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Polycystin-1 interacts with TAZ to stimulate osteoblastogenesis and inhibit adipogenesis

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The molecular mechanisms that transduce the osteoblast response to physical forces in the bone microenvironment are poorly understood. Here, we used genetic and pharmacological experiments to determine whether the polycystins PC1 and PC2 (encoded by Pkd1 and Pkd2) and the transcriptional coactivator TAZ form a mechanosensing complex in osteoblasts. Compound-heterozygous mice lacking 1 copy of Pkd1 and Taz exhibited additive decrements in bone mass, impaired osteoblast-mediated bone formation, and enhanced bone marrow fat accumulation. Bone marrow stromal cells and osteoblasts derived from these mice showed impaired osteoblastogenesis and enhanced adipogenesis. Increased extracellular matrix stiffness and application of mechanical stretch to multipotent mesenchymal cells stimulated the nuclear translocation of the PC1 C-terminal tail/TAZ (PC1-CTT/TAZ) complex, leading to increased runt-related transcription factor 2–mediated (Runx2-mediated) osteogenic and decreased PPARγ-dependent adipogenic gene expression. Using structure-based virtual screening, we identified a compound predicted to bind to PC2 in the PC1:PC2 C-terminal tail region with helix:helix interaction. This molecule stimulated polycystin- and TAZ-dependent osteoblastogenesis and inhibited adipogenesis. Thus, we show that polycystins and TAZ integrate at the molecular level to reciprocally regulate osteoblast and adipocyte differentiation, indicating that the polycystins/TAZ complex may be a potential therapeutic target to increase bone mass.

Introduction

Skeletal loading and unloading have major effects on skeletal homeostasis. In contrast to postmenopausal osteoporosis, in which bone resorption plays a major role in bone loss, and age-related osteoporosis and conditions of skeletal unloading, such as occurs due to microgravity, immobility, and/or sarcopenia, a decrease in osteoblast-mediated bone formation accompanied by a gain of marrow fat underlies the pathogenesis of osteopenia (1–4). On the other hand, mechanical loading from exercise, vibration, or pulsed electromagnetic fields leads to a gain in bone mass due to stimulation of osteoblastogenesis and inhibition of bone marrow adipogenesis (5–7). The molecular identity of the physiologically relevant mechanosensor in bone, and the cellular mechanisms responsible for the reciprocal control of osteoblastogenesis and adipogenesis during skeletal unloading, are uncertain (8–10).

Polycystin-1 (PC1, encoded by the PKD1 gene) and polycystin-2 (PC2, encoded by the PKD2 gene) form a prototypic mechanosensing complex in the kidney and other tissues. PC1, an adhesion-like receptor (11, 12), and PC2, a nonselective ion channel (13), interact through their respective coiled-coil domains to form a complex that senses fluid-shear stress in renal epithelial cells (14–17). Inactivating mutations of either PKD1 or PKD2 cause renal cystic disease in mice (18, 19) and autosomal dominant polycystic kidney disease (ADPKD) in humans (20, 21).

Polycystins are also expressed in other tissues (22, 23), and several extrarenal functions are being recognized (23, 24), including in the skeleton (25, 26), where recent evidence shows that the polycystin complex plays an important role in osteoblastogenesis to control bone formation (27–29). In this regard, osteoblast-specific deletion of Pkd1 or Pkd2 resulted in osteopenia, reduced runt-related transcription factor 2–dependent (Runx2-dependent) osteoblastogenesis, and impaired bone mechanosensing responses to in vivo mechanical loading in mice (30–32). While loss of Pkd1 and Pkd2 have concordant effects on osteoblast-mediated bone formation, they exhibit opposite effects on bone marrow adipogenesis (30–32). Indeed, loss of Pkd1 increases PPARγ-dependent adipogenesis, leading to increased bone marrow fat, while loss of Pkd2 results in decreased adipogenesis and suppression of both Runx2 and PPARγ. The discordant effects on adipogenesis suggest that PC1 and PC2 functions can be uncoupled and that alternative, PC2-independent pathways link PC1 to adipogenesis (30–32).

TAZ (transcriptional coactivator with a PDZ-binding domain; also known as WW domain–containing transcription regulator 1, or WWTR1), a downstream effector of the Hippo pathway, is a candidate for linking PC1 to adipogenesis. TAZ is regulated by extracellular mechanical stimuli that involve cytoskeletal-dependent nuclear shuffling in response to alterations in extracellular matrix.
stiffness (33–35). There is evidence that PC1 and TAZ may interact to cause polycystic kidney disease through a common pathway. Global Ta z knockout and conditional deletion of Taz from the kidney lead to cystic kidney disease in mice (36, 37); and in renal epithelial cells, TAZ binds to the PC1 C-terminal tail (PC1-CTT) to facilitate nuclear translocation (38–40), and binds to PC2-CTT to enhance PC2 degradation (41). TAZ also differentially regulates osteoblastogenesis and adipogenesis (42). Nuclear translocation of Taz in osteoblasts leads to increased osteoblast-mediated bone formation and decreased bone marrow adipogenesis (45); depletion of taz in zebrafish impairs bone development (42), and Taz−/− mice have small stature and ossification defects (36). These observations raise the possibility that TAZ may be an essential component of the polycystin mechanosensing pathway in bone that regulates bone mass through the reciprocal control of osteoblastogenesis and adipogenesis.

In the current study, we investigated whether TAZ mediates the differential effects of PC1 on osteoblastogenesis and adipogenesis in both in vivo mouse genetic models and in vitro cell culture models. We found that PC1-CTT interacts with TAZ in osteoblasts to integrate 2 distinct mechanosensing pathways, leading to stimulation of Runx2-mediated osteoblastogenesis and inhibition of PPARγ-mediated adipogenesis in the skeleton. Furthermore, using 3D ensemble docking algorithms, we identify a small molecule, predicted to bind to PC2 in the PC1:PC2 interacting region, that stimulated osteoblastogenesis and inhibited adipogenesis in vitro and in vivo. Hence, the polycystins/TAZ complex is a potential target for pharmaceutical development of small molecules to mimic mechanical loading to increase bone mass.

Results

Additive effects of combined Taz and Pkd1 deficiency on bone mass and lineage commitment. We explored genetic interactions between PC1 and TAZ in the skeleton by characterizing the effects of single- and compound-heterozygous loss of Pkd1 and Taz on bone mineral density (BMD). This was accomplished by crossing of heterozygous Ta z+/− mice with heterozygous Pkd1+/− mice to create double-heterozygous Ta z+/−;Pkd1+/− animals. These mice were born at the expected Mendelian frequency, and all genotypes had normal survival, gross appearance, and body weight indistinguishable from those of WT mice over the period of study. Neither single- nor compound-mutant mice developed cystic kidney disease by gross and histological inspection of the kidneys (data not shown).

Adult male and female Pkd1+/− mice exhibited a low BMD by dual-energy x-ray absorptiometry scan analysis. This was due to a reduction in trabecular bone volume (13.2%), cortical bone thickness (9.2%), and impaired osteoblast-mediated mineral apposition rates (26.6%), as previously reported in 6-week-old mice (46). In contrast, neither male nor female Taz−/− mice had abnormalities in bone mass, bone structure, and bone formation rate compared with WT mice (Figure 1). However, combined loss of 1 allele of Ta z and Pkd1 resulted in additive reductions in BMD, as evidenced by the 21% reduction in BMD in the double-heterozygous Ta z+/−;Pkd1+/− mice (Figure 1A). Micro-CT analysis revealed that double-heterozygous Ta z+/−;Pkd1+/− mice had greater loss in both trabecular (26.3%) and cortical bone (19.4%) than did single-heterozygous mice (Figure 1B). These reductions in bone volume were associated with a significant decrease in mineral apposition rates in single-heterozygous Ta z+/− and Pkd1+/− mice compared with age-matched WT mice and an even greater reduction (44.6%) in double-heterozygous Ta z+/−;Pkd1+/− mice (Figure 1C).

Finally, bone marrow exhibited an increased percentage of fat cells in single-heterozygous Pkd1+/− mice compared with age-matched WT mice and even greater increments in double-heterozygous Ta z+/−;Pkd1+/− mice. Consistent with low-turnover osteopenia with a reduction in osteoclast activity, histomorphometric analysis of the femurs by tartrate-resistant acid phosphatase (TRAP) immunostaining showed reduced osteoclast surface per bone surface in Pkd1+/− and Taz−/−;Pkd1+/− mice compared with WT and Taz+/− mice (Figure 1E).

To investigate whether combined TAZ and Pkd1 deficiency resulted in additive effects on gene expression profiles in bone, we examined by real-time reverse transcription PCR (RT-PCR) the expression levels of a panel of osteoblast-, osteoclast-, and chondrocyte-related mRNAs in the femurs of 8-week-old WT, heterozygous Ta z+/−, heterozygous Pkd1+/−, and double-heterozygous Ta z+/−;Pkd1+/− mice (Table 1). Single-heterozygous Ta z+/− showed normal bone gene expression profiles except 50% reduction of Taz transcripts (Table 1). However, bone derived from single-heterozygous Pkd1+/− mice had measurable reductions in osteocalcin, osteopontin, osteoprotegerin (Opg), RANK ligand (RankL), Mmp13, sclerostin (Sost), and Dmp1 mRNA levels in comparison with WT mice. Significantly greater reductions of osteoblasts and osteocyte-related genes were observed in double-heterozygous Ta z+/−;Pkd1+/− mice for osteocalcin, RankL, Mmp13, Sost, and Dmp1. Bone expression of Trap and Mmp9, markers of bone resorption, were reduced in heterozygous Pkd1+/− mice and to a greater extent in double-heterozygous Ta z+/−;Pkd1+/− mice compared with WT littermates (Table 1), suggesting that bone loss was mainly due to decreased osteoblast-mediated bone formation. These findings suggest that a low bone formation rate rather than increased bone resorption accounts for the low BMD and bone volume of femurs in single-heterozygous Pkd1+/− and double-heterozygous Ta z+/−;Pkd1+/− deficient mice. Pparγ, an adipocyte transcription factor, and adipocyte markers, including lipoprotein lipase (Lpl) and adipocyte fatty acid-binding protein 2 (aP2), were significantly increased in femurs of heterozygous Pkd1+/− mice and to a greater extent in double-heterozygous Ta z+/−;Pkd1+/− mice compared with WT littermates (Table 1). Transcripts of chondrocyte-related genes did not differ between single- and double-heterozygous Ta z+/−;Pkd1+/− mice (Table 1). Thus, Taz deficiency and Pkd1 deficiency are additive on both osteogenesis and adipogenesis.

Additive effects of combined Taz and Pkd1 deficiency on osteoblast differentiation. To explore the mechanism underlying the increase in osteoblastogenesis and decreased adipogenesis in double-heterozygous Ta z+/−;Pkd1+/− mice, we performed bone marrow-derived stromal cell (BMSC) cultures derived from age-matched WT, heterozygous Ta z+/−, heterozygous Pkd1+/−, and double-heterozygous Ta z+/−;Pkd1+/− mice grown in osteoblastic differen-
Figure 1. Effects of combined Pkd1 and Taz deficiency on bone mass in 8-week-old mice. (A) BMD in femurs by dual-energy x-ray absorptiometry scan. (B) Micro-CT analysis of distal femoral metaphysis and midshaft diaphysis. (C) Periosteal mineral apposition rate (MAR) by calcein double labeling. (D) OsO₄ staining of decalcified tibiae by micro-CT analysis. (E) TRAP staining (red color) for osteoclast activity. Data are presented as the mean ± SD from 6–8 individual mice (n = 6–8). P values were determined by 1-way ANOVA with Newman-Keuls multiple-comparisons test. *Significant difference from WT control mice, †significant difference from Taz+/− mice, ‡significant difference from Pkd1+/− mice at P < 0.05, respectively. BV/TV, bone volume density; Ct.Th, cortical thickness; Ad.V/Ma.V, adipocyte volume per marrow volume; Ad.N, adipocyte number; Oc.S/BS, osteoclast surface per bone surface.
in significant reductions in osteogenic markers such as Runx2 and osteocalcin and enhanced adipogenic markers such as Pparg and aP2 in vitro (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI93725DS1). In contrast, Pkd2-deficient osteoblasts exhibited coordinated reductions in both osteogenic and adipogenic markers during culture under osteogenic conditions (Supplemental Figure 1B). To verify these results, and to remove any confounding effects of the in vivo environment on the phenotype of osteoblasts isolated from knockout mice, we used adenovirus-CMV-iCre (Ad-CMV-iCre) and freshly isolated primary osteoblasts derived from the floxed Pkd1loxP and Pkd2loxP mice to examine whether loss of polycystins modulates osteoblast and adipocyte differentiation. We found that incubation of primary Pkd1loxP and Pkd2loxP osteoblasts with Ad-CMV-iCre ex vivo resulted in a 65% decrease in Pkd1 and Pkd2 message expression compared with Ad-CMV-null vector controls. The reduction in Pkd1 and Pkd2 transcripts resulted in significant reductions of osteogenic markers, such as Runx2 and alkaline phosphatase (ALPL) (Supplemental Figure 1C). However, Ad-CMV-iCre–mediated deletion of Pkd1 resulted in significant increases of adipogenic markers, such as Pparg and aP2 (Supplemental Figure 1C), whereas Ad-CMV-iCre–mediated deletion of Pkd2 ex vivo resulted in decreased adipogenic markers (Supplemental Figure 1D), consistent with observations in conditional Pkd1fl/fl and Pkd2fl/fl mice as we previously reported (30–32).

Evidence for a functional link between polycystins and TAZ in vitro. We assessed changes in TAZ protein expression and phosphorylation in Pkd1- and Pkd2-deficient osteoblasts. We found that the level of total TAZ protein was slightly decreased, but TAZ phosphorylation at Ser 89, which leads to TAZ cytoplasmic sequestration (47), was increased in both Pkd1- and Pkd2-deficient osteoblasts (Figure 3, A and B). Consistent with reductions in functional TAZ protein levels, we observed that TAZ-mediated activation of TEAD reporter, a measure of TAZ transcriptional activity, was markedly decreased in both Pkd1- and Pkd2-deficient osteoblasts (Figure 3C). Although both Pkd1 and Pkd2 deficiencies lead to decreased TEAD reporter activities, they may differentially regulate TAZ.

To explore TAZ interactions with PC1 and PC2, we overexpressed FLAG-tagged TAZ and full-length PC1 and PC2 in HEK-293T cells and performed coimmunoprecipitation (co-IP) with an anti-FLAG antibody. We found that TAZ forms a complex with PC1 and PC2, as evidenced by their co-IP (Figure 3D). We also substituted PC1-CTT for the full-length PC1 in these studies. We found that PC1-CTT was sufficient for binding to TAZ, since overexpression of PC1-CTT brought down TAZ and PC2 in the IP complex with an anti-myc antibody for PC1-CTT (Figure 3D). To test whether polycystins regulate TAZ function, we cotrans-

### Table 1. Gene expression profiles in femurs from 8-week-old mice by real-time quantitative reverse transcription PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Taz+/−</th>
<th>Pkd1+/−</th>
<th>Taz+/−:Pkd1+/−</th>
<th>P value</th>
</tr>
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<td>Osteoblast lineage</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pkd1</td>
<td>NM_013630.2</td>
<td>0.97 ± 0.19</td>
<td>0.50 ± 0.17</td>
<td>0.48 ± 0.08</td>
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<td>Taz</td>
<td>NM_133784.3</td>
<td>0.52 ± 0.14</td>
<td>1.02 ± 0.32</td>
<td>0.50 ± 0.13</td>
<td>&lt;0.0001</td>
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<tr>
<td>Rum2-Il</td>
<td>NM_009820.5</td>
<td>1.05 ± 0.22</td>
<td>0.74 ± 0.08</td>
<td>0.60 ± 0.16</td>
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<tr>
<td>Osteocalcin</td>
<td>NM_007541.2</td>
<td>0.99 ± 0.26</td>
<td>0.73 ± 0.08</td>
<td>0.47 ± 0.06</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>AF515708.1</td>
<td>0.95 ± 0.25</td>
<td>0.72 ± 0.12</td>
<td>0.49 ± 0.11</td>
<td>&lt;0.0001</td>
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<tr>
<td>Mmp13</td>
<td>NM_008607.2</td>
<td>0.93 ± 0.15</td>
<td>0.63 ± 0.16</td>
<td>0.41 ± 0.14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dpp</td>
<td>NM_008764.3</td>
<td>0.94 ± 0.20</td>
<td>0.64 ± 0.14</td>
<td>0.67 ± 0.16</td>
<td>0.0048</td>
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<tr>
<td>Rankl</td>
<td>NM_011613.3</td>
<td>0.94 ± 0.27</td>
<td>0.60 ± 0.23</td>
<td>0.67 ± 0.20</td>
<td>0.0033</td>
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<td>Sost</td>
<td>NM_024449.6</td>
<td>0.99 ± 0.16</td>
<td>0.72 ± 0.14</td>
<td>0.55 ± 0.09</td>
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<td>Dmp1</td>
<td>NM_016779.2</td>
<td>1.01 ± 0.12</td>
<td>0.73 ± 0.16</td>
<td>0.63 ± 0.14</td>
<td>&lt;0.0001</td>
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<td>Wnt10b</td>
<td>U61970.1</td>
<td>0.83 ± 0.14</td>
<td>0.85 ± 0.14</td>
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<td>Axin2</td>
<td>AF205889.1</td>
<td>0.83 ± 0.14</td>
<td>0.75 ± 0.14</td>
<td>0.68 ± 0.12</td>
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<td>Fzd2</td>
<td>NM_020510.2</td>
<td>0.83 ± 0.14</td>
<td>0.75 ± 0.14</td>
<td>0.68 ± 0.12</td>
<td>0.0033</td>
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<tr>
<td>Osteoclast</td>
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<td>Trop</td>
<td>NM_007388.3</td>
<td>0.93 ± 0.16</td>
<td>0.65 ± 0.06</td>
<td>0.56 ± 0.12</td>
<td>&lt;0.0001</td>
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<tr>
<td>Mmp9</td>
<td>NM_013599.3</td>
<td>0.95 ± 0.24</td>
<td>0.71 ± 0.05</td>
<td>0.47 ± 0.08</td>
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<td>Chondrocyte</td>
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<td>Type II collagen</td>
<td>NM_031163.3</td>
<td>1.02 ± 0.45</td>
<td>0.91 ± 0.27</td>
<td>0.94 ± 0.13</td>
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<td>Vegfa</td>
<td>NM_009505.4</td>
<td>0.90 ± 0.26</td>
<td>1.03 ± 0.24</td>
<td>1.05 ± 0.21</td>
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<td>Adipocyte</td>
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</tr>
<tr>
<td>Pparg</td>
<td>NM_009505.4</td>
<td>0.99 ± 0.24</td>
<td>1.51 ± 0.39</td>
<td>1.83 ± 0.37</td>
<td>0.0001</td>
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<tr>
<td>aP2</td>
<td>NM_024406.2</td>
<td>1.26 ± 0.29</td>
<td>1.71 ± 0.28</td>
<td>2.59 ± 0.84</td>
<td>&lt;0.0001</td>
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<td>Lpl</td>
<td>NM_008509.2</td>
<td>1.17 ± 0.20</td>
<td>1.57 ± 0.22</td>
<td>2.19 ± 0.73</td>
<td>&lt;0.0001</td>
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</table>

Data are mean ± SD from 6 femurs of 8-week-old individual mice (n = 6) and are expressed as the fold changes relative to the housekeeping gene cyclophilin A subsequently normalized to control mice. P values refer to the differences between group means by 1-way ANOVA. If the differences between the means are statistically significant (i.e., P < 0.05), the group means are compared by Newman-Keuls multiple-comparisons test. *Significant difference from WT control mice, †significant difference from Taz−/− mice, ‡significant difference from Pkd1+/− mice at P < 0.05, respectively.
The Journal of Clinical Investigation

affected either full-length PCI or PCI-CTT and full-length PC2 along with TEAD reporter constructs into C3H10T1/2 cells. We found that overexpression of either full-length PCI or PCI-CTT along with full-length PC2 markedly increased TAZ-induced activation of TEAD activity (Figure 3E), suggesting a functional link between polycystins and TAZ signaling. These findings are also consistent with the observations that PC2 increases PC1 C-terminal cleavage in COS-7 cells (48, 49), and subsequently enhances PC1-CTT nuclear translocation and TAZ signaling pathway. P

PCI C-tail interacts with TAZ to coregulate Runx2 and PPARγ activities. Next, we examined whether the PCI-CTT/TAZ complex coregulates Runx2 and PPARγ activities (33, 42). It is known that the PCI-CTT binds to TAZ, which is a coactivator for Runx2 and a corepressor for PPARγ activity (42, 44), but the effects of the PCI-CTT/TAZ complex on Runx2 and PPARγ activities have not been studied. Thus, we overexpressed FLAG-tagged TAZ, PCI-CTT, and Runx2 or Pparg in HEK-293T cells and performed co-IP with an anti-FLAG antibody (Figure 4, A and B). We found that TAZ was sufficient for binding to PCI-CTT, since overexpression of PCI-CTT brought down TAZ in the IP complex. We also found that TAZ and PCI-CTT form a complex with either Runx2 or PPARγ, as evidenced by their co-IP (Figure 4, A and B). Overexpression of TAZ, PCI-CTT, and Runx2 or PPARγ in multipotent C3H10T1/2 mesenchymal cells found that TAZ stimulated osteocalcin (Oc) promoter activity and was enhanced by PCI-CTT, and overexpression of Runx2. In contrast, we observed that TAZ inhibits PPARγ stimulation of aP2 promoter activity, and this effect was further suppressed by overexpression of PCI-CTT (Figure 4, C and D). These findings suggest that PCI-CTT modulates TAZ function through its binding to TAZ, which coactivates Runx2-mediated gene transcription and corepresses PPARγ-induced gene expression. To examine the effects of PCI C-tail cleavage on Oc and aP2 promoter reporter activities, we overexpressed membrane-bound PCI-CTT construct (26, 50) along with Oc or aP2 reporters in C3H10T1/2 cells. Overexpression of PCI-CTT stimulated Oc promoter reporter activities, but suppressed aP2 promoter reporter activities (Figure 4, E and F). As previously reported (51), PCI-CTT is released by a γ-secretase inhibitor, blocked the effects of PCI-CTT to stimulate Oc promoter, and reversed the inhibitory effect on aP2 promoter activities (Figure 4, E and F). These findings suggest that cleavage and release of the PCI C-tail from the membrane form that translocates to the nucleus play an essential role in the regulation of targeting gene transcription.

Next, we investigated the co-occupancy of endogenous Oc or aP2 promoter by PCI-CTT, TAZ, and Runx2 or PPARγ complex in C3H10T1/2 cells. OSE2 is a key osteoblast-specific cis-acting element in the proximal Oc promoter (52); Runx2 was shown to bind the OSE2 site and regulates mouse Oc promoter activity (53). Therefore, we examined whether PCI-CTT, TAZ, and Runx2 form a complex that binds the region of the proximal Oc promoter that contains the OSE2 site. Quantitative chromatin immunoprecipitation (ChIP) analyses using anti-FLAG for TAZ, anti-myc for PCI-CTT, and anti-Runx2 identified that TAZ, PCI-CTT, and Runx2 were specifically recruited to a region of the proximal Oc promoter containing the known OSE2 cis-element that is known to bind to Runx2 (Figure 4G). There was an approximately 3- to 7-fold increase in the ratio of the promoter sequence versus the coding region sequence in the anti-FLAG, anti-myc, or anti-Runx2 group compared with the IgG control group by quantitative real-time PCR. To establish that TAZ and PCI-CTT suppress PPARγ binding to the consensus ARE6 site in the proximal aP2 promoter (54), we performed quantitative ChIP analysis using an anti-FLAG for TAZ, an anti-myc for PCI-CTT, and an anti-PPARγ antibody. Real-time PCR on the immunoprecipitated DNA fragments with primers to amplify the region spanning the ARE6 sites con-

Figure 2. Assessment of osteoblastic differentiation in bone marrow-derived mesenchymal stem cell cultures from 8-week-old WT, Taz−/−, and Pkd1−/− mutant mice. (A) Histochemical staining of mineralization nodules with alizarin red S during 12-day cultures. (B) ALPL activity during 8-day cultures. (C) Osteogenic and adipogenic gene expressions during 8-day cultures. Data are presented as the mean ± SD from 3 independent experiments (n = 3). P-values were determined by 1-way ANOVA with Newman-Keuls multiple-comparisons test. *Significant difference from WT control mice, **significant difference from Taz−/− mice, ***significant difference from Pkd1−/− mice at P < 0.05, respectively.

jci.org Volume 128 Number 1 January 2018 161
firmed that TAZ, PC1-CTT, and PPARγ were specifically recruited to the segment of the proximal aP2 promoter containing the ARE6 site that is known to bind PPARγ (Figure 4H). There was an approximately 3- to 7-fold increase in the ratio of the promoter sequence versus the coding region sequence in the anti-FLAG, anti-myc, or anti-PPARγ group compared with the IgG control group by quantitative real-time PCR. Together, these findings indicate that PC1-CTT and TAZ colocalize to the proximal aP2 promoter to enhance Runx2-mediated osteocalcin gene transcription, and colocalize to the proximal aP2 promoter to enhance PPARγ-mediated aP2 gene transcription.

Matrix stiffness regulates Oc or aP2 promoter activities by PC1-CTT and TAZ nuclear translocation. Previous publications showed that matrix stiffness has an important impact on osteogenesis and adipogenesis (33). We examined whether matrix stiffness affects PCI-CTT and TAZ nuclear translocation to regulate osteogenic Oc promoter reporter and adipogenic aP2 promoter reporter activities in C3H10T1/2 cells transfected with PCI-CTT. Using C-terminal FLAG-tagged full-length PCI construct, we found that hard matrix promotes γ-secretase and PCI-CTT cleavage, whereas soft matrix inhibits γ-secretase and PCI-CTT cleavage (Figure 5, A and B). Using membrane-bound PCI-CTT construct, we observed that hard matrix (40 kPa) promotes PCI-CTT and TAZ translocation to the nucleus (Figure 5, C and D) and increases Oc promoter reporter activity (Figure 5E), whereas soft matrix (0.5 kPa) attenuates PCI-CTT and TAZ translocation to nucleus (Figure 5, C and D) and enhances aP2 promoter reporter activity (Figure 5F). In addition, DAPT, a γ-secretase inhibitor, blocked the effects of the transfected PC1-CTT to stimulate Oc promoter reporter, and reversed the inhibitory effect on aP2 promoter reporter activities (Figure 5, E and F). We observed that the TEAD, Oc, and aP2 promoter activity responded similarly in C3H10T1/2 cells expressing endogenous PC1 as compared with cells transfected with PCI-CTT (Supplemental Figure 2, A–C). In addition, real-time RT-PCR showed that soft matrix inhibited the expression of the osteogenic genes Runx2, Apl, and osteocalcin but promoted the expression of the adipogenic markers Pparg and aP2 in C3H10T1/2 cells, whereas hard matrix had the opposite effect on gene expression, which was reversed by treatment with the γ-secretase inhibitor.
These findings suggest that matrix stiffness regulates cleavage, release, and nuclear translocation of PC1-CTT from plasma membrane in the regulation of targeting gene transcription.

Mechanical stretch regulates Oc or aP2 promoter activities by PC1-CTT and TAZ nuclear translocation. Previous studies show that mechanical stretch promotes osteogenesis (55–57) and inhibits adipogenesis (1, 5, 58). We examined whether stretch affects PC1-CTT cleavage and TAZ nuclear translocation to regulate osteogenic Oc promoter reporter and adipogenic aP2 promoter reporter activities in C3H10T1/2 cells. Using C-terminal FLAG-tagged full-length PC1, we found that stretch stimulates γ-secretase activity and PC1-CTT cleavage, whereas the γ-secretase inhibitor DAPT abolished stretch-induced PC1-CTT cleavage (Figure 6, A and B). Using membrane-bound PC1-CTT, we observed that stretch promotes PC1-CTT and TAZ translocation to the nucleus (Figure 6, C and D) and increases TEAD and Oc promoter reporter activities (Figure 6, E and F), whereas DAPT blocks PC1-CTT-mediated reporter activities (Figure 6, G and H).
predicted to bind to the PC2:PC1 interacting region. From this screen of compounds predicted to modulate protein:protein interactions, Zinc01442821 (4-phenyl-1H-pyrrole-3-carboxylic acid, designated MS for molecular staple) was the best compound predicted to bind to the $\alpha$ regions of PC2, as shown in Figure 7A. In the computational model, Zinc01442821 binding forms hydrophobic interactions with Val880 and Leu881 that are essential for the PC1-PC2 coiled-coil stabilization and also with charged PC2 residues Arg877, Arg878, and Lys874, i.e., with the central region of the KRRE cluster that is essential to the PC1:PC2 interactions (Figure 7B) (17).

To assess engagement of Zinc01442821 with polycystins and TAZ, we performed co-IP assays using HEK-293T cell lysates. CTT and TAZ translocation to nucleus (Figure 6, C and D) and enhances aP2 promoter reporter activity (Figure 6G). Real-time RT-PCR confirmed that stretch enhanced the expression of the osteogenic genes Runx2, Alpl, and osteocalcin but inhibited expression of the adipogenic markers Pparγ and aP2 (Supplemental Figure 3). These findings suggest that mechanical stretch stimulates osteogenesis through regulation of PC1-CTT and TAZ nuclear translocation.

Figure 5. Matrix stiffness regulates Oc or aP2 promoter activities through PC1-CTT cleavage and TAZ nuclear translocation in C3H10T1/2 cells. (A) $\gamma$-Secretase activity. (B) A cleavage of PC1-CTT from full-length PC1. (C) Western blot analysis of cytoplasmic (Cyto) and nuclear-extracted (NE) proteins. (D) The quantification of NE TAZ or PC1-CTT abundance relative to that of Cyto TAZ or PC1-CTT in C3H10T1/2 cells transfected with PC1-CTT constructs. (E) Oc promoter reporter activity. (F) aP2 promoter reporter activity. Data are presented as the mean ± SD from 3 independent experiments ($n = 3$). $P$ values were determined by 1-way ANOVA with Newman-Keuls multiple-comparisons test. **$P < 0.01$, ***$P < 0.001$ compared with control group.
intracellular calcium and TAZ-mediated activation of the TEAD reporter. We observed that Zinc01442821 significantly increased the peak value of Fluo-4 intensity and intracellular calcium levels (Figure 8A), consistent with activation of PC1/PC2 signaling. In addition, Zinc01442821 significantly increased TEAD reporter activity, consistent with enhanced TAZ nuclear translocation (Figure 8B) in MC3T3-E1 osteoblastic cells. The above co-IP data and these signaling responses suggest that Zinc01442821 may interact with PC2 to enhance calcium signaling as well as disrupt PC1/TAZ interactions to promote TAZ signaling.

Next, we examined the dose-dependent effects of Zinc01442821 on osteoblastogenesis and adipogenesis in cotransfected with GFP-tagged full-length PC1, full-length PC2, and FLAG-tagged TAZ. Cells were treated with either Zinc01442821, its inactive analog MS4 (see below), or the DMSO vehicle control. We found that the FLAG antibody coprecipitated PC1, PC2, and TAZ in vehicle-treated cultures, consistent with the above studies showing that PC1, PC2, and TAZ form a trimeric complex (Figure 3). The addition of Zinc01442821 (10 μM) attenuated the incorporation of TAZ into this trimeric complex (Figure 7, C–E), but enhanced the co-IP of PC1 and PC2 complexes (Figure 7, F–H), consistent with stabilization of PC1 and PC2 interactions.

Using an MC3T3-E1 osteoblastic cell line that expresses endogenous PC1/PC2, we tested the effects of Zinc01442821 on intracellular calcium and TAZ-mediated activation of the TEAD reporter. We observed that Zinc01442821 significantly increased the peak value of Fluo-4 intensity and intracellular calcium levels (Figure 8A), consistent with activation of PC1/PC2 signaling. In addition, Zinc01442821 significantly increased TEAD reporter activity, consistent with enhanced TAZ nuclear translocation (Figure 8B) in MC3T3-E1 osteoblastic cells. The above co-IP data and these signaling responses suggest that Zinc01442821 may interact with PC2 to enhance calcium signaling as well as disrupt PC1/TAZ interactions to promote TAZ signaling.

Next, we examined the dose-dependent effects of Zinc01442821 on osteoblastogenesis and adipogenesis in
MC3T3-E1 osteoblasts in vitro. Zinc01442821 (about 0.5–10 μM) dose-dependently increased ALPL activity and calcium deposition during osteogenic cultures (Figure 8, C and D). Also, real-time RT-PCR analysis revealed that Zinc01442821 at 10 μM concentration markedly stimulated the expression of Pparg and its downstream gene osteocalcin, but significantly attenuated the expression of Ppard and its downstream gene aP2 (Figure 8E). To determine that PC1 and PC2 are mediating the response to Zinc01442821 in osteoblasts, we derived primary osteoblasts from Pkd1−/− and Pkd2-null mice. We found that primary osteoblasts responded to Zinc01442821, but Pkd1−/− and Pkd2-deficient osteoblasts lost Zinc01442821 stimulation of intracellular calcium (Figure 8F) and TEAD reporter activity (Figure 8G).

Finally, it is well known that the acute administration of bone active agents, such as parathyroid hormone, can lead to alterations in osteoblast-related gene expression within hours to days (61). Therefore, we administered Zinc01442821 to WT mice and assessed its effects on the expression of osteoblast and adipocyte markers in bone after 72 hours. Consistent with the in vitro osteoblast culture data, treatment of mice with Zinc01442821 (100 mg/kg) twice daily for 3 days significantly upregulated the expression of Runx2 message and its downstream gene osteocalcin, but inhibited the expression of Pparg message and its downstream gene aP2 (Figure 8H). These findings suggest that Zinc01442821 modulates PC1/PC2/TAZ signaling to regulate osteoblastogenesis and adipogenesis both in vitro and in vivo.

In vitro functional assays of Zinc01442821 analogs. We also synthesized 5 close analogs of Zinc01442821 (designated as MS1–MS5). MS1 is a methyl ester of Zinc01442821, MS2 is an N-methyl analog of MS1, and MS3, MS4, MS5 have the position of the nitrogen in this scaffold is important for function. Indeed, MS4, unlike Zinc01442821, failed to inhibit PC1/PC2/TAZ trimeric complex formation (Figure 7, C and D).

Discussion
In this study, we identify novel interactions between polycystins and TAZ in osteoblasts that create a molecular mechanism whereby the skeleton senses mechanical loading to regulate bone mass. PC1 forms a mechanosensing complex with PC2 (10, 31, 32), and also interacts with TAZ (33, 42) to regulate bone mass through the reciprocal control of osteoblastogenesis and adipogenesis. Accordingly, mice with compound deletion of Pkd1 and Taz exhibit additive effects to reduce bone mass through reduced osteoblastogenesis and increased bone marrow adipogenesis. An interaction between PC1 and TAZ was demonstrated by co-IP studies. Additionally, in vitro studies show that PC1-CTT and TAZ translocate to the nucleus to activate Runx2 and inhibit PPARγ-dependent gene transcription in response to matrix stiffness and mechanical stretch. Moreover, we discovered a novel small molecule that binds to PC2, enhances TAZ-mediated transcription, and mimics the effects of mechanical loading to stimulate osteoblastogenesis and inhibit adipogenesis in vitro.

Loss-of-function double-heterozygous mice indicate genetic interactions between PC1 and TAZ. Compound-heterozygous Pkd1+/− and Taz−/− mice exhibit additive effects to promote bone loss, reduce osteoblast-mediated bone formation, and increase bone marrow fat. Bone marrow stromal cell cultures derived from compound-heterozygous Pkd1+/− and Taz−/− mice confirm decreased osteogenesis and increased adipogenesis in vitro. In addition, in primary osteoblast cultures derived from Pkd1+/−/−, we observed reduced osteoblast markers and increased adipogenic markers. In contrast, primary osteoblast culture from Pkd1−/−/− mice exhibited a concordant decrease in both osteoblast and adipocyte gene expression. Ex vivo adenoviral Cre-mediated deletion of Pkd1 in primary osteoblasts isolated from Pkd1+/− mice also resulted in impaired osteoblast differentiation and increased adipogenic markers, whereas adenoviral Cre-mediated deletion of Pkd2 resulted in a concordant decrease in both osteogenic and adipogenic markers. These cell culture studies are consistent with our prior finding that conditional deletion of Pkd1 in the osteoblast lineage in mice results in inhibition of osteoblastogenesis and stimulation of adipogenesis in vivo, whereas conditional deletion of Pkd2 results in concordant inhibition of both osteoblastogenesis and adipogenesis (30, 32).

Gain-of-function studies demonstrate that PC1-CTT and TAZ stimulate osteoblast and inhibit adipocyte differentiation, respectively. Transfection of PCI-CTT, TAZ, or Runx2 individually stimulates Oe promoter activity, and cotransfection of all 3 constructs exhibits additive effects on osteocalcin gene transcription in C3H10T1/2 cells. Similarly, transfection of PCI-CTT, TAZ, or Pparg individually suppresses aP2 promoter activity, and cotransfection of all 3 constructs results in additive suppression of aP2 gene transcription in C3H10T1/2 cells. We also found that both hard matrix and mechanical stretch stimulated the polycystins/TAZ mechanosensing signaling complex in vitro, consistent with the possibility that this complex may integrate the response to different physical forces in the bone microenvironment (31, 33). Since these in vitro methods of mechanical loading are nonphysiological, additional studies to explore the response to mechanical loading in compound Pkd1 and TAZ heterozygous mutant mouse models are needed to further test our hypothesis in vivo. Furthermore, we found that pharmacological activation of PC1/
RESEARCH ARTICLE

A

B

C

D

Vehicle
Zinc01442821 (μM)

Vehicle Zinc01442821 (μM)

E

F

G

H

The Journal of Clinical Investigation

jci.org Volume 128 Number 1 January 2018

168
PC2/TAZ signaling by Zinc01442821 increased intracellular calcium levels and induced TAZ activation of the TEAD promoter in osteoblasts, in agreement with the effect of extracellular matrix (ECM) stiffness in inducing nuclear translocation of TAZ (33, 62). Zinc01442821 also promoted osteoblastogenesis and inhibited adipogenesis in osteoblast cultures. Finally, short-term administration to mice stimulates osteoblastic and inhibits adipocyte message expression in bone in vivo.

Co-IP experiments in both HEK-293T and C3H10T1/2 cells transfected with PCI, PC2, and TAZ cDNA constructs show that PCI/PC2 and TAZ form a trimeric complex. PCI-CTT also will pull down TAZ and PC2 in co-IP experiments. In addition, Runx2 and PPARγ are present in the PCI-CTT and TAZ co-IP complexes. The PCI-CTT/TAZ protein complexes translocate from the cytoplasmic compartment to the nucleus of C3H10T1/2 cells in response to culture on a hard ECM and mechanical stretch. Nuclear translocation of the PCI-CTT/TAZ complex is blocked by treatment with a γ-secretase inhibitor. The importance of polycystin/TAZ interactions is further shown by the effects on PCI/PC2/TAZ complex formation of Zinc01442821, a chemical probe developed using structure-based in silico screening (59, 60). Interactions showing that PC1-CTT also binds to TAZ highlight the complexity and competitive nature of the crosstalk between PC1, PC2, and TAZ. Other pathways may also be linked to polycystins. For example, interaction of PCI with other transcription factors (i.e., NFAT and STAT3) has been described in human osteoblasts that regulate cell proliferation/differentiation via induction of Runx2 (63, 64). PCI is also reported to mediate the response to mechanical strain via potentiation of intracellular calcium and Akt/β-catenin pathways in osteoblasts (65).

In conclusion, this study defines a new schema whereby PCI-CTT and TAZ form an integrative mechanosensing complex in cells within the osteoblast lineage that differentially regulates osteoblastogenesis and adipogenesis (Figure 10). This schema proposes that mechanical forces, such as ECM stiffness, flow, and mechanical stretch, activate the PCI/PC2 complex (13, 31, 50, 51, 63–66), leading to stimulation of intracellular calcium, induction of PCI-CTT cleavage, and TAZ nuclear translocation to enhance Runx2-mediated induction of osteoblast gene transcription and to inhibit PPARγ and adipogenesis (33, 39, 42, 44, 46, 67). Together this work identifies a new mechanosensing complex and the feasibility of targeting this complex for treating disorders caused by mechanical unloading and ageing.

Methods

Mice. All animal research was conducted according to guidelines provided by the NIH and the Institute of Laboratory Animal Resources, National Research Council. The University of Tennessee Health Science Center’s Animal Care and Use Committee approved all animal studies (protocol 15-137.0). We obtained the floxed Taz mouse (Taz^fl/fl), which harbors 2loxP sites flanking exon 2 of the Taz gene, from Jeff Wrana and Helen McNeill (Mount Sinai Hospital and Lunenfeld-Tanenbaum Research Institute, Toronto, Ontario, Canada) (68). We crossed the floxed Taz^fl/fl mice with CMV-Cre mice to generate global TAZ heterozygous mice (CMV-Cre;Taz^+/-). These mice were then crossed to WT mice to segregate the CMV-Cre transgene from the desired conditional null allele (Taz^+/+) as shown in Figure 1A, equivalent to the Taz null allele). These mice were bred and maintained on a C57BL/6J background for at least 6 generations. The heterozygous Pkd1^+/− mice were generated in our laboratory as previously described (46). These heterozygous Taz^+/− mice were mated with heterozygous Pkd1^+/− mice to generate WT, heterozygous Pkd1^+/+, heterozygous Taz^+/−, and double-heterozygous (Pkd1^+/−;Taz^+/−) mice lacking 1 functional allele of both Pkd1 and Taz. These mice were used for phenotypic and lifespan analysis. All animal studies were approved by the IACUC and performed in accordance with NIH guidelines for the care and use of laboratory animals. The mice were anesthetized with a halothane/oxygen mixture (1:1) for mice older than 4 weeks of age. Bladder function was assessed using transrectal catheterization. The mice were then killed by cervical dislocation. The organs were removed, weighed, and stored for histological analysis. The kidneys were fixed in 10% buffered formalin, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological examination.

TAZ also binds to PC2, leading to PC2 degradation (41), suggesting that interactions with TAZ may diminish PC2 signaling, and PC1 blocks the PC2/TAZ interaction to prevent TAZ-mediated degradation of PC2 (41). Thus, the current studies show that PCI-CTT also binds to TAZ highlight the complexity and competitive nature of the crosstalk between PC1, PC2, and TAZ. Other pathways may also be linked to polycystins. For example, interaction of PCI with other transcription factors (i.e., NFAT and STAT3) has been described in human osteoblasts that regulate cell proliferation/differentiation via induction of Runx2 (63, 64). PCI is also reported to mediate the response to mechanical strain via potentiation of intracellular calcium and Akt/β-catenin pathways in osteoblasts (65).

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RESEARCH ARTICLE

The Journal of Clinical Investigation

RESEARCH ARTICLE

170

jci.org Volume 128 Number 1 January 2018

Cells were removed with the first medium change, and then the adherent cells (representing BMSCs) received the subsequent treatments. For osteoblastic differentiation, the adherent cells were grown in osteogenic medium (α-MEM containing 10% vol/vol FBS supplemented with 5 mM β-glycerophosphate and 25 μg/ml of ascorbic acid) for periods of up to 12 days. ALPL activity and alizarin red S histochemical staining for mineralization were performed as previously described (69). For quantification of mineralization, alizarin red S was extracted with 10% cetylpyridinium chloride and assessed at 562 nm. Total DNA content was measured with a double-strand DNA quantitation reagent and kit (Molecular Probes). Protein concentrations of the supernatant were determined with a total protein assay kit (Bio-Rad). We also used TRIzol reagent to isolate the total RNAs for real-time RT-PCR analysis as previously described (46).

Osteoblastic cell cultures. Immortalized Pkd1null/null, Pkd2null/null, and MC3T3-E1 osteoblastic cell lines (ATCC) were cultured in α-MEM containing 10% FBS and 1% P/S as previously described (31, 32). For intracellular calcium measurements, Fluo-4 or Fluo-4 AM (Invitrogen) was used according to the manufacturer’s instructions (25). For TAZ signaling study, the immortalized Pkd1 null/null and Pkd2 null/null cells were cultured in the osteogenic differentiation media for 48 hours. Then the cells were lysed with 150 μl of T-PER with 1× Halt protease inhibitor and 1 mM PMSF per well. After 30-second sonication 3 times, total cell lysates were centrifuged at 13,000 g for 10 minutes, and supernatants were stored at –80°C until use for the following Western blot analysis.

Mechanical cell stretch. C3H10T1/2 (ATCC) cells were plated onto 6-well plates with Collagen Type I surface (Flexcell International) for 24 hours and then subjected to a 6-hour stretch regimen by software-controlled vacuum applied to a loading station housed in a humidified 5% CO₂ incubator at 37°C (Flexcell FX-4000T, Flexco Medical AG). A 3D image analysis was done to determine bone volume and cortical thickness as previously described (30–32).

Bone densitometry, histomorphometric, and micro-CT analysis. BMD of femurs was assessed at 8 weeks of age using a Lunar PIXimus bone densitometer (Lunar Corp.). Calcein (Sigma-Aldrich) double labeling of bone and histomorphometric analyses of periosteal mineral apposition rate in tibiae and osteoclast surface per bone surface (percent) in femurs by TRAP immunostaining were performed using the OsteoMeasure analysis system (OsteoMetrics). The distal femoral metaphyses were also scanned using a Scanco μCT 40 (Scanco Medical AG). A 3D image analysis was done to determine bone volume and cortical thickness as previously described (30–32).

OsO₄ staining and micro-CT analysis. Whole intact femurs and tibiae with encapsulated marrow were dissected from 8-week-old mice, fixed for 48 hours in phosphate-buffered paraformaldehyde, and decalcified in 14% EDTA for 2 weeks. Then the femurs and tibiae were stained for 2 hours in 2% aqueous osmium tetroxide (OsO₄). The bones were rinsed in water for 48 hours and then scanned at 6 μm resolution using a Scanco μCT 40, 45 kVp and 177 μA. Quantification of fat volume, density, and distribution throughout the marrow was registered to low-contrast decalcified bone as our laboratory previously described (30, 32).

BMS cultures. BMSCs from 8-week-old mice were prepared as previously described (69). Briefly, the femurs and tibiae were dissected, the ends of the bones were cut, and the marrow was flushed out with 2 ml of ice-cold α-minimum essential medium (α-MEM) containing 10% (vol/vol) FBS using a 22-gauge needle and syringe. Cells were seeded into 12-well plates or 60-mm plates at a density of 1 × 10⁷ cells/ml and cultured for 3 days in α-MEM supplemented with 15% vol/vol FBS, 1% penicillin/streptomycin (P/S), and kept in a humidified incubator with 5% CO₂ and 95% air at a temperature of 37°C. On day 3, all nonadherent cells were removed with the first medium change, and then the adherent cells (representing BMSCs) received the subsequent treatments. For osteoblastic differentiation, the adherent cells were grown in osteogenic medium (α-MEM containing 10% vol/vol FBS supplemented with 5 mM β-glycerophosphate and 25 μg/ml of ascorbic acid) for periods of up to 12 days. ALPL activity and alizarin red S histochemical staining for mineralization were performed as previously described (69). For quantification of mineralization, alizarin red S was extracted with 10% cetylpyridinium chloride and assessed at 562 nm. Total DNA content was measured with a double-strand DNA quantitation reagent and kit (Molecular Probes). Protein concentrations of the supernatant were determined with a total protein assay kit (Bio-Rad). We also used TRIzol reagent to isolate the total RNAs for real-time RT-PCR analysis as previously described (46).

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Figure 9. Effects of Zinc01442821 (MS) and its analogs on polycystins/TAZ signaling in osteoblast cultures. (A) Chemical structure of MS and its analogs. (B and C) Intracellular [Ca²⁺] response and TAZ-mediated activation of TEAD reporter activities in MC3T3-E1 osteoblasts with either MS analog (10 μM) or vehicle (DMSO) control. Data are expressed as the mean ± SD (n = 8). P values were determined by 1-way ANOVA with Newman-Keuls multiple-comparisons test. ***P < 0.001 compared with controls.
nuclear translocation in mesenchymal stem cells. Oc promoter activities through PC1-CTT and TAZ and PPARαP2 mediated γ-secretase activity, cleavage of PC1-CTT, and PC1-CTT and TAZ nuclear translocation at a completion of 6 hours of stretch. The cells were processed for luciferase activity assays after 18 hours when subjected to 6 hours of stretch.

γ-Secretase assay. Cellular γ-secretase activities were quantified using an assay based on a previous report (71). Briefly, C3H10T1/2 cells were harvested in cell lysis buffer A containing 20 mM HEPES (pH 7.0), 150 mM KCl, 2 mM EDTA, 1% [(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO; wt/vol; Sigma-Aldrich), and Protease Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were centrifuged at 9,300 g for 1 minute at 4°C to remove nuclei and large cell debris. Supernatants were collected, and the protein concentration was quantified. Fifty micrograms of protein were added to an opaque 96-well plate (Nunclon; Nunc) and made to a final volume of 200 μl with assay buffer containing 50 mM Tris-HCl (pH 6.8), 2 mM EDTA, and 0.25% CHAPSO (wt/vol); 10 mM of fluorogenic amyloid β-peptide precursor protein–derived probe acting as a γ-secretase substrate (Sigma-Aldrich) was added to the plate. Controls used were the peptide probe and cell lysates alone. Samples were incubated at 37°C for 18 hours in the dark. Fluorescence was measured using a Synergy H4 Hybrid Reader (BioTek Instruments Inc.), with excitation wavelength at 340 nm and emission wavelength at 460 nm. Background fluorescence of the peptide probe was subtracted from all readings.

Computational small-molecule discovery. Zinc01442821 (4-phenyl-1H-pyrrole-3-carboxylic acid, C11H9NO2) was identified by a computationally driven, structure-based, and virtual docking screening, following approaches previously described (59, 60). Briefly, we used the PC1:PC2 coiled-coil model of Zhu et al. (17) to perform a docking screening of 1,000 diverse fragment-like molecules from the ZINC database using the MOE docking facility (Chemical Computing Group) version 2015, in an induced-fit setting. Zinc01442821 ranks among the top molecules predicted to interact with residues in the binding pocket of PC2 in which PC1 binds. This compound was selected based on the number of similar compounds and potential chemical modifications that can be identified in the ZINC database for follow-up screening.

To induce osteoblastic differentiation, the MC3T3-E1 cells were plated at a density of 2 × 104 cells per well in a 12-well plate and 4 × 104 cells per well in a 6-well plate and grown up to 21 days in osteogenic medium in the presence of Zinc01442821 (0, 0.5, 1, and 10 μM). ALPL activity and alizarin red S histochemical staining for mineralization were performed as previously described (30, 32). Total DNA content was measured with a PicoGreen dsDNA quantitation reagent and kit (Molecular Probes). Protein concentrations of the supernatant were determined with a Bio-Rad protein assay kit.

Real-time quantitative RT-PCR and Western blot analysis. For real-time quantitative RT-PCR, 1.0 μg total RNA isolated from either the long bone of 6-week-old mice or BMSCs cultured for 8 days in differentiation media was reverse transcribed as previously described (30, 32). PCR reactions contained 20 ng template (cDNA), 375 nM each forward and reverse primers, and 1× EvaGreen Supermix (Bio-Rad) in 10 μl. The threshold cycle (Ct) of tested-gene product from the indicated genotype were determined with a total protein assay kit.

For Western blot analysis, protein concentrations of the supernatant were determined with a Bio-Rad protein assay kit (Bio-Rad). Equal quantities of protein were subjected to 4%-12% Bis-Tris or 3%-8% Tris-Acetate gradient gels (Invitrogen) and were analyzed with standard Western blot protocols as previously described (30, 32). Polyclin-1 antibody (7E12, sc-130554), polyclin-1 antibody (C-20, sc-10372), polyclin-2 antibody (H-280, sc-25749), and polyclin-2 antibody (YCE2, sc-47734) were purchased from Santa Cruz Biotechnology. Puriﬁed mouse TAZ antibody (no. 560236) was purchased from BD Biosciences. Phosphorylated TAZ2 (Ser89, sc-47610) and β-actin (sc-7778) antibodies were from Santa Cruz Biotechnology. The intensity of bands was quantified using ImageJ software (NIH; http://rsb.info.nih.gov/ij/).

Transient transfection, promoter reporter activity, and co-IP. C3H10T1/2 cells were cultured in minimum essential medium (MEM)
containing 10% FBS and 1% P/S. To examine the effect of PCI-CTT and TAZ interaction on Runx2 or PPARγ activity, 5 × 10^6 C3H10T1/2 cells were transfected with 1.0 μg of PCI-CTT, 1.0 μg of TAZ, and/or 1.0 μg of Runx2 or Pparg expression constructs in combination with 3.0 μg of mouse osteocalcin or aP2 promoter luciferase reporter and 0.6 μg of Renilla luciferase-null as internal control plasmid by electroporation using a Cell Line optimal transfection kit according to the manufacturer’s protocol (Amaxa Inc.). A total of 6.6 μg of plasmid DNAs was used for each electroporation. The transfected cells were plated in 12-well plates and harvested 32 hours after transfection. Cells were lysed in 1× reporter lysis buffer, a luciferase assay was performed using a dual luciferase assay kit (Promega), and activity was measured with a Synergy H4 Hybrid Reader. To test the effect of PCI C-tail cleavage on Oc or aP2 reporter activity, the transfected cells were treated with vehicle (DMSO) control or 50 μM DAPT (Sigma-Aldrich; a γ-secretase inhibitor) for the last 12 hours and harvested 36 hours after transfection.

For co-IP analyses, 5 × 10^6 HEK-293T cells were transfected with either 3.0 μg of FLAG-tagged TAZ, 3.0 μg of GFP-tagged full-length PCI, and 3.0 μg of myc-tagged full-length PC2 (Baltimore PKD Core Center) or 3.0 μg of FLAG-tagged TAZ, 3.0 μg of myc-tagged PCI-CTT, and 3.0 μg of Runx2 or Pparg expression constructs for 48 hours. The transfected cells were washed once with ice-cold 1× PBS and added to appropriate ice-cold 1× IP lysis buffer (Pierce Biotechnology) containing 1× protease inhibitors. Epitope Tag (DYKDDDDK) Antibody (FG4R, MA1-91878) was purified to the last 12 hours and harvested 36 hours after transfection. The cellular lysates were centrifuged at approximately 13,000 g for 10 minutes to pellet the cell debris at 4°C, and the supernatants were collected to appropriate ice-cold 1× reporter lysis buffer, a luciferase assay was performed using a dual luciferase assay kit (Promega), and activity was measured with a Synergy H4 Hybrid Reader. To test the effect of PCI C-tail cleavage on Oc or aP2 reporter activity, the transfected cells were treated with vehicle (DMSO) control or 50 μM DAPT (Sigma-Aldrich; a γ-secretase inhibitor) for the last 12 hours and harvested 36 hours after transfection.

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