

1 **TITLE:** Calcium calmodulin kinase II activity is required for cartilage homeostasis in
2 osteoarthritis

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27 **Running head:** Role of CaMKII in osteoarthritis

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34 **ABSTRACT:**

35 WNT ligands can activate several signalling cascades of pivotal importance during
36 development and regenerative processes. Their de-regulation has been associated with the
37 onset of different diseases. Here we investigated the role of the Wnt/Calcium Calmodulin
38 Kinase II (CaMKII) pathway in osteoarthritis. We identified Heme Oxygenase I (HMOX-1) and
39 Sox-9 as specific markers of the WNT/CaMKII signalling in articular chondrocytes through a
40 microarray analysis. We showed that the expression of the activated form of CaMKII, phospho-
41 CaMKII, was increased in human and murine osteoarthritis and the expression of HMOX-1
42 was accordingly reduced, demonstrating the activation of the pathway during disease
43 progression. To elucidate its function, we administered the CaMKII inhibitor KN93 to mice in
44 which osteoarthritis was induced by resection of the anterior horn of the medial meniscus and
45 of the medial collateral ligament in the knee joint. Pharmacological blockade of CaMKII
46 exacerbated cartilage damage and bone remodelling. Finally, we showed that CaMKII
47 inhibition in articular chondrocytes upregulated the expression of matrix remodelling enzymes
48 alone and in combination with Interleukin 1. These results suggest an important homeostatic
49 role of the WNT/CaMKII signalling in osteoarthritis which could be exploited in the future for
50 therapeutic purposes.

51 **Key words:** Osteoarthritis, WNT, Calcium Calmodulin Kinase II, cartilage

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63 Introduction

64 Osteoarthritis (OA) is a joint disease driven by pathological biomechanics and characterized
65 by cartilage breakdown, subchondral bone sclerosis, osteophytosis, and inconstant low
66 degree inflammation¹. OA is a leading cause of chronic disability worldwide: patients suffer
67 from joint pain and impaired motility, which reduces their independence ². No therapeutic
68 treatment other than symptomatic relief is currently available, leaving joint replacement
69 surgery as the ultimate option.

70 OA progression is driven by an imbalance between cartilage degradation and reparative,
71 homeostatic responses. Pathological biomechanical stimuli activate signalling pathways
72 including interleukin 1 beta (IL1B)³ and the WNT pathway⁴, which in turn upregulate matrix
73 metalloproteases (MMPs) and A Disintegrin and Metalloproteinase with Thrombospondin
74 motifs (ADAMTS) aggrecanases, which contribute to the degradation of the cartilage
75 extracellular matrix⁵.

76 Excessive activation of the WNT signalling pathway has been linked to the onset and
77 progression of cartilage degeneration in OA, both in human and in mice⁶⁻⁹. WNTs are a family
78 of 19 highly conserved morphogens capable of activating multiple signalling cascades. The
79 most characterised one is the WNT beta-catenin-dependent pathway. The WNT/beta-catenin
80 pathway (also known as canonical WNT pathway) is activated by the interaction of WNT
81 ligands with Frizzled (FZDs) and Lipoprotein-related Protein Receptor 5 and 6 (LRP5/6) which
82 leads to the intracellular accumulation of beta-catenin and its translocation to the nucleus.
83 Within the nucleus, beta-catenin interacts with T-cell factor/lymphoid enhancer-binding factor
84 (TCF/LEF) transcription factors promoting the transcription of target genes such as *AXIN2*¹⁰.
85 Other, so called “non-canonical” WNT pathways, are activated by the interaction of WNT
86 ligands with FZDs and other co-receptors such as Receptor Tyrosine Kinase (RYK) and
87 Retinoic Acid-Related Orphan Receptor (RORs), driving the activation of downstream
88 signalling cascades activated by intracellular calcium release and/or associated with
89 intercellular communication to coordinate polarity between adjacent cells¹¹.

90 Ca²⁺Calmodulin-dependent Kinase II (CaMKII) is an intracellular kinase that can be activated
91 by several stimuli including WNTs¹² and inflammatory stimuli^{13,14}. CaMKII is a Ser/Thr kinase
92 expressed in mammals in 4 isoforms, alpha, beta, gamma and delta¹⁵. Binding of
93 Ca²⁺/Calmodulin to CaMKII transiently activates it. Once activated, CaMKII phosphorylates its
94 substrates, including itself. The autophosphorylation of CaMKII in T287 results in its
95 constitutive activation, even in the absence of further stimulation¹⁶. WNT-induced CaMKII
96 activity regulates chondrocyte differentiation¹².

97 We showed that a single WNT ligand could simultaneously activate the beta catenin-
98 dependent and the CaMKII-dependent WNT pathways with distinct biological outcomes¹².

99 These data suggest an important and still uncharacterised role for the WNT/CaMKII pathway
100 in the maintenance of cartilage homeostasis. To test this hypothesis, the current study
101 investigates the role of the WNT/CaMKII pathway in the context of osteoarthritis.

102 **Methods**

103 **Patients**

104 Preserved (Mankin score ≤ 4 ¹⁷) human articular cartilage samples were obtained from the
105 femoral condyles removed during total knee replacement for osteoarthritis following informed
106 consent (4 male and 2 female patients aged between 51 and 76). All procedures were
107 approved by the East London and The City Research Ethics Committee 3 (ethics approval
108 REC N. 07/Q0605/29). Full thickness explants were prepared for histology, and chondrocytes
109 were isolated and expanded *in-vitro* as previously described¹². Normal cartilage samples were
110 retrieved from 3 patients (one 58 years-old, and two males aged respectively 14 and 16)
111 undergone lower limb amputation because of malignant bone tumours. The samples were
112 obtained upon favourable opinion of the Ethics Committee of the Ärztekammer Schleswig-
113 Holstein, Bad Segeberg, Germany. We performed all procedures in accordance to relevant
114 guidelines and regulations.

115

116 **Primary cells, cell lines and tissues**

117 Bovine chondrocytes were isolated from metatarsal joints obtained from a local abattoir within
118 24 hours from death. Experiments were performed when primary chondrocytes reached 80%
119 confluence (passage 0, P0) as previously described¹⁸. C3H/10T1/2 cells (ATCC) were cultured
120 according to the manufacturer's instructions.

121 Murine femoral heads and patellae were explanted from 1 month-old male 129/Sv mice and
122 processed for gene expression analysis (see below).

123 If not differently indicated, cells were stimulated in complete medium (DMEM/F-12 1:1 plus
124 GlutaMax, 10% FBS, 1 mM sodium pyruvate , and 2% antibiotic antimycotic solution
125 ThermoFisher Scientific) supplemented with human recombinant WNT3A (100ng/ml; R&D),
126 DKK1 (100ng/ml, R&D), IL1B (20ng/ml; InvivoGen), KN93 or its inactive control KN92 (10 μ M;
127 EMD Millipore), Autocamide-2 related inhibitory peptide II, AIP, 5 μ M; Calbiochem) or
128 respective vehicles as indicated.

129

130 **siRNA preparation and transfection**

131 Small interfering RNA for mouse CAMKII γ was prepared using the Silencer siRNA
132 Construction Kit (Life Technologies) following manufacturer's instructions. siCaMKII γ S:

133 AAGGGTTTAAGGGTTTCACTACCTCGTGCCTGTCTC; AS:
134 AAACACGAAAACACGAGGTAGTGAAACCCCCTGTCTC; scramble siRNA, S:
135 AATTCCGCAATTCCGCTCTGGACTGTAGTCCTGTCTC; AS:
136 AAGAACTTAAGAACTTGATCAACCAGATGCCTGTCTC. The siRNA were used at 20nM and
137 were transfected in C3H/10T1/2 cells using JetPRIME transfection reagent (Polyplus) as
138 described in¹⁸.

139

140 **RNA isolation and quantitative real time PCR**

141 RNA isolation and PCR were performed as previously described¹². For a complete list of
142 primers and conditions refers to Supplementary Table 1.

143

144 **Microarray analysis:**

145 Total RNA was quantified using a Nanodrop™ ND-1000 Spectrophotometer and assessed for
146 quality (QC) using the Agilent™ 2100 Bioanalyzer Pico kit. Five nanograms of total RNA with
147 RNA integrity numbers (RINs) higher than 9 were selected for the microarray study. A Poly-A
148 Spiking Control (Life Technologies) was used as amplification control.

149 Single primer isothermal amplification (SPIA) technology was used to generate cDNA using
150 NuGen Ovation Pico WTA System V2 kit, following manufacturer's instructions. The SPIA
151 cDNA was subjected to QC, fragmented and Biotin-labelled using the NuGen Encore Biotin
152 Module according to the manufacturer's instructions. The processed cDNA was subjected to
153 a further round of QC to assess fragmentation size (Agilent 2100 Bioanalyzer Nano kit;
154 fragment size < 200nt).

155 Hybridization cocktails were prepared according to Nugen's recommendations for Human
156 Gene 2.0 ST arrays and hybridization took place at 45°C for 16-20 hours in an Affymetrix
157 Genechip Hybridization oven 645.

158 The arrays were washed and stained using wash protocol FS450_0002 as recommended by
159 Affymetrix on the GeneChip Fluidics station 450. The arrays were scanned using the
160 Affymetrix GeneChip Scanner. CEL files were QC checked using the Expression Console
161 software package (Affymetrix, Thermo Fisher) by using standard metrics and guidelines for
162 the Affymetrix microarray system. Data were normalised together using the Robust Multi-array
163 Average (RMA) sketch algorithm.

164 The processing of all sample files was performed using R Programming Language (R). In
165 brief, raw data was inputted using the 'read.celfiles' function of the 'oligo' R package¹⁹, with
166 data then undergoing Robust Multiarray Average (RMA) normalisation using the 'rma' function.
167 For cross-sample comparisons, the study design model was first created by identifying sample
168 groupings based on KN92, KN92-WNT3a, and KN93-WNT3a status.

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170

171 **Immunohistochemical analysis**

172 CaMKII isoforms and phosphoCaMKII (pCaMKII) were detected by indirect
173 immunofluorescence as described in¹². For a list of retrieval methods, antibodies and dilutions
174 please refer to Supplementary Table 2. All images were acquired using identical settings on
175 an Olympus BX61 microscope and consistently modified for best rendering using Adobe
176 Photoshop. The fluorescent and dapi images of the same field were superimposed, or the
177 fluorescent and Nomarski images of the same field were inverted and superimposed
178 (subtraction).

179

180 **Instability-induced OA in mice**

181 All animal procedures were approved by the UK Home Office (PPL 70-7986). All the
182 experimental procedures were performed in accordance to relevant guidelines and
183 regulations. Mice were kept in an approved animal care facility, were housed 4 or 5 per cage
184 in standard cages and fed ad libitum. Ten week old, male C57BL/6 mice (Charles River) were
185 subjected to resection of the medial collateral ligament and of the anterior horn of the medial
186 meniscus (menisco-ligament injury – MLI) as previously described²⁰. In each cage, the animals
187 were randomized to receive either PBS or KN93 (see Supplementary Table 3). The
188 contralateral knee underwent sham surgery (arthrotomy but no damage to ligaments or
189 menisci). The joint capsule was sutured with Vicryl 6-0 and the skin with Ethylon 5-0 sutures
190 with atraumatic needles (Ethicon).

191

192 **Pharmacological blockade of CaMKII**

193 Four weeks after surgery the mice were randomized to receive either the water-soluble version
194 of the CaMKII inhibitor KN93 or PBS as control (15 animals per group). For the first 3 days
195 KN93 (10 μ mol/Kg/day) or PBS were administered by intraperitoneal injection. Subsequently,
196 ALZET[®] osmotic minipumps (Charles River Laboratories) were inserted subcutaneously on
197 the back of the mice allowing a continuous release of KN93 (5 μ mol/Kg/day) or PBS for
198 additional 28 days. The pumps were changed once during this time. The animals were then
199 killed by cervical dislocation.

200

201 **Histology and OA scoring**

202 Knee joints were dissected, the majority of the soft tissue was removed, and the joints were
203 fixed in 70% ethanol following which they were decalcified in formic acid, embedded in paraffin
204 and sectioned as described in⁷. The sections were stained with Safranin O (SO) (0.1%, pH4).
205 Images were taken using the same settings on an Olympus BX61 microscope and consistently
206 modified for best rendering using GIMP software. The extent of cartilage degradation was

207 scored by two blinded, independent investigators using the Osteoarthritis Research Society
208 International (OARSI) scoring system²¹. At least 5 sections/knee were scored.

209

210 **Histomorphometry**

211 Proteoglycan loss, osteophyte size and differentiation, and subchondral bone thickness were
212 measured by histomorphometry using ImageJ²². The mean values from at least 3, but on
213 average 5 sections from each knee (1 section every 50µm, spanning the entire depth of the
214 joint) were calculated and used for statistical analysis.

215 Proteoglycan loss was analysed by densitometry of the articular cartilage as previously
216 described⁷. In brief, images were rotated so the cartilage-bone junction in the middle of the
217 plateau was horizontal. This is important so that non-cartilage staining in the bone marrow
218 spaces can be eliminated. The tibial plateau was selected to include the growth plate, any
219 osteophytes and up to the end of the cartilage; and selections from every section were placed
220 on one canvas, so they were perfectly aligned horizontally. To isolate the metachromatic
221 Safranin O staining from the background and most of the orthochromatic staining (subchondral
222 bone), the RGB image was transformed into an HSB stack. On the 'Saturation' slice, a
223 rectangle 600microns wide and the full height of the canvas was placed over the first section
224 to include the tibial plateau, making sure to exclude any osteophytes and the "bulgy" part near
225 the intercondylar notch. Ctrl+1 was pressed, and then the rectangle was moved laterally to the
226 next section and Ctrl+2 was pressed. This was repeated until all sections were selected and
227 then Ctrl+3 plotted the density profile of each section. The first peak is the articular cartilage.
228 A horizontal line was drawn to cut residual background if any, and a lateral line to limit the
229 articular cartilage peak and eliminate any staining of bone marrow spaces. Using the wand
230 tool to select the inside of the density profile returned the area. These results were copied
231 from the measurements box to a statistics programme for further analysis.

232 Osteophytes consistently developed only on the medial tibial compartment of the operated
233 joints. On individual sections, osteophyte size was determined by selecting the osteophyte
234 with the polygon tool, and ensuring a global scale had been set, measuring the area.
235 Osteophyte differentiation was assessed by comparing the intensity of SO staining with the
236 staining of the growth plate, which was set as the threshold for comparison in Image J and
237 defined as the staining for fully differentiated cartilage. The percentage of the thresholded area
238 in each individual section was then normalised for the area of the osteophyte. The points in
239 the graph represent the average of the measurements for multiple sections (at least 3/mouse).
240 Subchondral bone thickness was determined by selecting the subchondral area underneath
241 the load-bearing area of the articular cartilage of the tibiae as described by Botter et al²³.

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243

244 **Micro-CT scanning**

245 Tibia were fixed in 70% ethanol and scanned with Skyscan 1174 Micro-CT scanner (Skyscan,
246 Antwerp, Belgium) at 53kV, 785 μ A, 0.7° rotation and 12.57 μ M isometric voxel resolution. The
247 individual slices were reconstructed using NRecon software (Skyscan) with an algorithm that
248 included maximum ring artefact correction, 70% beam-hardening correction and misalignment
249 compensation.

250

251 **Micro-CT analysis, Tibiae epiphyseal volume measurement**

252 The reconstructed scan was rotated and resliced using ImageJ²² in a coronal orientation.
253 Using CTAn software (Skyscan), the tibial epiphysis was reconstructed as a volume of interest
254 (VOI) by manually selecting the epiphysis on every 10th slice for the entire stack of images. A
255 threshold of 90 density units was set to distinguish mineralised from non-mineralised tissue
256 within the trabeculae.

257

258 **Statistical analysis**

259 Parametric data were compared with the t test or Anova with Tukey-Post test for multiple
260 comparisons. When possible, data transformation was applied to satisfy the assumptions of
261 parametric tests as described²⁴. Non-parametric data were analysed using the Wilcoxon–
262 Mann–Whitney test. p Values <0.05 were considered significant: *p<0.05; **p<0.01;
263 ***p<0.001. For the analysis of the microarray experiment, individual comparisons within the
264 study design model were conducted by fitting a linear model independently for each probe,
265 with group as the y variable, using 'lfit' ('limma' R package)²⁵. The linear fit for each
266 comparison was subsequently modified using the empirical Bayes ('eBayes') approach, which
267 aims to bring the probe-wise variances across samples to common values, resulting in
268 modified t-statistics, F-statistic, and log odds differential expression ratios. Finally, for each
269 comparison, log₂ fold-change (logFC), P value, and corrected P value (false discovery rate,
270 FDR) was output. A cut-off of 10% FDR was used. Volcano plots were generated using
271 'EnhancedVolcano' ('EnhancedVolcano' R package)²⁶.

272

273 **Results:**

274 **Transcriptional signature of the Wnt3a/CAMKII pathway in the articular chondrocytes**

275 We reported that WNT3A can activate both the Wnt/beta-catenin and the WNT/CaMKII
276 pathways in articular chondrocytes with distinct, dose-dependent biological outcomes¹². This
277 implies that the modulation of some transcriptional targets of WNT3A will be due to activation
278 of the WNT/beta-catenin-dependent pathway and others due to the activation of the CaMKII
279 pathway. To elucidate the specific transcriptional signature of the CaMKII pathway activation
280 by WNT3A, we performed an expression microarray analysis of human articular chondrocytes

281 treated with WNT3A in presence of KN93 or of its inactive control compound KN92 (Fig 1A-
282 C). We proposed that genes that are activated in a WNT/CaMKII-dependent manner will be
283 upregulated by WNT3A, but only in the absence of the CaMKII inhibitor KN93.

284 This analysis revealed that 44 genes were modulated by WNT3A in the absence of CaMKII
285 inhibitor (Fig. 1D), but only 3 were modulated by WNT3A in a CaMKII-dependent manner
286 (Fig1. D). The 3 genes modulated by WNT3A in a CaMKII-dependent manner included SRY-
287 Box Transcription Factor 9 (*SOX9*) (upregulated), the uncharacterized transcript *RP11-
288 474G23.3* (downregulated) and Heme-Oxygenase 1 (*HMOX-1*) (downregulated) mRNA. The
289 upregulation of *SOX9* confirmed our previous results¹². The downregulation of *HMOX-1* mRNA
290 in response to WNT/CaMKII activation was confirmed at qPCR level in human articular
291 chondrocytes (Fig. 1E). Consistent with the requirement for CaMKII activation, KN93
292 effectively rescued the downregulation of *HMOX1* induced by WNT-3A and significantly
293 upregulated its expression of this gene without exogenous WNT3A.

294 We previously showed that the WNT/beta-catenin pathway antagonizes the WNT/CaMKII
295 pathway in chondrocytes and that, therefore, its suppression using DKK1 results in hyper-
296 activation of the CaMKII-dependent targets¹². In keeping with this model, treatment of
297 chondrocytes with DKK1 alone decreased the expression of *HMOX-1* (Fig. 1F) and did not
298 rescue the downregulation of *HMOX-1* induced by WNT3A, confirming that *HMOX-1* is a
299 negative target of the WNT3A/CaMKII pathway but not of the beta-catenin-dependent WNT
300 pathway (Fig. 1F).

301 **Activated CaMKII and HMOX1 expression are inversely correlated in osteoarthritis.**

302 We next sought to confirm our *in vitro* findings in human cartilage and human OA pathology.
303 We mined transcriptomics data in publicly available OA cartilage databases²⁷. *CaMKII γ* and –
304 δ RNA were the most expressed *CaMKII* isoforms at mRNA levels in normal as well as in
305 osteoarthritic cartilage (Fig. 2C). A similar expression pattern was detected in a previously
306 reported expression microarray in human cartilage explants⁶ (Fig. 2A) and in cultured human
307 chondrocytes through *in vitro* culture (Fig.2B). Confirming a previous report²⁸, CaMKII
308 phosphorylation was increased in osteoarthritic cartilage as assessed with immunostaining
309 with an antibody directed to the T287 residue (Fig. 2D). In keeping with our *in vitro* data
310 showing that *HMOX-1* is a negative CaMKII target, *HMOX-1* expression decreased in cartilage
311 isolated from OA patients (Fig. 2E). Although *HMOX-1* expression can be modulated by
312 several cell stressors, our data suggest that the WNT/CaMKII pathway is activated in
313 osteoarthritic cartilage.

314 *CaMKII γ* and – δ were the most expressed isoforms in cartilage isolated from murine femoral
315 heads and patellae (Fig3. A-B), as previously seen in human cartilage. However, the different

316 CaMKII isoforms displayed a tissue specific-expression pattern in the murine joint. CaMKII γ
317 was expressed in the most superficial layer of the articular cartilage, with some limited
318 expression in the menisci (Fig3. H-J). CaMKII β and δ were also detectable in the surrounding
319 soft tissues and in the bone marrow (Fig.3 C-G and K-O). To further validate a direct
320 dependence of *HMOX-1* downstream the activation of CaMKII, we silenced *CaMKII γ* in the
321 murine fibroblast cell line C3H/10T1/2 by siRNA (Fig 3P-Q). Silencing *CaMKII γ* did not result
322 in compensation from the other isoforms (Fig. 3P) and was sufficient to significantly upregulate
323 *HMOX-1* mRNA in these cells (Fig. 3Q).

324 **Pharmacological inhibition of CaMKII promotes cartilage degeneration and** 325 **subchondral bone remodelling in a murine model of OA.**

326 To investigate if CaMKII inhibition affects the outcome of OA, we induced OA in adult mice by
327 meniscus-ligament injury (MLI)^{20,29} and 4 weeks later we inhibited CaMKII by systemic
328 administration of the CaMKII inhibitor KN93 or PBS as control for additional 4 weeks (Fig. 4A).
329 The weight and general health status of the animals was monitored throughout the entire
330 length of the stimulation and no significant difference between vehicle-treated vs inhibitor-
331 treated animals was noted (Supplementary Table 3). CaMKII phosphorylation was effectively
332 inhibited in KN93-treated animals (Supplementary Figure 1). Animals receiving KN93
333 developed more severe OA, as quantified using the OARSI scoring system (Fig. 4B-C) and a
334 corresponding decrease in proteoglycan content (Fig. 4 D and E), measured by
335 histomorphometry.

336 Bone changes were assessed by microCt. As expected, BV/TV of the entire tibial epiphysis
337 increased in the MLI-operated compared to the sham-operated knee, but no difference was
338 observed between treatment groups (Fig. 5A-B). Subchondral bone thickness, however, was
339 significantly less increased in KN93-treated mice (Fig. 5C). Osteophyte size and bone vs
340 cartilage ratio was not affected by KN93 treatment (Fig. 5C).

341 **CaMKII inhibition enhanced the capacity of IL1B to upregulate catabolic enzymes in** 342 **articular chondrocytes.**

343 To gain insight into the mechanisms leading to increased cartilage degeneration in mice
344 treated with KN93, we treated monolayer cultures of primary bovine articular chondrocytes
345 with IL1B, a potent driver of extracellular matrix degradation (25). CaMKII inhibition with KN93
346 (Fig. 6A, C, E, G) or AIP (Fig. 6B, D, F, H) induced the upregulation of the matrix-degrading
347 enzymes MMP3 and ADAMTS5 alone and in synergy with IL1.

348

349

350

351 Discussion

352 Deregulation of Wnt signalling is associated with OA in patients³¹ and in animal models^{8,9,32}.

353 We previously showed that WNT3A could simultaneously activate both the beta-catenin
354 dependent and the CaMKII-dependent pathways in chondrocytes, resulting in distinct
355 transcriptional and biological outcomes¹².

356 In this study we established the transcriptional signature of WNT/CaMKII signalling and
357 identified *HMOX-1* as a specific transcriptional target of this branch of the Wnt pathway (see
358 schematic representation in Figure 7). We showed that CaMKII phosphorylation was
359 increased in human and murine OA, while the expression of *HMOX-1* was decreased. CaMKII
360 blockade increased cartilage breakdown in an instability-induced OA model in mice. CaMKII
361 inhibition increased the capacity of IL1B to upregulate catabolic enzymes in chondrocytes.

362 The microarray analysis also confirmed *SOX9* mRNA upregulation in response to CaMKII
363 blockade, as we previously showed by qPCR¹². The mechanism by which CaMKII blockade
364 results in *SOX9* upregulation and, simultaneously, in upregulation of cartilage-degrading
365 enzymes is unknown. It could be speculated that *SOX9* upregulation occurs as a homeostatic
366 response to the increased levels of extracellular matrix degradation. *HMOX1* is a
367 cytoprotective enzyme exerting antioxidant effects and chondroprotective effects in vitro³³⁻³⁵.

368 The three CAMKII isoforms expressed in the joint had distinct expression patterns. CaMKII γ
369 was selectively expressed at the articular interface in the cartilage and in the menisci, while -
370 CaMKII β and CaMKII δ had a broader expression pattern. Unfortunately, because of their high
371 similarity across the phosphorylation site, no antibody specifically detecting the
372 phosphorylated form of the different isoforms is available, making impossible to determine if
373 only one isoform or all of them are activated in OA. Interestingly, however, we demonstrated
374 that downregulation of *CaMKII γ* alone is sufficient to mimic the effect of KN93 in inducing the
375 upregulation of *HMOX-1*, suggesting that this isoform might be downstream the activation of
376 the Wnt/CaMKII in the adult articular cartilage. The understanding of the differences in the
377 activity of the four isoforms is going to be important for targeting CaMKII for therapeutic
378 purposes.

379 In our *in vivo* experiments, CaMKII inhibition affected subchondral bone thickness. During
380 embryonic development CaMKII is a modulator of chondrocyte hypertrophic differentiation
381 during development^{36,37} and therefore we cannot exclude that altered skeletal morphogenesis
382 may have also contributed to the worsening of the chondropathy.

383 CaMKII appears to modulate the final outcomes of major homeostatic effects in cartilage
384 homeostasis in a highly context-dependent manner both embryonic development and in
385 adulthood^{12,28,36,37}. Saitta et al. also recently reported that CaMKII blockade reduced the
386 capacity of bone morphogenetic proteins (BMPs) to upregulate cartilage phenotypic
387 markers³⁸. Therefore, understanding the biology of CaMKII activation is going to be key to
388 successfully harness such pathways in musculoskeletal medicine.

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496 GN and FDA, conceived and designed the project, collected, analysed and interpret the data,
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498 AST, SEE and BLT, helped interpreting the data and in revising the manuscript; KB and SR
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502

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506 [Figure legends](#)

507

508 **Figure 1: *HMOX1* is a selective target of the Wnt/CaMKII pathway in the articular**
509 **chondrocytes.** A-D. Human articular chondrocytes isolated from OA patients were treated
510 with human recombinant WNT3A in presence of the CaMKII inhibitor KN93 or its inactive
511 control molecule KN92. A. Volcano plot showing genes regulated by WNT3A (KN92+WNT3A

512 vs KN92), B. genes modulated by CaMKII inhibition in the presence of WNT3A
513 (KN93+WNT3A vs KN92+WNT3A) and C. genes activated by WNT3A independently of
514 CaMKII (KN93+WNT3A vs KN92). D. Venn diagram showing the number of significantly
515 modulated genes in each group. E. qPCR confirming downregulation of *HMOX-1* in response
516 to WNT3A but not in the presence of the CaMKII inhibitor KN93. Microarray data were
517 obtained by testing samples from 4 human donors. For 2 donors all conditions were tested in
518 two independent cartilage explants; For one donor the condition KN92+WNT3A was tested in
519 a single explant; for the remaining donor all the conditions were tested in a single explant per
520 condition. F. qPCR showing that *HMOX-1* is a selective target of the WNT/CAMKII branch and
521 it is not modulated via activation of the WNT/beta-catenin pathway. n=3 human donors, 2
522 technical replicates per donor. A-D. data were analysed with a linear models with 10% FDR p
523 value correction. E-F. Data were analysed with one-way ANOVA followed by TukeyHSD post-
524 hoc test.

525 **Figure 2: CaMKII and HMOX1 expression are oppositely modulated in osteoarthritis.** A.
526 Relative expression of the four CaMKII mRNA isoforms as assessed by expression
527 microarrays in human cartilage explants⁶. B. Gene expression analysis by semiquantitative
528 PCR for *CaMKII* α , β , γ , δ , in P0 human articular chondrocytes (upper row). A pool of cDNA
529 from brain, placenta and lymph node was used as positive control for the PCR (lower row) (n
530 cycles= 40). All the samples were loaded and run in the same gel whose image was cut for
531 best rendering. A picture of the full original gel can be found in Supplementary Figure 3. C.
532 RNA expression of CaMKII isoforms in human cartilage retrieved from normal and OA human
533 cartilage as assessed by Soul and colleagues in a previously performed RNAseq²⁷ D.
534 Immunodetection of phospho-CaMKII in normal and OA articular cartilage. Image
535 representative of n=3. E. RNA deep sequencing analysis of *HMOX-1* expression in normal
536 and OA articular cartilage.

537 **Figure 3: Expression of CaMKII isoforms in murine cartilage.** A-B. qPCR analysis of
538 murine femoral heads and patellae removed from 1 month-old 129/Sv male mice (n=4). Gene
539 expression was normalized for the housekeeping gene beta-actin. C-O. Immunofluorescence
540 for CAMKII- β , γ and δ in the murine joint. P. mRNA expression of the CaMKII isoforms after
541 CAMKIIy silencing by siRNA in C3H/10T1/2 cells. n=4. Q. Downregulation of CAMKII by
542 siRNA was sufficient to upregulate *HMOX-1* in C3H/10T1/2 cells n=4. Data analysed by t-test.
543 F= femur; T=tibia; M=meniscus; BM=bone marrow; CL=cruciate ligament; SM=synovial
544 membrane.

545 **Figure 4: Effect of CAMKII inhibition *in vivo* in a murine model of OA.** A. Osteoarthritis
546 was induced by removal of the anterior horn of the medial meniscus and transection of the

547 medial collateral ligament (MLI model). Four weeks after surgery, mice were administered
548 KN93 (5uM) or PBS vehicle subcutaneously via a minipump for additional 4 weeks (the initial
549 minipump was replaced after 2 weeks). The total volume administered over 4 weeks was equal
550 to 400µl. n=15/treatment. B. Safranin O staining of representative sections of the medial
551 compartment of the knee of mice treated with PBS or KN93. C. OARSI score of the medial
552 and the lateral compartments of the MLI knees n=15/treatment D. Histomorphometry analysis
553 of the MLI knees e. Correlation of the OARSI score and histomorphometry analysis. MF:
554 medial femur; MT: medial tibia; LF: lateral femur; LT: lateral tibia. OARSI scores were analysed
555 with Mann-Whitney test with Wilcoxon post-test. Histomorphometry data were analysed by t-
556 test. Linear regression was used to compare the OARSI scores and histomorphometry data.

557 **Figure 5: CaMKII inhibition decreases subchondral thickness.** A-B. MicroCt analysis of
558 operated and contralateral sham-operated knee joints of mice treated with KN93 or PBS
559 vehicle upon surgical destabilization of the knee joint. A. Epiphyseal Bone volume / tissue
560 volume of the entire tibial epiphysis of the operated knees. B. Comparison of the percent
561 change of the BV/TV between the MLI and the sham operated knee in the two treatment
562 groups. C. Histomorphometrical assessment of osteophyte area, % of cartilage area and
563 histomorphometrical quantification of subchondral bone thickness. Data were analysed by t-
564 test. n=15/treatment.

565 **Figure 6: CaMKII inhibition enhanced the capacity of IL1B to upregulate catabolic**
566 **enzymes in the articular chondrocytes:** A-H. mRNA expression of *ADAMTS4/5* and
567 *MMP3/13* measured by qPCR of bovine articular chondrocytes stimulated for 24h with IL1B
568 (20ng/ml) in presence or absence of KN93 or its inactive analogue KN92 (both 10µM) (A, C,
569 E, G) or in the presence or absence of the CAMKII inhibitor AIP (B, D, F, H) (5µM). Data were
570 analysed with 2 ways ANOVA and Tukey post-test. n=3

571 **Figure 7:** Diagram summarising the transcriptional targets modulated by WNT3A in a CAMK2
572 dependent manner and the effect of the pharmacological blockade of CAMK2 on the
573 remodelling of joint tissues in osteoarthritis.

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