SUPPLEMENTAL METHODS:

RAAK study subjects

The ongoing RAAK study is approved by the ethical committee of the LUMC (P08.239) and is aimed at the bio-banking of joint materials (cartilage, bone and where available ligaments) and mesenchymal stem cells (hip joints only) and primary chondrocytes of patients and controls in the Leiden University Medical Center and collaborating outpatient clinics in the Leiden area. Informed consent was obtained from each patient. In the current study we used paired preserved and OA affected cartilage samples from 52 Caucasian end stage OA patients undergoing joint replacement surgery for primary OA (23 hips, 29 knees) in the Leiden University Medical Centre (**Table S1**). At the moment of collection (within 2 hours following surgery) tissue was washed extensively with phosphate buffered saline (PBS) to decrease the risk of contamination by blood, and cartilage was collected of the weight-baring area of the joint. Initial classification of cartilage was performed macroscopically and collected separately as OA affected or preserved regions. Classification was done according to predefined features for OA related damage based on color/whiteness of the cartilage, based on surface integrity as determined by visible fibrillation/crack formation, and based on depth and hardness of the cartilage upon sampling with a scalpel. During collection with a scalpel, care was taken to avoid contamination with bone or synovium. Collected cartilage was snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. We histologically assessed cartilage samples with the modified Mankin scoring system.

Nucleic acid isolation and genotyping

Snap frozen cartilage was powderized using a Retsch Mixer Mill 200 with continuous liquid nitrogen cooling. DNA was isolated using the Promega Wizard Genomic DNA Purification

26 kit according to the manufacturer's protocol. RNA was isolated using the Qiagen RNAeasy
27 Mini kits, followed by cDNA synthesis using 1 µg of RNA and random hexamer primers
28 (First Strand cDNA Synthesis Kit, Thermo Scientific). Samples were genotyped for rs225014
29 using restriction fragment length polymorphism analysis with RsaI (Forward primer (F): 5'-
30 AGTGGCAATGTGTTTAATGTGA-3', Reverse primer (R): 5'-
31 CACACACGTTCAAAGGCTACC-3').) DNA fragment length of wild type alleles were
32 called after gel electrophoresis and were 121, 30 and 389 base pairs. The risk allele C affects
33 the first cut site resulting in two fragments consisting of 151 and 389 base pairs. All primers
34 were ordered at Invitrogen.
35

36 **Electrophoretic mobility shift assay**

37 For Electrophoretic Mobility Shift Assays (EMSAs) synthetic oligonucleotides containing the
38 putative CTCF binding site were 5'-end labeled by γ-^{32}P-ATP and subsequently purified by
39 gel filtration on Sephadex G-25 Medium columns. The CTCF-11 zinc finger (11ZF) DNA
40 binding domain, full-length CTCF using pIVEX1.4 WG CTCF-11ZF and CTCF-FL
41 constructs were synthesized with the RTS 100 Wheat Germ CECF kit (5 PRIME). For
42 binding reactions, we used buffer containing standard PBS with 5 mM $MgCl_2$, 0.1 mM
43 $ZnSO_4$, 1 mM DTT, 0.05% NP40, 50 ng/µl poly(dI-dC) and 10% glycerol. The reaction
44 mixtures were incubated for 30 min at RT and analyzed by 5% native PAGE in 0.5x Tris-
45 borate-EDTA buffer. **Table S5** contains the sequences of the used probes. Expression of
46 *DIO2* was assessed using TaqMan probe Hs00988260_m1 (Applied Biosystems), normalized
47 for *GAPDH* expression (real-time PCR, F: 5'-TGCCATGTAGACCCCTTGAAG-3', R: 5'-
48 ATGGTACATGACAAGGTGCGG-3') and subsequently log transformed for downstream
49 analysis.
50

51 **Quantification of methylation**

52 Using the ZymoResearch EZ DNA Methylation kit isolated genomic DNA was treated with
53 sodium bisulphite (BS), thereby reducing unmethylated cytosine residues to uracil, while
54 methylated cytosines remain unchanged. The methylated fraction of CpG dinucleotides was
55 assessed with MALDI-TOF mass spectrometry (Epityper, Sequenom), a commonly applied
56 to quantify CpG methylation. Samples were randomly distributed on PCR plates prior to BS
57 treatment and PCR amplification. PCR amplification and MALDI-TOF measurements were
58 performed in triplicate as technical replicates. Using MethPrimer 9 amplicons (**Table S6**)
59 were designed upstream, downstream and intragenic of *DIO2*. Amplicons were designed to
60 cover conserved transcription factor binding sites (TFBS) according to Human Genome
61 Assembly, build 19. To avoid interference of SNPs in the MALDI-TOF measurements we
62 avoided SNPs in the amplified regions using dbSNP137. Methylation of several CpG
63 dinucleotides was measured redundantly by separate amplicons, measurements of CpG
64 dinucleotides with the most successful observations per amplicon were used for downstream
65 analyses. Finally, the 9 amplicons constituted 4 independent regions covering 23 unique CpG
66 dinucleotides.

67

68 **AZA treated cell cultures**

69 Primary articular chondrocytes were isolated from cartilage derived from three OA patients
70 who underwent total joint arthroplasty of the hip (RAAK study). Cartilage tissue was
71 incubated overnight in DMEM (high glucose; Gibco, Bleiswijk, The Netherlands)
72 supplemented with 10% fetal bovine serum (FBS; Gibco), antibiotics (100 U/ml penicillin,
73 100 µg/ml streptomycin; Gibco) and 2 mg/ml collagenase Type I at 37 ºC in a humidified 5%
74 CO_2 / 95% atmosphere. Subsequently, primary chondrocytes were resuspended and filtered
75 through a 100 µm mesh to remove undigested cartilage fragments and extracellular matrix

debris. Cells were expanded at 37 °C in a humidified 5% CO_2 / 95% atmosphere in DMEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 units/mL) and 0.5 ng/ml FGF-2 (PeproTech, Heerhugowaard, The Netherlands) for 2 passages. 24 Hours into the second passage 1.5 µM of the demethylating agent 5-aza-2 –deoxycytidine (AZA) (Sigma Aldrich; Zwijndrecht, The Netherlands) was added. Cells were harvested for DNA and RNA isolations after being grown to confluence, obtained after three more days.

Analysis of methylation data

Methylation of CpG dinucleotides with fewer than two out of three triplicate measurements or with a SD > 0.1 were discarded prior to analysis. CpG site-containing fragments that had equal or overlapping mass, making them irresolvable by mass spectrometry, and CpG sites containing fragments whose measurement was confounded by SNPs were removed prior to analysis[1]. Samples with bisulphite conversion rates < 98% were discarded.

All statistical analyses were performed by fitting Generalized Linear Mixed Models (GLMMs). To account for inter-individual differences a random effect for sample donor was added to each model. Homozygous carriers (N = 3) of the rs225014 “C” risk allele were pooled with heterozygous carriers (N = 27). Analyses were carried out using the R programming language with the *lme4* (GLMMs) package.[2] To assess the relation between the cartilage phenotype and methylation of separate features, we fitted the following model: $Methylation_i \sim (1|Donor) + Phenotype$. To identify functional CpG dinucleotides, we fitted the following model: $DIO2\ Expression \sim (1|)\ Donor + Methylation_i$. Where, in both models, $Methylation_i$ represents the methylated fraction of the *i*-th CpG feature. To explore other possible significant covariates, we fitted the following model: $DIO2\ Expression \sim (1|Donor) + Joint\ site + rs225014\ alleles + Methylation_{CpG\text{-}2031}$. Finally, p-values were adjusted for

100 multiple testing using Bonferroni correction. Analyses of differences in methylation and
101 expression in chondrocyte cultures after AZA treatment were done by paired-student T-tests.
102
103 **Cell culture and *in vitro* chondrogenic differentiation**
104 HEK 293T cells were grown in DMEM (Gibco; high glucose) supplemented with 10% fetal
105 calf serum (FBS; Gibco, Bleiswijk, The Netherlands) and antibiotics (100 U/ml penicillin,
106 100 µg/ml streptomycin; Gibco) at 37ºC in a humidified 5% CO_2 / 95% atmosphere.
107 Human bone marrow derived mesenchymal stem cells (hBMSCs) were isolated from hip
108 joints of 5 OA patients who underwent total hip arthroplasty as result of end stage OA as part
109 of the RAAK study at the Leiden University Medical Centre (LUMC). Cells were expanded
110 at 37ºC in a humidified 10% CO_2 / 90% atmosphere in DMEM (high glucose) supplemented
111 with 10% FBS, antibiotics, and 0.5 ng/ml FGF-2 (PeproTech, Heerhugowaard, The
112 Netherlands) for 5 passages. Subsequently, 3D pellets were formed using centrifugal forces
113 (1200 rpm; 5 min) on 2.5 x 10^5 cells in 15 ml polypropylene conical tubes. Chondrogenesis
114 was initiated in 1 ml serum-free chondrogenic differentiation medium (DMEM,
115 supplemented with Ascorbic acid (50 µg/ml; Sigma-Aldrich; Zwijndrecht, The Netherlands),
116 L-Proline (40 µg/ml; Sigma-Aldrich), Sodium Puryvate (100 µg/ml; Sigma-Aldrich),
117 Dexamethasone (0.1 µM; Sigma-Aldrich), ITS+, antibiotics, and TGF-β1 (10 ng/ml;
118 PeproTech)). Medium was changed every 3-4 days. From day 14 onwards cell pellets were
119 maintained either in the standard chondrogenic differentiation medium or in the presence of
120 T_3 (10 nM) or IOP (10 µM). Pellets were collected for immunohistochemistry and RNA
121 isolation at the time points indicated for up to 49 days total. Literature search on the use of T3
122 in mammalian cell-models showed that in a wide variety of cell-lines and phenotypes, a wide
123 range of concentrations is used (10 nM[3], 100 nM[4] and 1uM[5]). To induce OA-
124 symptoms in chondrogenesis models we tested two concentrations, namely 10 and 100 nM.

125 Using 10 nM T3 was shown sufficient in our model system to induce an OA phenotype
126 comparable to over expressing DIO2 (Data not shown).
127

128 **Lentiviral constructs and transduction**

129 C-terminal FLAG-tagged cys-D2 (kindly provided by Prof. Dr. Bianco) was digested with
130 *EcoR1* followed by Klenow-treatment and digestion with *Xba1*. Inserts were inserted into the
131 *EcoRV-XbaI* sites of the pLV-CMV-IRES-eGFP Lentiviral backbone (kindly provided by
132 Prof. Dr. R. Hoeben; Dept of Molecular Cell Biology; LUMC). Lentiviral production was
133 performed in HEK 293T cells as described previously.[6] In short, for lentiviral transduction,
134 hBMSCs (6 x 10^5 cells) were seeded onto 20 cm plates. After one day in culture
135 (approximately 80% confluence), cells were incubated for 16 hr with the corresponding
136 Lentiviruses at a multiplicity of infection (MOI) of 1 in the presence of 15 μg/ml Polybrene
137 (Sigma-Aldrich) before the medium was replaced to regular growth medium.
138

139 **Histochemistry and immunohistochemistry:**

140 hBMSC pellets were fixed in 4% formaldehyde upon harvesting and embedded in paraffin.
141 Sections were stained for GAGs with Alcian blue and counterstained with nuclear fast red
142 (Sigma-Aldrich).
143 For immunohistochemistry, paraffin sections were deparaffinized and rehydrated.
144 Endogenous peroxidase was blocked with MeOH / 0.3% H_2O_2, and antigen retrieval was
145 performed by incubation with proteinase K (5μl/ml; Sigma-Aldrich) for 10 minutes at 37ºC
146 and subsequently with Hyaluronidase (5mg/ml; Sigma-Aldrich) for 30 minutes at 37ºC.
147 Sections were blocked for 30 minutes at room temperature in TBST (2.5mM Tris-HCl pH
148 7.6, 7.5mM NaCl, 0.1% Tween-20) with 5% normal goat serum (R&D Systems, Abingdon,
149 Great Britain). Incubation with the primary antibodies was performed overnight at 4°C with

150 commercially available antibodies for collagen type 2 (COL2; MAB1330 Millipore;
151 Amsterdam, The Netherlands; 1:100 in TBST / 10% NGS) and collagen type 10 (COL10;
152 Clone X53, Quartett; Berlin, Germany; 1:100 in TBST/10% NGS). The next day,
153 Powervision (Immunologic; Duiven, The Netherlands) was applied and visualization was
154 carried out in a 3-diaminobenzidine (DAB)-solution (Sigma-Aldrich) for 1 and 10 minutes
155 for COL2 and COL10 respectively. The sections were then counterstained with
156 haematoxylin.
157

158 **Relative pixel intensity**

159 The relative pixel intensity was computed by loading the photos into ImageJ (v.1.47).[7],[8]
160 Automated thresholding (IsoData) was used to separate the glycosaminoglycans staining and
161 the background (vacuoles and surroundings), after using the concept of rolling ball algorithm
162 to correct for uneven illumination (**Fig. S5**, panel A-C). The regions depicted as
163 glycosaminoglycans by the automated thresholding were semi-automatically filled by using
164 the 4-connected background elements (**Fig. S5**, panel D). The glycosaminoglycans-staining
165 was separated from the background (**Fig. S5**, panel E) by splitting the image into separate
166 channels and only using the red channel. Every pixel of the image was then weighed for color
167 intensity (0 – 255) and the sum of al intensity was averaged by the number of pixels, giving
168 the average pixel intensity per condition.
169

170 **Surface area meusurements**

171 Surface area measurements were performed using the standard CellSens (Build 9164;
172 version1.5) software from Olympus. Within this microscope software, we used the option to
173 manually draw a border and let the software calculate the surface area within this border.
174 Measurements are derived from 3 individual pellets per condition, per time point, per donor.

175

176 **RNA isolation**

177 RNA isolation from the pellets was performed by pooling two pellets for every given
178 condition. The isolation of RNA was performed as described previously.[9] RNA quantity
179 was assessed using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc.,
180 Wilmington, USA).

181

182 **Real time quantitative reverse transcription PCR**

183 RNA was processed with the First Strand cDNA Synthesis Kit according to the
184 manufacturer's protocol (Roche Applied Science, Almere, The Netherlands). cDNA pre-
185 amplification was performed with a DNA Engine Tetrad® 2 Peltier Thermal Cycler (Bio-Rad)
186 for 5 cycles under standard PCR conditions and using the multiplexed primers (Table S8).
187 Primer efficiencies were verified in advance, by performing a concentration curve
188 experiment. Only primers showing a perfect correlation between Ct-value and concentration
189 and with a single peak in the melting-curve calculation (Lightcycler480 SW 1.5 software)
190 were used. The housekeeping gene GAPDH was used as a single reference gene for
191 qPCR.[10, 11] A custom sample-mix was made using the EvaGreen® dye (Biotium,
192 Amsterdam, The Netherlands), ROX reference dye (Life technologies), and FastStart Taq
193 polymerase (Roche Applied Science). Subsequently, qPCR was performed using the custom-
194 designed primers (Table S8) in triplicate with the using Biomark™ 96.96 Dynamic Arrays
195 (Fluidigm). Additional RT-qPCR measurements were performed on the Roche Lightcycler
196 480 II, using FastStart SYBR Green Master reaction mix according to the manufacturer's
197 protocol (Roche Applied Science).

198

199 **Gene expression and pixel intensity analyses**

200 Relative gene expressions were corrected for GAPDH by using the $2^{-\Delta\Delta Ct}$ method, in which
201 Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches
202 detection threshold. All values were calculated relative to the first timepoint measured in the
203 assay. For the hBMSC pellet-model, time point t = 7 days of treatment of the control was
204 taken as reference point for expression analysis.
205 The paired-student T-test was used to calculate the significance of changes in expression. The
206 same method was used to calculate significance of the changes in size of the pellets over
207 time. To calculate the significance of differences in pixel intensities relative to the control
208 conditions, the Wilcoxon–Mann–Whitney test was performed. All nominal P-values < 0.05
209 were considered statistically significant.

210 **References**

211
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