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1 **Title: The P2Y13 receptor regulates extracellular ATP metabolism and the osteogenic**
2 **response to mechanical loading.**

3
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17
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34

35 **Disclosure Page**

36 **All authors state that they have no conflicts of interest.**

37

38 **Abstract**

39 ATP release and subsequent activation of purinergic receptors has been suggested to be one of
40 the key transduction pathways activated by mechanical stimulation of bone. The P2Y₁₃ receptor,
41 recently found to be expressed by osteoblasts, has been suggested to provide a negative feedback
42 pathway for ATP release in different cell types. Therefore, we hypothesised that the P2Y₁₃
43 receptor may contribute to the mediation of osteogenic responses to mechanical stimulation by
44 regulating ATP metabolism by osteoblasts. To test this hypothesis, wild type (WT) and P2Y₁₃
45 receptor knock-out (P2Y₁₃R^{-/-}) mice were subject to non-invasive axial mechanical loading of the
46 left tibiae to induce an osteogenic response. Micro-Computed Tomography analysis showed
47 mechanical loading induced an osteogenic response in both strains of mice in terms of increased
48 total bone volume and cortical bone volume, with the P2Y₁₃R^{-/-} mice having a significantly
49 greater response. The extent of the increased osteogenic response was defined by dynamic
50 histomorphometry data showing dramatically increased bone formation and mineral apposition
51 rates in P2Y₁₃R^{-/-} mice compared with controls. In vitro, primary P2Y₁₃R^{-/-} osteoblasts had an
52 accumulation of mechanically induced extracellular ATP and reduced levels of hydrolysis. In
53 addition, P2Y₁₃R^{-/-} osteoblasts also had a reduction in their maximal alkaline phosphatase (ALP)
54 activity, one of the main ecto-enzymes expressed by osteoblasts which hydrolyses extracellular
55 ATP. In conclusion, deletion of the P2Y₁₃ receptor leads to an enhanced osteogenic response to
56 mechanical loading in vivo, possibly due to the reduced extracellular ATP degradation by ALP.
57 The augmented osteogenic response to mechanical stimulation, combined with suppressed bone
58 remodelling activities and protection from OVX-induced bone loss after P2Y₁₃ receptor
59 depletion as previously described, suggests a potential role for P2Y₁₃ receptor antagonist-based
60 therapy, possibly in combination with mechanical loading, for the treatment of osteoporosis.

61

62

63

64 **Key words:** P2Y13 receptor, osteogenic, mechanical loading, ATP release, ATP hydrolysis

65

66 **Introduction**

67 Bone integrity is maintained throughout life via bone remodelling where the balance between
68 bone resorption and formation is critical. Altered coupling of resorption and formation leads to
69 bone disorders such as osteoporosis which is characterized by higher resorption and lower
70 formation (1). Most current treatment strategies for osteoporosis have focused on anti-resorptive
71 therapies such as bisphosphonates and more recently antibodies to RANKL (Denosumab) which
72 can successfully reduce the risk of osteoporotic vertebral fractures (2,3). However, the only
73 current anabolic agent for osteoporosis treatment available at the moment is Parathyroid
74 hormone (PTH) (either as PTH1-34/ “teriparatide” or full-length PTH1-84). Due to the relatively
75 poor anti-fracture efficacy at some skeletal sites with these current agents, the need for new
76 anabolic targets is paramount. Mechanical loading of bone is widely accepted as a potent
77 anabolic stimulus for bone formation (4) and its use as a preventative measure or treatment for
78 osteoporosis is becoming increasingly attractive (5,6), especially in combination with drugs that
79 target the osteogenic response pathway (7,8). Bone osteogenic adaption to mechanical loading is
80 performed by regulating the activities of both osteoblasts and osteoclasts (9), mediated by the
81 osteocytes and bone lining cells that are thought to act as the principal mechanosensors (10). At
82 the cellular level, mechanical loading-induced osteogenic response is initiate via the release of
83 intracellular molecules such as nitric oxide (NO) and prostaglandins (PG), which are anabolic to
84 osteoblasts (11,12). Mechanical stimuli can also induce extracellular ATP release from a variety
85 of cells, including osteoblasts (13-15). This mechanism is now widely believed to be one of the
86 transduction pathways by which mechanical stimulation initiates a cellular response. Upon
87 stimulation, ATP not only mediates the secretion of other intracellular molecules such as PGs
88 (16), but also activates the purinergic receptors such as the P2X7 receptor which acts as fluid

89 flow sensor for ATP-dependent phosphorylation of ERK in osteoblasts in vitro (11,17)
90 stimulating proliferation (18). In vivo, P2X7 receptor knockout mice have been shown to have
91 ~70% reduction in the skeletal sensitivity to mechanical loading (19). Other purinergic receptors
92 are activated by extracellular ATP and have been demonstrated to play a role in integrating local
93 and systemic responses in the activation of bone remodelling (20). More recently the P2Y₁₃
94 receptor has been shown to be involved in the regulation of bone remodelling and protection of
95 mice from estrogen deficiency-induced bone loss (21). In addition, the P2Y₁₃ receptor was also
96 found to provide a negative feedback pathway to inhibit ATP release from human red blood cells
97 in response to low oxygen level (22). These findings suggest a role for P2Y₁₃ receptors in ATP
98 metabolism and potentially in the response to mechanical loading via other purinergic receptor
99 such as the P2X7 receptor. Indeed, there is evidence showing P2Y₁₃ and P2X7 receptors co-
100 mediate intracellular calcium responses to BzATP in rat cerebellar astrocytes (23). In addition, it
101 was recently shown that blocking the P2Y₁₃ receptor can mediate ERK1/2 involvement in β -cell
102 apoptosis (24). Interestingly, ERK1/2 signalling was demonstrated to be involved in osteoblastic
103 response upon mechanical strain and fluid flow (17,25).

104

105 Given the expression of P2Y₁₃ receptor by osteoblasts and the observed negative feedback
106 pathway for ATP release in red blood cells, we hypothesised that the P2Y₁₃ receptor would play
107 a role in the osteogenic response to mechanical stimulation via regulating ATP metabolism in
108 osteoblasts. To test this hypothesis, we examined the osteogenic response of P2Y₁₃ receptor
109 knockout (P2Y₁₃R^{-/-}) mice to mechanical stimuli in vivo. Non-invasive controlled axial
110 mechanical loading was performed on left tibiae of 4-month old P2Y₁₃R^{-/-} and wild type (WT)
111 mice in vivo (26,27). Microcomputed tomography (μ CT) analysis and dynamic

112 histomorphometry were used to determine the osteogenic response. ATP release and hydrolysis
113 by primary osteoblasts was determined.

114

115 **Materials and Methods**

116 **Mice**

117 P2Y₁₃R^{-/-} mice (28) were backcrossed onto the C57BL/6J background as previously described.
118 Sixteen week old P2Y₁₃R^{-/-} and WT mice were housed in the same environmentally controlled
119 conditions with a 12hr light/dark cycle at 22°C and free to access 2018 Teklad Global 18%
120 Protein Rodent Diet containing 1.01% Calcium (Harlan Laboratories, UK) and water ad libitum
121 in RB-3 cages. All procedures complied with the UK Animals (Scientific Procedures) Act 1986
122 and were reviewed and approved by the local Research Ethics Committee of the University of
123 Sheffield (Sheffield, UK).

124

125 **Mechanical loading in vivo**

126 In this study, the non-invasive axial loading tibial model (26) was used to examine responses to
127 mechanical loading in 16 week old WT and P2Y₁₃R^{-/-} mice. The peak load (15N) was selected to
128 induce bone formation in the loaded tibiae since evidence showed that similar peak load can
129 induce osteogenic response in female C57BL/6 mice (26,29,30). Briefly, a 14.5N dynamic load
130 was superimposed onto a 0.5N pre-load at rate of 160,000N/sec. Forty trapezoidal-waveform
131 load cycles (0.2 sec hold at 15N) with 10 sec interval between each cycle were applied to mice
132 tibiae, three times a week for 2 weeks. Mice were injected intraperitoneally with calcein (30
133 mg/kg) on the first (day 1) and last day (day 12) of loading. Mice were then euthanized on day
134 14 (27). Both tibiae were dissected and fixed in 70% ethanol for μ CT and dynamic

135 histomorphometry analysis. The contra-lateral non-loaded limb (right tibia) was treated as
136 internal control for loading [the functional adaption in both cortical and trabecular bone being
137 controlled locally and confined to the loaded bones (27,31)] and the osteogenic responses were
138 expressed as percentage change based on the non-loaded limb data ([Parameters of loaded tibia
139 (left)/ Parameters of own non-loading tibia (right)] x 100%)(32).

140

141 *μCT*

142 Fixed tibiae were scanned using a SkyScan 1172 desktop *μCT* machine at a resolution of 4.3 μ m
143 for the tibia proximal end and 17.3 μ m for the whole tibia, with the X-ray source operating at
144 50kV, 200 μ A and using a 0.5mm aluminium filter. Two-dimensional *μCT* images were captured
145 and reconstructed by Skyscan NRecon software at threshold of 0.0-0.16 and 0.0-0.14 for tibia
146 proximal end and whole tibia scan respectively. For the tibia proximal end scan, trabecular
147 morphometry was characterized by measuring structural parameters in a 1.0mm thick trabecular
148 region which is 0.2mm below the growth plate. Cortical morphometry was quantified from the
149 cortical regions locating in the proximal 20% (1.0mm thick, 1.0mm below the growth plate) and
150 the midshaft of tibiae (1.0mm thick, 7.0mm below the growth plate). Bone tissue mineral
151 densities (TMD) equal to grams of hydroxylapatite per cube centimetre were calculated based on
152 image greyscale with the following equation: $TMD = (0.012 \times \text{greyscale value}) - 0.296$ (21).
153 Nomenclature and symbols were used to describe the *μCT* derived bone morphometries
154 according to (33).

155

156 Linear-elastic finite element analysis (FEA)

157 Linear-elastic finite element models of the tibiae were generated to simulate compression of the
158 tibia and to verify strains induced by the 15N loading force in representative bones from WT and
159 P2Y₁₃R^{-/-} mice scanned post mortem. Briefly, cement blocks were added to the ends of the tibia
160 to facilitate even application of compressive force at the bone ends. Models were generated
161 directly from voxels of the whole contra-lateral non-loaded tibial μ CT scans using a cube-shaped,
162 8-node brick element with a side length of 0.0349 mm. Isotropic material properties were
163 assigned to the bone elements using the following empirical equations of Somerville et al (34).

164

$$\begin{aligned} \rho_{\text{ash}} &= 0.012\rho_{\text{CT}} - 0.296 \quad (\text{in g/cm}^3) \\ E &= 14.1\rho_{\text{ash}} - 2 \quad (\text{in GPa}) \end{aligned}$$

166

167 where ρ_{ash} and ρ_{CT} are ash density and bone density from μ CT respectively and E is modulus of
168 elasticity of bone. The modulus of elasticity for cement was assigned to 2 GPa. The Poisson's
169 ratio was set to 0.35 for bone and cement. The models were solved by a commercial FE package
170 ANSYS (ANSYS Inc., Canonsburg, PA, USA) for stress and strain at each element. The loading
171 induced average strain in the cortical and trabecular compartment were calculated on a 1.0 mm in
172 length region, 0.2 mm below the growth plate in tibia. An overall strain through the whole length
173 of the tibia was defined as the compressive displacement derived from the FEA ($L_1 - L'_1$)
174 divided by the original tibial length (L_1) in the non-loading state (Figure 1A).

175

176 Bone dynamic histomorphometry

177 Following μ CT analysis, tibiae were embedded into LR White resin (Taab Laboratory
178 Equipment Ltd). Sections were cut (at 10 μ m) longitudinally using a Leica Microsystems
179 Microtome and were examined under UV illumination using a DMRB microscope (Leica

180 Microsystems, Milton Keynes, UK). The bone histomorphometry software Osteomeasure
181 (Osteometrics) was used to measure the double labelled surface (dLS), single label surface (sLS),
182 the separation width between the two fluorescent labels (Ir.L.Th), and total bone surface (BS) on
183 a 3-mm length of both endocortical and periosteal surface, 0.25 mm from the growth plate (35).
184 The time separating the two labels (Ir.L.t) was the interval between the two IP injects of calcein
185 and was 12 days in all animals. Based on these measurements, mineralizing surface (MS),
186 mineral apposition rate (MAR), and bone formation rate (BFR/BS) were calculated and reported
187 in the results using nomenclature the based on the report of the ASBMR Histomorphometry
188 Nomenclature Committee (36).

189

190 Primary osteoblast isolation

191 Primary osteoblasts were isolated from neonatal mouse calvariae (less than 72 hours old, 5-7
192 pups per culture) as described before (21). Calvariae were dissected and the attached soft tissue
193 were digested in 1mg/ml Collagenase 1A (Sigma) for 15 mins. Calvariae were then subjected to
194 serial digestions in 1mg/mL Collagenase 1A for 30 mins; 0.25% Trypsin/EDTA (Gibco) for 15
195 mins; and 1mg/mL Collagenase 1A for 30 mins, at 37°C. All cells were harvested from the
196 digestion suspensions and seeded into a T75 flask and cultured until confluent in
197 DMEM+GLUTAMAX medium with sodium pyruvate (Gibco), 100 Units/mL Penicillin and 100
198 µg/mL Streptomycin (Gibco) and 10% foetal bovine serum (FBS) (Gibco).

199

200 Endogenous ATP release

201 Fluid flow-induced shear stress is a known stimulator for endogenous ATP release from cells
202 including osteoblasts (11,16). The mechanical disturbances caused by simple medium

203 displacement or replacement in vitro are widely accepted methods to induce fluid flow-induced
204 shear stress and stimulate ATP release (37,38) from cells including osteoblasts (39). Therefore
205 medium replacement on primary osteoblast was used to mimic mechanical loading in vitro. First
206 passage primary osteoblasts were seeded into 24 well plates at the density of 5×10^3 cell/well
207 and cultured until 70% confluence in growth medium: DMEM+GLUTAMAX medium with
208 sodium pyruvate (Gibco), 100 Units/mL Penicillin and 100 μ g/mL Streptomycin (P/S) (Gibco)
209 and 10% FCS (Gibco). The cells were washed three times with serum free medium:
210 DMEM+GLUTAMAX medium with sodium pyruvate, 100 Units/mL Penicillin and 100 μ g/mL
211 Streptomycin, and 25 mM HEPES buffer and replenished with 500 μ L serum free medium.
212 Samples were collected from four replicate wells at time points 0, 5, 10, 20, 30, 40, 50, and 60
213 mins. ATP concentration was then determined using the HS ViaLight Kit (Lonza, Slough, UK)
214 as previously described. To confirm that ATP release was not caused by cell death, the cell lysis
215 marker lactate dehydrogenase was measured from non-heat inactivated medium samples using
216 the CytoTox 96 well Non-Radioactive Cytotoxicity Assay (Promega, Southampton, UK) on a
217 SpectraMAX M5e plate reader at 492nm. Samples showing increased LDH release were
218 removed from analysis. Samples for luciferase assay were heated at 98°C for 2 mins to inactivate
219 soluble ATPases and frozen down immediately in liquid nitrogen and stored at -80°C. Samples
220 for LDH Assay were directly frozen down in liquid nitrogen and stored at -80°C.

221

222 Exogenous ATP hydrolysis

223 Following sample collection for endogenous ATP release measurement, the media was removed
224 completely from the wells. Fresh serum free medium (500 μ L) was carefully added into each
225 well and the plate incubated for 60 mins at 37°C to return the medium pH and extracellular ATP

226 concentration to basal levels. Medium samples were collected from four replicate wells per time
227 point for both luciferase and LDH assay prior (t=-1 mins) to the addition of 300nM ATP (Sigma:
228 99.9% pure by HPLC, reconstituted in 25 mM HEPES buffer) and at time point t=0(immediately
229 after addition), 5, 10, 20, and 30 mins.

230

231 Alkaline Phosphatase (ALP) assay

232 First passage primary osteoblast cells isolated from P2Y₁₃R^{-/-} and WT neonatal calvariae were
233 seeded at 1.5x10⁴ cells per well in a 12-well cell culture plates and cultured for six days. At the
234 end of this time period the cells were washed with PBS and harvested by addition of nuclease-
235 free water into each well and the samples snap frozen at -80°C. Cell lysates were obtained after
236 three freeze thaw cycles. Alkaline Phosphatase (ALP) activity was measured using p-nitrophenyl
237 phosphate (pNPP) (Sigma) as the chromogenic ALP substrate in the presence of Mg²⁺ ions in a
238 buffered solution. The absorbance was read at 405nm using the SpectraMax M5e Microplate
239 Reader. The ALP activity was then normalized to DNA content quantified using Quant-iT™
240 PicoGreen dsDNA Assay Kit (Invitrogen) according to the manufacturer's instructions.

241

242 Statistical analysis

243 All data are expressed as mean ± SEM. Statistical significance was tested for using either
244 univariate analysis of variance (PASW Statistics, NY) or a t-test (Prism 5, GraphPad, La Jolla).

245

246 **Results**

247 Osteogenic response of whole tibia

248 After 2 weeks axial loading of the left tibiae of 16 week-old mice, μ CT analysis at the level of
249 the whole bone demonstrated that the loaded tibia of the P2Y₁₃R^{-/-} mice had a significant greater
250 increase in total bone volume (BV) than WT in response to mechanical loading, when compared
251 to the BV of the non-loaded control ($126.7\% \pm 1.2$ versus $121.6\% \pm 1.4$, $p = 0.0140$) (Figure 1
252 B). The morphological changes were compared on the loaded and non-loaded tibia of WT and
253 P2Y₁₃R^{-/-} mice using μ CT 3D models of the whole bone (Figure 1 C). The FEA showed that
254 there was no significant difference in the simulated loading-induced strain through the full length
255 of the tibia between WT and P2Y₁₃R^{-/-} mice (5081 ± 254.4 versus 5048 ± 258.8 microstrain, $p =$
256 0.9306) (Figure 1 D). The FEA based average strain across the trabecular (696.0 ± 60.0 versus
257 693.4 ± 94.5 , $p = 0.9820$) and cortical compartments (757.8 ± 20.3 versus 758.2 ± 20.2 , $p =$
258 0.9894) were also not significantly different between WT and P2Y₁₃R^{-/-} mice (Figure 1 E, 1 F).

259

260 Osteogenic response of trabecular bone

261 Analysis of the trabecular bone structure of the tibial region by μ CT demonstrated that both
262 P2Y₁₃R^{-/-} and WT mice had significantly increased trabecular bone volume (BV/TV), trabecular
263 thickness (Tb.Th), trabecular number (Tb.N), and trabecular pattern factor (Tb.Pf) in loaded tibia
264 compared to internal non-loaded controls. The quantitative data are summarized in Table 1 and
265 thicker trabeculae were clearly visible in images of 3D models of the loaded tibia trabecular bone
266 from both P2Y₁₃R^{-/-} and WT mice (Figure 2 A).

267

268 When compared to the parameters from the contra-lateral non-loaded tibia, P2Y₁₃R^{-/-} mice
269 showed a significant higher Tb.Th increase compared to the increase in WT mice ($134.1 \pm 1.9\%$
270 versus $126.3 \pm 3.0\%$, $p = 0.0316$) (Figure 2 B), whilst the increase of BV/TV of P2Y₁₃R^{-/-} was

271 not significantly higher than WT (149.1 ± 5.1 % versus 146.4 ± 4.1 %, $p = 0.6982$) (Figure 2 C).
272 $P2Y_{13}R^{-/-}$ mice had almost 21% lower Tb.Pf decreases in the loaded tibia (80.1 ± 3.7 % versus
273 66.2 ± 3.8 %, $p = 0.0185$) (Figure 2 D). More interestingly, the $P2Y_{13}R^{-/-}$ trabecular bone had
274 positive changes to the structure model index (SMI) compared to negative changes in the WT
275 (107.0 ± 2.8 % versus 95.4 ± 3.7 %, $p = 0.0189$) (Figure 2 E).

276

277 Osteogenic response of cortical bone

278 Cortical bone volume of the tibia at 20% proximal and at the mid-shaft (Figure 3 A) was
279 measured by μ CT and demonstrated that both $P2Y_{13}R^{-/-}$ and WT had significantly increased
280 cortical bone volume (Ct.V) in the loaded tibia (Table 1). Compared to the osteogenic response
281 of WT, $P2Y_{13}R^{-/-}$ mice showed significantly greater responses in both regions (Figure 3 B, 3 C),
282 including significantly increased Ct.V response in both the proximal 20% region (136.4 ± 2.3 %
283 versus 128.2 ± 1.5 %, $p = 0.0130$) (Figure 3 D) and the mid-shaft region (148.3 ± 4.1 % versus
284 136.6 ± 2.8 %, $p = 0.0362$) (Figure 3 E).

285

286 Rate and extent of mineralization induced by mechanical loading of the tibia

287 Two distinctive calcein labels (14 and 2 days prior to sacrifice respectively) on both 20%
288 proximal and midshaft endocortical surfaces of tibiae can be visualized using a fluorescent
289 microscope and confirmed the endocortical lamellar bone formation (Figure 4 A). Calcein labels
290 on both endocortical and periosteal surfaces were measured to calculate the parameters including
291 MAR, BFR/BS, and MS. $P2Y_{13}R^{-/-}$ mice showed a significant increase in all three parameters in
292 both endocortical and periosteal surfaces of loaded tibiae, compared to non-loaded control tibiae.

293 Whilst WT mice only showed significant changes in periosteal BFR/BS and MAR on both
294 periosteal and endocortical surfaces. The quantitative data are summarized in Table 2.

295

296 To determine if the response of the P2Y₁₃R^{-/-} mice was different to WT, the loaded tibia data was
297 compared to contra-lateral non-loaded tibia. In the endocortical surfaces, loaded tibia of P2Y₁₃R^{-/-}
298 showed more than a two-fold increased response in MAR (355.4 ± 88.4 % versus 140.5 ±
299 16.4 %, p = 0.0276) (Figure 4 B), a 5-fold increased response in BFR/BS (714.7 ± 235.4 %
300 versus 171.1 ± 41.1 %, p = 0.0338) (Figure 4 C), and almost a 2-fold higher response in MS
301 (186.6 ± 30.8 % versus 115.6 ± 16.1 %, p = 0.0599) (Figure 4 D). The same trend was found on
302 the periosteal surface, but only the increased response in MAR by P2Y₁₃R^{-/-} mice reached
303 statistical significance (973.7 ± 108.2 % versus 586.6 ± 116.4 %, p = 0.0402) (Figure 4 E).

304

305 Endogenous ATP release from primary osteoblasts in vitro

306 Endogenous ATP release after medium change from primary osteoblasts was examined using the
307 luciferase assay. LDH assay was used to exclude ATP release due to cell lysis. After medium
308 change (t₀), the initial extracellular ATP released from P2Y₁₃R^{-/-} osteoblasts into the medium
309 showed no significant difference compared to WT cells (18.6nM ± 3.6 versus 20.5nM ± 3.4, p =
310 0.7063). The extracellular ATP concentration in the medium of WT osteoblast cultures gradually
311 returned to basal level 60 mins (t₆₀) after medium change (t₀ = 20.5nM ± 3.4 versus t₆₀ = 9.6nM ±
312 1.6, p = 0.0227). However, the extracellular ATP concentration in the medium of P2Y₁₃R^{-/-} cells
313 did not return to baseline and demonstrated a trend towards accumulation instead of degradation,
314 with the ATP concentration being significantly higher than the initial concentration from 50 mins
315 onwards (t₀ = 18.6nM ± 3.6 versus t₅₀ = 32.7nM ± 4.2, p = 0.0182). The extracellular ATP

316 concentration in the medium of P2Y₁₃R^{-/-} osteoblast cultures was also significantly higher than
317 that of WT cultures from 50 mins after medium change (32.7nM ± 4.2 versus 15.6nM ± 2.6, p =
318 0.0023) (Figure 5 A).

319

320 Exogenous ATP hydrolysis by primary osteoblasts

321 After measuring endogenous ATP release, primary osteoblast cells were incubated in serum free
322 medium to let ATP concentration and pH settle back to basal levels. Exogenous ATP (300nM)
323 was added into each well and the concentration of ATP in the medium determined over a time
324 course. The hydrolysis of exogenous ATP in P2Y₁₃R^{-/-} osteoblast cultures was slower than that in
325 WT cultures. The ATP concentration in WT osteoblast cultures reduced by 50% within 5 mins,
326 whilst the ATP concentration of P2Y₁₃R^{-/-} cultures was significantly higher than WT from 5
327 mins and remained at 200 nM level even after 30 mins (Figure 5 B).

328

329 ALP activity of primary osteoblasts

330 ALP is a nucleotidase highly expressed by osteoblasts that is capable of hydrolysing extracellular
331 ATP. The basal level of ALP activity was measured in primary osteoblast cultures using the
332 pNPP assay. P2Y₁₃R^{-/-} mice showed a 15% reduction in ALP activity compared to osteoblasts
333 from WT mice when normalized to DNA content (0.72 ± 0.02 versus 0.85 ± 0.03, p = 0.0002,
334 Figure 5 C).

335

336 **Discussion**

337 The P2Y₁₃ receptor has been suggested to be involved in ATP metabolism in different cell types
338 and ATP release and purinergic signalling is one of the main transduction pathways of

339 mechanical stimulation. Therefore, we hypothesised that the P2Y₁₃ receptor would play a role in
340 regulating ATP metabolism by osteoblasts and in mediating the osteogenic response upon
341 mechanical stimulation. To test this hypothesis, we examined the osteogenic response of P2Y₁₃R^{-/-}
342 mice subject to mechanical stimuli both in vivo and in vitro. The results provide compelling
343 evidence for a role for the P2Y₁₃R in bone homeostasis. Whilst the effect of the deletion of the
344 P2Y₁₃R on the normal bone phenotype is modest, the response to loading in vivo is dramatically
345 enhanced in the KO mice, possibly due to the lack of a P2Y₁₃R regulated negative feedback
346 pathway for ATP release, as demonstrated in vitro.

347

348 Non-invasive axial mechanical loading at peak loading force of 15N was performed on left tibiae
349 of both P2Y₁₃R^{-/-} and WT mice in vivo using a method as described before (26,27). Compared to
350 the contra-lateral non-loaded right tibia, the total bone volume of loaded tibia demonstrated
351 significant increases in both WT and P2Y₁₃R^{-/-} mice although bone length did not change. This
352 indicated that mechanical loading successfully induced osteogenic response mainly in the tibia
353 cross-sectional dimensions (40). High resolution μ CT analysis showed that trabecular bone in
354 both WT and P2Y₁₃R^{-/-} loaded tibia had significantly increased BV/TV, Tb.Th and Tb.N. Similar
355 increases in Ct.V were also found in cortical bone. Therefore, the total BV increase was a
356 combined result of new bone formation activities from both trabecular and cortical bone. This
357 was confirmed with the increased BFR and MAR in both WT and P2Y₁₃R^{-/-} loaded tibiae using
358 dynamic histomorphometry analysis, especially the lamellar bone formation on the endocortical
359 bone surfaces. In addition, increased bone remodeling activities led to coarse surface which was
360 observed specifically in the periosteal surface of tibial proximal end 3D μ CT image. This result
361 was consistent with previous findings that there was a greater osteogenic response in the

362 corticocancellous proximal metaphysis (41) and periosteal formation surface was predominantly
363 woven bone (42,43).

364

365 To compare the extent of the osteogenic response between $P2Y_{13}R^{-/-}$ and WT mice, the
366 parameters from loaded tibia were compared to those from the corresponding contra-lateral non-
367 loaded tibia controls. The $P2Y_{13}R^{-/-}$ mice had a further 20% response in total BV increase in the
368 loaded tibiae compared to WT. This was mainly the result of the increased osteogenic response
369 of cortical bone because $P2Y_{13}R^{-/-}$ had a significant greater response in the increases in Ct.V but
370 not in trabecular BV/TV over that of WT. The higher osteogenic response in $P2Y_{13}R^{-/-}$ mice
371 under mechanical stimulation mainly involved osteoblastic bone forming activities. This was
372 confirmed by the results of fluorochrome double labelling in the cortical compartment which
373 showed dramatically higher MAR and BFR increases in $P2Y_{13}R^{-/-}$ bones compared to WT,
374 indicate enhanced activities of osteoblasts (36).

375

376 The trabecular structure of $P2Y_{13}R^{-/-}$ mice after loading did not alter towards the ideal load
377 bearing architecture as the WT mice did; the $P2Y_{13}R^{-/-}$ mice showed less of a decrease in Tb.Pf
378 and significantly increased SMI, indicating that the trabecular did not improve connectivity in
379 any great extent and remained a rod-like structure (44,45). However, WT mice showed better
380 structure alteration with significantly decreased Tb.Pf and slightly reduced SMI. The reduced
381 change in Tb.Pf could be the result of a weaker primary trabecular structure in $P2Y_{13}R^{-/-}$ bones,
382 whereas, the possible reason for an increased SMI could be due to a failure in osteoclast
383 resorption of the $P2Y_{13}R^{-/-}$ mice as demonstrated previously (21). This would lead to an

384 abnormal capacity to remodel the trabecular structure since osteoclasts are suggested to control
385 the conversion of trabecular from plate elements to rod elements (45).

386

387 One possible explanation for the different osteogenic response could have been that the lower
388 bone volume in the $P2Y_{13}R^{-/-}$ mice led to an increase in the strains engendered by the 15N
389 loading. However, our FEA studies, a widely recognized method to predict loading induced
390 strain (46), demonstrated that this is not the case because the bones of the WT and $P2Y_{13}R^{-/-}$
391 mice experienced the same overall strains and average strain across trabecular and cortical
392 compartments under modelled loading. The overall strains calculated were in the region of 5,000
393 microstrain and are relatively higher than previous studies using strain gauge to measure strain
394 (26,27,30) but are consistent with other new findings using FEA (43). This is because applying
395 the 15N loads to the tibia in silico is not the same as loading tibia in vivo, where several layers of
396 other tissues including skin, subcutaneous tissues, and at least two thicknesses of cartilage are
397 compressed as well. The important issue is therefore not the absolute values derived from the
398 FEA measurement but the lack of strain difference between WT and $P2Y_{13}R^{-/-}$ bones and hence
399 the observed enhanced osteogenic response to mechanical loading in $P2Y_{13}R^{-/-}$ mice is real.

400

401 Another possible cause of the different osteogenic response could have been the result of
402 enhanced woven bone formation due to an increased inflammatory response (47). However, our
403 dynamic histomorphometry results clearly show lamellar bone formation on the endocortical
404 bone surface, where the increases in both MAR and BFR/BS in loaded tibiae were significantly
405 higher in $P2Y_{13}R^{-/-}$ than those in WT mice. On the periosteal surface, where woven bone
406 formation was predominant, there is a similar trend of enhanced bone formation in $P2Y_{13}R^{-/-}$

407 mice but it is not as dramatic as on the endocortical bone surfaces and only the MAR reached
408 statistical significance at this site. Therefore, there may be an element of an inflammatory
409 response but we believe it is not the main cause of the different adaption to mechanical loading
410 between WT and P2Y₁₃R^{-/-} mice.

411

412 Many mechanisms has been suggested to be involved in the alteration of osteogenic response to
413 mechanical loading in mice, including aging and changes in other signalling pathways such as
414 Wnt, ER and BMP/TGFβ pathways (48,49). The in vitro findings in this study may provide a
415 possible explanation for the reason why P2Y₁₃R^{-/-} mice had enhanced osteogenic response to
416 mechanical loading. The constitutive endogenous ATP release was investigated in the primary
417 osteoblasts isolated from neonatal mice calvariae using luciferase assay. After medium change,
418 the extracellular ATP concentration in the medium of P2Y₁₃R^{-/-} osteoblast cultures showed a
419 trend towards accumulation of ATP instead of gradually degrading ATP as in WT osteoblast
420 cultures. As a result, P2Y₁₃R^{-/-} osteoblasts showed three fold higher extracellular ATP
421 concentration than WT cells one hour after medium change. This confirms that the deletion of
422 P2Y₁₃R results in a lack of the negative feedback pathway for ATP release in P2Y₁₃R^{-/-}
423 osteoblasts. Interestingly, when a higher concentration of exogenous ATP was added to the
424 primary osteoblasts, P2Y₁₃R^{-/-} cells have a decreased capacity to hydrolyse ATP, whilst WT
425 osteoblasts degraded the exogenous ATP back to basal levels within 5 minutes. Thirty minutes
426 after exogenous ATP treatment, extracellular ATP concentration of P2Y₁₃R^{-/-} osteoblasts was
427 double that of WT cells. Osteoblasts are known to have numerous membrane-bound
428 nucleotidases which are responsible for breaking down ATP to adenosine and are critical in the
429 ATP turnover process (50). One particular nucleotidase, ALP, is highly expressed by osteoblasts

430 and interestingly, the ALP activity in vitro was found to be 15% lower in P2Y₁₃R^{-/-} osteoblasts
431 than WT under basal conditions, possibly due to the down regulation of RhoA/ROCK I
432 signalling pathway as a consequence of P2Y₁₃R deletion (21,51). Therefore, one possible
433 mechanism leading to the observed higher osteogenic response to mechanical loading in P2Y₁₃R^{-/-}
434 mice may be as a result of a reduction in nucleotidase activity. Under basal conditions, it
435 appears that the reduced level of ATP hydrolysis to ADP is still sufficient to provide a negative
436 feedback pathway to regulate ATP release. However, under mechanical stimulation, increased
437 and sustained ATP release may not be matched by hydrolysis to ADP due to basal reduced ALP
438 levels, and therefore a lack of the negative feedback loop leads to extracellular ATP
439 accumulation. This extracellular ATP accumulation may in turn trigger other P2 receptor
440 signalling pathways and cause an increased osteogenic response possibly via ATP-dependent
441 phosphorylation of ERK (11,17), which then stimulates osteoblastic proliferation and drives the
442 osteogenic response (18).

443

444 In conclusion, this study examined the role of P2Y₁₃ receptor in bone osteogenic response to
445 mechanical loading in vivo and in vitro. Deletion of the P2Y₁₃R leads to higher bone formation,
446 mainly in cortical compartment, than WT upon mechanical loading in vivo, possibly due to the
447 lack of P2Y₁₃R regulated negative feedback pathway for ATP release. This was further
448 supported by our in vitro findings of abnormal extracellular ATP accumulation from primary
449 osteoblast under mechanical stimulation. Reduced ALP activity caused by P2Y₁₃R gene deletion
450 and the following reduction in extracellular ATP degradation might be one reason for this
451 phenomenon. This augmented osteogenic response to mechanical stimulation, combined with
452 suppressed bone remodelling activities and protect from OVX induced bone loss after P2Y₁₃R

453 depletion as recently described (21), suggests a potential role for P2Y₁₃R antagonist-based
454 therapy, possibly in combination with mechanical loading, for the treatment of osteoporosis in
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456

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465

466 **Authors' roles:** AG, TS and JMB conceived the project. NW and RR performed the experiments.
467 NW and LY performed the FEA. BR generated the P2Y₁₃R^{-/-} mice. Data analysis and
468 interpretation: NW, RR, LY, TS, JMB and AG. NW and AG wrote the draft manuscript, with
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470

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- 627
- 628

Tables

Table 1. Quantitative results of tibia trabecular and cortical bone after mechanical loading using μ CT analysis.

	WT			P2Y ₁₃ R ^{-/-}		
	n=9		p value	n=12		p value
	Loaded	Non-loaded		Loaded	Non-loaded	
TMD (g/cm³)	1.12 ± 0.00	1.13 ± 0.01	b	1.12 ± 0.00	1.13 ± 0.00	a
BV/TV	12.30 ± 0.39	8.42 ± 0.19	c	8.60 ± 0.37	5.80 ± 0.24	c
BS/BV (1/mm)	65.00 ± 1.05	85.41 ± 1.33	c	67.80 ± 0.60	91.50 ± 1.24	c
Tb.Th (mm)	0.064 ± 0.001	0.051 ± 0.001	c	0.065 ± 0.001	0.048 ± 0.001	c
Tb.N (1/mm)	1.93 ± 0.07	1.66 ± 0.04	a	1.33 ± 0.06	1.20 ± 0.05	a
Tb.Pf (1/mm)	16.93 ± 0.95	25.62 ± 0.57	c	24.38 ± 0.89	30.72 ± 0.94	c
Tb.Sp (mm)	0.25 ± 0.01	0.26 ± 0.01		0.29 ± 0.01	0.31 ± 0.01	
SMI	2.06 ± 0.06	2.16 ± 0.03		2.53 ± 0.05	2.37 ± 0.05	a
DA	2.04 ± 0.08	2.28 ± 0.10		1.66 ± 0.05	1.90 ± 0.06	a
Proximal 20% Ct.V (mm³)	1.16 ± 0.02	0.91 ± 0.01	c	1.17 ± 0.02	0.86 ± 0.01	c
Midshaft Ct.V (mm³)	0.98 ± 0.02	0.71 ± 0.01	c	1.00 ± 0.02	0.67 ± 0.01	c

Values are mean ± SEM, ^a p < 0.05, ^b p < 0.01, ^c p < 0.001 (paired t-test)

Table 2. Quantitative results of endocortical and periosteal tibia dynamic histomorphometry.

	WT			P2Y₁₃R^{-/-}		
	n=6		p value	n=5		p value
	Loaded	Non-loaded		Loaded	Non-loaded	
Endocortical MS (%)	85.68 ± 6.26	78.20 ± 6.85		88.83 ± 0.87	51.97 ± 6.77	b
Endocortical MAR (µm/day)	1.87 ± 0.16	1.38 ± 0.11	a	2.60 ± 0.59	0.80 ± 0.15	a
Endocortical BFR/BS (µm ³ /µm ² /day)	1.58 ± 0.14	1.11 ± 0.16		2.32 ± 0.55	0.44 ± 0.13	a
Periosteal MS (%)	94.91 ± 3.47	78.83 ± 11.01		92.09 ± 3.71	52.53 ± 9.12	b
Periosteal MAR (µm/day)	3.62 ± 0.56	0.65 ± 0.06	b	3.28 ± 0.39	0.34 ± 0.03	b
Periosteal BFR/BS (µm ³ /µm ² /day)	3.45 ± 0.57	0.54 ± 0.01	b	3.05 ± 0.42	0.19 ± 0.05	b

Values are mean ± SEM, ^a p < 0.05, ^b p < 0.01, ^c p < 0.001 (paired t-test)

Legends

Figure 1 Whole bone response to mechanical loading

(A) Finite element models of the mice tibia showing the loading and constraint conditions and length changes before (L_1) and under compressive load (L'_1). (B) Percentage change in whole tibial bone volume of the loaded compared to unloaded internal control. All values are mean \pm SEM, P2Y₁₃R^{-/-} n=12; WT n=9. ^a p<0.05 (unpaired t-test). (C) The 3D models of whole tibia from P2Y₁₃R^{-/-} and WT loaded and non-loaded animals were constructed from μ CT images, scale bar = 2.0mm. (D) The overall strain based on compressive displacement of the whole tibia was analysed by FEA and compared between WT and P2Y₁₃R^{-/-}. The average strain in the (E) trabecular and (F) cortical compartment were also calculated from a 1.0 mm in length region, 0.2 mm below the growth plate in tibia. n=5 (unpaired t-test).

Figure 2. Trabecular bone response to mechanical loading

(A) Three dimensional images of a region of 1.0mm thick trabecular bone 0.2mm below the growth plate of mechanical loaded and non-loaded tibiae, scale bar = 0.5 mm. The contra-lateral non-loaded right tibiae were used as internal controls. The percentage change of (B) trabecular thickness (Tb.Th), (C) trabecular bone volume (BV/TV), (D) trabecular pattern factor (Tb.Pf), and (E) structure model index (SMI) for loaded tibia compared to unloaded controls. All values are mean \pm SEM, P2Y₁₃R^{-/-} n=12; WT n=9. ^a p<0.05 (unpaired t-test).

Figure 3. Cortical bone response to mechanical loading.

(A) Mouse tibial 3D models indicating the two regions analysed for determining cortical bone parameters, including proximal 20% and the mid-shaft of tibiae (1.0mm in thickness, 1.0mm and

7.0mm below the growth plate respectively). The cross section μ CT images of loaded and non-loaded tibiae were compared between WT and P2Y₁₃R^{-/-} at **(B)** 2.0mm and **(C)** 8.0mm below the growth plate. The Ct.V in loaded tibiae normalized to contra-lateral non-loaded right tibiae at **(D)** the proximal 20% region and **(E)** the mid-shaft region. All values are mean \pm SEM, P2Y₁₃R^{-/-} n=12; WT n=9. ^a p<0.05 (unpaired t-test).

Figure 4. Rate and extent of mineralization induced by mechanical loading of the tibia

Double calcein labelling was used to determine the bone formation activities on both endocortical and periosteal surface. **(A)** Clear double labelling of calcein on endocortical surfaces confirmed lamellar bone formation at this site. The percentage change of loaded tibia compared to contra-lateral non-loaded right tibiae of **(B)** Mineral apposition rate (MAR), **(C)** bone formation rate (BFR/BS), and **(D)** mineralizing surface (MS%) on the endocortical surface. **(E)** MAR, **(F)** BFR/BS, and **(G)** MS on the periosteal surface. All values are mean \pm SEM, P2Y₁₃R^{-/-} n = 5, WT n = 6, ^a p<0.05 (unpaired t-test).

Figure 5. Regulation of extracellular ATP levels in osteoblast cultures.

(A) A time course of ATP release and degradation in osteoblast cultures following medium change. P2Y₁₃R^{-/-} osteoblasts showed a trend of extracellular ATP accumulation compared to the gradual degradation seen in WT cultures. All values are mean \pm SEM, n = 4 per experiment, with 3 independent experiments, ^a p<0.05, ^b p<0.01 (unpaired t-test). **(B)** Exogenous ATP (300nM) was hydrolyzed to half the amount within 5 mins in WT osteoblast cultures. However, the degradation of exogenous ATP in P2Y₁₃R^{-/-} osteoblasts was slower than WT, with extracellular ATP concentration in the P2Y₁₃R^{-/-} cultures being significantly higher than WT from 5 mins

onwards. All values are mean \pm SEM, n = 4 per experiment, with 3 independent experiments, ^b p<0.01, ^c p<0.001 (unpaired t-test). **(C)** ALP activity of WT and P2Y₁₃R^{-/-} osteoblast cultures was measured using pNPP assay and normalized to dsDNA content. All values are mean \pm SEM, n=3 repeat experiments with 12 replicates per experiment, ^c p < 0.001, (Univariate analysis of variance).

Figure 1

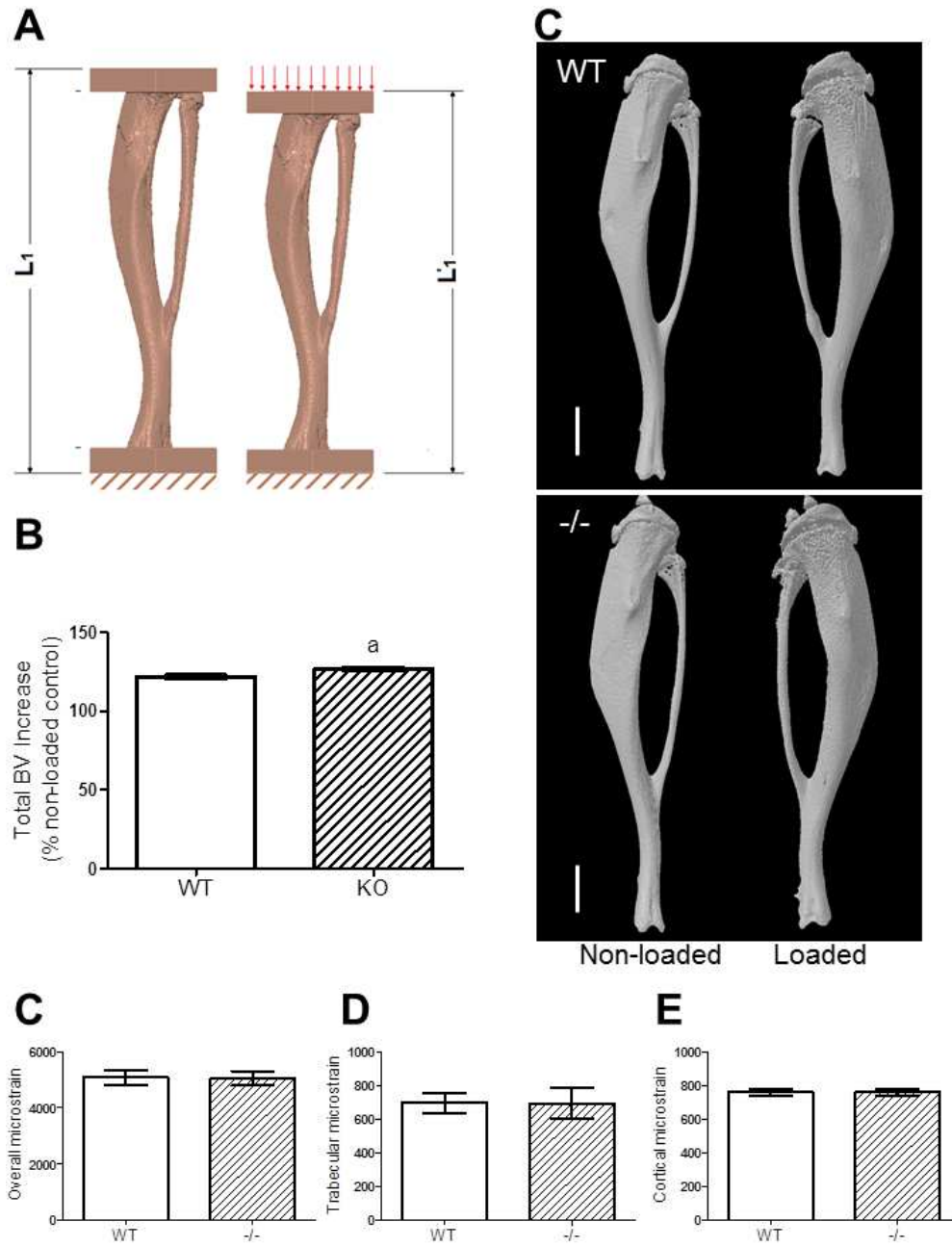


Figure 2

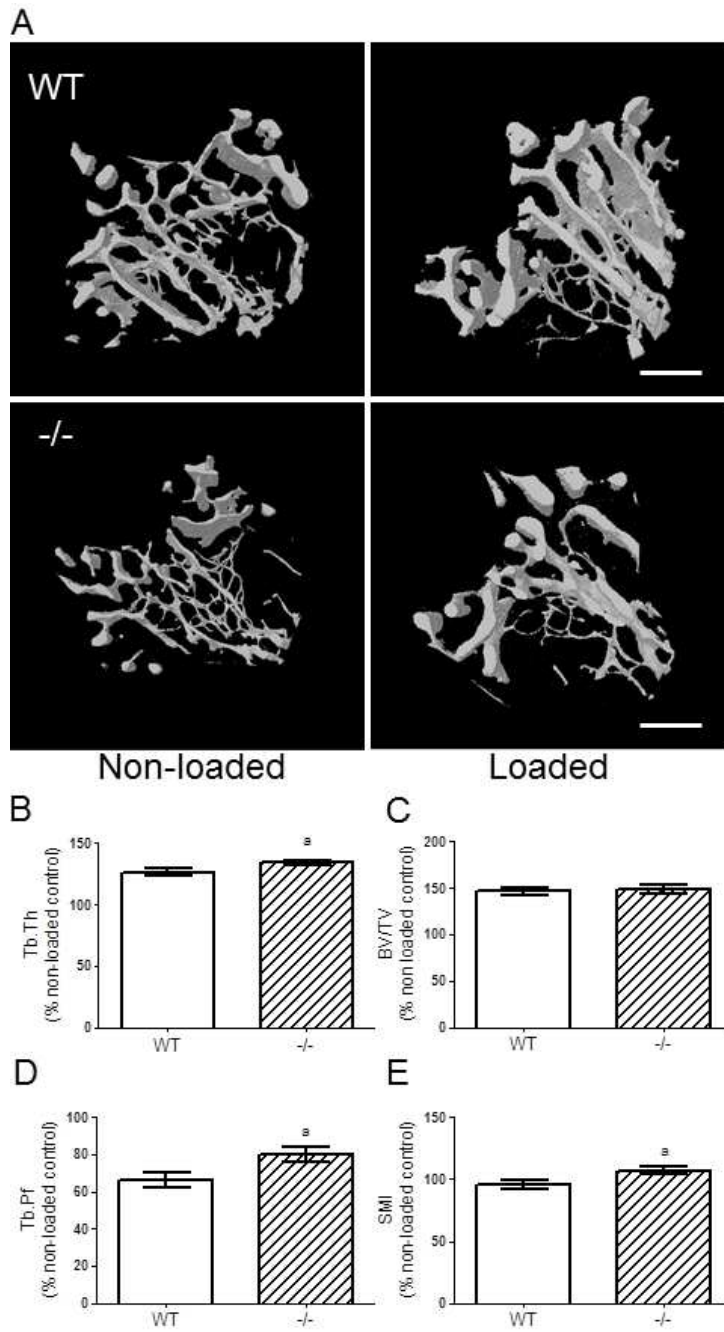


Figure 3

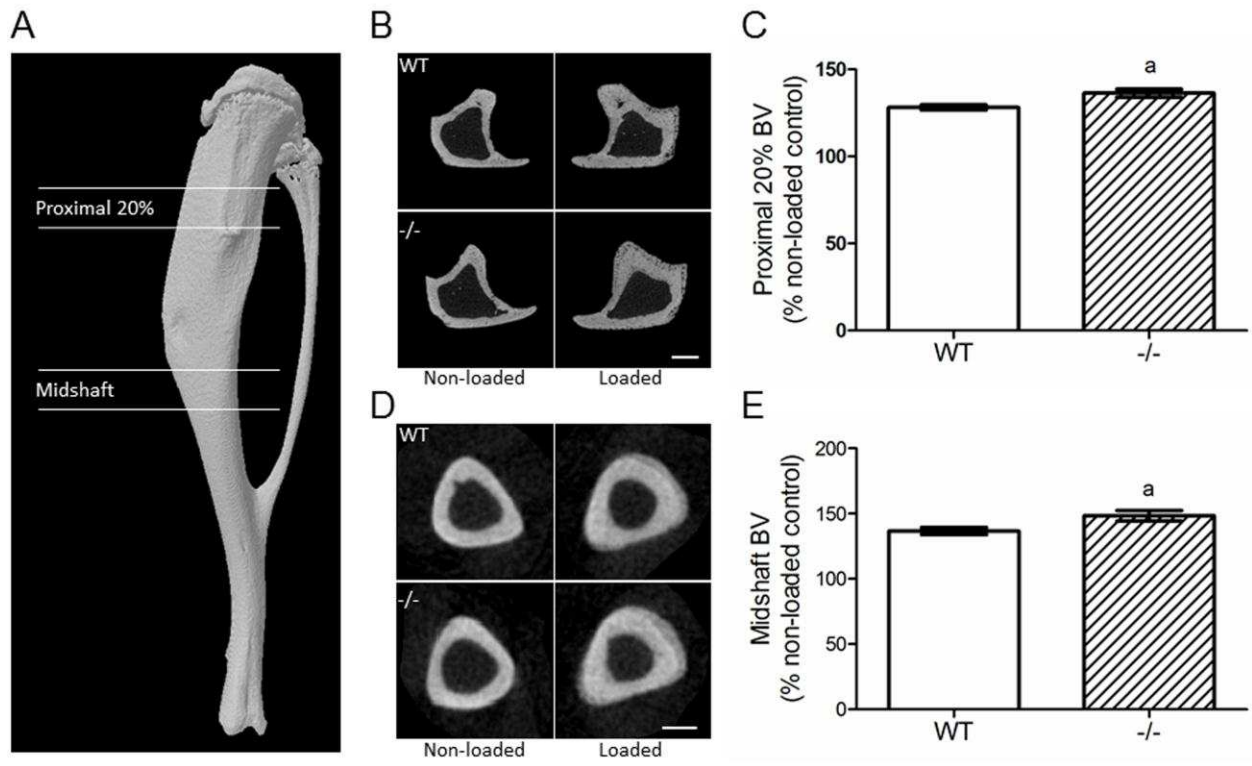


Figure 4

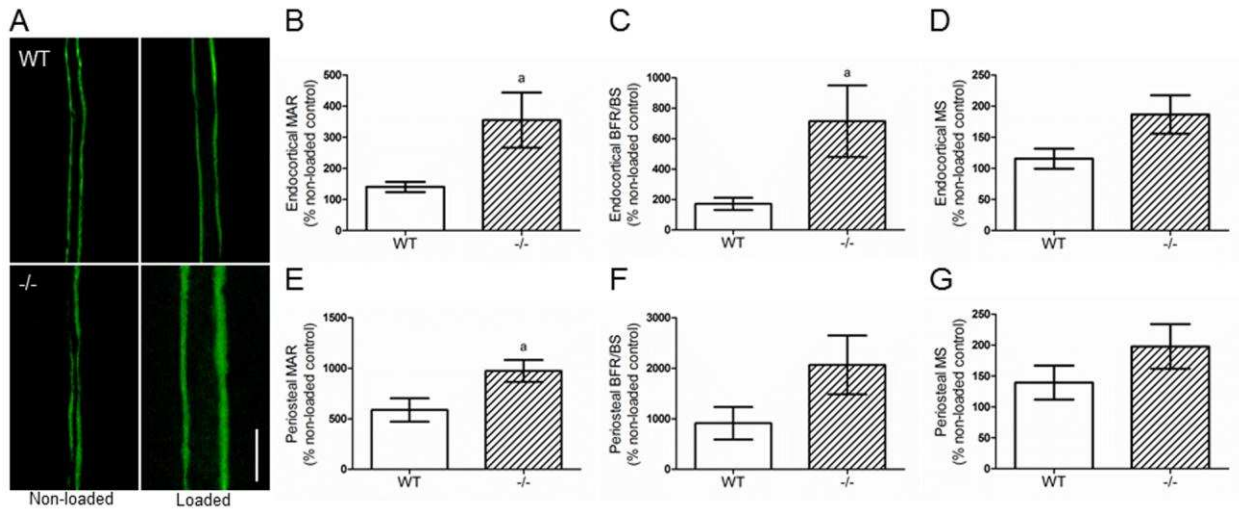


Figure 5

