

1 **Skeletal cell YAP and TAZ redundantly promote bone development by regulation of collagen I**
2 **expression and organization.**

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16
17 **ABSTRACT**

18 The functions of the transcriptional co-activators YAP and TAZ in bone are controversial. Each has
19 been observed to either promote or inhibit osteogenesis *in vitro*, while their roles in bone development
20 are unknown. Here we report that combinatorial YAP/TAZ deletion from skeletal cells in mice caused
21 osteogenesis imperfecta with severity dependent on targeted cell lineage and allele dosage. Osteocyte-
22 conditional deletion impaired bone accrual and matrix collagen, while allele dosage-dependent deletion
23 from all osteogenic lineage cells caused spontaneous fractures, with neonatal lethality only in dual
24 homozygous knockouts. We identified putative target genes whose mutation in humans causes
25 osteogenesis imperfecta and which contain promoter-proximate binding domains for the YAP/TAZ co-
26 effector, TEAD4. Two candidates, Col1a1 and SerpinH1, exhibited reduced expression upon either
27 YAP/TAZ deletion or YAP/TAZ-TEAD inhibition by verteporfin. Together, these data demonstrate that
28 YAP and TAZ redundantly promote bone matrix development and implicate YAP/TAZ-mediated
29 transcriptional regulation of collagen in osteogenesis imperfecta.

30 INTRODUCTION

31 Bone is a living hierarchical composite whose form and function depend not only on the
32 composition of the matrix, but also its microstructure, which are controlled during development by
33 skeletal cell lineage progression and by osteocyte-coordinated matrix deposition and remodeling.
34 Various genetic, hormonal, or environmental abnormalities can impair these processes, leading to
35 debilitating diseases including osteoporosis and osteogenesis imperfecta. However, the molecular
36 mechanisms governing cell fate and matrix production in bone remain poorly understood, limiting
37 therapeutic intervention. Several transcriptional programs have been described as essential regulators
38 of bone development, but current understanding is insufficient to fully explain the heterogeneity found in
39 congenital and acquired bone diseases¹⁻³. In this study, we sought to define the function of the Hippo
40 pathway effectors YAP and TAZ in bone development.

41

42 *YAP/TAZ functional diversity*

43 Yes-associated protein (YAP) and Transcriptional co-activator with PDZ-binding motif (TAZ;
44 also known as WWTR1) are paralogous transcriptional co-activators that display either equivalent or
45 divergent functions, depending on cell type and context⁴. While they possess transcription activation
46 domains, they lack DNA-binding domains and require interaction with co-factors for transcriptional
47 activity⁵. Their most well-studied interactions are with the transcriptional enhancer activator-domain
48 (TEAD) family proteins, which themselves lack activation domains, providing specificity for YAP/TAZ-
49 TEAD signaling⁶. Many other co-effectors are known, including Runx2⁷, β -catenin⁸⁻¹⁰, and Smad2/3^{11,12},
50 each of which is known to contribute to bone development and osteoprogenitor lineage progression¹³⁻¹⁷.
51 Thus, independent pathways that regulate coincident activation of these various binding partners could
52 provide additional layers of contextual specificity in bone. Further, as paralogs, the YAP and TAZ
53 proteins also possess structural differences (reviewed in Ref.¹⁸) that enable distinct protein interactions
54 to confer unique physiological functions of YAP vs. TAZ. Notably, global YAP deletion in mice is
55 embryonic lethal (E8.5) due to impaired yolk sac vasculogenesis¹⁹, while the global TAZ knockout lives
56 to maturity with modest skeletal defects²⁰ demonstrating conclusive gene-specific functions. However,

57 in other contexts, they exhibit clear functional homology, with either protein capable of compensation
58 for the other^{21,22}.

59

60 *YAP and TAZ function in bone: conflicting evidence*

61 Roles for YAP and TAZ in osteogenesis were first described in 2004 and 2005, respectively^{23,24}.
62 YAP was reported to suppress osteoblastic differentiation through sequestration and transcriptional
63 repression of Runx2²³, while TAZ was identified as a Runx2 co-activator and an inhibitor of the
64 adipogenic nuclear receptor, PPAR γ ^{24,25}. A subsequent study found that overexpression of a
65 constitutively-active YAP mutant in marrow stromal cells (MSCs) promoted osteogenic differentiation
66 even under conditions more favorable for adipogenesis²⁶. In contrast, another report identified that YAP
67 overexpression inhibited osteogenesis in MSCs by suppressing activation of WNT target genes²⁷. The
68 role of TAZ in osteogenic differentiation appears similarly complicated with a recent report
69 demonstrating that overexpression of active-mutant TAZ inhibited canonical WNT/ β -catenin signaling²⁸,
70 while another suggested that TAZ promoted osteogenic differentiation through the canonical WNT
71 pathway²⁹. *In vivo*, osteoblast-specific overexpression of TAZ promoted bone formation with higher
72 expression levels of Runx2³⁰, while YAP overexpression in chondrocytes impaired endochondral bone
73 formation³¹. These data suggest the importance of these proteins in osteogenic differentiation and
74 mineralized matrix production, but their combinatorial physiological roles in bone development remain
75 unknown.

76

77 **RESULTS**

78 *YAP/TAZ expression and deletion in bone*

79 To determine YAP/TAZ expression profiles in bone, we immunostained YAP and TAZ in the
80 growth plate and cancellous and cortical bone of 8 week-old C57Bl6/J mouse femora. YAP and TAZ
81 immunolocalized in hypertrophic chondrocytes, osteoblasts, and osteocytes with minimal detectable
82 expression in quiescent or proliferating chondrocytes (Fig. S1A). Based on these expression patterns,
83 we chose to assess the physiological roles of YAP and TAZ by combinatorial conditional ablation²² at

84 two stages in the skeletal cell sequence: 1) all cells of the skeletal lineage using Osterix1-GFP::Cre
 85 (Osx1-Cre)³² and 2) osteocytes using 8kb-DMP1-Cre (Dmp1-Cre)^{33,34}. We selected a breeding strategy
 86 that yielded littermates with variable YAP/TAZ allele dosage (Table S1). To verify Cre-mediated
 87 recombination and deletion of YAP and TAZ, we assessed mRNA expression in femoral bone
 88 preparations by qPCR (Fig. S1D-F), and verified the absence of expression in bone cells in conditional
 89 knockout mice by IHC. YAP/TAZ expression was reduced by 50-80% in bone preparations from
 90 homozygous knockout mice with either Osx1-Cre- or DMP1-Cre-mediated excision (Fig. S1B-G).

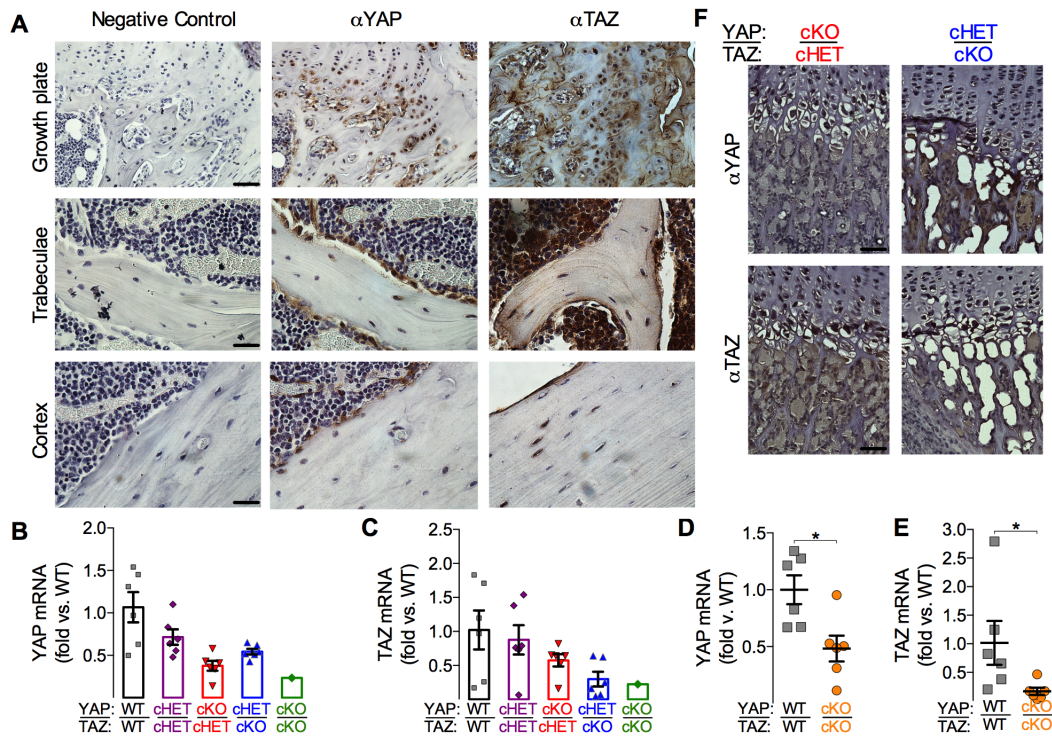
91 **Table S1: Genotypes evaluated**

Osx1-GFP::Cre	YAP/TAZ alleles	8kb-DMP1-Cre	YAP/TAZ alleles
Yap ^{fl/fl} ;Taz ^{fl/fl}	YAP ^{WT} ;TAZ ^{WT}	Yap ^{fl/fl} ;Taz ^{fl/fl}	YAP ^{WT} ;TAZ ^{WT}
Yap ^{fl/+} ;Taz ^{fl/+} ;Osx1 ^{cre/+}	YAP ^{CHET} ;TAZ ^{CHET}	Yap ^{fl/+} ;Taz ^{fl/+} ;DMP1 ^{cre/+}	YAP ^{CHET} ;TAZ ^{CHET}
Yap ^{fl/fl} ;Taz ^{fl/+} ;Osx1 ^{cre/+}	YAP ^{CKO} ;TAZ ^{CHET}	Yap ^{fl/fl} ;Taz ^{fl/+} ;DMP1 ^{cre/+}	YAP ^{CKO} ;TAZ ^{CHET}
Yap ^{fl/+} ;Taz ^{fl/fl} ;Osx1 ^{cre/+}	YAP ^{CHET} ;TAZ ^{CKO}	Yap ^{fl/+} ;Taz ^{fl/fl} ;DMP1 ^{cre/+}	YAP ^{CHET} ;TAZ ^{CKO}
Yap ^{fl/fl} ;Taz ^{fl/fl} ;Osx1 ^{cre/+}	YAP ^{CKO} ;TAZ ^{CKO}	Yap ^{fl/fl} ;Taz ^{fl/fl} ;DMP1 ^{cre/+}	YAP ^{CKO} ;TAZ ^{CKO}

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Supplementary Figure 1. Immunolocalization of YAP/TAZ expression and YAP/TAZ

97 **recombination efficiency.** *In vivo* YAP/TAZ expression and knockdown efficiency were assessed by
98 immunohistochemistry (IHC) and quantitative (qPCR). **A**) YAP/TAZ were expressed in hypertrophic
99 chondrocytes, osteoblasts, and osteocytes in bone. Scale bars in the first column are 50 microns for the
100 growth plate, and 100 microns for magnified images of trabecular and cortical bone. YAP (**B,D**) and
101 TAZ (**C,E**) transcript expression in Osterix- and DMP1-conditional knockout mouse bone. (**F**) IHC
102 verification of YAP and TAZ knockdown at the protein level in Osterix-conditional cKO bone. Scale bars
103 in the first column are 50 microns.

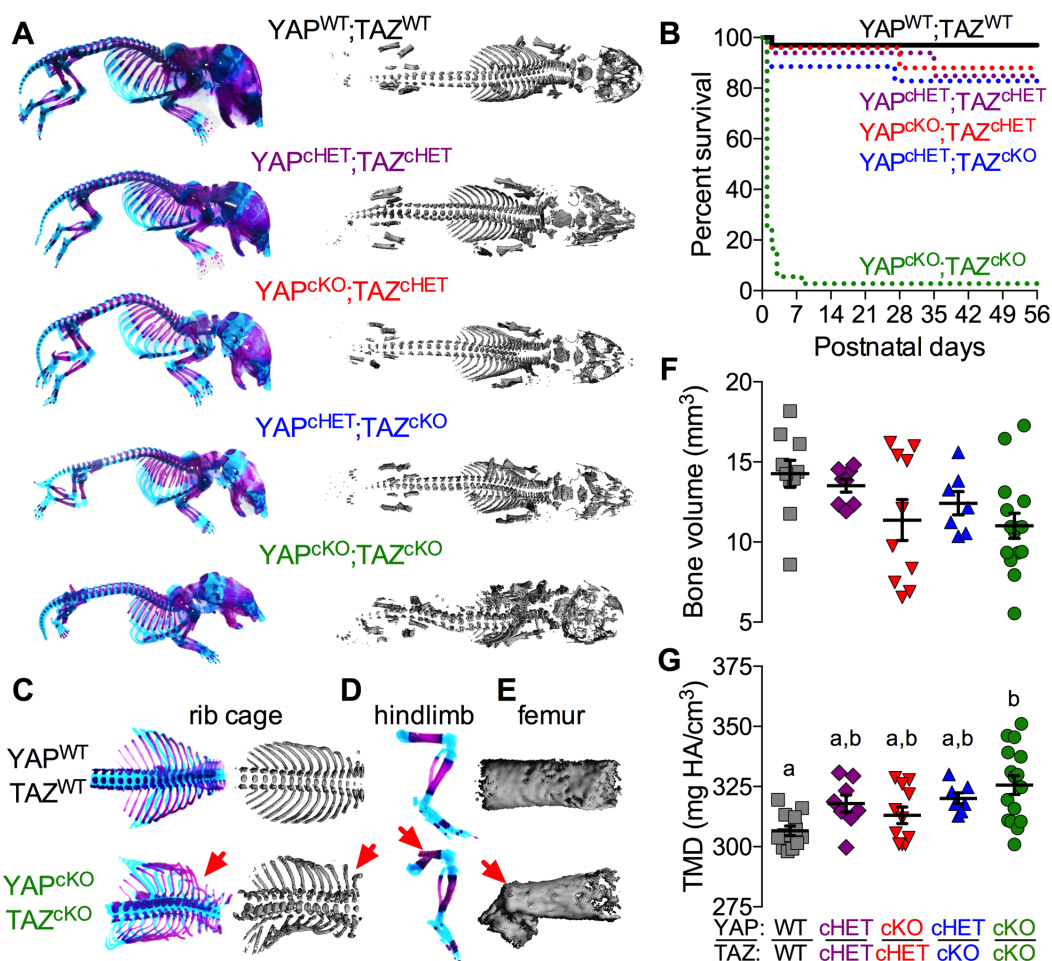
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105 **Osterix-conditional YAP/TAZ cKO: neonatal lethality and hypermineralization**

106

All osterix-conditional knockouts and littermate controls were born at expected Mendelian ratios,
107 but dual homozygous conditional deletion ($YAP^{cKO};TAZ^{cKO}$) caused neonatal asphyxiation secondary to
108 ribcage malformation and fracture (Fig. 1A-C), resulting in 75% mortality at postnatal day 0 (P0) and
109 99% by P7 (Fig. 1B). Only one female $YAP^{cKO};TAZ^{cKO}$ mouse lived to P56 for each endpoint analysis.
110 $YAP^{cKO};TAZ^{cKO}$ neonates exhibited spinal scoliosis, cranial vault deformity, and spontaneous fractures
111 of the ribs, tibia, femur, radius and ulna (Fig. 1A,C-E). Spontaneous extremity fractures were not
112 present in other genotypes at P0 (Fig. 1A). Littermate neonates displayed a trend of reduced whole-

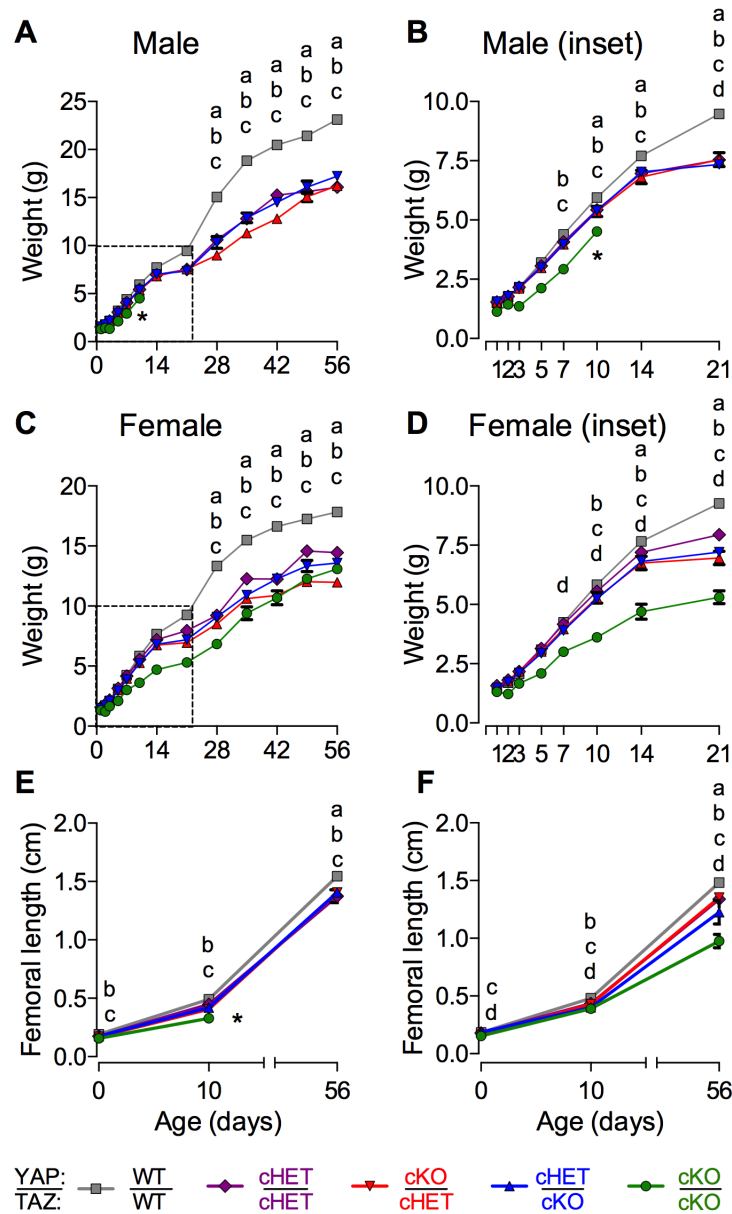
113 skeleton bone volume (Fig. 1B; $p=0.05$, ANOVA) and significantly elevated bone tissue mineral density
 114 (Fig. 1C; $p<0.01$, ANOVA) with decreasing YAP/TAZ allele dosage. Osterix-conditional YAP/TAZ
 115 deletion also significantly reduced birth weight and intact femoral length in an allele dosage-dependent
 116 manner (Fig. S2A). Males and females exhibited similar phenotypes (Fig. S2B).



117

118 **Figure 1. Combinatorial YAP/TAZ ablation from Osterix-expressing cells caused allele dosage-**
 119 **dependent perinatal skeletal deformity and lethality.** Skeletal structures of littermate mice were
 120 evaluated at postnatal day 0 (P0). **A)** Whole body skeletal preparations of osterix-conditional YAP/TAZ
 121 knockouts and controls stained with Alcian blue/Alizarin red and microCT reconstructions reveal
 122 progressive skeletal malformation with decreasing allele dosage. **B)** Survival curves for each genotype
 123 show 99% lethality of $YAP^{cKO};TAZ^{cKO}$ mice by P56. **C-E)** Skeletal preparations and microCT
 124 reconstructions of rib cages, hindlimbs, and femora, respectively, illustrate spontaneous perinatal
 125 fractures in $YAP^{cKO};TAZ^{cKO}$ mice. **F)** P0 whole skeleton bone volume was not significantly altered by
 126 YAP/TAZ deletion. **G)** P0 whole skeleton tissue mineral density (TMD) increased with YAP/TAZ allele

127 deletion. Data are presented as individual samples with lines corresponding to the mean and standard
 128 error of the mean (SEM). Sample sizes, n = 7-15.



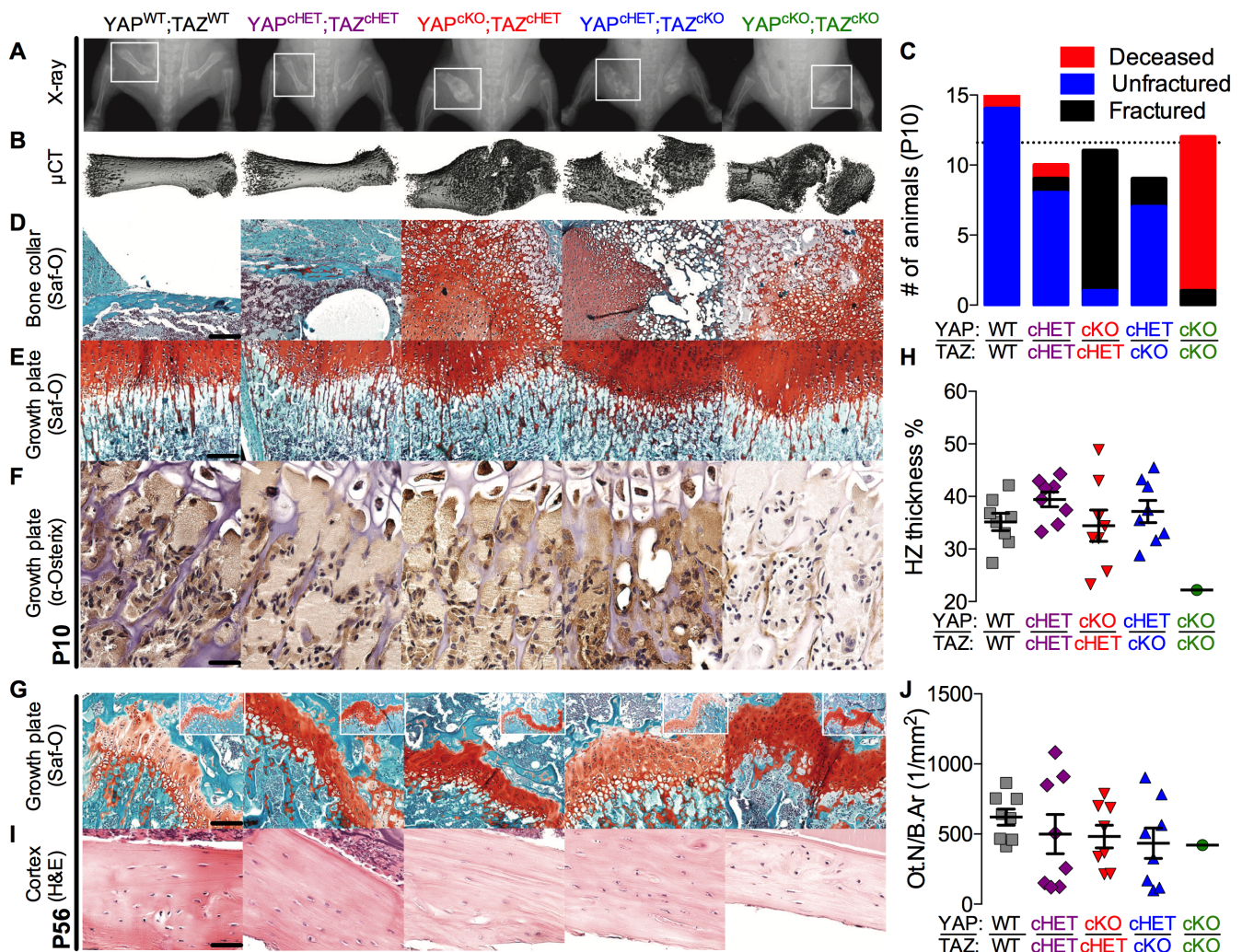
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130 **Supplementary Figure 2. Allele-dose dependent YAP/TAZ ablation from Osterix-expressing cells**
 131 **in 8-week-old mice reduced overall and long bone growth independent of sex.** Allele-dosage-
 132 dependent YAP/TAZ ablation from Osterix-expressing cells reduced body weight in male (**A, B**) and
 133 female (**C, D**) mice and reduced femoral length (**E, F**). Sample sizes, n = 8 for both genders and
 134 genotypes except n = 1 for YAP^{ckO};TAZ^{ckO} male and n = 2 for YAP^{ckO};TAZ^{ckO} females. * indicates death
 135 of male YAP^{ckO};TAZ^{ckO} mouse at P10.

136

137 ***Osterix-conditional YAP/TAZ cKO: spontaneous neonatal long bone fractures & defective***
138 ***endochondral bone formation***

139 A single copy of either gene rescued neonatal lethality, with 83 and 85% of YAP^{CHET};TAZ^{CKO} and
140 YAP^{CKO};TAZ^{CHET} mice surviving to terminal analysis at P56, respectively. However, between P1 and
141 P10, both YAP^{CHET};TAZ^{CKO} and YAP^{CKO};TAZ^{CHET} mice sustained spontaneous femoral and other bone
142 fractures (Fig. 2A-B), with significantly increased femoral fracture incidence in the YAP^{CKO};TAZ^{CHET} mice
143 ($p < 0.01$, χ^2 test; Fig. 2C). Fractures healed by endochondral repair in all groups, though
144 YAP^{CHET};TAZ^{CKO} and YAP^{CKO};TAZ^{CKO} calluses exhibited numerous empty lacunae in the hypertrophic
145 transition zone, suggesting increased hypertrophic chondrocyte death or insufficient progenitor cell
146 recruitment (Fig. 2C,D). Consistently, staining of Osterix-positive cells was qualitatively reduced in the
147 transition zone of YAP^{CKO};TAZ^{CKO} growth plates, but we found no significant differences in growth plate
148 morphology at P10 (Fig. 2E,F). Histomorphometric analysis of P56 growth plates did not reveal
149 significant differences in proliferative or hypertrophic zone thickness, though the single YAP^{CKO};TAZ^{CKO}
150 mouse that survived to P56 exhibited a lower relative hypertrophic zone thickness (Fig. 2G,H). Osterix-
151 conditional YAP/TAZ deletion did not alter osteocyte density (Ot.N/B.Ar) at P56 (Fig. 2I,J).



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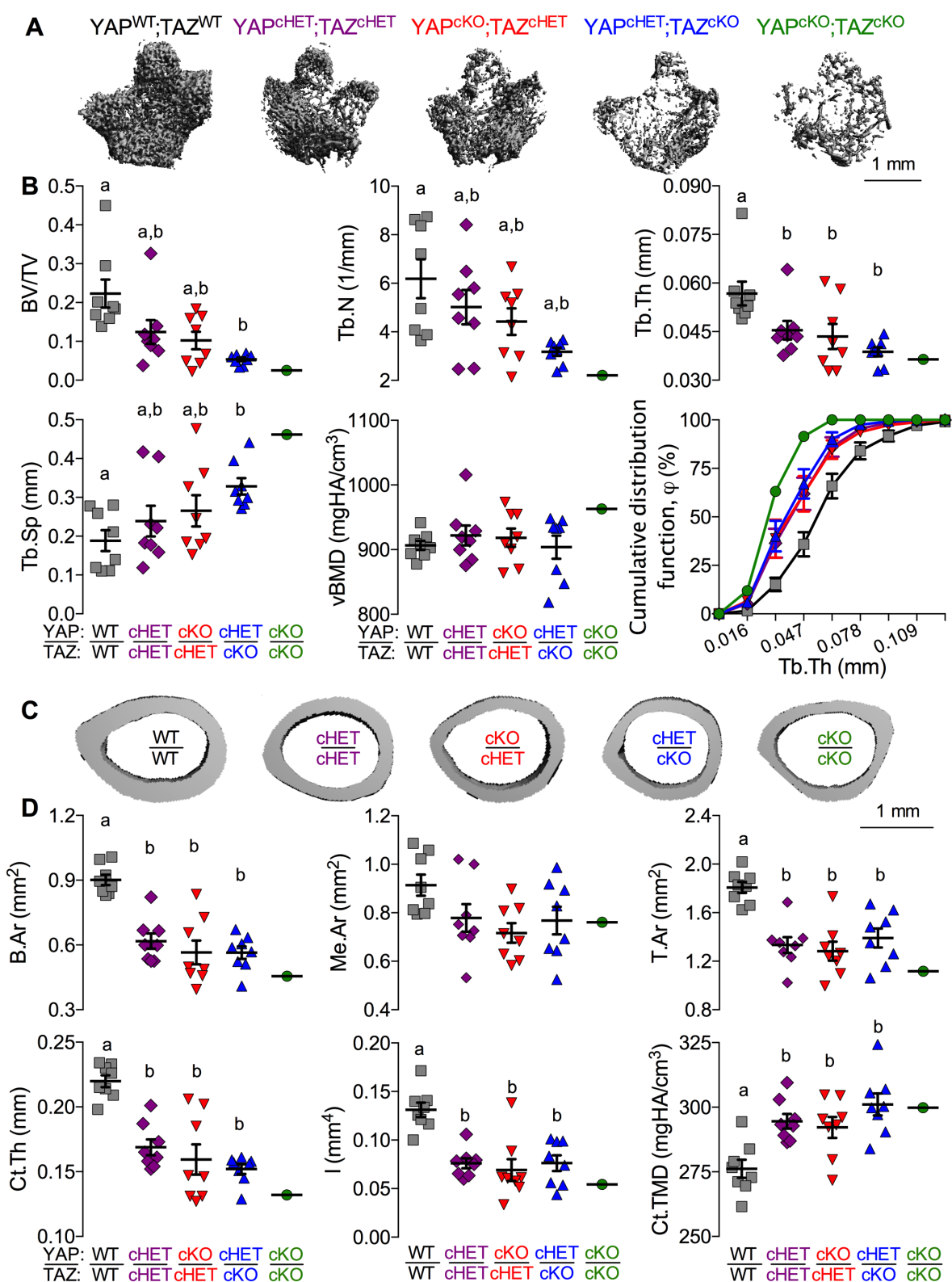
153 **Figure 2. YAP/TAZ ablation from Osterix-expressing cells induced spontaneous neonatal**
 154 **femoral fractures and impaired endochondral bone formation.** Representative radiographs (A) with
 155 matched microCT reconstructions of femoral fracture calluses at P10 (B). C) Quantification of the
 156 number of femoral fractures demonstrated significantly increased fracture incidence in YAP^{cKO};TAZ^{cHET}
 157 mice. D) Safranin-O/fast green (Saf-O) staining of mid-diaphysis bone collar or fracture callus and (E)
 158 growth plates of matched P10 femora. Scale bar = 50 μ m. F) Representative micrographs of P10 distal
 159 femur growth plates immunostained for osterix-positive cells (brown). Scale bar = 25 μ m. G)
 160 Representative Safranin-O/fast green-stained distal femoral growth plates at P56. Scale bar = 50 μ m.
 161 Inset: Saf-O/fast green (Inset width = 1.5 mm). H) Histomorphometric quantification of P56 hypertrophic
 162 zone thickness as percentage of total growth plate thickness (HZ thickness %). I) Representative
 163 micrographs of P56 mid-diaphyseal cortical bone stained by H&E. J) Histomorphometric quantification
 164 of osteocyte number per bone area (Ot.N/B.Ar). Data presented as individual samples with lines
 165 corresponding to the mean and standard error of the mean (SEM). Sample sizes, n = 3-6 at P10, n = 8
 166 at P56, except YAP^{cKO};TAZ^{cKO} n = 1.

167

168 ***Osterix-conditional YAP/TAZ cKO: reduced cortical and cancellous microarchitectural***
169 ***properties***

170 YAP/TAZ deletion from all cells of the skeletal lineage reduced cancellous (Fig. 3A,B) and
171 cortical bone (Fig. 3C,D) volume and shape, according to allele dosage. Distal femur metaphyseal
172 cancellous bone exhibited reduced trabecular bone volume fraction (BV/TV), thickness (Tb.Th), and
173 number, and increased spacing and structural model index (SMI, indicative of more rod-like trabeculae)
174 (Fig. 3B, Fig. S3A). The cumulative distribution of trabecular thicknesses shifted in an allele dosage-
175 dependent manner, indicating reduced numbers of both small and large trabeculae (Fig. 3B, S3B).
176 Volumetric bone mineral density was not altered, suggesting an increase in local tissue mineral density
177 proportional to the decrease in trabecular bone volume (Fig. 3B). Mid-diaphyseal femoral cortical bone
178 (Fig. 3C,D) similarly exhibited reduced thickness, area, and moment of inertia (I) in cKO mice,
179 attributable primarily to reduced periosteal bone accumulation as indicated by moderate reductions in
180 medullary area (Me.Ar) compared to cortical bone area (B.Ar) and total area (T.Ar). Consistent with the
181 observations of vBMD in the cancellous compartment, cortical tissue mineral density (Ct.TMD) was
182 significantly increased in an allele-dosage dependent manner; however, unlike the cancellous bone, the
183 increase in tissue mineral density of the cortical bone was insufficient to normalize bone mass lost by
184 reduced bone volume. Together, these data demonstrate that both YAP and TAZ contribute positively
185 to bone quantity and microarchitecture in both cancellous and cortical compartments.

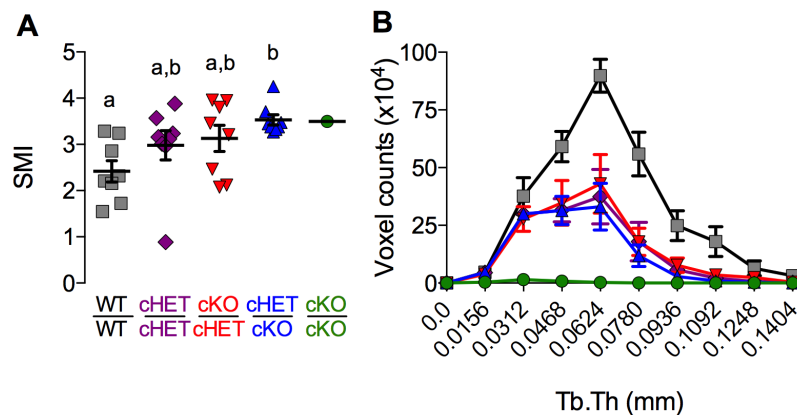
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187

188 **Figure 3. YAP/TAZ ablation from Osterix-expressing cells altered bone microarchitectural**
 189 **properties in a manner dependent on allele dosage.** Femora from 8 week-old osterix-conditional
 190 YAP/TAZ littermates were evaluated by microCT analysis. **A)** Representative microCT reconstructions

191 of metaphyseal cancellous bone, arranged in decreasing allele dosage. **B)** Cancellous bone
 192 microarchitectural parameters were impaired according to YAP/TAZ allele dosage: bone volume
 193 fraction (BV/TV), trabecular thickness (Tb.Th), number (Tb.N) and spacing (Tb.Sp), and volumetric
 194 bone mineral density (vBMD). The cumulative distribution function, ϕ , demonstrated a reduced
 195 percentage of trabeculae smaller than a given thickness (on the abscissa) with increasing allele
 196 deletion. **C)** Representative microCT reconstructions of the mid-diaphyseal cortex, arranged in
 197 decreasing allele dosage. **D)** Cortical cross-sectional properties were reduced in cKO mice: bone area
 198 (B.Ar), medullary area (Me.Ar), total area (T.Ar), cortical thickness (Ct.Th), moment of inertia in the
 199 direction of bending (I), and cortical tissue mineral density (Ct.TMD). Data are presented as individual
 200 samples with lines corresponding to the mean and standard error of the mean (SEM). Sample sizes, n=
 201 8 except YAP^{ckO};TAZ^{ckO} n = 1. Scale bars indicate 1 mm for microCT reconstructions.

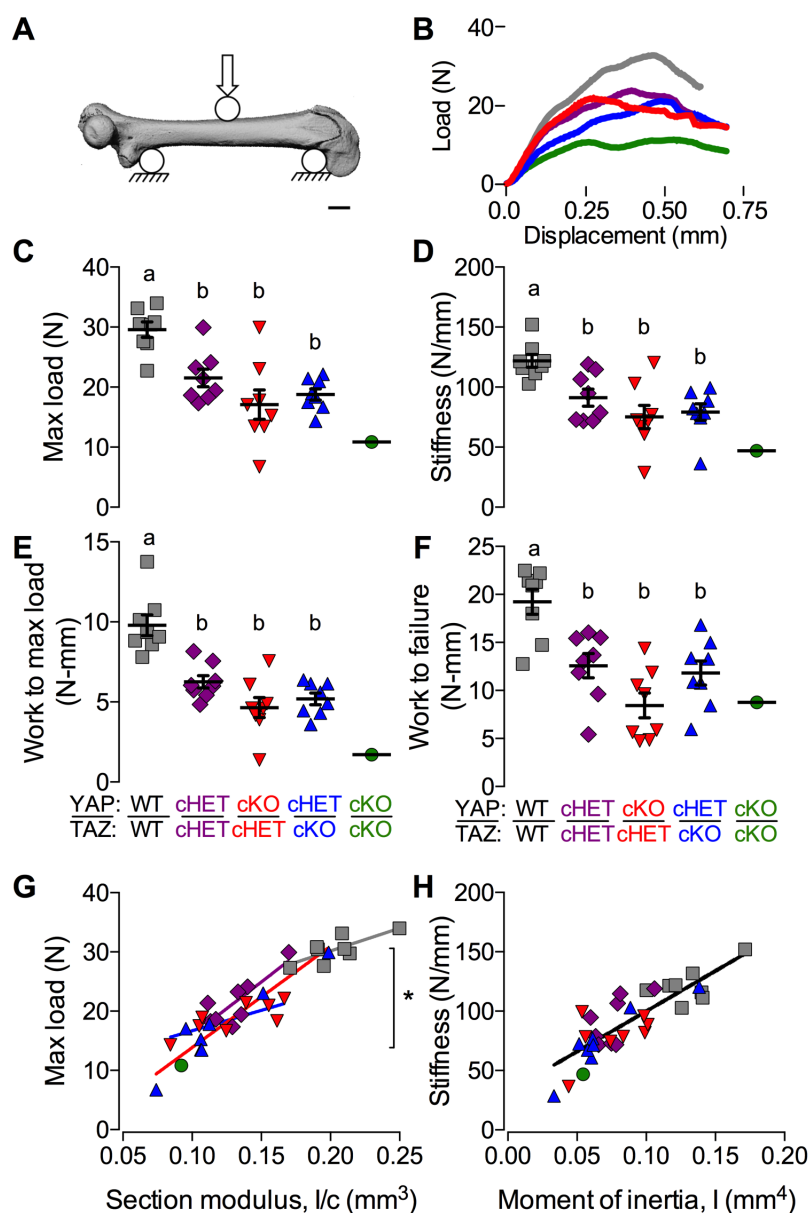


202
 203 **Supplementary Figure 3. Allele-dose dependent YAP/TAZ ablation from Osterix-expressing cells**
 204 **in 8-week-old mice altered trabecular architecture. A)** Structural model index (SMI) **B)** Trabecular
 205 thickness (Tb.Th) histogram distributions. Sample sizes, n= 8 except YAP^{ckO};TAZ^{ckO} n = 1.

206
 207 ***Osterix-conditional YAP/TAZ cKO: reduced intrinsic bone mechanical properties and matrix***
 208 ***collagen content and microstructure***

209 To determine whether Osterix-conditional YAP/TAZ deletion impaired adolescent bone matrix
 210 quality, we performed three-point bend testing to failure (Fig. 4A,B) on each femur previously analyzed
 211 by microCT. The extrinsic bone properties (i.e., maximum load at failure, bending stiffness, work to max
 212 load, and work to failure; Fig. 4C-F, respectively) depend on both the intrinsic mechanical properties of
 213 the bone matrix and the bone amount and cross-sectional distribution. Since the assumptions of Euler-
 214 Bernoulli beam theory are decidedly not met in three-point bend testing of mouse long bones^{35,36}, we
 215 performed an analysis of covariance (ANCOVA) using linear regression (Fig. 4G,H) to decouple the

216 contributions of bone quantity and distribution from the mechanical behavior³⁷. If the variability in
217 extrinsic mechanical properties is best predicted by individual regression lines for each genotype, this
218 would indicate differences in intrinsic matrix mechanical properties between genotypes; however, a
219 best-fit by a single regression line for all groups would indicate that differences in extrinsic behavior are
220 sufficiently described merely by changes in bone geometry³⁷. We found that individual regression lines
221 for each genotype best predicted maximum load at failure, indicating significant differences in intrinsic
222 failure properties (Fig. 4G). In contrast, a single regression line best fit the stiffness data (Fig. 4H),
223 indicating that the differences in stiffness can be attributed to changes in moment of inertia rather than
224 intrinsic matrix elastic properties.



225

226 **Figure 4. YAP/TAZ ablation from Osterix-expressing cells reduced intrinsic bone failure**
 227 **properties.** Femora from 8 week-old osterix-conditional YAP/TAZ littermates were tested in three point
 228 bending (**A**) to failure. **B**) Representative load-displacement curves collected during testing. **C-F**)
 229 YAP/TAZ deletion reduced extrinsic mechanical properties measured from the load-displacement
 230 curves including maximum load (**C**), stiffness (**D**), work to maximum load (**E**) and work to failure (**F**).
 231 ANCOVA analysis accounting for bone geometry revealed significant differences in intrinsic failure
 232 properties (**G**), but not intrinsic elastic properties (**H**). Data are presented as individual samples with
 233 lines corresponding to the mean and standard error of the mean (SEM). Sample sizes, $n = 8$ except
 234 $YAP^{cKO};TAZ^{cKO} n = 1$.

235

236 As a composite material, bone mechanical behavior is determined predominantly by its two
237 primary matrix components: mineral and collagen. By microCT scanning prior to mechanical testing, we
238 noted above that femora from mice with Osterix-conditional YAP/TAZ deletion exhibited moderate
239 hypermineralization (Fig. 3D). Next, to characterize the bone matrix collagen in these same samples,
240 we performed polarized light microscopy and second harmonic imaging microscopy (SHIM)³⁸. Both
241 polarized-light microscopy of Picrosirius red-stained sections (Fig. 5A) and second harmonic imaging
242 (Fig. 5B) demonstrated that YAP/TAZ deletion significantly reduced collagen content and organization
243 (Fig. 5C), and produced a trend of reduced fiber directionality (Fig. S3A,B). Therefore, to determine the
244 contributions of geometry, mineralization, and collagen content and microstructure to bone mechanical
245 behavior, we performed a best-subsets correlation analysis to identify significant predictors based on
246 Akaike's information criterion (AIC)^{39,40}. For both elastic (Fig. 5D) and failure (Fig. 5E) properties, bone
247 tissue mineral density was not a significant predictor; however, moment of inertia and SHG intensity
248 significantly improved model capability to explain variation ($R_{\text{adj}}^2 = 73$ and 88% for stiffness and max
249 load, respectively) and reduced AIC (Fig. S4A,C). Addition of TMD did not improve predictive power or
250 model quality (Fig. S4B,D).

251 These data suggested that YAP and TAZ may regulate skeletal cell expression of collagen-
252 related genes. To evaluate potential YAP/TAZ transcriptional targets, we first identified candidate genes
253 whose mutations in humans cause osteogenesis imperfecta with similar penetrance to the observed
254 mouse phenotypes (Table S2). For each candidate gene, we searched published ChIP-seq data in the
255 UCSC Genome Browser⁴¹ for DNA binding domains of known YAP/TAZ co-transcription factors
256 proximate (within 10 kb) to the transcription start site (TSS) (Table S2). We identified functional TEAD-
257 binding MCAT elements (5'-CATTCCA/T-3') in multiple OI-related genes. Based on the motif score⁴²
258 and TSS-proximity of each identified MCAT element, we selected Col1a1, Col1a2, and SerpinH1 as
259 putative YAP/TAZ target genes. We performed quantitative PCR amplification of candidate mRNA
260 transcripts isolated from 8 week-old femoral cortical bone preparations. Osterix-conditional YAP/TAZ
261 deletion significantly reduced Col1a1 and SerpinH1 expression in a manner dependent on allele
262 dosage (Fig. 5F,G). Differences in Col1a2 expression did not reach significance (Fig. 5H).

263

264 **Table S2: UCSC Genome Browser candidate gene identification.** Identification of functional TEAD-

265 binding MCAT elements (5'-CATTCCA/T-3') in multiple OI-related genes. *indicate statistically

266 significant motif scores ($p < 0.05$). TSS = Transcriptional Start Site. + and - indicate downstream and

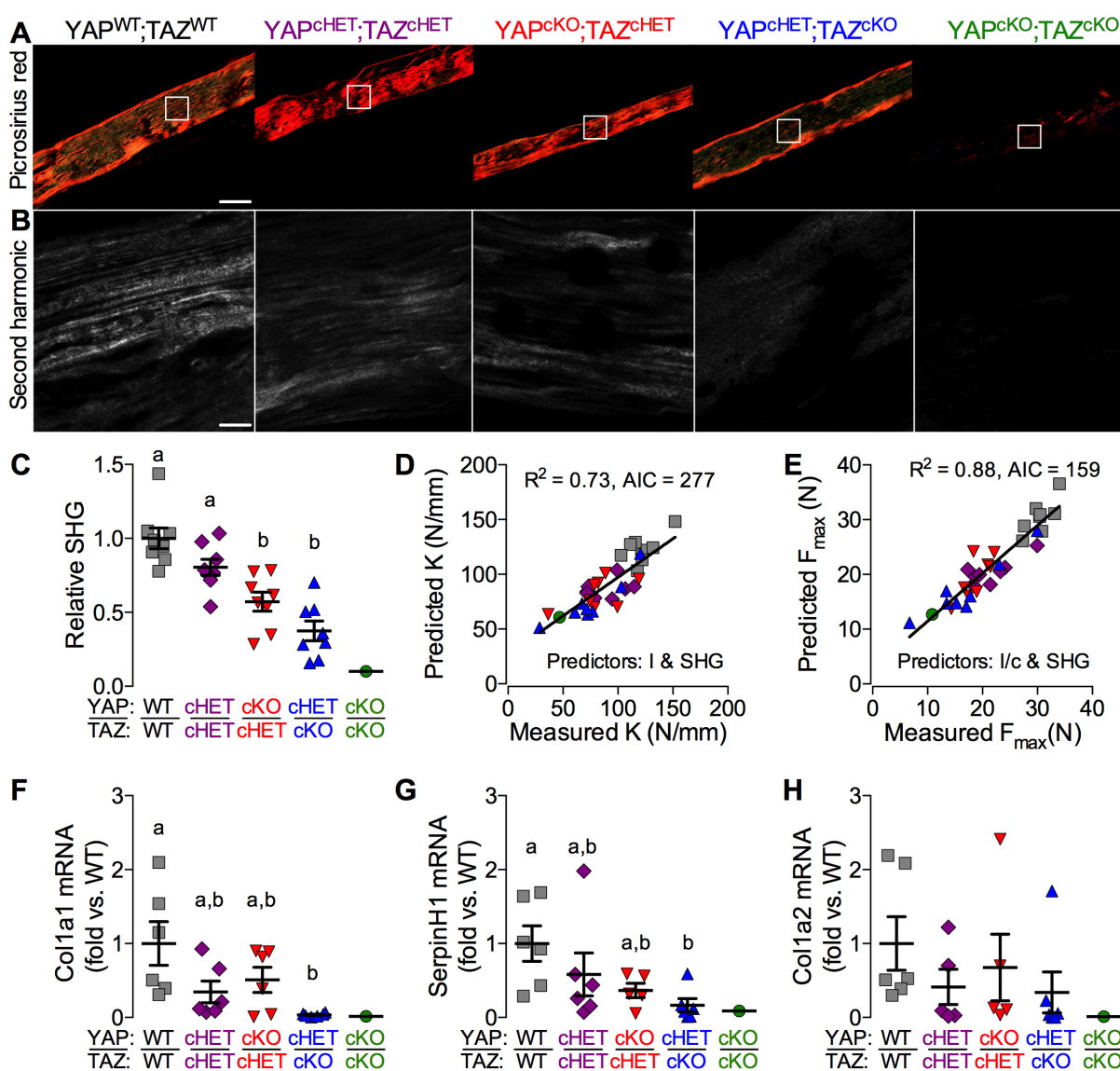
267 upstream of TSS, respectively.

Gene (location)	OI-related gene function ⁴³	TEAD binding motif?	Motif score ⁴²	Distance to TSS (kb)
<i>COL1A1</i> (Chr17: 48,261,457)	Classical Sillence types	Yes	1.22	+0.353
<i>COL1A2</i> (Chr7: 94,023,873)		Yes	1.96* 1.72*	-1.303 -2.529
<i>SERPINH1</i> (Chr11: 75,271,275)	Collagen chaperone defects	Yes	1.58* 1.96*	+1.408 -2.570
<i>FKBP10</i> (Chr17: 39,966,270)		Yes	1.27	+8.071
<i>CRTAP</i> (Chr3: 43,212,006)	3-Hydroxylation defects	Yes	1.27	+8.071
<i>LEPRE1</i> (Chr1: 43,212,006)		Yes	1.27	+8.071
<i>PIIB</i> (Chr15: 64,448,014)		No		
<i>OSTERIX/Sp7</i> (Chr12: 53,717,808)	Osteoblast maturation defects	No		

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272 **Figure 5. YAP/TAZ ablation from Osterix-expressing cells reduced bone matrix collagen content,**

273 **organization, and gene expression.** Imaging of matrix collagen and quantification of collagen-

274 associated gene expression were performed on two sets of femora from 8 week-old osterix-conditional

275 YAP/TAZ littermates. **A,B)** Representative polarized light (**A**) and second harmonic generation (SHG;

276 **B)** microscopy images from cortical bone tissue sections of three-point bend tested femora. **C)** Second

277 harmonic generation (SHG) intensity, relative to WT, was reduced according to allele-dosage. **D,E)**

278 Best subsets regression analyses indicating significant contributions of both bone geometry and

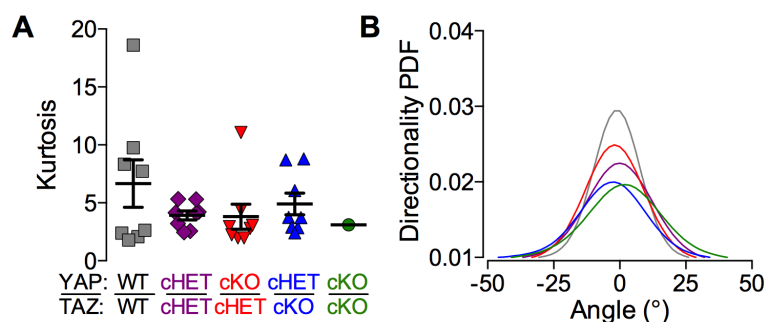
279 collagen content and microstructure, but not tissue mineral density, to both elastic (**D**) and failure (**E**)

280 mechanical properties. **F-H)** Quantitative PCR amplification of genes identified as bone matrix collagen

281 regulators with putative YAP/TAZ co-factor binding domains. Osterix-conditional YAP/TAZ deletion

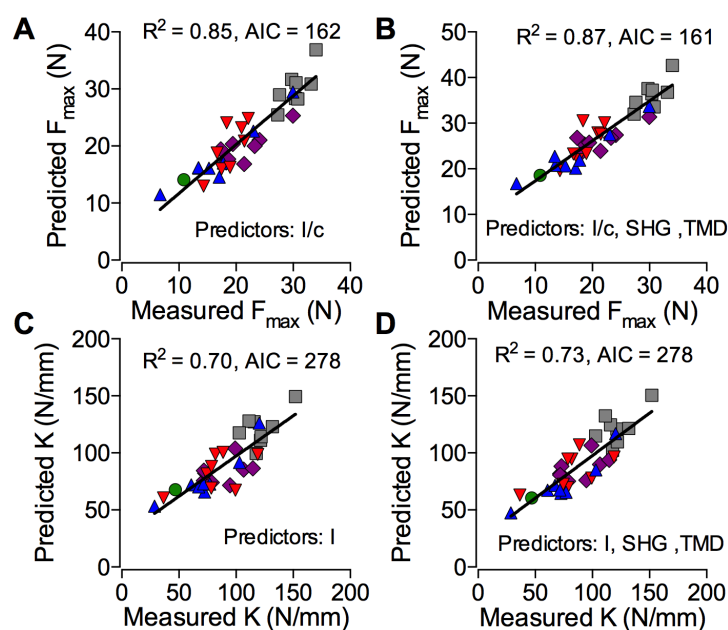
282 reduced mRNA expression of both Col1a1 (**F**) and SerpinH1 (**G**), but not Col1a2 (**H**). Sample sizes, n =

283 8 and 6 for SHG and qPCR analyses, respectively; except in both cases $YAP^{ckO};TAZ^{ckO}$ $n = 1$. Scale
 284 bars indicate 100 μ m and 25 μ m in Picosirius red and SHG images, respectively.



285

286 **Supplementary Figure 3. Allele-dose dependent YAP/TAZ ablation from Osterix-expressing cells**
 287 **in 8-week-old mice did not significantly alter collagen fiber directionality. (A)** Kurtosis
 288 quantification of fiber directionality distributions with **(B)** accompanying mean directionality distribution
 289 probability density function (Directionality PDF). Sample sizes, $n = 8$ except $YAP^{ckO};TAZ^{ckO}$, $n = 1$



290

291 **Supplementary Figure 4. Best subsets analysis on morphological parameters from the YAP/TAZ**
 292 **ablation in Osterix-expressing cells from 8-week-old mice demonstrated increased goodness of**
 293 **fit parameters by accounting for collagen content and organization.** Within the mice with Osterix
 294 driven deletion of YAP/TAZ, we compared experimental ultimate load (F_u) with predicted model values
 295 with either **(A)** only section modulus (I/c) as a predictor or **(B)** the best model from the multivariate
 296 analysis, which incorporated both I/c and second harmonic generated (SHG) signal intensity. Similarly,
 297 we comparing experimental to predicted stiffness with either **(C)** only moment of inertia or **(D)** the best
 298 model from the multivariate analysis, which incorporated both moment of inertia and second harmonic

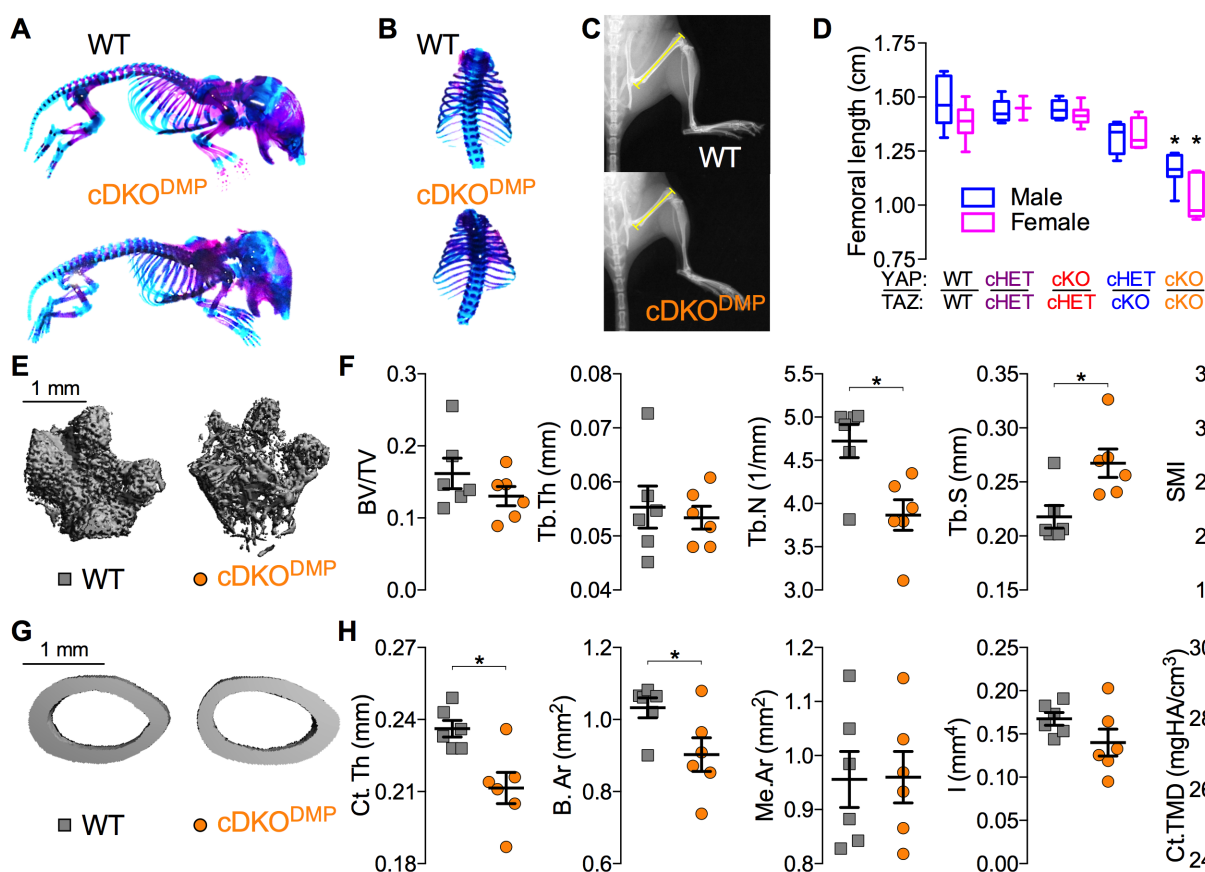
299 generated signal intensity. Akaike's information criterion (AIC) decreased while R^2 increased in both
300 regressions for maximum load and stiffness, suggesting a better goodness of fit with the addition of
301 SHG. See methods for multivariate best subset analysis full details. Sample sizes, $n = 8$ except
302 $YAP^{cKO};TAZ^{cKO}$, $n = 1$

303

304 ***YAP and TAZ are functionally redundant in DMP1-expressing cells and promote cortical and***
305 ***cancellous bone development***

306 To determine the roles of YAP and TAZ late in the skeletal cell sequence, we next used 8kb-
307 $Dmp1-Cre$ ³³ mice to preferentially delete YAP and TAZ from osteocytes^{33,34}. We used the same
308 breeding strategy as above to generate YAP/TAZ allele dosage-dependent DMP1-conditional
309 knockouts. All pups were born at expected Mendelian ratios, and unlike the Osterix-conditional
310 knockouts, double homozygous DMP1-cKO mice ($cDKO^{DMP1}$) exhibited grossly normal skeletal
311 structure and composition at P0 (Fig. 6A), without ribcage malformation (Fig. 6B) or spontaneous
312 fractures. Femoral lengths, measured at 12 weeks of age (Fig.6C), revealed no effect of YAP/TAZ
313 allele dosage on long bone growth, except for significantly reduced length in the double homozygous
314 knockouts for both sexes (Fig.6D). A single copy of either gene was sufficient to rescue this defect,
315 suggesting functional redundancy. Therefore, for further analyses, we selected for comparison
316 littermate wild type (WT) and $cDKO^{DMP1}$ mice. In metaphyseal cancellous bone of the distal femur,
317 YAP/TAZ deletion from DMP1-expressing cells reduced trabecular number and increased trabecular
318 spacing (Fig.6E-F). Femoral cortical bone exhibited reduced thickness and area, without changes in
319 medullary area, indicating reduced periosteal bone in $cDKO^{DMP1}$ mice (Fig.6G-H). Unlike Osterix-
320 conditional knockouts, YAP/TAZ deletion from DMP1-expressing cells did not significantly alter cross
321 sectional moment of inertia or tissue mineral density (Fig. 6H).

322



323

324 **Figure 6. YAP/TAZ ablation from DMP1-expressing cells moderately impaired cancellous and**
 325 **cortical bone development.** Littermate mice at P0 and P84 were selected for skeletal morphology
 326 analysis by skeletal preparation and microCT analysis. Whole body (A) and rib cage (B) skeletal
 327 preparations stained with Alcian blue/Alizarin red demonstrated normal skeletal structure in cDKO^{DMP} at
 328 P0. C) Representative radiographs illustrating femoral length at week 12. D) Femoral length was
 329 reduced only dual homozygous knockouts for both males and females. E) Representative microCT
 330 reconstructions of distal metaphyseal microarchitecture at week 12. F) YAP/TAZ deletion moderately
 331 altered cancellous bone microarchitecture: bone volume fraction (BV/TV), trabecular thickness (Tb.Th),
 332 number (Tb.N), spacing (Tb.Sp), and structural model index (SMI). G) Representative microCT
 333 reconstructions of femoral mid-diaphysis. H) YAP/TAZ deletion reduced cortical microarchitectural
 334 properties: bone area (B.Ar), medullary area (Me.Ar), cortical thickness (Ct.Th), moment of inertia in the
 335 direction of bending (I), and cortical tissue mineral density (Ct.TMD). Data are presented as individual
 336 samples with lines corresponding to the mean and standard error of the mean (SEM). Sample sizes, n
 337 = 6. Scale bars indicate 1 mm for microCT reconstructions.

338

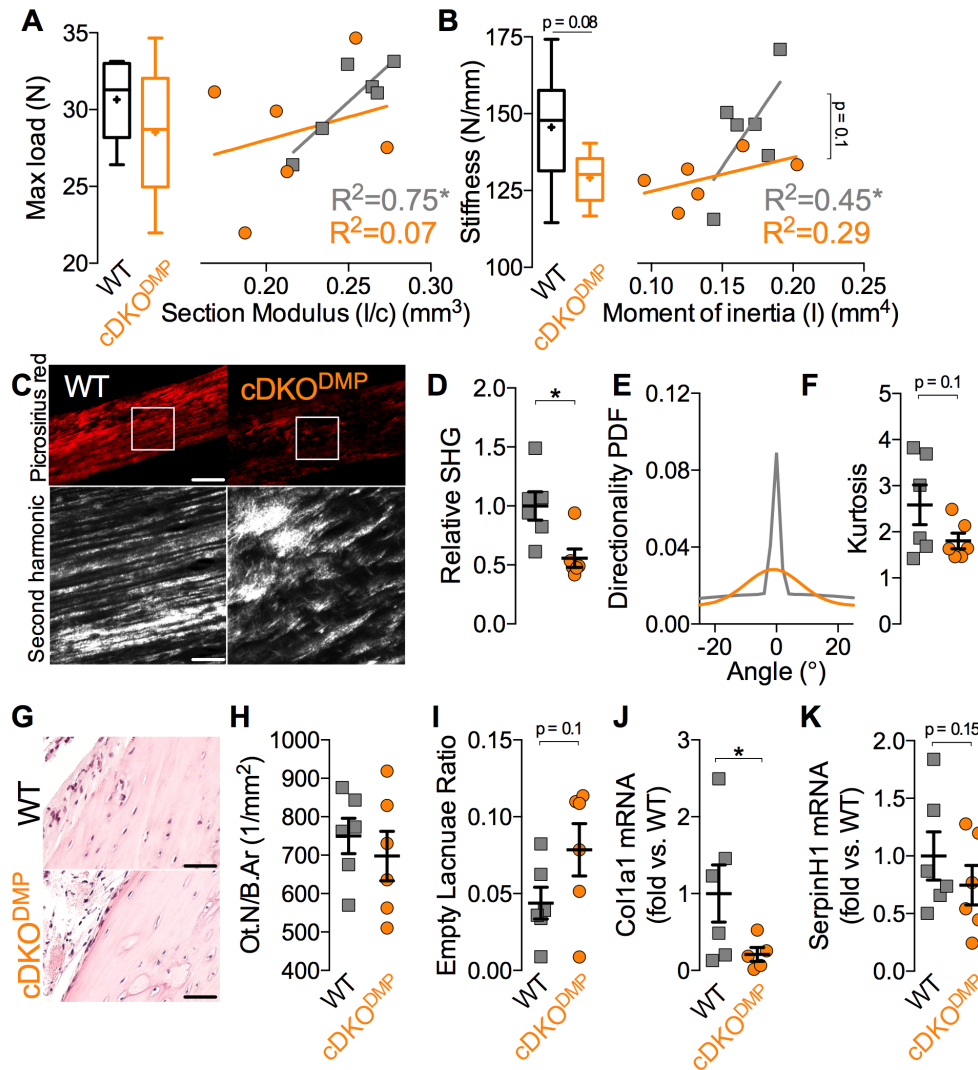
339

340

341 ***DMP1-conditional YAP/TAZ deletion impairs bone matrix collagen content, organization, and***
342 ***collagen-associated gene expression.***

343 Homozygous YAP/TAZ deletion from DMP1-expressing cells did not significantly reduce
344 maximum load (Fig. 7A; $p = 0.3$) or bending stiffness (Fig. 7B; $p = 0.08$) in three-point bend testing to
345 failure. Correlation of extrinsic properties with bone cross-sectional geometry (Fig. 7A,B) revealed
346 significantly linear correlations for WT, but not for cDKO^{DMP} bones, though differences in regression
347 lines between WT and cDKO^{DMP} did not reach statistical significance ($p=0.4$ and 0.1 for maximum load
348 and stiffness, respectively). Matrix collagen content was significantly reduced in cDKO^{DMP} cortical bone
349 while differences in the distribution of collagen fiber alignment did not reach statistical significance ($p =$
350 0.1) (Fig. 7C-F). To assess osteocyte density, the number osteocytes per bone area (Ot.N/B.Ar) were
351 quantified by histomorphometry in cortical bone of the same limbs evaluated for microCT, mechanical
352 testing, and SHIM. Cortical Ot.N/B.Ar was not significantly altered ($p = 0.5$), and differences in the
353 percentage of empty lacunae were not significant ($p = 0.1$) (Fig. 7G-I). Best subsets regression analysis
354 revealed significant contributions of both bone geometry (section modulus and moment of inertia) and
355 collagen matrix properties (SHG) in predicting mechanical behavior (Fig. S5A-D).

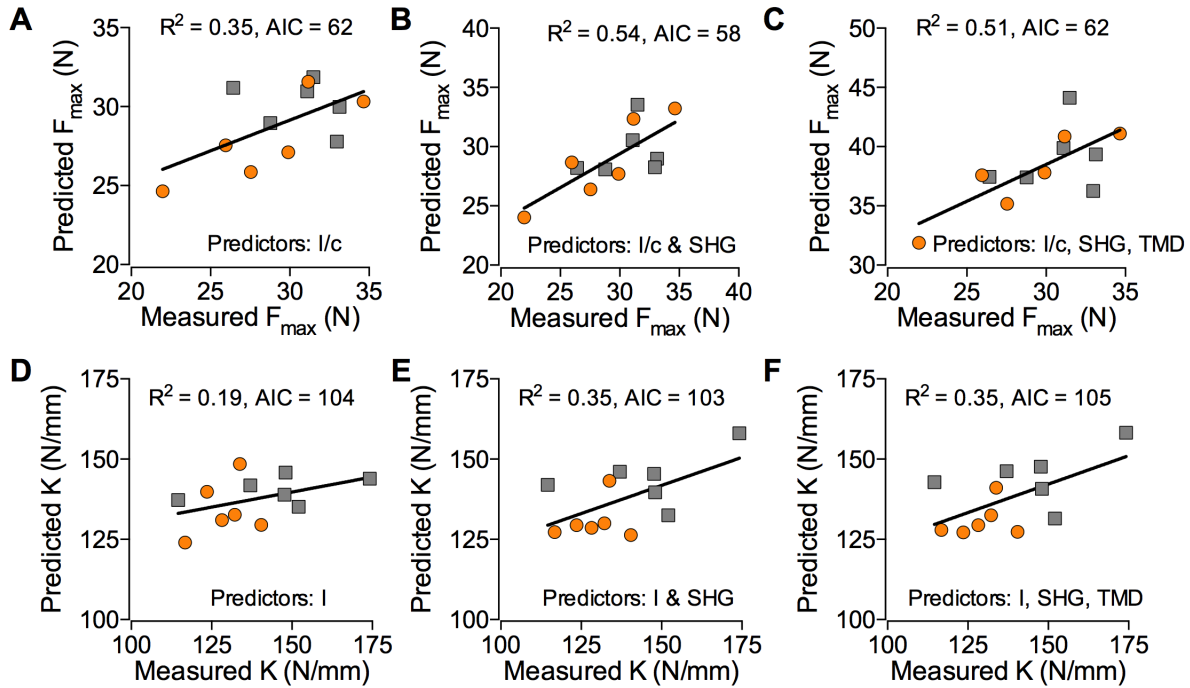
356 Like YAP/TAZ deletion from the full skeletal lineage, DMP1-conditional ablation reduced Col1a1
357 expression *in vivo* (Fig. 7J), with a similar trend for SerpinH1 ($p = 0.15$; Fig. 7K). Col1a2 was not
358 significantly reduced ($p = 0.8$; data not shown). Unlike *Osx*-dependent knockouts, DMP1-mediated
359 deletion also significantly reduced Runx2 expression (Fig. S6G).



360

361 **Figure 7. Dual homozygous YAP/TAZ deletion from DMP1-expressing cells reduced matrix**
 362 **collagen content, organization, and collagen-associated gene expression.** Femora from 12 week-
 363 old littermate mice were evaluated by mechanical testing, second harmonic imaging microscopy,
 364 histomorphometry, and gene expression analyses. **A)** DMP1-conditional YAP/TAZ deletion did not
 365 significantly reduce maximum load or intrinsic failure properties. Reductions in extrinsic stiffness and
 366 intrinsic elastic properties did not reach significance ($p = 0.08$ and 0.1 , respectively). Polarized light
 367 microscopy and second harmonic generation demonstrated reduced matrix collagen content and
 368 microstructural organization (**D**). The kurtosis (**E**) of the fiber directionality distributions (**F**) were not
 369 statistically significant ($p = 0.01$). Histological tissue sections of cortical bone stained with hematoxylin
 370 and eosin (H&E) (**G**) could not detect significant differences in osteocyte density expressed as
 371 osteocyte number per bone area (**H**) or the ratio of empty to total lacunae (**I**). YAP/TAZ deletion
 372 significantly reduced mRNA expression levels of Col1a1 (**J**), but SerpinH1 was not significant ($p=0.15$)

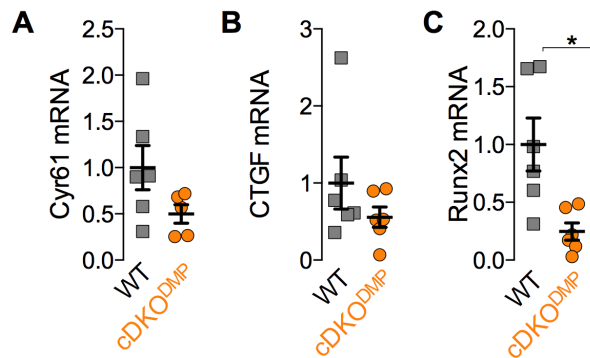
373 (K). Sample sizes, $n = 6$ for all analyses. Scale bars equal 100, 25, and 10 microns in the Picosirius
374 red, SHG, and H&E images, respectively.



375

376 **Supplementary Figure 5. Best subsets analysis on morphological parameters from the YAP/TAZ**
377 **ablation in DMP1-expressing cells from 12-week-old mice demonstrated enhanced goodness of**
378 **fit parameters by accounting for collagen content and organization.** Within the mice with DMP1-
379 driven deletion of YAP/TAZ, we similarly compared experimental ultimate load (F_u) with predicted
380 model values with only moment of inertia as a predictor (**A**), moment of inertia and second harmonic
381 generated signal intensity (**B**), or moment of inertia, second harmonic generated signal intensity, and
382 tissue mineral density (**C**). Similarly, we compared experimental to predicted stiffness using the same
383 predictors, respectively (**D-F**). Akaike's information criterion (AIC) decreased while R^2 increased in both
384 regressions with maximum load and stiffness, suggesting optimal goodness of fit with the addition of
385 SHG. See methods for full details of multivariate best subset analysis. Sample size, $n = 6$.

386



387

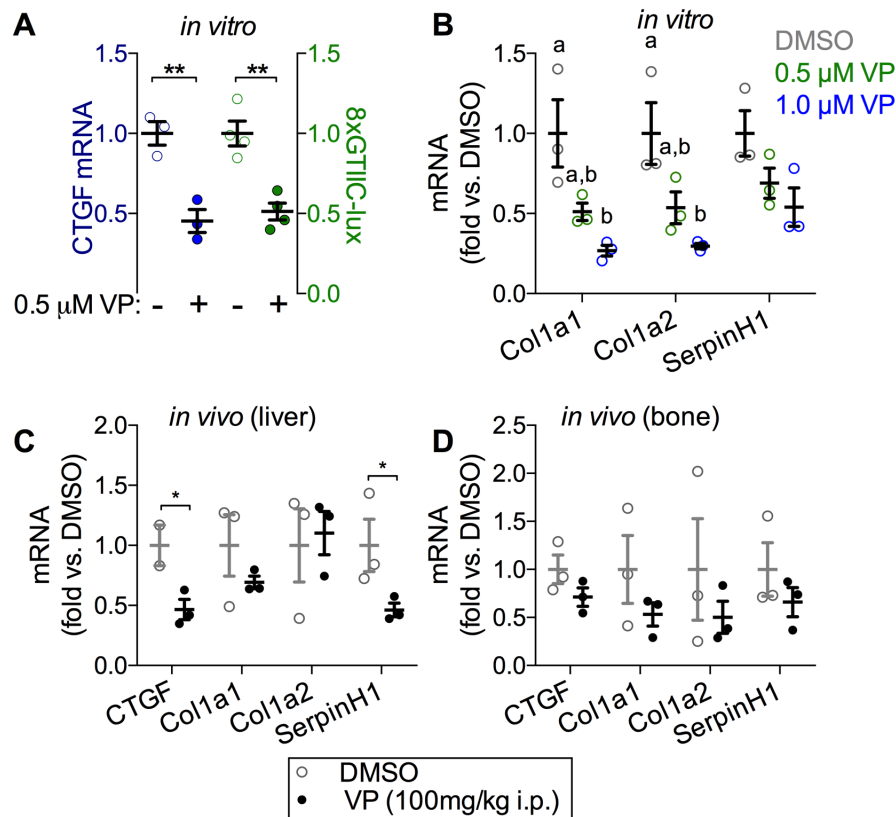
388 **Supplementary Figure 6. YAP/TAZ ablation in DMP1-expressing cells decreases trends in bone**
389 **mRNA expression levels of canonical YAP/TAZ target genes. (A) CTGF, and (B) Cyr61 expression**
390 **were down, yet not significant. (C) Runx2 expression was only significantly down. mRNA was isolated**
391 **from bone cell enriched lysates from femurs with DMP1-drive deletion of YAP/TAZ. Sample size, n = 6.**
392

392

393

394 ***Acute YAP/TAZ-TEAD inhibition reduced collagen and collagen-associated gene expression***

395 Since changes in gene expression could result either from direct YAP/TAZ regulation or from
396 developmental changes in bone cell populations, we next tested whether acute pharmacological
397 YAP/TAZ inhibition using the small molecule verteporfin (VP) would also reduce YAP/TAZ activity and
398 Col1a1 and SerpinH1 expression *in vitro* and *in vivo*. VP treatment of osteoblast-like UMR-106 cells *in*
399 *vitro* reduced expression of the canonical YAP/TAZ-TEAD target gene, CTGF, and reduced synthetic
400 YAP/TAZ-TEAD-responsive promoter activity (Fig. 8A)²⁶. VP treatment *in vitro* dose-dependently
401 reduced Col1a1 and Col1a2 expression, and SerpinH1 showed a similar but non-significant trend
402 (p=0.1; Fig. 8B). WT mice were then treated every other day with intraperitoneal VP injection
403 (100mg/kg) for two weeks. VP delivery significantly reduced expression of CTGF and SerpinH1 in liver
404 tissue *in vivo*, but reductions in Col1a1 expression in liver and CTGF, Col1a1, and SerpinH1 in bone
405 did not reach statistical significance (Fig. 8C,D). Consistent with genetic YAP/TAZ deletion, Col1a2 was
406 not significantly reduced in either tissue.



407

408 **Figure 8. YAP/TAZ-TEAD inhibition with verteporfin reduced Col1a1 and SerpinH1 expression.**

409 **A)** Verteporfin (VP) treatment of UMR-106 cells reduced mRNA expression of the canonical YAP/TAZ-
 410 TEAD target gene *CTGF* commensurate with synthetic TEAD (8xGTIIC) reporter activity, demonstrating
 411 VP effectiveness. 8xGTIIC reporter activity was normalized to Renilla luciferase expression and
 412 expressed as fold vs. DMSO. **(B)** VP treatment dose dependently reduced mRNA levels of *Col1a1*,
 413 *Col1a2*, and *SerpinH1* UMR-106 cells in comparison to DMSO. **(C,D)** 16-week-old wild type mice
 414 received intraperitoneal injections of VP (100 mg/kg) every two days. Livers and femora were harvested
 415 after 14 days to quantify mRNA expression. VP treatment significantly reduced hepatocyte expression
 416 of *CTGF* and *SerpinH1* **(C)** and exhibited similar trends in bone **(D)**, but differences were not
 417 statistically significant. Sample sizes, n = 3-4.

418

419 Discussion

420 YAP and TAZ have been implicated as regulators of osteogenesis for over a decade^{23,24}, and
 421 have been studied extensively *in vitro*; however, existing data on the differential roles of YAP vs. TAZ
 422 are strikingly contradictory^{23-30,44-46}, and their combinatorial functions in bone have not been studied *in*
 423 *vivo*. To solve this apparent conflict in a physiological context, we used combinatorial skeletal cell-

424 conditional YAP/TAZ deletion in mice to dissect the roles of YAP and TAZ both early and late in the
425 skeletal cell differentiation sequence. Our data reveal that YAP and TAZ have mutually redundant,
426 positive roles in both osteoblast progenitors and committed bone cells during development and regulate
427 bone matrix mechanical properties putatively through transcriptional control of collagen expression and
428 matrix organization.

429

430 *YAP/TAZ deletion from skeletal cells phenocopies osteogenesis imperfecta*

431 YAP/TAZ deletion phenocopied osteogenesis imperfecta (OI), with severity dependent on
432 targeted cell lineage and allele dosage. OI is a highly heterogeneous group of diseases characterized
433 by bone fragility and deformity, whose severity varies from mildly increased fracture risk to perinatal
434 lethality⁴⁷. Though heritable through either autosomal dominant or recessive forms, mutations in the
435 structure, organization, or quantity of collagen deposited in the bone matrix cause 85-90% of all OI
436 cases^{47,48}. The specific genetic mutations that cause the various types of OI remain poorly understood,
437 though new putatively causal mutations continue to be discovered⁴⁹. YAP/TAZ signaling has not been
438 associated with OI, but elucidation of this pathway in bone may contribute new insights into the
439 heterogeneity and/or etiology of the disease. Because global YAP deletion is embryonic lethal in animal
440 models, loss-of-function mutations in YAP/TAZ are unlikely to cause human OI. However, there are
441 many pathways that converge on YAP/TAZ which could place this signaling axis upstream of the
442 human disease, including TGF β -Smad2/3^{11,12} and WNT- β -catenin^{9,10}. Further research will be required
443 to evaluate whether YAP/TAZ signaling is causally linked to OI.

444 Mice possessing only a single copy of either gene in osterix-lineage cells rescued the lethality
445 found in dual homozygous knockouts, demonstrating mutually compensatory function in skeletal cells.
446 This contradicts some observations in the literature implicating divergent roles of YAP^{23,26,27} and
447 TAZ^{25,29,30} in osteogenesis, but corroborate other reports of equivalent function^{24,26,44,50}. These data also
448 indicate that future loss-of-function studies aiming to understand the roles of these paralogs in bone
449 must account for the activity of both genes in concert.

450

451 *YAP/TAZ regulate endochondral ossification*

452 The spontaneous long bone fractures observed in Osterix-conditional YAP^{CKO};TAZ^{CHET} and
453 YAP^{CHET};TAZ^{CKO} mice healed through natural endochondral repair similar to experimental neonatal
454 fractures, which straighten and heal through bidirectional growth plate formation controlled by forces
455 exerted by the adjacent muscle⁵¹. This suggests that this mechanotransductive realignment mechanism
456 remained functional despite prior demonstration that YAP negatively regulates chondrogenesis both *in*
457 *vitro*⁵² and *in vivo*³¹. However, the Osterix-Cre transgene is not strongly expressed in early or
458 proliferating chondrocytes³², which are responsible for bone fragment re-alignment⁵¹. Instead, YAP/TAZ
459 deletion from Osterix-expressing cells impaired endochondral ossification, potentially through reduced
460 osteoprogenitor motility²⁶, proliferation⁵², or survival⁵³. Further research outside the scope of this study
461 will be required to elucidate these mechanisms.

462

463 *YAP/TAZ regulate bone matrix collagen*

464 Osterix-conditional knockout mice mimicked clinical observations of Types II and III OI and
465 several established OI mouse models⁴³, characterized by reduced bone volume, hypermineralization,
466 and reduced intrinsic mechanical properties. For example, the human Col1a1 minigene mouse^{54,55},
467 which expresses a human transgene with a clinically-observed mutation in pro- $\alpha_1(I)$ collagen, dose-
468 dependently reproduces the phenotypes seen here with Osterix1-conditional YAP/TAZ deletion. Like
469 YAP^{CKO};TAZ^{CKO} mice, high dose pro- $\alpha_1(I)$ transgenics exhibited neonatal lethality, while mice with
470 moderate transgene dose mimicked the heterozygous knockouts with spontaneous femoral fractures
471 and reduced failure, but not elastic bone material properties. Similarly, the naturally-occurring *oim*
472 mouse, caused by a frameshift mutation in pro- $\alpha_2(I)$ collagen, also features reduced bone mechanical
473 properties and increased fracture incidence despite elevated mineral density^{56,57}.

474 Notably, YAP/TAZ deletion later in the skeletal sequence (from DMP1-expressing cells)
475 exhibited a similar but milder phenotype with moderate defects in cortical and cancellous bone accrual
476 and matrix collagen content and organization. A single intact allele of either gene in DMP1-expressing
477 cells sufficiently rescued the defect in long bone growth, indicating mutual YAP/TAZ redundancy.

478

479 *YAP/TAZ-dependent gene expression*

480 Using a candidate-based approach selected from disease-causing mutations in human OI, we
481 identified Col1a1 and SerpinH1 as YAP/TAZ-regulated genes in bone, with consistently reduced
482 expression either by genetic YAP/TAZ ablation at multiple stages in the skeletal cell lineage or by acute
483 YAP/TAZ-TEAD inhibition *in vitro* and *in vivo*. VP-induced reductions in CTGF, Col1a1, and SerpinH1
484 mRNA in bone did not reach significance, likely due to the small sample size and the less efficient
485 biodistribution of porphyrins to bone compared to liver and other tissues^{58,59}. However, known YAP/TAZ
486 target genes and SerpinH1 were significantly reduced in livers of VP-treated mice, indicating generality.
487 These genes clearly do not constitute a sufficient set of all YAP/TAZ target genes with potential
488 importance in bone development, and many other pathways are likely involved through other putative
489 YAP/TAZ co-effectors¹³⁻¹⁷. Further research will be required to identify all YAP/TAZ-regulated genes in
490 bone.

491 Both Osterix-Cre⁶⁰ and DMP1-Cre⁶¹ transgenes have reports of targeting non-skeletal-lineage
492 cell populations, which must be taken under consideration. However, the consistency in the phenotypes
493 observed through *in vivo* YAP/TAZ ablation in DMP1-expressing cells and in Osterix-expressing cells,
494 combined with the allele dosage-dependent response in bone phenotype severity, suggest the
495 importance of skeletal cell YAP/TAZ. Taken together, these data suggest that YAP and TAZ
496 redundantly promote bone matrix quality during development through transcriptional co-activation of
497 TEAD to regulate Col1a1 and SerpinH1 expression *in vivo* and suggest that the YAP/TAZ signaling axis
498 could have a role in osteogenesis imperfecta.

499

500 **MATERIALS AND METHODS**

501 *Animals*

502 Mice harboring loxP-flanked exon 3 alleles in both YAP and TAZ were kindly provided by Dr. Eric Olson
503 (University of Texas Southwestern Medical Center). The tetracycline responsive B6.Cg-Tg(Sp/7-
504 tTA,tetO-EGFP/cre)1AMc/J (Jackson laboratories) mice (Osx-Cre) were raised, bred, and evaluated

505 without tetracycline administration to induce constitutive gene recombination in osteoprogenitor cells
506 and their progeny³². Mature osteoblasts and osteocytes were separately targeted using Cre-
507 recombination under the control of an 8kb fragment of the Dmp1 promoter (Dmp1-Cre)³³. Mice with
508 homozygous floxed alleles for both YAP and TAZ (YAP^{fl/fl};TAZ^{fl/fl}) were mated with double heterozygous
509 conditional knockout mice (YAP^{fl/+};TAZ^{fl/+};Osx-Cre and YAP^{fl/+};TAZ^{fl/+};Dmp1-Cre) to produce eight
510 possible genotypes in each litter (Table S1). Both male and female mice were evaluated. No
511 differences in skeletal phenotype were observed based on sex (Fig. S2A,B). All mice were fed regular
512 chow ad libitum and housed in cages containing 2-5 animals each. Mice were maintained at constant
513 25°C on a 12-hour light/dark cycle. Mice were tail or ear clipped after weaning or prior to euthanasia
514 and genotyped by PCR or using a third party service (Transnetyx Inc.) All protocols were approved by
515 the Institutional Animal Care and Use Committee at the University of Notre Dame in adherence to
516 federal guidelines for animal care.

517

518 *Skeletal preparations*

519 Skeletal preparations stained with Alizarin red and Alcian blue were performed as described
520 previously⁶². Briefly, mice were euthanized via CO₂ asphyxiation at postnatal day 0 (P0) and skin,
521 viscera, and adipose tissues were dissected quickly. Dissected skeletons were fixed in 80% ethanol
522 and dehydrated in 95% ethanol overnight at room temperature immediately followed by incubation in
523 100% acetone for two days to remove remaining adipose tissue. Prepared skeletons were stained at
524 room temperature using Alcian blue (A3157; Sigma) and Alizarin red (A5533; Sigma) for two days.
525 Stained skeletons were incubated in 95% ethanol for 1 hour then remaining tissue was cleared through
526 incubation in a 1% potassium hydroxide (KOH) solution. Skeletal preparations were stabilized in a 1%
527 KOH / 20% glycerol solution and imaged in 1% KOH / 50% glycerol solution with a Nikon D7100
528 camera.

529

530 *μCT and mechanical testing*

531 Femora were harvested at 8 or 12 weeks of age and stored at -20°C until evaluation. The frozen
532 specimens were thawed and imaged in PBS using a vivaCT 80 scanner (Scanco Medical) to determine
533 trabecular and cortical femoral bone architecture prior to mechanical testing to failure in three-point
534 bending. The mid-diaphysis and distal femur were imaged with an X-ray intensity of 114 μ A, energy of
535 70 kVp, integration time of 300 ms, and resolution of 10 μ m. Mid-diaphyseal and distal femoral 2D
536 tomograms were manually contoured, stacked and binarized by applying a Gaussian filter (sigma =1,
537 support = 1) at a threshold value of 250 mg HA/cm³. Mechanical testing analysis of the femurs was
538 carried out by three-point bend testing. The femurs were loaded with the condyles facing down onto the
539 bending fixtures with a lower span length of 4.4 mm. The upper fixture was aligned with the mid-
540 diaphysis. The femora were loaded to failure at a rate of 0.5 mm/s using the ElectroForce 3220 Series
541 testing system (TA Instruments). Sample sizes: n = 8 animals for each group except n = 1 animal for
542 the YAP^{ckO};TAZ^{ckO}.

543

544 *Histology*

545 Bone samples were fixed in 10% neutral buffered formalin overnight at 4°C, decalcified in a 7:3
546 mixture of 14% neutral buffered EDTA solution and 10% neutral buffered formalin for 3 weeks at 4°C,
547 and transferred to 70% ethanol for storage at 4°C. Paraffin sections of 5 μ m thickness for both
548 histological staining and immunohistochemistry were deparaffinized and rehydrated in a graded series
549 of ethanol solutions and washed three times in PBS. For immunohistochemistry, heat induced antigen
550 retrieval in a 1x sodium citrate buffer preceded the quenching of endogenous peroxides with
551 PeroxoBlock (Invitrogen). Primary antibodies were compared to negative control sections. Anti-Osterix
552 (abcam: Cat# ab22552, 1:250), anti-YAP (Cell Signaling: Cat# 14074, 1:400), anti-TAZ (Cell Signaling:
553 Cat# 4883, 1:400) were both applied overnight at 4°C and compared to sections receiving only
554 secondary antibody. Colorimetric detection using the DAB Peroxidase HRP-linked Substrate Kit (Vector
555 Labs) allowed for immunohistochemical detection of YAP, TAZ, or Osterix-positive cells. Hematoxylin
556 and eosin stains (H&E) were used to detect nuclei and tissue morphology, Safranin O/Fast Green (Saf-
557 O) to visualize sulfated glycosaminoglycans, and Picrosirius Red to visualize collagen fibers. Osteocyte

558 number per bone area and empty lacunae ratio (empty lacunae over both empty and full lacunae were
559 manually counted using ImageJ (NIH) on H&E stained sections. Hypertrophic chondrocyte zone
560 percent thickness (HZ thickness %) was calculated manually in ImageJ (NIH). Three separate lines
561 were drawn across the area of positive Saf-O staining, normalized to the respective length of the
562 hypertrophic chondrocyte zone within each line, and averaged together for each image. Three images
563 per animal were taken at the growth plate. Sample sizes: n = 8 animals for each group except n = 1
564 animal for the YAP^{ckO};TAZ^{ckO}.

565

566 *Imaging*

567 Histological and immunohistochemical sections were imaged on a 90i Upright/Widefield
568 Research Microscope (Nikon Instruments) at the 4x, 10x, 20x, and 40x objectives. Three-point bend
569 femur sections stained with Picro-sirius Red were imaged under polarized light using an Eclipse ME600
570 Microscope (Nikon Instruments) at the 20x objective while second harmonic image microscopy (SHIM)
571 images were taken on a multiphoton-enabled Fluoview Research Microscope (Olympus) at 875nm with
572 the 25x objective on sections oriented in the same direction for all groups. All SHIM images were
573 quantified using ImageJ (NIH) and reported as mean pixel intensity within the cortical region of the
574 femoral bone. Mean pixel intensities across four separate regions of interest within each image of the
575 cortex were averaged as technical replicates for a given histological section. Directionality was
576 assessed by using the FIJI macro to ImageJ (NIH). Raw directionality histograms were imported from
577 FIJI to MATLAB (v2015.b). The histograms from each image were averaged and then fit with a
578 Gaussian function assuming a normal distribution. The generated average probability density functions
579 were reported for each genotype.

580

581 *Verteporfin delivery in vivo*

582 Six littermate control (four male and two female) mice (YAP^{WT};TAZ^{WT}) were aged until 16
583 weeks. Two males and one female were then either administered Verteporfin (100 mg/kg) in 0.9%
584 saline or dimethyl sulfoxide (DMSO) in 0.9% saline by intraperitoneal injection every other day for 2

585 weeks. Livers and femurs from both verteporfin-treated and DMSO-treated mice were harvested on the
586 day of the last injection.

587

588

589

590 *RNA isolation and qPCR*

591 Total bone and liver samples were snap frozen in liquid nitrogen cooled isopentane for 1 minute before
592 quickly being store at -80°C until processed. Upon processing, tissue was homogenized via mortar and
593 pestle and RNA from the sample was collected using Trizol Reagent (Life Technologies Inc.) followed
594 with centrifugation in chloroform. Upon layer separation, the RNA sample was purified using the RNA
595 Easy Kit (Qiagen) and quantified by spectrophotometry using a NanoDrop 2000. Reverse transcriptase
596 polymerase chain reaction (RT-PCR) was performed on 0.5 ug/ul concentration of RNA using the
597 TaqMan Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction
598 (qPCR) assessed mRNA amount using a CFX Connect (Bio-Rad) relative to the internal control of
599 glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented using the $\Delta\Delta C_t$ method.
600 Specific mouse primer sequences are listed in Table S2. n = 6 animals for each group except n = 1
601 animal for the YAP^{ckO};TAZ^{ckO}.

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Table S3: Mouse primer sequences for qPCR.

Gene		Primer Sequence (5' to 3')
<i>Gapdh</i>	F R	TCACTGCCACCCAGAAGAC TGTAGGCCATGAGGTCCAC
<i>Yap</i>	F R	TGGACGTGGAGTCTGTGTTG AAGCGGAACAACGATGGACA
<i>Taz</i>	F R	GTCCATCACTTCCACCTC TTGACGCATCCTAATCCT
<i>Col1a1</i>	F R	GCTCCTCTTAGGGGCCACT CCACGTCTCACCATTGGGG
<i>Col1a2</i>	F R	GTA ACTTCGTGCCTAGCAACA CCTTTGTCAGAATACTGAGCAGC
<i>SerpinH1</i>	F R	AGCCGAGGTGAAGAAACCC CATCGCCTGATATAGGCTGAAG
<i>Runx2</i>	F R	AGCCTCTTCAGCGCAGTGAC CTGGTGCTCGGATCCCAA
<i>Osterix</i>	F R	CTGGGGAAAGGAGGCACAAAGAAG GGGTTAAGGGAGCAAAGTCAGAT
<i>Ctgf</i>	F R	GGGCCTTTCTGCGATTC ATCCAGGCAAGTGCATTGGTA
<i>Cyr61</i>	F R	CTGCGCTAAACA ACTCAACGA GCAGATCCTTTCAGAGCGG

618

619

620 *Rat osteosarcoma cell line*

621 UMR-106 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) modified to

622 contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium

623 bicarbonate and 10% FBS according to the American Type Culture Collection (ATCC; Catalog #: 30-

624 2002) recommendations. UMR-106 cells at 50 % confluence were transfected with the TEAD-
625 responsive luciferase reporter plasmid 8XGTIIC-Luciferase (Addgene) and a control Renilla plasmid,
626 both kindly provided by Munir Tanas, MD, University of Iowa. Forty-eight hours after transfection, cells
627 were treated with either DMSO, 0.5 μ M, or 1 μ M verteporfin in serum-free conditions for 1 hour. All
628 verteporfin experiments were carried out in the dark. Cells were then lysed immediately using the Dual-
629 Luciferase Reporter Assay System according to manufactures instructions (Promega). Luciferase
630 activity was measured on a VICTOR 3 (Perkin Elmer) plate reader and normalized to baseline renilla
631 activity as previously described⁶³. Separately cultured UMR-106 cells were simultaneously treated with
632 either DMSO, 0.5 μ M, or 1 μ M verteporfin and then cultured under serum-free conditions for one hour.
633 mRNAs were then isolated and purified with the RNA-easy Mini-Kit (Qiagen) following the
634 manufacturer's instructions. mRNA (0.5 μ g) was reverse transcribed using the TaqMan Reverse
635 Transcription Kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) assessed
636 mRNA amount using a CFX Connect (Bio-Rad) relative to the internal control of glyceraldehyde 3-
637 phosphate dehydrogenase (GAPDH). Data are presented using the $\Delta\Delta$ Ct method. Specific rat primer
638 sequences are listed in Table S3. Sample size, n = 3 independent experiments.

639 **Table S4: Rat primer sequences for qPCR.**

Gene	Primer Sequence (5' to 3')	
<i>Gapdh</i>	F	CATGGCCTTCCGTGTTCCCTA
	R	GCGGCACGTCAGATCCA
<i>Col1a1</i>	F	ACAGCGTAGCCTACATGG
	R	AAGTTCCGGTGTGACTCG
<i>Col1a2</i>	F	ATGGTGGCAGCCAGTTTG
	R	GCTGTTCTTGCAGTGGTAGG
<i>SerpinH1</i>	F	TCATGGTGACCCGCTCCTAC
	R	GCTTATGGGCCAAGGGCATC
<i>Ctgf</i>	F	ATCCCTGCGACCCACACAAG
	R	CAACTGCTTTGGAAGGACTCGC
<i>Cyr61</i>	F	AGAGGTGTTGAGCATCGTGGAG
	R	AACTGCGACTGCGTTACTGTCC

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641 *Statistics and regression*

642 All statistics and linear regressions including the ANCOVA were performed in GraphPad Prism.
643 Comparisons between two groups were made using the independent t-test while comparisons between
644 3 or more groups were made using a one-way ANOVA with post-hoc Bonferroni's multiple comparisons
645 test if the data were normally distributed according to D'Agostino-Pearson omnibus normality test and
646 homoscedastic according to Bartlett's test. When parametric test assumptions were not met, data were
647 log-transformed prior and residuals were evaluated. If necessary, the non-parametric Kruskal-Wallis
648 test with post-hoc Dunn's multiple comparisons was used. A p-value of less than 0.05 (adjusted for
649 multiple comparisons) was considered significant. Data are represented as individual samples with
650 mean \pm standard error of the mean (s.e.m.). Multivariate correlations were performed using the *bestglm*
651 package (available at <https://cran.r-project.org/web/packages/bestglm/> version 0.36) in the open-source
652 GNU statistical package R (Version 2.13.1). We followed a modified procedure previously described⁴⁰.
653 Briefly, we chose to use an exhaustive best subsets algorithm to determine the best predictors of
654 maximum load and stiffness from a subset of morphological parameters measured, which included
655 moment of inertia (I) or section modulus (I/c), tissue mineral density (TMD), and second harmonic
656 generation (SHG) intensity based on the Akaike's information criterion (AIC).³⁹ The lowest AIC selects
657 the best model while giving preference to less complex models (those with fewer explanatory
658 parameters). Finally, the overall "best" model for each predicted mechanical property was compared to
659 the prediction from only the moment of inertia (I/c or I for maximum load and stiffness, respectively)
660 using Type II general linear regression.

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