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gp130 in late osteoblasts and osteocytes is required for PTH-induced osteoblast differentiation

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Abstract

Parathyroid hormone (PTH) treatment stimulates osteoblast differentiation and bone formation, and is the only currently approved anabolic therapy for osteoporosis. In cells of the osteoblast lineage, PTH also stimulates expression of members of the IL-6 cytokine superfamily. Although the similarity of gene targets regulated by these cytokines and PTH suggest cooperative action, the dependence of PTH anabolic action on IL-6 cytokine signaling is unknown.

To determine whether cytokine signaling in the osteocyte through glycoprotein 130 (gp130), the common IL-6 superfamily receptor subunit, is required for PTH anabolic action, male mice with conditional gp130 deletion in osteocytes (*Dmp1Cre.gp130$^{f/f}$*) and littermate controls (*Dmp1Cre.gp130$^{w/w}$*) were treated with hPTH(1-34) (30µg/kg 5x/week for 5 weeks). PTH dramatically increased bone formation in *Dmp1Cre.gp130$^{w/w}$* mice, as indicated by elevated osteoblast number, osteoid surface, mineralizing surface, and increased serum N-terminal propeptide of type I collagen (P1NP). However, in mice with DMP1Cre-directed deletion of gp130, PTH treatment changed none of these parameters.

Impaired PTH anabolic action was associated with a 50 percent reduction of *Pth1r* mRNA levels in *Dmp1Cre.gp130$^{f/f}$* femora compared to *Dmp1Cre.gp130$^{w/w}$*. Furthermore, lentiviral cre infection of *gp130$^{f/f}$* primary osteoblasts also lowered *Pth1r* mRNA levels to 16% of that observed in infected C57/BL6 cells.

In conclusion, osteocytic gp130 is required to maintain PTH1R expression in the osteoblast lineage**,** and for the stimulation of osteoblast differentiation that occurs in response to PTH.

Introduction

Intermittent administration of parathyroid hormone (PTH) to animal models and humans (Teriparatide (Forteo)) increases bone mass (Lindsay, et al. 2007; Neer, et al. 2001; Reeve, et al. 1980), and is the only approved treatment for osteoporosis capable of inducing bone formation (reviewed in (Hodsman, et al. 2005; Khosla, et al. 2008)). However, the mechanisms by which intermittent PTH increases bone mass remain unclear, and identifying downstream targets of this pathway may aid in the design of improved anabolic therapies.

The effects of PTH on bone mass are likely to be mediated by cells of the osteoblast lineage. This lineage includes committed pre-osteoblasts, matrix-producing osteoblasts, bone lining cells, and matrix-embedded osteocytes. PTH acts directly at each stage of differentiation, as follows. PTH promotes pre-osteoblast differentiation (Dobnig and Turner 1995), inhibits osteoblast apoptosis (Jilka, et al. 1999), and reactivates quiescent lining cells to become active osteoblasts (Kim, et al. 2012). PTH also acts directly on osteocytes to reduce their expression of the Wnt antagonist sclerostin, an inhibitor of bone formation (Bellido, et al. 2005; Keller and Kneissel 2005).

PTH also stimulates expression of receptor activator of NF-kappa-B ligand (RANKL) by early osteoblast lineage cells, thereby promoting osteoclast differentiation (Udagawa, et al. 1999). However, the stages of osteoblast differentiation most important for the actions of PTH remain controversial, since

expression of RANKL by matrix-embedded osteocytes is also stimulated by PTH (Xiong, et al. 2011).

PTH also acts on the osteoblast lineage to rapidly promote expression of IL-6 family cytokines and receptors. These include interleukin-6 (IL-6) (Greenfield, et al. 1996), interleukin-11 (IL-11), oncostatin M (OSM) receptor (OSMR), leukemia inhibitory factor (LIF) and cytokine receptor-like factor 1 (CRLF1)(Walker, et al. 2012). These cytokines all depend on the promiscuous co-receptor gp130 for signaling (reviewed in (Sims and Walsh 2010)), and gp130 expression by the osteoblast lineage is also stimulated by PTH (Romas, et al. 1996).

Many of the actions and gene targets of IL-6 family cytokines are common to those of PTH. As is the case with PTH, the cytokines IL-6, IL-11, OSM, LIF and cardiotrophin (CT-1) promote osteoblast differentiation *in vitro* (Walker, et al. 2008; Walker, et al. 2010) and OSM, LIF and CT-1 stimulate bone formation *in vivo* (Cornish, et al. 1993; Walker et al. 2008; Walker et al. 2010). Family members IL-11, LIF, OSM, CT-1 and CNTF also inhibit osteocytic sclerostin expression (Johnson, et al. 2014b; Walker et al. 2010). In addition, IL-6, IL-11, OSM, LIF and CT-1 stimulate osteoblast lineage expression of RANKL (O'Brien, et al. 1999; Palmqvist, et al. 2002; Walker et al. 2008) and promote osteoclastogenesis when precursors are co-cultured with osteoblasts *in vitro* (Richards, et al. 2000; Tamura, et al. 1993). These similar effects, and the upregulation of IL-6 family cytokines in osteoblasts by PTH suggest that this cytokine family may play a role in the actions of PTH on the osteoblast lineage.

Hence, in this study we examined the requirement of gp130 signaling in osteocytes for the anabolic action of PTH, using mice with DMP1Cre-directed deletion of gp130 in osteocytes (*Dmp1Cre.gp130$^{f/f}$*) (Johnson, et al. 2014a) and mature osteoblasts (Torreggiani, et al. 2013; Xiong et al. 2011). We found that gp130 in these cells is required for PTH to increase osteoblast number and bone forming surfaces, and to maintain PTH1R expression in the osteoblast lineage.

Materials and methods

Mice

All animal procedures were conducted with approval of the St. Vincent's Health Melbourne Animal Ethics Committee. DMP1Cre mice were obtained from Lynda Bonewald (University of Kansas, Kansas City, USA) (Lu, et al. 2007). Floxed gp130 mice backcrossed onto C57/BL6 were obtained from Rodger McEver (Oklahoma Medical Research Foundation) (Betz, et al. 1998). Mice hemizygous for the Cre transgene were crossed with the gp130 flox mouse in which the transmembrane domain (exon 15) was flanked by loxP sites, resulting in ablation of intracellular gp130 signalling, as previously reported (Betz et al. 1998) and confirmed at the mRNA level in bone (Johnson et al. 2014a). For all experiments, DMP1.Cre+ cousins were used as controls.

Six week old male DMP1Cre+gp130 wildtype (*Dmp1Cre.gp130$^{w/w}$*) or DMP1Cre+gp130 floxed (*Dmp1Cre.gp130$^{f/f}$*) mice were injected i.p. with 30µg/kg human parathyroid hormone 1-34 (hPTH 1-34) or vehicle 5 days a week for 5 weeks (n=9/10 per group). This dose and duration of PTH treatment was chosen because it provides a robust increase in lamellar bone formation rate and osteoblast surface in male mice without increasing osteoclastogenesis (Takyar, et al. 2013; Tonna, et al. 2014; Walker et al. 2012). Mice were also injected with calcein (20mg/kg) 7 and 2 days prior to tissue collection. Bones were collected one hour after the last PTH injection. The mice were fasted for 12 hours prior to anaesthesia with ketamine/xylazine and a final blood sample was collected by

cardiac puncture. Blood samples were centrifuged for 10 minutes at 4,000 rpm and serum was removed to a fresh tube and stored at -80°C until analysis for cross-linked C-terminal telopeptide of type I collagen (CTX-1), N-terminal propeptide of type I collagen (P1NP) (Immunodiagnostic Systems Limited, Boldon, Tyne & Wear, UK) and PTH (Immunotopics, San Clemente, CA) as per manufacturer's instructions. One femur was flushed of marrow and the bone shaft was collected for RNA analyses as previously described (Walker et al. 2012). Briefly, bones were homogenized with a LS-10-35 Polytron homogenizer in Trizol for 4 x 5 second bursts and stored at -80C. RNA from each bone was purified using the RNeasy lipid tissue minikit (Qiagen), according to manufacturer's instructions.

The other femur was analysed by micro-computed tomography as previously described (Johnson et al. 2014a) using the SkyScan 1076 system (Bruker-microCT, Kontich, Belgium). Images were acquired using the following settings: 9µm voxel resolution, 0.5mm aluminium filter, 48kV voltage and 100µA current, exposure time, rotation 0.5°, frame averaging =1. Images were reconstructed and analysed using SkyScan software programs NRecon (version 1.6.3.3), DataViewer (version 1.4.4) and CT Analyser (version 1.12.0.0). Femoral trabecular analysis region of interest (ROI) was determined by identifying the distal end of the femur and calculating 15% of the total femur length towards the femora mid-shaft, where we then analysed an ROI of 12.6% of the total femur length. Analysis of bone structure was completed using adaptive thresholding (mean of min and max values) in CT Analyser. Thresholds for analysis were

149 determined based on multilevel Otsu thresholding of the entire data set, and
150 were set at 45-255 for trabecular bone. Cortical analyses were performed 35%
151 above the distal end of the femur toward the femora mid-shaft, also with a 12.6%
152 ROI with the threshold values set at 100-255.
153
154 Tibiae were collected for histomorphometric analyses as previously described
155 (Sims, et al. 2006). Briefly, trabecular histomorphometry was carried out on
156 undecalcified sections in the secondary spongiosa of the proximal tibia, in a
157 region 370μm below the proximal edge of the hypertrophic zone of the growth
158 plate, extending 1.11 mm in the proximal direction. Periosteal
159 histomorphometry was carried out on the antero-fibular side of the tibia,
160 commencing 1.11mm below the chondro-osseus junction of the growth plate,
161 and extending 1.11 mm in the proximal direction. Nomenclature is as previously
162 described (Parfitt, et al. 1987).
163
164 *Lenti-cre viral infection*
165 Calvarial osteoblasts were collected from C57/BL6 wildtype and *gp130*$^{f/f}$
166 neonates by digesting calvaria in 1:2 collagenase II/dispase solution at 37°C on a
167 shaker (1 x 5 minutes, 4 x 10 minute digestions). The cells were resuspended in
168 culture media, and allowed to adhere overnight before being frozen and stored
169 in liquid nitrogen. When required, isolated cells were thawed and expanded in
170 culture and infected with a GFP-tagged lenti-Cre virus synthesised as previously
171 described (Tonna et al. 2014) for 24 hours with polybrene in maintenance
172 media. Following infection, media was changed and cells were evaluated for GFP

expression by microscopy; >30-60% transfection efficiency was observed (n=3 independent experiments). Cells were expanded in culture for 2-3 weeks in maintenance media and GFP positive cells (fluorescence driven by Cre transgene expression) were sorted on a FACS Aria (BD Biosciences, San Jose, California) for GFP. Sorted cells were harvested for RNA in Trizol (Life Technologies, Carlsbad, California) and separated and precipitated using chloroform and isopropanol. Extracted RNA was DNase treated using Ambion TURBO DNA-free kit (Life Technologies) and quantified on a NanoDrop ND1000 spectrophotemeter (Thermo Scientific, Wilmington, DE).

Semi-quantitative real-time PCR (qPCR)

cDNA synthesis from 50-100 ng DNase-treated RNA from each femur or cell culture preparation was performed using AffinityScript (Agilent Technologies, Santa Clara, California, USA) per the manufacturer's instructions. Stock cDNA was diluted to a concentration of 5 ng/µl and semi-quantitative real-time PCR was performed on 12.5 ng cDNA in a reaction volume of 10 µl using in-house master mix of 10X AmpliTaq Gold with SYBR Green nucleic acid gel stain (Life Technologies). *Dkk1* primers were designed using NCBI Primer Blast: Forward-GAGGGGAAATTGAGGAAAGC; Reverse-ACGGAGCCTTCTTGTCCTTT. Other primers were as previously described for *Pth1r, Hprt1, Sost, Tnfsf11, Il6* (Allan, et al. 2008), *B2m* (McGregor, et al. 2010), and *Hmbs* (Johnson et al. 2014a).

Samples were dispensed onto optically clear 96-well plates (Thermo Scientific) and run on a Stratagene Mx3000P (Agilent Technologies). Cycling conditions

were 95˚C for 10 min, (95˚C for 30 sec, 58˚C for 1min, 72˚C for 30 sec) X 40 cycles, followed by dissociation step (95˚C for 1min, 55˚C for 30 sec, 95˚C for 30 sec). Post-run samples were analysed using Stratagene software MxPro and reported using linear ΔCT values normalized to the geometric mean of the two housekeeping genes (HKG) hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and hydroxymethylbilane synthase (*Hmbs*) or to β-2 microglobulin (*B2m*) as indicated.

Statistics

All graphs are presented as the mean/genotype + standard error of the mean. N = 5-10 animals/group as indicated on the graph or in the figure legend. For *in vitro* experiments, data shown is the average of 3 independent biological replicates. Statistical significance was considered when $p<0.05$. In all figures *=p≤0.05, **=p≤0.01, ***=p≤0.001. Differences between groups were analysed by 2-way ANOVA and post hoc Šidak multiple comparison test. Skewed variables (mRNA data in Figure 4) were transformed using the natural logarithm before statistical analyses. For the lenti-viral Cre infected primary calvarial osteoblasts, Student's t-test was used to assess significance. Statistical analyses were performed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, La Jolla, California, USA).

Results

DMP1Cre.gp130$^{f/f}$ mice show no increase in trabecular osteoblast number in response to PTH

PTH treatment at 30µg/kg/day significantly increased osteoblast number / bone perimeter (NOb/BPm) on trabecular bone in *Dmp1Cre.gp130$^{w/w}$* mice by 76% (Figure 1A). Osteoblast surface / bone surface (ObS/BS) (Figure 1B) and osteoid surface / bone surface (OS/BS)(Figure 1C) were also elevated by PTH treatment to similar extents. We detected no significant changes in osteoid thickness in *Dmp1Cre.gp130$^{w/w}$* mice after PTH treatment (Figure 1D).

In contrast to *Dmp1Cre.gp130$^{w/w}$* mice, PTH treatment did not increase osteoblast or osteoid-derived parameters in age- and sex-matched *Dmp1Cre.gp130$^{f/f}$* mice (Figure 1A-D). Two-way ANOVA revealed that the effects of PTH treatment on both NOb/BPm and ObS/BS were significantly reduced in the *Dmp1Cre.gp130$^{f/f}$* mice compared to *Dmp1Cre.gp130$^{w/w}$* controls (interaction p=0.039, and 0.043, respectively). This indicates that the effect of PTH on osteoblast differentiation is dependent on gp130 expression in osteocytes.

In line with the effects on osteoblast numbers, bone forming surfaces, indicated by incorporation of calcein labels, including both double labeled surface (Figure 1E) and single labeled surface (p≤0.05, not shown) were significantly greater in PTH treated *Dmp1Cre.gp130$^{w/w}$* mice compared to controls. Again, this was not

observed in *Dmp1Cre.gp130$^{f/f}$* mice. Mineral apposition rate (MAR) was significantly greater in both *Dmp1Cre.gp130$^{f/f}$* and *Dmp1Cre.gp130$^{w/w}$* mice treated with PTH compared to their vehicle-treated controls (Figure 1F), indicating that an increase in mineralization rate in response to PTH is retained on those surfaces on which bone formation occurs in *Dmp1Cre.gp130$^{f/f}$* mice.

PTH treated *Dmp1Cre.gp130$^{w/w}$* mice had significantly higher serum P1NP levels than *Dmp1Cre.gp130$^{w/w}$* untreated controls. In contrast, in *Dmp1Cre.gp130$^{f/f}$* mice there was no significant effect of PTH on P1NP levels compared to vehicle-treated *Dmp1Cre.gp130$^{f/f}$* mice (Figure 1G); interaction p-value=0.009 by two-way ANOVA. These results are consistent with the histomorphometry data and confirm that at a systemic level, the effect of PTH on bone formation is blunted in *Dmp1Cre.gp130$^{f/f}$* mice.

In both *Dmp1Cre.gp130$^{f/f}$* and *Dmp1Cre.gp130$^{w/w}$* mice, intermittent human PTH treatment led to reduced production of endogenous circulating murine PTH levels (Figure 1H), demonstrating that negative feedback at the parathyroid induced by exogenous PTH administration was maintained in both groups of mice.

Although this dose of PTH significantly increased all markers of bone formation in *Dmp1Cre.gp130$^{w/w}$* mice, we did not detect a significant increase in trabecular bone mass by micro-computed tomography with this short time course of low dose treatment (Table 1).

No effect of intermittent PTH treatment on bone resorption

This protocol of intermittent PTH treatment did not significantly change osteoclast number / bone perimeter (NOc/BPm) (Figure 2A), osteoclast surface / bone surface (OcS/BS) (Figure 2B) or serum levels of cross-linked C-terminal telopeptide of type I collagen (CTX1) (Figure 2C) in either *Dmp1Cre.gp130$^{f/f}$* or *Dmp1Cre.gp130$^{w/w}$* mice. This confirms our previous observations using similar protocols over 4 weeks of treatment (Takyar et al. 2013; Tonna et al. 2014; Walker et al. 2012).

Effects of PTH on cortical bone

Although periosteal double labeled surface (dLS/BS) was not altered by PTH treatment in either genotype (Figure 3A), periosteal mineral apposition rate (Figure 3B) and periosteal perimeter (Figure 3C) were all significantly greater in PTH-treated *Dmp1Cre.gp130$^{w/w}$* mice compared with untreated mice. None of these parameters were significantly increased by PTH treatment in *Dmp1Cre.gp130$^{f/f}$* mice compared to genotype-matched vehicle controls (Figure 3B), indicating that periosteal growth in response to PTH may also be impaired in the absence of osteocytic gp130.

Normal response of osteoclastic genes, but lack of inhibition of Wnt signaling inhibitors by PTH treatment in DMP1Cre.gp130$^{f/f}$ mice

RANKL (gene name *Tnfsf11*) and IL-6 (*Il6*) are both potent stimuli of osteoclast formation, and PTH increases their expression in cells of the osteoblast lineage

(Greenfield, et al. 1995; Udagawa et al. 1999). Indeed, in marrow-flushed femoral samples collected 1 hour after the last of these 5 weeks of injections, mRNA levels of *Tnfsf11* and *Il6* were significantly higher in both genotypes after PTH treatment (Figure 4A and 4B); this increase was not significantly affected by the genotype (two-way ANOVA interaction p values = 0.365 and 0.314, respectively). This indicated that among cells in the flushed femora, which would include osteoblasts at different stages of differentiation as well as osteocytes, are some cells that retain normal responses of these genes to PTH.

Wingless (Wnt)-signaling is important for osteoblast differentiation and bone formation, and PTH has been shown to stimulate Wnt signaling by suppressing Dickopf1 (*Dkk1)* and sclerostin (*Sost)* expression in the osteoblast lineage (Keller and Kneissel 2005; Yao, et al. 2011). For this reason, we quantified mRNA levels of *Dkk1* and *Sost* in flushed femurs. As expected, *Dkk1* mRNA levels were significantly lower in PTH-treated *Dmp1Cre.gp130$^{w/w}$* femurs compared with untreated mice. However, *Dkk1* was not lower in femurs from PTH-treated *Dmp1Cre.gp130$^{f/f}$* mice compared to controls (Figure 4C). *Sost* mRNA levels were slightly, but not significantly, lowered in response to PTH in *Dmp1Cre.gp130$^{w/w}$* femora. *Dmp1Cre.gp130$^{f/f}$* femora showed a lower level of *Sost* mRNA compared to vehicle treated *Dmp1Cre.gp130$^{w/w}$* controls; with PTH treatment these mice showed a significant increase in *Sost* mRNA levels (Figure 4D). These differences in effects of PTH treatment on gene expression were significant by two-way ANOVA for both *Dkk1* (interaction p=0.01) and *Sost* (interaction p=0.003). Thus, PTH treatment does not decrease Wnt antagonist expression in *Dmp1Cre.gp130$^{f/f}$*

mice, implying that gp130 signalling in osteocytes is important for the PTH effect on Wnt signaling inhibitors.

Pth1r expression is reduced in DMP1Cre.gp130$^{f/f}$ mice and gp130 deficient osteoblasts

Since many effects of PTH were blocked in *Dmp1Cre.gp130$^{f/f}$* mice we quantified *Pth1r* mRNA levels in flushed femurs from untreated 12-week-old *Dmp1Cre.gp130$^{f/f}$* and *Dmp1Cre.gp130$^{w/w}$* mice. Surprisingly, *Pth1r* mRNA expression was 47% lower in *Dmp1Cre.gp130$^{f/f}$* compared to *Dmp1Cre.gp130$^{w/w}$* femurs (p=0.03) (Figure 5A).

These findings were supported by *in vitro* data, where C57/BL6 and *gp130$^{f/f}$* calvarial osteoblasts were infected with lentiviral cre-recombinase. In cre-infected *gp130$^{f/f}$* osteoblasts, *gp130* was significantly lowered by 52%, and *Pth1r* mRNA was 84% lower than in infected C57/BL6 cells (Figure 5 B,C). mRNA levels of *Runx2, Osx* and *Alpl* were not significantly altered by cre-infection of *gp130$^{f/f}$* osteoblasts (Figure 5C), consistent with previously published mRNA levels of these genes in the femora of DMP1Cre.gp130$^{f/f}$ mice (Johnson, et al. 2014a). This suggests that cells of the osteoblast lineage require signals mediated by gp130 to maintain PTH1R expression, and that a lack of PTH1R in DMP1Cre expressing cells is responsible for the reduced response to anabolic PTH treatment.

Discussion

This work demonstrates that PTH-induced osteoblast differentiation is dependent on gp130 expression in mature osteoblast lineage cells. gp130 is needed to maintain *Pth1r* expression in osteoblasts, and is required for PTH to suppress the Wnt-antagonists *Dkk1* and *Sost*. In contrast, gp130 expression by osteocytes is not required for PTH to stimulate mRNA levels of the pro-osteoclastogenic factors RANKL (*Tnfsf11)* and *Il6* in bone.

The stimulatory effect of PTH on trabecular osteoblast numbers and mineralizing surface was completely ablated in *DMP1Cre.gp130$^{f/f}$* mice. This may, at least partly, be explained by the lack of a reduction in both Wnt signaling inhibitors *Sost* and *Dkk1* in response to PTH. Wnt signaling stimulates osteoblast differentiation, and it has been postulated that this is one pathway through which PTH stimulates bone formation (Kulkarni, et al. 2005), a hypothesis supported by impaired PTH responses in mice overexpressing sclerostin or Dkk1 (Guo, et al. 2010; Kramer, et al. 2010). PTH directly inhibits *Sost* via cAMP-PKA signaling (Keller and Kneissel 2005). IL-6 family cytokines also rapidly inhibit *Sost,* although the mechanism remains unknown (Walker et al. 2010). Whether the reduction in effect of PTH on Wnt signaling is entirely due to the reduced PTH1R expression or results from some dependence on gp130 cytokines on this same pathway in osteoblasts and osteocytes remains unclear.

In contrast to the effect on Wnt-antagonists, both *Dmp1Cre.gp130$^{w/w}$* and *Dmp1Cre.gp130$^{f/f}$* mice demonstrated increased femoral *Rankl* and *Il6* mRNA

levels in response to PTH. Despite these increases in both genotypes, osteoclast numbers were unchanged, as we have previously reported with this low dose of intermittent PTH treatment (Takyar et al. 2013; Tonna et al. 2014), likely because the inductions of RANKL and IL-6 are transient (Ma, et al. 2001; Walker et al. 2012). Il-6 and RANKL are expressed by a wide range of cells in the bone, including osteoblast lineage cells as well as osteocytes (Dai, et al. 2006; Lee and Lorenzo 1999; Nakashima, et al. 2011; Xiong et al. 2011), but also cells within the bone marrow, including T-cells (Horwood, et al. 1999; Matthews, et al. 2014) and, in the case of IL-6, macrophages (Balic and Mina 2011). Although PTH has recently been suggested to directly promote RANKL expression in osteocytes (Xiong et al. 2011), our findings suggest that the major cellular targets that produce these pro-osteoclastogenic factors in response to PTH are not osteocytes. Notably, although PTH was unable to increase osteoblast numbers or mineralizing surface in the *Dmp1Cre.gp130*$^{f/f}$ mice, on those surfaces where double calcein labels were incorporated into the bone matrix, the distance between them (MAR) was significantly greater in PTH treated mice, regardless of genotype. This suggests that those bone-forming osteoblasts that are on the bone surface in *Dmp1Cre.gp130*$^{f/f}$ mice retain sufficient PTHR expression to respond to PTH with increased matrix production. Since marrow was flushed from the femora, and *Pth1r* levels were dramatically reduced in undifferentiated cultured cre-expressing cells, we suggest that the key PTH-responsive cells producing RANKL and IL-6 in this model are less differentiated osteoblasts, not expressing DMP1Cre, on the bone surface.

383 *Pth1r* mRNA was lower in cortical bone from mice lacking gp130 in osteocytes
384 (*Dmp1Cre.gp130$^{f/f}$*) compared with littermate controls, an effect that was
385 reproduced when gp130 was deleted in cultured primary calvarial osteoblasts.
386 There are two ways to understand this: firstly, since osteoblast differentiation is
387 impaired in the *Dmp1Cre.gp130$^{f/f}$* mice (Johnson et al. 2014a), and PTH1R
388 expression in the osteoblast lineage is higher in more mature osteoblasts (Allan
389 et al. 2008; Allan, et al. 2003; Balic, et al. 2010), there may be fewer mature
390 PTH1R-expressing osteoblasts present within the bone of these mice. Another
391 interpretation is that gp130 is needed to maintain the expression of PTH1R in
392 the osteoblast lineage. This latter hypothesis is supported by our *in vitro* data,
393 since we observed that a reduction of gp130 by about 50% in calvarial
394 osteoblasts cultured *in vitro* reduced *Pth1r* mRNA by nearly 80%. This further
395 suggests that as well as maintaining PTH1R levels in the osteocyte, gp130 may
396 maintain PTH1R expression in throughout the osteoblast lineage.
397
398 Although *Pth1r* levels were low in the femora of *Dmp1Cre.gp130$^{f/f}$* mice, their
399 phenotype is strikingly different to mice with a conditional deletion of PTH1R in
400 osteocytes (Ocy-PPRKO), generated using the same DMP1Cre (Saini, et al. 2013).
401 Ocy-PPRKO mice showed a greater trabecular bone mass than controls with no
402 significant alteration in osteoblast numbers, indicating that the underlying cause
403 of bone fragility in the *Dmp1Cre.gp130$^{f/f}$* mice is not simply low PTH1R
404 expression in the osteocyte. As observed in *Dmp1Cre.gp130$^{f/f}$* mice, Ocy-PPRKO
405 mice failed to reduce *Sost* in response to PTH treatment. However, in direct
406 contrast to *Dmp1Cre.gp130$^{f/f}$* mice, Ocy-PPRKO lacked a *Tnfsf11* response to PTH.

This suggests that the *Dkk1/Sost* and *Tnfsf11/Il6st* responses to PTH occur in different cell populations, and it is only the former that is affected by DMP1Cre-mediated gp130 deletion. Alternatively, the *Dkk1/Sost* induction may require a higher level of PTH1R expression than the *Tnfsf11/Il6st* response; the low level of PTH1R expression in the *Dmp1Cre.gp130$^{f/f}$* mice may be sufficient for the latter.

In addition to mediating the response of osteoblasts to exogenous PTH treatment, PTH1R also acts as a receptor for parathyroid hormone related protein (PTHrP). Although first identified as the mediator of humoral hypercalcemia of malignancy (Suva, et al. 1987), PTHrP is also produced by the osteoblast lineage (Kartsogiannis, et al. 1997; Sackmann 1995). This local PTHrP production is essential for normal osteoblast differentiation, as indicated by studies of an osteoblast-lineage PTHrP null mouse (Miao, et al. 2005). This suggests that basal defects in osteoblast differentiation in our model lacking gp130 in osteocytes may relate specifically to a lack of PTHrP signal. Notably, and in direct contrast to our model, the osteoblast-lineage knockout of PTHrP also exhibited a significant impairment in osteoclastogenesis (Miao et al. 2005), a finding that may relate to the difference in the gene driving expression of the Cre-recombinase. The PTHrP$^{f/f}$ deletion was driven by the Col2.3Cre, which would delete expression in osteocytes, but also in less mature osteoblasts than the DMP1Cre that we have used. Again, this suggests that the PTH-induced expression of RANKL is likely to occur in less mature osteoblasts.

In conclusion, in addition to the recently described role of osteocytic gp130 in maintaining bone formation and strength (Johnson et al. 2014a), the current study has revealed a new role for gp130 in the osteoblast lineage in bone: it is needed to maintain PTH1R expression and to increase osteoblast numbers in response to anabolic PTH treatment.

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Author contributions

Study design: NAS, TS. Study conduct and data collection: TS, RWJ, NEM, IJP, PWMH. Data analysis: TS, RWJ, NEM, IJP, PWMH, NAS. Data interpretation: TS, RWJ, NEM, IJP, PWMH, TJM, NAS. Drafting manuscript: TS and NAS. Revising manuscript content: TS, NAS, RWJ, TJM. All authors approved the final version of manuscript. NAS takes responsibility for the integrity of the data analysis.

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Figure legends

Figure 1. Osteocytic gp130 is required for PTH to increase osteoblast numbers and bone formation in trabecular bone. Male mice were treated with hPTH (1-34) at 30μg/kg/day for 5 weeks. Shown are (A) numbers of osteoblasts / bone perimeter (N.Ob/B.Pm), (B) osteoblast surface / bone surface

(Ob.S/BS) and (C) osteoid surface / bone surface (OS/BS), (D) osteoid thickness (OTh), (E) Double calcein labeled surface (dLS/BS) and (F) mineral apposition rate (MAR) from trabecular bone in the proximal tibial secondary spongiosa in *Dmp1Cre.gp130$^{w/w}$* (gp130 w/w) and *Dmp1Cre.gp130$^{f/f}$* (gp130 f/f) mice. (G) Serum levels of procollagen type 1 amino-terminal propeptide (P1NP) and (H) endogenous murine PTH measured at the end of the treatment protocol are also shown. Bars are mean + SEM, n=8-10 per group. * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, PTH-treated compared to genotype-matched vehicle treated mice.

Figure 2. No effect of intermittent PTH on bone resorption. Male mice were treated with hPTH (1-34) at 30µg/kg/day for 5 weeks. Shown are (A) numbers of osteoclasts per unit bone perimeter (NOc/BPm), (B) osteoclast surface per unit bone surface (OcS/BS) measured in the proximal tibial secondary spongiosa, and (C) serum levels of cross-linked C-terminal telopeptide of type I collagen (CTX1) in PTH and vehicle treated *Dmp1Cre.gp130$^{w/w}$* (gp130 w/w) and *Dmp1Cre.gp130$^{f/f}$* (gp130 f/f) mice. Bars are mean + SEM, n=8-10 per group.

Figure 3. PTH effects on cortical bone are impaired in *Dmp1Cre.gp130$^{f/f}$* mice.

Male mice were treated with hPTH (1-34) at 30µg/kg/day for 5 weeks. Shown are (A) tibial periosteal double-labeled surface (Ps.dLS/BS), periosteal mineral apposition rate (Ps.MAR) in the tibial diaphysis and (C) periosteal perimeter (Ps.Pm) of the femoral diaphysis in PTH and vehicle treated *Dmp1Cre.gp130$^{w/w}$* (gp130 w/w) and *Dmp1Cre.gp130$^{f/f}$* (gp130 f/f) mice. Bars are mean + SEM, n=8-

10 per group. ** p≤ 0.01, ns, p>0.05 (not statistically significant) in PTH-treated compared to genotype-matched vehicle-treated mice. +, p<0.05, vehicle-treated *Dmp1Cre.gp130$^{f/f}$* compared to vehicle-treated *Dmp1Cre.gp130$^{w/w}$*.

Figure 4. PTH effects on Wnt-inhibitor, but not osteoclastogenic, mRNA levels are impaired in *DMP1Cre.gp130$^{f/f}$* mice. RNA was isolated from femurs flushed of bone marrow and expression of PTH target genes was examined by relative quantitative PCR. *Tnfs11* mRNA (A), *Il-6* mRNA (B), *Dkk1* mRNA (C) and *Sost* mRNA (D) in *Dmp1Cre.gp130$^{w/w}$* and *Dmp1Cre.gp130$^{f/f}$* mice treated for five weeks with PTH, collected one hour after the final injection. All values are shown relative to housekeeping (HKG) - the geometric mean of hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and hydroxymethylbilane synthase (*Hmbs*)). Bars are mean + SEM, n=5-8 bones per group, with mRNA prepared and analysed separately. ** p≤ 0.01, *** p≤ 0.001, PTH-treated compared to genotype-matched vehicle treated mice; +, p<0.05, vehicle-treated *Dmp1Cre.gp130$^{f/f}$* compared to vehicle-treated *Dmp1Cre.gp130$^{w/w}$*.

Figure 5. PTH1R expression is reduced in *DMP1Cre.gp130$^{f/f}$* mice and gp130 deficient cultured osteoblasts. (A) *Pth1r* mRNA quantified by qPCR in femurs flushed of bone marrow obtained from untreated 12-week old *Dmp1Cre.gp130$^{w/w}$* and *Dmp1Cre.gp130$^{f/f}$* mice, normalized to *Hmbs;* n=8 samples per group. (B) *gp130* (*Il6st*) and *Pth1r* mRNA levels in primary calvarial osteoblasts obtained from *gp130$^{f/f}$* or C57/BL6 wild type neonates infected with lentiviral Cre-recombinase; levels are shown normalised to beta-2-microglobulin

690 (*B2m*) (n=3 biological replicates). * p≤ 0.05; ** p≤ 0.01, vs gp130 w/w or

691 C57/BL6.

Table 1. Effects of PTH on trabecular and cortical bone in femora from *Dmp1Cre.gp130$^{w/w}$* and *Dmp1Cre.gp130$^{w/w}$* mice.

	Dmp1Cre.gp130$^{w/w}$		***Dmp1Cre.gp130$^{f/f}$***	
	Vehicle (n=9)	PTH (n=10)	Vehicle (n=9)	PTH (n=9)
BV/TV (%)	24.86 ± 0.42	21.54 ± 1.32	17.70 ± 1.55^{+++}	20.61 ± 0.96
Tb.Th (µm)	57.61 ± 1.80	56.49 ± 2.22	57.92 ± 3.01	57.42 ± 3.83
Tb.N (/mm)	4.35 ± 0.15	3.80 ± 0.16	3.03 ± 0.15^{+++}	3.64 ± 0.18
Tb.Sp (µm)	122.71 ± 2.96	144.39 ± 14.04	171.05 ± 15.74^{+++}	158.98 ± 10.37
Ct.Ar (mm^2)	0.60 ± 0.02	0.65 ± 0.02	0.63 ± 0.02	0.67 ± 0.03

Fixed nondemineralized femora from vehicle or PTH-treated mice were analyzed by µCT. Effect of PTH treatment: *, p<0.05 vs *Dmp1Cre.gp130w/w.* Effect of gp130$^{f/f}$ transgene: *** p< 0.001; *, p<0.05 vs *Dmp1Cre.gp130$^{w/w}$* (2-way ANOVA with Šidak multiple comparisons test). BV/TV: bone volume per total volume of the region of interest, Tb. Th: trabecular thickness, Tb. N: trabecular number, Tb.Sp: trabecular separation; Ct.Ar: cortical area.

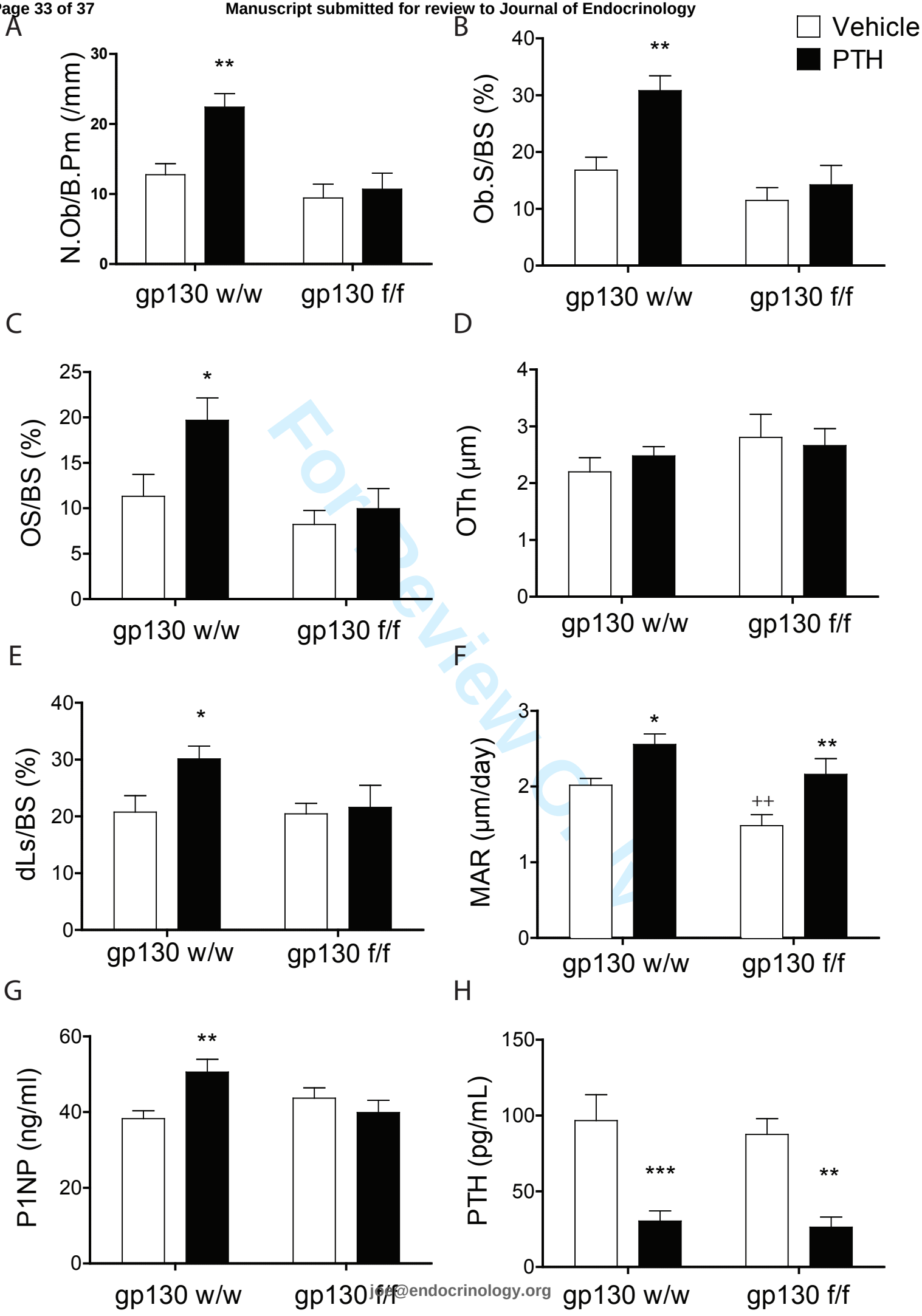

Figure 1

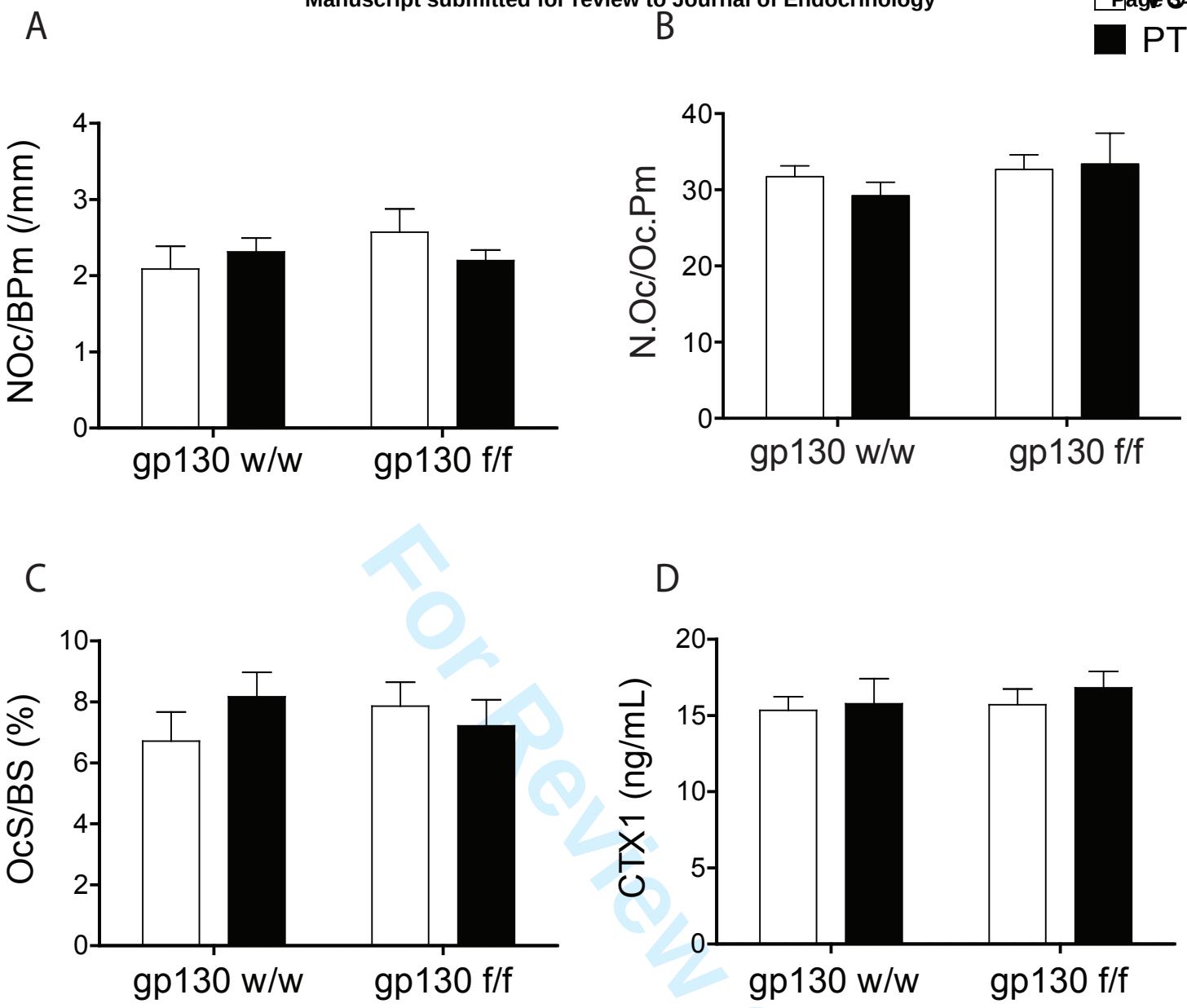

Figure 2

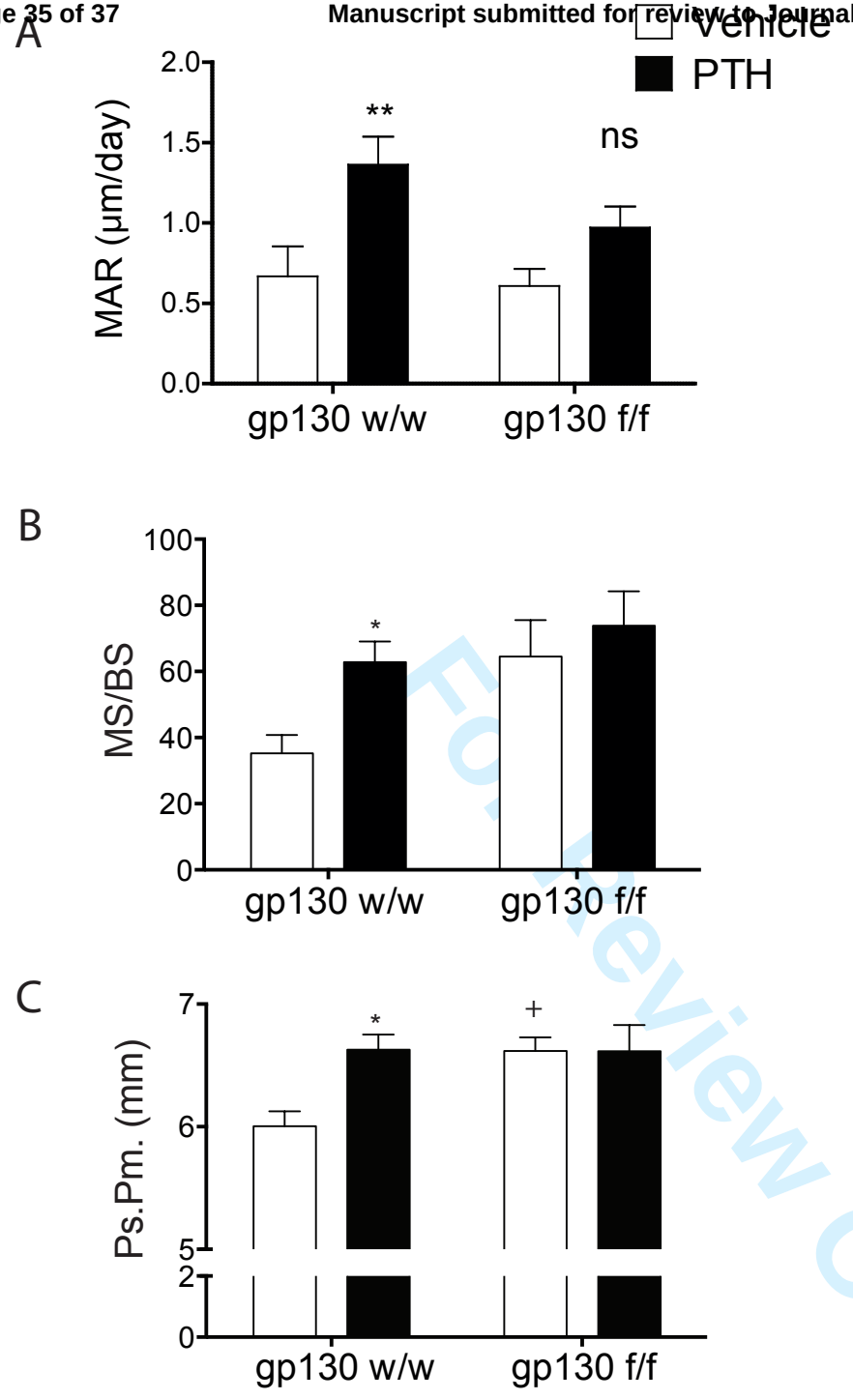

Figure 3

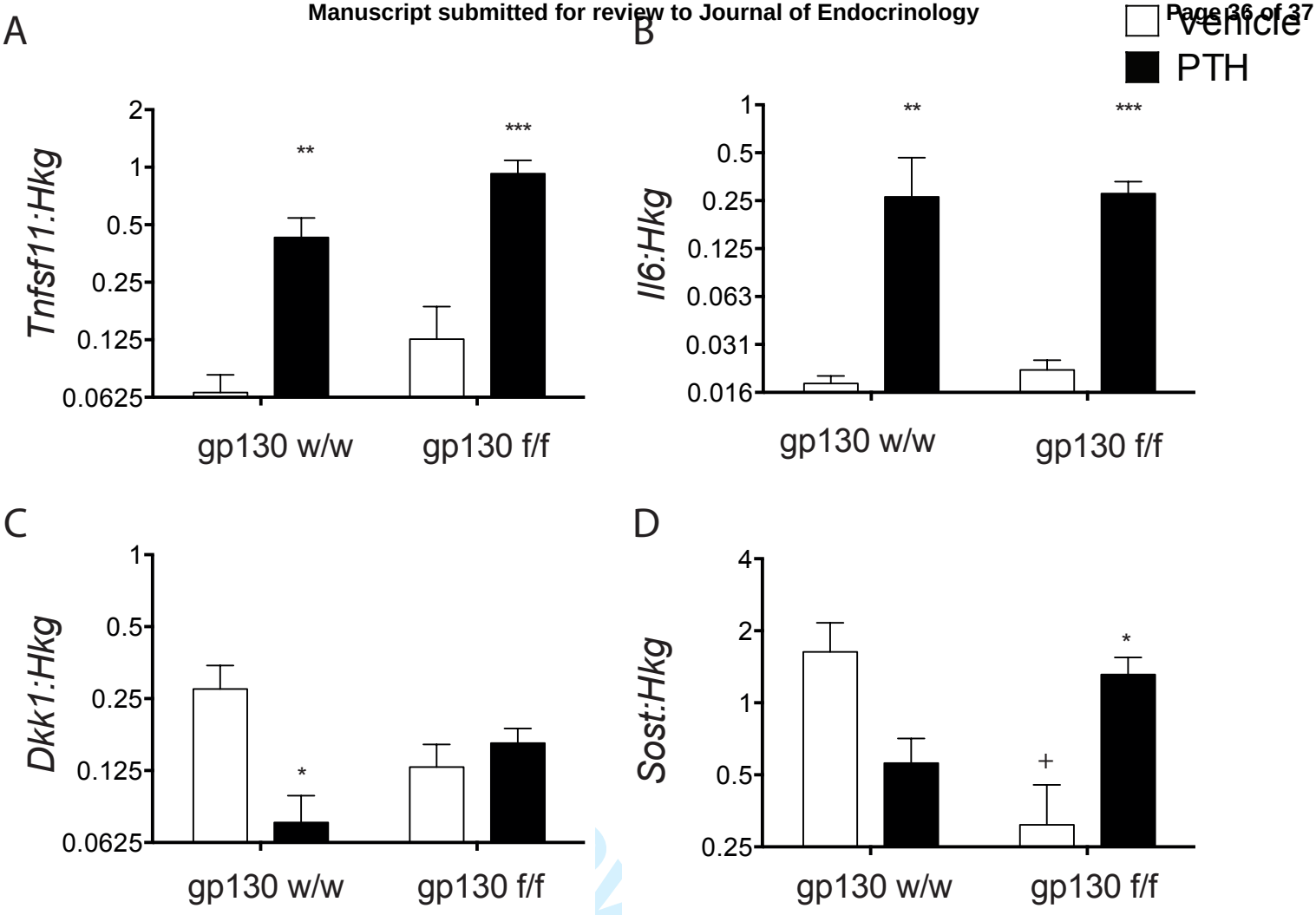

Figure 4

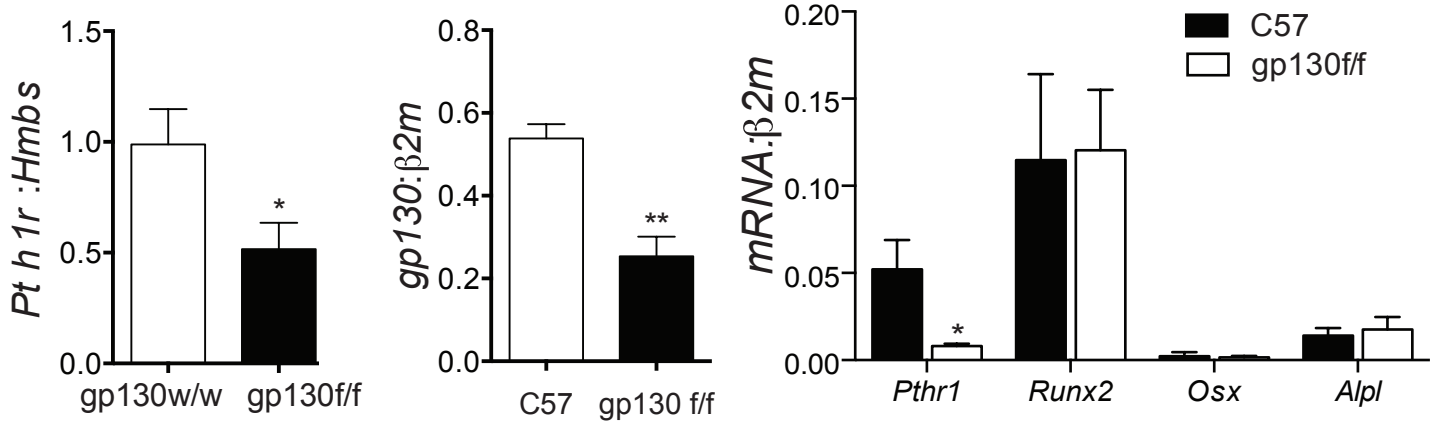

Figure 5