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Yanmei Yang  
*Thomas Jefferson University*

Hong Lei  
*Thomas Jefferson University; Nanjing University of Finance and Economics*

Ya-Wei Qiang  
*University of Arkansas for Medical Sciences*

Bin Wang  
*Thomas Jefferson University*

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# Ixazomib enhances parathyroid hormone–induced $\beta$ -catenin/T-cell factor signaling by dissociating $\beta$ -catenin from the parathyroid hormone receptor

Yanmei Yang<sup>a</sup>, Hong Lei<sup>a,b</sup>, Ya-wei Qiang<sup>c</sup>, and Bin Wang<sup>a,\*</sup>

<sup>a</sup>Center for Translational Medicine, Department of Medicine, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA 19107; <sup>b</sup>College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing 210023, China; <sup>c</sup>Myeloma Institute, University of Arkansas for Medical Sciences, Little Rock, AR 72205

**ABSTRACT** The anabolic action of PTH in bone is mostly mediated by cAMP/PKA and Wnt-independent activation of  $\beta$ -catenin/T-cell factor (TCF) signaling.  $\beta$ -Catenin switches the PTH receptor (PTHr) signaling from cAMP/PKA to PLC/PKC activation by binding to the PTHr. Ixazomib (Izb) was recently approved as the first orally administered proteasome inhibitor for the treatment of multiple myeloma; it acts in part by inhibition of pathological bone destruction. Proteasome inhibitors were reported to stabilize  $\beta$ -catenin by the ubiquitin-proteasome pathway. However, how Izb affects PTHr activation to regulate  $\beta$ -catenin/TCF signaling is poorly understood. In the present study, using CRISPR/Cas9 genome-editing technology, we show that Izb reverses  $\beta$ -catenin–mediated PTHr signaling switch and enhances PTH-induced cAMP generation and cAMP response element–luciferase activity in osteoblasts. Izb increases active forms of  $\beta$ -catenin and promotes  $\beta$ -catenin translocation, thereby dissociating  $\beta$ -catenin from the PTHr at the plasma membrane. Furthermore, Izb facilitates PTH-stimulated GSK3 $\beta$  phosphorylation and  $\beta$ -catenin phosphorylation. Thus Izb enhances PTH stimulation of  $\beta$ -catenin/TCF signaling via cAMP-dependent activation, and this effect is due to its separating  $\beta$ -catenin from the PTHr. These findings provide evidence that Izb may be used to improve the therapeutic efficacy of PTH for the treatment of osteoporosis and other resorptive bone diseases.

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## INTRODUCTION

Long after Bauer and colleagues discovered the anabolic effect of parathyroid hormone (PTH) in 1929 (Bauer *et al.*, 1929), recombinant PTH(1-34) (teriparatide) was approved as the first anabolic agent for the treatment of osteoporosis in the United States in 2002,

and no other anabolic drugs are on the market. The optimal use of PTH therapy in osteoporosis depends in part on our understanding of the regulation of PTH signaling to maintain the anabolic actions of PTH while mitigating its catabolic effects. PTH action in bone is mediated by type 1 PTH receptor (PTHr), a member of the G protein–coupled receptor superfamily. Stimulation of PTHr by PTH on osteoblasts leads to activation of  $G_{\alpha s}$  and  $G_{\alpha q}$ , with consequent induction of cAMP/protein kinase A (PKA) and phospholipase C (PLC)/PKC signaling pathways, which results in both osteoblast formation and osteoclast resorption (Qin *et al.*, 2004; Cheloha *et al.*, 2015). Whereas anabolic PTH effects in bone are mediated mostly through the cAMP/PKA signaling pathway, PLC/PKC signaling has been shown to be inhibitory to the osteoanabolic actions of PTH (Ogata *et al.*, 2011). Wnt/ $\beta$ -catenin signaling controls bone formation and homeostasis by increasing osteoblast differentiation and inhibiting osteoclastogenesis (Gaur *et al.*, 2005; Glass *et al.*, 2005;

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\*Address correspondence to: Bin Wang ([bin.wang@jefferson.edu](mailto:bin.wang@jefferson.edu)).

Abbreviations used: CREB, cAMP response element–binding protein; CRE-luc, cAMP response element–luciferase; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; GST, glutathione-S-transferase; Izb, ixazomib; PKA, protein kinase A; PLC, phospholipase C; PTH, parathyroid hormone; PTHr, PTH receptor; TCF, T-cell factor.

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Liu *et al.*, 2015). Our data and those from other groups show that  $\beta$ -catenin interacts with the PTHR and switches the PTHR signaling from  $G\alpha s/cAMP$  to  $G\alpha q/PLC$  activation (Yano *et al.*, 2013; Yang and Wang, 2015). There is cross-talk between PTHR and frizzled receptor to regulate PTH action in bone (Wan *et al.*, 2008; Romero *et al.*, 2010; Revollo *et al.*, 2015). The low-density lipoprotein receptor-related protein 6 (LRP6), one of frizzled's coreceptors, is a key element of the PTH signaling for regulating osteoblast activity (Wan *et al.*, 2008; Revollo *et al.*, 2015). PTHR directly interacts with Dishevelled to regulate  $\beta$ -catenin signaling and osteoclast differentiation (Romero *et al.*, 2010). PTH facilitates canonical Wnt signaling via inactivation of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) in osteoblasts to inhibit  $\beta$ -catenin sequestration (Suzuki *et al.*, 2008). These findings highlight the role of  $\beta$ -catenin/T-cell factor (TCF) signaling in anabolic action of PTH in bone. It is unclear how  $\beta$ -catenin directly contributes to the osteoanabolic action of PTH.

$\beta$ -Catenin is a multifunctional protein and does not contain a transmembrane domain (Nelson and Nusse, 2004; Stepniak *et al.*, 2009; Marie *et al.*, 2014). Cadherins, which have a single-pass transmembrane domain, bind to  $\beta$ -catenin through their carboxyl-terminal domain at the cytoplasmic tail. Thus  $\beta$ -catenin serves as a link between cadherins and the actin cytoskeleton to mediate the role of cadherins in cell adhesion (Nelson and Nusse, 2004; Stepniak *et al.*, 2009; Marie *et al.*, 2014). In addition,  $\beta$ -catenin also binds to numerous other proteins in a cadherin-independent manner (Bienz and Clevers, 2003; Yano *et al.*, 2013; Yang and Wang, 2015). In bone, N-cadherin associates with  $\beta$ -catenin at the cell membrane to regulate osteoblastogenesis by limiting Wnt signaling. Revollo *et al.* (2015) reported that N-cadherin modulated LRP6-PTHR interaction, restrained the intensity of PTH-induced  $\beta$ -catenin signaling, and reduced bone formation in response to intermittent PTH administration. Moreover, N-cadherin restrains PTH's repressive effects on sclerostin/SOST by regulating LRP6-PTHR interaction (Yang *et al.*, 2016). The interaction of N-cadherin with  $\beta$ -catenin causes increased cadherin abundance on the cell surface and results in  $\beta$ -catenin sequestration at the plasma membrane (Stepniak *et al.*, 2009; Marie *et al.*, 2014). The outcome reduces  $\beta$ -catenin nuclear translocation and decreases TCF/lymphoid enhancer factor-dependent transcriptional activity. Accordingly, blockade of  $\beta$ -catenin degradation may enhance PTH stimulation of  $\beta$ -catenin/TCF signaling.

The ubiquitin-proteasome pathway plays an important role in regulating and controlling bone metabolism (Murray *et al.*, 1998; Garrett *et al.*, 2003; Yang *et al.*, 2015). The first-generation proteasome inhibitor bortezomib has been used as an effective therapy for the treatment of multiple myeloma, a disease characterized by an increase in the activity of osteoclasts and a decrease in the function of osteoblasts adjacent to tumor cells in the bone marrow (Pennisi *et al.*, 2009). Carfilzomib, a next-generation selective proteasome inhibitor, exhibits potent antimyeloma efficacy compared with bortezomib (Herndon *et al.*, 2013; Berenson *et al.*, 2014). We previously reported that carfilzomib suppressed PTH stimulation of osteoclast differentiation and bone-resorptive activity to mitigate the catabolic effects of PTH (Yang *et al.*, 2015). A significant limitation of treatment with bortezomib and carfilzomib is that both drugs are administered intravenously or subcutaneously, which is inconvenient for patients and increases treatment cost. Ixazomib (Izb) is a small-molecule proteasome inhibitor that overcomes these limitations. In November 2015, Izb became the first orally administered proteasome inhibitor approved in the United States as an effective therapy for multiple myeloma. This effect is mediated in part through inhibition of pathologic bone destruction (Kupperman *et al.*, 2010; Garcia-Gomez *et al.*, 2014; Muz *et al.*, 2016). Proteasome inhibitors such as bortezomib

and carfilzomib directly stabilize  $\beta$ -catenin protein and increase free cytosolic  $\beta$ -catenin and  $\beta$ -catenin nuclear translocation (Qiang *et al.*, 2009; Hu *et al.*, 2013). However, how proteasome inhibition regulates the complex interplay between PTHR and  $\beta$ -catenin to in turn regulate  $\beta$ -catenin/TCF signaling has not been elucidated.

In the present study, we show that Izb reverses the  $\beta$ -catenin-mediated PTHR signaling switch by enhancing PTH-induced cAMP formation and cAMP response element-luciferase (CRE-luc) reporter gene activity and reducing PTH-stimulated intracellular calcium mobilization in osteoblasts. Izb enhances PTH-induced  $\beta$ -catenin/TCF signaling by separating  $\beta$ -catenin from the PTHR and promoting  $\beta$ -catenin translocation.

## RESULTS

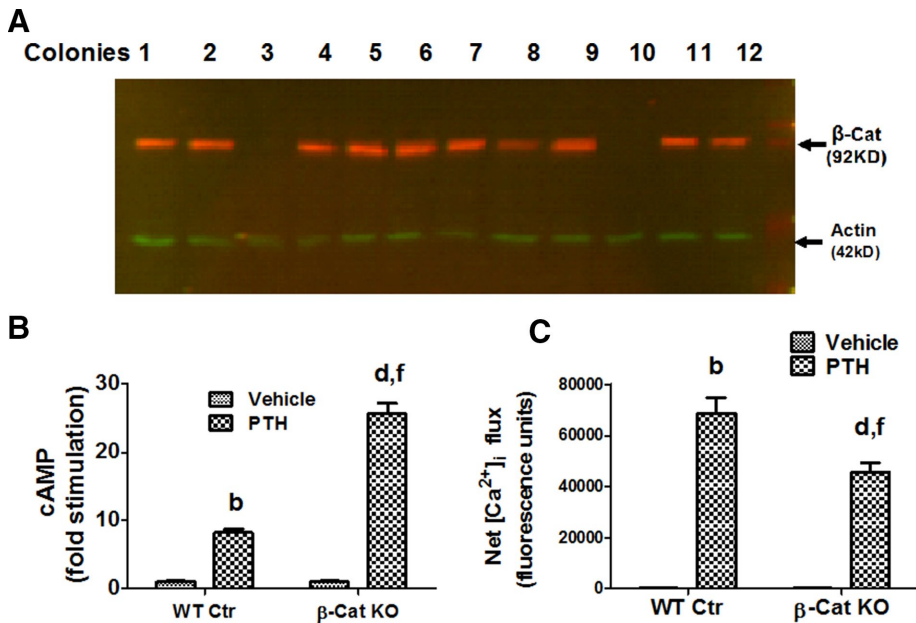
### Knockout of $\beta$ -catenin increases PTH-induced cAMP formation and reduces intracellular calcium in osteoblasts

Recent findings show that  $\beta$ -catenin switches the PTHR signaling by binding to the intracellular carboxyl-terminal region of the PTHR in both chondrocytes and HEK293 cells (Yano *et al.*, 2013; Yang and Wang, 2015). In an initial set of experiments, we examined whether  $\beta$ -catenin also switched the PTHR signaling in osteoblasts. Saos2 osteoblastic cells endogenously express both  $\beta$ -catenin and the PTHR (Wang *et al.*, 2009). These cells were transfected with a clustered regularly interspaced short palindromic repeats (CRISPR) construct or CRISPR control plasmid. After 48 h of transfection, the cells were transferred into a 96-well plate with one cell per well by a serial dilution. Two single colonies were identified for knockout of  $\beta$ -catenin expression in Saos2 cells (hereafter referred to as Saos2- $\beta$ -Cat-KO-3 and Saos2- $\beta$ -Cat-KO-10 cells; Figure 1A), whereas no  $\beta$ -catenin expression was reduced in cells transfected with control plasmid (Saos2- $\beta$ -Cat-Ctr; Supplemental Figure S1).

To assess the effect of  $\beta$ -catenin on PTH-induced  $G\alpha s/cAMP$  signaling, we conducted PTH stimulation of cAMP generation in Saos2- $\beta$ -Cat-KO-3 cells ( $\beta$ -Cat KO) and their control cells (Saos2- $\beta$ -Cat-Ctr-1, WT Ctr). Knockout of  $\beta$ -catenin significantly increased PTH(1-34) (hereafter referred to as PTH) stimulation of cAMP formation (Figure 1B). To evaluate PTHR-mediated  $G\alpha q/PLC$  signaling, we measured intracellular calcium mobilization ( $[Ca^{2+}]_i$ ), an index of PLC activity, in Saos2- $\beta$ -Cat-KO-3 cells and Saos2- $\beta$ -Cat-Ctr-1 cells loaded with the calcium-sensitive dye Fluo-4 AM. Knockout of  $\beta$ -catenin markedly inhibited PTH-induced  $[Ca^{2+}]_i$  (Figure 1C). Similar results also occurred in Saos2- $\beta$ -Cat-KO-10 and Saos2- $\beta$ -Cat-Ctr-2 cells (unpublished data). Collectively these data clearly demonstrate that knockout of  $\beta$ -catenin reverses the PTHR signaling switch to increase  $G\alpha s/cAMP$  signaling and reduce  $G\alpha q/PLC$  activation, which favors the anabolic PTH action in bone.

### Izb enhances PTH-induced cAMP formation in a time- and concentration-dependent manner

We previously reported that proteasome inhibitors stabilized  $\beta$ -catenin by the ubiquitin-proteasome pathway (Qiang *et al.*, 2009; Hu *et al.*, 2013). Because  $\beta$ -catenin switches PTHR signaling, we next determined whether Izb regulated PTHR activation. Izb alone was not able to stimulate cAMP formation (Supplemental Figure S2). However, pretreatment of Izb enhanced PTH stimulation of cAMP formation in Saos2 cells, which exhibited time and concentration dependence. Maximal stimulation by Izb was achieved at 3 h (Figure 2A), but prolonged Izb treatment tended to reduce its effect on PTH-induced cAMP formation. At the 3 h, Izb elicited a concentration-dependent increase in PTH-induced cAMP formation over the range 12.5–100 nM (Figure 2B), but higher concentrations of Izb also decreased its effect on PTH stimulation of cAMP production.



**FIGURE 1:** Knockout of  $\beta$ -catenin switches the PTHR signaling from  $G\alpha_q$ /PLC to  $G\alpha_s$ /cAMP activation. Saos2 cells in a six-well plate were transfected with CRISPR construct or control plasmid. After 48 h, the cells, which expressed only GFP reporter as visualized using an Evos machine, were transferred into a 96-well plate with one cell per well. (A) After  $\sim 3$  wk, two single colonies (Saos2- $\beta$ -Cat-KO-3 and Saos2- $\beta$ -Cat-KO-10 cells) were identified for knockout of  $\beta$ -catenin expression in Saos2 cells by Western blotting. Immunoblotting was performed with primary antibodies of mouse monoclonal  $\beta$ -catenin ( $\beta$ -Cat) antibody plus rabbit polyclonal actin antibody and then with secondary antibodies (goat anti-mouse antibody [red] plus goat anti-rabbit antibody [green]). Actin was used for loading control. (B, C) Saos2- $\beta$ -Cat-KO-3 cells ( $\beta$ -Cat KO) and control Saos2- $\beta$ -Cat-Ctr-1 cells (WT Ctr) were set up in a 24- or 96-well plate. After 24 h of culture, cells were serum starved overnight. PTH (100 nM) stimulation of intracellular cAMP accumulation (B) or PTH (1  $\mu$ M) induction of intracellular calcium (C) was measured as described in *Materials and Methods*. Data are summarized as mean  $\pm$  SE of triplicate measurements.  $n = 4$ . <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with WT Ctr cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with WT Ctr cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with  $\beta$ -Cat KO cells treated with vehicle.

Similar to other proteasome inhibitors (Qiang *et al.*, 2009; Hu *et al.*, 2013), lzb but not PTH caused slight cytotoxicity when the cells were treated with it for a prolonged time or at higher concentrations (Supplemental Figure S3, A–C). To reduce the cytotoxicity of lzb, we pretreated Saos2 cells with lzb or vehicle for the first 3 h, followed by an additional 5 h of culture in lzb-free medium, which mimics the pulse treatment used in the clinic and avoids unselective cytotoxicity of proteasome inhibition (Boissy *et al.*, 2008). As expected, this regimen of lzb not only enhanced PTH stimulation of cAMP formation, but it also reduced its cytotoxicity (Figure 2, C and D). To verify these results, we also found that lzb enhanced PTH-induced cAMP generation in mouse primary osteoblasts (Supplemental Figure S4).

### lzb promotes PTH stimulation of cAMP formation by facilitating the dissociation of $\beta$ -catenin from the PTHR

There are different cellular pools of  $\beta$ -catenin in the plasma membrane, cytosol, and nucleus. In most cells, the majority of  $\beta$ -catenin is located at the plasma membrane in a complex with cadherins or other proteins (Stepniak *et al.*, 2009; Marie *et al.*, 2014). Previous findings reported that both bortezomib and carfilzomib increased free and active forms of  $\beta$ -catenin in Saos2 cells (Qiang *et al.*, 2009; Hu *et al.*, 2013). We used an affinity-based E-cadherin–glutathione-S-transferase (GST) pull-down assay to separate the free  $\beta$ -catenin from the pool of membrane-bound  $\beta$ -catenin. Saos2 cells were treated

with lzb, carfilzomib, or vehicle for the first 3 h, followed by an additional 5 h of culture in lzb- or carfilzomib-free medium. lzb and carfilzomib exhibited increases in the free cytosolic  $\beta$ -catenin level in a concentration-dependent manner (Figure 3A). In addition, lzb increased nuclear  $\beta$ -catenin expression (Figure 3B). An independent experiment performed by confocal microscopy showed that lzb induced  $\beta$ -catenin translocation in Saos2 cells, as shown by its accumulation in cytosol and nucleus (Supplemental Figure S5).

The PTHR is a seven-transmembrane domain protein, whereas  $\beta$ -catenin does not contain any transmembrane domain in its structure. Because lzb increases active forms of  $\beta$ -catenin and promotes  $\beta$ -catenin translocation, we asked whether lzb was able to separate  $\beta$ -catenin from the PTHR at the plasma membrane. Saos2 cells were transfected with pCDNA3.1 vector, hemagglutinin (HA)-PTH, and/or Flag- $\beta$ -catenin as indicated. After 48 h of transfection, the cells were treated with vehicle or lzb (100 nM) for 3 h, followed by an additional 5 h of culture in lzb-free medium. The membrane proteins were isolated, and the interaction of Flag- $\beta$ -catenin with HA-PTH was performed by immunoprecipitation assay. The result in Figure 3C show that treatment with lzb reduced the interaction of  $\beta$ -catenin with the PTHR at the plasma membrane, further confirming that lzb promotes  $\beta$ -catenin translocation to cytosol and nucleus. As a complementary experiment, Saos2 cells were transiently transfected with green fluorescent protein (GFP)-PTH. After 48 h of transfection, the cells were treated with vehicle or lzb as before. There was a colocalization of  $\beta$ -catenin with the PTHR at the plasma membrane in the absence of lzb, whereas lzb reduced this colocalization (Figure 3D). Collectively lzb enhanced PTH-induced cAMP generation due to the separation of  $\beta$ -catenin from the PTHR.

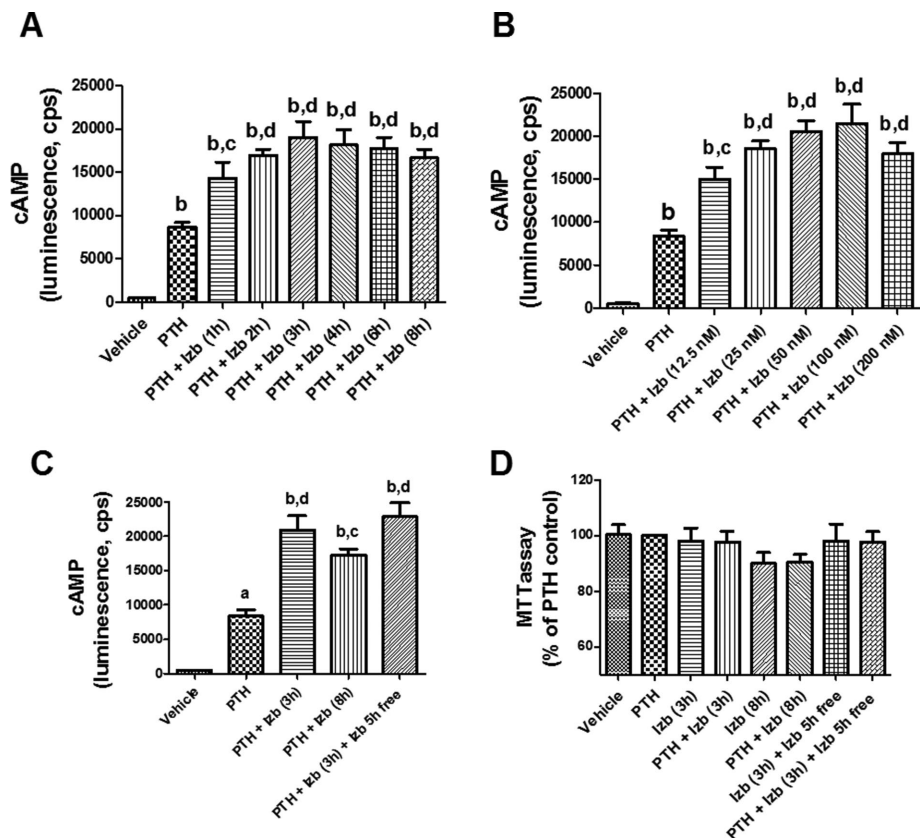
### lzb regulation of the PTHR signaling is $\beta$ -catenin dependent

To test whether the specificity of lzb effect on PTH-induced cAMP production is due to separation of  $\beta$ -catenin from the PTHR, we used both wild-type and  $\beta$ -catenin-knockout Saos2 cells. Indeed, lzb failed to enhance PTH-induced cAMP formation and reduce intracellular calcium mobilization in  $\beta$ -catenin-knockout cells compared with that of wild-type Saos2 control cells (Figure 4).

### lzb promotes PTH-induced CRE-luc activity and cAMP response element-binding phosphorylation

We next determined the effect of lzb on cAMP/PKA downstream signaling in osteoblasts. The active catalytic subunit of PKA stimulates CRE-luc activity (Castellone *et al.*, 2005). Wild-type Saos2 control cells or Saos2- $\beta$ -Cat-KO cells were infected with lentiviral particles containing CRE-luc reporter gene. The cells were pretreated with lzb (100 nM for 1 h) before PTH (100 nM) was added to the culture for another 2 h. The CRE-luc activity was then measured. As shown in Figure 5A, lzb itself was not able to stimulate CRE-luc activity in wild-type control or  $\beta$ -catenin-knockout cells. However,





**FIGURE 2:** Izb enhances PTH-induced cAMP formation in a time- and concentration-dependent manner. (A) Time course of Izb on PTH-stimulated cAMP formation. Saos2 cells were seeded onto a six-well plate. After 90% confluence, the cells were transfected with Glosensor cAMP reporter plasmid (pGS-22F) using Lipofectamine 2000. At 36 h after transfection, cells were transferred into a 96-well plate and cultured for 14 h. The cells were serum starved overnight and then treated with vehicle or Izb (100 nM) for the indicated times, and PTH-induced cAMP formation was measured. The peak response time for cAMP formation is 14 min. (B) Concentration response of Izb on PTH-stimulated cAMP formation. At 3 h, Izb (12.5–100 nM) concentration dependently increased PTH stimulation of cAMP formation. (C) Pulse treatment of Izb increases of PTH-stimulated cAMP formation. Saos2 cells were treated with Izb (100 nM) for 8 h or for the first 3 h followed by an additional 5 h of culture in Izb-free medium in Saos2 cells. (D) Pulse treatment of Izb reduces cytotoxicity. Saos2 cells were treated with Izb (100 nM) for 8 h or for the first 3 h followed by an additional 5 h of culture in Izb-free medium in the presence of PTH (100 nM) in Saos2 cells. Cytotoxicity was assessed by MTT assay as described in *Materials and Methods*. Data are summarized as mean  $\pm$  SE of triplicate measurements.  $n = 3$ .  $^a p < 0.05$ ,  $^b p < 0.01$ , compared with cells treated with vehicle;  $^c p < 0.05$ ,  $^d p < 0.01$ , compared with cells treated with PTH.

knockout of  $\beta$ -catenin significantly increased PTH-induced CRE-luc activity. Izb failed to enhance PTH stimulation of CRE-luc activity in  $\beta$ -catenin-knockout cells compared with wild-type control cells.

cAMP response element-binding (CREB) protein signaling plays a key role in regulating osteoblast activity and bone formation (Long *et al.*, 2001). PTH can induce phosphorylation of CREB through the cAMP/PKA signaling pathway (Pearman *et al.*, 1996; Revollo *et al.*, 2015). Saos2 wild-type cells were pretreated with Izb (100 nM for 2 h) before PTH (100 nM) was added to the culture for another 1 h. PTH markedly induced CREB phosphorylation (Figure 5B). Izb by itself had no effect on phosphorylation of CREB but facilitated PTH stimulation of CREB phosphorylation. Total CREB abundance was unaffected in each group. PKA inhibitor H89 or PKC inhibitor bisindolylmaleimide I (Bis I) alone had no effect on CREB phosphorylation (Supplemental Figure S6). However, H89 but not Bis I blocked Izb enhancement of PTH-induced CREB phosphorylation (Figure 5B). In addition, PTH significantly stimulated CREB phosphorylation in  $\beta$ -catenin-knockout

cells, and this change was not affected by Izb in these cells (Figure 5C), further confirming that effects of Izb on PTH downstream events are  $\beta$ -catenin dependent and mediated by the cAMP/PKA signaling pathway.

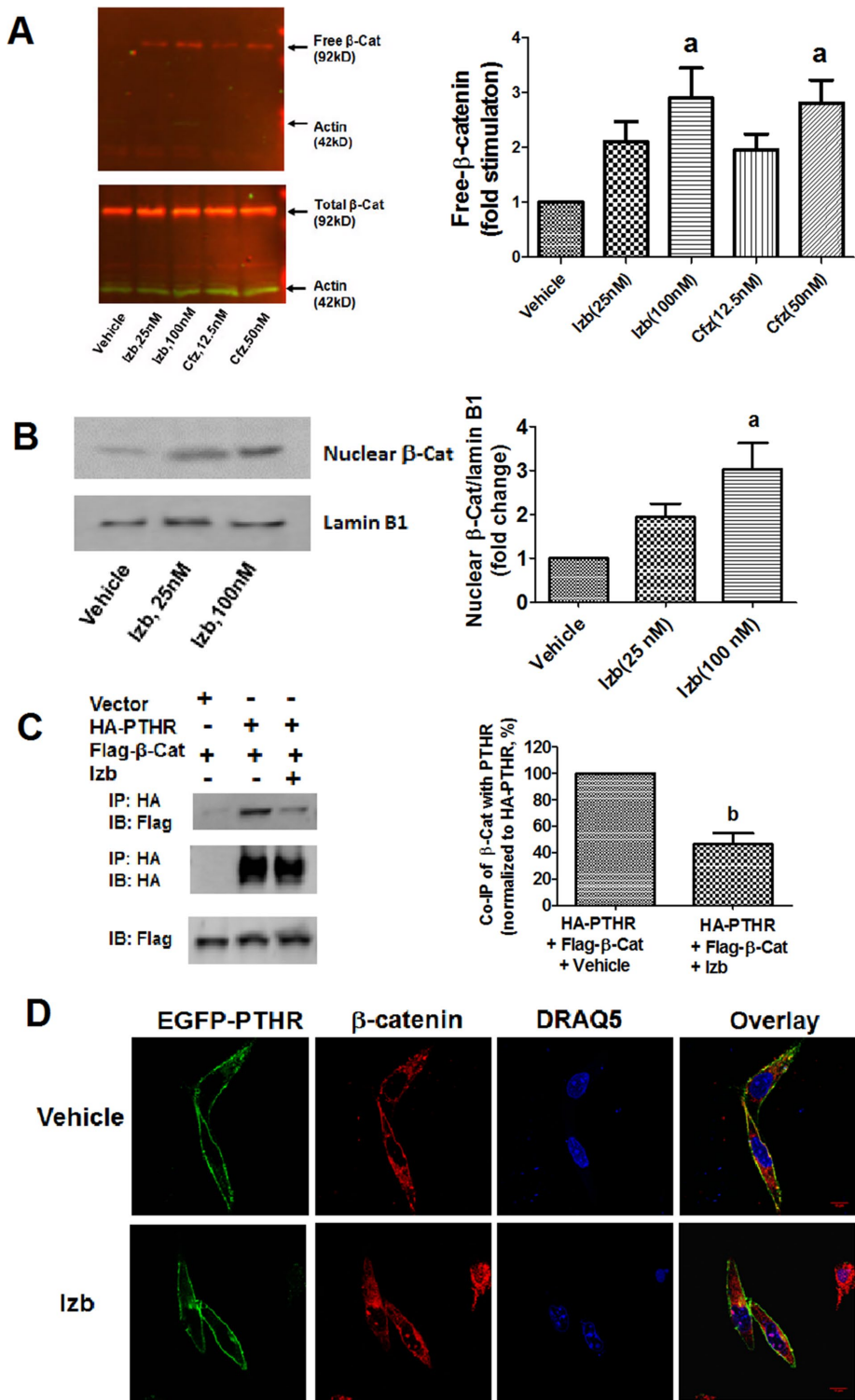
### Izb enhances PTH stimulation of GSK3 $\beta$ phosphorylation at Ser-9 and $\beta$ -catenin phosphorylation at Ser-675

The stability of  $\beta$ -catenin is regulated by a multiprotein complex, which includes adenomatous polyposis coli, GSK3 $\beta$ , and axin. PKA phosphorylates GSK3 $\beta$  at the amino acid Ser-9, which inhibits its kinase activity and then inactivates the  $\beta$ -catenin destruction complex (Castellone *et al.*, 2005; Suzuki *et al.*, 2008). PTH induces  $\beta$ -catenin/TCF signaling via inactivation of GSK3 $\beta$  in osteoblasts (Suzuki *et al.*, 2008). Saos2 cells were pretreated with Izb (100 nM) for 2.5 h, and PTH (100 nM) was then added for another 30 min. GSK3 $\beta$  functions upstream of  $\beta$ -catenin activation. As expected, Izb by itself has no effect on GSK3 $\beta$  phosphorylation at Ser-9 (Figure 6A). PTH treatment increased GSK3 $\beta$  phosphorylation, and Izb further enhanced PTH stimulation of GSK3 $\beta$  phosphorylation.

PKA-phosphorylated  $\beta$ -catenin at Ser-675 increases  $\beta$ -catenin accumulation in the cytosol, thereby promoting  $\beta$ -catenin downstream signaling (Taurin *et al.*, 2006; Revollo *et al.*, 2015). We hypothesized that Izb enhanced PTH-induced phosphorylated  $\beta$ -catenin at this site. To test this idea, we pretreated Saos2 cells with Izb for 1 h. Then we added 100 nM PTH to culture for another 2 h. The data in Figure 6B show that PTH stimulated  $\beta$ -catenin phosphorylation at Ser-675. Izb but not PTH slightly increased total  $\beta$ -catenin abundance. However, Izb and PTH synergistically increased  $\beta$ -catenin (Ser-675) phosphorylation, although Izb on its own had no notable effect on the phosphorylation of  $\beta$ -catenin.

### Izb promotes PTH-induced $\beta$ -catenin/TCF signaling in osteoblasts

PTH-facilitated  $\beta$ -catenin/TCF signaling is cAMP/PKA dependent (Kulkarni *et al.*, 2005; Suzuki *et al.*, 2008). Our data show that Izb enhanced PTH-induced cAMP formation and promoted PTH stimulation of GSK3 $\beta$  and  $\beta$ -catenin phosphorylation. These findings suggest an additional mechanism by which Izb enhances PTH-induced  $\beta$ -catenin/TCF signaling in osteoblasts. To test this idea, we transfected Saos2 cells with TOPflash or FOPflash plasmid. The cells were treated with Izb for the first 3 h, followed by an additional 5 h culture in Izb-free medium in the presence or absence of PTH for 8 h. After treatment, the TOPflash and FOPflash reporter activities were measured. Both bortezomib and carfilzomib induce osteoblast differentiation via Wnt-independent induction of  $\beta$ -catenin translocation and activation of  $\beta$ -catenin/TCF signaling (Qiang *et al.*, 2009; Hu *et al.*, 2013). As we predicted, Izb by itself stimulated nuclear  $\beta$ -catenin expression and  $\beta$ -catenin/TCF signaling (Figure 7, A and B). Izb and PTH



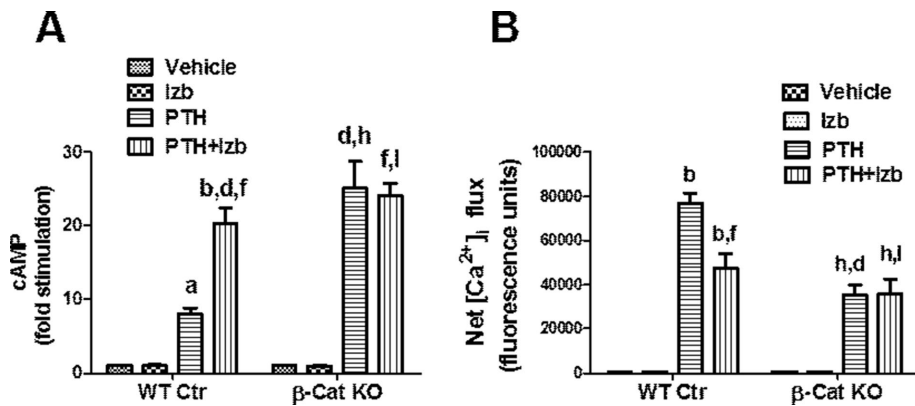
**FIGURE 3:** Izb facilitates the separation of  $\beta$ -catenin from PTHR. (A) Izb increases free  $\beta$ -catenin protein levels. Left, top, Saos2 cells treated with vehicle, Izb (25, 100 nM), or carfilzomib (Cfz, 12.5, 50 nM) for the first 3 h followed by an additional 5 h of culture in Izb- or Cfz-free medium. GST-E-cadherin was bound to free  $\beta$ -Cat, and the complexes were purified using GST beads. Immunoblotting was then performed with primary antibodies of mouse monoclonal  $\beta$ -Cat antibody plus rabbit polyclonal actin antibody and then with secondary antibodies (goat anti-mouse antibody and goat anti-rabbit antibody). Lack of actin expression suggests that the beads were washed completely. Left, bottom, immunoblotting conducted with total lysates with the same primary antibodies and secondary antibodies. Right, quantified free  $\beta$ -catenin levels in three independent experiments presented as mean  $\pm$  SE. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle. (B) Izb increases nuclear  $\beta$ -catenin expression. Saos2 cells were treated

synergistically increased nuclear  $\beta$ -catenin levels and  $\beta$ -catenin/TCF activation. Similar results were also identified in mouse osteoblasts (Supplemental Figure S7). PKA inhibitor H89 but not PKC inhibitor Bis I inhibited PTH-stimulated TCF reporter activity (Figure 7B), confirming that PTH-stimulated  $\beta$ -catenin/TCF signaling is cAMP/PKA dependent. However, H89 only partially reversed the effect of Izb on PTH stimulation of  $\beta$ -catenin/TCF activation. Furthermore, H89 failed to affect Izb increase of free cytosolic  $\beta$ -catenin level, nuclear  $\beta$ -catenin expression (Supplemental Figure S8, A and B), and  $\beta$ -catenin/TCF signaling (Supplemental Figure S8D). H89 also had no effect on  $\beta$ -catenin phosphorylation at Ser-675 in the presence of Izb (Supplemental Figure S8C). These data clearly demonstrate that the effect of Izb alone on  $\beta$ -catenin translocation and  $\beta$ -catenin/TCF signaling is not related to a cAMP/PKA pathway. Together the results show that, in addition to being capable of promoting  $\beta$ -catenin translocation, Izb enhances PTH stimulation of  $\beta$ -catenin/TCF signaling via an increase in cAMP-dependent signaling.

## DISCUSSION

Although PTHR signaling pathways are being studied in increasing detail, the regulation of PTHR functions has not been fully elucidated. The anabolic PTH effects on bone are mostly mediated by the  $G_{\alpha s}$ /cAMP signaling pathway, whereas  $G_{\alpha q}$ /PLC activation may antagonize these osteoanabolic actions (Datta and Abou-Samra, 2009;

with vehicle or Izb as in A. Left, nuclear proteins were prepared and  $\beta$ -catenin expression analyzed by immunoblotting. Right, quantified nuclear  $\beta$ -catenin levels in three independent experiments presented as mean  $\pm$  SE. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle. (C) Saos2 cells were transfected with pCDNA3.1 vector, HA-PTHR, and/or Flag- $\beta$ -Cat as indicated. After 48 h of transfection, the cells were treated with vehicle or Izb (100 nM) as before. Left, the plasma membrane proteins were isolated, and the interaction of Flag- $\beta$ -Cat with HA-PTHR measured. Right, coIP of  $\beta$ -catenin with PTHR in three independent experiments normalized to HA-PTH band. <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle. (D) Saos2 cells were transfected with GFP-PTH. After 48 h, the cells were treated with vehicle or Izb (100 nM) as before. The cells were fixed, stained, and visualized for colocalization of PTHR with  $\beta$ -catenin by confocal microscopy. Representative of three independent experiments performed with similar results. Scale bar, 10  $\mu$ m.



**FIGURE 4:** Izb blocks  $\beta$ -catenin–mediated PTHR signaling switch. Saos2- $\beta$ -Cat-KO-3 cells ( $\beta$ -Cat KO) and their control cells (Saos2- $\beta$ -Cat-Ctr-1, WT Ctr) were seeded onto a 24- or 96-well plate. After confluence, the cells were serum starved overnight and then pretreated with Izb (100 nM) for 3 h. PTH stimulation of cAMP formation (A) and intracellular calcium (B) were measured. Data are summarized as mean  $\pm$  SE of triplicate measurements.  $n = 3$ . <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with WT Ctr cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with WT Ctr cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with WT Ctr cells treated with Izb; <sup>g</sup> $p < 0.05$ , <sup>h</sup> $p < 0.01$ , compared with  $\beta$ -Cat KO cells treated with vehicle; <sup>i</sup> $p < 0.05$ , <sup>j</sup> $p < 0.01$ , compared with  $\beta$ -Cat KO cells treated with PTH; <sup>k</sup> $p < 0.05$ , <sup>l</sup> $p < 0.01$ , compared with  $\beta$ -Cat KO cells treated with Izb.

Ogata *et al.*, 2011). Additional anabolic signaling by PTH action involves the canonical  $\beta$ -catenin pathway, which is independent of Wnt. Therefore manipulating PTHR signaling by shifting  $G\alpha_q$ /PLC to  $G\alpha_s$ /cAMP activation and increasing  $\beta$ -catenin/TCF signaling represents a means of maintaining the anabolic actions of PTH.

Sequences located at the carboxyl terminus of PTHR control its signaling (Datta and Abou-Samra, 2009; Romero *et al.*, 2011). PTHR has a PSD-95/Discs large/ZO-1 (PDZ) motif located in the last four amino acids, which interact with NHERF1 and NHERF2 (Romero *et al.*, 2011; Vilardaga *et al.*, 2011). As originally described by Mahon *et al.* (2002), NHERF2, a NHERF1 homologue, markedly inhibits adenylyl cyclase by stimulating inhibitory  $G\alpha_i$  proteins in PS120 cells transfected with the PTHR. In contrast, no differences of PTH-stimulated cAMP formation were noted between wild-type and NHERF1-null proximal tubule cells (Cunningham *et al.*, 2005) in the presence or absence of NHERF1. In ROS17/2.8 cells or primary osteoblasts, NHERF1 increases PTH-stimulated cAMP accumulation (Wheeler *et al.*, 2008; Wang *et al.*, 2013). Moreover, truncation of the carboxyl-terminal region of the PTHR that lacks determinants for stable  $\beta$ -arrestin association (PTHR-480stop) enhances PTH stimulation of adenylyl cyclase but not PLC (Iida-Klein *et al.*, 1995; Wang *et al.*, 2007). In contrast to the aforementioned regulatory proteins,  $\beta$ -catenin specifically binds to the intracellular carboxyl-terminal region of the PTHR and switches the PTHR signaling from  $G\alpha_s$ /cAMP to  $G\alpha_q$ /PLC activation in chondrocytes and HEK293 cells (Yano *et al.*, 2013; Yang and Wang, 2015). As an initial step, we determined whether this signaling switch by  $\beta$ -catenin also occurred in osteoblasts. To that end, we generated a  $\beta$ -catenin–knockout cell line in osteoblasts using CRISPR/Cas9 genome-editing technology. Our data showed that knockout of  $\beta$ -catenin (*i.e.*, zero expression of  $\beta$ -catenin) significantly increased PTH-induced cAMP formation, whereas PTH-stimulated PLC activity was markedly reduced in these knockout cells compared with cells transfected with CRISPR/Cas9 control plasmid (Figure 1). These data are consistent with a previous study, using small interfering RNA, demonstrating that knockdown of  $\beta$ -catenin (*i.e.*, reduction of  $\beta$ -catenin expression) inhibited the PTHR signaling switch (Yano *et al.*, 2013). These findings raised an

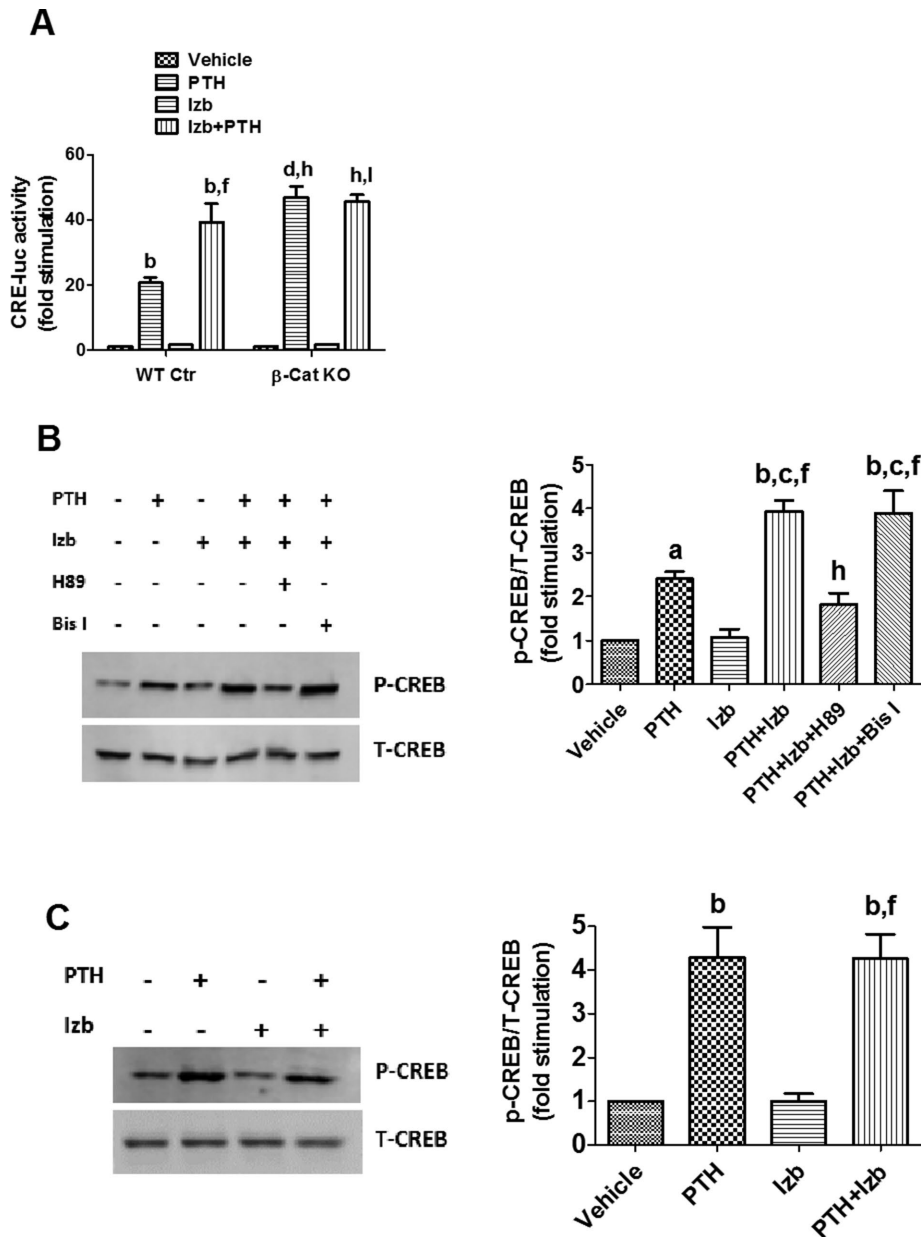
important question of whether the intervention of  $\beta$ -catenin interaction with PTHR in osteoblasts has a similar effect to alter PTH-induced PTHR signaling. Proteasome inhibitors such as bortezomib and carfilzomib, which have been used to treat multiple myeloma patients, were shown to directly stabilize  $\beta$ -catenin protein and increase  $\beta$ -catenin nuclear translocation (Qiang *et al.*, 2009; Hu *et al.*, 2013). In the present study, we chose Izb, the first orally administered proteasome inhibitor for the treatment of multiple myeloma, because it has the potential to treat resorptive bone diseases such as osteoporosis. We found that Izb increased active  $\beta$ -catenin expression and translocation (Figure 3 and Supplemental Figure S5). Most importantly, we found, using coimmunoprecipitation (coIP) and confocal microscopy, that Izb was able to separate  $\beta$ -catenin from the PTHR at the cell membrane. Pretreatment of Izb increased PTH-stimulated cAMP production and reduced PTH-induced PLC activity (Figure 4 and Supplemental Figure S4), demonstrat-

ing that Izb can inhibit the PTHR signaling switch to increase cAMP formation in osteoblasts by promoting the dissociation of  $\beta$ -catenin from the PTHR. To the best of our knowledge, this is the first report showing how a proteasome inhibitor regulates PTHR signaling by dissociating  $\beta$ -catenin from the PTHR.

It is established that PTH-induced  $\beta$ -catenin/TCF signaling is cAMP/PKA dependent (Kulkarni *et al.*, 2005; Suzuki *et al.*, 2008). CRE-luc activity and CREB phosphorylation are downstream events that depend on cAMP/PKA activation. We demonstrated that Izb by itself had no effect on CRE-luc activity and CREB phosphorylation but enhanced PTH stimulation of CRE-luc activity and phosphorylation of CREB (Figure 5). Furthermore, Izb and PTH synergistically increased GSK3 $\beta$  phosphorylation at Ser-9 and phosphorylated  $\beta$ -catenin at Ser-675 (Figure 6), the outcome of which results in  $\beta$ -catenin translocation to the nucleus. It is known that PTH-induced  $\beta$ -catenin/TCF activation takes  $>6$  h. Similar to other proteasome inhibitors, Izb treatment in cells for  $>3$  h has some cytotoxicity (Boissy *et al.*, 2008). To maintain Izb biological activity and avoid its toxicity, Saos2 cells received Izb for the first 3 h, followed by an additional 5 h of culture in Izb-free medium. Our findings show that Izb enhanced PTH-induced  $\beta$ -catenin/TCF signaling without inducing cytotoxicity (Figures 2 and 7). Therefore our work reveals a novel role for Izb in regulating PTH-induced  $\beta$ -catenin/TCF signaling via the cAMP/PKA-dependent pathway.

The increase of cytosolic and nuclear  $\beta$ -catenin by proteasome inhibition is via its specific inhibition of  $\beta$ -catenin degradation but independent of Wnt ligands and not related to GSK3 $\beta$  phosphorylation (Qiang *et al.*, 2009). In the present study, we demonstrated that Izb by itself does not affect GSK3 $\beta$  phosphorylation at Ser-9 and  $\beta$ -catenin phosphorylation at Ser-675. Thus the underlying mechanisms by which Izb induces  $\beta$ -catenin/TCF signaling are not fully understood. Proteasome inhibition can stabilize numerous proteins. PTHR directly interacts with Dishevelled to regulate  $\beta$ -catenin signaling (Romero *et al.*, 2010). Zhou *et al.* (2016) recently reported that ubiquitin-specific protease 4 strongly inhibited Wnt/ $\beta$ -catenin signaling by removing lysine-63 linked polyubiquitin chain from Dishevelled and antagonized osteoblast differentiation





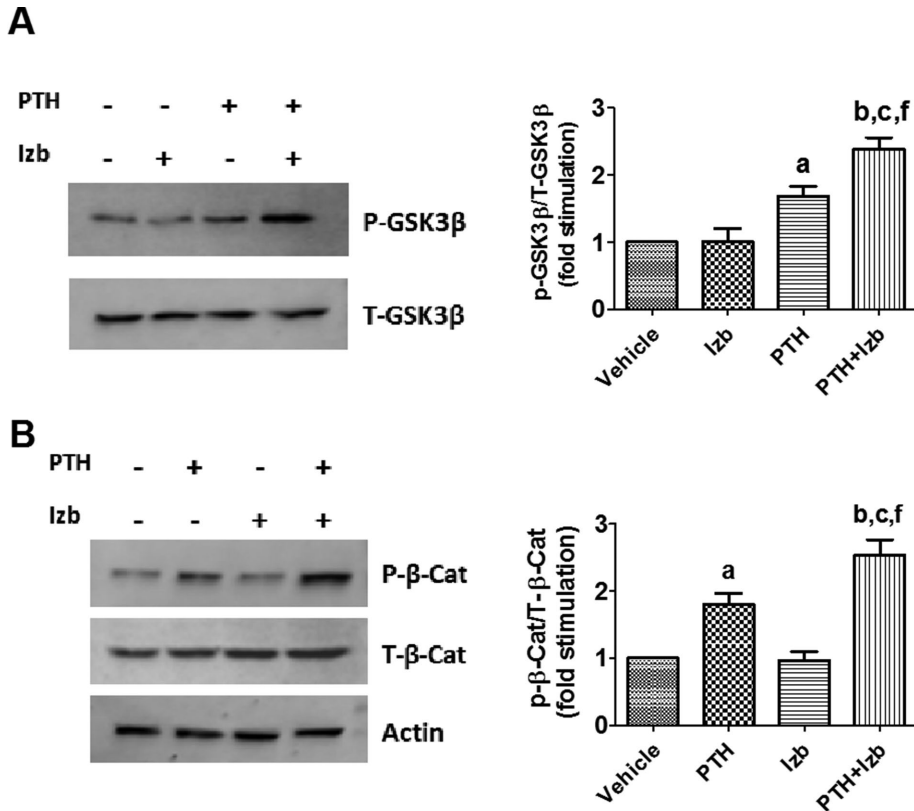
**FIGURE 5:** Izb enhances PTH-induced CRE-luc activity and CREB phosphorylation. (A) Knockout of  $\beta$ -catenin increases PTH-induced CRE-luc activity. Saos2- $\beta$ -Cat-KO-3 cells ( $\beta$ -Cat KO) and their control cells (Saos2- $\beta$ -Cat-Ctr-1, WT Ctr) were seeded onto a 24-well plate. After cells reached 80% confluence, the cells were infected with lentiviral particles of CRE-luc reporter and cultured for 48 h. The cells were serum starved overnight and then pretreated with Izb (100 nM for 1 h) before PTH (100 nM) was added to the culture for another 2 h. PTH induction of CRE-luc activity in each group was measured as described in *Material and Methods*. Data are summarized as mean  $\pm$  SE of triplicate measurements.  $n = 3$ . <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with WT Ctr cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with WT Ctr cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with WT Ctr cells treated with Izb; <sup>g</sup> $p < 0.05$ , <sup>h</sup> $p < 0.01$ , compared with  $\beta$ -Cat KO cells treated with vehicle; <sup>i</sup> $p < 0.05$ , <sup>j</sup> $p < 0.01$ , compared with  $\beta$ -Cat KO cells treated with PTH; <sup>k</sup> $p < 0.05$ , <sup>l</sup> $p < 0.01$ , compared with  $\beta$ -Cat KO cells treated with Izb. (B) Izb enhancement of PTH-induced CREB phosphorylation is cAMP/PKA dependent. Saos2- $\beta$ -Cat-Ctr-1 cells (WT Ctr) were pretreated with Izb (100 nM for 2 h) before PTH (100 nM) was added to the culture for another 1 h in the presence or absence of PKA inhibitor H89 (10  $\mu$ M) or PKC inhibitor Bis I (10  $\mu$ M) for 1 h and 15 min. Left, PTH-induced CREB phosphorylation and total CREB expression (loading control). Right, quantified CREB phosphorylation and total CREB expression in four independent experiments presented as mean  $\pm$  SE. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with cells treated with Izb; <sup>g</sup> $p < 0.05$ , <sup>h</sup> $p < 0.01$ , compared with cells treated with PTH plus Izb. (C) Izb fails to affect PTH stimulation of CREB

and mineralization through Dishevelled degradation. Therefore we do not exclude the possibility that Izb enhancement of PTH stimulation of  $\beta$ -catenin/TCF signaling may be related to its inhibition of Dishevelled degradation. In addition, PTHR activation, desensitization, endocytosis, and recycling proceed in a cyclical manner. We previously reported that PTHR recycling was complete by 2 h after stimulation with PTH(1-34), a biologically active peptide fragment, suggesting the PTHR trafficking is different from that of  $\beta$ -catenin. However, PTH(7-34), which does not activate the PTHR but promotes receptor internalization, down-regulates the PTHR by a ubiquitin-sensitive pathway (Sneddon *et al.*, 2003; Alonso *et al.*, 2011). Continuous and high-dose PTH(1-34) causes bone resorption and hypercalcemia (Qin *et al.*, 2004; Cheloha *et al.*, 2015). Future investigations will need to assess the roles of proteasome inhibition on prolonged PTH treatment in induction of both cAMP formation and  $\beta$ -catenin/TCF signaling in bone cells.

On the basis of the present findings and generally accepted roles of proteasome inhibitor and  $\beta$ -catenin/TCF, we propose a model (Figure 8) for the effect of Izb on regulation of PTHR signaling cascade in bone. According to this notion, Izb regulates PTHR signaling by dissociating  $\beta$ -catenin from the PTHR to enhance PTH-induced  $\beta$ -catenin/TCF activity. Such an effect is cAMP/PKA dependent. Izb has been applied for the treatment of both the tumor burden and bone loss associated with multiple myeloma. Whereas our previous study emphasized that proteasome inhibition mitigated the catabolic effect of PTH on bone by suppressing PTH-induced osteoclast differentiation and bone-resorptive activity (Yang *et al.*, 2015), the present study demonstrates that Izb can regulate the PTHR signaling in osteoblasts, which favors anabolic PTH action in bone. These findings warrant further investigation of this combination of PTH and Izb on osteoblast differentiation

phosphorylation in  $\beta$ -catenin-knockout cells. Saos2- $\beta$ -Cat-KO-3 cells ( $\beta$ -Cat KO) cells were pretreated with Izb (100 nM for 2 h) before PTH (100 nM) was added to the culture for another 1 h. Left, PTH stimulation of CREB phosphorylation. Right, quantified CREB phosphorylation and total CREB expression in three independent experiments presented as mean  $\pm$  SE. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with cells treated with Izb.





**FIGURE 6:** Izb facilitates PTH stimulation of GSK3 $\beta$  and  $\beta$ -catenin phosphorylation. (A) Saos2 cells were seeded onto a six-well plate. After confluence, the cells were serum starved overnight and then pretreated with Izb (100 nM) for 2.5 h, and PTH (100 nM) was then added to the culture for another 30 min. Left, PTH-induced GSK3 $\beta$  phosphorylation and total GSK3 $\beta$  expression. Right, quantified GSK3 $\beta$  phosphorylation at Ser-9 and total GSK3 $\beta$  expression in four independent experiments presented as mean  $\pm$  SE. (B) Saos2 cells were grown in a six-well plate and cultured as before. The cells were pretreated with Izb (100 nM) for 1 h, and PTH (100 nM) was then added to the culture for another 2 h. Left, PTH-induced  $\beta$ -catenin phosphorylation at Ser-675 and total  $\beta$ -catenin expression. Actin was used for loading control. Right, quantified  $\beta$ -catenin phosphorylation and total  $\beta$ -catenin expression in four independent experiments presented as mean  $\pm$  SE. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with cells treated with Izb.

and osteogenesis in vitro and therapeutic efficacy in animal models of osteoporosis.

## MATERIALS AND METHODS

Human [Nor<sup>β,18</sup>,Tyr<sup>34</sup>]PTH(1-34) was purchased from Bachem California (Torrance, CA). Carfilzomib was purchased from LC Laboratories (Woburn, MA), and Izb was obtained from Selleck Chemicals (Houston, TX). Both drugs were prepared in a 10 mM stock solution in dimethyl sulfoxide (DMSO) and diluted in plain medium just before use. Antibodies of  $\beta$ -catenin phosphorylation at Ser-675, phospho-GSK3 $\beta$  (Ser-9), GSK3 $\beta$ , phospho-CREB, and CREB were obtained from Cell Signaling Technology (Danvers, MA). HA.11 ascites monoclonal antibody (mAb) and HA.11 monoclonal affinity matrix were purchased from Covance (Berkeley, CA). Actin polyclonal antibody and lamin B1 mAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal  $\beta$ -catenin antibody and Fluo-4 AM were obtained from BD Biosciences (San Jose, CA). Lentiviral particles of reporter gene CRE-luc were from Qiagen (Germantown, MD). GeneArt CRISPR Nuclease Vector with orange fluorescent protein (OFP) reporter kit (A21174),  $\alpha$ -MEM, Opti-MEM, and Lipofectamine 2000 were purchased from Invitrogen

(Carlsbad, CA). Glosensor cAMP reporter plasmid (pGS-22F) was purchased from Promega (Madison, WI). Protease inhibitor cocktail Set I, H89, and Bis I were from Calbiochem (San Diego, CA). The Cyclic AMP Direct EIA Kit was purchased from Arbor Assays (Ann Arbor, MI). Ionomycin was from EMD Millipore (Billerica, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

## Cell culture

Saos2 cells, a human osteoblast-like cell line, were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM/F-12 with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. MC4 osteoblastic cells (subclone 4 of MCT3-E1; ATCC) were cultured in  $\alpha$ -MEM with 10% FBS in the presence of 50  $\mu$ g/ml ascorbic acid for 5 d and then used in the experiments. All cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

## Primary osteoblast culture

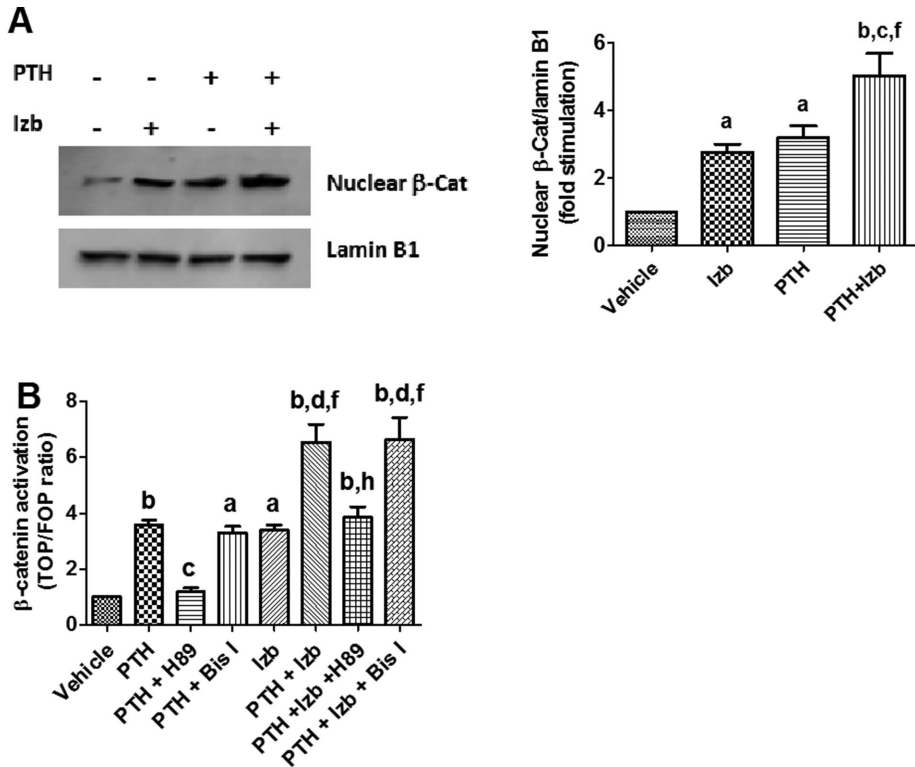
All of the experiments using mice for generation of primary osteoblasts were performed according to the protocol approved by the Animal Care and Use Committee of Thomas Jefferson University. Generation of primary osteoblast cultures was described previously (Yang *et al.*, 2015). Briefly, calvariae were removed from 2- to 3-d-old C57BL/6 mice and digested with 1 mg/ml collagenase type II and 0.25% trypsin-EDTA. Cells released from the second and third digestions were grown in  $\alpha$ -MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. After trypsinization of the confluent cells, differentiating osteoblasts were

cultured in the presence of 50  $\mu$ g/ml ascorbic acid for 7 d and used in the experiments.

## cAMP formation and detection

Saos2 cells were transfected with Glosensor cAMP reporter plasmid (pGS-22F) in a six-well plate by Lipofectamine 2000 following the manufacturer's instruction. After 36 h of transfection, cells were transferred into a 96-well plate. The confluent cells were then treated with vehicle or Izb (100 nM) at the indicated time points. After treatment, cells were incubated with luciferin for 20 min at room temperature, and then PTH or vehicle was added. Luminescence arising from intracellular cAMP binding to the Glosensor reporter protein was measured in real time at 2-min intervals for 60 min using a plate reader (Synergy-2; Gidon *et al.*, 2014; Carter *et al.*, 2015). The peak response time occurred by 16 min.

Additional cAMP measurements were performed in primary osteoblasts and Saos2- $\beta$ -Cat-KO cells and Saos2- $\beta$ -Cat-Ctr cells. These cells were treated with vehicle or Izb (100 nM for 3 h). PTH (100 nM for 15 min)-induced cAMP formation was detected using the cyclic AMP Direct EIA Kit (Arbor Assays) following the supplier's instructions. The samples were analyzed by using a cAMP standard curve.



**FIGURE 7:** Izb facilitates PTH-induced Wnt/ $\beta$ -catenin signaling. (A) Izb enhances PTH-stimulated nuclear  $\beta$ -catenin expression. Saos2 cells were treated with Izb (100 nM) for the first 3 h, followed by an additional 5 h of culture in Izb-free medium in the presence or absence of PTH (100 nM) for 8 h. Left, nuclear proteins were prepared and  $\beta$ -catenin expression was analyzed by immunoblotting. Right, quantified nuclear  $\beta$ -catenin expression in four independent experiments presented as mean  $\pm$  SE. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with cells treated with Izb. (B) Izb promotes PTH-induced TCF reporter activity. Saos2 cells were seeded into a 12-well plate. After 90% confluence, the cells were transfected with TOPflash or FOPflash plasmid. At 36 h after transfection, the cells were serum starved for 12 h and then treated with Izb for the first 3 h, followed by an additional 5 h of culture in Izb-free medium in the presence or absence of PTH (100 nM), PKA inhibitor H89 (5  $\mu$ M), or PKC inhibitor Bis I (5  $\mu$ M) for 8 h. The  $\beta$ -catenin/TCF activation was measured as described in *Materials and Methods*. Data are summarized as mean  $\pm$  SE of triplicate measurements.  $n = 3$ . <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , compared with cells treated with vehicle; <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$ , compared with cells treated with PTH; <sup>e</sup> $p < 0.05$ , <sup>f</sup> $p < 0.01$ , compared with cells treated with PTH plus Izb.

### Intracellular calcium assay

Saos2 cells were seeded into poly-D-lysine-coated 96-well plates and grown to confluence. The cells were then treated with vehicle or Izb (100 nM) for 3 h. After treatment, the cells were loaded with 2  $\mu$ M Fluo-4 AM according to the manufacturer's protocol. PTH or vehicle was added by an automated pipetting system in triplicate, and the 525-nm signals were generated by excitation at 485 nm with a Flex Station II (Molecular Devices, Sunnyvale, CA) as previously reported (Deshpande et al., 2014). Maximal  $Ca^{2+}$  response was determined by stimulating the cells with 1  $\mu$ M ionomycin. The net peak  $Ca^{2+}$  response was calculated using the equation (maximum agonist-induced fluorescence units) – (basal fluorescence units).

### CRE-luc assay

Saos2 cells were infected with lentiviral particles of CRE reporter. After 48 h, the cells were serum starved overnight and then treated with Izb, PTH, or vehicle at the indicated time points. The CRE-luc activity was detected as previously described (Yan et al., 2011).

### Cell viability assay

Cell viability was measured as described previously (Yang et al., 2015). Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a final concentration of 500  $\mu$ g/ml. The cells were further incubated for 1 h at 37°C in a 5%  $CO_2$  atmosphere, and the liquid in the wells was subsequently removed. DMSO was then added to each well, and the absorbance was measured at 570 nm.

### Knockout of $\beta$ -catenin gene expression in Saos2 cells

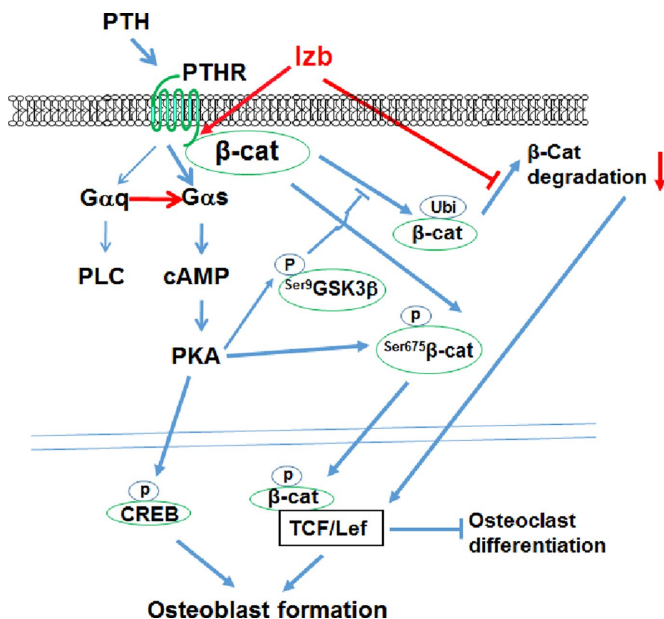
Constitutive  $\beta$ -catenin expression in Saos2 cells was knocked out using the CRISPR/Cas9 genome-editing technique. Table 1 lists the double-stranded oligonucleotides encoding a CRISPR targeting RNA (crRNA) of  $\beta$ -catenin that allows sequence-specific targeting of the Cas9 nuclease. The double-stranded oligonucleotides were cloned into linearized GeneArt CRISPR nuclease vectors with an OFP reporter following the manufacturer's protocol. Saos2 cells were seeded into a six-well plate. After cells reached 90% confluence, CRISPR plasmid or control plasmid was transfected into the cells using Lipofectamine 2000 following the manufacturer's protocol. Two days after the transfection, the cells were counted and seeded into a 96-well plate with one cell per well by a serial dilution. OFP reporter was applied to monitor transfection efficiency in cells for fluorescence-based tracking using an Evos machine (Invitrogen). After  $\sim 3$  wk, Western blotting was used to screen  $\beta$ -catenin expression in cells. Zero expression of  $\beta$ -catenin was identified in two single colonies when the cells were transfected with CRISPR construct, whereas  $\beta$ -catenin expression was not changed in cells transfected with the control plasmid.

### Cell fractionation

Membrane and nuclear proteins were prepared by fractionating cell lysates using differential centrifugation at 4°C as described previously (Wang et al., 2008; Hu et al., 2013). Briefly, cells treated with Izb, PTH, or vehicle were harvested in hypotonic buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 1 mM  $MgCl_2$ , 0.5 mM  $CaCl_2$ , 1 mM EDTA) supplemented with protease inhibitor cocktail set I and then homogenized by 30 strokes of a Dounce homogenizer. After removal of the nuclear pellet, lysates were separated into cytosolic and membrane fractions by ultracentrifugation (100,000  $\times g$ ) for 60 min. The membrane proteins were used for the interaction of  $\beta$ -catenin with the PTHR, and the nuclear proteins were subjected to immunoblot analysis for  $\beta$ -catenin expression.

### Immunoprecipitation and immunoblot analysis

The plasma membrane proteins were solubilized in immunoprecipitation assay buffer (1% Nonidet P-40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with



**FIGURE 8:** Model of Izb regulation of PTH signaling cascade in bone. Both PTH and Izb are able to induce  $\beta$ -catenin ( $\beta$ -Cat)/TCF signaling. Izb stabilizes  $\beta$ -Cat by inhibiting its proteasome degradation.  $\beta$ -Cat-mediated PTH signaling switch is blocked by Izb due to its promoting of  $\beta$ -Cat translocation, thereby dissociating  $\beta$ -Cat from the PTHR. Izb facilitates PTH stimulation of CREB phosphorylation,  $\beta$ -Cat (Ser-675) phosphorylation, GSK3 $\beta$  (Ser-9) phosphorylation, and TCF/Lef activity through a cAMP/PKA signaling pathway. Collectively these findings suggest that the combination of PTH and Izb favors the anabolic PTH action in bone.

protease inhibitor cocktail set I. HA beads were added to equal amounts of soluble membrane proteins in each group. Immunoprecipitated proteins, nuclear proteins, or total lysates (Wang *et al.*, 2008; Hu *et al.*, 2013) were analyzed by SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semidry method (Bio-Rad). Membranes were blocked with 3% bovine serum albumin in Tris-buffered saline/Tween 20 buffer at room temperature for 1 h and incubated with different antibodies overnight at 4°C. The membranes were then washed and incubated with IRDye 800CW goat anti-rabbit immunoglobulin G (IgG) or IRDye 680RD goat anti-mouse IgG at room temperature for 1 h. Band intensity was quantified using the LI-COR Odyssey system (Yang and Wang, 2015).

Double-stranded cloning Oligo	Sequence	Accession number
$\beta$ -Catenin	Forward: 5'-TCCCACTAAT-GTCCAGCGTT Reverse: 5'-AACGCTGGA-CATTAGTGGGA	NM_001904
Control	Forward: 5'-CATTCT-CAGTGCTATAGA Reverse: 5'-TCTATAG-CACTGAGAAATG	

**TABLE 1:** CRISPR sequences for knockout of human  $\beta$ -catenin.

### GST-E-cadherin pull-down assay

The GST-E-cadherin binding assay was performed as described (Qiang *et al.*, 2009). The  $\beta$ -catenin-binding site of E-cadherin was cloned as a GST-fusion protein, and complexes were purified using GST beads. GST-E-cadherin was used to precipitate uncomplexed  $\beta$ -catenin from 500  $\mu$ g of cell lysates. The free  $\beta$ -catenin fraction detected by immunoblotting represents active forms of  $\beta$ -catenin (Qiang *et al.*, 2009).

### Fluorescence staining

Saos2 cells were grown on glass coverslips and then transfected with GFP-PTHR. After 40 h, the cells were incubated with vehicle or Izb (100 nM) for 3 h, followed by an additional 5 h of culture in Izb-free medium. The cells were rinsed in phosphate-buffered saline (PBS), fixed on 4% paraformaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Blocking was performed by incubating the cells for 1 h at room temperature in 10% goat serum in PBS. Anti- $\beta$ -catenin mouse mAb diluted (1:250) in blocking buffer was applied to the specimens for 1 h at room temperature. Alexa Fluor 546-tagged goat anti-mouse secondary antibody diluted 1:1000 was applied under the same conditions as the primary antibody. Nucleus staining was performed with DRAQ5. Coverslips were mounted for confocal microscopy (Wang *et al.*, 2008).

### TOPflash/FOPflash activity assay

Saos2 cells or MC4 cells were transfected with TOPflash or FOPflash in a 12-well plate by using Lipofectamine 2000 according to the manufacturer's protocol. After 36 h of transfection, cells were serum starved for 12 h and then treated with the indicated reagents. Eight hours after treatment, cells were lysed with reporter lysis buffer (Promega). The cell lysates were then drawn four times through a 21-gauge needle attached to a 1-ml syringe. The supernatants were transferred to a 96-well plate. The luminescence of each well was recorded after addition of BioGlo Luciferase substrate (Promega). The ratio of TOP/FOP signal was calculated and normalized to control conditions.

### Statistical analysis

The data are presented as mean  $\pm$  SE of triplicate measurements. In the figures, *n* indicates the number of independent experiments. Statistical analysis between control and treated groups was performed using Student's *t* test. Multiple comparisons in one or two types of cells were evaluated by one-way or two-way analysis of variance followed by Bonferroni's posttest (Prism; GraphPad). *p* < 0.05 was considered sufficient to reject the null hypothesis.

### ACKNOWLEDGMENTS

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