

2 **Title: Agrin induces long term osteochondral regeneration by supporting repair**
3 **morphogenesis**

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5 **Single Sentence Summary:** Agrin recruits joint stem cells and induces the formation of cartilage
6 and bone to heal joint surface defects in mice and sheep.

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32 **Abstract:** Cartilage loss leads to osteoarthritis, the most common cause of disability for which
33 there is no cure. Cartilage regeneration, therefore, is a priority in medicine. We report that agrin
34 is a potent chondrogenic factor and that a single intra-articular administration of agrin induced
35 long-lasting regeneration of critical-size osteochondral defects in mice, with restoration of tissue
36 architecture and bone-cartilage interface. Agrin attracted joint resident progenitor cells to the site
37 of injury and, through simultaneous activation of CREB and suppression of canonical WNT
38 signaling downstream of β -catenin, induced expression of the chondrogenic stem cell marker
39 GDF5 and differentiation into stable articular chondrocytes, forming stable articular cartilage. In
40 sheep, an agrin-containing collagen gel resulted in long-lasting regeneration of bone and
41 cartilage, which promoted increased ambulatory activity. Our findings support the therapeutic
42 use of agrin for joint surface regeneration.

43 **Introduction**

44 Articular cartilage overlies subchondral bone at the joint surface and enables the frictionless
45 movement of joints. Whereas bone has a high turnover and heals well, cartilage is avascular, has
46 a low turnover, and often fails to repair after injury (1). This results in further cartilage loss and
47 osteoarthritis, the most common form of arthritis, which causes pain and disability. Currently,
48 there is no pharmacological therapy to restore cartilage or slow cartilage loss. Osteoarthritis is
49 therefore, along with cardiovascular disease, the leading cause of chronic disability, costing around
50 1.5-2% of the gross domestic product (GDP) for westernized countries (2).

51 Joint surface defects are common(3, 4) and, when exceeding a critical size, heal poorly. When
52 successful, the repair of small osteochondral defects involves trafficking of specialized
53 mesenchymal stem cells (MSCs) ontogenetically derived from the growth differentiation factor 5
54 (GDF5)-expressing cells of the embryonic joint interzone to the injury site (5, 6). During
55 embryonic development, MSCs are recruited from SOX9-expressing progenitor cells, transiently
56 express GDF5, and give rise to the articular cartilage, menisci, and ligaments (7). In adulthood,
57 GDF5-lineage progenitor cells persist in the synovial membrane (SM-MSCs) (5, 6). At the bottom
58 of the defect, the repair cartilage is invaded by vessels and replaced by bone through endochondral
59 bone formation, which proceeds towards the surface of the defect and stops at the level of the
60 osteochondral junction (8–10). The most superficial layer of cartilage remains avascular and is
61 resistant to endochondral bone formation (8, 9). Although this morphogenetic process takes place
62 over several weeks in rodents and several months in humans, at the molecular level, the
63 mesenchyme becomes patterned long before morphological changes become obvious (10). Such

64 patterning displays striking similarity to that of developing joints during embryonic
65 morphogenesis.

66 During skeletal development, the chondrogenic mesenchyme forming the skeletal templates
67 becomes segmented by the joint interzones, where specific molecular markers — including
68 WNT9A and GDF5, a member of the bone morphogenetic protein (BMP) family of morphogens
69 — indicate the location where joints will form (6, 11–13). Through the process of cavitation, a
70 fissure forms in the center of the joint interzones, eventually separating the skeletal elements.
71 Meanwhile, the center of the cartilaginous template undergoes vascular invasion and chondrocytes
72 undergo hypertrophy (expressing markers such as COL10A1) and are eventually replaced by bone.
73 In the long bones, this process starts at the center (diaphysis) and proceeds towards the growth
74 plate. Secondary ossification centers then form near the joints, in the epiphysis, to form the
75 subchondral spongiosa containing bone marrow. The last few layers of chondrocytes closest to the
76 joint cavity are spared from undergoing endochondral bone formation and form the articular
77 cartilage. Lineage tracking experiments have established that the cells that form the articular
78 cartilage (which persists throughout life), and those of the epiphyseal cartilage (destined to be
79 replaced by bone), derive from distinct lineages, the former expressing GDF5 during embryonic
80 joint formation (7, 12, 14).

81 Members of the WNT family of morphogens (WNT4, WNT9A, and WNT16) are the earliest
82 markers of the joint interzone (11, 15). The activation of the WNT pathway is both required (11,
83 15) and sufficient to initiate the process of joint morphogenesis (15). The ectopic expression of
84 Wnt9a was shown to trigger the ectopic expression of other joint interzone markers including *Gdf5*
85 (11). During development, WNT signaling prevents the premature differentiation of the joint

86 interzone cells into mature chondrocytes. In adulthood, WNT signaling maintains a population of
87 chondroprogenitors at the surface of the cartilage by preventing their differentiation into mature
88 chondrocytes (12, 16). Due to its anti-chondrogenic effect however, excessive activation of WNT
89 signaling within the joint predisposes to osteoarthritis (17, 18).

90 WNTs are secreted signaling molecules involved in the regulation of cell proliferation, polarity,
91 morphogenesis and differentiation (19) during both development and adulthood. In the absence of
92 WNTs, the intracellular protein β -catenin is constitutively phosphorylated by GSK-3 β and is
93 degraded through the proteasome pathway. In the presence of so called “canonical” WNTs such
94 as WNT1, WNT3A, or WNT8, the heterodimerization of frizzled (FZD) receptors and their co-
95 receptors LRP5 and 6 results in de-activation of GSK-3 β and consequent stabilization of β -catenin.
96 Stabilized β -catenin is transported to the nucleus where it interacts with the transcription factors
97 TCF/LEF and activates transcription of target genes (19). Other WNT ligands, such as WNT5A,
98 activate other calcium-dependent pathways, collectively denominated “non-canonical”. One of
99 these non-canonical pathways is mediated by the intracellular kinase CaMKII and the transcription
100 factor CREB(20). In many cells, including articular chondrocytes, activation of the non-canonical
101 WNT signaling results in inhibition of the canonical pathway (21).

102 In this study we report that AGRIN , a signaling proteoglycan (encoded by the gene *AGRN*) best
103 known for its role at the neuromuscular junction, where it stabilizes the clustering of the
104 acetylcholine receptors (22) by binding to its receptor LRP4 (23), is an orchestrator of repair
105 morphogenesis at the joint surface by modulating multiple signaling pathways. Agrin is composed
106 of a large N-terminal portion that binds to components of the basal membrane and a biologically
107 active C-terminal portion encompassing three globular domains separated by EGF-like repeats

108 (24). Agrin is expressed in a splice isoform devoid of the y and z motifs, playing a role not only in
109 differentiation of mature articular chondrocytes (25) but also in chondrogenesis and in the repair
110 of osteochondral defects.

111 **Results**

112 **AGRIN is upregulated in injured cartilage and induces chondrogenesis in MSCs**

113 In the context of screening for genes upregulated after acute cartilage injury (26), we discovered
114 that agrin was upregulated at the mRNA and protein level twenty-four hours after mechanical
115 injury to human articular cartilage explants *ex vivo* (Fig. 1, A to C). *AGRN* mRNA was also
116 upregulated in C28/I2 human chondrocytes by treatment with IL-1 β (Fig. 1D) and TNF- α (Fig.
117 1E), two inflammatory cytokines released by injured cartilage (26–29). Agrin upregulation in adult
118 primary human articular chondrocytes after IL-1 β treatment was confirmed as assessed by mining
119 a publicly accessible gene expression dataset (30) (fig. S1A). Compared to green fluorescent
120 protein (GFP), used hereafter as transfection control, agrin overexpression in human adult synovial
121 membrane-derived mesenchymal stem cells (SM-MSCs) (5, 31) resulted in their differentiation
122 into cartilage as assessed by increased production of cartilage-specific Alcian blue-positive
123 extracellular matrix and upregulation of the cartilage master transcription factor *SOX9* mRNA
124 (Fig. 1, F and G). Together, these data show that agrin is upregulated in injured cartilage and
125 induces chondrogenic differentiation in SM-MSCs that normally reside in the joint.

126

127 **Agrin induces chondrogenesis by suppressing WNT signaling downstream of β -catenin**

128 Next, we investigated the molecular pathway underlying the chondrogenic effect of agrin. The
129 agrin receptor LDL receptor related protein 4 (LRP4) mediates chondrocytic differentiation in the
130 murine chondrocytic cell line ATDC5 by inhibiting canonical WNT signaling (32). We found that
131 overexpression of either LRP4 (Fig. 2A) or agrin itself (Fig. 2B), both in the absence and presence
132 of WNT3A, in primary bovine chondrocytes upregulated *SOX9* mRNA. LRP4 is known to bind to
133 and mediate the function of WNT inhibitory molecules such as DKK1 and SOST (33); therefore,
134 we investigated whether agrin is required for the chondrogenic function of LRP4. Silencing *AGRN*
135 in C28I/2 chondrocytes prevented the *SOX9* upregulation induced by LRP4 overexpression (Fig.
136 2C). These data indicate that agrin is necessary for the chondrogenic effects of LRP4. Agrin
137 silencing did not alter expression of *DKK1* mRNA (fig. S1B). *SOST* is not expressed in articular
138 chondrocytes and was not detected by PCR.

139 Canonical WNT signaling is known to suppress chondrogenesis (12, 16). Agrin overexpression
140 blocked the capacity of WNT3A and WNT9A to activate the WNT/ β -catenin-dependent reporter
141 assay TOPFlash (34) (Fig. 2, D and E). Overexpression of either LRP4 or agrin suppressed
142 TOPFlash activation in a WNT3A dose-response curve and co-overexpression of LRP4 and agrin
143 was synergistic further inhibited TOPFlash activation (Fig. 2F). Moreover, agrin failed to induce
144 extracellular matrix formation in C28I/2 chondrocytes in which activation of canonical WNT
145 signaling was achieved directly in the nucleus by overexpressing a constitutively active form of
146 LEF1 (caLEF1) composed of the transactivation domain of VP16 and the DNA-binding domain
147 of LEF1 (35) (Fig. 2G). Therefore, the capacity of agrin to suppress canonical WNT signaling is

148 essential to its chondrogenic effect. Taken together, these data indicate that agrin induces
149 chondrogenesis by suppressing canonical WNT signaling.

150 To test at what level in the signaling cascade agrin inhibits WNTs, we activated WNT signaling in
151 COS7 cells using either the GSK-3 β inhibitor BIO (36) or the inhibitor of AXIN/GSK3- β
152 interaction SKL2001 (37). Disruption of the β -catenin destruction complex with either compound
153 resulted in ligand/receptor-independent activation of the TOPFlash reporter assay; however, agrin
154 overexpression was still able to inhibit such activation (Fig 3, A and B), thereby demonstrating
155 that the capacity of agrin to inhibit canonical WNT signaling resides downstream of the β -catenin
156 destruction complex. Similarly, agrin inhibited the activation of the TOPFlash reporter assay
157 induced by overexpression of constitutively active β -catenin [CTNNB1(Δ ex3), caCTNNB1] (38)
158 (Fig 3C). In keeping with the notion that agrin acts downstream of the β -CATENIN destruction
159 complex, agrin enhanced extracellular matrix production in C28/I2 chondrocytes even in the
160 presence of SKL2001 (fig. S2). However, when COS7 cells were transfected with caLEF1, Agrin
161 was unable to prevent activation of the TOPFlash reporter (Fig.3D). Taken together, these data
162 suggest that agrin suppresses canonical WNT signaling downstream of β -catenin.

163

164 **Agrin activates CREB signaling**

165 Agrin was previously reported to activate the Calcium/CaMKII/CREB signaling pathway in
166 neurons (39–41). We therefore hypothesized that agrin might be blocking canonical WNT
167 signaling downstream of β -catenin by activating the CaMKII/CREB pathway (21). Agrin
168 transfection or exogenous recombinant agrin resulted in phosphorylation and consequent

169 activation of CREB (pCREB) in C28/I2 chondrocytes (Fig. 3, E to H and fig. S3) and activation
170 of a CREB reporter assay (42) (Fig 3I). Conversely, silencing endogenous *AGRN* in C28/I2 cells
171 using siRNA resulted in a decrease in the number of phosphorylated CREB-positive cells, while
172 total CREB-positive cells remained unchanged (Fig. 3, J to L).

173 In the presence of the CREB inhibitor 666-15 (43), agrin failed to suppress the capacity of WNT3A
174 to activate the TOPFlash reporter assay (Fig 4A), suggesting that the capacity of agrin to inhibit
175 WNT signaling is mediated by CREB. Confirming the epistasis of CaMKII in the CREB activation
176 cascade (39, 40), the CaMKII inhibitors KN93 and AIP negated the capacity of agrin to suppress
177 the activation of the TOPFlash reporter assay induced by WNT3A when compared to KN92
178 (inactive control) or vehicle control respectively (Fig. 4B andC). Several signaling pathways
179 converge onto the CREB pathway with distinct, context-dependent transcriptional and biological
180 outcomes (44–47). Therefore, we investigated whether the capacity to suppress canonical WNT
181 signaling is specific to agrin or is a general effect of CREB activation. Forskolin, an activator of
182 adenylyl cyclase and CREB agonist, failed to inhibit activation of the TOPFlash reporter assay after
183 WNT3A treatment (Fig. 4D). Therefore, CREB activation is required but not *per se* sufficient for
184 the capacity of agrin to suppress WNT signaling. We next tested whether agrin-induced CREB
185 activation is essential for its chondrogenic capacity. In keeping with this hypothesis, agrin or LRP4
186 lost the capacity to enhance extracellular matrix formation in C28I/2 chondrocytes in the presence
187 of the CREB inhibitor 666-15 (Fig. 4E to H). Taken together, these data demonstrate that agrin
188 activates the CaMKII/CREB cascade and that these events are essential for its capacity to inhibit
189 WNT signaling and to induce cartilage formation.

190

191 **Agrin supports the repair of critical size osteochondral joint surface defects in mice.**

192 To test if exogenous agrin is sufficient to improve the outcome of joint surface repair *in vivo*, we
193 generated cylindrical osteochondral defects in the lateral femoral condyle of adult mice. Defects
194 were 0.78 ± 0.042 mm wide and 1.79 ± 0.056 mm deep (mean \pm SD) and extended into the
195 subchondral spongiosa. Without treatment, such defects result in partial healing of the bone, but
196 not of the articular cartilage or the subchondral plate, after 8 weeks (fig. S4A-B). A type I collagen
197 gel containing either human full-length agrin or GFP as control was injected into the joint surface
198 defect immediately after it was generated. Eight weeks after surgery, the cartilage layer
199 regenerated significantly better in the agrin group (Fig. 5A) both in terms of glycosaminoglycan
200 content (48) ($P=0.04141$) (Fig. 5B) and Pineda injury score (49) ($P=0.04083$) (Fig. 5C). The size
201 of the residual bone defect was also reduced in the agrin group (Fig. 5D), however no evidence of
202 ectopic bone formation was observed by μ CT (fig. S4C). Whereas in the agrin group most of the
203 repair tissue was composed of either bone or cartilage, in the GFP group there was a larger amount
204 of non-differentiated fibroblast-like mesenchyme (Fig. 5E).

205

206 **Agrin induces GDF5 upregulation in a CREB-dependent manner**

207 We previously reported that the cells that contribute to the repair of cartilage defects derive from
208 a lineage of progenitor cells that, during skeletal development, express the joint interzone marker
209 GDF5 (5). During skeletal development, WNT9A induces the expression of GDF5 in the
210 mesenchymal cells residing in the portion of the skeletal elements that will give rise to the articular
211 cartilage, menisci, and ligaments, and that are resistant to endochondral bone formation (7, 11,

212 13). In adulthood, joint-specific progenitor cells derived from the GDF5 lineage persist within the
213 synovial membrane and are the main contributors to the regeneration of cartilage defects, which,
214 when small in size, repair spontaneously (5, 9). Unstimulated human SM-MSCs did not express
215 detectable GDF5, however, 24 hr after agrin transfection, many of the cells highly expressed GDF5
216 (Fig. 6A). In addition, agrin transfection induced GDF5 upregulation in C28/I2 human
217 chondrocytes at protein (Fig. 6B-C) and mRNA levels (Fig. 6D). Conversely, silencing of
218 endogenous *AGRN* using siRNA in C28/I2 cells resulted in a reduction of GDF5 expression at
219 protein (Fig. 6 E and F) and mRNA levels (Fig. 6G). This loss of GDF5 was rescued with the
220 addition of exogenous rAgrin (Fig. 6E and F). Strikingly, agrin was unable to induce GDF5
221 expression in bone marrow-derived MSCs (fig. S5). This suggests that the capacity of agrin to
222 induce GDF5 is restricted to cells of the GDF5-derived lineage, such as chondrocytes and synovial
223 membrane-derived MSCs (5, 12, 14).

224 During embryonic development, WNT9A is sufficient to induce GDF5 expression in the joint
225 interzones. Agrin and WNT9A alone or in combination induced GDF5 protein expression (Fig.
226 6H to I) and activated the CREB reporter assay (Fig 6J). Interestingly, agrin and WNT9A in
227 combination induced activation of the CREB reporter assay more than agrin or WNT9A alone.
228 The CREB inhibitor 666-15 negated the capacity of agrin to induce *GDF5* mRNA upregulation in
229 C28/I2 cells (Fig. 6K), suggesting that the capacity of agrin to upregulate *GDF5* is CREB-
230 dependent. Finally, overexpression of constitutively active LEF1, but not WNT3A, also negated
231 agrin-induced *GDF5* upregulation (Fig. 6L to M). This does not necessarily mean that suppression
232 of WNT signaling drives activation of *GDF5*, because caLEF1 overexpression also suppressed the
233 capacity of agrin to induce CREB phosphorylation. Taken together, these results indicate that agrin

234 activates GDF5 expression and prompts chondrogenesis through activation of CREB-dependent
235 transcription and suppression of canonical WNT signaling.

236

237 **Agrin induces local accumulation of *Gdf5*-lineage joint stem cells and phosphorylation of**
238 **CREB *in vivo***

239 To study whether the *Gdf5*-lineage of joint-specific MSCs contribute to agrin induced joint surface
240 repair, we used transgenic mice harboring a tdTomato (Tom) cassette preceded by a LoxP-flanked
241 stop cassette within the ROSA26 locus and Cre recombinase under the control of the *Gdf5*
242 responsive elements active during embryonic development (5, 50). In these *Gdf5*-Cre;Tom reporter
243 mice, the progeny of cells that at any point during embryonic development have expressed *Gdf5*
244 will express Tom, regardless of whether they still express *Gdf5*. Similar to our findings in wild
245 type mice, agrin enhanced joint surface regeneration in *Gdf5*-Cre;Tom reporter mice (Fig 7A).
246 Three weeks after surgery there was a marked increase in the number of Tom+ cells within the
247 superficial portion of the repair tissue as well as in the synovial membrane of the mice that received
248 agrin compared to controls (Fig. 7B to D). Co-immunofluorescence staining for Tom and collagen
249 type II at eight weeks after injury revealed the presence of Tom+ chondrocytes embedded in a
250 collagen type II-containing matrix along the joint surface of the repair tissue (Fig. 7E).

251 In keeping with our *in vitro* data, three weeks after surgery we detected a higher percentage of
252 cells positive for pCREB within the repair tissue of agrin-treated animals (Fig. 7F and G). Dose
253 response experiments using recombinant agrin revealed that concentrations between 1 and 1000
254 ng/ml suppressed WNT signaling and activated CREB signaling to a similar extent as COS7-

255 AGRIN cell lysates (fig. S6A and B). An injection of a collagen gel containing 100 ng/ml rAGRIN
256 into osteochondral defects also led to increased Tom+ cells in the repair mesenchyme three weeks
257 after surgery compared to PBS control (fig. S6, C to E), as observed with the COS7-AGRIN cell
258 lysates.

259

260 **Intra-articular agrin delivery improves long-term repair of critical size osteochondral**
261 **defects and improves joint function in sheep.**

262 Finally, we tested whether agrin could also support long-term cartilage repair in a large animal
263 model. A critical-size osteochondral defect (8mm diameter and 5mm deep) was generated in the
264 weight-bearing region of the medial femoral condyle of adult sheep. The defect was filled with a
265 type I collagen gel containing either human full-length agrin or GFP as control. At 6 months post-
266 surgery, μ CT analysis revealed that bone repair was better in the agrin than the control group, as
267 noted by reduced defect volume (Fig. 8A and B). The Pineda injury score revealed superior healing
268 of the defect in the agrin group (Fig. 8C and D). Sheep that received the agrin-containing gel spent
269 more time playing and less time resting throughout the study (Fig. 8E and F), suggesting that the
270 improved repair was associated with improved function.

271 **Discussion**

272 We demonstrated that joint surface injury triggers expression of agrin, which in turn recruits
273 chondrogenic GDF5 lineage joint-resident progenitor cells to the repair mesenchyme and enables
274 the morphogenesis of joint surface. In critical size defects, which do not heal spontaneously,
275 exogenous agrin induced GDF5 expression in joint-resident MSCs and triggered their
276 chondrocytic differentiation by inhibiting WNT signaling downstream of β -catenin in a CREB-
277 dependent manner (fig. S7). Tissue patterning requires temporal and spatial coordination of cell
278 migration, proliferation and differentiation (6, 10). The WNT, BMP and CREB-dependent
279 signaling pathways are key players in the patterning and morphogenesis of synovial joints(6, 51)
280 during embryonic development. Whereas the modulation of these pathways individually failed to
281 result in morphogenesis -- for instance, BMP2 is chondrogenic but leads to ectopic cartilage and
282 bone formation(52) -- exogenous agrin resulted in harmonious postnatal repair morphogenesis.

283 During embryonic development WNT9A is sufficient (11) but not required (53) to induce joint
284 formation whereas GDF5 is required (at least for some joints) but not sufficient (13, 54), because
285 disruption of *Gdf5* in mice is not associated with joint fusion. It was previously thought that the
286 *GDF5* lineage of progenitor cells was established early in development and that cells later migrated
287 to the joint interzones, thereby contributing to the formation of the articular cartilage and ligaments
288 (12, 14). This concept was challenged by subsequent lineage-tracking experiments using an
289 inducible system allowing genetic labeling of *Gdf5*-positive cells at different stages of
290 development (7). Such experiments demonstrated a continuous recruitment of *Gdf5*-lineage cells
291 to the joint interzones throughout development. Cells entering the *Gdf5* lineage at different
292 developmental stages contributed to different tissue structures within the joints. This new paradigm

293 is in keeping with our data showing recruitment of *Gdf5*-lineage cells to the site of injury induced
294 by agrin even in adulthood. Agrin failed to induce GDF5 in bone marrow-derived MSCs, thereby
295 suggesting that its function is specific to *GDF5*-lineage cells. This may explain why agrin, as
296 opposed to other chondrogenic molecules such as BMPs and TGF- β (55, 56), did not induce
297 ectopic cartilage or bone formation.

298 Although both WNT9A and agrin induced GDF5 upregulation, the former is an activator of the
299 canonical WNT signaling and inhibits chondrogenesis whereas the latter is an inhibitor of
300 canonical WNT signaling and promotes chondrogenesis. WNT9A enhanced the capacity of agrin
301 in activating CREB in HEK293 cells. The presence of a cAMP response element (CRE) in the
302 *GDF5* promoter (47) suggests that CREB is a critical element for the capacity of agrin to
303 upregulate GDF5.

304 Agrin inhibited canonical WNT signaling downstream of β -catenin. Such mechanism is
305 independent of the ligands moiety and the WNT receptor repertoire and therefore overrides all
306 other upstream regulation including activating mutations of β -catenin which result in cancer (57).
307 This property of agrin may open therapeutic opportunities for its use in other conditions such as
308 osteoarthritis (58) and cancer (57), in which downregulation of canonical WNT signaling is
309 desirable without incurring compensatory mechanisms. Notably, WNT inhibition is currently
310 being tested as a treatment for osteoarthritis (59–62).

311 The capacity of agrin to induce long-term cartilage regeneration after a single administration
312 makes it an excellent candidate for clinical use. One problem in clinical translation is
313 manufacturing. In its fully glycosylated state, agrin is a large, poorly soluble molecule of ~500-
314 600kD which is difficult to purify to clinical grade in a biologically active form. We have shown

315 that a purified C-terminal deletion mutant of only ~95kD is sufficient to induce chondrogenesis *in*
316 *vitro* at least as potently as the full-length molecule, but the efficacy of such deletion needs to be
317 confirmed *in vivo*, since the N-terminus contains domains responsible for binding to the
318 extracellular matrix. Such domains, and the capacity of agrin to bind to the extracellular matrix,
319 may be responsible for its remarkable long-term efficacy.

320 Another limitation of our study is that we applied agrin therapeutically shortly after surgical
321 generation of osteochondral defects in otherwise normal knees. In human patients, cartilage defects
322 are often associated with meniscal/ligament damage and sometimes with some degree of
323 osteoarthritis. It is still to be proven whether agrin will be able to induce cartilage regeneration
324 under these circumstances in which joint instability might be compromised, or in the presence of
325 inflammation. Finally, accurate pharmacokinetic studies and dose responses will be needed to
326 identify the optimal administration regimen.

327 No ectopic cartilage was observed after intraarticular delivery despite the chondrogenic capacity
328 of agrin. This is in contrast with the abundant ectopic cartilage and bone formation observed after
329 delivery of TGF- β or BMP2 (56, 63). In addition, the chondrogenic and anabolic capacity of agrin
330 could be detected consistently even in the presence of 10% fetal bovine serum, which overrides
331 the anabolic capacity of TGF- β and BMPs (64, 65). The capacity of agrin to preserve the
332 architecture of the native tissue is distinct and of important translational relevance. We anticipate
333 that the optimization of delivery will be key for the clinical translation in cartilage repair strategies.

334

335 **Materials and Methods**

336 **Study design**

337 The overall scope of this controlled laboratory study was to assess the effect of agrin in the
338 regeneration of osteochondral defects and its mechanism of action. Human primary cells were
339 obtained from patients undergoing joint replacement as described below according to ethics
340 approval REC N. 07/Q0605/29. Cell lines were acquired commercially. Treatments, for each
341 experiment, are detailed in the figure legends. Sample size of in vitro and in vivo experiments was
342 determined by power calculations based on previous similar experiments to ensure a power of at
343 least 0.8 in detecting an effect size of 0.5.

344 **In vivo studies.**

345 *Preliminary efficacy study in Fig 5.*

346 Wild type, 10 week old male C57BL/6 mice (4 animals per group, 4 joints analysed) were
347 subjected to the generation of osteochondral defects as described below and the defect was filled
348 immediately with either a collagen gel containing GFP (crude cell extract from transduced COS7
349 cells) or a collagen gel containing full length agrin (crude cell extract from transduced COS7 cells).
350 The animals were killed 8 weeks after surgery.

351 *Efficacy study in figure 7.*

352 Female *Gdf5-Cre;Tom* reporter mice (age 10 week old, 8 mice per group) were subjected to the
353 generation of bilateral osteochondral defects. The defects were filled immediately with either a
354 collagen gel containing GFP (crude cell extract from transduced COS7 cells) or a collagen gel

355 containing full length agrin (crude cell extract from transduced COS7 cells). Three mice per group
356 were killed 3 weeks after surgery and 5 mice per treatment group were killed after 8 weeks. One
357 joint from the control group at 8 week time point was excluded from analysis because of an
358 accidental cortical fracture during surgery.

359 *Confirmation of recruitment of Gdf5-Tom+ cells using recombinant agrin (fig S6).*

360 Eight *Gdf5-Cre;Tom* mice (2 females and 6 males; 3 males and 1 female per treatment group) were
361 subjected to the generation of bilateral osteochondral defects. The defects were filled immediately
362 with either a collagen gel or a collagen gel containing 100 ng/ml of recombinant C-terminal agrin
363 (rAGRIN). Animals were killed after 3 weeks and one joint per animal was processed for analysis.

364 *Efficacy study in sheep (Fig. 8).*

365 Twelve female sheep aged 2.9 years \pm 0.41 (SD) were subjected to the generation of an
366 osteochondral defect. The defects were filled immediately with either a collagen gel containing
367 GFP (crude cell extract from transduced COS7 cells) or a collagen gel containing full length agrin
368 (crude cell extract from transduced COS7 cells). In the GFP group 2 animals were excluded from
369 the histological analysis, one because of osteomyelitis and one because of a subchondral cyst. All
370 animals were killed 6 months after surgery.

371 In all animal studies, neither the operator nor the assessors were aware of the treatment. To
372 minimize the risk that fights within individual cages skewed biased the results, treatment was
373 randomized in each cage in the experiment with wild type mice. All sheep were kept in the same
374 flock. The treatment table and the outcome tables were kept in separate databases until the
375 outcomes had been recorded and only merged at the time of statistical analysis. Conditions to stop

376 collection of data and humane endpoints for mice included weight loss >15% or evidence of
377 excoriating dermatitis for more than 1 week or of ulcerative dermatitis for any length but were
378 never met. No mouse, therefore, was killed early or excluded from analysis. Three sheep developed
379 large subchondral cysts as a complication of surgery, which were detected radiographically and
380 were excluded from further analysis.

381

382 **Cells, cell lines and expression vectors**

383 Adult human articular cartilage and synovial membrane were obtained following informed consent
384 from patients who underwent joint replacement for knee OA after obtaining informed consent (5
385 men and 3 women, with a mean \pm SD age of 68 ± 7 years). All procedures were approved by the
386 East London and The City Research Ethics Committee 3 (ethics approval REC N. 07/Q0605/29).

387 Articular chondrocytes and synovial membrane mesenchymal stem cells were isolated and
388 expanded as previously described (25, 31). Bovine chondrocytes were isolated from the metatarsal
389 joints of 18-month-old bovine, obtained within 6hrs of death from a local abattoir, as previously
390 described (25), chondrocytes from three joints were pooled. C28/I2 chondrocytes (66) were a kind
391 gift from Dr Mary Goldring (HSS Research Institute, Hospital for Special Surgery, New York,
392 New York). COS-7 cells were a kind gift from Dr Michael Ferns (UC Davis Health system, USA).
393 HEK293 cells were purchased from ATCC.

394 All cells were cultured in complete medium (DMEM/F-12, containing 10% FBS and 1% antibiotic
395 antimycotic solution) (Thermo Fisher Scientific). COS-7 feeders producing agrin or GFP or TGF-

396 β were obtained as previously described (25). Transfections were performed using JetPrime
397 (Polyplus) according to the manufacturer's instructions.

398 With all cells, chondrogenesis was assessed in micromass culture as previously described (25, 48,
399 67). Extracellular matrix deposition was quantified by staining with Alcian Blue 8 GS (Merck) at
400 pH 0.2 followed by extraction in 8 M guanidine HCl (Thermo Fisher Scientific) and
401 spectrophotometric quantitation at a wavelength of 630nm (25, 48, 67). DNA was quantified using
402 the Sybr Green method according to manufacturer's instruction (Origene).

403 The Rat Agrin plasmid (68) was a kind gift from Dr Michael Ferns (UC Davis Health system,
404 USA). The Lrp4 plasmid (23) was a kind gift of Dr Lin Mei (Medical College of Georgia, Augusta,
405 USA). TGF- β plasmid was a kind gift from Dr. Gerhard Gross. The caLEF1 and the caCTNNB1
406 plasmids were a kind gift from Dr. Carles Gasson-Massuet.

407 siRNA oligonucleotide sequences can be found in table S1. A Stealth RNAi negative control
408 duplex of low guanine-cytosine (GC) content (Invitrogen) was used as a negative control for
409 *AGRN* siRNA.

410

411 **Generation of agrin-expressing COS7 cells and agrin-containing collagen gel**

412 The full-length coding sequence of human agrin (accession N. AB191264) was cloned into the
413 BamHI and Kpn1 site of the pLNTSFFV. The agrin sequence was synthesized in 3 parts by Gene-
414 Art (Life Technologies). The 5' fragment was ligated into the BamHI/XhoI sites of the vector. The
415 3' fragment was the ligated into this plasmid at the XhoI and Kpn1 sites. Finally, the XhoI

416 fragment comprising the central portion of the gene was ligated into the XhoI site of the vector to
417 give the complete cDNA. Lentiviruses were packaged in HEK 293T cells using standard
418 procedures. The agrin lentivirus (or GFP lentivirus as control) was used to transduce COS7 cells,
419 which were then cloned by limiting dilution. After three passages, the clone with the highest
420 expression of agrin as determined by immunofluorescence was selected and used for further
421 studies.

422 To generate collagen gel containing agrin (or GFP as control), agrin or GFP-overexpressing COS7
423 cells were washed twice in ice-cold PBS, detached mechanically with a cell scraper, resuspended
424 in PBS, pelleted at 10000 g for 20 minutes and resuspended in an equal volume of PBS. The
425 samples were subjected to 5 cycles of freeze-thawing alternating between liquid nitrogen and a
426 37°C water bath and finally diluted 1:1 in a 5 mg/ml solution of ice-cold type I rat tail collagen at
427 pH 7.5 (Corning - 354249) prepared according to the manufacturer's instruction. The preparation
428 was kept on ice to prevent polymerization until injected.

429

430 **Generation of recombinant Agrin**

431 Recombinant human non-neuronal C-terminal Agrin (rAGRIN) was generated as follows. The C-
432 terminal portion of Agrin (AA 1244-2045 from GeneBank accession number BAD52440) was
433 cloned by PCR from the backbone of the full-length human non-neuronal Agrin adenovirus and
434 subcloned into a 3rd generation lentivirus gene expression vector backbone downstream of the
435 CMV promoter, an IgG kappa signal peptide and followed by an enterokinase cleavage site,
436 thermostable alkaline phosphatase, Myc and 10X His tags and finally by a stop codon. The

437 lentivirus backbone was transiently transfected into Expi293 cells (Thermo Fisher Scientific) using
438 the Expi293 Expression System (Thermo Fisher Scientific) as per manufacturer's instructions. At
439 day 3 post transfection, cell-free supernatant was collected and recombinant Agrin was recovered
440 using His SpinTrap columns (GE Healthcare), according to manufacturer's instructions.

441

442 **Animals and animal procedures**

443 All animal procedures were subjected to local ethical approval and Home Office Licensing. Mouse
444 experiments were regulated by PPL no. 70/7986 and 60/4528, sheep experiments by PPL no.
445 70/7740. C57BL/6 mice were purchased from Charles River UK. *Gdf5*-Cre;Tom mice (5) were
446 generated by crossing *Gdf5*-Cre transgenics (Tg(*Gdf5*-Cre-ALPP)1Kng) (14) (Kind gift of Dr D.
447 Kingsley, Stanford, CA, USA) with Cre-inducible tdTomato (Tom) reporter mice (B6.Cg-
448 Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J) (Jackson Laboratories). *Gdf5*-Cre;Tom mice were
449 on a mixed FVB/C57BL/6 background. All mice were maintained in isolator cages or standard
450 housing in groups of 3-5 and fed ad libitum.

451

452 **Murine model of osteochondral defect repair**

453 Mice were anesthetized with isoflurane. The knees were shaved and disinfected with 70%
454 ethanol. The skin was cut with fine scissors and separated from the underlying tissue by blunt
455 dissection. The femur was placed so that the shaft was perfectly vertical, with the knee flexed at
456 90°. A 25G needle (Terumo Agani G25, cannula 0.5mm, length 25mm, bevel 11°) was placed on

457 the lateral condyle in correspondence of the intersection of a vertical line tangent to the lateral
458 margin of the patella and a horizontal line tangent to the inferior margin of the patella. By applying
459 gentle pressure and rotation, the needle was driven through the joint capsule, the cartilage, and the
460 bone, while aiming for the center of the femoral shaft. As soon as the bevel of the needle was
461 completely buried, the G25 needle was retracted and replaced with a G21 needle (Terumo Agani,
462 G21, cannula 0.8 mm, length 50 mm, bevel 11°). The G21 needle was again gently rotated and
463 advanced until its bevel was completely hidden. The G21 was retracted while still turning to extract
464 the bone debris and leaving a cavity. If there was any bleeding, this was blotted with sterile gauze.
465 Liquid collagen type I gel containing the lysate of COS7 cells overexpressing full-length human
466 agrin(crude extract from transduced COS7 cells)accession No. AB191264) or recombinant C-
467 terminal agrin as indicated, or GFP (crude extract from transduced COS7 cells) or PBS as
468 indicated, was injected using a pulled glass pipette tip with a diameter of approximately 10µm
469 mounted at the end of a regular 2 µl pipette tip until the defect was full. After waiting
470 approximately 20 seconds to allow the gel to set, the joint capsule was closed with a single suture
471 with Vycril 6-0 and the skin was closed with an interrupted suture (Ethilon 5-0 a-traumatic needle).
472 After recovery mice, fed *ad libitum* in individually filtered cages (3-5 mice per cage). For wildtype
473 mice, treatments were randomized within each cage. The animals were monitored post-operatively
474 for signs of suffering and local infection. The operator and the scorers were blind to the treatment.

475 At the stated time points mice were killed, the joint dissected and processed for histology. Sagittal
476 sections through the center of the defect were identified as the first section that, starting from the
477 lateral side, intersected the lateral margin of the patellar bone. Such sections were stained with
478 Safranin O and scored using the Pineda score (49).

479

480 **Ovine model of osteochondral defect repair**

481 Adult [aged 2.9 years \pm 0.41 (SD); individual ages can be found in table S1] female sheep were
482 anesthetized with isoflurane. Following a sterile preparation of the skin, the joint was opened using
483 a lateral para-patella approach. An 8 mm diameter, 5 mm deep osteochondral defect was created
484 using a hand drill. The defect was lavaged to remove debris. Defects were filled with liquid
485 collagen type I gel containing the lysate of COS7 cells overexpressing full length human agrin or
486 GFP as control. After waiting about 20 seconds to allow the gel to set, the capsule was closed using
487 3M Monocryl in an interrupted mattress pattern. The skin was closed with 2M Vicryl. Sheep were
488 recovered and then housed for two weeks post-surgery indoors in pens. Carprofen was
489 administered at a dose of 4 mg/kg at the time of surgery then 4 mg/kg once a day for three days
490 post-surgery. After this time, sheep were kept in one flock in a field to allow free and natural
491 movement. At 6 months post-surgery sheep were killed, the knees processed for μ CT and
492 subsequently processed for histology. Mid-defect sections were stained and scored as described
493 above.

494 For μ CT analysis, sheep knee joints were scanned using a Nikon XT H 225 ST CT scanner.
495 Reconstruction was done using CT Pro V2.2 Nikon software (Nikon Metrology UK Ltd) and the
496 images were saved as a tif series. These were then viewed using Dataviewer v1.5 software (Bruker,
497 Kontich). To allow subsequent analysis the data was then resaved as a transaxial (x,y) dataset. This
498 new dataset was then opened in CTAn (v1.13) (Bruker, Kontich). Before analysis was carried out
499 the true pixel value from the Nikon scan was manually added using the image properties option,
500 as the calibration was not automatically saved. A region of interest was drawn to define the defect

501 area in each joint, from which the defect volume was determined. The person analyzing the μ CT
502 data was blinded to the study groups.

503

504 **Histology and immunostainings**

505 All samples were fixed in 4% paraformaldehyde at 4°C overnight, decalcified in 10% EDTA in
506 PBS for 2 weeks at 4°C (*Gdf5*-Cre;Tom) or in 33% Formic Acid for 24hrs and then washed for
507 24hrs in water at room temperate (wildtype), dehydrated in an ethanol series, embedded in paraffin
508 and 5 μ m sections were obtained. Safranin O staining (pH 4.2) or toluidine blue (pH 4.5) was
509 performed according to standard protocols.

510 Immunofluorescence and immunohistochemical, staining was carried out as previously described
511 (25, 48). For antigen retrieval on paraffin sections pepsin digestion was performed. Where
512 phosphatase treatment was carried out, sections were incubated with Lambda phosphatase for 2hrs
513 at 37°C according to manufacturer's instructions (CST). Antibodies and dilutions used are
514 provided in table S2. Tissue staining was carried out using an overnight incubation of the primary
515 antibody at 4°C, immunocytochemistry was performed following 1hr incubation at room
516 temperature. Sections were counterstained with hematoxylin or with 4',6-diamidino-2-
517 phenylindole (DAPI) (Life Technologies). Slides were mounted in Mowiol (EMD Millipore,
518 Darmstadt), and images were acquired with a fluorescence microscope (BX61; Olympus) using a
519 Uplan-Fluor 40 \times NA 0.85 objective lens, a Zeiss 710 META Laser-Scanning Confocal
520 Microscope (Carl Zeiss Ltd), or a Zeiss Axioscan Z1 slide scanner (Carl Zeiss Ltd). Images were
521 acquired by using an F-View II Soft Imaging Solutions (SIS) camera and Cell P software

522 (Olympus), or using ZEN software (Carl Zeiss Ltd). Image contrast was modified with Photoshop
523 7.0 for best graphic rendering, equally for all treatments.

524

525 **Histomorphometry**

526 Histomorphometry was performed with ImageJ software (NIH). The number of cells positive for
527 phospho-CREB (pCREB) was calculated as follows. Images of immunohistochemistry
528 counterstained with hematoxylin were opened in ImageJ (69). All cells (positive and negative)
529 were selected using the color threshold tool (Image>Adjust>Color threshold). The tool was set on
530 the RGB color space and all three (red, blue and green) channels were passed, ensuring that the
531 blue channel (hematoxylin positive cells) was passed with the upper limit on the peak of the
532 histogram. The passed component of the image was sampled and pasted on a new image. Such
533 image contained all cells, positive (brown) and negative (blue) and no background. This image
534 was converted to 8 bit and thresholded in such a way to maximize separation of adjacent cells
535 while still selecting every cell. A further deconvolution of overlapping cells was obtained using
536 the watershed tool (Process>binary>watershed). Total cells were then counted with the Analyze
537 Particles tool (Analyze>Analyze Particles). Care was taken to optimize the size of the particles to
538 count so to exclude specks that did not reach the minimum size of a cell. In our case we used 100
539 px~infinity. The positive cells were counted in the same way except that during colour
540 thresholding, the upper limit of the blue channel was placed immediately to the left of the blue
541 histogram, so that all blue cells were thresholded out and the resulting image only contained brown
542 cells. The counts were expressed as (positive/total cells) x100.

543 The number of cells positive for Tomato in immunohistochemistry could not be quantified in the
544 same way because the cytoplasmic staining of neighboring cells could not always reliably be
545 deconvoluted. Therefore, the area occupied by brown (immunohistochemistry) or blue
546 (hematoxylin) staining was considered as proportional to the positive and negative cells. Image
547 processing for this analysis was similar to that described above for phospho-CREB staining, with
548 the following differences. First, after color thresholding, the second round of thresholding was
549 performed so to include the entire histogram of the 8-bit images so not to alter the area occupied
550 by any positive staining in the 8-bit images. Second, instead of the particle count, we used the
551 “total area” of the results from “Analyze Particles” as (total area total cells/total area positive
552 cells) x100.

553 **Western blotting**

554 Cells were washed in ice-cold PBS and lysed in ice-cold RIPA Buffer in the presence of protease
555 and phosphatase inhibitors (Sigma) for 20 mins on ice. Protein concentrations were determined by
556 bicinchoninic acid protein assay (Pierce). Samples were prepared for SDS-PAGE on 10% (wt/vol)
557 Bis-Tris NuPAGE gels (Invitrogen) and transferred to nitrocellulose membrane. Blots were
558 blocked in 5% BSA in 0.1% TBS-Tween) and incubated with primary antibodies at the
559 concentrations stated in supplementary table I overnight at 4°C. After three washes in 0.1% TBST,
560 blots were incubated for one hour at room temperature with HRP-conjugated secondary IgG
561 (Dako). After further three washes, protein bands were visualized by chemiluminescence
562 (Luminata Forte; Merk Millipore) using FluorChem E imaging system (Protein Simple).
563 Measurements of band densitometry and quantification of protein expression was conducted using

564 ImageJ (NIH) (69). Phospho protein expression was normalized to total protein levels and to α -
565 TUBULIN (endogenous loading control).

566

567 **Reporter assays**

568 Subconfluent cells were co-transfected with SUPER8XTOPFlash (34) TCF/LEF–firefly luciferase
569 reporter vector (Addgene) and CMV-Renilla luciferase vector (in a ratio 1:100). 24hrs after
570 transfection, the medium was replaced and the cells were treated for 24hrs as specified. Luciferase
571 activity was measured using the Dual Luciferase Reporter Assay System (Promega) in a TD-20/20
572 Luminometer (Turner Designs). Firefly luciferase activity was normalized by Renilla luciferase
573 activity and expressed as relative luciferase units. See table S3 for all reagents.

574 **Gene expression analysis**

575 RNA extraction was performed using Trizol (Invitrogen) according to the manufacturer's
576 instruction. Reverse transcription and real-time PCR were performed as previously described (25).
577 Primers and amplicon length are listed in table S4.

578 Microarray data from previously published datasets (30) were accessed through the Gene
579 Expression Omnibus database at NIH (GEO accession GSE75181). Briefly, normalized data were
580 downloaded from GEO as an expression dataset; the samples of interest (IL-1 β -treated and control)
581 were selected and gene expression was compared by fitting a linear model independently for each
582 probe, with group as the y variable, using 'lmfit' ('limma' R package). The linear fit for each
583 comparison was subsequently modified using the empirical Bayes ('eBayes') approach. For each

584 comparison, \log_2 fold-change (logFC), P value, and adjusted P value (false discovery rate, FDR
585 for multiple comparisons) was output. Individual samples expression data for agrin were extracted
586 from the expression dataset and the statistics obtained from the statistics output and used to build
587 the graph. To facilitate the reproduction of the data, an R script is supplied in supplementary
588 materials to obtain the raw data, select the samples of interest, perform the statistical analysis and
589 generate the graph. Pre-processed, normalized data for individual genes were obtained using the
590 GEO2R functionality.

591

592 **Statistical analysis**

593 Means of parametric data were compared with a student's t test or with ANOVA followed by
594 Tukey HSD post hoc test for multiple comparisons. When necessary, log or square root
595 transformation was applied to correct skewed distributions in order to satisfy the assumptions of
596 parametric tests. Non-parametric data were analyzed with the Mann-Whitney U test or, for
597 multiple comparisons, the Kruskal Wallis test followed by the Dunn test. Dose response curves
598 and repeated measures were assessed by two-way ANOVA and, if different treatments were
599 applied, ANCOVA followed by Tukey HSD for multiple comparisons. Statistical analysis was
600 performed using either R or GraphPad Prism software. Data shown as box and whisker blot. Box
601 extends from the 25th to 75th percentiles. Error lines represent max to min points. *P* values
602 <0.05 were considered significant.

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822

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832 H.W., M.K., G.Z., H.L., S.C., D.S., A.A., K.S., J.W., B.F.F. and F.H. performed experiments. Data
833 was interpreted by S.E.E., F.D., A-S.T., A.J.R., C.D.B., F.H. and B.L.T. S.E.E., F.D., A.J.R. and
834 C.D.B. contributed to writing the manuscript.

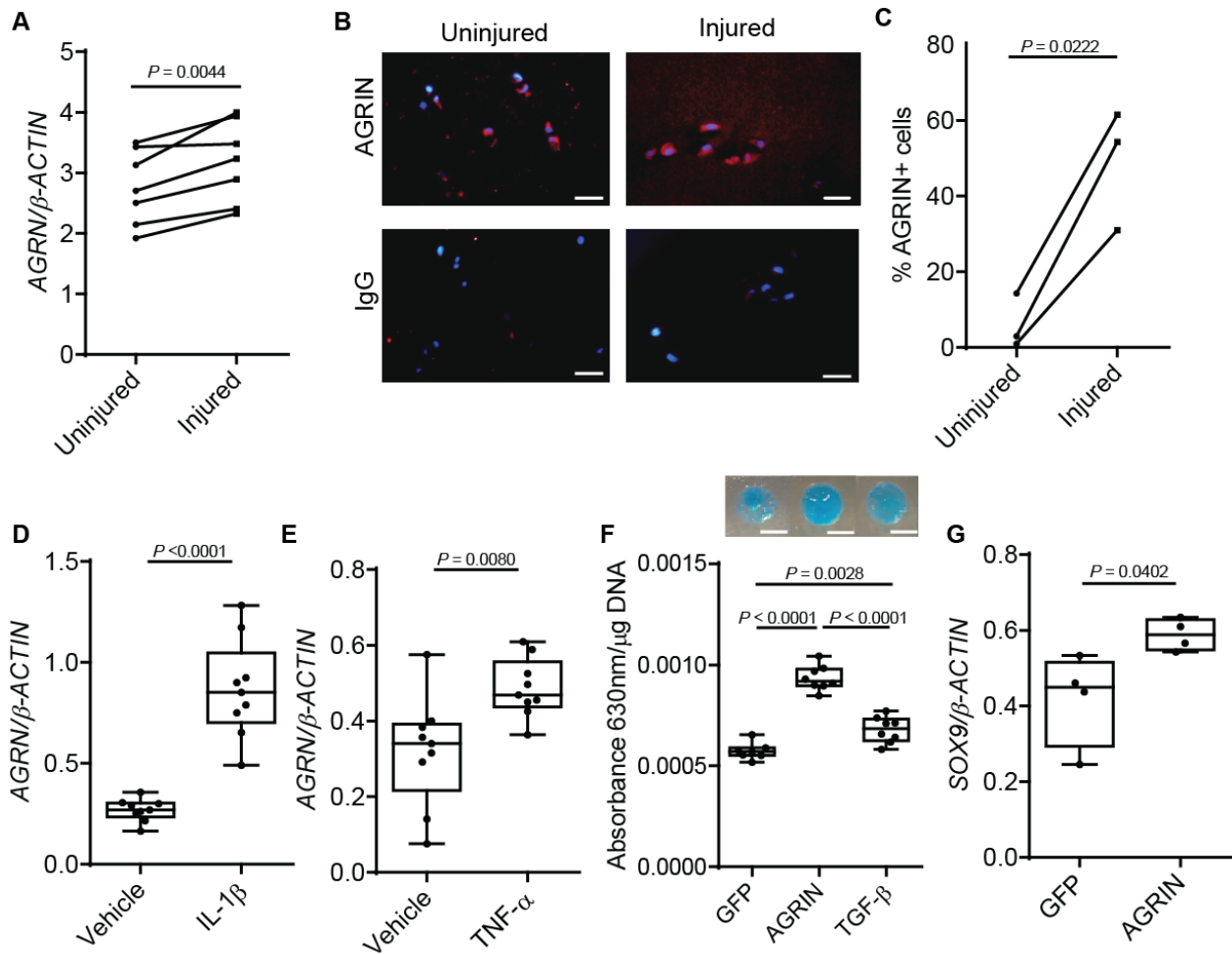
835 **Competing interests:** FD has received consultancy fees from Samumed and UCB. FD and SE
836 have filed a patent application for the use of agrin for cartilage regeneration.

837

838 **Data and materials availability:** All data associated with this study are present in the paper or
839 the Supplementary Materials. Plasmids are available upon request.

840 **Figure legends**

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842

843 **Fig. 1. Agrin is upregulated after cartilage injury, induces chondrogenesis in MSCs, and is**

844 **chondrogenic in joint-resident MSCs. (A) RT-PCR for *AGRN* of human adult articular cartilage**

845 **explants after mechanical injury or in control conditions ($n = 7$), paired t-test $P = 0.0044$. Individual**

846 **values plotted. (B) AGRIN immunostaining (red) of human adult articular cartilage explants after**

847 **mechanical injury or in control conditions; bars 50 μm , counterstained with DAPI (blue). (C)**

848 **Quantification of AGRIN staining normalized for number of cells ($n = 3$), paired t-test $P = 0.0222$.**

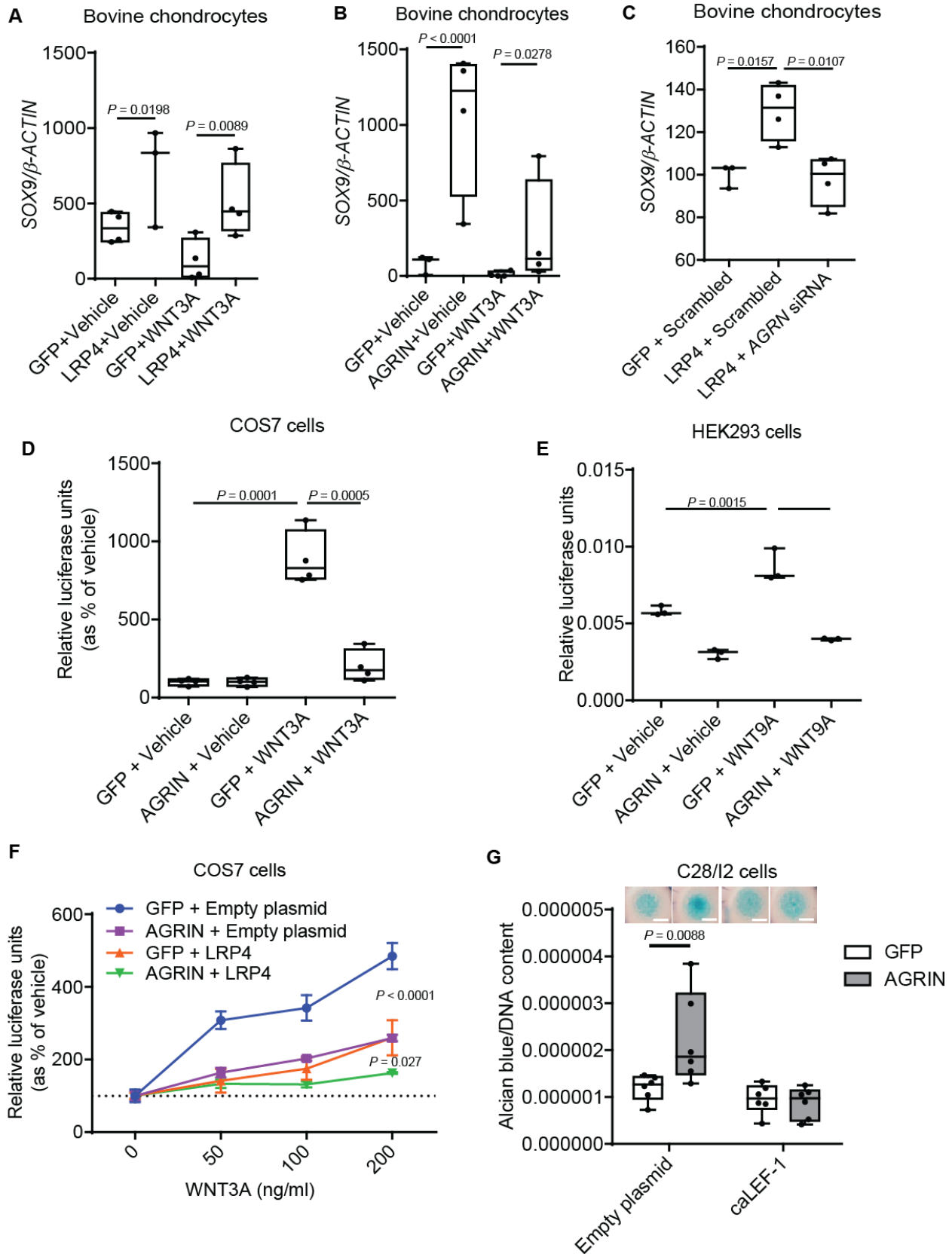
849 **Individual values plotted. (D) RT-PCR for *AGRN* in C28/I2 chondrocytes treated for 3 days with**

850 **IL-1 β (20 ng/ml, $n = 9$, t-test $P < 0.0001$) or (E) TNF- α (20 ng/ml, $n = 8$, t-test $P = 0.0080$). (F)**

851 **Alcian blue staining and spectrophotometric quantitation of glycosaminoglycans in micromasses**

852 **of SM-MSCs over a feeder of growth-arrested COS7 cells overexpressing AGRIN ($n = 8$), GFP**

853 ($n = 7$), or TGF- β ($n = 8$) for 6 days, one-way ANOVA with Tukey's HSD post-hoc GFP vs
854 AGRIN $P < 0.0001$, GFP vs TGF- β $P = 0.0028$, AGRIN vs TGF- β $P < 0.0001$; bars 0.5mm (G) RT-
855 PCR for *SOX9* of SM-MSC micromasses overexpressing AGRIN or GFP ($n = 4$), t-test $P = 0.0402$.
856 Box and whiskers plots show all values, boxes extend from the 25th to 75th percentiles, error bars
857 span max to min values.



859 **Fig. 2. Agrin/LRP4 signaling activates chondrogenesis by inhibiting WNT signaling.**

860 (A-C) RT-PCR for *SOX9* of primary bovine chondrocytes transfected with (A and C) LRP4 or (B)

861 AGRIN and treated with (A-B) recombinant WNT3A or (C) co-transfected with *AGRN* siRNA (n

862 = 3, lined bars; n =4, square bars)); (A) *SOX9* levels were compared using a generalized linear

863 model followed by pairwise comparison within each WNT3A treatment (Tukey correction),

864 GFP+Vehicle vs LRP4+vehicle $P=0.0198$, GFP+WNT3A vs LRP4+WNT3A $P=0.0089$. (B)

865 Square root transformed *SOX9* levels were compared using a generalized linear model followed

866 by pairwise comparison within each WNT3A treatment (Tukey correction), AGRIN+Vehicle vs

867 GFP+Vehicle $P<0.0001$, AGRIN+WNT3A vs GFP+WNT3A $P= 0.0278$; (C) t-test,

868 GFP+Scrambled vs LRP4+Scrambled $P=0.0157$, LRP4+Scrambled vs LRP4+*AGRN* siRNA

869 $P=0.0107$. (D) TOPFlash reporter assay in COS7 cells transduced with AGRIN or GFP and treated

870 with recombinant WNT3A (100 ng/ml) ($n = 4$); t-test GFP vehicle vs GFP WNT3A $P=0.0001$,

871 GFP WNT3A vs AGRIN WNT3A $P=0.0005$. (E) TOPFlash reporter assay in HEK293 cells

872 transfected with AGRIN or GFP and treated with recombinant WNT9A (200 ng/ml) ($n = 3$); one

873 way ANOVA with Tukey GFP + Vehicle vs GFP+WNT9A $P=0.0015$, GFP+WNT9A vs

874 AGRIN+WNT9A $p<0.0001$. (F) TOPFlash reporter assay of COS7 cells stably expressing AGRIN

875 or GFP and transfected with either Empty plasmid or LRP4 plasmid and treated with increasing

876 doses of recombinant WNT3A ($n = 4$; two-way ANOVA – Tukey HSD. AGRIN vs GFP

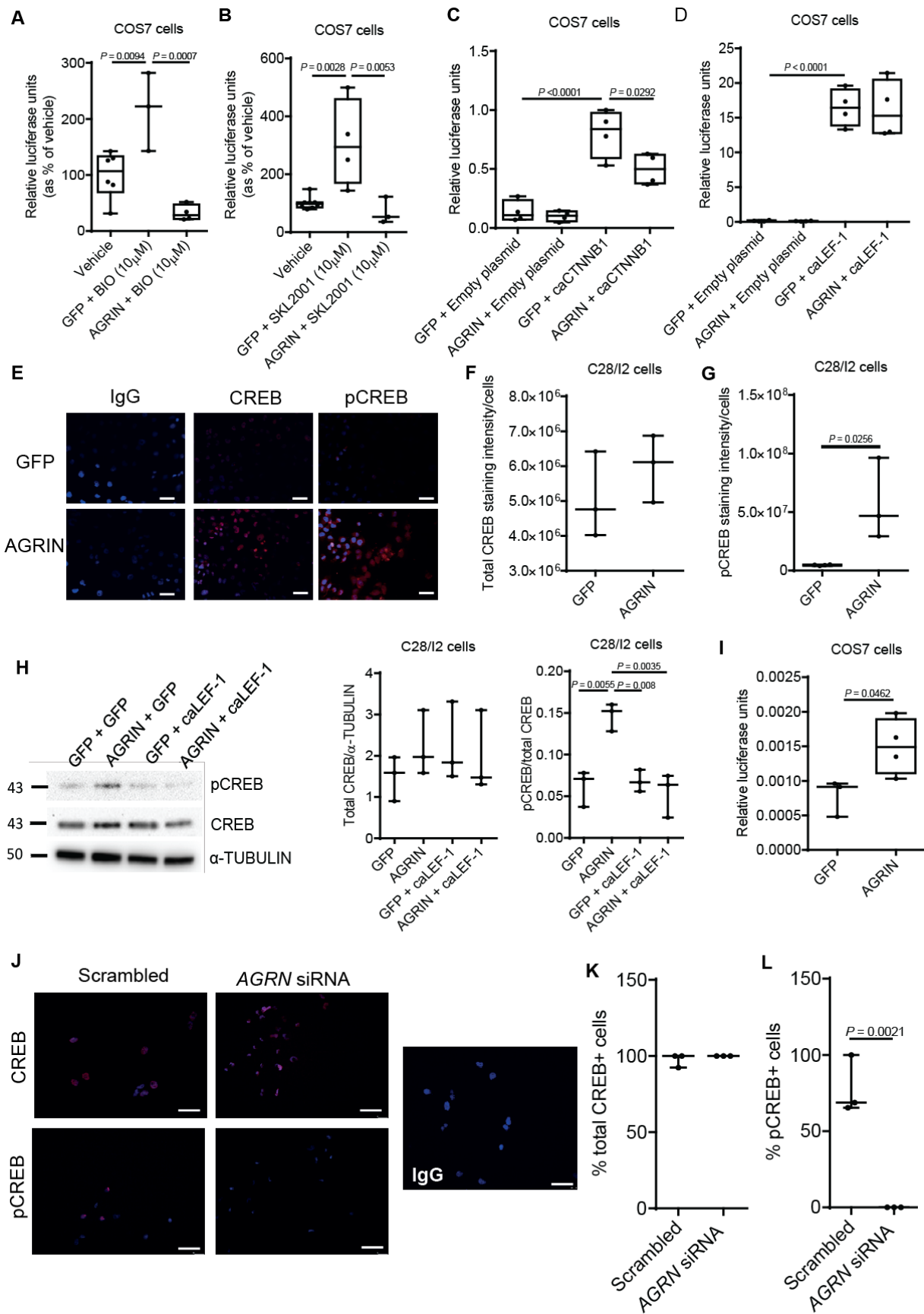
877 $P<0.0001$; AGRIN+LRP4 vs AGRIN $P= 0.027$. Mean values with SEM are plotted. (G) Alcian

878 blue staining and quantification of C28I/2 chondrocytes in micromass culture 4 days after

879 transfection with AGRIN or GFP with or without caLEF-1 ($n = 4$) two-way ANOVA $P=0.0088$;

880 bars 0.5 mm. Box and whiskers plots show all values, boxes extend from the 25th to 75th

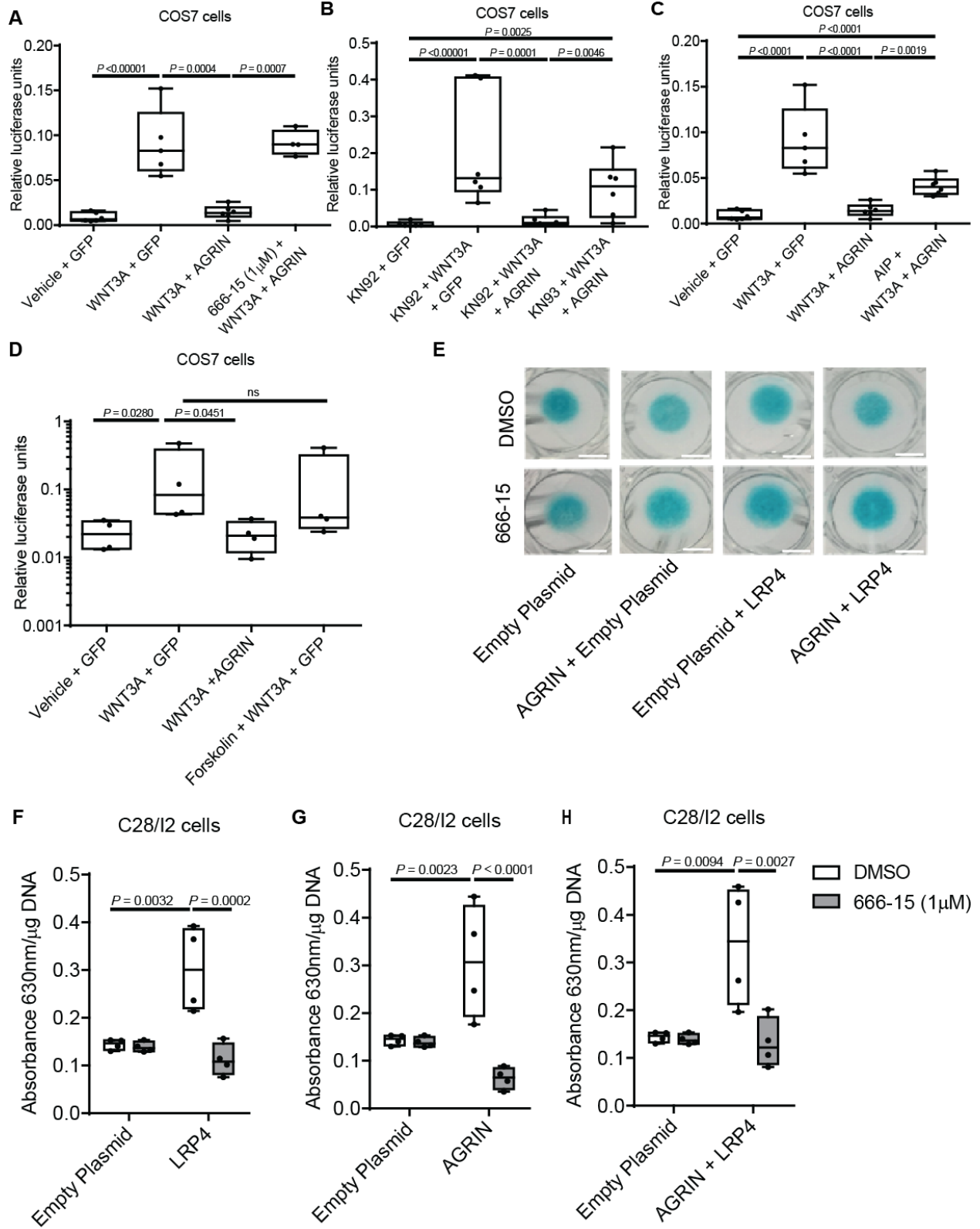
881 percentiles, error bars span max to min values.



884 **Fig. 3. Agrin inhibits canonical WNT signaling downstream of β -catenin and activates**
885 **CREB-dependent transcription.**

886 (A) TOPFlash reporter assay in COS7 cells transduced with AGRIN or GFP treated with BIO,
887 (GFP+ Vehicle, $n = 6$, GFP + BIO $n = 3$, AGRIN+ BIO $n = 4$), (B) SKL2001, (GFP + Vehicle n
888 = 8, GFP + SKL2001 $n = 4$, AGRIN + SKL2001 $n = 3$), (C) transfected with caCTNNB1(Δ ex3)
889 ($n = 4$) or (D) constitutively active LEF-1 ($n = 4$); (A to D) one-way ANOVA followed by Tukey's
890 HSD post-hoc. (A) Vehicle vs GFP+BIO $P=0.0094$, GFP+BIO vs AGRIN+BIO $P=0.0007$, (B)
891 Vehicle vs GFP+SKL2001 $P=0.0028$, GFP+SKL2001 vs AGRIN+SKL2001 $P=0.0053$, (C)
892 GFP+Empty plasmid vs AGRIN+Empty plasmid $P<0.0001$, GFP+caCTNNB1 vs
893 AGRIN+caCTNNB1 $P=0.0292$, (D) GFP+Empty plasmid vs GFP+caLEF1 $P<0.0001$. (E)
894 Immunostaining for CREB or phosphorylated CREB (pCREB) in C28/I2 cells 24 hr after
895 transfection with AGRIN or GFP (red) with DAPI counterstain (blue). Quantification in (F and G)
896 ($n = 3$); (G) t-test $P=0.0256$. (H) C28/I2 chondrocytes were cultured for 3 days in micromass,
897 transfected as indicated, and CREB phosphorylation (p-CREB) was assessed by western blotting
898 ($n = 3$); two-way ANOVA GFP vs AGRIN $P=0.0055$, AGRIN vs GFP+caLEF1 $P=0.008$, AGRIN
899 vs AGRIN+caLEF1 $P=0.0035$. (I) CREB reporter assay in COS7 cells transfected with AGRIN
900 ($n = 4$) or GFP ($n = 3$); t-test $P=0.0462$. (J) Immunostaining for CREB and phosphorylated CREB
901 (pCREB) (red) with DAPI counterstain (blue) in C28/I2 cells 24hr after transfection with
902 Scrambled or *AGRN* siRNA and quantification in (K and L) ($n = 3$); t-test $P=0.0021$. Box and
903 whiskers plots show all values, boxes extend from the 25th to 75th percentiles, error bars span max
904 to min values.

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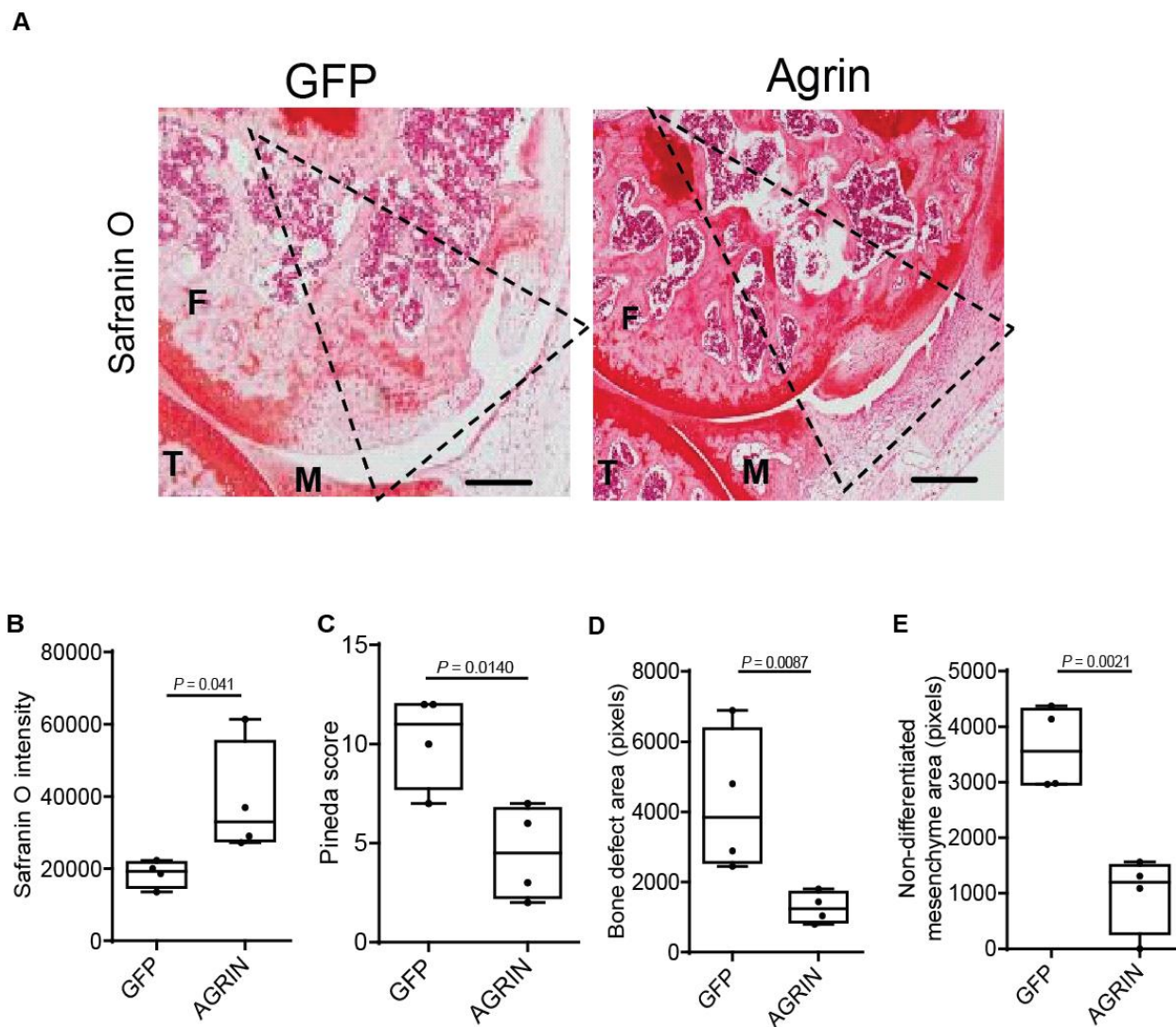


907 **Figure 4. Agrin requires CREB for its capacity to suppress WNT signaling and induce**
908 **chondrogenesis.**

909 (A) TOPFlash reporter assay in COS7 cells transfected with Agrin or GFP 24 hours after treatment
910 with WNT3A in the presence or the absence of the CREB inhibitor 666-15 ($n = 4$). (B and C)
911 TOPFlash reporter assay in COS7 cells transfected with AGRIN or GFP 24 hrs after WNT3A (200
912 ng/ml) treatment in the presence or in the absence of (B) the CaMKII inhibitor KN93 or its inactive
913 control KN92 or (C) the CaMKII inhibitor AIP; (A to C) two-way ANOVA Tukey's HSD post-
914 hoc, (A) Vehicle+GFP vs WNT3a+GFP $P < 0.00001$, WNT3a+GFP vs WNT3a+AGRIN
915 $P = 0.0004$, WNT3a+AGRIN vs 666-15+WNT3a+AGRIN $P = 0.0007$; (B) KN92+GFP vs
916 KN92+WNT3A+GFP $P < 0.00001$, KN92+WNT3A+GFP vs KN92+WNT3A+AGRIN $P = 0.0001$,
917 KN92+WNT3A+AGRIN vs KN93+WNT3A+AGRIN $P = 0.0046$, KN92+GFP vs
918 KN93+WNT3A+AGRIN $P = 0.0025$, (C) Vehicle+GFP vs WNT3A+GFP $P < 0.0001$,
919 Vehicle+GFP vs AIP+WNT3A+AGRIN $P < 0.0001$, WNT3A+GFP vs WNT3A+AGRIN
920 $P < 0.0001$, WNT3A+AGRIN vs AIP+WNT3A+AGRIN $P = 0.0019$. (D) TOPFlash reporter assay
921 in COS7 cells transfected with either AGRIN or GFP treated with WNT3A (200 ng/ml) and/or
922 Forskolin (10 μ M); Kruskal-Wallis, overall $P = 0.0168$. *Multiple comparison was carried out*
923 *using a Dunn test, P values obtained with the Benjamini-Hochberg correction* Vehicle vs WNT3A
924 $P = 0.0280$, WNT3A vs WNT3A+AGRIN $P = 0.0451$, Vehicle vs Forskolin + WNT3A +GFP. (A to
925 D $n = 4$). (E) Representative images of Alcian blue staining of C28/I2 chondrocytes in micromass
926 culture transfected with either empty plasmid or AGRIN and LRP4 in the presence or the absence
927 of the CREB inhibitor 666-15; bars 0.5 mm. (F to H) Glycosaminoglycans quantification from the
928 experiment in E ($n = 4$); log transformed values, one-way ANOVA, Tukey's HSD post-hoc, (F)
929 Empty plasmid vs LRP4 $P = 0.0032$, LRP4 vs LRP4+666-15 $P = 0.0002$, (G) Empty plasmid vs

930 AGRIN $P=0.0023$, AGRIN vs AGRIN+666-15 $P<0.0001$, (H) Empty plasmid vs LRP4+AGRIN
 931 $P=0.0094$, AGRIN+LRP4 vs AGRIN+LRP4+666-15 $P=0.0027$. Box and whiskers plots show all
 932 values, boxes extend from the 25th to 75th percentiles, error bars span max to min values.

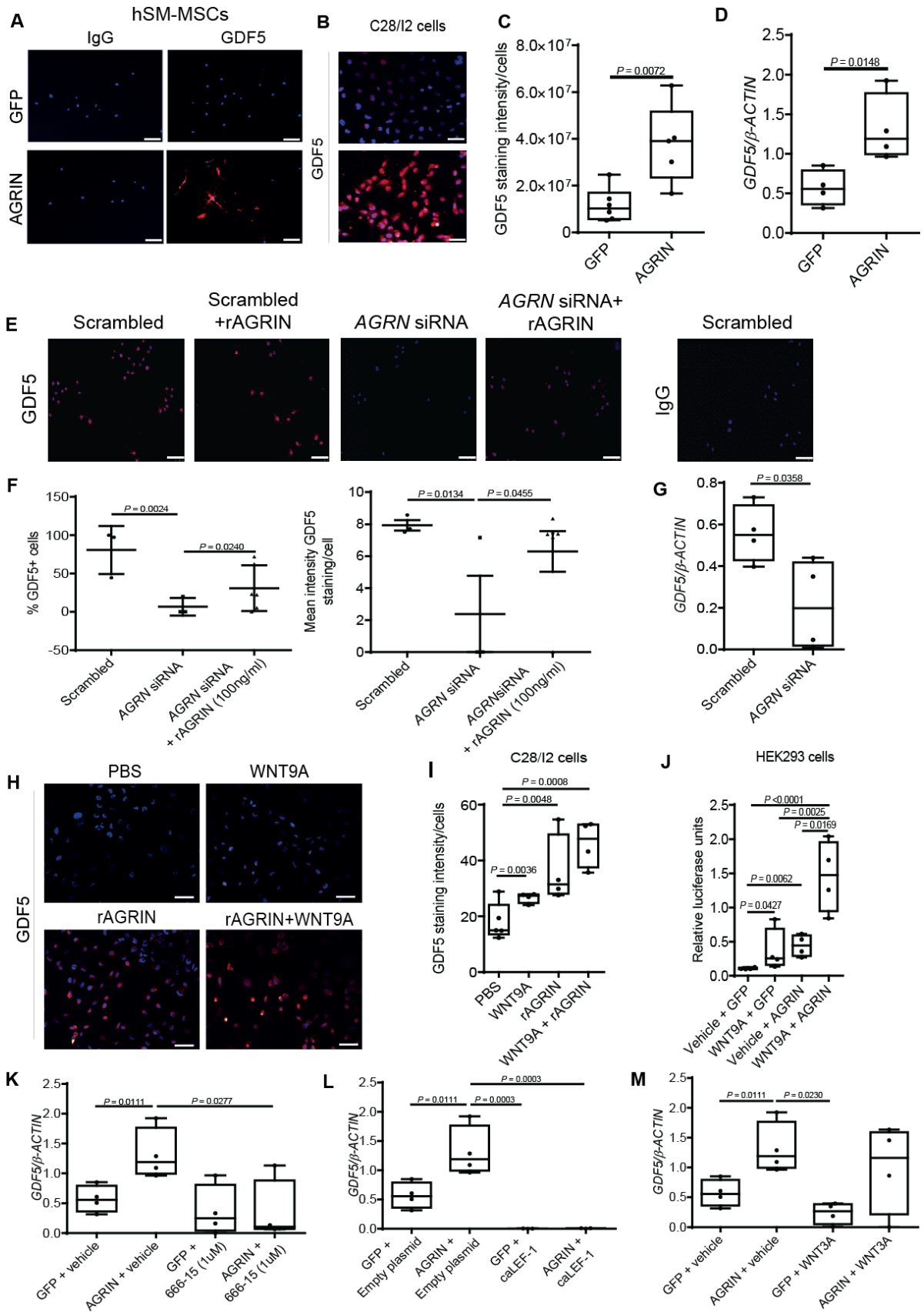
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935 **Fig. 5. Agrin supports repair morphogenesis and articular cartilage formation in vivo.**

936 (A) Representative safranin O staining of the femoral condyle of C57BL/6 mice 8 weeks after the
937 generation of an osteochondral defect filled with a collagen gel containing either AGRIN or GFP
938 ($n = 4$); bars 200 μm . The dotted lines represent the approximate location of the original defect. M
939 = meniscus; F = Femur; T = tibia (B) Quantification of Safranin O staining in the repair cartilage
940 layer ($n = 4$). T-test after logarithmic transformation $P=0.041$. (C) Pineda score of osteochondral
941 defect repair after 8 weeks (lower scores indicate better repair) ($n = 4$), Mann-Whitney U test,
942 $P=0.0140$. (D) Histomorphometric quantification of the residual bone defect. ($n = 4$; Welch Two
943 Sample t-test following log transformation; $P=0.0087$). (E) Quantification of the non-
944 differentiated (fibroblast-like) portion of the repair mesenchyme ($n = 4$; Welch Two Sample t-test
945 following log transformation; $P=0.0021$). Box and whiskers plots show all values, boxes extend
946 from the 25th to 75th percentiles, error bars span max to min values.

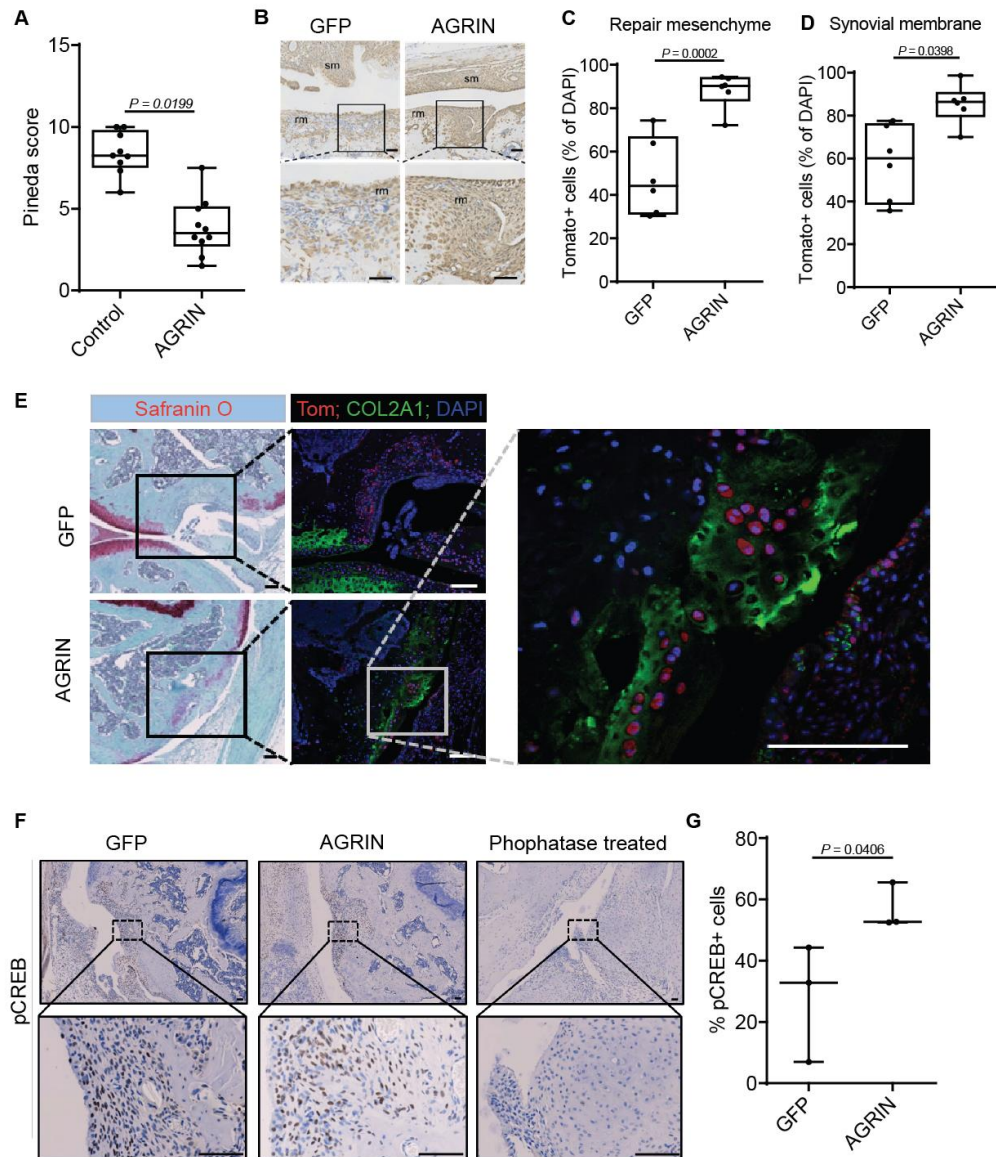


948 **Fig. 6. Agrin supports GDF5 expression in synovial membrane MSCs**

949 (A and B) GDF5 immunocytochemistry of SM-MSCs (A) or C28/I2 cells (B) transfected with
950 AGRIN or GFP (red) and cultured in monolayer for 24 hrs ($n = 4$); bars 50 μ m. DAPI counterstain.
951 (C) Quantification of GDF5 staining intensity in (B) normalized by number of cells; t-test
952 $P=0.0072$. (D) RT-PCR for *GDF5* in C28/I2 cells transfected with AGRIN or GFP and cultured
953 for 3 days in micromass ($n = 4$); t-test $P=0.0148$. (E) GDF5 immunocytochemistry (red)
954 counterstained with DAPI (blue) of C28/I2 cells cultured in monolayer for 24 hrs following
955 transfection with Scrambled or *AGRN* siRNA in the presence or absence of rAGRIN; bars 50 μ m.
956 (F) Quantification of % GDF5+ cells from (E) Scrambled $n = 3$; *AGRN* siRNA $n = 3$; *AGRN*
957 siRNA+rAGRIN $n = 6$) one-way ANOVA Scrambled vs *AGRN* siRNA $P=0.0024$, *AGRN* siRNA
958 vs *AGRN* siRNA+rAGRIN $P=0.024$; and mean intensity per cell, one-way ANOVA using
959 generalized linear model followed by pairwise comparison within each Scrambled vs *AGRN*
960 siRNA $P=0.0134$, *AGRN* siRNA vs *AGRN* siRNA+rAGRIN $P=0.0455$. (G) RT-PCR for *GDF5*
961 in C28/I2 cells transfected with Scrambled or *AGRN* siRNA cultured for 3 days in micromass (n
962 = 4); t-test $P= 0.0358$. (H) Immunostaining for GDF5 in C28/I2 chondrocytes treated with
963 recombinant AGRIN (300 ng/ml) and/or WNT9A (200 ng/ml) for 24 hrs; bars 50 μ m; and (I)
964 quantification ($n = 4$); after reciprocal transformation values were compared by one-way ANOVA
965 with Tukey HSD post-hoc for multiple comparisons $P=0.0008$; PBS vs WNT9A $P=0.0036$, PBS
966 vs rAGRIN $P=0.0048$, PBS vs WNT9A+AGRIN $P=0.0008$. (J) CREB reporter assay in HEK293
967 cells treated with recombinant AGRIN (300 ng/ml)and/or WNT9A (200 ng/ml) ($n = 4$); one-way
968 ANOVA with Tukey test for multiple comparisons on log transformed values $P=$ Vehicle+GFP
969 vs WNT9A+GFP $P=0.0427630$, Vehicle+GFP vs Vehicle+AGRIN $P=0.0063$, Vehicle+GFP vs
970 WNT9A+AGRIN $P<0.0001$, Vehicle+AGRIN vs WNT9A+AGRIN $P=0.0169$, WNT9A+GFP vs

971 WNT9A+AGRIN $P=0.0025$. (**K** to **M**) RT-PCR for *GDF5* mRNA in C28/I2 cells transfected with
972 AGRIN or GFP plasmids, cultured in micromass for 4 days and treated in the presence of (**K**) 666-
973 15 (1 μM) or vehicle or (**L**) co-transfected with caLEF1 plasmid or (**M**) recombinant WNT3A
974 (200 ng/ml) ($n = 4$); one-way ANOVA with Tukey's HSD post-hoc (**K**) GFP vs AGRIN $P=0.0111$,
975 AGRIN+vehicle vs AGRIN666-15 $P=0.0277$, (**L**) GFP vs AGRIN $P=0.0111$, AGRIN vs
976 GFP+caLEF1 $P=0.0003$, AGRIN vs AGRIN+caLEF1 $P=0.00033$, (**M**) AGRIN+vehicle vs
977 GFP+WNT3A $P=0.0230$. Box and whiskers plots show all values, boxes extend from the 25th to
978 75th percentiles, error bars span max to min values.

979

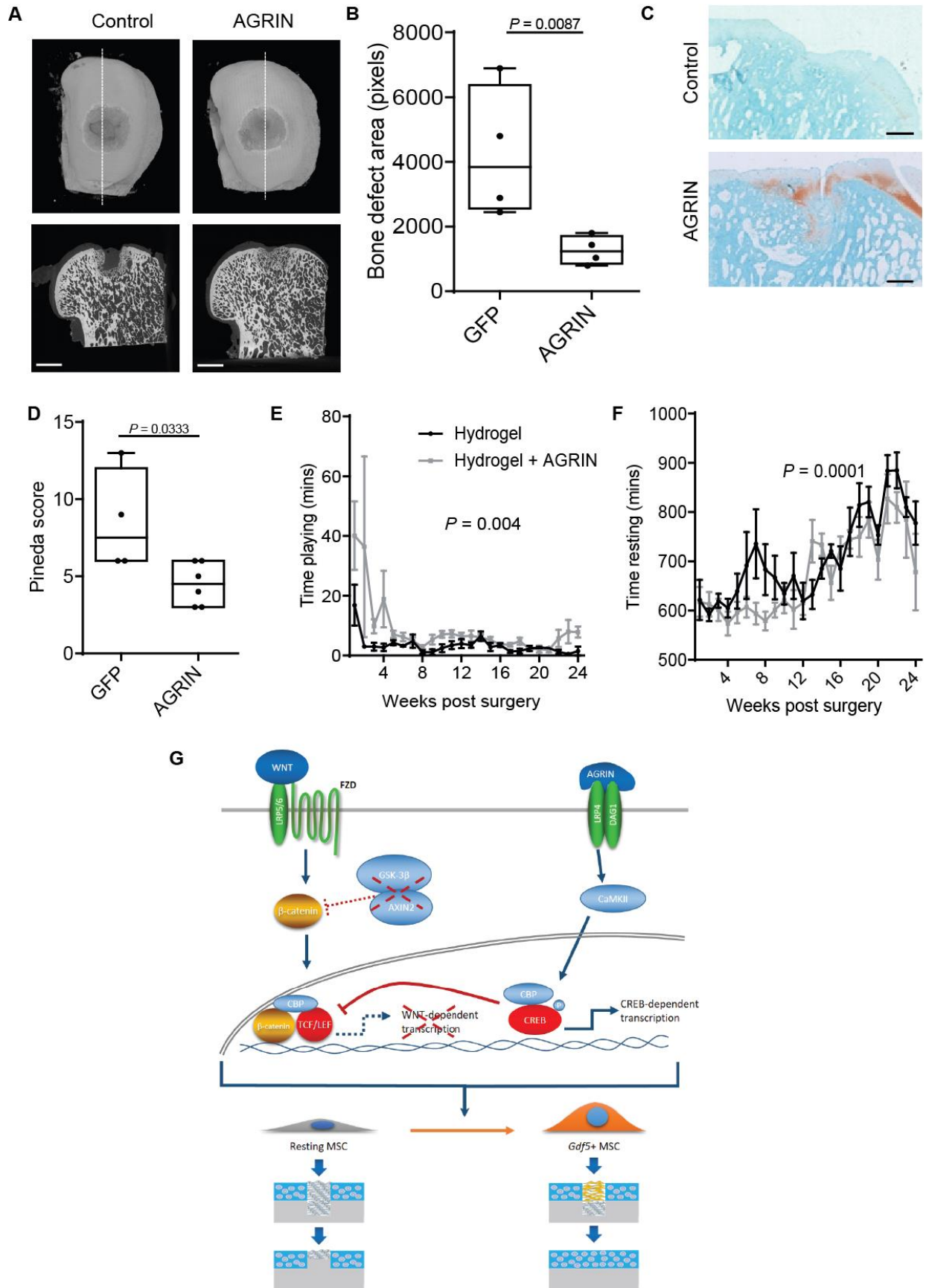


980

981 **Fig. 7. Agrin-induced joint surface repair is associated with increased recruitment of Gdf5-**
 982 **lineage joint stem cells and CREB phosphorylation in the repair mesenchyme.**

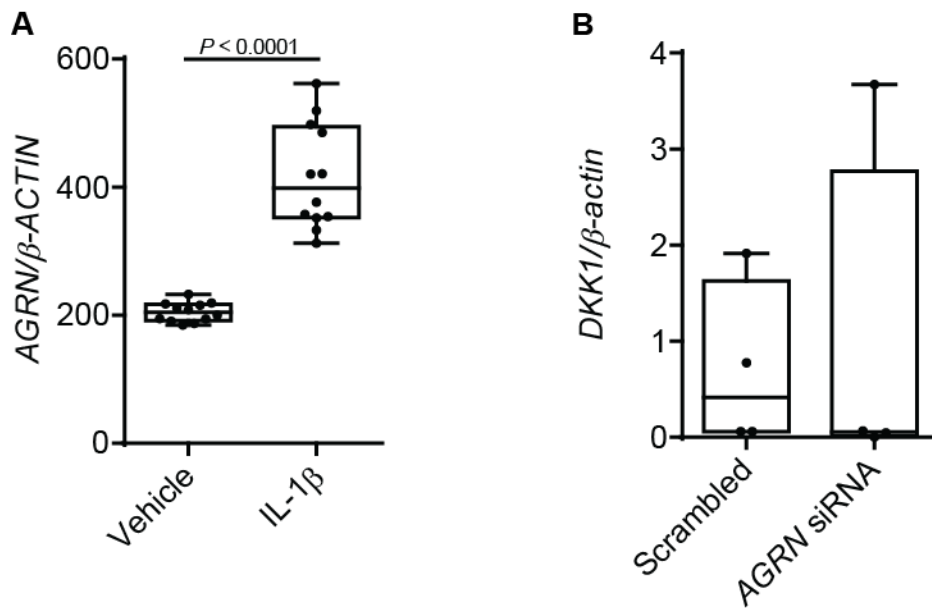
983 (A) Pineda score of *Gdf5*:Tom transgenic mice 8 weeks after the generation of an osteochondral
 984 defect filled with either agrin or GFP ($n = 9$ GFP, $n = 10$ AGRIN; Mann-Whitney U test;
 985 $P=0.01994$). (B) Immunohistochemistry for Tomato in the defect of *Gdf5*:Tom transgenic mice 3

986 weeks after the generation of an osteochondral defect filled with a collagen gel containing either
987 AGRIN or GFP; sm=synovial membrane; rm=repair mesenchyme ($n = 6$). Boxed region shown at
988 higher magnification below; bars 50 μm . (C) Quantification of Tom⁺ cells in the repair
989 mesenchyme and (D) in the synovial membrane; t-test (C) $P=0.0002$, (D) $P=0.0398$. Box and
990 whiskers plots show all values, boxes extend from the 25th to 75th percentiles, error bars span max
991 to min values. (E) Safranin O (left panels) and immunofluorescence (right) for Tomato (red) and
992 Collagen type 2 (green) 8 weeks post-surgery, counter stained for DAPI (blue). Boxed region
993 shown at higher magnification below; bars 100 μm . Immunohistochemistry (F) and quantification
994 (G) of phospho-CREB (pCREB) in the repair mesenchyme of *Gdf5*;Tom mice treated with AGRIN
995 or control, 3 weeks after the generation of an osteochondral defect ($n = 3$); phosphatase treatment
996 was used as staining control; (F) Welch two sample t-test of squared values $P=0.04058$; bars 100
997 μm . Mean values with SEM are plotted.



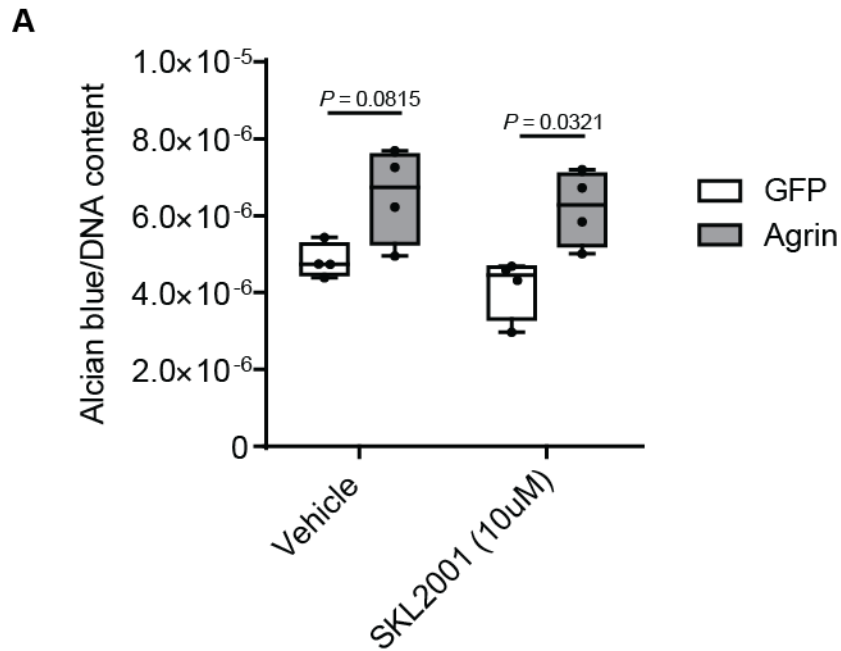
999 **Figure 8. A single administration of agrin in critical size joint surface defects in sheep**
1000 **regenerates the articular cartilage.**

1001 Sheep underwent the generation of an osteochondral defect that was filled with a collagen gel
1002 containing AGRIN or GFP and killed after 6 months ($n = 6$). (A) Representative μ CT images of
1003 defects at 6-month time-point ($n = 6$; scale bar 5mm) and (B) quantification of the residual non-
1004 calcified defect area ($n = 6$ control and $n = 5$ Agrin; Welch t-test after log transformation,
1005 $P=0.0134$). (C) Safranin O staining of the joint surface defect area, bars 200 μ m. (D) Pineda
1006 score ($n = 4$ controls and $n = 6$ AGRIN; Mann-Whitney U test $P=0.0333$). (E) Time spent playing
1007 (two-way ANOVA, Treatment $P=0.00495$) and (F) time spent resting (two-way ANOVA,
1008 Treatment $P=0.00043$). (G) AGRIN promotes the morphogenesis of the repair mesenchyme at
1009 the site of cartilage injury. This process involves the activation of CREB-dependent upregulation
1010 of *Gdf5* and suppression of WNT signaling downstream of β -catenin.



1015

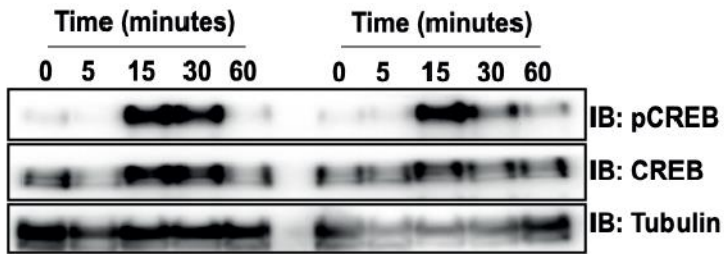
1016 **Supplementary figure 1.** (A) Microarray human chondrocytes – (mined from GEO accession
1017 GSE75181³⁰), $P < 0.0001$ ($n = 12$). (B) RT-PCR for *DKK1* in C28/I2 cells transfected with
1018 Scrambled or *AGRN* siRNA cultured for 3 days in micromass ($n = 4$).



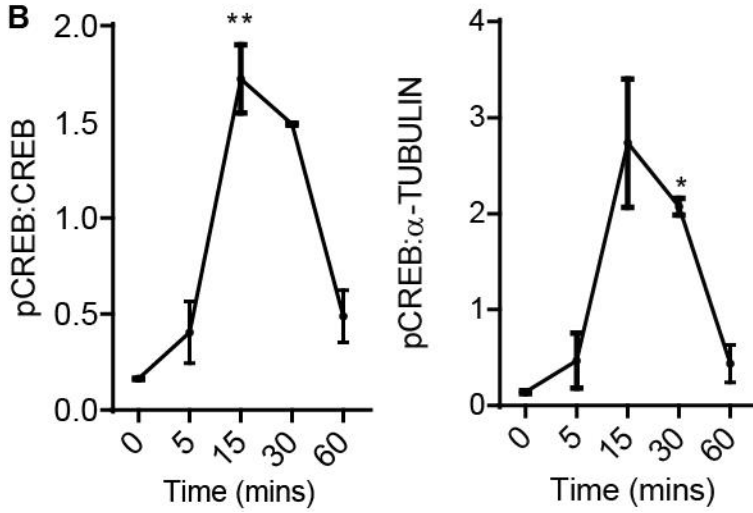
1019

1020 **Supplementary figure 2.** Alcian blue staining and quantification of C28I/2 chondrocytes in
1021 micromass culture 4 days after transfection with GFP or AGRIN with or without SKL2001 ($n =$
1022 4), two-way ANOVA $P = 0.0013$, GFP + Vehicle vs AGRIN + Vehicle $P = 0.0815$, GFP +
1023 SKL2001 vs Agrin + SKL2001 $P = 0.0321$.

A

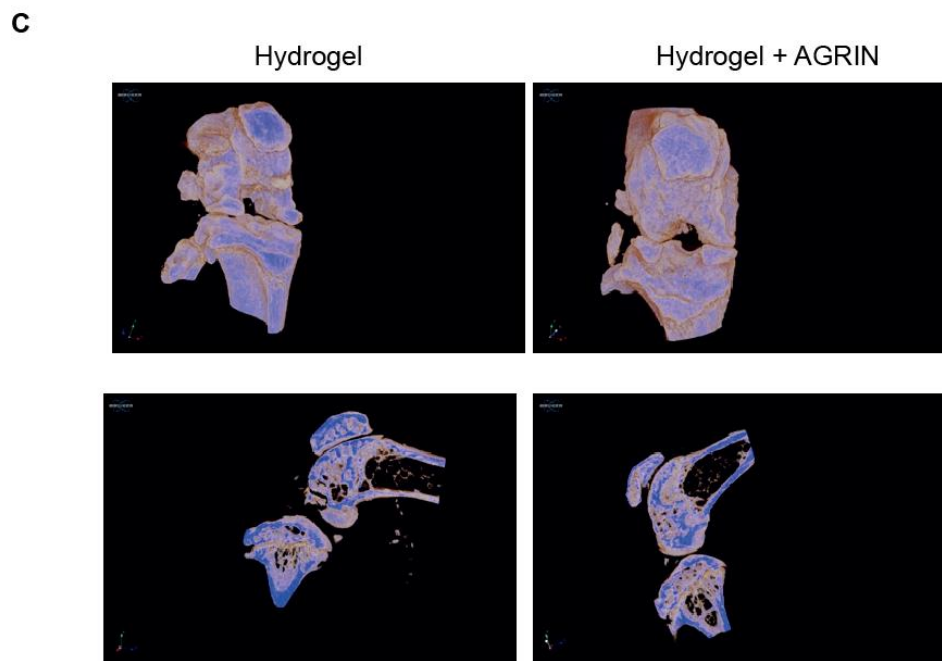
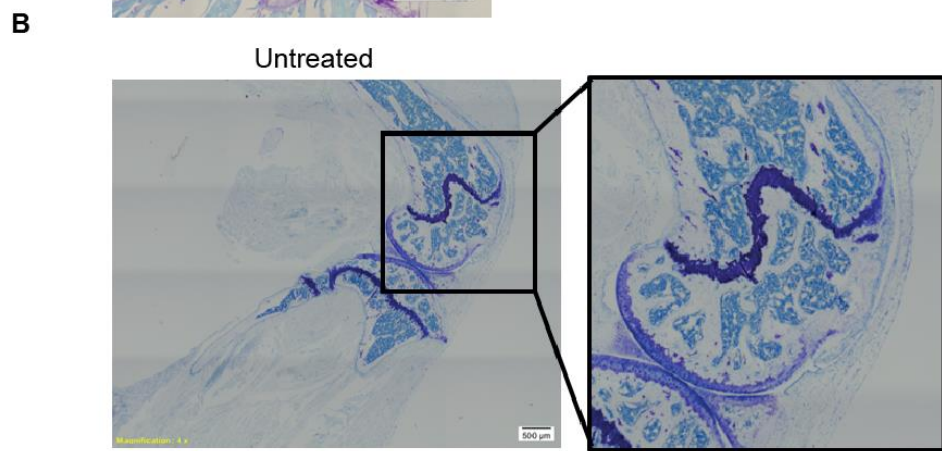
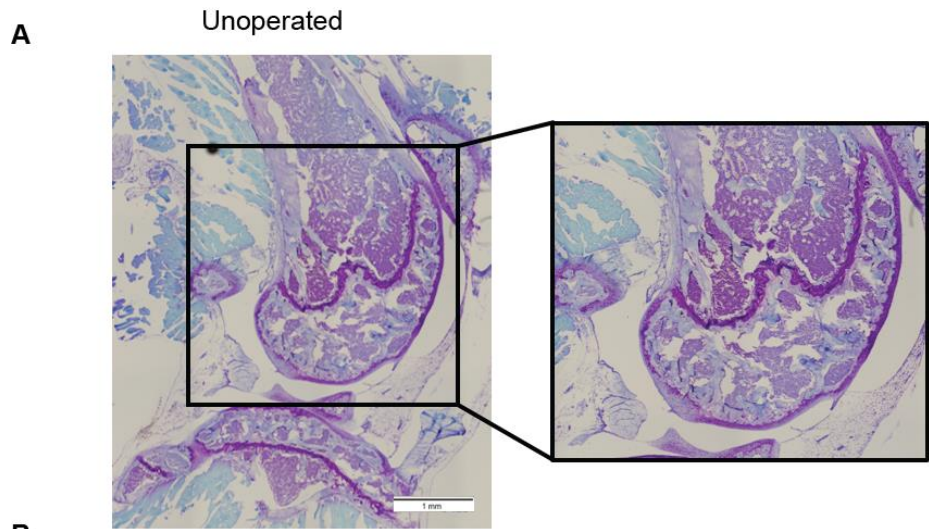


B

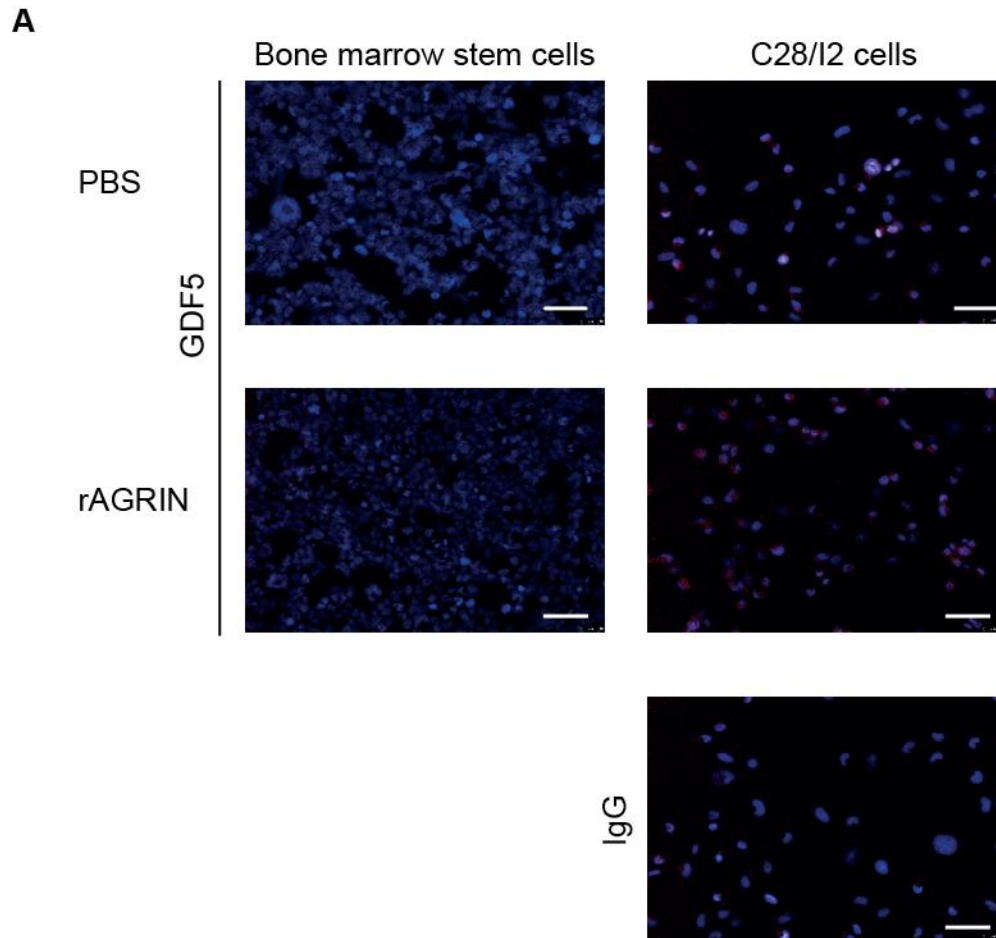


1024

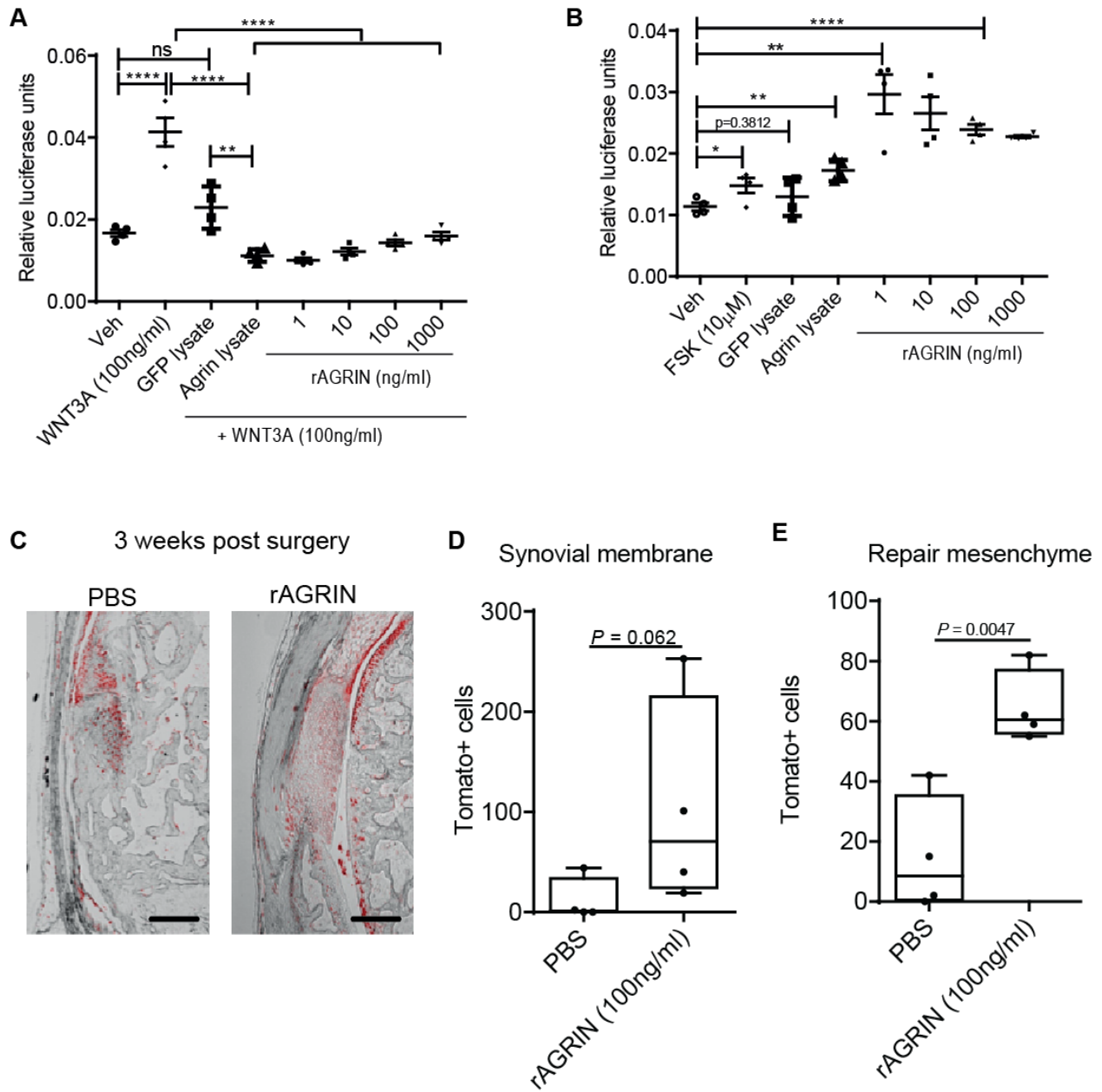
1025 **Supplementary figure 3. (A)** Temporal analysis by western blot of pCREB in C28/I2 cells
1026 treated with 100 ng/ml rAgrin normalized to **(B)** CREB and tubulin (one-way ANOVA followed
1027 by Dunnett's multiple comparison test) ($n = 2$).



1029 **Supplementary figure 4.** (A) Unoperated joint of a mouse stained with Toluidine blue. (B)
1030 Untreated subchondral defect in mice 8 weeks post-surgery stained with toluidine blue. (C)
1031 MicroCT of *Gdf5*-Cre;Tom mice 8 weeks post-surgery.



1033 **Supplementary figure 5.** Immunofluorescence for GDF5 in murine bone marrow derived stem
1034 cells treated with rAgrin (100 ng/ml) compared to C28/I2 chondrocytes as positive control; bars
1035 50 μ m ($n = 4$).



1036

1037 **Supplementary figure 6.** (A) TOPFlash reporter assay of HEK293 cells cultured in the presence
 1038 of COS7-AGRIN or COS7-GFP cell lysate (used at ratio equal to in vivo) and compared to
 1039 increasing doses of recombinant Agrin (rAgrin) in the presence of WNT3A ($n = 4$). (B) CREB
 1040 reporter assay of HEK293 cells cultured in the presence of COS7-AGRIN or COS7-GFP cell

1041 lysate (used at ratio equal to in vivo) and compared to increasing doses of recombinant AGRIN
1042 (rAGRIN), forskolin was used as a positive control ($n = 4$); (**A** and **B**) one-way ANOVA,
1043 $*=p<0.05$, $**=p<0.01$, $***=p<0.001$, $****=p<0.0001$. (**C**) Immunofluorescence for Tomato in
1044 the defect of *Gdf5*:Tom transgenic mice 3 weeks after the generation of a joint surface defect
1045 filled with a collagen gel containing either rAGRIN (100 ng/ml) or PBS; bars 200 μm .
1046 Quantification of the number of Tom+ cells in (**D**) the synovial membrane and (**E**) repair
1047 mesenchyme in the defect site.

1048

1049

1050 **Supplementary table 1.** Ages of operated sheep. COS7-GFP treated group mean age 2.9 years \pm
1051 0.45 (SD); COS7-AGRIN treated group mean age 2.95 years \pm 0.40 (SD).

Treatment group	Age	Exclusion
COS7-GFP	2.6	
COS7-GFP	2.5	
COS7-GFP	3.3	
COS7-GFP	3.6	Osteomyelitis in the forelimb, CT analysis only
COS7-GFP	2.6	Cyst, CT analysis only
COS7-GFP	2.8	
COS7-AGRIN	3.4	
COS7-AGRIN	2.5	
COS7-AGRIN	2.5	
COS7-AGRIN	2.8	
COS7-AGRIN	3.3	
COS7-AGRIN	3.2	

1052

1053

1054 **Supplementary table 2.** Antibody supplier and usage information.

Antibodies	Species	Code	Supplier	Western Blotting	Immunofluorescence
AGRIN	Rabbit	H300	Santa Cruz Biotechnology		1:100
CREB	Rabbit	9197	Cell Signaling	1:1000	1:800
pCREB	Rabbit	9198	Cell signaling	1:1000	1:800
GDF5	Rabbit	93855	Abcam		1:1000
IgG	Rabbit	37415	Abcam		1:100-1:800
AlexaFluro55 5	Goat	150078	ThermoFisher		1:300
α -TUBULIN	Mouse	T5168	Sigma	1:5000	
COL2A1	Rabbit	Ab212 91	Abcam		1:100
Tomato	Goat	Ab008 1	Sicgen		1:100
Tomato	Rabbit	600- 401- 379	Rockland		1:600

1055

1056 **Supplementary table 3.** Recombinant proteins and reagents supplier and usage information.

Recombinant	Concentration	Resuspended in	Code	Supplier
IL-1 β	20 ng/ml	0.1% BSA in PBS	201-LB	R&D systems
TNF- α	20 ng/ml	0.1% BSA in PBS	210-TA	R&D systems
WNT3A	50-200 ng/ml	0.1% BSA in PBS	5036- WN	R&D systems
Wnt-9A	200 ng/ml	0.1% BSA in PBS	8148- WN	R&D systems
SKL2001	10 μ M	DMSO	681667	Calbiochem
BIO	10 μ M	DMSO	3194	Tocris
MeBIO	10 μ M	DMSO	3873	Tocris
Forskolin	10 μ M	DMSO	F6886	Sigma
666-15	1 μ M	DMSO	5661	Tocris
KN92	10 μ M	DMSO	4130	Tocris
KN93	10 μ M	DMSO	1278	Tocris
AIP	5 μ M	DMSO	5959	R&D systems

1057

1058

1059 **Supplementary table 4.** Primer and siRNA sequences

	Sense	Antisense	Amplicon (bps)
<i>hβ-ACTIN</i>	TGACGGGGTCACCCACACTGTG CCCATCTA	CTAGAAGCATTGCGGTGGACG ATGGAGG	661
<i>hAGRN</i>	CCTGACCCTCAGCTGGCCCT	AGATACCCAGGCAGGCGGCA	136
<i>hDKK</i>	ATTCCAACGCTATCAAGAAC	CCAAGGTGCTATGATCATTACC	384
<i>hGDF5</i>	AGGCAACAGCAGCGTGAAGT	GGTCATCTTGCCCTTTGTCAA	77
<i>hSOX9</i>	GAACGCACATCAAGACGGAG	TCTCGTTGATTCGCTGCTC	631
<i>bβ-ACTIN</i>	AGGAGTCGGTTGGATCGAGCA	GGGAAGGCAAAGGACTTCCTGT AAC	136
<i>bSOX9</i>	ACTCTGGGCAAGCTCTGGAGAC T	GGCGCGGCTGGTACTTGTAGTC C	121
<i>hAGRN</i> siRNA	CCUUUGUCGAGUACCUCAACGC UGU	ACAGCGUUGAGGUACUCGACA AAGG	

1060 A Stealth RNAi negative control duplex of low GC content was used as a negative control

1061 (Invitrogen). h:human, b:bovine.

1062 **R script for download and statistical analysis of microarray data from Comblain et al. (30)**

1063 # Version info: R 3.2.3, Biobase 2.30.0, GEOquery 2.40.0, limma 3.26.8

1064 # R scripts generated Wed Sep 4 06:31:58 EDT 2019

1065

1066 #####

1067 # Differential expression analysis with limma

1068 library(Biobase)

1069 library(GEOquery)

1070 library(limma)

1071

1072 # load series and platform data from GEO

1073

1074 gset <- getGEO("GSE75181", GSEMatrix =TRUE, AnnotGPL=TRUE)

1075 if (length(gset) > 1) idx <- grep("GPL10558", attr(gset, "names")) else idx <- 1

1076 gset <- gset[[idx]]

1077

```
1078 # make proper column names to match toptable

1079 fvarLabels(gset) <- make.names(fvarLabels(gset))

1080

1081 # group names for all samples

1082 gsms <- "000000000000XXXXXXXXXXXXXXXX111111111111XXXXXXXXXXXXXXXX"

1083 sml <- c()

1084 for (i in 1:nchar(gsms)) { sml[i] <- substr(gsms,i,i) }

1085

1086 # eliminate samples marked as "X"

1087 sel <- which(sml != "X")

1088 sml <- sml[sel]

1089 gset <- gset[ ,sel]

1090

1091 # log2 transform

1092 ex <- exprs(gset)

1093 qx <- as.numeric(quantile(ex, c(0., 0.25, 0.5, 0.75, 0.99, 1.0), na.rm=T))
```



```

1094 LogC <- (qx[5] > 100) ||
1095     (qx[6]-qx[1] > 50 && qx[2] > 0) ||
1096     (qx[2] > 0 && qx[2] < 1 && qx[4] > 1 && qx[4] < 2)
1097 if (LogC) { ex[which(ex <= 0)] <- NaN
1098     exprs(gset) <- log2(ex) }
1099
1100 # set up the data and proceed with analysis
1101 sml <- paste("G", sml, sep="") # set group names
1102 fl <- as.factor(sml)
1103 gset$description <- fl
1104 design <- model.matrix(~ description + 0, gset)
1105 colnames(design) <- levels(fl)
1106 fit <- lmFit(gset, design)
1107 cont.matrix <- makeContrasts(G1-G0, levels=design)
1108 fit2 <- contrasts.fit(fit, cont.matrix)
1109 fit2 <- eBayes(fit2, 0.01)

```

```

1110 tT <- topTable(fit2, adjust="fdr", sort.by="B", number=250)

1111

1112 tT                                     <-                               subset(tT,
1113 select=c("ID","adj.P.Val","P.Value","t","B","logFC","Gene.symbol","Gene.title"))

1114 write.table(tT, file=stdout(), row.names=F, sep="\t")

1115

1116

1117 #####

1118 # Boxplot for selected GEO samples

1119 library(Biobase)

1120 library(GEOquery)

1121

1122 # load series and platform data from GEO

1123

1124 gset <- getGEO("GSE75181", GSEMatrix =TRUE, getGPL=FALSE)

1125 if (length(gset) > 1) idx <- grep("GPL10558", attr(gset, "names")) else idx <- 1

```

```

1126  gset <- gset[[idx]]

1127

1128  # group names for all samples in a series

1129  gsms <- "000000000000XXXXXXXXXXXX111111111111XXXXXXXXXXXX"

1130  sml <- c()

1131  for (i in 1:nchar(gsms)) { sml[i] <- substr(gsms,i,i) }

1132  sml <- paste("G", sml, sep="") set group names

1133

1134  # eliminate samples marked as "X"

1135  sel <- which(sml != "X")

1136  sml <- sml[sel]

1137  gset <- gset[ ,sel]

1138

1139  # order samples by group

1140  ex <- exprs(gset)[ , order(sml)]

1141  sml <- sml[order(sml)]

```

```
1142 fl <- as.factor(sml)

1143 labels <- c("Control", "IL-1")

1144

1145 # set parameters and draw the plot

1146 palette(c("#dfeaf4", "#f4dfdf", "#AABBCC"))

1147 dev.new(width=4+dim(gset)[[2]]/5, height=6)

1148 par(mar=c(2+round(max(nchar(sampleNames(gset)))/2),4,2,1))

1149 title <- paste ("GSE75181", '/', annotation(gset), " selected samples", sep =")

1150 boxplot(ex, boxwex=0.6, notch=T, main=title, outline=FALSE, las=2, col=fl)

1151 legend("topleft", labels, fill=palette(), bty="n")

1152

1153
```