CYLD, a Mechanosensitive Deubiquitinase, Regulates TGFβ for Load-induced Bone Formation and Mediates PGE2-dependent Repression of TGFβ Signaling in Osteocytes

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Abstract

Osteoporosis is a bone degenerative disease that stems from an imbalance of bone formation and bone resorption, which must be coordinated to maintain bone homeostasis. Lack of mechanical stimulus on bone is a significant contribution to osteoporosis experienced by astronauts and bed-bound patients. Many pathways involved in bone homeostasis have been shown to regulate mechanotransduction in bone and specifically osteocytes, the central cell mediating bone mechanotransduction. TGFβ signaling is essential for coordinating osteoblast and osteoclast activities and is rapidly repressed in osteocytes within 5 hours following mechanical loading on the mice tibiae. PGE2 is a well-known mechanotransduction mediator in osteocytes that immediately responds to mechanical stimulus within 5 minutes. Although the importance of TGFβ and PGE2 in osteocyte mechanotransduction is established, the mechanism of TGFβ signaling in mechanosensitive bone remains to be determined, which may involve crosstalk with the PGE2 pathway. Here, we used an in vivo mice

hindlimb loading system to identify mechanosensitive molecules in the TGFβ pathway and determined the role of PGE2 in regulating TGFβ signaling. We found phosphorylated Smad3, an effector of TGFβ, is reduced as early as 3 hours after mechanical stimulus in the tibiae. PGE2 and its receptor, EP2, repress pSmad3 expression and activity on Pai1 transcription in osteocytes. Interestingly, PGE2 and EP2 regulate pSmad3 turnover through a proteasomal-dependent mechanism and in particular through CYLD, a deubiquitinase. CYLD protein level is reduced within 3 hours after mechanical loading in the tibiae and significantly CYLD is required for the rapid load-mediated repression of pSmad3 and load-induced bone formation. Our data introduces CYLD as a novel mechanosensitive deubiquitinase and provides a framework for PGE2 repression of TGFβ signaling in osteocytes. The data here suggests CYLD may be a plausible target for controlling mechanosensitive protein turnover in osteocytes and bone formation.

Introduction

Osteoporosis predisposes 125 million people worldwide to the risk of fractures and consequently lowers quality of life [1]. Mechanical loading on bone is critical for the maintenance of bone mass and prevention of low bone mass diseases such as osteoporosis. Evidently in astronauts, sedentary adults, mice with hindlimb unloading, the absence or reduction of mechanical stimulus resulted in deregulation of mineral homeostasis and bone loss [2] [3] [4]. Osteocytes are the centralized bone cells that sense the mechanical stimulus and transduce this signal to osteoblast and osteoclast to regulate their activity [5]. For example, mechanical loading represses the expression of

an osteocyte-secreted protein, called Sclerostin, which binds the Wnt coreceptor, Lrp5/6, on osteoblast and inhibits osteoblastic acitivity [6] [7]. Previously, we found that mice with reduced TGFβ signaling in osteocytes cannot relay the load-mediated repression of *SOST* expression and, consequently, have reduced load-induced bone formation [8]. Although we have shown that TGFβ is required for load-induced bone formation, the mechanism by which mechanical load regulates TGFβ signaling in osteocytes remains unclear.

Prostaglandins E2, or PGE2, is one of the early responses to mechanical stimulus in osteocytes and elicit signal transduction in these cells for cell-cell communication and ultimately transcription of genes that elicit bone formation. PGE2 facilitates cell-cell communication by moving between cells through gap channels, like connexin43. Fluid flow on osteocytes induces connexin43 translocation to the cell membrane allowing for PGE2 to move into the adjacent cell [9]. PGE2 can also act on the extracellular side and bind to the EP2 receptor leading to the activation of effectors PKA and PI3K to repress GSK3β, a Wnt effector, in osteocytes [10]. Another Wnt pathway molecule, Sclerostin, is repressed by PGE2 in cultured osteoblasts [11]. In addition, PGE2 produced from mechanically stimulated osteocytes protects osteocytes from glucocorticoid-induced apoptosis [12]. Clearly, PGE2 has a pleiotrophic effect on many pathways that maintain osteocyte viability and function.

Although crosstalk between PGE2 and TGFβ signaling is not defined in osteocytes, in other tissues PGE2 plays an important role in matrix deposition and TGEbeta signaling. For example, PGE2 represses TGFβ through an EP2/PKA mechanism to induce fibroblast activity [13]. PGE2-activated EP4 signaling in prostate

cancer cells is dependent on TGFβ to allow for migration and metastasis of these cancer cells [14]. PGE2-activated EP2 regulates TGFβ -induced mammary fibrosis and onocogenesis [15]. Since TGFβ mediates PGE2 effects in multiple biological systems, we hypothesized that PGE2 regulates TGFβ signaling in osteocytes and possibly during mechanical stimulus.

The rapid load-mediated downregulation of Smad3 phosphorylation in bone suggests a posttranslational mechanism that reduces TGFβ signaling in osteocytes. Protein degradation by ubiquitin-dependent proteasomal degradation is one method to rapidly reduce levels of signaling molecules. Furthermore, ubiquitin has previously been implicated as a mechanosensitive mechanism in other cell types. For example, in muscle cells, the ubiquitin ligase, CHIP, marks damaged proteins for degradation when cells are subjected to tensional pull [16]. *C. elegans* utilizes the E3 ubiquitin ligase, MTB1, to degrade a mechanosensitive channel called MEC4 allowing for the organism to sense touch [17]. Mechanosensitive control of ubiquitin in osteocyte has not been evaluated.

Ubiquitin is also an important regulatory mechanism in TGFβ signaling and bone homeostasis. In particular, CYLD is a deubiquitinase that indirectly targets Smad3 for degradation in lung cells to regulate matrix deposition and fibrosis [18]. In addition to regulating the TGFβ pathway, CYLD targets NEMO, a subunit of IKK, and represses NF-kB signaling [19]. The NF-kB pathway is important for regulating RANK signaling for osteoclastogenesis. CYLD deficient mice have increased osteoclast numbers and activities and, consequently, low bone mass [20]. Since Smad3 is rapidly downregulated in mechanically stimulated bone, we hypothesized that CYLD

participates in load-mediated repression of TGFβ signaling in osteocytes and in load-induced bone formation.

Although the importance of TGFβ and PGE2 in osteocyte mechanotransduction is established, the mechanism of TGFβ signaling in mechanosensitive bone remain to be determined, which may involve crosstalk with the PGE2 pathway. Signaling complexes turnover is essential to control signal transduction and cellular activities. However, the mechanisms of ubiquitin and protein turnover in osteocyte mechanotransduction are not well-studied. Therefore, examining the role of CYLD in controlling PGE2 and TGFβ pathways in osteocyte will uncover novel signaling mechanisms that act rapidly in osteocyte mechanotrandusction.

Materials and Methods

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 [21]. C57BL/6 wild type littermates were used as comparative controls.

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 [22]. 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 $\mu\varepsilon$ 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.

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.

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 TGFβ1 (Peprotech, 100-36E), 3.2uM Butaprost (EP2 agonist, Cayman), 10uM GWX627368X (EP4 antagonist, Cayman), 50uM MG132 (proteasome inhibitor, Sigma), 20uM AH6809 (EP2 antagonist, Cayman), or 20uM PGE1 alcohol (EP4 agonist, Cayman) for the indicated times. All agents were resuspended in solvents suggested by product manufacturers. Vehicle was added to cells in controls using the same conditions.

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 hours after transfection. RNA and protein were extracted as indicated.

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.

Table 2.1: Primers for QPCR of genes of interests.

Gene	Forward Sequence	Reverse Sequence
Mouse L19	ACGGCTTGCTGCCTTCGCAT	AGGAACCTTCTCTCGTCTTCCGGG
Mouse TGFβ1	AGCCCGAAGCGGACTACTAT	TCCCGAATGTCTGACGTATTG

Mouse TGFβ2	GAAATACGCCCAAGATCGAA	TGTCACCGTGATTTTCGTGT
Mouse TGFβ3	TGAATGGCTGTCTTTCGATG	ATTGGGCTGAAAGGTGTGAC
Mouse TβRI	TCGACGCTGTTCTATTGGTG	CAACCCATGGATCAGAAGGT
Mouse TβRII	AGGACCATCCATCCACTGAA	TGTCGCAAGTGGACAGTCCT
Mouse Smad3	TGAAGCGCCTGCTGGGTTGG	GCTCGTCCAACTGCCCCGTC
Mouse CYLD	GGACAGTACATCCAAGACCG	GAACTGCATGCGGTTGCTC
Mouse Pai1	AACCAATTTACTGAAAAACTGCACAA	TCCGGTGGAGACATAACAGAT
Mouse Htra1	CAGTCACCACTGGGATCGTC	ATCCCAATCACCTCGCCATC
Mouse MCSF	CCCATATTGCGACACCGAA	AAGCAGTAACTGAGCAACGGG
Mouse MMP2	GATAACCTGGATGCCGTCGTG	CTTCACGCTCTTGAGACTTTGGTTC

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-

pSmad3 (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. 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.

Statistical Analyses:

Statistics were performed using GraphPad Prism 5. For the animal studies, at

least 5 animals were used for the statistical 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.

Results

Rapid post-transcriptional control of TGFβ mechanosensitivity

We previously reported the rapid mechanosensitive repression of TGFβ signaling within 5 hours of in vivo hindlimb loading [8]. Although the mechanoregulation of TGFβ signaling was essential for load-induced bone formation, the mechanisms responsible for TGFβ mechanosensitivity in bone remain unclear. To elucidate these mechanisms, we examined the effect of short-term in vivo hindlimb loading on components of the TGFβ signaling pathway, including TGFβ ligands, receptors, Smads, and other known TGFβ effectors. Consistent with our prior findings at 5 hours after hindlimb loading [8], Smad 3 and Smad3 phosphorylation were decreased within 3 hours of load, relative to nonloaded tibiae (Figure 1A). However, transcriptional analysis of TGFβ ligands (TGFβ1, TGFβ2, TGFβ3), receptors (TβRI and TβRII), and Smads (Smad3) show no significant differences between nonloaded and loaded tibiae (Figure 1C). Other factors implicated in the post-transcriptional regulation of TGFβ signaling, including HtRA1 and CYLD, also show no load-dependent differences in mRNA expression (Figure 1C).

Interestingly, the expression of CYLD, a deubiquitinase implicated in TGFβ signaling in the lung [18], was significantly reduced by at least 50% within 3 hour of loading (Figure 1B). These findings, as well as the absence of transcriptional differences in TGFβ signaling components at this time point, collectively focused our attention on post-transcriptional mechanisms responsible for TGFβ mechanosensitivity in bone.

Prostaglandins repress Smad3 phosphorylation and CYLD expression in an EP2-dependent mechanism

To further dissect the mechanisms involved in the mechanoregulation of TGFβ signaling, we evaluated the regulation of TGFβ signaling by prostaglandins in MLO-Y4 osteocyte-like cells. PGE2 is an established early mediator of mechanotransduction in osteocyte and is rapidly increased within 10 minutes of fluid flow stimulation in osteocytes [23]. Blocking EP2 receptor by the antagonist, AH6809, inhibits fluid flow stimulated PGE2 activity in osteocytes [24]. In addition, PGE2 represses TGFβ signaling in mammary tumor cells through EP2 for tumorigenesis [15].

To determine the role of prostaglandins in TGFβ signaling in osteocytes, we treated MLO-Y4 cells with 5μM PGE2 for 3 hours. While TGFβ stimulates a robust induction of Smad3 phosphorylation in MLO-Y4 cells, PGE2 treatment suppresses both basal and TGFβ-inducible Smad3 phosphorylation (Figure 2A). Following TGFβ stimulation, phosphorylated Smad3 activates transcription of TGFβ target genes such as Serpine1, Pai1, (Figure 2B). However, PGE2 significantly reduces TGFβ-inducible Serpine1 mRNA expression. Prostaglandins signal through EP2. Butaprost, a pharmacologic agonist of the EP2 receptor, mimics the effects of PGE2 treatment, repressing basal and TGFβ-inducible Smad3 phosphorylation (Figure 2C), as well as suppressing TGFβ-inducble Serpine1 mRNA expression (Figure 2D).

Prostaglandins suppress Smad3 phosphorylation in a ubiqutination-dependent mechanism

The common regulation of Smad3 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 Smad3 and CYLD. Since CYLD is a deubiquitinase that regulates proteolytic degradation [18], we tested the effect of proteosome inhibition on the regulation of Smad3 phosphorylation by TGFβ and prostaglandins. As in Figure 2, prostaglandins block TGFβ inducible Smad3 phosphorylation (Figure 3A). However, the inhibitory effect of prostaglandins on Smad3 phosphorylation is reversed in MLO-Y4 cells treated with the proteasome inhibitor MG132 (Figure 3A). This suggests that prostaglandin repression of Smad3 phosphorylation in osteocytes relies on proteosomal activity.

Since Smad3 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 Smad3 phosphorylation, both PGE2 (Figure 3B) and the EP2 agonist (Figure 3C) reduced CYLD protein expression within 3 hour, in basal conditions as well as in the presence of TGFβ. Therefore, prostaglandin signaling through EP2 receptors in cultured osteocytes is sufficient to mimic the rapid repression of Smad3 phosphorylation and CYLD expression in hindlimb-loaded bones.

CYLD is required for PGE2/EP2 repression of TGFβ signaling in osteocytes

To test the hypothesis that the deubiquitinase CYLD controls the level of Smad3 phosphorylation in osteocytes, we ablated 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) [25]. We tested the hypothesis that CYLD is required for prostaglandin-dependent repression of Smad3 phosphorylation. As expected, the EP2 agonist significantly represses TGFβ-inducible Smad3 phosphorylation in control siRNA-transfected MLO-Y4 cells (Figure 4D). However, this repression is lost in cells with reduced CYLD activity due to CYLD siRNA transfection. Likewise, the ability of prostaglandins to block TGFβ-inducible Serpine1 mRNA expression is abrogated in CYLD siRNA transfected cells (Figure 4E). Therefore, prostaglandins antagonize TGFβ signaling through Smad3 in a CYLD-mediated mechanism in osteocytes.

CYLD is required for load-mediated repression of pSmad3 and load-induced bone formation

The rapid epistatic control of Smad3 by prostaglandins and CYLD led us to hypothesize that CYLD is required for mechanosensitive control of TGFβ signaling in bone anabolism. This hypothesis is consistent with the mechanosensitive repression of CYLD expression in loaded bone (Figure 1C). To test this hypothesis, we applied mechanical stimulation to tibiae of CYLD knockout mice and the wild type littermates

[21]. As expected, within 3 hour after mechanical load, loaded tibiae of wild type mice showed reduced levels of Smad3 phosphorylation relative to nonloaded contralateral tibia. In CYLD-deficient mice, however, loading caused no significant changes in the level of Smad3 phosphorylation (Figure 5A and 5B).

To test the functional effects of CYLD-deficiency, histomorphometric analysis were used to assess the effect of load on 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 5C). However, no further increase was observed in loaded tibiae of CYLD knockout mice compared to control. 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 5D and 5F). 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 Smad3 phosphorylation, as well as for load-induced bone formation.

Discussion

In summary, we found that TGFβ signaling is mechanoregulated as early as 3 hour after load. TGFβ-inducible phosphorylated Smad3 is repressed at the protein level within 3 hour after mechanical loading on the tibia. This indicates a post-translational

mechanism of load-mediated repression of TGFβ signaling and we further investigated this possibility in osteocytes. We found that PGE2 and EP2 repress pSmad3 and the downstream Pai1 expression in osteocytes. PGE2 and EP2 destabilize TGFβ signaling through a proteasomal-mediated mechanism specifically by CYLD, a deubiquitinase. Furthermore, CYLD is mechanosensitive in bone and is critical for load-induced bone formation (Figure 6).

Mechanical stimulation by compression, tension, fluid flow shear stress, and other physical cues regulates TGFβ signaling in many biological systems. Fluid flow shear stress in particular exerts diverse effects on TGFβ signaling. In renal epithelial cells flow reduces protein expression of total and active TGFβ ligand, but induces Smad2/3 activation within 6 hours of stimulus [26]. Interstitial fluid flow on dermal fibroblasts for 2 hours increases TGFβ1 secretion but also decreases TβRII protein expression [27]. Periosteal cells in rat hindlimb subjected to 4-point bending had an increase in TGFβ1 mRNA expression 4 hours after mechanical load application [28]. We showed in the tibiae of loaded wild type mice TGFβ ligands mRNA expressions are not significantly altered although pSmad3 is reduced 3 hours after load. Our data provide another evidence that mechanical stimulation rapidly and transiently affect TGFβ pathway components.

PGE2 is vital to maintaining bone homeostasis and so it functions in many biological processes including mechanotransduction. The inflammatory mediator signals through four different G-protein receptors, EP1-4, with active EP2 and EP4 inducing bone formation [29] [30]. Inhibiting PGE2 synthesis with Naproxen, an NSAID, treatment in mice impairs load-induced bone formation and reduces NGF expression

[31]. EP4 functions downstream of PGE2 to regulate processes for bone homeostasis including crosstalk between the nervous system and bone formation [32]. However, EP4 agonist, ONO4819, additively enhances load-mediated bone formation in rats [33]. In osteocytes, the centralized cells that modulate mechanoresponse in bone, fluid flow stimulation induces PGE2 and EP2 production [24] [12]. EP2 and TGFβ are both mechanosensitive in osteocytes and this led us to hypothesize that EP2 and TGFβ may crosstalk in these cells [8]. We showed here that PGE2 and EP2 repress TGFβ signaling in osteocytes through a proteasomal-mediated mechanism specifically through CYLD activity. Further study on the dependency of TGFβ signaling in EP2-mediated mechanotransduction in osteocytes and bone would definitively align these two pathways in osteocyte-dependent mechanoregulation.

CYLD and TGFβ signaling intersect at many levels. CYLD regulates the stability of TβRI, Smad7, and Smad3 [34] [18] [25]. In bone, CYLD inhibits osteoclastogenesis and CYLD knockout mice were reported to be sensitive to RANKL-induced osteoclastogenesis thus exhibit low trabecular bone mass but only with slight reduction in cortical bone mass and no change in BV/TV [20]. Our findings showed that CYLD is repressed with load stimulation and is important for load-mediated bone formation in the cortical bone by dynamic histomorphometry. This introduces CYLD as one of the few deubiquitnases to be mechanosenstive, including CHIP and MEC-4, and further study investigating the mechanism of mechanical-dependent regulation of CYLD may suggest CYLD as a possible therapeutic target for osteoporosis [16] [17] . While we uncovered the role of CYLD in regulating TGFβ signaling in mechanically stimulated osteocytes, CYLD regulates effectors of other pathways such as the Wnt pathway [35]. Studying

how CYLD intersects with other mechanosensitive pathways can uncover how signaling is dampened and controlled for proper homeostasis.

To our knowledge, this is the first study to uncover CYLD mechanosensitivity in bone and provide insights into CYLD's role in the PGE2 pathway. Future studies elucidating CYLD rapid activity in osteocyte-dependent mechanotransduction may support CYLD as a target for osteoporosis treatment.

Figure Legends

Figure 1: Rapid post-transcriptional control of TGFβ mechanosensitivity. (A) Protein levels of TGFβ receptor type 1 (TβRI), phosphorylated Smad3, and Smad3 are reduced in loaded tibiae as compared to nonloaded tibiae (n>6). Activated Smad3 are reduced by 25% in loaded tibiae within 3hr of the mechanical stimulation (n>6, *p<0.05). CYLD is reduced in loaded tibia 3 hours after stimulus (n>6, *p<0.05) (B). (C) TGFβ ligands, receptors, and Smad3 mRNA levels are not significantly regulated by load within 3hr (n>5).

Figure 2: Prostaglandins repress Smad3 phosphorylation and CYLD expression in an EP2-dependent mechanism. PGE2 inhibits pSmad3 and TGFβ responsive genes PAi1 in MLOY4 cells (A-B). Butaprost, an EP2 receptor agonist, also represses pSmad3 and Pai1 transcriptional activity in MLOY4 cells (C-D). All experiments were repeated at least three times and two technical replicates for the RNA analysis (* p<0.05).

Figure 3: Prostaglandins suppress Smad3 phosphorylation in a ubiqutination-dependent mechanism. MG132, a proteasome inhibitor, stabilizes TGFβ-activated pSmad3 expression in the presence of PGE2 in osteocytes (* p<0.05) (A). PGE2 represses CYLD protein expression in MLOY4 cells (B). Stimulation of EP2 in MOY4 cells also down-regulates CYLD protein expression in MLOY4 cells (C) (* p<0.05).

Figure 4: CYLD is required for PGE/EP2 repression of TGFβ in osteocytes. CYLD siRNA effectively knockdown CYLD mRNA expression by at least 50% (A). CYLD protein expression is significantly reduced in MLOY4 cells containing CYLD siRNA (B). Knockdown of CYLD leads to increase in MMP2 and MCSF (p<0.05) (C). EP2 prevents TGFβ transactivation of pSmad3 in control MLOY4 cells, while knockdown of CYLD rescues TGFβ phosphorylation of Smad3 even in the presence of EP2 stimuation (D). Likewise, reduced CYLD activity in CYLD siRNA MLOY4 cells alleviates EP2-mediated repression of Pai1 mRNA expression (E).

Figure 5: CYLD is required for load-mediated repression of pSmad3 and load-induced bone formation. pSmad3 activation is reduced in loaded wild type control mice, but pSmad3 in CYLD mutants is non-responsive to mechanical stimulus on the tibia (A). Quantification of pSmad3 protein bands in wild type and CYLD mutant mice in response to load shows pSmad3 expression is significantly reduced, while in the CYLD knockout mice pSmad3 expression remains unchanged in the presence of load (B). Calcein labeling of newly formed bone with response to load in wild type and CYLD

knockout mice tibae shows dramatic increase in calcein incorporation in stimulated wild type mice but no clear increase in CYLD knockout mice (C). 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 tibae of CYLD knockout mice (D-E) (* $p<0.05$).

Figure 6: Mechanosensitive CYLD mediates PGE2 repression of TGFβ signaling.
Load represses pSmad3 and CYLD expressions in bone, while stimulating PGE2 production. PGE2 signals through EP2 in osteocytes to repress CYLD for proteasomal-dependent repression of pSmad3.

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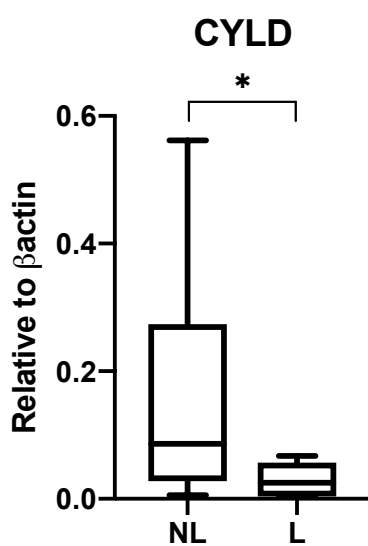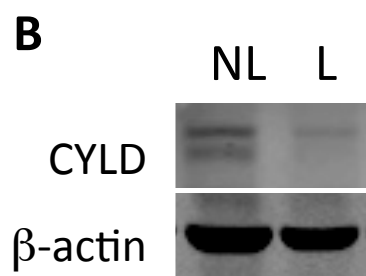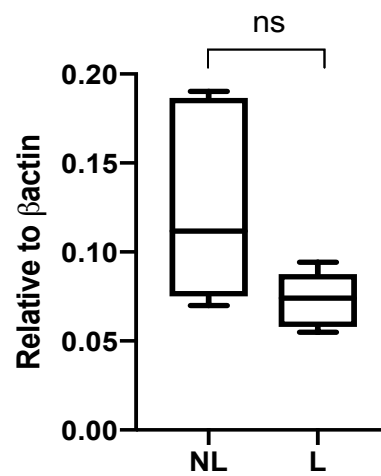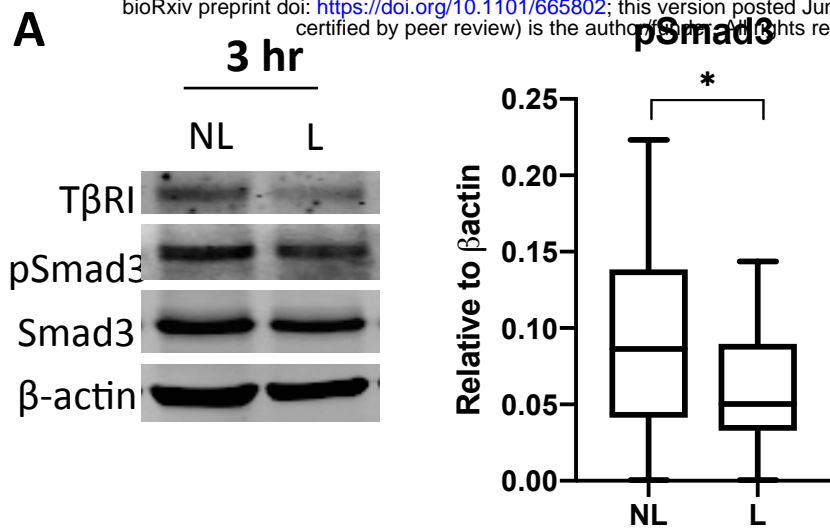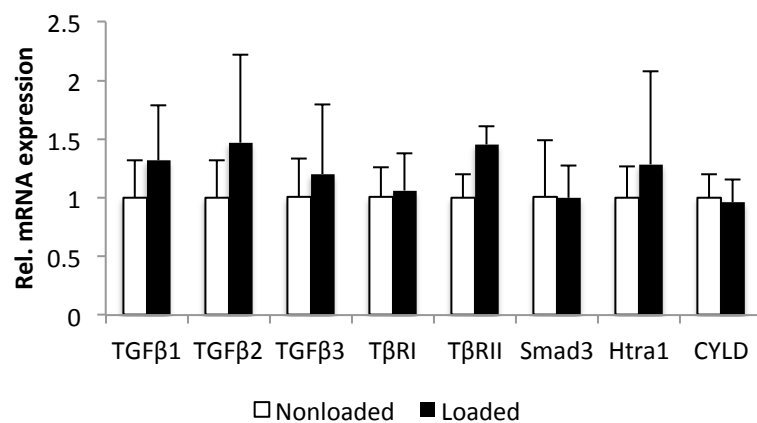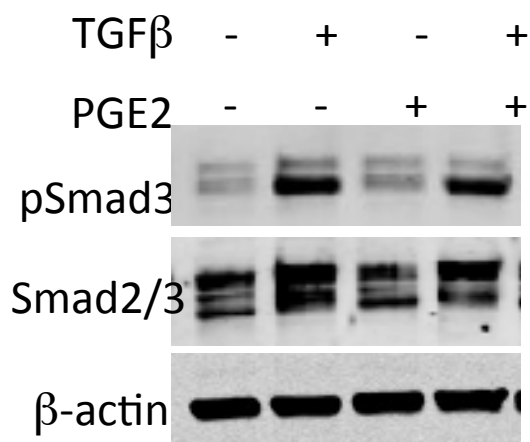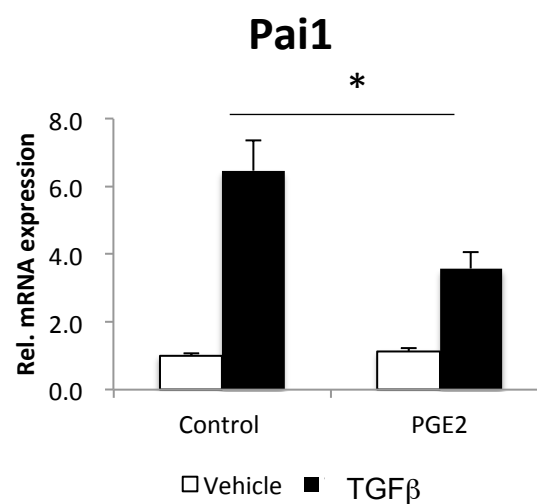
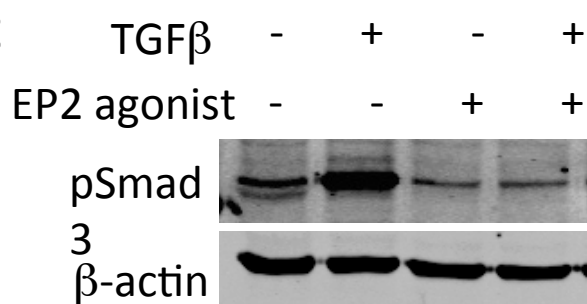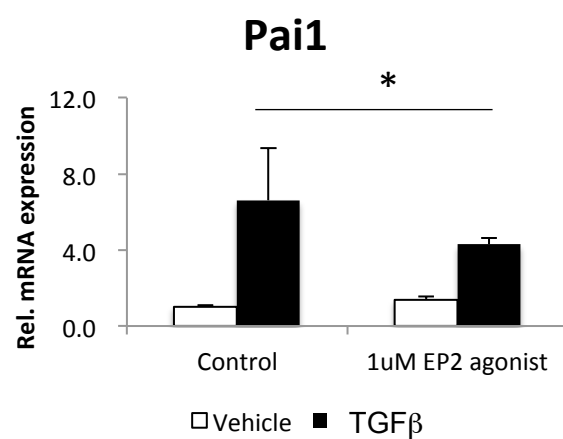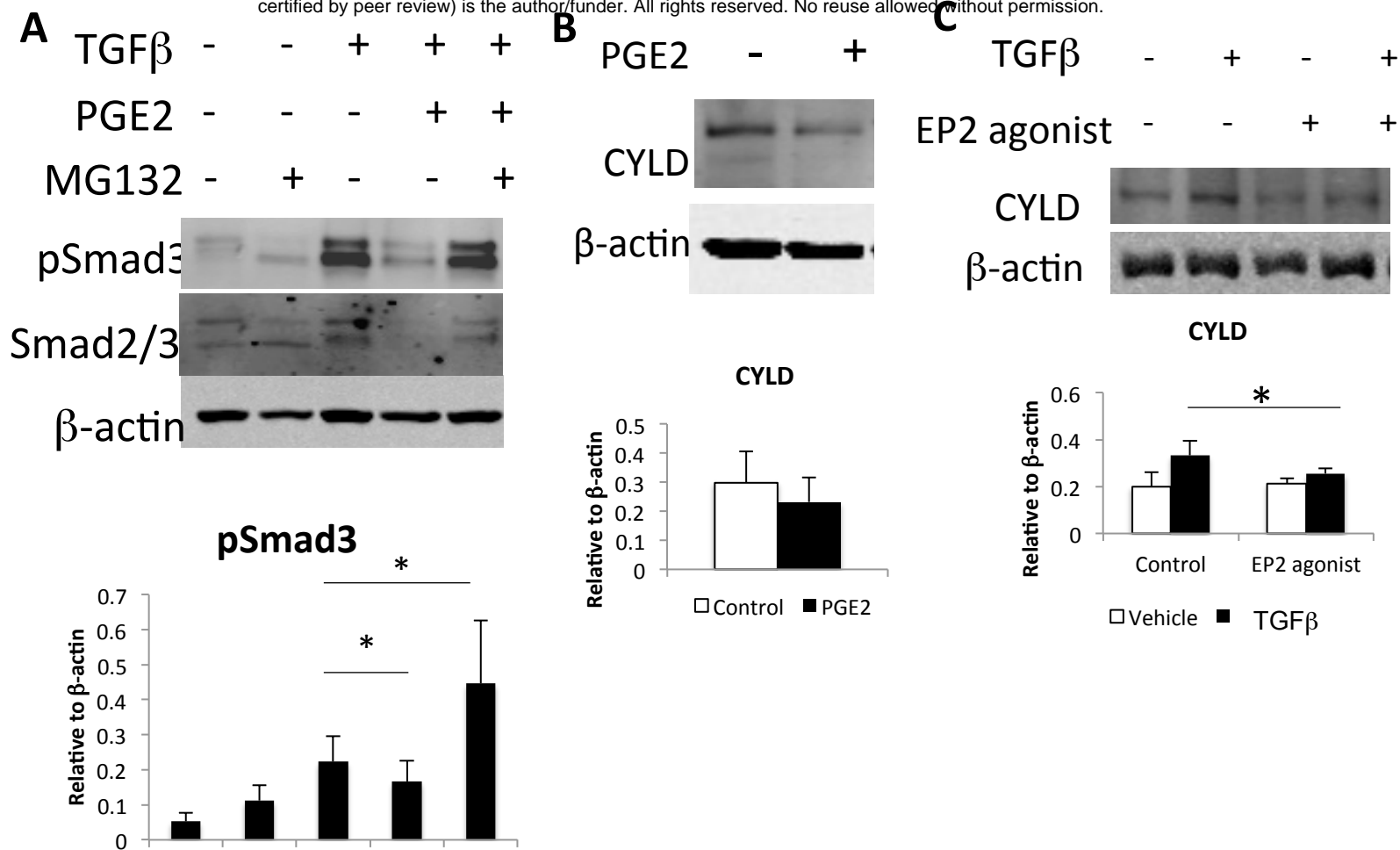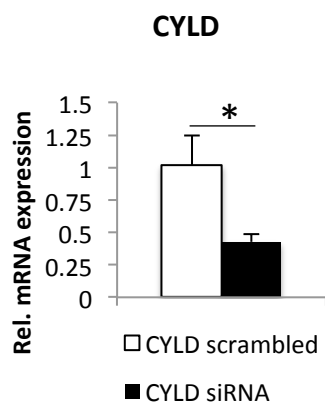**Figure 1: Rapid post-transcriptional control of TGFβ mechanosensitivity**

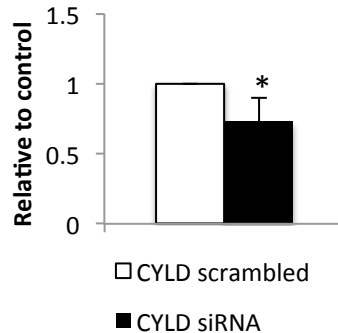**Figure 2: Prostaglandins repress Smad3 phosphorylation and CYLD expression in an EP2-dependent mechanism**

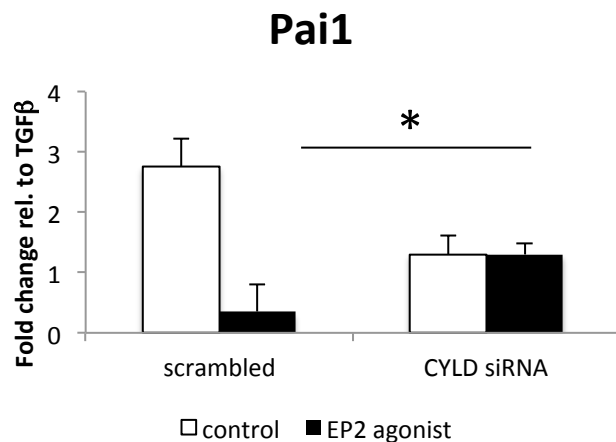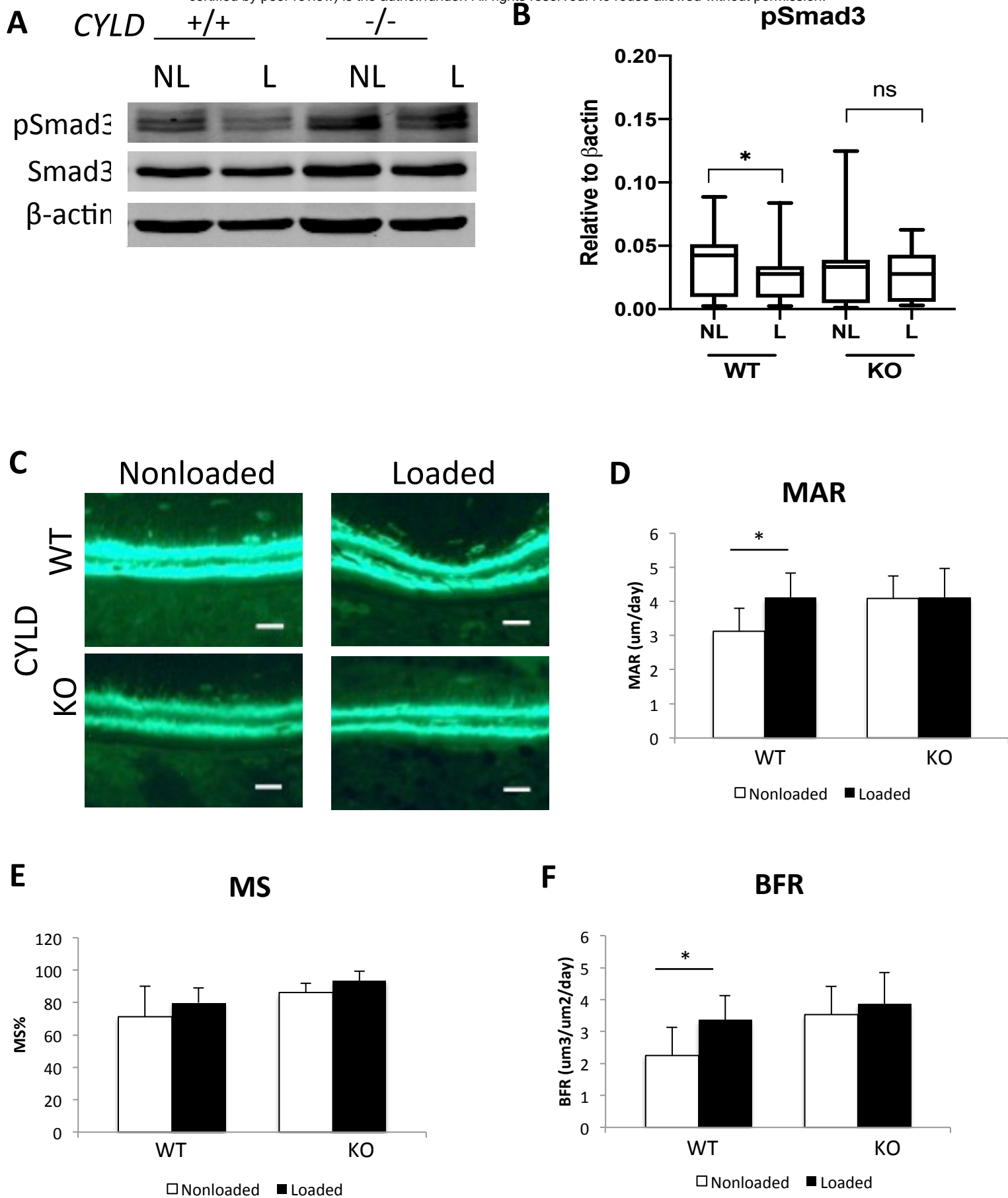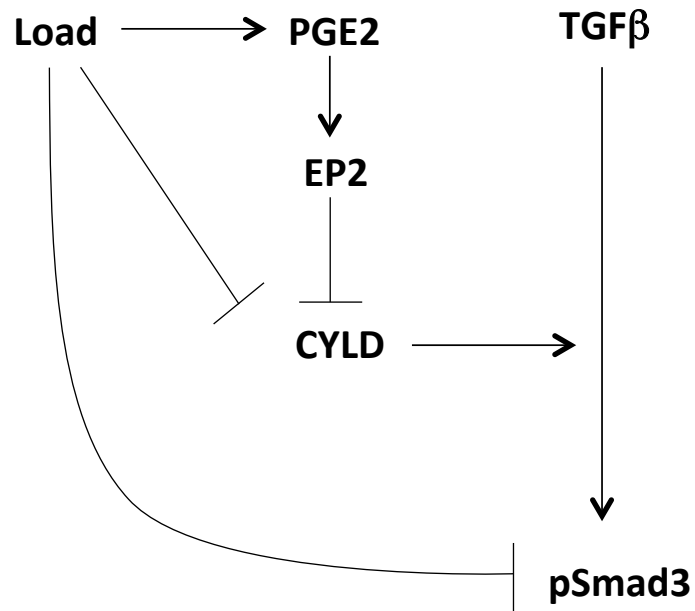**Figure 3: Prostaglandins suppress Smad3 phosphorylation in a ubiqutination-dependent mechanism**
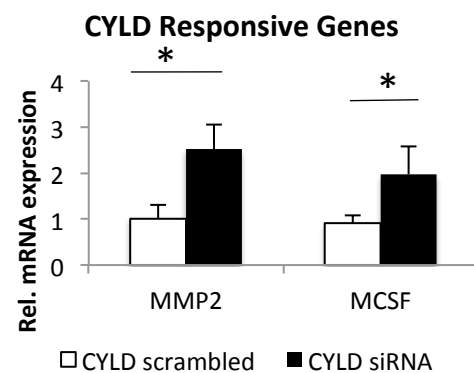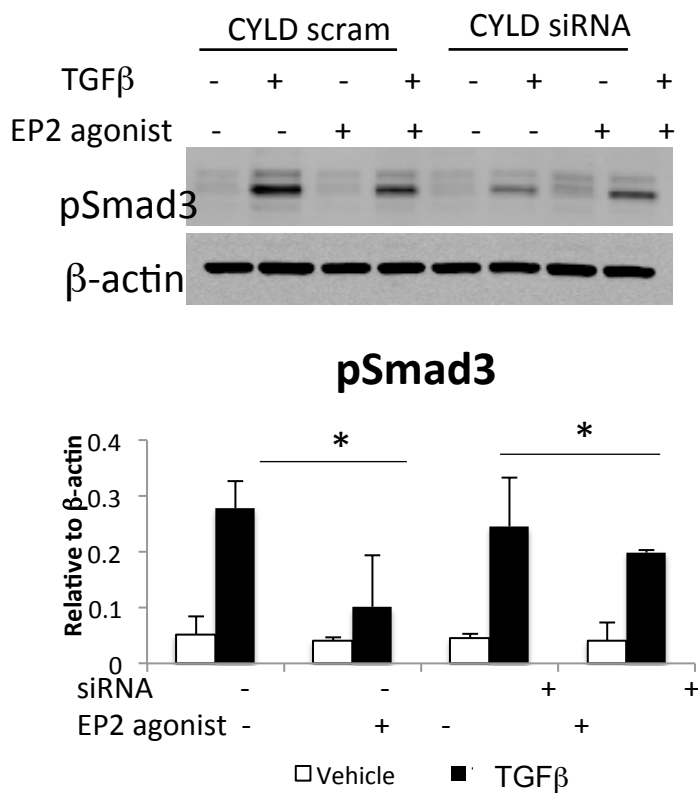
Figure 4 : CYLD is required for PGE/EP2 repression of TGFβ signaling in osteocytes.

Figure 5: CYLD is required for load-mediated repression of pSmad3 and load-induced bone formation.

Figure 6: Mechanosensitive CYLD mediates PGE2 repression of TGFβ signaling.