

# Mice lacking AMP-activated protein kinase $\alpha 1$ catalytic subunit have increased bone remodelling and modified skeletal responses to hormonal challenges induced by ovariectomy and intermittent PTH treatment

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## Abstract

AMP-activated protein kinase (AMPK) is a key regulator of cellular and body energy homeostasis. We previously demonstrated that AMPK activation in osteoblasts increases *in vitro* bone formation while deletion of the *Ampk $\alpha 1$*  (*Prkaa1*) subunit, the dominant catalytic subunit expressed in bone, leads to decreased bone mass *in vivo*. To investigate the cause of low bone mass in the *Ampk $\alpha 1$ <sup>-/-</sup>* mice, we analysed bone formation and resorption in the tibia of these mice by dynamic histomorphometry and determined whether bone turnover can be stimulated in the absence of the *Ampk $\alpha 1$*  subunit. We subjected 12-week-old *Ampk $\alpha 1$ <sup>+/+</sup>* and *Ampk $\alpha 1$ <sup>-/-</sup>* mice to ovariectomy (OVX), intermittent PTH (iPTH) administration (80  $\mu\text{g}/\text{kg}$  per day, 5 days/week) or both OVX and iPTH hormonal challenges. Tibiae were harvested from these mice and bone micro-architecture was determined by micro-computed tomography. We show for the first time that *Ampk $\alpha 1$ <sup>-/-</sup>* mice have a high bone

turnover at the basal level in favour of bone resorption. While both *Ampk $\alpha 1$ <sup>+/+</sup>* and *Ampk $\alpha 1$ <sup>-/-</sup>* mice lost bone mass after OVX, the bone loss in *Ampk $\alpha 1$ <sup>-/-</sup>* mice was lower compared with controls. iPTH increased trabecular and cortical bone indexes in both ovariectomised *Ampk $\alpha 1$ <sup>+/+</sup>* and *Ampk $\alpha 1$ <sup>-/-</sup>* mice. However, ovariectomised *Ampk $\alpha 1$ <sup>-/-</sup>* mice showed a smaller increase in bone parameters in response to iPTH compared with *Ampk $\alpha 1$ <sup>+/+</sup>* mice. By contrast, non-ovariectomised *Ampk $\alpha 1$ <sup>-/-</sup>* mice responded better to iPTH treatment than non-ovariectomised *Ampk $\alpha 1$ <sup>+/+</sup>* mice. Overall, these data demonstrate that *Ampk $\alpha 1$ <sup>-/-</sup>* mice are less affected by changes in bone turnover induced by OVX but respond better to the anabolic challenge induced by iPTH. These results suggest that AMPK $\alpha 1$  activation may play a role in the hormonal regulation of bone remodelling.

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## Introduction

AMP-activated protein kinase (AMPK) is a sensor and regulator of energy homeostasis not only at the cellular level but also at the whole-body level where it mediates the central and peripheral effects of many hormones on the metabolisms of appetite, fat and glucose (Minokoshi *et al.* 2002, Yamauchi *et al.* 2002, Andersson *et al.* 2004, Banerjee *et al.* 2004, Minokoshi *et al.* 2004, Han *et al.* 2005, Kola *et al.* 2005, Yamauchi *et al.* 2008 and for reviews see Hardie *et al.* 2006, Kola *et al.* 2006 and Lage *et al.* 2008). It is a highly

conserved, ubiquitously expressed serine/threonine heterotrimeric protein kinase consisting of a catalytic  $\alpha$  subunit and regulatory  $\beta$  and  $\gamma$  subunits, all of which have several isoforms with differential tissue-specific expression patterns (Hardie *et al.* 2006, Kola *et al.* 2006, Steinberg & Kemp 2009, Viollet *et al.* 2010, Hardie *et al.* 2011). AMPK senses the AMP/ATP ratio within the cell and is activated in response to environmental or nutritional stress factors that deplete intracellular ATP levels (Hardie *et al.* 2006, Hardie 2008, Steinberg & Kemp 2009). AMP binding activates AMPK by two mechanisms, phosphorylation of the Thr-172 residue in

the  $\alpha$  subunit by upstream kinases and inhibition of dephosphorylation of Thr-172 by phosphatases (Hawley *et al.* 1996, 2003, 2005, Xiao *et al.* 2011). Once activated, it switches on catabolic pathways that generate ATP and switches off anabolic pathways that consume ATP.

In the last few years, several *in vitro* and *in vivo* studies have established that the AMPK signalling pathway could also play a role in bone physiology (Kanazawa *et al.* 2008, 2009, Kasai *et al.* 2009, Lee *et al.* 2010, Molinuevo *et al.* 2010, Quinn *et al.* 2010, Shah *et al.* 2010, Zhen *et al.* 2010, Jang *et al.* 2011a,b, Mai *et al.* 2011, Wu *et al.* 2011). We demonstrated that the AMPK $\alpha$ 1 subunit is the dominant catalytic isoform expressed in bone and that AMPK activators stimulate *in vitro* bone nodule formation (Shah *et al.* 2010). Several other studies have confirmed that the two main AMPK activators, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and metformin, are osteogenic *in vitro*. They stimulate proliferation, differentiation and mineralisation of MC3T3-E1 osteoblastic cells (Kanazawa *et al.* 2008, 2009, Jang *et al.* 2011a,b, Mai *et al.* 2011), primary osteoblasts (Shah *et al.* 2010, Zhen *et al.* 2010) and bone marrow progenitor cells (Molinuevo *et al.* 2010, Wu *et al.* 2011). However, it was also reported that osteoblast differentiation is functionally associated with decreased AMPK activity (Kasai *et al.* 2009). The relationship between AMPK activation and bone resorption is also unclear. Activation of AMPK was shown to inhibit osteoclast formation and bone resorption *in vitro*, AMPK acting as a negative regulator of RANKL (Lee *et al.* 2010). By contrast, *in vivo* studies have shown that AICAR stimulates bone loss and bone turnover in male mice (Quinn *et al.* 2010). The evidence for a role of AMPK signalling in the regulation of bone mass is best supported by genetic studies. Our work has shown that *Ampkα1* (*Prkaa1*) knockout (*Ampkα1*<sup>-/-</sup>) mice have a very low bone mass compared with the WT (*Ampkα1*<sup>+/+</sup>) mice, both cortical and trabecular bone compartments being smaller in the *Ampkα1*<sup>-/-</sup> mice (Shah *et al.* 2010). Similarly, Quinn *et al.* (2010) showed that germline deletions of either the AMPK $\beta$ 1 or  $\beta$ 2 subunit resulted in reduced trabecular bone density and mass.

The underlying mechanism for the low bone mass in *Ampkα1*<sup>-/-</sup> mice and the exact role of AMPK in bone remodelling *in vivo* have not yet been investigated. Bone remodelling occurs constantly at multiple locations within the skeleton and bone needs to balance energy in response to nutrient availability with growth and turnover. To address the role of AMPK in bone turnover, we subjected *Ampkα1*<sup>-/-</sup> and *Ampkα1*<sup>+/+</sup> mice to two types of hormonal challenges that increase bone turnover, ovariectomy (OVX) that induces a negative bone balance and intermittent PTH (iPTH) treatment that is anabolic, and examined their effects on bone architecture in these mice. We show that *Ampkα1*<sup>-/-</sup> mice have high bone turnover at basal level and that bone turnover in *Ampkα1*<sup>-/-</sup> mice is altered in response to OVX and iPTH, suggesting that AMPK activation may modulate the hormonal regulation of bone remodelling.

## Materials and Methods

### Animals

*Ampkα1* knockout mice were generously provided by Dr Benoit Viollet (INSERM, U1016, Paris, France) and were generated as described previously (Jorgensen *et al.* 2004). *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice in C57BL/6 $\times$ 129/Sv mixed background were used. All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. All mice were maintained under a controlled temperature (21 °C) and lighting with 12 h light:12 h darkness cycle and received a standard mouse chow diet and water *ad libitum*. The knockout mice do not show any obvious metabolic phenotypes and their body weight was unaffected (Jorgensen *et al.* 2004).

### Hormonal challenges

Three independent experiments were carried out to determine the response of bone to hormonal challenges in *Ampkα1* knockout mice. In study 1, 12-week-old *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice ( $n=7$ /group) were either ovariectomised or sham operated. Tibiae were collected from these mice 6 weeks after OVX for micro-computed tomography (micro-CT) analysis. For measurement of dynamic bone formation parameters, mice in this study were i.p. injected with calcein (Sigma-Aldrich) and alizarin red complexone (Sigma-Aldrich), at days 6 and 3, respectively, before killing. For study 2, 12-week-old *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice ( $n=8$ /group) were all ovariectomised and immediately treated for 4 weeks with s.c. injection of either 80  $\mu$ g/kg per day, 5 days/week, PTH (human PTH (1–34; Bachem, Inc., Torrance, CA, USA) dissolved in 1 mM HCl containing 0.2% BSA) or saline. For study 3, 12-week-old *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice ( $n=10$ –11/group) were treated with s.c. injection of either 80  $\mu$ g/kg per day, 5 days/week, iPTH or saline. For all mice, body weight was measured at the beginning of week 13 (i.e. on day of sham operation, OVX or at the beginning of iPTH treatment) and at the end of the experiment. Left and right tibiae were harvested from these mice for micro-CT (studies 1, 2 and 3) and bone histomorphometric analyses (study 1) respectively. Femora were collected for western blot and RT-PCR analyses.

### Histomorphometry analysis of tibia

Right tibia from sham-operated *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice from study 1 were fixed in 10% neutral-buffered formalin for 24–72 h, dehydrated and embedded in pure methyl methacrylate at low temperature to preserve enzymatic activity (Chappard *et al.* 1987). Unstained 8  $\mu$ m-thick sections were used for fluorescence

microscopy to assess mineral apposition rate (MAR,  $\mu\text{m}/\text{day}$ ). Mineralising surfaces were expressed as double + half single labelled surfaces per bone surfaces (MS/BS, %) and the bone formation rate was calculated as  $\text{MS/BS} \times \text{MAR}$  (BFR/BS,  $\mu\text{m}^3/\mu\text{m}^2$  per day; Chavassieux *et al.* 1997). Alternatively, sections were stained for tartrate-resistant acid phosphatase (TRAP; Leucognost SP, Merck) and counterstained with Mayer's hemalum solution. Goldner's trichrome staining was performed to determine adipocyte number per tissue area. Histomorphometric parameters were measured on the trabecular bone of the metaphysis, on a region of interest consisting of 2 mm width below the growth plate. Measurements were performed using an Image Analysis Software (Bone, Explora Nova, La Rochelle, France). Histomorphometric parameters were reported in accordance with the ASBMR Committee nomenclature (Parfitt *et al.* 1987).

#### Micro-CT analysis of tibia

Left tibia was fixed in 10% neural-buffered formalin for 24–72 h and stored in 70% ethanol at 4 °C. They were scanned with high-resolution (5  $\mu\text{m}$  pixel size) micro-CT (Skyscan 1172, Kontich, Belgium), as described previously (Shah *et al.* 2010). The whole tibia was reconstructed using NRecon v.1.4.4.0 (Skyscan) and bone histomorphometric analyses in 2- and 3-dimensions (2D and 3D) were performed by Skyscan Software (CT-Analyser v.1.5.1.3). For the analysis of trabecular bone, the cortical shell was excluded by operator-drawn regions of interest and 3D algorithms were used to determine the relevant parameters that included bone volume (BV) percentage (BV/tissue volume (TV), %), direct trabecular thickness (Tb.Th) and spacing, trabecular number (Tb.N), structure model index (SMI), trabecular bone pattern factor (TBPf) and the degree of anisotropy (DA). Analysis of cortical bone was performed using a 0.49 mm long segment (or 100 tomograms) at 37% of the tibias' length from the proximal end. For analysis of the cortical bone compartment, 2D computation was used and parameters were determined for each one of the 100 tomograms and then averaged. They included periosteal perimeter (Ps.Pm), endosteal perimeter (Ec.Pm) and cortical thickness (Ct.Th).

#### RNA extraction and RT-PCR analysis

Total RNA was isolated from femora and femoral muscles of *Ampk $\alpha$ 1<sup>+/+</sup>* and *Ampk $\alpha$ 1<sup>-/-</sup>* mice and amplified using subunit-specific primers, as described previously (Shah *et al.* 2010).

#### Protein extraction and western blot analysis

For the isolation of total proteins, right and left femora from *Ampk $\alpha$ 1<sup>+/+</sup>* and *Ampk $\alpha$ 1<sup>-/-</sup>* mice were carefully dissected, all their surrounding musculature removed leaving the periosteum intact. The cartilaginous ends of the bones were separated and the remaining femoral shafts were flushed

with PBS to remove the marrow. The femoral shafts were then snap-frozen and pulverised under liquid nitrogen using a mortar and pestle and then lysed in cold denaturing lysis buffer (2% SDS, 2 M urea, 8% sucrose, 20 mM sodium glycerophosphate, 1 mM sodium fluoride, and 5 mM sodium orthovanadate). Proteins were denatured by boiling for 5 min and concentrations were determined by BCA protein assay. Two micrograms of proteins were size fractionated using SDS-PAGE and electrotransferred onto Protran nitrocellulose membranes (Schliecher and Schuell, Dassel, Germany). Membranes were blocked for 1 h in 0.2% (w/v) I-block (Topix, Bedford, MA, USA), before being incubated with primary antibodies. The blots were incubated overnight at 4 °C with antibodies against total AMPK $\alpha$ 1/2 (tAMPK  $\alpha$ 1/2, rabbit), phospho-(Thr-172)-AMPK $\alpha$ 1/2 (pAMPK $\alpha$ 1/2, rabbit) (New England Biolabs, Hitchin, UK) and  $\beta$ -actin (goat) (Dako, Ely, UK), all added at a 1:1000 dilution. The following secondary antibodies were used: goat anti-rabbit (New England Biolabs) against tAMPK and pAMPK $\alpha$ 1/2 and rabbit anti-goat (Dako) against  $\beta$ -actin antibody, both at 1:2500 dilution at room temperature for 1 h. Proteins were visualised using the enhanced chemiluminescence (ECL) detection system (GE Healthcare UK Ltd., Little Chalfont, UK). The intensity of the specific bands was quantified by densitometry using Image J Software.

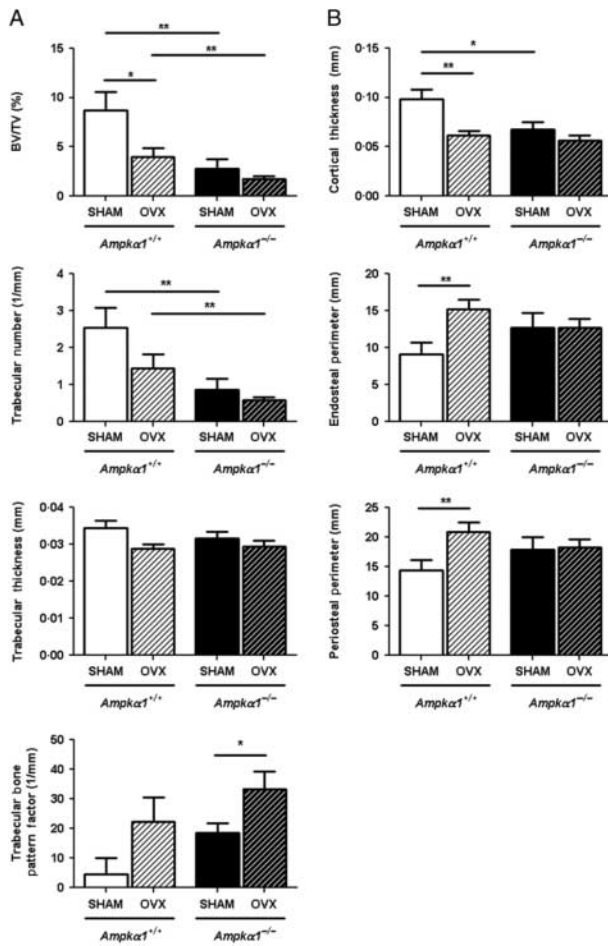
#### Statistical analysis

The results are presented as mean  $\pm$  s.e.m. Comparisons between groups for all the data were performed using nonparametric Mann-Whitney *U* test. Differences were considered statistically significant at  $P < 0.05$ . All statistical analyses were performed using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### Female *Ampk $\alpha$ 1<sup>-/-</sup>* mice have decreased bone mass and increased bone remodelling

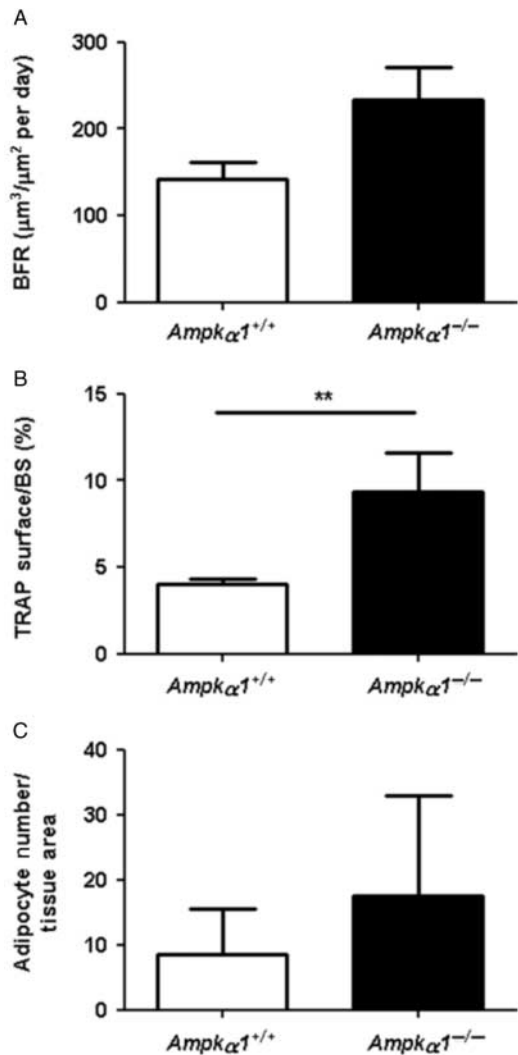
While our previous analysis of bone architecture in *Ampk $\alpha$ 1<sup>-/-</sup>* mice was done in males (Shah *et al.* 2010), we confirmed with the present experiments that female *Ampk $\alpha$ 1<sup>-/-</sup>* mice have a similar low bone mass phenotype compared with *Ampk $\alpha$ 1<sup>+/+</sup>* mice. The micro-CT measurements of trabecular and cortical parameters in tibia of non-OVX adult female mice (Fig. 1A and B) showed that the *Ampk $\alpha$ 1<sup>-/-</sup>* mice had significantly lower BV/TV, Tb.N and Ct.Th compared with *Ampk $\alpha$ 1<sup>+/+</sup>* mice. *Ampk $\alpha$ 1<sup>-/-</sup>* mice also showed a significant increase in SMI, a parameter reflecting trabecular shape, plate to rod elements (*Ampk $\alpha$ 1<sup>+/+</sup>*,  $1.19 \pm 0.15$  vs *Ampk $\alpha$ 1<sup>-/-</sup>*,  $1.69 \pm 0.07$ ;  $P < 0.05$ ), compared with *Ampk $\alpha$ 1<sup>+/+</sup>* mice. There were statistically non-significant increases in trabecular separation (*Ampk $\alpha$ 1<sup>+/+</sup>*,  $0.30 \pm 0.05$  mm vs *Ampk $\alpha$ 1<sup>-/-</sup>*,  $0.47 \pm 0.05$  mm;  $P = 0.073$ ) and TBPf (Fig. 1A) in *Ampk $\alpha$ 1<sup>-/-</sup>*



**Figure 1** Effect of ovariectomy (OVX) on trabecular and cortical bone parameters in tibia of *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. (A) Trabecular bone parameters in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice that have undergone OVX or sham operation. (B) Cortical bone parameters in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice subjected to OVX or sham operation. Values are mean  $\pm$  S.E.M. of  $n=7$  mice/group, \* $P<0.05$ , \*\* $P<0.01$ .

mice, suggesting poor trabecular interconnection. The DA reflecting trabecular structure (*Ampkα1*<sup>+/+</sup>,  $1.64 \pm 0.10$  vs *Ampkα1*<sup>-/-</sup>,  $2.57 \pm 0.90$ ;  $P=1.000$ ) and Ps.Pm and Ec.Pm (Fig. 1B) were not significantly different between the *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. These changes in the trabecular and cortical parameters of female *Ampkα1*<sup>-/-</sup> mice are similar to those observed in male *Ampkα1*<sup>-/-</sup> mice (Shah *et al.* 2010). The trabecular architecture in *Ampkα1*<sup>-/-</sup> mice, characterised by increased SMI, trabecular separation and TBPf and lower BV/TV are similar to the changes seen during ageing and osteoporosis, suggesting an altered bone remodelling at basal level in these mice. To determine the cause of the low bone mass in *Ampkα1*<sup>-/-</sup> mice and whether bone remodelling is affected in these mice at basal level, we examined bone formation and resorption in the tibia of those mice, using bone histomorphometry. Analysis of BFR using

double fluorescence labelling showed that the *Ampkα1*<sup>-/-</sup> mice had a higher BFR than *Ampkα1*<sup>+/+</sup> mice (Fig. 2A), but this was not significant ( $P=0.095$ ). The percentage of TRAP-positive surfaces (osteoclasts surfaces) was significantly higher in the *Ampkα1*<sup>-/-</sup> mice compared with *Ampkα1*<sup>+/+</sup> mice (Fig. 2B). These results suggest that the *Ampkα1*<sup>-/-</sup> mice have a higher bone turnover compared with their *Ampkα1*<sup>+/+</sup> littermates in favour of bone resorption. In addition, the *Ampkα1*<sup>-/-</sup> mice had twice the number of adipocytes than the *Ampkα1*<sup>+/+</sup> mice within the bone marrow but, due to a large variation between animals, the difference was not significant (Fig. 2C).



**Figure 2** Dynamic bone histomorphometry analyses in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. (A) BFR, (B) percentage of TRAP surfaces and (C) number of adipocytes per tissue area in 16-week-old *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. Values are mean  $\pm$  S.E.M. of  $n=7$  mice/group, \*\* $P<0.01$ .

### Ovariectomy (OVX) induces bone loss in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice

To investigate the skeletal response of *Ampkα1*<sup>-/-</sup> mice to OVX, known to stimulate bone remodelling, OVX or sham operations were performed in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. All the mice from these groups have gained weight throughout the treatment period. However, comparison of weight changes (from the day of sham or OVX operation to day of sacrifice, i.e. over the 6-week period) between the groups did not detect any statistically significant differences (sham-*Ampkα1*<sup>+/+</sup>, 1.37±0.61 g vs OVX-*Ampkα1*<sup>+/+</sup>, 1.66±0.42 g; sham-*Ampkα1*<sup>-/-</sup>, 1.76±0.25 g vs OVX-*Ampkα1*<sup>-/-</sup>, 1.54±0.30 g). In addition, there was no significant difference in weight change between sham-*Ampkα1*<sup>+/+</sup> and sham-*Ampkα1*<sup>-/-</sup> mice. Tibial bone length was also not significantly different between *Ampkα1*<sup>+/+</sup> or *Ampkα1*<sup>-/-</sup> mice (data not shown).

As expected, OVX induced bone loss in *Ampkα1*<sup>+/+</sup> mice. These mice showed a significant decrease in BV/TV after OVX (Fig. 1A). Tb.N ( $P=0.097$ ) and thickness ( $P=0.073$ ) were also decreased in the *Ampkα1*<sup>+/+</sup> mice after OVX, while TBPf was increased ( $P=0.165$ ), although these parameters were not statistically significant (Fig. 1A). Analysis of cortical parameters demonstrated a significant increase in Ec.Pm and Ps.Pm in *Ampkα1*<sup>+/+</sup> mice after OVX (Fig. 1B). By contrast, Ct.Th was significantly decreased after OVX in these mice (Fig. 1B).

Ovariectomy also induced alterations in both the trabecular and cortical bones in the *Ampkα1*<sup>-/-</sup> mice, but the effects were moderate, indicating that the bone response to OVX in these mice is attenuated (Fig. 1). *Ampkα1*<sup>-/-</sup> mice showed a significant increase in TBPf, but there were non-statistically significant decreases in BV/TV, Tb.N and thickness after OVX in these mice (Fig. 1A). Cortical parameters were not significantly affected in the *Ampkα1*<sup>-/-</sup> mice after OVX (Fig. 1B).

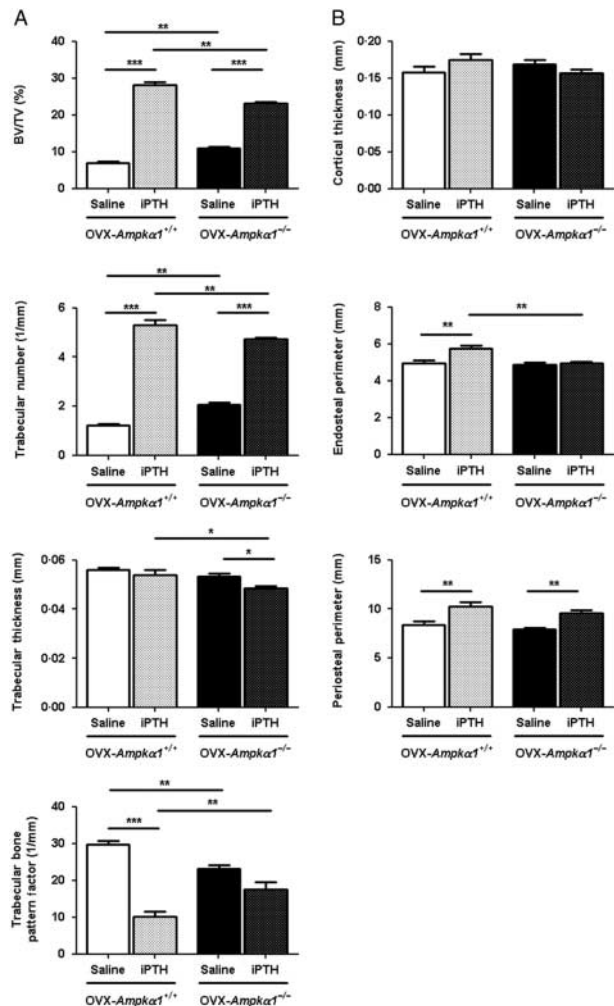
### iPTH increases bone formation in ovariectomised *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice

To determine whether PTH treatment overcomes the bone loss induced by OVX in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice, those mice were ovariectomised and then treated with iPTH or saline for 4 weeks. There was no difference in weight due to iPTH treatment in the ovariectomised *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice (data not shown). Our results demonstrate that iPTH treatment alters trabecular and cortical bone indexes in both OVX-*Ampkα1*<sup>+/+</sup> and OVX-*Ampkα1*<sup>-/-</sup> mice (Fig. 3).

In OVX-*Ampkα1*<sup>+/+</sup> mice, iPTH induced a significant increase in BV/TV and Tb.N and a significant decrease in TBPf (Fig. 3A), trabecular separation (Saline OVX-*Ampkα1*<sup>+/+</sup>, 0.46±0.03 mm vs iPTH OVX-*Ampkα1*<sup>+/+</sup>, 0.20±0.01 mm;  $P<0.0005$ ) and SMI (Saline OVX-*Ampkα1*<sup>+/+</sup>, 2.37±0.04 vs iPTH OVX-*Ampkα1*<sup>+/+</sup>,

1.38±0.09;  $P<0.0005$ ). In the cortical compartment, Ps.Pm and Ec.Pm were significantly increased as a result of iPTH treatment in the OVX-*Ampkα1*<sup>+/+</sup> mice, while Ct.Th was not affected (Fig. 3B).

Similarly, in OVX-*Ampkα1*<sup>-/-</sup> mice, iPTH significantly increased BV/TV and Tb.N, but significantly decreased Tb.Th (Fig. 3A), separation (Saline OVX-*Ampkα1*<sup>-/-</sup>, 0.29±0.01 mm vs iPTH OVX-*Ampkα1*<sup>-/-</sup>, 0.23±0.01 mm;  $P<0.05$ ) and SMI (Saline OVX-*Ampkα1*<sup>-/-</sup>, 2.01±0.05 vs iPTH OVX-*Ampkα1*<sup>-/-</sup>, 1.53±0.13;  $P<0.05$ ). Within the cortical compartment of these mice, Ps.Pm was also significantly increased by iPTH



**Figure 3** Effect of 4 weeks of iPTH treatment on trabecular and cortical bone parameters in tibia of ovariectomised *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. (A) Trabecular bone parameters in ovariectomised *Ampkα1*<sup>+/+</sup> (OVX-*Ampkα1*<sup>+/+</sup>) and *Ampkα1*<sup>-/-</sup> (OVX-*Ampkα1*<sup>-/-</sup>) mice that have been treated with iPTH or saline. (B) Cortical bone parameters in OVX-*Ampkα1*<sup>+/+</sup> and OVX-*Ampkα1*<sup>-/-</sup> mice subjected to iPTH or saline treatment. Values are mean±S.E.M. of  $n=8$  mice/group, \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

treatment but not the other parameters (Fig. 3B). Our results illustrate that the ovariectomised *Ampkα1*<sup>-/-</sup> mice have an attenuated response to iPTH treatment compared with *Ampkα1*<sup>+/+</sup> mice.

#### iPTH increases bone formation in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice

We then analysed the effect of iPTH in non-ovariectomised *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. Four weeks of iPTH treatment in the non-ovariectomised *Ampkα1*<sup>+/+</sup> mice induced mild increases in bone formation in the trabecular and cortical compartments (Fig. 4). Within the trabecular compartment, the only statistically significant change

induced by iPTH was a decrease in DA (saline-*Ampkα1*<sup>+/+</sup>,  $1.81 \pm 0.04$  vs iPTH-*Ampkα1*<sup>+/+</sup>,  $1.69 \pm 0.04$ ;  $P < 0.05$ ). There were non-significant increases in BV/TV ( $P = 0.057$ ), Tb.N ( $P = 0.076$ ) and thickness ( $P = 0.066$ ; Fig. 4A). iPTH also induced a significant increase in Ct.Th in the *Ampkα1*<sup>+/+</sup> mice (Fig. 4B), while the other cortical bone parameters were not significantly affected. By contrast, in the *Ampkα1*<sup>-/-</sup> mice, iPTH induced a significant increase in BV/TV and Tb.N and a significant decrease in TBPf (Fig. 4A), trabecular separation (saline-*Ampkα1*<sup>-/-</sup>,  $0.25 \pm 0.01$  mm vs iPTH-*Ampkα1*<sup>-/-</sup>,  $0.19 \pm 0.01$  mm;  $P < 0.005$ ), SMI (saline-*Ampkα1*<sup>-/-</sup>,  $2.07 \pm 0.08$  vs iPTH-*Ampkα1*<sup>-/-</sup>,  $1.12 \pm 0.18$ ;  $P < 0.0005$ ) and DA (saline-*Ampkα1*<sup>-/-</sup>,  $1.98 \pm 0.05$  vs iPTH-*Ampkα1*<sup>-/-</sup>,  $1.59 \pm 0.07$ ;  $P < 0.005$ ). In the cortical compartment, it significantly increased Ec.Pm but had no effect on the other parameters (Fig. 4B). Surprisingly, in this experiment, Ct.Th was not decreased in the saline *Ampkα1*<sup>-/-</sup> mice compared with *Ampkα1*<sup>+/+</sup> mice, in contrast to Fig. 1 and our previous results (Shah *et al.* 2010).

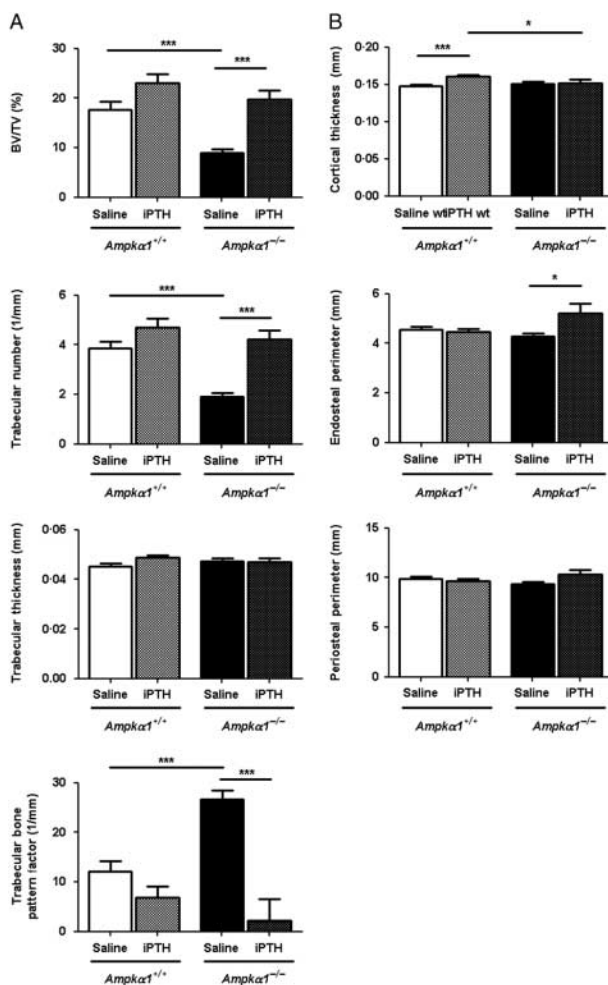
The comparison of changes in trabecular and cortical parameters due to iPTH treatment between *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice shows that PTH induces a greater increase in bone in the non-ovariectomised *Ampkα1*<sup>-/-</sup> mice.

#### iPTH induces AMPKα phosphorylation in *Ampkα1*<sup>+/+</sup> mice

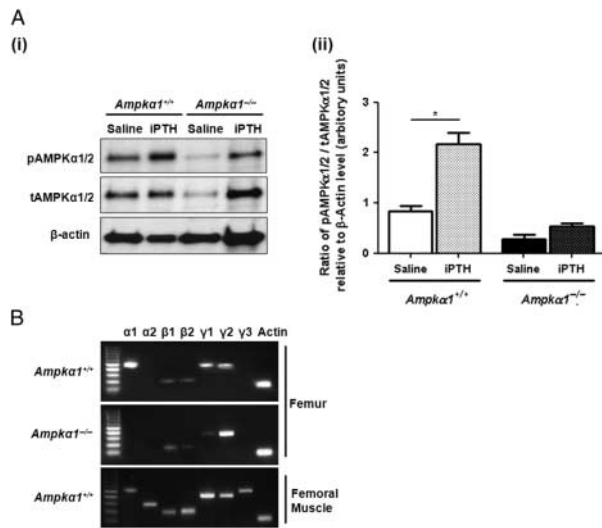
To determine whether iPTH treatment could affect bone AMPK activity, AMPKα1/2 subunit phosphorylation was determined by western blot analysis of proteins extracted from femora of mice from study 2 treated with saline or iPTH. The antibodies we used against phosphorylated AMPKα and total AMPKα do not differentiate between the α1 and α2 subunits. Our western blot analysis showed that iPTH induced a significant increase in pAMPKα1/2 levels in *Ampkα1*<sup>+/+</sup> mice but not in *Ampkα1*<sup>-/-</sup> mice (Fig. 5Ai and ii). We previously showed that there is a very low level expression of α2 subunit in bone at basal conditions (Shah *et al.* 2010). To confirm that the absence of α1 transcript in the *Ampkα1*<sup>-/-</sup> mice does not induce a compensatory increase in α2 expression in bone, we examined the expression of α subunits in bones from *Ampkα1*<sup>-/-</sup> mice and showed that there was no overexpression of α2 in the bones of these mice (Fig. 5B).

## Discussion

We show in this study that the low bone mass observed in male *Ampkα1*<sup>-/-</sup> mice is also observed in female *Ampkα1*<sup>-/-</sup> mice and is due to an increase in bone formation and resorption with an imbalance in favour of resorption. In addition, our results reveal that bone turnover induced by OVX and iPTH hormonal challenges is moderately reduced in the *Ampkα1* subunit knockout mice. However, the skeletal responses to OVX and iPTH in these mice were different



**Figure 4** Effect of 4 weeks of iPTH treatment on trabecular and cortical bone parameters in tibia of *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. (A) Trabecular bone parameters in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice that have been treated with either iPTH or saline for 4 weeks. (B) Cortical bone parameters in *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice treated with iPTH or saline for 4 weeks. Values are mean  $\pm$  S.E.M. of  $n = 10$ – $11$  mice/group, \* $P < 0.05$ , \*\*\* $P < 0.001$ .



**Figure 5** Effect of iPTH treatment on AMPK $\alpha$ 1/2 phosphorylation in bone of ovariectomised *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. (A, i) Western blot analysis of pAMPK $\alpha$ 1/2 and tAMPK $\alpha$ 1/2. Proteins were extracted from femora of 16-week-old OVX-*Ampkα1*<sup>+/+</sup> and OVX-*Ampkα1*<sup>-/-</sup> mice from study 2 and probed with polyclonal antibodies directed against pAMPK $\alpha$ 1/2, tAMPK $\alpha$ 1/2 and  $\beta$ -actin. Representative immunoblots are shown, which were repeated three times with similar results. (ii) Graph showing the ratio of pAMPK  $\alpha$ 1/2 to tAMPK $\alpha$ 1/2 relative to  $\beta$ -actin determined by densitometry analysis of western blot data using Image J Software. Proteins were extracted from femora of two mice per group and western blot analyses were carried out in triplicates. Values are mean  $\pm$  S.E.M., \* $P$  < 0.05. (B) RT-PCR analysis of RNA extracted from femora of *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice showing differential subunit expression pattern. Expression pattern of AMPK subunits in femoral muscle from *Ampkα1*<sup>+/+</sup> mice was carried as a control.

from their WT littermate controls, suggesting that AMPK activation mediates the effects of these hormones on bone turnover.

The increased BFR and resorption surfaces in the *Ampkα1*<sup>-/-</sup> mice suggest increased bone remodelling. This accelerated bone turnover in favour of bone resorption could explain their low bone mass at basal level. Interestingly, deletion of AMPK $\beta$  subunits in mice also reduced bone mass and the authors did not observe any reduction in osteoblast or osteoclast numbers in these mice, suggesting that the low bone mass observed in *Ampkβ1* and 2 knockout mice could be due to changes in bone cellular functions (Quinn *et al.* 2010). Our results, although not statistically significant, show an increase in BFR and MS, reflecting active bone formation, which could be due to an increase in the birth of new remodelling units and/or an increase in the lifespan of these remodelling units. At the cellular level, this could be the result of an enhanced differentiation of osteoprogenitors into mature osteoblasts or an increased lifespan of osteoblasts. This increase in bone formation was an unexpected finding as several *in vitro* studies indicate that AMPK activation stimulates bone formation (Kanazawa *et al.* 2008, 2009,

Molinuevo *et al.* 2010, Shah *et al.* 2010, Jang *et al.* 2011a,b, Mai *et al.* 2011, Wu *et al.* 2011). We also show in this paper a trend towards an increase in marrow adipocyte numbers in bones of *Ampkα1*<sup>-/-</sup> mice, which could suggest a potential interaction between AMPK signalling in fat and bone, and this will need to be further investigated. Our results indicate that bone resorption is increased in *Ampkα1*<sup>-/-</sup> mice, suggesting that AMPK $\alpha$ 1 activation inhibits bone resorption. Indeed, it has been shown that AMPK acts as a negative regulator of RANKL in bone marrow macrophages, and inhibition of AMPK increases RANKL-dependent formation of TRAP-positive multinucleated cells and bone resorption area (Lee *et al.* 2010). This could therefore explain the increase in percentage of TRAP surfaces in *Ampkα1*<sup>-/-</sup> mice.

To clarify whether changes in basal bone cellular activities in *Ampkα1*<sup>-/-</sup> mice affect their responses to changes in bone turnover, we first submitted these mice to OVX. Loss of bone mass, trabecular thinning and increased trabecular separation are general features of bone after OVX (Parfitt *et al.* 1987, Compston *et al.* 1989). This is due to increased bone resorption, which exceeds bone formation at the initial stages (Lambers *et al.* 2012). Consistent with this, we observed deteriorated trabecular bone architecture in the *Ampkα1*<sup>+/+</sup> mice after OVX. Furthermore, OVX caused endosteal bone resorption and periosteal bone apposition in these mice, which is consistent with the known effect of OVX on cortical bone architecture (Turner *et al.* 1987a,b). Weight gain is typically observed after OVX in rats and in humans after menopause (Lobo 2008, Tezval *et al.* 2011a). Although all mice gained weight during the 6-week experimental period, OVX did not induce significant weight gain in both the *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice. Body weight gain after OVX in mice is not always observed and may depend on the genetic background, as previously reported (Andersson *et al.* 2001, Boussein *et al.* 2005, Li *et al.* 2005, Iwaniec *et al.* 2006).

Our results illustrate that *Ampkα1*<sup>-/-</sup> mice, similar to *Ampkα1*<sup>+/+</sup> mice, can lose bone after OVX, although this bone loss was moderate compared with *Ampkα1*<sup>+/+</sup> mice. This suggests that AMPK $\alpha$ 1 is likely to play a role in bone resorption and remodelling induced by OVX. As bone mass is already very low at basal levels in the *Ampkα1*<sup>-/-</sup> mice, there may be a protective mechanism to reduce the level of bone loss induced by OVX and to preserve the bone architecture without causing deleterious effects. One possibility for this mechanism could be the fact that there is less surface area for bone resorption. A study investigating OVX-induced bone loss in different inbred mouse strains revealed indeed that strains with low basal trabecular bone mass lose less bone compared with mice with high basal bone mass (Boussein *et al.* 2005). Alternatively, as bone remodelling is already very high in *Ampkα1*<sup>-/-</sup> mice, they may be less sensitive to an increase in bone remodelling induced by oestrogen withdrawal.

Intermittent administration of PTH is known to increase bone mass and improve bone architecture (Iida-Klein *et al.* 2002, Jiang *et al.* 2003, Brouwers *et al.* 2009, Recker *et al.* 2009). At the cellular level, iPTH has been shown to increase bone remodelling (Fox *et al.* 2006, Wade-Gueye *et al.* 2010) with bone formation exceeding resorption. To understand the bone responses of *Ampkα1*<sup>-/-</sup> mice to anabolic (iPTH) stimuli, we first subjected ovariectomised *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice to iPTH treatment for 4 weeks (study 2). Our results confirm previous studies showing that iPTH (1–34) can increase bone formation in ovariectomised rodents (Alexander *et al.* 2001, Fox *et al.* 2006, Wade-Gueye *et al.* 2010, Tezval *et al.* 2011a). We show that similar to OVX–*Ampkα1*<sup>+/+</sup> mice, OVX–*Ampkα1*<sup>-/-</sup> mice have an increase in trabecular BV and number as well as an augmented cortical bone mass in response to iPTH, confirming that osteoblast function is not severely affected in these *Ampkα1*<sup>-/-</sup> mice. OVX–*Ampkα1*<sup>-/-</sup> mice were, however, less affected than OVX–*Ampkα1*<sup>+/+</sup> mice by the changes in bone turnover induced by iPTH, possibly because they lost less bone after OVX than their WT controls. We indeed found in this study that the trabecular bone mass in OVX–*Ampkα1*<sup>-/-</sup> mice was higher than that of OVX–*Ampkα1*<sup>+/+</sup> mice (Fig. 3), which contrasts with our first study (Fig. 1) where OVX–*Ampkα1*<sup>-/-</sup> mice had lower trabecular bone mass than OVX–*Ampkα1*<sup>+/+</sup> mice. This suggests that the *Ampkα1*<sup>-/-</sup> mice in study 2 did not lose as much bone mass after OVX as those in study 1. This discrepancy in the amount of bone loss after OVX between our two studies may be due to the time after OVX. In study 1, bone mass was determined 6 weeks after OVX while in study 2 it was investigated after 4 weeks, and there are studies supporting a time-dependent bone loss after OVX (Li *et al.* 2005, Iwaniec *et al.* 2006). It is also possible that the absence of AMPKα1 may have contributed to this delay in bone loss after OVX in the *Ampkα1*<sup>-/-</sup> mice.

To further investigate whether *Ampkα1*<sup>-/-</sup> mice responded to the sole anabolic effect of iPTH, non-ovariectomised *Ampkα1*<sup>+/+</sup> and *Ampkα1*<sup>-/-</sup> mice were treated with iPTH for 4 weeks (study 3). In non-ovariectomised *Ampkα1*<sup>+/+</sup> mice, iPTH induced a smaller increase in bone mass compared with mice that were ovariectomised, suggesting that OVX enhances the anabolic effect of iPTH on bone mass, as previously demonstrated (Andersson *et al.* 2001, Tezval *et al.* 2011b). By contrast, iPTH elicited a larger increase in bone mass in non-ovariectomised *Ampkα1*<sup>-/-</sup> mice compared with *Ampkα1*<sup>+/+</sup> mice, possibly due to the low basal level of bone mass in these mice, enhancing the effect of iPTH. Interestingly, the comparison of the percentage of increased bone mass induced by iPTH in OVX–*Ampkα1*<sup>-/-</sup> mice (study 2) and non-OVX–*Ampkα1*<sup>-/-</sup> mice (study 3) showed that it is similar, in contrast to *Ampkα1*<sup>+/+</sup> mice where there is about a tenfold decrease in the response to iPTH in non-OVX mice.

Our data suggest that the presence of the AMPKα1 subunit, and consequently AMPK activation in bone, is not

essential for bone turnover but may contribute to the modulation of this process. We previously showed that the α2 subunit, in contrast to other tissues (Stapleton *et al.* 1996, Quinn *et al.* 2009, Shah *et al.* 2010), is not highly expressed in bone (Quinn *et al.* 2010, Shah *et al.* 2010). Furthermore, our results demonstrate that there is no compensatory up-regulation of α2 in bones of *Ampkα1*<sup>-/-</sup> mice. This is in contrast to the demonstration of an up-regulation of α2 in the soleus and extensor digitorum muscle in the *Ampkα1*<sup>-/-</sup> mice (Jorgensen *et al.* 2004). We cannot exclude, however, that this up-regulation of α2 in muscle and possibly in other tissues in these mice may have indirectly affected bone (Jorgensen *et al.* 2004).

As our study was performed with *Ampkα1*<sup>-/-</sup> mice that are conventional whole-body knockout, we cannot exclude that the effects of the deletion of the α1 subunit observed in bone may be the result of indirect effects of AMPK deletion in other tissues. *Ampkα1*<sup>-/-</sup> mice have been reported to have significantly reduced inguinal and epididymal fat weights compared with *Ampkα1*<sup>+/+</sup> mice and a tendency for lower body weights (Daval *et al.* 2005), although this was not observed in our study or in other studies (Jorgensen *et al.* 2004, Viollet *et al.* 2009). No other metabolic phenotype was reported in these *Ampkα1*<sup>-/-</sup> mice and these mice have no changes in oestrogen levels. It is, however, unknown whether PTH levels are altered in these mice.

While the hormonal regulation of AMPK activation is well characterised in several tissues (Xue & Kahn 2006, Dzamko & Steinberg 2009, Lim *et al.* 2010), it has not been extensively studied in bone. Our previous work has shown that AMPK activity in bone cells could be regulated by the same hormones that regulate food intake and energy expenditure through AMPK activation in the brain and peripheral tissues (Shah *et al.* 2010). While our preliminary data in the osteoblastic cell line UMR-106 have shown no effect of oestrogen on AMPK activation (data not shown), our results show for the first time that iPTH increased the level of pAMPKα1/2 *in vivo* in the ovariectomised *Ampkα1*<sup>+/+</sup> mice, suggesting that PTH may activate AMPK signalling in bone, although this signalling pathway does not seem essential for the effect of PTH on bone formation. Further studies must, however, be carried out to elucidate downstream pathways and mechanism of action. PTH also induced a non-significant effect on phosphorylation of AMPKα1/2 in the *Ampkα1*<sup>-/-</sup> mice, likely due to the phosphorylation of the α2 subunit, expressed at very low levels in bone.

In conclusion, we demonstrate that *Ampkα1*<sup>-/-</sup> mice have an increased bone turnover compared with *Ampkα1*<sup>+/+</sup> mice and can increase and decrease bone mass in response to anabolic and catabolic hormonal challenges, although these responses are modified. Taken together, our results indicate that AMPKα1 activity is not essential for bone turnover but may contribute to the regulation of bone remodelling.



## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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