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CYLD, a Mechanosensitive Deubiquitinase, Regulates TGF β Signaling in Load-Induced Bone Formation

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Abstract

Many signaling pathways involved in bone homeostasis also participate in the anabolic response of bone to mechanical loading. For example, TGF β signaling coordinates the maintenance of bone mass and bone quality through its effects on osteoblasts, osteoclasts, and osteocytes. TGF β signaling is also essential for the mechanosensitive formation of new bone. However, the mechanosensitive mechanisms controlling TGF β signaling in osteocytes remain to be determined, particularly those that integrate TGF β signaling with other early responses to mechanical stimulation. Here, we used an *in vivo* mouse hindlimb loading model to identify mechanosensitive molecules in the TGF β pathway, and MLOY4 cells to evaluate their interactions with the prostaglandin E2 (PGE2) pathway, which is well-known for its rapid response to mechanical stimulation and its role in bone anabolism. Although mRNA levels for several TGF β ligands, receptors, and effectors were unchanged, the level of phosphorylated Smad2/3 (pSmad2/3) was reduced in tibial bone as early as 3 hrs after early mechanical stimulation. We found that PGE2 and its receptor, EP2, repress pSmad2/3 levels and transactivation of Serpine1 in osteocytes. PGE2 and EP2 control the level of pSmad2/3 through a proteasome-dependent mechanism that relies on the deubiquitinase CYLD. CYLD protein levels were also reduced in the tibiae within 3 hrs of mechanical loading. Using CYLD-deficient mice, we found that CYLD is required for the rapid load-mediated repression of pSmad2/3 and for load-induced bone formation. These data introduce CYLD as a mechanosensitive deubiquitinase that participates in the prostaglandin-dependent repression of TGF β signaling in osteocytes.

Keywords

TGF β signaling; mechanotransduction; osteocyte

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1. Introduction

Mechanical loading on bone is required for the maintenance of bone mass. In astronauts, sedentary adults, or in mice with unloaded hindlimbs, the reduced mechanical stimulation deregulates mineral homeostasis and causes bone loss [1] [2] [3]. Since low bone mass diseases such as osteoporosis predispose 125 million people worldwide to the risk of fractures [4], understanding the cellular and molecular mechanisms by which mechanical load maintains bone homeostasis is clinically significant. Osteocytes are the bone-embedded cells that sense mechanical stimuli and respond by regulating osteoblast and osteoclast activity [5]. For example, mechanical loading represses the expression of an osteocyte-secreted protein, called Sclerostin, which binds and inhibits the Wnt coreceptor, Lrp5/6 on osteoblasts. Thus, loading stimulates Wnt-mediated bone anabolism by repressing Sclerostin levels [6] [7].

Previously, we found that mice with reduced TGF β signaling in osteocytes cannot relay the load-mediated repression of Sclerostin expression and, consequently, have impaired load-induced bone formation [8]. The mechanoregulation of Sclerostin expression and new bone formation required the active phosphorylated form of the TGF β effector Smad3, which was repressed within 5 hrs of loading. However, the mechanosensitive mechanisms regulating TGF β signaling in osteocytes remain unclear, particularly those that occur rapidly in response to loading. Here we sought to elucidate early events in the anabolic response of bone to mechanical load by focusing on the mechanisms responsible for the repression of phosphorylated Smad3 within 5h of mechanical stimulation.

A rapid increase in prostaglandin E2, or PGE2, is one of the first responses of osteocytes to mechanical stimulation. Through a variety of mechanisms, PGE2 leads to increased transcription of genes that elicit bone formation. PGE2 facilitates cell-cell communication by moving between cells through gap channels, like connexin43, to maintain bone homeostasis [9]. Fluid flow on osteocytes induces connexin43 translocation to the cell membrane, allowing for PGE2 to move into the adjacent cell [10]. PGE2 also acts extracellularly by binding to transmembrane receptors. PGE2 binding to the EP2 receptor in osteocytes stimulates PKA and PI3K signaling to repress the antagonist of Wnt/ β -catenin signaling, GSK3 β [11] [12]. PGE2 further induces Wnt signaling by repressing Sclerostin expression [13]. In addition, PGE2 produced from mechanically stimulated osteocytes protects osteocytes from glucocorticoid-induced apoptosis [14]. These are some of the mechanisms by which PGE2 rapidly coordinates the activity of multiple signaling pathways to maintain osteocyte function and bone homeostasis in response to mechanical load.

Although crosstalk between PGE2 and TGF β signaling is not defined in osteocytes, in other tissues PGE2 plays an important role in the control of matrix deposition and TGF β signaling. For example, PGE2 represses TGF β through an EP2/PKA-dependent mechanism to stimulate fibroblast activity [15]. PGE2-activated EP4 signaling in prostate cancer cells relies on TGF β signaling to stimulate cell migration and metastasis [16]. PGE2-activated EP2 regulates TGF β -induced mammary fibrosis and oncogenesis [17]. Since TGF β mediates PGE2 effects in multiple biological systems, we hypothesized that PGE2 regulates

TGF β p signaling in osteocytes, possibly as part of a signaling cascade that occurs rapidly after mechanical stimulation.

Among the possible mechanisms to rapidly reduce the level of signaling molecules is ubiquitin-dependent proteasomal degradation. In other cell types, this process has been implicated as a mechanosensitive mechanism. In muscle cells, the ubiquitin ligase, CHIP, marks damaged proteins for degradation when cells are subjected to tensional pull [18]. *C. elegans* utilizes the E3 ubiquitin ligase, MTB1, to direct the degradation of a mechanosensitive channel called MEC4, which allows the organism to sense touch [19]. However, mechanosensitive control of ubiquitin-dependent proteasomal activity in osteocytes has not been evaluated.

Ubiquitin is also an important regulatory mechanism in TGF β signaling and in bone homeostasis. In particular, CYLD is a deubiquitinase that indirectly targets Smad3 for degradation in lung cells to regulate matrix deposition and fibrosis [20]. In addition to regulating the TGF β pathway, CYLD targets NEMO, a subunit of IKK, and represses NF- κ B signaling [21][22]. The NF- κ B pathway is important for regulating RANK signaling in osteoclastogenesis. CYLD deficient mice have increased osteoclast numbers and activity and, consequently, low trabecular bone mass [23]. Since Smad3 is rapidly downregulated in mechanically-stimulated bone, we hypothesized that CYLD participates in load-mediated repression of TGF β signaling in osteocytes and in load-induced bone formation.

Although the importance of TGF β in osteocyte mechanotransduction is established, the mechanosensitive mechanisms controlling TGF β signaling in bone, particularly its interaction with PGE2, remain to be determined. The regulated turnover of signaling complexes is an essential mechanism for the control of signal transduction and cellular function. However, the role of ubiquitin-dependent proteasomal degradation in osteocyte mechanotransduction is not well-studied. Therefore, we investigated mechanosensitive mechanisms controlling Smad2/3 phosphorylation and load-induced bone formation, with a focus on PGE2 and the deubiquitinase CYLD.

2. Materials and Methods

2.1 Mice:

Treatments and protocols used for the animal studies were approved by the University of California, San Francisco Institution Animal Care and Use Committee and were designed to minimize discomfort to the animals. This study used 8-9 week-old male CYLD-deficient mice on a C57BL/6 background [24]. Wild type littermates were used as comparative controls.

2.2 In Vivo Mechanical Loading:

Axial compressive loads equivalent to 10 times the mouse's body weight were delivered by a Bose Electroforce ELF3200 desktop load frame (Bose, MN, USA) fitted with two custom-made hemi-spherical fixtures that gripped the mouse knee and ankle [8]. Similar methods of in vivo loading have been shown to upregulate bone anabolism [25]. In our analysis of these ex vivo limb loading conditions using in situ strain rosettes, this range of parameters

produces maximum principal strains in the range of 1500-2500 $\mu\epsilon$ on the mid-diaphyseal surface of the tibiae. For each mouse, only the right hind limb was loaded, while the left hind limb was not loaded to serve as the contralateral control (nonloaded). Each round of loading consisted of 600 cycles of axial compression at 1 Hz for 10 minutes administered under general injectable anesthesia (ketamine). The anesthesia recovery agent, atipamezole, was not used. Unless otherwise indicated, mice were subjected to this loading regimen once and were euthanized at the indicated time points after loading. For analysis of load-induced bone formation in CYLD-deficient mice, mice were loaded for 5 consecutive days as described below.

2.3 Histomorphometry:

Mice were loaded for 5 consecutive days (with one bout of loading each day) with calcein injected at day 1 and day 5 of the loading regimen. Two days after the last loading, mice were euthanized and the tibiae were collected and stored in 4% paraformaldehyde in PBS. Bones were processed and embedded in methyl methacrylate for sectioning to generate 5 μm sections for analysis of calcein labeling with an epifluorescent microscope at 20X magnification. Calcein labels were measured using ImageJ.

2.4 Cell Culture:

MLO-Y4 cells (gift from Dr. Lynda Bonewald), a mature mouse osteocyte cell line, were used for the in vitro studies. MLO-Y4 cells were cultured as described on plastic tissue culture plates coated with rat-tail collagen I (BD). These cells are cultured in 2.5% heat-inactivated Fetal Bovine Serum (Characterized, Hyclone) and 2.5% heat-inactivated Bovine Calf Serum (UCSF CCF) in alpha-MEM media containing nucleosides (UCSF CCF). At 80% confluence, MLO-Y4 cells were treated with the following reagents in serum-free media: 5 ng/ml TGF β 1 (Peprotech, 100-36E), 0.1 μM PGE2 (Sigma), 1 μM Butaprost (EP2 agonist, Cayman), 50 μM MG132 (proteasome inhibitor, Sigma) for the indicated times. All agents were resuspended in solvents suggested by product manufacturers. For controls, vehicle was added to cells in the same conditions.

2.5 siRNA or shRNA knockdown of CYLD:

CYLD knockdown was achieved using a stably expressed lentiviral pLKO.1 puro mouse CYLD shRNA construct (Sigma, MC5) and nontarget shRNA (Sigma, SHC002). Lentiviral vectors were packaged into the virus by the UCSF RNAi Core. MLOY4 cells were grown to 80% confluency and infected with CYLD or nontarget lentivirus. MLOY4 cells with the shRNA vectors were selected with 5 μM Puromycin and maintained under selection throughout the experiments. We were able to achieve about a 50% knockdown verified through qPCR of the nontarget and CYLD shRNA transfected cells.

CYLD knockdown using siRNA was achieved using mouse CYLD siRNA (AUAACUAGAUACGUAAGACUU) and scrambled (GUCUUACGUAUCUAGUUAUUU). siRNA oligos were transfected into MLOY4 cells using Lipofectamine2000. Cells were treated as indicated 48-72 hrs after transfection. RNA and protein were extracted as indicated.

2.6 Quantitative PCR:

RNA from the tibia bone was extracted by first removing the epiphyses, and subsequently the bone was centrifuged to remove the marrow. The bones were snap frozen in liquid nitrogen and pulverized with a mortar and pestle. Bone powder was transferred to Trizol (Invitrogen) and RNA was purified using the Invitrogen Purelink system according to the manufacturer's instructions. RNA was extracted from cells using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. RNA concentration was measured using a Nanodrop ND1000 Spectrophotometer. cDNA was generated from up to 1 µg of RNA with the iScript cDNA Synthesis kit (Bio-Rad). Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) with the primers on the table below. Each gene was normalized against the housekeeping gene, L19. Analysis used the $\Delta\Delta$ CT method to compare the load vs nonloaded samples from the same animal.

2.7 Western Blot Analysis:

Bone protein was isolated from liquid nitrogen snap frozen tibia by pulverizing the bone using a mortar and pestle and transferring the powder to radioimmunoprecipitation buffer (RIPA) containing 10 mM Tris, pH 8, 1 mM EDTA, 1 mM EGTA, 140 mM sodium chloride, 1% sodium pyrophosphate, 100 mM sodium fluoride, 500 µM PMSF, and 5 mg/mL eComplete Mini protease inhibitor tablet (Roche). Lysates were sonicated for 10 seconds 8 times in an ice-cold water bath and centrifuged to separate the protein extract. Bradford assay was used to quantify the amount of proteins and similar amount of proteins were loaded onto the gel. The protein supernatant was loaded onto a 10% SDS-PAGE gel, separated, and transferred to a nitrocellulose membrane. Similarly, MLO-Y4 whole cell lysate was collected in RIPA buffer, sonicated for 10 seconds 3 times in ice-cold water bath, for separation on a 10% SDS-PAGE as above. Westerns were probed using the following antibodies: mouse anti-beta actin (Abcam), rabbit anti-pSmad2/3 (gift from Dr. E. Leof), rabbit anti-Smad2/3 (Santa Cruz Biotech or abcam), rabbit anti-TβRI (Santa Cruz Biotech), and rabbit anti-CYLD (Sigma). Fluorescently-tagged secondary antibodies were used to detect the primary antibodies above and the blots were imaged on an Odyssey LiCor Imaging System. For pSmad2/3, this doublet of bands was quantified and analyzed together. The protein bands were normalized to the housekeeping protein, beta-actin, and the intensity was measured on the LiCor imaging software according to manufacture's instructions.

2.8 Statistical Analyses:

Statistics were performed using GraphPad Prism 5. For the animal studies, at least 5 animals were used for each analysis. For the in vitro studies, each experiment had two technical replicates and was repeated at least three times. T-tests were used to compare the differences between groups of normally distributed data. Nonparametric tests were used for non-normally distributed data as stated in each figure. Significance of comparisons is defined by p-values equal to or less than 0.05.

3. Results

3.1 Rapid post-transcriptional control of TGF β mechanosensitivity

We previously reported the rapid mechanosensitive repression of TGF β signaling within 5 hrs of in vivo hindlimb loading [8]. Although the mechanoregulation of TGF β signaling was essential for load-induced bone formation, the mechanisms responsible for TGF β mechanosensitivity in bone remain unclear. To elucidate these mechanisms, we examined the effect of short-term in vivo hindlimb loading on components of the TGF β signaling pathway, including TGF β ligands, receptors, Smads, and other known TGF β effectors. Consistent with our prior findings at 5 hrs after hindlimb loading [8], the levels of Smad2/3 phosphorylation were decreased at 3 hrs after load, relative to nonloaded tibiae (Figure 1A). T β RI protein levels were also reduced with load, but the protein reduction was not statistically significant as compared to pSmad2/3 levels. However, transcriptional analysis of mRNA encoding TGF β ligands (TGF β 1, TGF β 2, TGF β 3), receptors (T β RI and T β RII), and Smads (Smad3) show no significant differences between nonloaded and loaded tibiae (Figure 1B). Other factors implicated in the post-transcriptional regulation of TGF β signaling, including HtRA1, also show no load-dependent differences in mRNA expression (Figure 1B).

Interestingly, the expression of CYLD, a deubiquitinase implicated in TGF β signaling in the lung [20], was rapidly reduced by loading. Within 3 hrs of loading, CYLD protein levels were significantly reduced (Figure 1D). Although not statistically significant, a reduction in CYLD expression was consistently detected 1 hr after loading showed in the loaded limb. CYLD mRNA expression at 1 hr and 3 hr, however, were unchanged with load (Figure 1C). These findings, as well as the absence of transcriptional differences in TGF β signaling components at this time point, collectively focused our attention on post-transcriptional mechanisms responsible for TGF β mechanosensitivity in bone.

3.2 Prostaglandins repress Smad2/3 transcriptional activity

To further dissect the mechanisms involved in the mechanoregulation of TGF β signaling, we evaluated the regulation of TGF β signaling by prostaglandins in MLO-Y4 osteocyte-like cells. PGE2 is an established early mediator of mechanotransduction and is rapidly increased within 10 minutes of fluid flow stimulation in osteocytes [26]. Blocking PGE2 signaling with a chemical antagonist of the EP2 receptor, AH6809, inhibits downstream effects of fluid flow stimulation in osteocytes [21]. Since PGE2 can repress TGF β signaling in mammary tumor cells through EP2 [17], we hypothesized that PGE2 is sufficient to antagonize TGF β signaling in osteocytes.

To test this hypothesis, we treated MLO-Y4 cells with 5 μ M PGE2 for 3 hrs. Following TGF β stimulation, phosphorylated Smad2/3 activates transcription of TGF β target genes such as Serpine1 (Figure 2A). However, PGE2 significantly reduces TGF β -inducible Serpine1 mRNA expression (Figure 2A). PGE2 signals through EP2 and EP4 receptors. Butaprost, a pharmacologic agonist of the EP2 receptor, mimics the effects of PGE2 treatment by suppressing TGF β -inducible Serpine1 mRNA expression (Figure 2B). Similar effects were observed with an EP4 agonist (not shown).

3.3 Prostaglandins suppress CYLD and Smad2/3 phosphorylation through a proteasome-dependent mechanism

Since Smad2/3 phosphorylation and CYLD protein expression were regulated similarly in response to mechanical loading *in vivo*, we hypothesized that CYLD would also be regulated by prostaglandins *in vitro*. In the same conditions used to evaluate Smad2/3 phosphorylation, both PGE2 (Figure 3A) and the EP2 agonist (Figure 3B) reduced CYLD protein expression within 3 hrs. Therefore, prostaglandin signaling through EP2 receptors in cultured osteocytes is sufficient to repress Smad3 phosphorylation and CYLD expression in a manner that mimics the rapid repression in hindlimb-loaded bones.

The common regulation of Smad2/3 phosphorylation and CYLD expression by load in bone, and by prostaglandins in MLO-Y4 osteocyte-like cells, led us to investigate the epistatic interaction of Smad2/3 and CYLD. Since CYLD is a deubiquitinase that regulates proteolytic degradation [20], we tested the effect of proteasome inhibition on the regulation of Smad2/3 phosphorylation by TGF β and prostaglandins. As in Figure 2, prostaglandins block TGF β inducible Smad2/3 phosphorylation (Figure 3C). However, the inhibitory effect of prostaglandins on Smad2/3 phosphorylation is reversed in MLOY4 cells treated with the proteasome inhibitor MG132 (Figure 3C). This suggests that the repressive effect of prostaglandins on Smad2/3 phosphorylation in osteocytes relies on proteasomal activity.

3.4 CYLD is required for PGE/EP2 repression of TGF β signaling in osteocytes

To test the hypothesis that the deubiquitinase CYLD controls the level of Smad2/3 phosphorylation in osteocytes, we suppressed CYLD expression in MLO-Y4 cells. CYLD mRNA expression was 50% lower in MLO-Y4 cells transfected with CYLD siRNA, relative to cells transfected with a scrambled siRNA control (Figure 4A). Likewise, CYLD siRNA significantly reduced CYLD protein expression (Figure 4B) and increased the expression of CYLD-repressed genes, MMP2 and MCSF (Figure 4C) [28]. Using this approach, we found that CYLD is required for prostaglandin-dependent repression of Smad2/3 phosphorylation. As expected, the EP2 agonist significantly represses TGF β -inducible Smad2/3 phosphorylation in control siRNA-transfected MLOY4 cells (Figure 4D). However, this repression is lost in cells transfected with CYLD siRNA. Likewise, the ability of prostaglandins to block TGF β -inducible Serpine1 mRNA expression is abrogated in CYLD siRNA transfected cells (Figure 4E). Therefore, prostaglandins antagonize TGF β signaling through Smad2/3 in a CYLD-mediated mechanism in osteocytes.

3.5 CYLD is required for load-mediated repression of pSmad2/3 and load-induced bone formation

The rapid epistatic control of Smad2/3 by prostaglandins and CYLD led us to hypothesize that CYLD participates in the mechanosensitive control of TGF β signaling in bone anabolism. This hypothesis is consistent with the mechanosensitive repression of CYLD expression in loaded bone (Figure 1D). To test this hypothesis, we applied mechanical stimulation to tibiae of CYLD knockout mice and their wild type littermates [24]. As expected, within 3 hrs after mechanical load, loaded tibiae of wild type mice showed reduced levels of Smad2/3 phosphorylation relative to nonloaded contralateral tibia (Figure

5A). In CYLD-deficient mice, however, loading caused no significant changes in the level of Smad2/3 phosphorylation (Figure 5A).

Dynamic histomorphometry was used to test the functional effects of CYLD-deficiency on load-induced bone formation in wild type and CYLD-deficient mice. Calcein labeling showed an increase in bone formation in loaded tibiae of wild type mice, relative to the non-loaded contralateral tibia (Figure 5B). However, no further increase was observed in loaded tibiae of CYLD knockout mice compared to control (Fig. 5B). Accordingly, the matrix apposition rate (MAR) and the bone formation rate (BFR) are significantly increased in loaded bone of wild type mice compared to control but not in loaded tibiae of CYLD knockout mice compared to control (Figure 5C and 5D). No significant load-dependent differences in the mineralization surface were observed in either wild type or CYLD-deficient bones (Figure 5E). Together these data indicate that CYLD is necessary for the rapid mechanosensitive repression of Smad2/3 phosphorylation, as well as for load-induced bone formation.

4. Discussion

Here we describe a new mechanosensitive mechanism that regulates TGF β signaling in bone rapidly after hindlimb loading. TGF β -inducible phosphorylated Smad2/3 was repressed within 3 hrs after mechanical loading on the tibia. We found that prostaglandin signaling, acting through the EP2 receptor, is sufficient to repress pSmad2/3 and its transactivation of Serpine1 in osteocytes. PGE2 and EP2 suppress TGF β signaling through a proteasomal mechanism that depends on the deubiquitinase CYLD. Furthermore, CYLD is mechanosensitive in bone and is required for load-induced bone formation (Figure 6). To our knowledge, this is the first study to uncover CYLD mechanosensitivity and to implicate CYLD as a target of the PGE2 pathway.

TGF β signaling is tightly regulated by many physical cues, including compression, tension, substrate stiffness, and fluid flow shear stress [8] [29] [30] [31]. This regulation occurs at multiple levels of the TGF β pathway. In some cases, the same physical stimulus simultaneously exerts distinct and competing effects on TGF β ligands, receptors, and intracellular effectors. For example, after 6h of fluid flow shear stress, renal epithelial cells produce lower levels of total and active TGF β 1 ligand, but possess elevated levels of TGF β 1 mRNA and increased levels of phosphorylated Smad2/3 [32]. On the other hand, interstitial fluid flow on dermal fibroblasts for 2 hrs increases TGF β 1 secretion but also decreases T β RII protein expression [33]. With respect to bone, periosteal cells from rat hindlimbs possess increased levels of TGF β 1 mRNA expression within 4 hrs of 4-point bending [34]. In tibiae of loaded wild type mice, we find that TGF β ligand mRNA expression is not significantly altered, although pSmad2/3 is reduced 3 hrs after load. Consistent with evidence throughout the field of mechanobiology, the effect of physical cues on TGF β signaling depends on the cell type, the physical perturbation, the time points, and the cellular microenvironment, among other factors. However, the molecular mechanisms responsible for the diversity of these responses remain unclear. Within this context, we implicate a novel CYLD-dependent mechanism by which cells modulate TGF β signaling in response to physical cues.

Prostaglandin signaling is vital to maintaining bone homeostasis and it functions in many biological processes including mechanotransduction. This inflammatory mediator signals through four different G-protein receptors, EP1-4, with active EP2 and EP4 inducing bone formation [35] [36]. The role of prostaglandin signaling in bone and in mechanotransduction has been probed using pharmacologic agonists and antagonists of this pathway. For example, inhibiting PGE2 synthesis with Naproxen, an NSAID, impairs load-induced bone formation in mice and reduces NGF expression [37]. EP4 regulates crosstalk between the nervous system and bone formation [38] and the EP4 agonist, ONO4819, additively enhances load-mediated bone formation in rats [39]. In osteocytes, the cells that modulate the mechanoresponse in bone, fluid flow stimulation induces PGE2 and EP2 production [27] [14]. Since prostaglandin and TGF β signaling are both mechanosensitive in osteocytes, we hypothesized that prostaglandins may participate in the mechanoregulation of TGF β function in these cells [8]. We show here that prostaglandins can act through the EP2 receptor to repress TGF β signaling in osteocytes through a proteasome-mediated mechanism. Three hours after PGE2 treatment, PGE2-mediated repression of the deubiquitinase CYLD can be detected (Figure 3A), though this repression may be more apparent at earlier time points. In addition, the prostaglandin-dependent repression of pSmad2/3 functionally depends on CYLD. Depletion of CYLD levels blunts the prostaglandin-dependent repression of Smad2/3 phosphorylation in osteocytes (Figure 4D). Future in vitro studies with fluid flow shear stress will help to dissect the molecular epistasis of PGE2, EP2, CYLD, and TGF β signaling in osteocyte mechanotransduction, and could also identify the roles of the EP4 receptor and specific ubiquitin modifications in this process.

CYLD and TGF β signaling intersect at many levels. CYLD regulates the stability of T β RI, Smad7, and Smad3 [40] [20] [28]. In bone, CYLD inhibits osteoclastogenesis. With hyperresponsivity to RANKL, CYLD-deficient mice thus exhibit low trabecular bone volume, but no significant change in cortical bone volume [23]. We found that CYLD is repressed with mechanical stimulation and is important for load-mediated bone formation. This introduces CYLD as one of the few deubiquitinases to be mechanosensitive, including CHIP and MEC-4 [18] [19]. While we uncovered the role of CYLD in regulating TGF β signaling in mechanically stimulated osteocytes, CYLD regulates effectors of other pathways such as the Wnt pathway [41]. Studying how CYLD intersects with other mechanosensitive pathways can uncover how signaling is dampened and controlled for proper homeostasis. Further study of the role of CYLD in bone mechanobiology may reveal CYLD to be an attractive therapeutic target for osteoporosis.

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References

- [1]. Lang T, LeBlanc A, Evans H, Lu Y, Genant H, Yu A, Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight, *Journal of Bone and Mineral Research* 19(6) (2004) 1006–1012. [PubMed: 15125798]

- [2]. Gabel L, Macdonald HM, Nettlefold L, McKay HA, Physical Activity, Sedentary Time, and Bone Strength From Childhood to Early Adulthood: A Mixed Longitudinal HR-pQCT study, *Journal of Bone and Mineral Research* 32(7) (2017) 1525–1536. [PubMed: 28326606]
- [3]. Lescale C, Schenten V, Djeghloul D, Bennabi M, Gaignier F, Vandamme K, Strazielle C, Kuzniak I, Petite H, Dosquet C, Fripiat J-P, Goodhardt M, Hind limb unloading, a model of spaceflight conditions, leads to decreased B lymphopoiesis similar to aging, *Faseb Journal* 29(2) (2015) 455–463. [PubMed: 25376832]
- [4]. Black DM, Rosen CJ, Postmenopausal Osteoporosis, *New England Journal of Medicine* 374(3) (2016) 254–262. [PubMed: 26789873]
- [5]. Bonewald LF, The Amazing Osteocyte, *Journal of Bone and Mineral Research* 26(2) (2011) 229–238. [PubMed: 21254230]
- [6]. Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, Mantila SM, Gluhak-Heinrich J, Bellido TM, Harris SE, Turner CH, Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin, *Journal of Biological Chemistry* 283(9) (2008) 5866–5875. [PubMed: 18089564]
- [7]. Morse A, McDonald MM, Kelly NH, Melville KM, Schindeler A, Kramer I, Kneissel M, van der Meulen MCH, Little DG, Mechanical Load Increases in Bone Formation via a Sclerostin-Independent Pathway, *Journal of Bone and Mineral Research* 29(11) (2014) 2456–2467. [PubMed: 24821585]
- [8]. Nguyen J, Tang SY, Nguyen D, Alliston T, Load Regulates Bone Formation and Sclerostin Expression through a TGF beta-Dependent Mechanism, *Plos One* 8(1) (2013).
- [9]. Davis HM, Aref MW, Aguilar-Perez A, Pacheco-Costa R, Allen K, Valdez S, Herrera C, Atkinson EG, Mohammad A, Lopez D, Harris MA, Harris SE, Allen M, Bellido T, Plotkin LI, Cx43 overexpression in osteocytes prevents osteocyte apoptosis and preserves cortical bone quality in aging mice, *JBMR plus* 2(4) (2018) 206–216. [PubMed: 29978155]
- [10]. Siller-Jackson AJ, Burra S, Gu S, Xia X, Bonewald LF, Sprague E, Jiang JX, Adaptation of connexin 43-hemichannel prostaglandin release to mechanical loading, *Journal of Biological Chemistry* 283(39) (2008) 26374–26382. [PubMed: 18676366]
- [11]. Xia X, Batra N, Shi Q, Bonewald LF, Sprague E, Jiang JX, Prostaglandin Promotion of Osteocyte Gap Junction Function through Transcriptional Regulation of Connexin 43 by Glycogen Synthase Kinase 3/beta-Catenin Signaling, *Molecular and Cellular Biology* 30(1) (2010) 206–219. [PubMed: 19841066]
- [12]. Loisel AE, Lloyd SAJ, Paul EM, Lewis GS, Donahue HJ, Inhibition of GSK3 beta Rescues the Impairments in Bone Formation and Mechanical Properties Associated with Fracture Healing in Osteoblast Selective Connexin 43 Deficient Mice, *Plos One* 8(11) (2013) 10.
- [13]. Galea GL, Sunter A, Meakin LB, Zaman G, Sugiyama T, Lanyon LE, Price JS, Sost down-regulation by mechanical strain in human osteoblastic cells involves PGE2 signaling via EP4, *Febs Letters* 585(15) (2011) 2450–2454. [PubMed: 21723865]
- [14]. Kitase Y, Barragan L, Qing H, Kondoh S, Jiang JX, Johnson ML, Bonewald LF, Mechanical Induction of PGE(2) in Osteocytes Blocks Glucocorticoid-Induced Apoptosis Through Both the beta-Catenin and PKA Pathways, *Journal of Bone and Mineral Research* 25(12) (2010) 2381–2392.
- [15]. Penke LRK, Huang SK, White ES, Peters-Golden M, Prostaglandin E-2 Inhibits alpha-Smooth Muscle Actin Transcription during Myofibroblast Differentiation via Distinct Mechanisms of Modulation of Serum Response Factor and Myocardin-related Transcription Factor-A, *Journal of Biological Chemistry* 289(24) (2014) 17151–17162. [PubMed: 24802754]
- [16]. Vo BT, Morton D Jr., Komaragiri S, Millena AC, Leath C, Khan SA, TGF-beta Effects on Prostate Cancer Cell Migration and Invasion Are Mediated by PGE(2) through Activation of PI3K/AKT/mTOR Pathway, *Endocrinology* 154(5) (2013) 1768–1779. [PubMed: 23515290]
- [17]. Tian M, Schiemann WP, PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-beta signaling during mammary tumorigenesis, *Faseb Journal* 24(4) (2010) 1105–1116. [PubMed: 19897661]
- [18]. Ulbricht A, Eppler FJ, Tapia VE, van der Ven PFM, Hampe N, Hersch N, Vakeel P, Stadel D, Haas A, Saftig P, Behrends C, Fuerst DO, Volkmer R, Hoffmann B, Kolanus W, Hoehfeld J,

- Cellular Mechanotransduction Relies on Tension-Induced and Chaperone-Assisted Autophagy, *Current Biology* 23(5) (2013) 430–435. [PubMed: 23434281]
- [19]. Chen X, Chalfie M, Regulation of Mechanosensation in *C. elegans* through Ubiquitination of the MEC-4 Mechanotransduction Channel, *Journal of Neuroscience* 35(5) (2015) 2200–2212. [PubMed: 25653375]
- [20]. Lim JH, Jono H, Komatsu K, Woo C-H, Lee J, Miyata M, Matsuno T, Xu X, Huang Y, Zhang W, Park SH, Kim Y-I, Choi Y-D, Shen H, Heo K-S, Xu H, Bourne P, Koga T, Xu H, Yan C, Wang B, Chen L-F, Feng X-H, Li J-D, CYLD negatively regulates transforming growth factor-beta-signalling via deubiquitinating Akt, *Nature Communications* 3 (2012).
- [21]. Sun SC, CYLD: a tumor suppressor deubiquitinase regulating NF-kappa B activation and diverse biological processes, *Cell Death and Differentiation* 17(1) (2010) 25–34. [PubMed: 19373246]
- [22]. Massoumi R, Ubiquitin chain cleavage: CYLD at work, *Trends in Biochemical Sciences* 35(7) (2010) 392–399. [PubMed: 20347313]
- [23]. Jin W, Chang M, Paul EM, Babu G, Lee AJ, Reiley W, Wright A, Zhang M, You J, Sun S-C, Deubiquitinating enzyme CYLD negatively regulates RANK signaling and osteoclastogenesis in mice, *Journal of Clinical Investigation* 118(5) (2008) 1858–1866. [PubMed: 18382763]
- [24]. Massoumi R, Chmielarska K, Hennecke K, Pfeifer A, Faessler R, Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappa B signaling, *Cell* 125(4) (2006) 665–677. [PubMed: 16713561]
- [25]. Fritton JC, Myers ER, Wright TM, van der Meulen MCH, Loading induces site-specific increases in mineral content assessed by microcomputed tomography of the mouse tibia, *Bone* 36(6) (2005) 1030–1038. [PubMed: 15878316]
- [26]. Kleinnulend J, Vanderplas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH, SENSITIVITY OF OSTEOCYTES TO BIOMECHANICAL STRESS IN-VITRO, *Faseb Journal* 9(5) (1995) 441–445. [PubMed: 7896017]
- [27]. Cherian PP, Cheng BX, Gu SM, Sprague E, Bonewald LF, Jiang JX, Effects of mechanical strain on the function of gap junctions in osteocytes are mediated through the prostaglandin EP2 receptor, *Journal of Biological Chemistry* 278(44) (2003) 43146–43156. [PubMed: 12939279]
- [28]. Shinriki S, Jono H, Maeshiro M, Nakamura T, Guo J, Li J-D, Ueda M, Yoshida R, Shinohara M, Nakayama H, Matsui H, Ando Y, Loss of CYLD promotes cell invasion via ALK5 stabilization in oral squamous cell carcinoma, *Journal of Pathology* 244(3) (2018) 367–379. [PubMed: 29235674]
- [29]. Rys JP, Monteiro DA, Alliston T, Mechanobiology of TGF beta signaling in the skeleton, *Matrix Biology* 52-54 (2016) 413–425. [PubMed: 26877077]
- [30]. Allen JL, Cooke ME, Alliston T, ECM stiffness primes the TGF beta pathway to promote chondrocyte differentiation, *Molecular Biology of the Cell* 23(18) (2012) 3731–3742. [PubMed: 22833566]
- [31]. Klingberg F, Chow ML, Koehler A, Boo S, Buscemi L, Quinn TM, Costell M, Alman BA, Genot E, Hinz B, Prestress in the extracellular matrix sensitizes latent TGF-beta 1 for activation, *Journal of Cell Biology* 207(2) (2014) 283–297. [PubMed: 25332161]
- [32]. Kunnen SJ, Leonhard WN, Semeins C, Hawinkels LJAC, Poelma C, ten Dijke P, Bakker A, Hierck BP, Peters DJM, Fluid shear stress-induced TGF-beta/ALK5 signaling in renal epithelial cells is modulated by MEK1/2, *Cellular and Molecular Life Sciences* 74(12) (2017) 2283–2298. [PubMed: 28168444]
- [33]. Nithiananthan S, Crawford A, Knock JC, Lambert DW, Whawell SA, Physiological Fluid Flow Moderates Fibroblast Responses to TGF-beta 1, *Journal of Cellular Biochemistry* 118(4) (2017) 878–890. [PubMed: 27748539]
- [34]. Raabculen DM, Thiede MA, Petersen DN, Kimmel DB, Recker RR, MECHANICAL LOADING STIMULATES RAPID CHANGES IN PERIOSTEAL GENE-EXPRESSION, *Calcified Tissue International* 55(6) (1994) 473–478. [PubMed: 7895187]
- [35]. Yoshida K, Oida H, Kobayashi T, Maruyama T, Tanaka M, Katayama T, Yamaguchi K, Segi E, Tsuboyama T, Matsushita M, Ito K, Ito Y, Sugimoto Y, Ushikubi F, Ohuchida S, Kondo K, Nakamura T, Narumiya S, Stimulation of bone formation and prevention of bone loss by

- prostaglandin E EP4 receptor activation, Proceedings of the National Academy of Sciences of the United States of America 99(7) (2002) 4580–4585. [PubMed: 11917107]
- [36]. Paralkar VM, Borovecki F, Ke HZ, Cameron KO, Lefker B, Grasser WA, Owen TA, Li M, DaSilva-Jardine P, Zhou M, Dunn RL, Dumont F, Korsmeyer R, Krasney P, Brown TA, Plowchalk D, Vukicevic S, Thompson DD, An EP2 receptor-selective prostaglandin E-2 agonist induces bone healing, Proceedings of the National Academy of Sciences of the United States of America 100(11) (2003) 6736–6740. [PubMed: 12748385]
- [37]. Park J, Fertala A, Tomlinson RE, Naproxen impairs load-induced bone formation, reduces bone toughness, and diminishes woven bone formation following stress fracture in mice, Bone 124 (2019) 22–32. [PubMed: 30998998]
- [38]. Chen H, Hu B, Lv X, Zhu S, Zhen G, Wan M, Jain A, Gao B, Chai Y, Yang M, Wang X, Deng R, Wang L, Cao Y, Ni S, Liu S, Yuan W, Chen H, Dong X, Guan Y, Yang H, Cao X, Prostaglandin E2 mediates sensory nerve regulation of bone homeostasis, Nature Communications 10 (2019).
- [39]. Hagino H, Kuraoka M, Kameyama Y, Okano T, Teshima R, Effect of a selective agonist for prostaglandin E receptor subtype EN (ONO-4819) on the cortical bone response to mechanical loading, Bone 36(3) (2005) 444–453. [PubMed: 15777678]
- [40]. Zhao Y, Thornton AM, Kinney MC, Ma CA, Spinner JJ, Fuss IJ, Shevach EM, Jain A, The Deubiquitinase CYLD Targets Smad7 Protein to Regulate Transforming Growth Factor beta (TGF-beta) Signaling and the Development of Regulatory T Cells, Journal of Biological Chemistry 286(47) (2011) 40520–40530. [PubMed: 21931165]
- [41]. Tauriello DVF, Haegerbarth A, Kuper I, Edelmann MJ, Henraat M, Canninga-van Dijk MR, Kessler BM, Clevers H, Maurice MM, Loss of the Tumor Suppressor CYLD Enhances Wnt/beta-Catenin Signaling through K63-Linked Ubiquitination of Dvl, Molecular Cell 37(5) (2010) 607–619. [PubMed: 20227366]

Highlights

- The deubiquitinase, CYLD, is mechanosensitive in bone and is required for load-induced bone formation.
- Prostaglandins and the EP2 receptor represses TGF β signaling in osteocytes.
- CYLD mediates the prostaglandin-dependent repression of TGF β signaling in osteocytes.

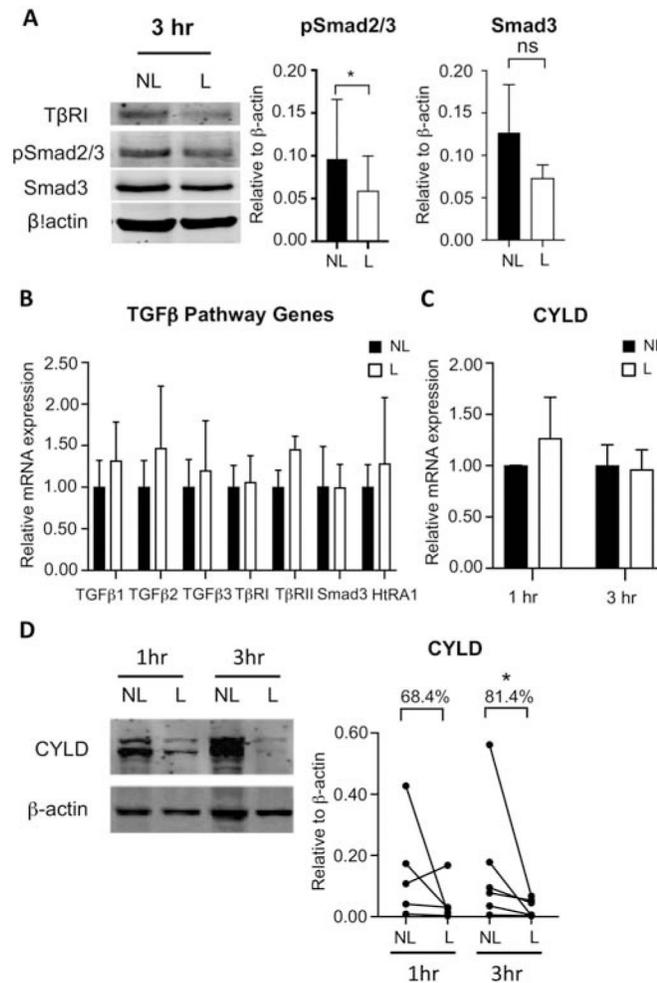


Figure 1: Rapid post-transcriptional control of TGFβ mechanosensitivity.

(A) Protein levels of TGFβ receptor type 1 (TβRI), phosphorylated Smad2/3 (pSmad2/3), and Smad3 are reduced in loaded tibiae as compared to nonloaded tibiae (n>6). Phosphorylated Smad3 is reduced by 25% in loaded tibiae at 3hr of the mechanical stimulation. Error bars shown are standard deviation and the non-parametric Wilcoxon test was used for statistical analysis (n>6, *p<0.05). (B) mRNA levels for TGFβ ligands, receptors, Smad3, and HtrA1 are not significantly regulated by load at 3 hr. Data shown are averages with standard error of the mean and the Wilcoxon test was used for statistical analysis (n>5). (C) CYLD mRNA expression is not changed at 1 hr or 3 hr after load (n=6). (D) CYLD protein levels are significantly reduced in loaded tibia 3 hr after stimulus. Error bars shown are standard deviation and the Wilcoxon test was used for statistical analysis (n=6, *p<0.05). The average percent repression of CYLD expression is indicated above the 1 hr and 3 hr bars.

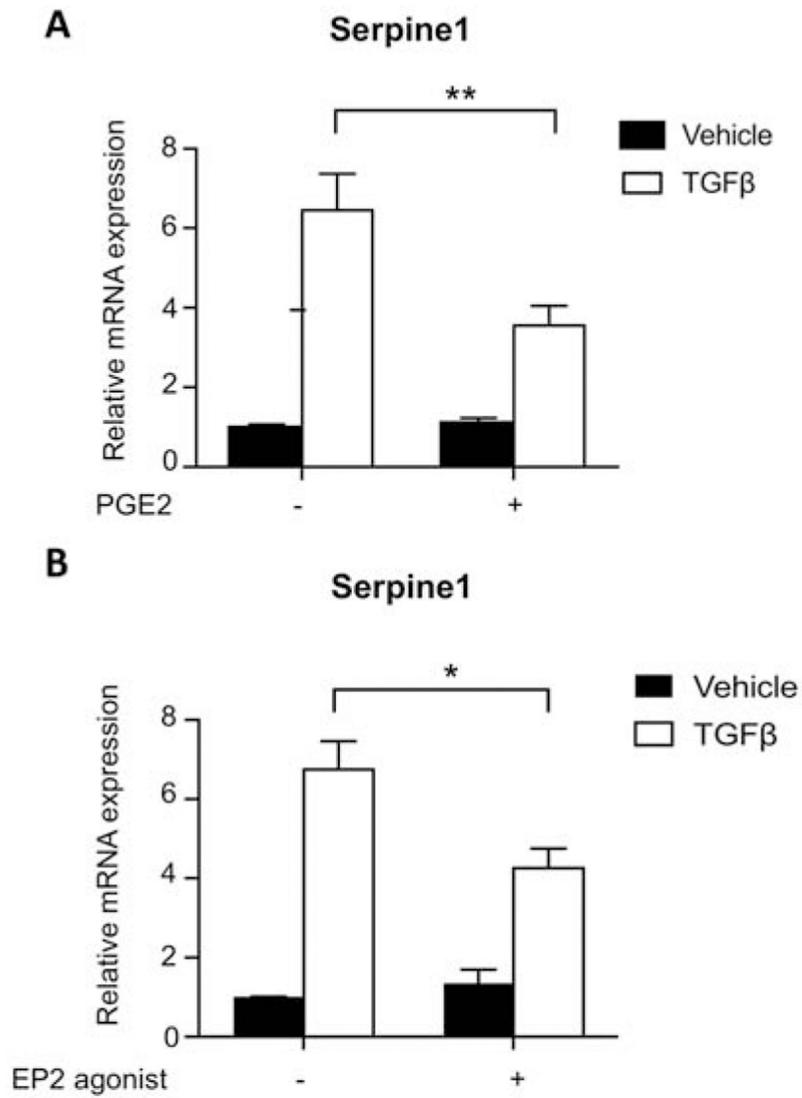


Figure 2: Prostaglandins repress Smad2/3 transcriptional activity. PGE2 inhibits the TGFβ responsive genes Serpine1 in MLOY4 cells within 3 hrs (A). Butaprost, an EP2 receptor agonist, also represses Serpine1 transactivation in MLOY4 cells within 3 hrs (B). All experiments were repeated at least three times, with two technical replicates for the RNA analysis. Standard error of the mean are shown and two-way ANOVA analysis was completed (* p<0.05, ** p<0.005).

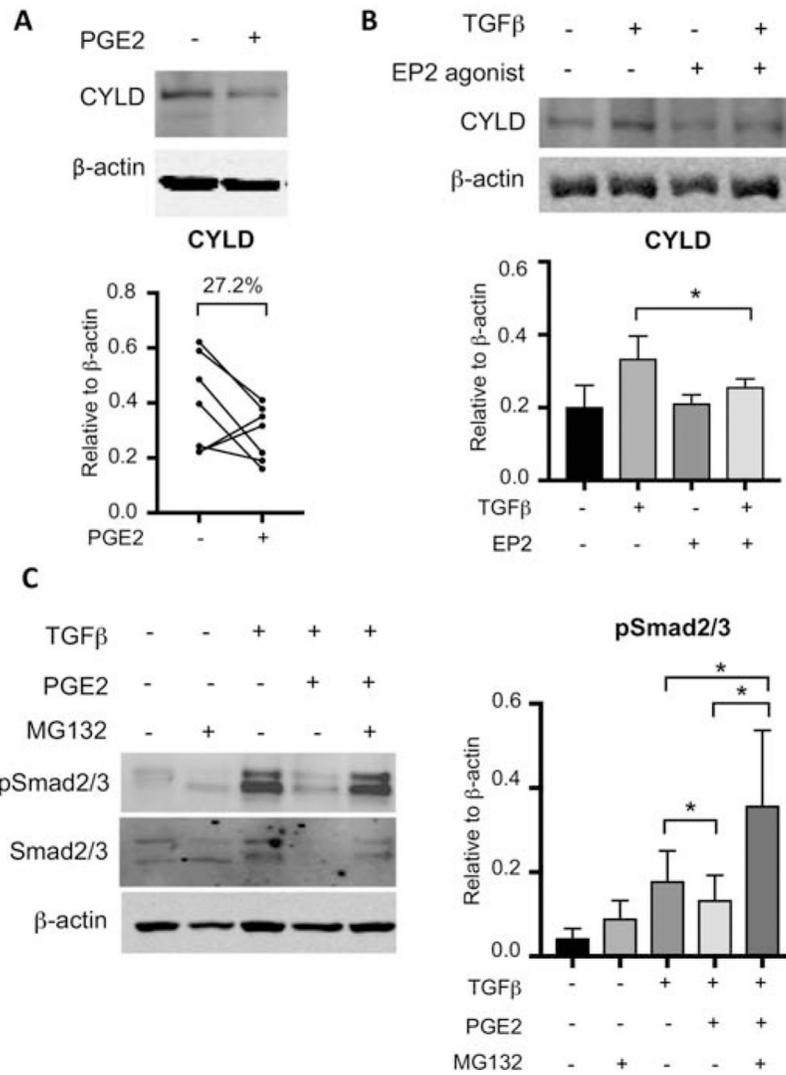


Figure 3: Prostaglandins suppress CYLD expression and Smad2/3 phosphorylation in a proteasome-dependent mechanism.

(A) PGE2 represses CYLD protein expression in MLOY4 cells within 3 hrs. The average percent repression is indicated above the data bar. (B) Stimulation of EP2 with butaprost for 3 hrs in MLOY4 cells down-regulates CYLD protein expression. (C) MG132, a proteasome inhibitor, stabilizes TGFβ-activated pSmad2/3 levels during a 3 hrs exposure to PGE2 in MLOY4 cells. Data shown are averages of at least three experiments with standard deviation shown (* p<0.05).

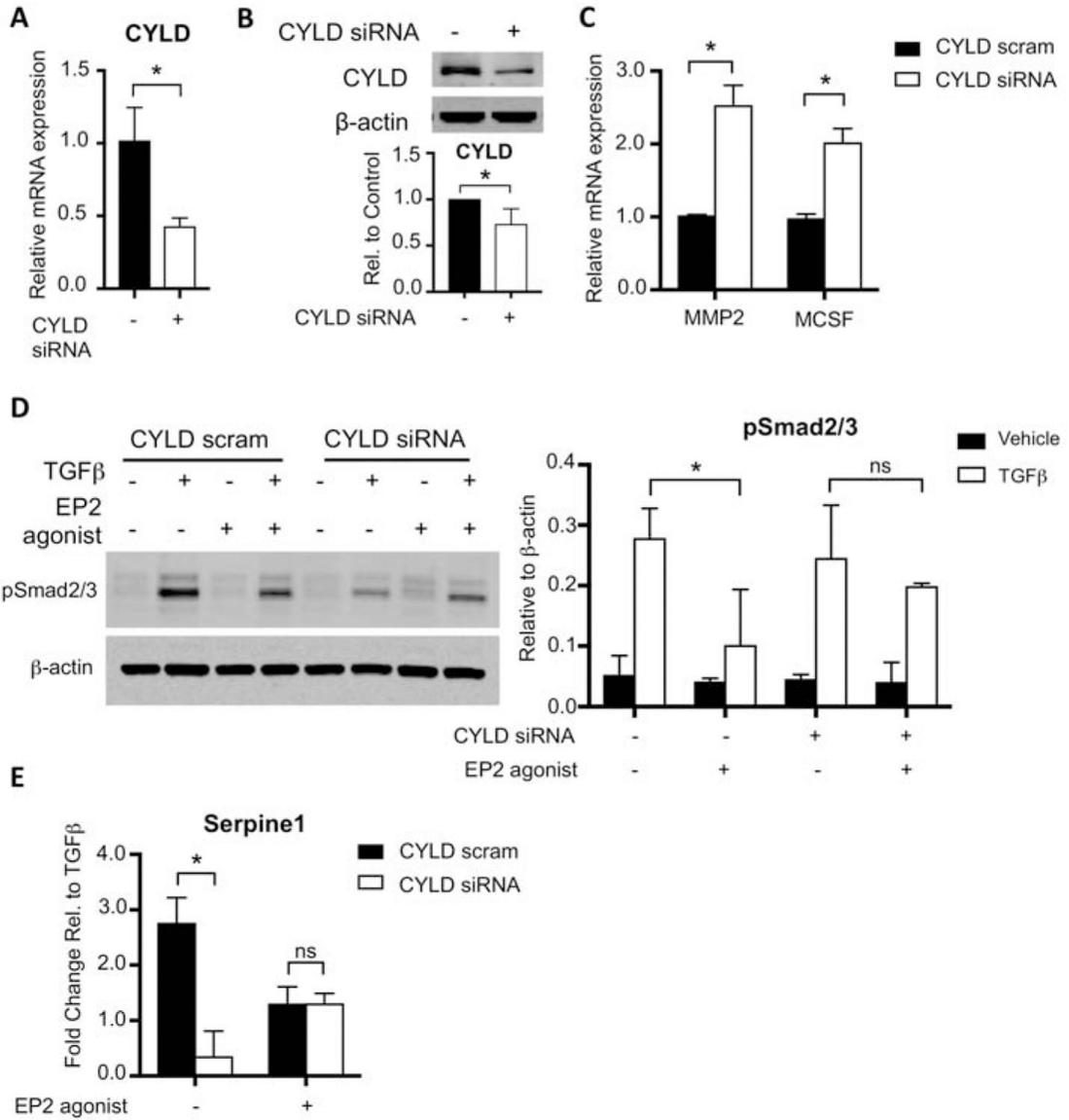


Figure 4: CYLD is required for PGE/EP2 repression of TGFβ activity in osteocytes. CYLD siRNA effectively reduced CYLD mRNA (A) and protein (B) expression relative to a scrambled control siRNA. CYLD protein expression is significantly reduced in MLOY4 cells containing CYLD siRNA (B). Relative to a scrambled control siRNA, siRNA for CYLD leads to increased expression of MMP2 and MCSF, known targets of CYLD repression (C). The EP2 agonist butaprost prevents TGFβ transactivation of pSmad2/3 in control siRNA MLOY4 cells, while CYLD siRNA rescues TGFβ-inducible Smad2/3 phosphorylation in the presence of EP2 stimulation (D). Likewise, reduced CYLD activity in CYLD siRNA MLOY4 cells prevents EP2-mediated repression of Serpine1 mRNA expression. This is a representative figure of at least three different experiments and average fold changes along with standard error of the mean are shown (E). All experiments were repeated at least three times and averages along with standard deviation (B, D) or standard error of the mean (A, C) are shown (p<0.05)

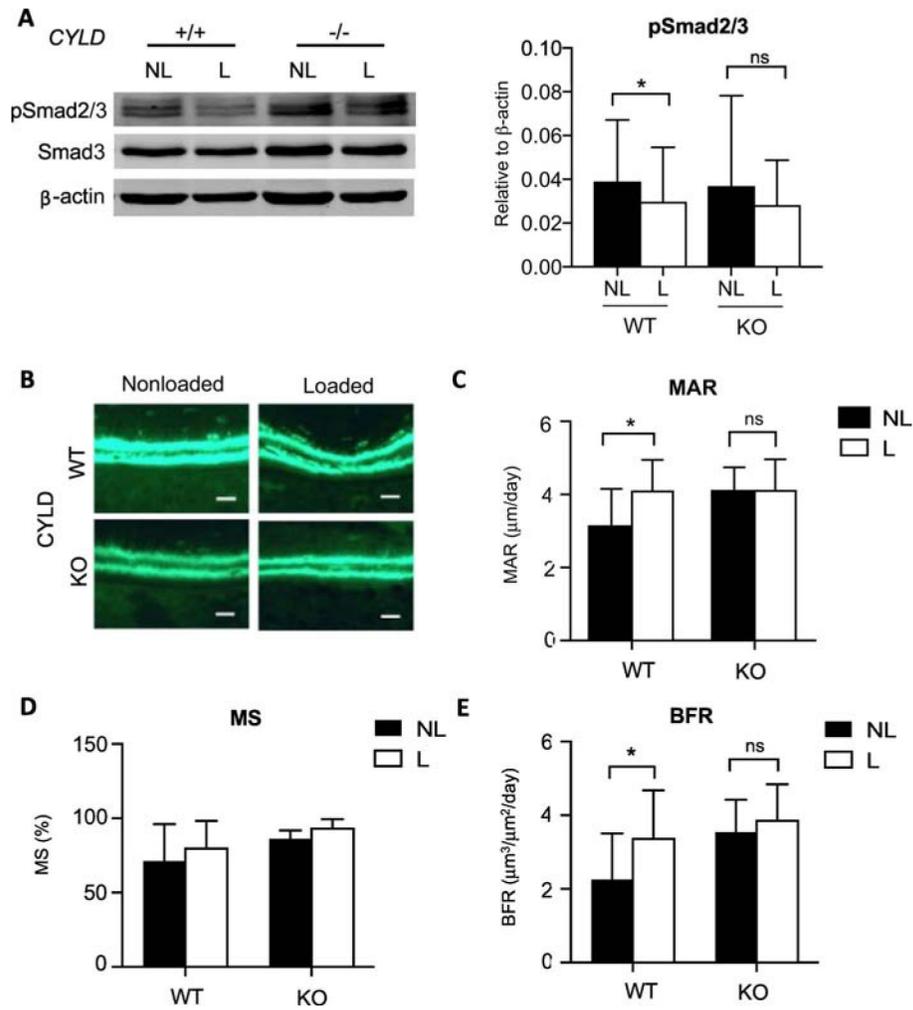


Figure 5: CYLD is required for load-mediated repression of pSmad2/3 and load-induced bone formation.

pSmad2/3 activation is reduced in tibia from loaded (L) relative to non-loaded limbs from wild type (+/+) mice, but not from their CYLD-deficient (-/-) littermates 3 hrs after loading (A). Data shown are averages along with standard deviation and the Wilcoxon test was used for statistical analysis (WT n=8, KO n=7, *p<0.05). Calcein labeling of newly formed bone shows a load-dependent increase in calcein incorporation in wild type mice, but not in CYLD-deficient littermates. Line indicates 20μm magnification. (B). Dynamic histomorphometry measurements of mineral apposition rate (MAR), mineralizing surfaces (MS), and bone formation rate (BFR) showed a significant increase in MAR and BFR in loaded tibiae of wild type mice, but not in loaded tibiae of their CYLD-deficient littermates (C-E). Data shown are averages along with standard deviation and paired t-test was used for statistical analysis (WT n=6, KO n=5, * p<0.05).

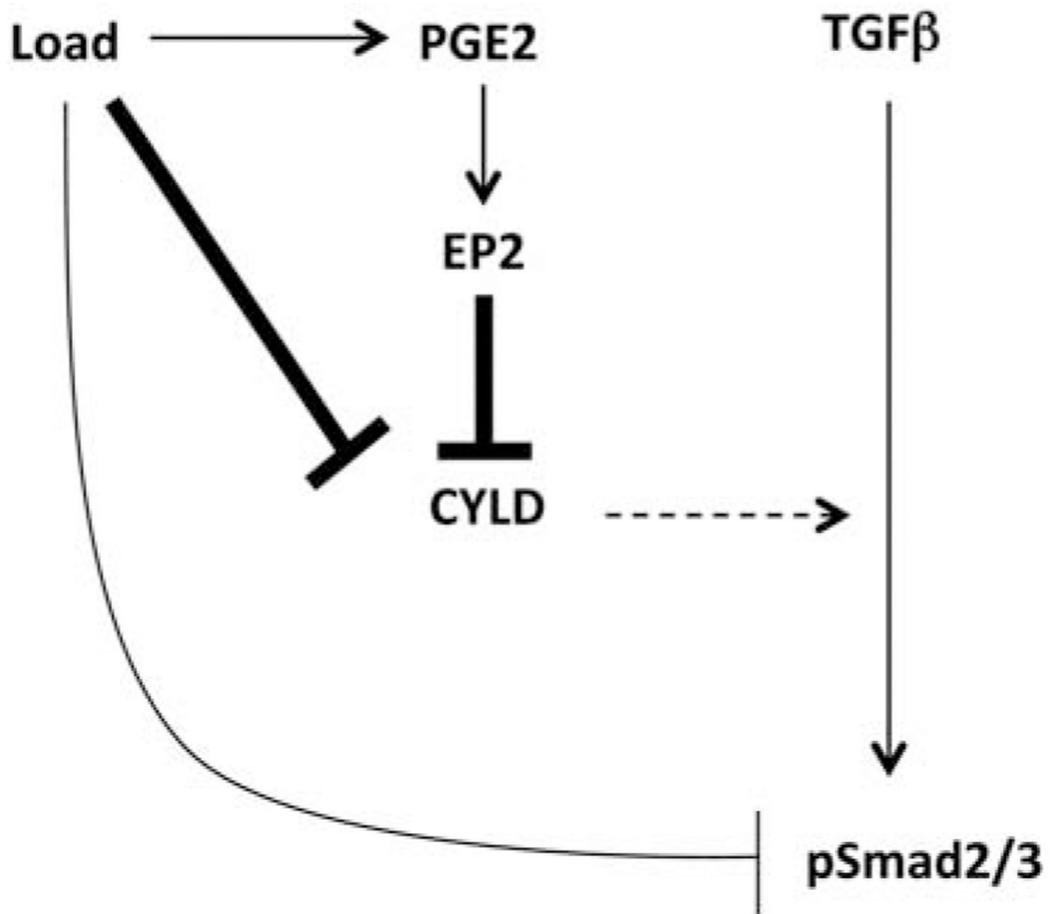


Figure 6: Mechanosensitive CYLD mediates PGE2 repression of TGFβ signaling. This study builds on our prior understanding of mechanisms by which load regulates TGFβ signaling in bone (light lines) by showing (bold lines) the sensitivity of CYLD expression to mechanical load and to prostaglandin signaling, and the requirement for CYLD in the regulation of Smad2/3 phosphorylation and load-induced bone formation.

Table 2.1:

Primers for QPCR of genes of interests.

Gene	Forward Sequence	Reverse Sequence
L19	ACGGCTTGCTGCCTTCGCAT	AGGAACCTTCTCTCGTCTTCCGGG
TGFβ1	AGCCCGAAGCGGACTACTAT	TCCCGAATGTCTGACGTATTG
TGFβ2	GAAATACGCCCAAGATCGAA	TGTCACCGTGATTTTCGTGT
TGFβ3	TGAATGGCTGTCTTTCGATG	ATTGGGCTGAAAGGTGTGAC
TβRI	TCGACGCTGTTCTATTGGTG	CAACCCATGGATCAGAAGGT
TβRII	AGGACCATCCATCCACTGAA	TGTCGCAAGTGGACAGTCCT
Smad3	TGAAGCGCTGCTGGGTTGG	GCTCGTCCAAGTCCCGGTC
CYLD	GGACAGTACATCCAAGACCG	GAACTGCATGCGGTTGCTC
Serpine1	AACCAATTTACTGAAAACTGCACAA	TCCGGTGGAGACATAACAGAT
HβRA1	CAGTCACCACTGGGATCGTC	ATCCCAATCACCTCGCCATC
MCSF	CCCATATTGCGACACCGAA	AAGCAGTAACTGAGCAACGGG
MMP2	GATAACCTGGATGCCGTCGTG	CTTCACGCTCTTGAGACTTTGGTTC