7	0.032282	24	6
33	ENSMUSG00000087433.1	Gm14167	0.032282	24	12
34	ENSMUSG00000081289.1	Gm14857	0.032282	24	15
35	ENSMUSG00000047307.1	Pcdhb13	0.032282	24	4
36	ENSMUSG00000027547.11	Sall4	0.032282	24	17
37	ENSMUSG00000086884.1	Gm16225	0.032282	24	16
38	ENSMUSG00000080775.1	Gm6368	0.032282	24	18
39	ENSMUSG00000033405.3	Nudt15	0.032282	24	3
40	ENSMUSG00000040904.4	Gm21988	0.032282	24	13
41	ENSMUSG00000029608.7	Rph3a	0.032282	24	17
42	ENSMUSG00000003410.7	Elavl3	0.032282	24	16
43	ENSMUSG00000062257.6	Opcml	0.032282	24	4
44	ENSMUSG00000035211.8	Xrra1	0.032282	24	6
45	ENSMUSG00000094248.1	Hist1h2ao	0.032282	24	15
46	ENSMUSG00000078087.4	Rps12l1	0.032282	24	22
47	ENSMUSG00000070271.5	Gm13268	0.032282	24	16
48	ENSMUSG00000062417.5	Hist1h3g	0.032282	24	12
49	ENSMUSG00000057626.1	Gm5621	0.032282	24	0
50	ENSMUSG00000082163.1	Gm14276	0.032282	24	12
51	ENSMUSG00000031995.2	St14	0.032282	24	16

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2	ENSMUSG00000022144.3	Gdnf	0.042414	24	6
3	ENSMUSG00000030307.7	Slc6a11	0.042414	24	16
4	ENSMUSG00000009214.3	Tmem8c	0.042414	24	6
5	ENSMUSG00000034584.3	Exph5	0.042414	24	6
6	ENSMUSG00000044081.7	4930441O14Rik	0.042414	24	6
7	ENSMUSG00000036264.9	Fstl4	0.042414	24	18
8	ENSMUSG00000031376.9	Atp2b3	0.042414	24	5
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7 **The intervertebral disc contains intrinsic circadian clocks that are regulated by age**
8 **and cytokines and linked to degeneration**
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39 **Running Title: IVD clock and degeneration**

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41 **Key words: circadian clock, intervertebral disc, cytokine, ageing, *Bmal1***
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Abstract

Objectives: The circadian clocks are internal timing mechanisms that drive ~24 hr rhythms in a tissue-specific manner. Many aspects of the physiology of the intervertebral disc (IVD) show clear diurnal rhythms. However, it is unknown whether IVD tissue contains functional circadian clocks and if so, how their dysregulation is implicated in IVD degeneration.

Methods: Clock gene dynamics in *ex vivo* IVD explants (from PER2::LUC reporter mice) and human disc cells (transduced with lentivirus containing *Per2::luc* reporters) were monitored in real-time by bioluminescence photon counting and imaging. Temporal gene expression changes were studied by RNAseq and qRT-PCR. IVD pathology was evaluated by histology in a mouse model with tissue-specific deletion of the core clock gene *Bmal1*.

Results: Here we show the existence of the circadian rhythm in mouse IVD tissue and human disc cells. This rhythm is dampened with ageing in mice and can be abolished by treatment with IL-1 β but not TNF α . Time series RNAseq revealed 607 genes with 24 hr patterns of expression representing several essential pathways in IVD physiology. Mice with conditional knockout of *Bmal1* in their disc cells demonstrated age-related degeneration of IVDs.

Conclusions: We have established autonomous circadian clocks in mouse and human IVD cells which respond to age and cytokines, and control key pathways involved in the homeostasis of IVDs. Genetic disruption to the mouse IVD molecular clock predispose to IVD degeneration. These results support the concept that disruptions to circadian rhythms may be a risk factor for degenerative IVD disease and low back pain.

Introduction

The circadian clocks are internal timing mechanisms which drive ~24 hr rhythms in physiology and behaviour. In mammals, the central pacemaker Suprachiasmatic Nuclei (SCN) in the hypothalamus synchronizes peripheral clocks in most major body organs.¹⁻³ Circadian rhythms coordinate tissue-specific physiology with light/darkness, rest/activity feeding cycles and body temperature fluctuations.¹⁴ Disruptions to circadian rhythms (during ageing or in shift workers) have been linked to increased risk of diseases (e.g. obesity, diabetes, cardiovascular disease, and osteoarthritis).^{5,6} At the molecular level, the circadian clock consists of a network of transcriptional activators (*Clock*, *Bmal1*) and repressors (*Per1/2* and *Cry1/2*) organized in a negative feedback loop.⁶ This core oscillator generates 24 hour rhythms in the expression of not only its core components but also a myriad of clock controlled genes (CCGs). Depending on the tissue, expression of 3-16% of the whole transcriptome exhibits a circadian rhythm.⁷

The spine is comprised of bony vertebral bodies alternating with fibro-cartilagenous intervertebral discs (IVD). IVD degeneration is among the most prevalent musculoskeletal disorders affecting one in five people under 60 and more than half of the people above 60 years of age.⁸ Low back pain, which is often associated with IVD degeneration, is the number one cause of Years Lived with Disability in the developed countries.⁹ Existing evidence suggests that the IVD is a highly rhythmic tissue, experiencing a diurnal cycle of higher loading (activity phase),^{10,11} followed by a period of low-load recovery (resting phase). Under high load the pressurized interstitial fluid flows to regions of lower pressure through the outer annulus fibrosus (AF) and the cartilaginous end plate (CEP), resulting in decreased disc height, AF outward bulging and an increase in osmolarity of the central gelatinous nucleus pulposus (NP). During the recovery period, the process is reversed by high osmotic pressure inside the disc causing fluid flow to the NP.¹² Exchange of nutrients/metabolites that occurs with fluid flow during this cycle maintains disc cell homeostasis.¹³

Consistent with the rhythmic nature of IVD tissue, shift work (a factor known to disrupt circadian rhythms) was reported to be associated with higher risk of LBP and IVD degeneration.¹⁴⁻¹⁸ We have previously shown that environmental disruption of circadian rhythm in mice, when combined with high fat diet, leads to degeneration of the lumbar IVD tissue in mice.¹⁹ More recently, changes in the expression of circadian clock genes have been identified in rat IVD tissues following passive smoking (a risk factor for LBP).²⁰ However, no studies have examined whether IVD cells express intrinsic circadian clocks, how these IVD clocks are regulated, what their targets are, and whether genetic disruption to the IVD clock impact on tissue homeostasis and susceptibility to degeneration.

In this study, we systemically characterized the molecular circadian clock mechanisms in mouse and human IVD tissue/cells. Moreover, by generating a tissue-specific *Bmal1* KO mouse model, our study provides the first genetic evidence linking a core clock factor to IVD degeneration.

Results

Intervertebral disc possesses a functional, temperature entrainable circadian clock

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7 To test whether the IVD contains a molecular circadian clock capable of driving circadian
8 rhythm of gene expression we monitored the dynamics of PER2::Luc protein in IVD explant
9 cultures isolated from PER2::Luc reporter mice.²¹ Real-time bioluminescence photon
10 counting demonstrated robust circadian rhythm of PER2::Luc activity which lasted for more
11 than 5 days, with a period of 23.93 +/- 0.10 hrs (mean +/- SEM, n=6, Fig. 1A). As the IVD
12 comprises two distinct cell types, the NP and AF cells, we wanted to know if both regions
13 exhibit circadian rhythms. Live imaging of the mouse IVD explants using high sensitivity EM-
14 CCD camera revealed rhythmic PER2::Luc signals from both AF and NP cells (see
15 Supplementary videos 1-3). To extend these studies to humans, primary human NP cells
16 were transiently transfected with a vector carrying the luciferase gene under the control of
17 the *Per2* promoter. This approach revealed cell autonomous circadian oscillations of
18 *Per2::luc* expression, indicating the operation of a functional clock machinery in these human
19 disc cells (Fig. 1B). IHC staining of human NP tissue sections using antibodies against
20 BMAL1 and CLOCK confirmed presence of these essential circadian clock components in
21 human discs (Fig. 1C).

22
23 One of the key properties of a peripheral circadian clock is their ability to respond to time
24 cues that are controlled by the SCN clock, such as hormones or changes in body
25 temperature. Since the IVDs are not vascularised or innervated (except in pathological
26 conditions)²², we hypothesised that daily body temperature oscillations may be a mechanism
27 of clock entrainment for IVDs. To test this, IVD explants from the same mouse were placed
28 in different incubators programmed to have oppositely phased cyclic temperature changes
29 for 4 days (38.5°C for 12 hrs/35.5°C for 12 hrs, or vice versa), before returning to a constant
30 37°C. As a control, another IVD explant from the same mouse was incubated under constant
31 37°C. The PER2::Luc rhythms in IVD explants were all in similar circadian phase for the first
32 3 days before the temperature protocol (Fig. 1D). Once the antiphasic protocol was
33 introduced, the oscillations were driven 180 ° out of phase with each other. Interestingly, the
34 antiphasic oscillations were maintained for at least three more days after the tissues were
35 released to constant temperature. In contrast, the IVD explant that remained at constant
36 temperature gradually lost its ability to oscillate by day 7, mainly due to desynchronization
37 in culture (Fig. 1D). These results clearly indicate that temperature cycles that approximate
38 body temperature changes are capable of not only entraining the circadian phase of the IVD
39 oscillation, but enhancing the oscillation amplitude.

40 **Aging affects the circadian rhythm of IVDs**

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42 Daily systemic time cues in body temperature and hormone release are known to be altered
43 with aging.²³ In addition, intrinsic properties of the clock oscillator could deteriorate with age
44 as well.^{23 24} Indeed, we have previously demonstrated that the amplitude of circadian
45 oscillations in cartilage and tendon tissues dampen with aging.^{25 26} Therefore, we
46 hypothesized that circadian rhythms may change in aging disc, compromising the daily
47 control of IVD physiology. To assess this, we compared the oscillations of PER2::Luc
48 expression in mouse IVD explant cultures from animals aged 2 and 12 months (Fig. 2A,
49 supplementary Video 1). The amplitude of oscillations in IVDs from 12 months old mice was
50 severely reduced (by ~60%) as compared to 2 month old mice. Additionally, the average
51 period of oscillations was significantly lengthened by 1.6 hrs in IVDs from 12 month old mice
52 (Fig. 2A). IHC staining showed decreased expression of the core circadian transcription
53 factors BMAL1 and CLOCK in 12 month (Fig. S1) and 24 month old mice as compared to 2
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7 month old (Fig. 2B). These data demonstrate that the IVD clock becomes dysregulated with
8 ageing.

11 The circadian rhythm of IVD is disrupted by IL-1 β in a NF- κ B dependent manner

12 Chronic inflammation is a known factor associated with IVD degeneration and lower back
13 pain.²⁷ To investigate the effects of catabolic cytokines on disc circadian clock, we treated
14 IVD explants from the PER2::Luc reporter mice with IL-1 β , LPS and TNF α . Tissues were
15 under continuous bioluminescence recording. Treatment with IL-1 β (or LPS, Fig. S2A)
16 resulted in complete disruption of the PER2::Luc circadian rhythm, associated with
17 significant changes of clock genes (*Bmal1*, *Per2* and *Nr1d1*) (Fig. 3A, Fig.S3). The disrupted
18 rhythm could be reinstated by ~~the addition of~~ dexamethasone (an anti-inflammatory
19 glucocorticoid, Fig. 3A) or IL-1RA (an antagonist of IL-1, Fig.S2B), but not by forskolin (a
20 clock synchronising agent without anti-inflammatory properties, Fig. ~~S2B~~S2C). NF κ B is one
21 of the classic pathways through which IL-1 β can mediate its effects. To evaluate the
22 involvement of NF κ B, we used the IKK1/2 inhibitor BMS-345541 to block the activation of
23 NF κ B. The clock-disrupting effect of IL-1 β was blocked by pre-treating the IVD explant with
24 BMS-345541, supporting a role of NF κ B pathway in the IL-1 β -mediated clock disruption. In
25 contrast to IL-1 β , treatment of IVD explants with TNF α had no effect on their circadian
26 rhythms (Fig. 3B). In contrast, both IL-1 β and TNF α elicited a strong induction of NF κ B
27 signalling in a lung epithelial cell line, suggesting a possible cell-type specific response
28 (Fig.S2D). Next, we took advantage of a transgenic mouse strain expressing the p65-
29 DsRedXP protein fusion construct²⁸ to observe the nuclear translocation of p65, one of the
30 major components of the NF κ B complex. Live imaging showed that treatment of IVD
31 explants with IL-1 β caused rapid nuclear translocation of p65 both in AF and NP cells.
32 However, addition of TNF α (up to 40 ng/mL) had no effect on p65 translocation (Fig.3C).

34 There are at least two potential mechanisms through which IL-1 β could disrupt the IVD
35 circadian rhythm. Individual cells may still have robust clocks but become desynchronised,
36 with their clocks being in different phases, leading to reduced oscillation amplitude; or
37 individual cells may have lost their pacemaking properties. To distinguish between these
38 two possibilities, we used a high sensitivity EM-CCD camera to visualize the PER2::Luc
39 bioluminescence signals from individual cells in the presence or absence of IL-1 β .
40 Consistent with the lack of effect of forskolin, this imaging approach revealed loss of
41 bioluminescence at single cell level, excluding the desynchronization hypothesis (Fig. 3D
42 and Supplementary video 2). Therefore, disruption to the IVD clock could be a hitherto
43 undiscovered response to pro-inflammatory cytokines.

46 Identification of the first IVD circadian transcriptome

47 Circadian clocks in different tissues exert their local functions through regulating diverse yet
48 highly tissue-specific set of target genes. To reveal the extent of rhythmic genes in IVD
49 tissue under physiological conditions, we performed a time-series RNAseq study using IVD
50 tissues (collected every 4 hours for 48 hours) from mice kept in 12 hr light/12 hr darkness.
51 We used a well-recognized JTKCycle²⁹ algorithm to pick out rhythmic genes. Using
52 $P_{\text{adjust}} < 0.05$ as a cut-off, we identified 607 genes (3.5% of expressed genes in IVD) with
53 rhythmic 24 hr expression patterns (Figure 4A, Supplementary Table 1). Further phase
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7 clustering analysis of these rhythmic genes using R package revealed 4 main clusters (Fig.
8 [S3S4](#)), with more than 70% of these genes peaking at night time points (representing the
9 active phase of mouse). Gene ontology (GO)-term analysis using topGO revealed dozens of
10 overrepresented functional groups with an adjusted $p < 0.01$, including “fatty acid metabolic
11 process”, “circadian rhythm”, “intracellular protein transmembrane transport”, “intrinsic
12 apoptotic signaling pathway”, “carboxylic acid metabolic process”, and “response to
13 endoplasmic reticulum stress”. We next compared the IVD rhythmic gene list to that of the
14 mouse cartilage and tendon we published earlier.^{25 26} There was a very small number of
15 genes (6-16%) overlapping between any two of these skeletal tissues, with only 16 genes
16 common to all three, supporting the tissue-specific function of the peripheral clocks (Figure
17 4B). Of these 16 common genes, 8 were core circadian clock genes. The expression
18 profiles of canonical clock genes (*Bmal1*, *Per2*, *Dbp*) and selected target genes *Follistatin* (a
19 BMP antagonist)³⁰ and *Timp4* (a tissue inhibitor of MMPs)³¹ relevant to IVD physiology and
20 catabolism were validated by temporal qRT-PCR in mouse IVD tissues (Figure 4C, [Figure](#)
21 [S45](#)).

22 23 24 Targeted deletion of *Bmal1* causes age-dependent IVD degeneration

25 *Bmal1* is an essential circadian clock component for the generation of 24 hr rhythms. The
26 global *Bmal1* knock-out mouse shows multi-tissue pathologies, including ectopic calcification
27 of IVDs.³² However, the severe disruption to whole body circadian rhythms confounds
28 interpretation of phenotype. To evaluate the function of local IVD clocks, we produced a
29 conditional KO mouse model (*Col2a1-Bmal1* KO, cKO) with a cell type-specific abolition of
30 the transcription factor *Bmal1* in $\alpha 1(\text{II})$ collagen expressing cells, including NP and AF cells,
31 and chondrocytes.³³ We have previously shown that the central SCN clock and behavioural
32 locomotion rhythms in the cKO mice are not affected.³³ IHC staining of IVDs confirmed loss
33 of BMAL1 expression in the majority of the AF cells and chondrocytes of the cartilaginous
34 end plate in cKO mice (Fig. 5A). The cKO mouse was crossed with the PER2::Luc mouse to
35 enable real-time tracking of clock rhythms. Photon counting of PER2::Luc bioluminescence
36 demonstrated a lack of circadian oscillations in the cKO IVDs, with no response to
37 dexamethasone treatment (Fig. 5B). Bioluminescence imaging of the cKO IVDs confirmed
38 lack of circadian oscillations of PER2::Luc in both AF and NP cells (Fig. 5C and
39 Supplementary video 3).

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41 Histological analysis revealed early signs of degeneration of the lumbar IVDs in cKO mouse
42 at 6 months of age, such as thinning of the growth plate of vertebral body (Fig. 6A), and
43 gradual disappearance of the CEP (Fig. [S4S56](#)). At 12 months, there was widespread
44 degeneration of lumbar IVDs in cKOs. Bone bridges appeared within the growth plate, the
45 CEP was almost completely replaced by bone (Fig. 6A, black arrow), and the height of the
46 disc was significantly reduced in cKO IVDs (Fig. 6A). In addition, staining with Safranin O
47 and picrosirius red revealed disorganisation of the outer annulus structure and signs of
48 fibrosis (with organized collagen bundles) appearing at the periphery of the IVDs (Fig. 6A-C,
49 asterisk). Finally, using X-ray studies, the cKO mice showed clear signs of calcification and
50 narrowing of spaces between vertebrae at 6 months (in tail IVDs, data not shown) and 12
51 months (in lumbar IVDs, Fig. 6C, [red and yellow arrows](#)). No signs of degeneration were
52 evident in age-matched WT mice up to the age of 12 months (Fig. 6B). However, similar
53 degenerative changes to the cKO mutants were visible in WT mice at 24 months of age (Fig.
54 [S5S67](#)), suggesting the possibility that loss of *Bmal1* and/or circadian rhythm in IVD cells
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7 leads to accelerated ageing of the tissue. [TUNEL assay and qPCR were performed to explore the underlying mechanisms for the observed phenotype. There were no obvious signs of apoptosis, although significant upregulation of catabolism-related genes \(*Adamts1*, *Adamts5*, *Adamts15* and *Follistatin*\) were observed in cKO IVDs \(Fig.S8, S9\).](#) Together, these results indicate the essential role of the locally expressed core clock factor BMAL1 in IVD homeostasis, loss of which led to profound tissue degeneration.

16 Discussion

18 Low back pain is amongst the most prevalent spinal diseases associated with increasing age, with over 80% of the UK population predicted to experience back pain within their lifetime. Progressive degeneration of the IVD tissue, partly caused by increased catabolism driven by inflammatory/catabolic cytokines, is a major contributing factor in LBP.³⁴ It has long been known that the physiology of IVD is under strong influence by a diurnal rhythm associated with the rest/activity cycles, i.e., daily cycles of loading (activity phase) and low-load recovery (resting phase).¹⁰⁻¹³ Exchange of nutrients/metabolites that occurs with fluid flow during this cycle maintains disc cell homeostasis. Recent epidemiological and experimental studies have linked shift work (in humans) and chronic disruption of circadian rhythms (in mice) to higher risk of IVD degeneration.^{14 15 17-19} However, our study represents the first critical analysis of the molecular and cellular mechanisms of the IVD clock under physiological and pathological conditions. Using the clock gene reporter mouse/cell models, as well as a conditional *Bmal1* KO mouse model that had disrupted IVD clock, we established autonomous circadian clocks in mouse and human IVD cells that respond to temperature cycles, dampen with age and become dysregulated by catabolic cytokines. Genetic disruption to the mouse IVD molecular clock predisposes to IVD degeneration. Global *Bmal1* KO also showed a phenotype in the skeletal system, including the spine. However, our conditional KO model allows us to conclude the essential role of locally expressed BMAL1 or circadian rhythm in maintaining IVD homeostasis. These results support the notion that disruptions to circadian rhythms during ageing or in shift workers may be a contributing factor for the increased susceptibility to degenerative IVD diseases and low back pain.

41 We also revealed for the first time the circadian transcriptome of the IVD tissue. Of particular interest are the genes and pathways that have been previously implicated in IVD physiology and pathology, such as genes involved in matrix homeostasis/repair (e.g. *Follistatin*, *Timp4*, *Adamts1*, *Adamts5*, *Adamts15* and *Adam17*),^{30 31} mitochondria function and fatty acid metabolism (e.g. *Pex1*, *Pex2*, *Pex5*, *Pex15*, *Adipoq*, *Adipor2*, *Fasn*).^{35 36} Although glucose and anaerobic glycolysis represent major metabolic pathways in IVD, there is evidence that mitochondria in the NP are functional and they retain the capacity to metabolise fatty acids through mitochondrial oxidative metabolism.³⁵ Other relevant pathways include ER stress and apoptosis (e.g. *Aifm1*, *Atf6*, *Chac1*, *Bak1*, *Bbc3*, *Opa1* and *Fas*).^{37 38} The diverse clock-controlled pathways identified by this approach implicate circadian rhythm as a critical regulatory mechanism for IVD biology.

52 Using IVD tissue explants, we have identified the disruption of the circadian clock in IVD as hitherto undiscovered response to pro-inflammatory cytokines. Similar clock disruptions by

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7 inflammatory cytokines have been found in other cell types, such as in macrophages,³⁹
8 synovial fibroblasts,⁴⁰ and chondrocytes.²⁸ The involvement of NFκB pathway in mediating
9 the effects of IL-1 is consistent with our earlier findings in chondrocytes, where NFκB
10 interferes with the core clock complex to disrupt circadian pacemaking.²⁸ Given the diverse
11 pathways controlled by the IVD clock, cytokine-mediated circadian disruption may be
12 involved in driving key aspects of the catabolic response of IVD to chronic inflammation.
13 Therefore, there is the possibility of stabilizing IVD clock rhythm as a novel strategy to
14 combat tissue catabolism. [Although the concentration we used for IL-1β \(5 ng/mL\) in these
15 tissue explant studies was higher than that in degenerative IVD \(~50 pg/mL\), this dose is in
16 line with most ex vivo/in vitro studies.](#)

17 We also identified a lack of response of the IVD clock (and cartilage clock)²⁸ to TNFα,
18 possibly due to the defective NFκB nuclear translocation. These findings suggest IL-1 and
19 TNFα may act on distinct downstream pathways and regulate different target genes within
20 the IVD, as seen in chondrocytes. In SW1353 chondrocyte-derived cells, catabolic genes
21 such as IL-6, BMP-2, MMP13 and COX-2 only respond to IL-1, with almost no response to
22 TNFα.^{41 42} Such results are intriguing because we have shown that IL-1β plays a more
23 prominent role in driving disc degeneration than TNFα.^{43 44} Therefore, anti-inflammatory
24 drugs that selectively target IL-1 are more likely to bring therapeutic benefits.

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26 In conclusion, our results provide a firm basis for future studies that aim to elucidate the
27 functional implication and therapeutic potential of the human IVD circadian rhythm in health
28 and disease of the spine.
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Figure legends

Figure 1. IVDs possess an autonomous circadian clock. (A) Representative PER2::Luc bioluminescence trace of mouse IVD explant culture (period = $23.93 \pm 0.247\text{h}$; mean \pm SD; n=6); (B) Representative trace of human NP cells transduced with a *Per2::luc* reporter (period= $22.52 \pm 0.39\text{h}$; mean \pm SD; n=3); (C) IHC of BMAL1 and CLOCK on NP biopsy of human IVDs (magnification 5x left, 10x right); n=3. (D) Temperature entrainment (n=4). Two IVD explant cultures (represented by red and blue traces) from the same animal were held under antiphase temperature cycles (alternating 12-hour cycles of $38.5^\circ\text{C}/35.5^\circ\text{C}$; baseline temperature = 37°C). Third IVD explant culture from the same animal was kept at a constant temperature of 37°C (Purple trace below).

Figure 2. Circadian rhythm of IVD is dampened during aging. (A) Representative bioluminescence traces of young (2 months) and ageing (12 months) IVDs from PER2::Luc mice. The period was significantly lengthened in older mice ($p < 0.05$) and the amplitude was significantly dampened ($p < 0.05$) (two-tailed nonparametric Mann-Whitney test; n=4); (B) IHC of BMAL1 and CLOCK on young (3 months) and aged (24 months) mouse IVDs; n=4. Magnification 10x. The Safranin O staining panel on the right was included to ease visualization of the different structures of the IVD. NP- nucleus pulposus; AF- annulus fibrosus; OAF- outer annulus fibrosus; CEP- cartilaginous end plate.

Figure 3. IL-1 β , but not TNF α , disrupts the circadian rhythm of IVDs. (A) Representative bioluminescence traces of PER2::Luc mouse IVD explants. Arrows indicate time of treatment with IL-1 β (5 ng/mL), IKK inhibitor (BMS-345541, 10 μM) and dexamethasone (100 nM). Red trace - treated with IL-1 β , green trace - pre-treated with IKK inhibitor before addition of IL-1 β , blue trace - vehicle control; n=3. (B) Representative bioluminescence traces treated with TNF α (red trace, 40 ng/mL) or control (blue trace). Arrows indicate time of treatments; n=3. (C) Live fluorescence imaging of p65DsRed reporter in mouse IVDs by confocal microscopy before and after treatment with IL-1 β or TNF α . Scale bar 20 μm . Arrows indicate the nuclei. AF- annulus fibrosus; NP- nucleus pulposus; (D) Live bioluminescence imaging of an IVD tissue from PER2::Luc mouse, treated with IL-1 β (at 48h), followed by dexamethasone (at 96h).

Figure 4. Circadian transcriptome in mouse IVD identified by time series RNA sequencing. (A) Heat map depicting the expression patterns of the 607 rhythmic genes (3.5 % of the IVD transcriptome) identified by JTKCycle. Genes were organized according to timing of peak expression. White bars represent the day; black bars represent the night. (B) Venn diagram comparing the number of rhythmic genes of IVD, cartilage and tendon. (C) qPCR validation of time-dependent expression of clock genes (*Bmal1*, *Per2* and *Dbp*) and target genes (*Follistatin* and *Timp4*) in mouse IVDs normalized to *Gapdh*. Mean and SEM (n = 6). Grey shadow indicates the night phase.

Figure 5. Conditional deletion of *Bmal1* in *Col2a1*-expressing cells results in disruption of the circadian rhythms in mouse IVDs. (A) IHC of BMAL1 in 3 month old WT and KO mice (magnification: upper panels 10x and lower panels 40x); n=3. (B) Representative bioluminescence traces of WT (blue) and *Bmal1* cKO (red) mouse IVD explant cultures; n=6. Arrow indicates treatment with dexamethasone. (C) Live

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bioluminescence imaging of IVDs from WT and *Bmal1* cKO IVDs from mice on a PER2::Luc background.

Figure 6. Loss of *Bmal1* leads to degeneration of IVDs and cartilaginous tissues of the spine. (A) Safranin O staining of 12 month old WT and *Bmal1* cKO mouse lumbar IVDs; n=4. Red arrow-loss of CEP; Black arrow- fragmentation of growth plate; *-fibrosis (magnification 2.5x). Analysis of the IVD height and growth plate thickness was shown (two-tailed nonparametric Mann-Whitney test; n=4) * - p<0.05; *** - p<0.001. (B) Picrosirius red staining of lumbar IVDs from 12 month old WT and *Bmal1* cKO mouse showing organisation of collagen (magnification 2.5x left and 5x right panels); n=4. Images were visualized under brightfield or polarized light. (C) X-ray radiography of 12 month old WT and *Bmal1* cKO mouse spines; n=3. Yellow arrows- calcification of IVDs; Red arrows- calcification of tissues surrounding the IVDs.

Supplementary figure legends

Figure S1. Reduced expression of BMAL1 and CLOCK in ageing IVDs. IHC of BMAL1 and CLOCK on sections of IVDs from 3 and 12 months old mice; n=4. Magnification, 5x left and middle panels, 10x right panels. BMAL1 staining was visible in the AF, but not the CEP, of 12 month old mice. CLOCK staining was largely absent in both AF and CEP in 12 month old mice.

Figure S2. Effects of LPS, IL-1RA and, forskolin and TNF α on IVD oscillations. Representative bioluminescence traces of PER2::Luc mouse IVD explants; n=3. (A) LPS treatment (1 μ g/mL, red trace) disrupted the rhythm, which could be rescued by treatment with dexamethasone (100 nM). (B, C) Disrupted circadian rhythm by IL-1 β treatment (5 μ g/mL, red trace) was not rescued by application of forskolin (10 μ M), but by pre-treatment with IL-1RA (1 μ g/mL) could not be rescued by forskolin, a clock synchronising agent. Arrows indicate time of treatment. (D) Both IL-1 β (5 ng/mL) and TNF α (40 ng/mL) induced strong NF κ B signalling in lung epithelial cells stably transfected with NF κ B::luc reporter. Representative, n=3.

Figure S3. Effects of IL-1 β on endogenous clock gene expression. qPCR of several clock genes, *Adams1* and *JL-6* in IVD explants upon IL-1 β treatment (5 ng/mL for 4 hours). *, p<0.05; ***, p<0.001, n=4.

Figure S3S4. Phase clustering analysis of rhythmic genes in mouse IVDs. Clustering analysis was performed using cluster (A) and Rtsne (B) of R package. These analyses revealed 4 main clusters with different peak times (two at night and two during the day). Example genes for each cluster were highlighted. There was a good concordance between these two methods of analysis.

Figure S4S5. Time course qPCR of β -actin in mouse IVDs. Note the lack of circadian rhythms. Mean and SEM (n = 6).

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Figure S6. Early onset of IVD degeneration in IVDs from 6 month old cKO mice. Safranin O/methyl green staining revealed gradual disappearance of CEP in the *Bmal1* cKO mouse (black arrow); n=4.

Figure S7. Spontaneous degeneration of IVDs from aged WT mice. Picrosirius red staining and polarised light microscopy were performed on IVDs from 3 and 24 month old wild type mice; n=4. The aged WT mouse IVDs display a phenotype similar to *Bmal1* cKO IVDs, with fibrosis of the outer AF composed of bundles of organised collagen visible under polarised light microscope.

Figure S8. TUNEL staining of IVDs from 12 months old WT and *Bmal1* cKO mice. Note there were no detectable signs of apoptosis in either WT or cKO IVDs. N=4.

Figure S9. Time course qPCR of catabolic genes in IVDs from 3 months old WT and *Bmal1* cKO mice. Mean and SEM (n = 4). *, p<0.05; **, p<0.01; *, p<0.001.**

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Supplementary table and videos

Supplementary Table 1. List of rhythmic IVD genes with a ~24 hr period.

Supplementary Video 1, Live bioluminescence imaging of the PER2::Luc mouse IVD explants (2 month on the left and 12 month on the right) using high sensitivity EM-CCD camera.

Supplementary Video 2, Live bioluminescence imaging of the PER2::Luc mouse IVD explants treated with IL-1 β (at 48 hr), followed by Dex (at 96 hr).

Supplementary Video 3, Live bioluminescence imaging of the mouse IVD explants from a WT mouse (left) and a *Bmal1* cKO mouse (right).

Supplementary Online Methods

Animals

All animal studies were performed in accordance with the 1986 UK Home Office Animal Procedures Act. Approval was provided by the local ethics committee. Mice were maintained at 20-22°C, with standard rodent chow available *ad libitum* and under 12:12 hr light dark schedule (light on at 7 am; light off at 7 pm). *Bmal1*^{fllox/fllox} mice⁴⁵ were crossed onto a PER2::luc background. The PER2::Luc mice carry the firefly luciferase gene fused in-frame with the 3' end of the *Per2* gene, creating a fusion protein reporter.²¹ *Bmal1*^{fllox/fllox} - PER2::Luc mice were subsequently crossed with *Col2a1*^{cre} mice expressing cre recombinase under the control of the *Col2a1* promoter⁴⁶ to generate cartilage/IVD specific *Bmal1* KO. All mice were bred in-house at the University of Manchester. Genotyping of the *Col2a1-Bmal1*^{-/-} mice was described before.³³

Reagents and antibodies

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7 IL-1 β and TNF α were purchased from R&D, lipopolysaccharides (LPS), BMS-345541,
8 dexamethasone (Dex), Forskolin (FSK) were purchased from Sigma. The following
9 antibodies were used in this study, BMAL1 (mouse monoclonal)⁴⁷ and CLOCK (Abcam
10 ab3517).

11 **Tissue explant cultures, bioluminescence recording and imaging**

12
13 Organotypic IVD tissue explants were prepared as described before.²⁸ Explants were
14 cultured on 0.4- μ m cell culture inserts (Millipore), and bioluminescence was recorded in real
15 time using a LumiCycle apparatus (Actimetrics). Baseline subtraction was carried out using a
16 24-hour moving average. Amplitude was measured at second peak from the start of
17 recording and period was determined from three peaks using LumiCycle analysis software.
18 For temperature entrainment the incubator housing the LumiCycle after 37°C three day initial
19 phase were set to oscillate the temperature from to 35.5°C for 12 hours to 38.5°C for 12
20 hours for four cycles. Another incubator was cycling the temperature in opposite phase.
21 Third incubator was kept at constant 37°C temperature as control.
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24 For live tissue bioluminescence imaging, intervertebral discs of the WT and *Col2a1-Bmal1*^{-/-}
25 mice (on a PER2::Luc background) were imaged using a self-contained Olympus
26 Luminoview LV200 microscope (Olympus) and recorded using a cooled Hamamatsu
27 ImageEM C9100-13 EM-CCD camera.³³ Images were taken every hour for the duration of
28 the experiment and combined in ImageJ.

29 **Time course sample collections, mRNA extraction, RNAsequencing and quantitative** 30 **real-time PCR**

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32 The circadian transcriptome studies in mouse IVD were performed as described before.²⁵
33 Intervertebral discs were obtained from 8-12 weeks old mice kept under 12 hr/12 hr
34 light/darkness conditions. IVDs were collected every 4 hr for 48 hrs, starting at 9 am
35 (zeitgeber time ZT2). 3-4 lumbar discs of the same animal were pooled to obtain sufficient
36 material. The tissues were immediately snap frozen in liquid nitrogen, and then stored at -
37 80°C until mRNA extraction. Tissues were homogenised using a Mikro-Dismembrator S
38 (Satorius Stedim Biotech) with the barrel and ball of the dismembrator pre-cooled in liquid
39 nitrogen. mRNA was extracted using RNeasy micro kit (Qiagen) according to the
40 manufacturer's protocol. Quality and integrity of total RNA samples were assessed using a
41 2100 Bioanalyzer or a 2200 TapeStation (Agilent Technologies) according to the
42 manufacturer's instructions. Thus prepared mRNA was used for RNAseq and qPCR
43 analysis.
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45 For RNA sequencing, RNA-seq libraries were generated using the TruSeq® Stranded
46 mRNA assay (Illumina, Inc.) according to the manufacturer's protocol. Briefly, total RNA (0.1-
47 4 μ g) was used as input material from which polyadenylated mRNA was purified using poly-
48 T, oligo-attached, magnetic beads. The mRNA was then fragmented using divalent cations
49 under elevated temperature and then reverse transcribed into first strand cDNA using
50 random primers. Second strand cDNA was then synthesised using DNA Polymerase I and
51 RNase H. Following a single 'A' base addition, adapters were ligated to the cDNA fragments,
52 and the products then purified and enriched by PCR to create the final cDNA library. Adapter
53 indices were used to multiplex libraries, which were pooled prior to cluster generation using
54 a cBot instrument. The loaded flow-cell was then paired-end sequenced (101 + 101 cycles,
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7 plus indices) on an Illumina NextSeq instrument. Demultiplexing of the output data (allowing
8 one mismatch) and BCL-to-Fastq conversion was performed with CASAVA 1.8.3.
9 101bp×101bp paired-end reads were generated from each sample. Up to 82M total reads
10 were obtained in each sample.

11 For qPCR, RNA concentrations were determined using NanoDrop 2000 (Thermo Scientific),
12 equal amounts of RNA were converted to cDNA using the High Capacity cDNA Reverse
13 Transcription (RT) Kit (Applied Biosystems). Taqman based qPCR was carried out using a
14 StepOne Plus Real-Time PCR System (Applied Biosystems) with Fast Blue qPCR
15 MasterMix (Eurogentec). Taqman primers and probes were purchased from Applied
16 Biosystems. Gene names and probe IDs are as follows, *Gapdh*: Mm99999915_g1; *Arntl*,
17 Mm00500226_m1; *Per2*, Mm00478113_m1; *Dbp*, Mm01194021_m1; *Follistatin*,
18 Mm00514982_m1; *Timp4*, Mm01184417_m1.
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20 21 **Bioinformatic analysis of the RNAseq data**

22 The fastq files were analysed with FastQC and any low quality reads and contaminated
23 barcodes were trimmed with Trimmomatic. All libraries were aligned to GRCm38.p2
24 assembly of mouse genome using Tophat-2.1.0 and only matches with the best score were
25 reported for each read. The mapped reads were counted by genes with HTSeq against
26 gencode.vM2.annotation.gtf. Genes with very low expressed (with average read across all
27 time point <10) were filtered out. Time-dependent genes were identified by JTKCycle.²⁹ The
28 rhythmic genes with a 24 hr period and an adjusted p value less than 0.05 were selected for
29 further validation. GO analysis of the JTK_CYCLE identified circadian genes was performed
30 using topGO of the R package.⁴⁸ These genes were also clustered with cluster and Rtsne of
31 the R package.⁴⁹ Raw data were deposited in EMBL-EBI Array Express (accession number
32 pending).
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34 35 **Human tissues and cells and lentiviral transduction**

36 Adult human IVD specimens were obtained with informed consent from the Intervertebral
37 Disc Tissue Bank at the University of Manchester, using surgical specimens from patients
38 undergoing disc surgery for treatment of disc herniation or IVD degeneration, in accordance
39 with local ethical committee approval. Tissue was processed for cell extraction and
40 representative samples of all tissues containing intact AF and NP regions were formalin-
41 fixed, paraffin-embedded and sections histologically graded as previously reported⁵⁰ with
42 samples graded as follows: non-degenerate (grade 0-4); mildly degenerate (grade 5-7); and
43 severely degenerate (grade 8-12). Only low grade samples (grade 0-2) were used in this
44 study. NP tissue from each sample was macroscopically dissected from AF, and finely
45 minced prior to enzymatic digestion in a solution of 0.1% (w/v) type II collagenase and 0.1%
46 (w/v) hyaluronidase in serum-free DMEM overnight at 37°C with agitation. Isolated cells
47 were cultured in DMEM supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate,
48 10,000U/ml penicillin, 10mg/ml streptomycin, 25µg/ml amphotericin B and 1mM ascorbate
49 under standard conditions (37°C, 21% O₂, 5% CO₂). Cells were then expanded in monolayer
50 and used at passage <2. Grade 2 cells were used for *Per2::luc* lentiviral transduction and
51 bioluminescence photon counting.
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Lentiviral transduction of primary human NP cells was performed using methods previously described.²⁵ Briefly, lentiviral particles containing a *Per2::luc* reporter were produced in HEK 293T packaging cells and used to transduce the human NP cells. Cells were then synchronized with forskolin before lumicycle recording.

Histology and immunohistochemistry

Mouse spines were dissected and fixed in PBS 4% paraformaldehyde solution followed by decalcification in 20% EDTA pH 7.4. Decalcified tissues were processed and embedded in paraffin. Frontally embedded lumbar spine paraffin blocks were sectioned on a microtome to 5 μ m thickness, 3-4 sections per slide. Each block yielded 40-50 slides. Every 5th slide was stained with Safranin O and the sections were examined for the presence of disc degeneration. H&E, picrosirius red and Safranin O staining were performed according to standard protocols. Safranin O stained sections were imaged using Zeiss Observer D1 Axiocam 105 color camera and measurements of disc height and growth plate thickness were performed in ImageJ. Picrosirius red stained slides were imaged under brightfield or polarized light. The latter allows us to incorporate the birefringent properties of fibrillar collagen, in order to visualize the more organized collagen molecules.

IHC was performed using DAB staining method as described previously.²⁵ Briefly, the slides were deparaffinised and rehydrated. Antigen retrieval was performed using 1mg/mL Trypsin in PBS (Sigma) digestion for 10 min. Slides were washed and blocked with blocking solution (3% donkey serum in PBS TritonX 0.1%) for 1 h at room temperature. The slides were then incubated with primary antibody diluted in blocking solution overnight at 4°C. Subsequently slides were washed and incubated with fluorescent secondary antibody diluted in blocking solution for 1 h at 4°C. One of the sections on each slide was used as no primary antibody control.

Live imaging of p65-DsRed

Intervertebral discs from p65-DsRed mouse were cut in half to expose both the AF and NP tissue and embedded in the Matrigel matrix (BD Biosciences) in 35-mm glass bottom Cellview dishes (Greiner Bio-one). Images were acquired with a Zeiss LSM 780 Confocal Inverted Microscope in a humidified CO₂ incubator (at 37°C, 5% CO₂) with a C-Apochromat 40 \times /1.2 W Korr objective. During imaging tissue was treated with IL-1 β (5–20 ng/mL) or TNF α (up to 40 ng/mL). DsRedXP tagged p65 was visualized by excitation with a green helium neon laser (543 nm) and detection through both a 545-nm dichroic mirror and a 560-nm long pass filter. Data capture was performed using ZEN2010B software (Zeiss).

Statistical analysis

Data were evaluated using Two-tailed Student's *t*-test, two way ANOVA or non-parametric, two-tailed Mann-Whitney test. Results were presented as mean \pm SEM from at least three independent experiments. Differences were considered significant at the values of **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Funding

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Supplementary Online Methods

Animals

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IL-1 β and TNF α were purchased from R&D, lipopolysaccharides (LPS), BMS-345541, dexamethasone (Dex), Forskolin (FSK) were purchased from Sigma. The following antibodies were used in this study, BMAL1 (mouse monoclonal)⁴⁷ and CLOCK (Abcam ab3517).

Tissue explant cultures, bioluminescence recording and imaging

Organotypic IVD tissue explants were prepared as described before.²⁸ Explants were cultured on 0.4- μ m cell culture inserts (Millipore), and bioluminescence was recorded in real time using a LumiCycle apparatus (Actimetrics). Baseline subtraction was carried out using a 24-hour moving average. Amplitude was measured at second peak from the start of recording and period was determined from three peaks using LumiCycle analysis software. For temperature entrainment the incubator housing the LumiCycle after 37°C three day initial phase were set to oscillate the temperature from to 35.5°C for 12 hours to 38.5°C for 12 hours for four cycles. Another incubator was cycling the temperature in opposite phase. Third incubator was kept at constant 37°C temperature as control.

For live tissue bioluminescence imaging, intervertebral discs of the WT and *Col2a1-Bmal1^{-/-}* mice (on a PER2::Luc background) were imaged using a self-contained Olympus Luminoview LV200 microscope (Olympus) and recorded using a cooled Hamamatsu ImageEM C9100-13 EM-CCD camera.³³ Images were taken every hour for the duration of the experiment and combined in ImageJ.

Time course sample collections, mRNA extraction, RNAsequencing and quantitative real-time PCR

The circadian transcriptome studies in mouse IVD were performed as described before.²⁵ Intervertebral discs were obtained from 8-12 weeks old mice kept under 12 hr/12 hr light/darkness conditions. IVDs were collected every 4 hr for 48 hrs, starting at 9 am (zeitgeber time ZT2). 3-4 lumbar discs of the same animal were pooled to obtain sufficient material. The tissues were immediately snap frozen in liquid nitrogen, and then stored at -80°C until mRNA extraction. Tissues were homogenised using a Mikro-Dismembrator S (Satorius Stedim Biotech) with the barrel and ball of the dismembrator pre-cooled in liquid

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3 nitrogen. mRNA was extracted using RNeasy micro kit (Qiagen) according to the
4 manufacturer's protocol. Quality and integrity of total RNA samples were assessed using a
5 2100 Bioanalyzer or a 2200 TapeStation (Agilent Technologies) according to the
6 manufacturer's instructions. Thus prepared mRNA was used for RNAseq and qPCR
7 analysis.
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10 For RNA sequencing, RNA-seq libraries were generated using the TruSeq® Stranded mRNA
11 assay (Illumina, Inc.) according to the manufacturer's protocol. Briefly, total RNA (0.1-4 µg)
12 was used as input material from which polyadenylated mRNA was purified using poly-T,
13 oligo-attached, magnetic beads. The mRNA was then fragmented using divalent cations
14 under elevated temperature and then reverse transcribed into first strand cDNA using
15 random primers. Second strand cDNA was then synthesised using DNA Polymerase I and
16 RNase H. Following a single 'A' base addition, adapters were ligated to the cDNA fragments,
17 and the products then purified and enriched by PCR to create the final cDNA library. Adapter
18 indices were used to multiplex libraries, which were pooled prior to cluster generation using a
19 cBot instrument. The loaded flow-cell was then paired-end sequenced (101 + 101 cycles,
20 plus indices) on an Illumina NextSeq instrument. Demultiplexing of the output data (allowing
21 one mismatch) and BCL-to-Fastq conversion was performed with CASAVA 1.8.3.
22 101bp×101bp paired-end reads were generated from each sample. Up to 82M total reads
23 were obtained in each sample.
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26 For qPCR, RNA concentrations were determined using NanoDrop 2000 (Thermo Scientific),
27 equal amounts of RNA were converted to cDNA using the High Capacity cDNA Reverse
28 Transcription (RT) Kit (Applied Biosystems). Taqman based qPCR was carried out using a
29 StepOne Plus Real-Time PCR System (Applied Biosystems) with Fast Blue qPCR MasterMix
30 (Eurogentec). Taqman primers and probes were purchased from Applied Biosystems. Gene
31 names and probe IDs are as follows, *Gapdh*: Mm99999915_g1; *Arntl*, Mm00500226_m1;
32 *Per2*, Mm00478113_m1; *Dbp*, Mm01194021_m1; *Follistatin*, Mm00514982_m1; *Timp4*,
33 Mm01184417_m1.
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38 **Bioinformatic analysis of the RNAseq data**

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40 The fastq files were analysed with FastQC and any low quality reads and contaminated
41 barcodes were trimmed with Trimmomatic. All libraries were aligned to GRCm38.p2
42 assembly of mouse genome using Tophat-2.1.0 and only matches with the best score were
43 reported for each read. The mapped reads were counted by genes with HTSeq against
44 gencode.vM2.annotation.gtf. Genes with very low expressed (with average read across all
45 time point <10) were filtered out. Time-dependent genes were identified by JTKCycle.²⁹ The
46 rhythmic genes with a 24 hr period and an adjusted p value less than 0.05 were selected for
47 further validation. GO analysis of the JTK_CYCLE identified circadian genes was performed
48 using topGO of the R package.⁴⁸ These genes were also clustered with cluster and Rtsne of
49 the R package.⁴⁹ Raw data were deposited in EMBL-EBI Array Express (accession number
50 pending).
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53 **Human tissues and cells and lentiviral transduction**

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55 Adult human IVD specimens were obtained with informed consent from the Intervertebral
56 Disc Tissue Bank at the University of Manchester, using surgical specimens from patients
57 undergoing disc surgery for treatment of disc herniation or IVD degeneration, in accordance
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3 with local ethical committee approval. Tissue was processed for cell extraction and
4 representative samples of all tissues containing intact AF and NP regions were formalin-
5 fixed, paraffin-embedded and sections histologically graded as previously reported⁵⁰ with
6 samples graded as follows: non-degenerate (grade 0-4); mildly degenerate (grade 5-7); and
7 severely degenerate (grade 8-12). Only low grade samples (grade 0-2) were used in this
8 study. NP tissue from each sample was macroscopically dissected from AF, and finely
9 minced prior to enzymatic digestion in a solution of 0.1% (w/v) type II collagenase and 0.1%
10 (w/v) hyaluronidase in serum-free DMEM overnight at 37°C with agitation. Isolated cells were
11 cultured in DMEM supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 10,000U/ml
12 penicillin, 10mg/ml streptomycin, 25µg/ml amphotericin B and 1mM ascorbate under
13 standard conditions (37°C, 21% O₂, 5% CO₂). Cells were then expanded in monolayer and
14 used at passage <2. Grade 2 cells were used for *Per2::luc* lentiviral transduction and
15 bioluminescence photon counting.
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20 Lentiviral transduction of primary human NP cells was performed using methods previously
21 described.²⁵ Briefly, lentiviral particles containing a *Per2::luc* reporter were produced in HEK
22 293T packaging cells and used to transduce the human NP cells. Cells were then
23 synchronized with forskolin before lumicycle recording.
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27 **Histology and immunohistochemistry**

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29 Mouse spines were dissected and fixed in PBS 4% paraformaldehyde solution followed by
30 decalcification in 20% EDTA pH 7.4. Decalcified tissues were processed and embedded in
31 paraffin. Frontally embedded lumbar spine paraffin blocks were sectioned on a microtome to
32 5 µm thickness, 3-4 sections per slide. Each block yielded 40-50 slides. Every 5th slide was
33 stained with Saffranin O and the sections were examined for the presence of disc
34 degeneration. H&E, picosirius red and Safranin O staining were performed according to
35 standard protocols. Safranin O stained sections were imaged using Zeiss Observer D1
36 Axiocam 105 color camera and measurements of disc height and growth plate thickness
37 were performed in ImageJ. Picosirius red stained slides were imaged under brightfield or
38 polarized light. The latter allows us to incorporate the birefringent properties of fibrillar
39 collagen, in order to visualize the more organized collagen molecules.
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42 IHC was performed using DAB staining method as described previously.²⁵ Briefly, the slides
43 were deparaffinised and rehydrated. Antigen retrieval was performed using 1mg/mL Trypsin
44 in PBS (Sigma) digestion for 10 min. Slides were washed and blocked with blocking solution
45 (3% donkey serum in PBS TritonX 0.1%) for 1 h at room temperature. The slides were then
46 incubated with primary antibody diluted in blocking solution overnight at 4°C. Subsequently
47 slides were washed and incubated with fluorescent secondary antibody diluted in blocking
48 solution for 1 h at 4°C. One of the sections on each slide was used as no primary antibody
49 control.
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53 **Live imaging of p65-DsRed**

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55 Intervertebral discs from p65-DsRed mouse were cut in half to expose both the AF and NP
56 tissue and embedded in the Matrigel matrix (BD Biosciences) in 35-mm glass bottom
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3 Cellview dishes (Greiner Bio-one). Images were acquired with a Zeiss LSM 780 Confocal
4 Inverted Microscope in a humidified CO₂ incubator (at 37°C, 5% CO₂) with a C-Apochromat
5 40×/1.2 W Korr objective. During imaging tissue was treated with IL-1β (5–20 ng/mL) or
6 TNFα (up to 40 ng/mL). DsRedXP tagged p65 was visualized by excitation with a green
7 helium neon laser (543 nm) and detection through both a 545-nm dichroic mirror and a 560-
8 nm long pass filter. Data capture was performed using ZEN2010B software (Zeiss).
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10 11 12 **Statistical analysis**

13 Data were evaluated using Two-tailed Student's *t*-test, two way ANOVA or non-parametric,
14 two-tailed Mann-Whitney test. Results were presented as mean ± SEM from at least three
15 independent experiments. Differences were considered significant at the values of **P* < 0.05,
16 ***P* < 0.01 and ****P* < 0.001.
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