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Microtubules tune mechanotransduction through NOX2 and TRPV4 to decrease sclerostin abundance in osteocytes

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Abstract

The adaptation of the skeleton to its mechanical environment is orchestrated by mechanosensitive osteocytes, largely by regulating the abundance of sclerostin, a secreted inhibitor of bone formation. We defined a microtubule-dependent mechanotransduction pathway that linked fluid shear stress to reactive oxygen species (ROS) and calcium (Ca^{2+}) signals that led to a reduction in sclerostin abundance in cultured osteocytes. We demonstrated that microtubules stabilized by detyrosination, a reversible posttranslational modification of polymerized α -tubulin, determined the stiffness of the cytoskeleton, which set the mechanoresponsive range of cultured osteocytes to fluid shear stress. We showed that fluid shear stress through the microtubule network activated NADPH oxidase 2 (NOX2)–generated ROS that target the Ca^{2+} channel TRPV4 to elicit Ca^{2+}

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SUPPLEMENTARY MATERIALS

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Competing interests: J.S.L., J.P.S., and C.W.W. are named as inventors on a provisional patent application (U.S. provisional patent no. 62/422,717) that has been filed on the use of MT-manipulating drugs to treat osteoporosis. J.P.S., C.W.W., and J.S.L. are named as inventors on a U.S. patent (no. 15/466,255) for a multifunctional fluid flow device used in this study. C.W.W. and R.J.K. are named on a U.S. patent (no. 14/284,736) on cytoskeletal-targeted therapeutics for the treatment of muscular conditions and muscular dystrophies. All other authors declare that they do not have any competing interests.

influx. Furthermore, tuning the abundance of dephosphorylated tubulin affected cytoskeletal stiffness to define the mechanoresponsive range of cultured osteocytes to fluid shear stress. Finally, we demonstrated that NOX2-ROS elicited Ca^{2+} signals that activated the kinase CaMKII to decrease the abundance of sclerostin protein. Together, these discoveries may identify potentially druggable targets for regulating osteocyte mechanotransduction to affect bone quality.

INTRODUCTION

Bone dynamically remodels to adapt to mechanical loads to maintain its structural integrity. Bone-embedded osteocytes that reside in the fluid-filled lacunar-canalicular system are central to skeletal mechanoresponsiveness (1). In response to mechanical load, osteocytes experience fluid shear stress (FSS), which triggers calcium (Ca^{2+}), extracellular adenosine triphosphate (ATP), nitric oxide, and prostaglandin E_2 (PGE_2) signals (2, 3), and orchestrate bone remodeling through effector molecules, such as sclerostin, RANKL, and osteoprotegerin (1–3). These effectors act on bone-forming osteoblasts and bone-resorbing osteoclasts to add, remove, and replace bone to accommodate mechanical demands. Sclerostin (which is encoded by *Sost*) is an osteocyte-specific secreted glycoprotein that suppresses bone formation by antagonizing canonical Wnt/ β -catenin signaling, reducing osteoblast differentiation and bone formation (4, 5). In an important response to mechanical load, osteocytes reduce sclerostin abundance, leading to “derepression” of osteoblastogenesis and stimulation of de novo bone formation (6, 7).

In humans, sclerostin deficiency leads to the high bone mass disorders sclerosteosis and van Buchem disease (8, 9), and genetic ablation of *Sost* in mice results in increased bone mass (10). Although therapeutically targeting sclerostin is effective at improving bone quality in animal models and in humans (11, 12), the mechanotransduction pathways linking FSS to the decrease in sclerostin abundance remain undefined. Similarly, despite the mechanoresponsive nature of osteocytes, the identity of the “mechanosensor” is controversial. Furthermore, although integrin-associated mechanosomes, osteocyte cell processes, primary cilia, and connexin 43 (Cx43) hemichannels have been implicated as mechanosensors and in mechanoactivated Ca^{2+} influx in bone cells (13–18), they have not been mechanistically linked to sclerostin down-regulation.

The cytoskeleton, composed of microtubules (MTs), actin, and intermediate filaments, is a dynamic structure that forms an interconnected three-dimensional framework of molecular struts and cables within the cell (19). The cytoskeleton is critical for the cellular response to the mechanical environment, because it integrates and transduces mechanical energy to mechanosensitive proteins that generate biological signals in various cell types (20, 21). Here, we demonstrated an MT-dependent mechanotransduction pathway linking FSS to sclerostin down-regulation in osteocytes.

MTs arise from the polymerization of α - and β -tubulin dimers (19). The MT network is a dynamic structure whose density and stability is regulated by posttranslational modifications (such as dephosphorylation, acetylation, and phosphorylation) and microtubule-associated proteins (MAPs) that affect the equilibrium between MT filament growth, disassembly, and association with other cytoskeletal elements (22, 23). We have shown that when the α -

tubulin subunit of MTs is detyrosinated, this subset of modified MTs defines the mechanosensitivity of osteocytes by stiffening the cytoskeleton (24–26).

Here, we demonstrated that a threshold amount of FSS to the osteocyte acted through the MT network to activate NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase 2 (NOX2) to generate reactive oxygen species (ROS). These NOX2-dependent ROS signals targeted TRPV4 channels to elicit Ca^{2+} influx, activate Ca^{2+} /calmodulin-dependent kinase II (CaMKII), and decrease sclerostin abundance in the osteocyte. In summary, we identified the subset of MTs, stabilized by detyrosination, that tune cytoskeletal stiffness to define the mechanosensitivity of osteocytes to FSS, leading to activation of this mechanotransduction pathway to affect sclerostin bioavailability.

RESULTS

Ocy454 cells respond to FSS with a rapid increase in intracellular Ca^{2+} that is required for CaMKII phosphorylation and the mechanically induced decrease in sclerostin

Unlike some of the commonly used osteocyte cell lines, the Ocy454 osteocyte line, which is derived from the Immortomouse, reliably produces detectable sclerostin protein and is sensitive to mechanical stimuli (27). In Ocy454 cells loaded with the Ca^{2+} indicator dye Fluo-4-AM, FSS at 4 dynes/cm² elicited a rapid, transient increase in intracellular Ca^{2+} concentration in ~84% of cells (Fig. 1, A and B), resulting in activation of CaMKII and a concomitant threefold decrease in sclerostin protein observed within 5 min after FSS (Fig. 1C). The FSS-induced CaMKII phosphorylation and decrease in sclerostin protein were inhibited when Ca^{2+} signaling was blocked by loading the cells with BAPTA AM and performing the experiment in Ca^{2+} -free fluid flow buffer (Fig. 1C), demonstrating that Ca^{2+} was required for CaMKII phosphorylation and the decrease in sclerostin. Inhibition of CaMKII signaling with KN-93 (Fig. 1D) or by overexpression of a dominant negative CaMKII (T286A) construct (Fig. 1E) prevented the FSS-induced sclerostin decrease.

MTs are present in the putative mechanosensitive structures of Ocy454 cells

The cytoskeleton, composed of actin, MT, and intermediate filament networks, is a dynamic structural and signaling scaffold within all cells. A key function of the cytoskeleton is to transmit mechanical forces to proteins and enzymes that generate biological signals during mechanotransduction. In other cell types, MTs have been implicated in mechanotransduction-elicited Ca^{2+} signaling (28–30). In bone cells, an intact MT network is required for mechanosensation by osteoblasts or osteocytes in culture (31–34), and the MT network of osteocytes remodels and reorients itself in response to FSS (34–36). In addition, MTs are an important component of the primary cilia, which has been proposed to be a mechanosensor in osteocytes (16, 37). Another putative mechanosensitive component is the long cellular process, extending from the cell body of the osteocyte, which is sensitive to FSS application (14). Immunofluorescence labeling of Ocy454 cells revealed abundant MTs within the cell processes and primary cilia of Ocy454 cells (Fig. 2A). Similarly, the labeling of MTs with SiR-tubulin revealed distinct fluorescence within osteocyte cell processes in murine femurs, indicating the presence of MTs within the proposed mechanosensitive structures of osteocytes (Fig. 2B).

MTs are required for the osteocyte response to FSS

The MT network is dynamically unstable, with MT end-binding proteins and posttranslational modifications promoting MT filament disassembly or growth. Colchicine, a drug that binds tubulin and promotes MT depolymerization, inhibits extracellular signal-regulated kinase (ERK) signaling and cell proliferation and alters the expression of genes encoding osteopontin, collagen, and matrix metalloproteinases in osteoblasts and osteocytes exposed to mechanical cues (31–34). Consistent with these reports, we observed that reduction of the MT network density in Ocy454 cells with colchicine reduced responses to FSS. In response to either 4 or 16 dynes/cm² FSS, colchicine treatment decreased the number of cells responding (suggesting decreased mechanosensitivity) while also reducing the magnitude (peak $\Delta F/F$) of the Ca²⁺ response (suggesting decreased mechanoresponsiveness) in cells that did respond (Fig. 2, C and D). Likewise, MT network disruption with colchicine eliminated the FSS-induced increase in CaMKII phosphorylation and decrease in sclerostin protein (Fig. 2E). Immunofluorescence labeling validated that MTs were disrupted after colchicine treatment (Fig. 2F). These data demonstrated that an intact MT network was required for mechanotransduction-elicited Ca²⁺ influx, CaMKII phosphorylation, and decrease in sclerostin in osteocytes.

MT stabilization alters the set point for FSS-induced Ca²⁺ influx, CaMKII activation, and sclerostin abundance

We next sought to determine the effect of the MT network on regulating osteocyte mechanotransduction. The drug Taxol binds to and stabilizes the MT filament against depolymerization, thereby increasing MT network density. Real-time Ca²⁺ imaging of Ocy454 cells treated with Taxol showed a statistically significant decrease in the percentage of cells responding to 4 dynes/cm² FSS and a decrease in the magnitude (peak $\Delta F/F$) of their response (Fig. 3A). However, unlike the effect of colchicine-mediated MT depolymerization, the Taxol-induced suppression of both mechanoresponsiveness and mechanosensitivity was restored at 16 dynes/cm² FSS (Fig. 3B). Consistent with the impact of increased MT density on Ca²⁺ signaling, Taxol-treated Ocy454 cells subjected to 4 dynes/cm² FSS had reduced FSS-induced CaMKII phosphorylation and a blunted decrease in sclerostin protein, both of which were restored at 16 dynes/cm² FSS (Fig. 3C). Immunofluorescence labeling of the MT network confirmed the increase in MT density after Taxol treatment (Fig. 3D). These results implied that increases in the density or stability of the MT network raised the threshold for FSS-induced activation of Ca²⁺ influx, CaMKII signaling, and sclerostin abundance.

The abundance of detyrosinated tubulin in the MT network defines the mechanosensitivity of Ocy454 cells to FSS

Taxol-induced MT stabilization is associated with an increase in the fraction of detyrosinated tubulin in the MT filament. Detyrosinated tubulin arises from the enzymatic cleavage of a C-terminal tyrosine residue of α -tubulin by tubulin tyrosine carboxypeptidase (TTCP; protein identity unknown), leaving a glutamate (38). This reaction can be reversed by the ligation of tyrosine back to the glutamate by a tubulin tyrosine ligase. Because

detyrosinated tubulin contributes to MT-dependent mechanotransduction in cardiac and skeletal muscle (39), we examined its impact on osteocyte mechanotransduction.

To profile the presence of detyrosinated tubulin in the osteocyte MT network, Ocy454 cells and murine femurs were examined by Western blotting and immunofluorescence. Detyrosinated tubulin was observed in the osteocyte cell process and primary cilia of Ocy454 cells (Fig. 4A) and in the cell processes of osteocytes in situ in formaldehyde-fixed paraffin-embedded sections of murine cortical bone (Fig. 4B). As observed in other tissues, Taxol treatment of Ocy454 cells in vitro or murine cortical bone ex vivo markedly increased the amount of detyrosinated tubulin (Fig. 4, C and D).

The abundance of detyrosinated tubulin within the MT network can be effectively reduced by parthenolide (PTL), a sesquiterpene lactone that inhibits the activity of the TTCF enzyme responsible for detyrosination (40). In striated muscle, the PTL-induced reduction in detyrosinated tubulin inhibits mechanosignaling (39), suggesting that the abundance of detyrosinated tubulin is the main determinant of mechanoactivation. Real-time Ca^{2+} imaging of Ocy454 cells treated with PTL and exposed to FSS showed a statistically significant reduction in mechanosensitivity (as assessed by the percentage of cells responding) and mechanoresponsiveness (as assessed by the magnitude of the cellular response, peak $\Delta F/F$) at both 4 and 16 dynes/cm² FSS (Fig. 4, E and F). In addition, PTL treatment blunted both the FSS-induced phosphorylation of CaMKII and decrease in sclerostin abundance at both 4 and 16 dynes/cm² FSS (Fig. 4G). These effects of PTL on mechanoresponsiveness occurred with a reduction in detyrosinated tubulin without affecting the overall structure of the MT network (Fig. 4, G and H). These data suggested that the amount of detyrosinated tubulin plays a key role in modulating osteocyte mechanotransduction.

Because Taxol increases both the density of the MT network and the amount of detyrosinated tubulin, we sought to determine the respective contributions of these alterations. To this end, we simultaneously treated cells with PTL and Taxol to promote an increase in MT density while eliminating the concomitant increase in detyrosinated tubulin. Compared to cells treated with Taxol or PTL individually (Figs. 3 and 4), combination treatment restored mechanoresponsiveness, as indicated by the restoration of FSS-induced Ca^{2+} response, CaMKII phosphorylation, and decrease in sclerostin abundance at 4 dynes/cm² (Fig. 5, A and B, and fig. S1). Immunofluorescence microscopy of treated Ocy454 cells confirmed that the combination of Taxol and PTL resulted in the expected Taxol-driven increase in MT density, with PTL preventing the concomitant enhancement in detyrosinated tubulin (Fig. 5, B and C). In total, these data supported that detyrosinated tubulin, rather than MT density, was the dominant regulator of the osteocyte response to FSS.

The abundance of detyrosinated tubulin determines cytoskeletal stiffness in Ocy454 cells

Detyrosinated tubulin promotes MT interactions with other cytoskeletal elements (such as actin, intermediate filaments, and MAPs), which increase the stiffness of the cytoskeleton (24–26, 39). Accordingly, we interrogated cytoskeletal stiffness in Ocy454 cells. Nanoindentation atomic force microscopy (AFM) revealed that Taxol treatment increased the elastic modulus, reflecting increased cytoskeletal stiffness (Fig. 5D and fig. S2). Western

blotting confirmed a marked increase in the deetyrosinated tubulin in the Taxol-treated cells (Fig. 5E and fig. S2). In contrast, PTL-treated cells showed a decrease in cytoskeletal stiffness and nearly undetectable amounts of deetyrosinated tubulin (Fig. 5, D and E, and fig. S2). The combination of PTL and Taxol, which increased MT density while maintaining a modest amount of deetyrosinated tubulin (Fig. 5, C and E), resulted in an intermediate amount of cell stiffness, with an increase in the elastic modulus (increased cytoskeletal stiffness) over PTL treatment alone, yet less than that caused by Taxol treatment (Fig. 5D). When examined in the context of FSS-stimulated Ca^{2+} influx, CaMKII phosphorylation, and decreased sclerostin abundance, these AFM data support a model in which cytoskeletal stiffness, which was affected by deetyrosinated tubulin abundance, defined a permissive range for both the sensitivity and responsiveness of osteocytes to FSS.

Ocy454 FSS-induced Ca^{2+} influx is mediated by TRPV4

We used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to establish the expression profile of mRNAs encoding Ca^{2+} channel(s) implicated in osteocyte Ca^{2+} signaling (41–43). *Trpv4* was particularly abundant at the mRNA level (fig. S3) and was an attractive candidate, given evidence that TRPV4 has been implicated in MT-dependent mechanotransduction in other cell types (44–46). Consistent with the abundance of *Trpv4* transcript, immunofluorescence staining of Ocy454 cells and paraffin-embedded murine cortical bone sections showed the presence of TRPV4 in osteocytes (Fig. 6A). Western blot analysis of Ocy454 cells and murine long bone extracts confirmed the presence of TRPV4 protein (Fig. 6B).

To determine the impact of TRPV4 on FSS-triggered mechanotransduction, we treated Ocy454 cells with the TRPV4 antagonist GSK2193874, which decreased mechanosensitivity (as assessed by the percentage of cells responding) and mechanoresponsiveness (as assessed by peak Ca^{2+} response) in GSK2193874-treated Ocy454 cells (Fig. 6C). In addition, transfection of Ocy454 cells with TRPV4 targeting small interfering RNA (siRNA) (Fig. 6D) yielded similar results to the pharmacological antagonist, supporting the conclusion that TRPV4 was a major contributor to Ca^{2+} influx pathway acutely activated by FSS.

Consistent with TRPV4 as the source of FSS-induced Ca^{2+} influx, Ocy454 cells treated with the TRPV4 antagonist or transfected with TRPV4 siRNA showed a reduction in FSS-induced CaMKII phosphorylation and a blunted FSS-induced down-regulation of sclerostin (Fig. 6, E and F). Conversely, treating Ocy454 cells with the TRPV4 agonist GSK1016790A recapitulated the mechanoresponse, including the reciprocal activation of CaMKII and reduction in sclerostin protein independently of FSS (Fig. 6G), demonstrating that TRPV4 activation was sufficient to result in phosphorylation of CaMKII and decrease in sclerostin.

TRPV4 opens in response to FSS-induced ROS

TRPV4 can be activated by mechanical stimuli through direct tethering to the cytoskeleton (44) or by ROS-dependent oxidation (47, 48). To assess the impact of ROS-mediated activation, we treated Ocy454 cells with the ROS scavenger *N*-acetylcysteine (NAC). Real-time, live cell Ca^{2+} imaging showed that NAC treatment abrogated the FSS-induced

response at both 4 and 16 dynes/cm² (Fig. 7A and fig. S4A). Likewise, we observed a reduction in CaMKII phosphorylation and a blunting of the FSS-induced decrease in sclerostin protein in NAC-treated cells (Fig. 7B). Hydrogen peroxide (H₂O₂) challenge to Ocy454 cells reciprocally increased phosphorylation of CaMKII and reduced sclerostin protein independently of FSS (Fig. 7C), thus supporting ROS as the signal downstream of mechanosensation. To confirm this observation, we simultaneously imaged Ocy454 cells for ROS using CellROX and Ca²⁺ using Fluo-4. Treatment with H₂O₂ stimulated both ROS and intracellular Ca²⁺ (Fig. 7D). Treatment with a TRPV4 antagonist blunted the H₂O₂-induced Ca²⁺ influx without affecting ROS (Fig. 7D). Activation of TRPV4 in Ocy454 cells with the TRPV4 agonist was insufficient to induce ROS production (fig. S4B). In aggregate, these data established ROS as a necessary, upstream regulator of TRPV4-dependent Ca²⁺ influx.

FSS-induced ROS signaling is mediated by the mechanosensitive ROS-generating enzyme NOX2

NOX2 is a mechanosensitive ROS-generating enzyme implicated in MT-dependent ROS signaling (39, 49–51). Western blot confirmed the presence of NOX2 in Ocy454 cells (Fig. 8A). Exposure of Ocy454 cells to 4 dynes/cm² FSS elicited the production of ROS, as measured by CellROX, an effect that was blunted by inhibition of tubulin dephosphorylation with PTL or the NOX2 inhibitor GP91ds-TAT (Fig. 8B). Likewise, when GP91ds-TAT-treated Ocy454 cells were subjected to FSS and monitored for intracellular Ca²⁺, both mechanosensitivity (as assessed by the percentage of cells responding) and mechanoresponsiveness (as assessed by peak Ca²⁺ response) were attenuated (Fig. 8C). Unlike stiffening the MT network with Taxol, which could be overcome with increased FSS, the NOX2 inhibition persisted at higher flow rates (fig. S4C), confirming NOX2 as a convergence point in this mechanotransduction cascade. In addition, inhibition of NOX2 with GP91ds-TAT blocked the phosphorylation of CaMKII by FSS and reduced the FSS-induced decrease in sclerostin at both 4 and 16 dynes/cm² (Fig. 8D). These data implicated NOX2 as the source of ROS that activates TRPV4-dependent Ca²⁺ influx during FSS.

DISCUSSION

Here, we report a mechanotransduction pathway in osteocytes that links FSS to the activation of Ca²⁺ influx that drives the mechanically induced suppression of sclerostin abundance. Central to our discovery was that the MT network, and more specifically the abundance of dephosphorylated tubulin that defined the cytoskeletal stiffness, determined the mechanosensitivity of osteocytes to FSS. Upon a threshold amount of FSS, MT-dependent activation of NOX2 elicited ROS that activated TRPV4-dependent Ca²⁺ influx signals and CaMKII phosphorylation, driving sclerostin down-regulation in osteocytes (Fig. 8E). Our data revealed new molecular players and provided insights into osteocyte mechanotransduction.

Our data showed that MTs are required for mechanosignaling, consistent with reports on other mechanosignaling events in the bone (31–34). We build upon this concept by demonstrating that the MT network, and specifically its abundance of dephosphorylated tubulin, was a critical regulator of cytoskeletal stiffness, which tuned the mechanoresponsive range at

which osteocytes were activated by FSS. We revealed that a targeted reduction in detyrosinated tubulin abundance (induced through PTL treatment) decreased MT-dependent cytoskeletal stiffness, impairing the ability of osteocytes to sense and transduce mechanical cues (Fig. 8E). In contrast, driving up the abundance of detyrosinated tubulin increased cytoskeletal stiffness, which increased the amount of FSS needed to activate the mechanotransduction pathway. Thus, the cytoskeleton is a dynamic integrator of mechanical cues, affecting the mechanical set point at which an osteocyte can respond to a given mechanical load. Our discovery that MTs were central to this mechanotransduction pathway may unify several models of osteocyte mechanosensing. The primary cilia hypothesis (16, 18, 37), the integrin-based mechanosome (14, 15), and perhaps even the opening of Cx43 hemi-channel response to mechanical activation of integrins (17) are all based on structures linked to the MT network.

Another finding was that TRPV4 was a major pathway for the initial and rapid FSS-induced Ca^{2+} influx that drives sclerostin down-regulation in osteocytes. Unlike modifications of the MT network, which fully abrogated mechanosensitivity (as shown by Ca^{2+} influx, CaMKII phosphorylation, and decreased sclerostin abundance), we still observed residual FSS-induced Ca^{2+} influx with pharmacologic or molecular inhibition of TRPV4. Although several other Ca^{2+} influx pathways have been identified in osteocytes, our results suggested that these pathways are likely activated downstream or in parallel to the initial Ca^{2+} influx through TRPV4. Oscillating Ca^{2+} waves can be driven by ATP release and purinergic receptor activation in mechanoactivated osteocytes (52–54) as well as Ca^{2+} influx through T-type voltage-gated calcium channels (41, 55). Regardless, our data suggested that TRPV4 activity is obligatory even if other Ca^{2+} pathways are also involved in mechanosensing.

The involvement of TRPV4 in osteocyte mechanosensing was consistent with the demonstration of TRPV4 as a mediator of mechanically induced Ca^{2+} influx in the primary cilia of bone cells (37). Likewise, TRPV4 plays an important role in chondrocyte mechanotransduction because blocking TRPV4 prevents an anabolic response to load, whereas activating the receptor mimics load (56). In contrast to our prediction, global TRPV4 knockout mice have increased bone mass; however, the interpretation is complicated by a severe osteoclast defect that contributes to the skeletal phenotype (57). Despite higher trabecular and cortical bone mass, male TRPV4 knockout mice have reduced bone matrix mineralization, increased cortical porosity, a lower ultimate stress, and reduced elastic modulus (58). Although these reports do not preclude a role of TRPV4 in osteocyte mechanoresponsiveness, they also do not definitively support our hypothesis. Regardless, TRPV4 plays a role in the skeleton as numerous gain-of-function TRPV4 mutations cause skeletal dysplasias with a breadth of severity (59). A single-nucleotide polymorphism in the human *TRPV4* locus is associated with a 30% increased risk of nonvertebral fractures in males in a prospective, population-based cohort study and has been confirmed in subsequent meta-analysis (58).

Consistent with reports in striated muscle (39, 49, 51), our data showed an important role for mechanoactivated, NOX2-dependent ROS in the osteocyte response to FSS. Aged mice globally deficient in p47^{phox}, a subunit of the NOX2 enzyme, have decreased bone mass and strength due to deficits in osteoblast differentiation, osteoblast number, and accelerated cell

senescence (60). This phenotype is not observed in 6-week-old mice, which have increased bone mass. Whether changes in mechanosensing or sclerostin bioavailability contribute to the worsening skeletal phenotype has not been assessed, and these mice have not been studied in the context of mechanical loading.

Our data not only aligned with the reports that implicate MTs in mechanotransduction but also are consistent with the remodeling and reorientation of the MT network of osteocytes in response to FSS (34–36). It is reasonable to speculate that the FSS-dependent remodeling of MTs is itself a mechanoadaptation event that adjusts the homeostatic set point for mechanotransduction. As mentioned above, our data also suggested a unifying basis for how various known mechanosensitive elements (such as primary cilia, cell processes, integrin-mediated mechanosomes, and Cx43 hemichannels) may integrate mechanical signals into biological responses through the cytoskeleton. Further, our data mechanistically linked the mechanoactivated Ca^{2+} influx to the decrease in sclerostin abundance. The implications of ROS as a fundamental driver of mechanoresponses may also extrapolate to known deficits in bone mechanoresponsiveness in conditions of aberrant redox buffering capacity, including aging (61).

In summary, we have defined the MT-dependent mechanotransduction pathway linking FSS to NOX2-generated ROS that elicits TRPV4-dependent Ca^{2+} influx signals that activate CaMKII to decrease sclerostin protein in osteocytes. Given the fundamental nature of osteocyte mechanoresponsiveness to bone turnover throughout the life span, these mechanistic insights may provide a new perspective for understanding diseases and conditions that manifest through altered skeletal structure and properties. Furthermore, given the impact of the MT network on the fundamental regulation of Ca^{2+} signaling and sclerostin production in osteocytes, we propose the MT network as a target for manipulating the osteocyte response to mechanical cues for therapeutic interventions in bone.

MATERIALS AND METHODS

Chemicals and reagents

Taxol, colchicine, GSK2193874, GSK1016790A, NAC, and PTL were purchased from Sigma. BAPTA AM ester was from Cayman Chemical. GP91ds-TAT was from AnaSpec. SiR-tubulin was from Cytoskeleton Inc. CellROX Deep Red Reagent and Fluo-4-AM ester were purchased from Thermo Fisher Scientific.

Cell culture and treatments

Osteocyte-like Ocy454 cells (provided by P. Divieti-Pajevic, Boston University) were cultured on type I rat tail collagen (BD Biosciences)-coated dishes in α -minimum essential medium (MEM) supplemented with 10% fetal bovine serum (27). Cells were maintained at 33°C and 5% CO_2 . Before experiments, cells were seeded into a tissue culture-treated vessel and maintained at 37°C and 5% CO_2 overnight. For alteration of the MT network, cells were pretreated with 0.1% dimethyl sulfoxide (control), colchicine (2 μM , 20 min), Taxol (1 μM , 2 hours), or PTL (25 μM , 2 hours). In the case of the combined treatment, cells were dosed with PTL for 30 min before Taxol was added to the same medium for an additional 1.5 hours

for a total incubation time of 2 hours. To modulate TRPV4 activity, the cells were treated with the TRPV4 antagonist GSK2193874 (15 μ M, 30 min) or the TRPV4 agonist GSK1016790A (15 μ M, 30 min) before the stimulation of the cells. To modulate ROS, the cells were treated with NAC (10 mM, 15 min), H₂O₂ (100 μ M, 30 min), or GP91ds-TAT (10 μ M, 30 min) before the stimulation of the cells.

Transient transfections

Ocy454 cells were transfected with jetPRIME reagent (Polypus), as previously described (62). ON-TARGETplus mouse TRPV4 siRNA and ON-TARGETplus Non-targeting siRNA were purchased from Dharmacon. siRNAs were used at 0.42 μ g/cm². Cell exposure to FSS was started 48 hours after transfection.

Fluid flow

Cells in culture were exposed to fluid flow using a custom FSS device (63). Cell medium was removed, and cells were rinsed in Hepes-buffered Ringer solution containing 140 mM NaCl, 4 mM KCl, 1 mM MgSO₄, 5 mM NaHCO₃, 10 mM glucose, 1.8 mM CaCl₂, and 10 mM Hepes (pH 7.3). Ringer solution was also used as fluid flow buffer. For calcium-free conditions, Hepes-buffered manganese Ringer solution, containing 140 mM NaCl, 4 mM KCl, 1 mM MgSO₄, 5 mM NaHCO₃, 10 mM glucose, 2 mM MnCl, and 10 mM Hepes (pH 7.3), was used, and cells were loaded with BATPA AM ester (10 μ M, 30 min).

Calcium and ROS imaging

Cells were seeded into optically clear 96-well plates (Corning), incubated overnight at 37°C and 5% CO₂, and treated as indicated. For Ca²⁺ imaging, cells were loaded with Fluo-4-AM ester (5 μ M; Thermo Fisher Scientific) for 30 min, washed, and allowed to rest for 15 min to allow dye de-esterification, as described (39). For ROS imaging, cells were loaded with CellROX (5 μ M; Thermo Fisher Scientific) for 30 min and then washed three times per the manufacturer's recommendations. Individual wells were imaged as previously described (39). Time-lapse fluorescence intensity measurements were collected using ImageJ Time Series Analyzer plug-in, and data were analyzed and plotted using OriginPro software. Final results represent a minimum of three independent experiments performed on separate days with new cultures ($n > 700$ cells per treatment group). All conditions were run with controls on each experimental day.

Atomic force microscopy

Ocy454 cells were plated onto 22 mm $\times$ 22 mm glass coverslips and allowed to grow for 16 to 24 hours at 37°C and 5% CO₂ with α -MEM. Thereafter, cells were washed with phosphate-buffered saline (PBS) before being incubated with pharmacological agents as indicated for 2 hours at 37°C and 5% CO₂ in α -MEM. After each treatment, cells were transferred to 60-mm culture dishes with prewarmed Hepes-based medium containing identical concentrations of the previously mentioned agents. Cells were probed with an MFP-1D atomic force microscope (Asylum Research) (64, 65) using MLCT cantilevers (Bruker) with a nominal spring constant of $k = 0.01$ N/m. The pull distance used was 2 μ m with a tip velocity of 4 μ m/s to generate ~ 1 to 2 nN of force onto the cell corresponding to

~1- μ m indentation, ensuring that the cytoskeleton was effectively being probed. The elastic moduli (stiffness) of the cells were calculated using the Sneddon-Hertz model, which has been described (66).

Immunofluorescence

Ocy454 cells seeded and grown on glass coverslips were fixed and permeabilized as described (67). For histological sections of bone, decalcified, paraffin-embedded sections were processed as described (68). Coverslips were incubated in SuperBlock PBS (Life Technologies) for 1 hour before the addition of primary antibodies. Primary antibodies were diluted in SuperBlock PBS and added to the coverslips for an overnight incubation at 4°C. Secondary antibodies were diluted in SuperBlock PBS and incubated at room temperature for 6 hours. Coverslips were mounted using ProLong Diamond with DAPI (Life Technologies). The antibodies used were α -tubulin (Sigma, T9026), detyrosinated tubulin (Abcam, ab48389), and TRPV4 (Abcam, ab39260). Goat anti-mouse Alexa Fluor 488 and 647 and goat anti-rabbit Alexa Fluor 488 and 568 were purchased from Life Technologies. Actin was stained using phalloidin-tetramethyl rhodamine isothiocyanate (Molecular Probes). Slides were imaged as described (69).

SiR-tubulin labeling and confocal imaging

Murine long bones (tibia and fibula) were isolated, flushed of marrow, and placed in 60-mm Fluo-dish glass-bottom plates. These long bones were then incubated in α -MEM containing the live cell tubulin stain SiR-tubulin (1 μ M; 37°C and 5% CO₂ for 2 hours). Confocal fluorescence imaging [Nikon A1R; 40 $\times$ objective water lens; numerical aperture (NA), 1.4] was used to profile the structure of the MT network in the bone-embedded osteocytes as previously described (51).

Western blotting

Western blotting of whole-cell extracts isolated from cells in culture after FSS or extracts isolated from murine long bone was done as previously described (68, 70). Equal amounts of protein were loaded and electrophoresed on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% nonfat dry milk (unless otherwise stated) and probed with the indicated primary antibodies overnight at 4°C. Antibodies were detected with the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and enhanced chemiluminescence detection reagent (Bio-Rad). The antibodies used were sclerostin (R&D Systems, AF1589), α -tubulin (Sigma, T9026), detyrosinated tubulin (Abcam, ab48389), phospho-CamKII Thr²⁸⁶ (Cell Signaling Technology, 12716S), total CaMKII (Cell Signaling Technology, 11945S), and GAPDH (Millipore, MAB374). Blots were acquired using an EpiChem gel documentation system (UVP Bioimaging Systems) and analyzed using ImageJ software.

Quantitative RT-PCR

RNA extraction was done by Direct-zol RNA MiniPrep (Zymo Research). RNA was reverse-transcribed with either iScript (Bio-Rad) or RevertAid (Fermentas) reverse

transcription master mix according to the manufacturer's directions. qRT-PCR was carried out by SYBR Green Master Mix from Quanta using an Applied Biosystems 7300 sequence detection system. A melting curve was performed to ensure amplification of a single PCR product. For each sample, the relative gene expression was determined by simultaneously normalizing the gene of interest with three housekeeping genes (*Rpl13*, *Hprt*, and *Gapdh*) by the $2^{-\Delta\Delta C_t}$ method, using geNorm version 3.5 software (Ghent University Hospital Ghent, Belgium) as described (67). Primer sequences are available upon request.

Statistical analysis

Experiments were repeated a minimum of three times with triplicate samples, unless indicated otherwise. Graphs show averages, with error bars indicating SE. Data normality was assessed by GraphPad Prism 6 software by D'Agostino-Pearson omnibus normality test. For normally distributed data, samples were compared by an ANOVA for unpaired samples with a Holm-Sidak post hoc test, as appropriate, using GraphPad Prism 6 software. For nonparametric data, a two-tailed Mann-Whitney test or Kruskal-Wallis test was performed, as indicated. A *P* value of <0.05 was used as a threshold for statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The FSS-induced Ca²⁺ response is required for CaMKII phosphorylation and reduction in sclerostin

(A) Ca²⁺ imaging of Ocy454 cells exposed to 4 dynes/cm² FSS. Pseudocolored images are shown. $n = 5$ independent experiments. Scale bars, 100 μ m. (B) Ca²⁺ responses in Ocy454 cells exposed to 4 dynes/cm² FSS. Trace indicates Fluo-4 fluorescence changes over time. Average trace of all cells (>200 cells in $n = 3$ independent experiments) shown in bold. Representative individual cell traces are shown in gray. “% Cells responding” indicates the number of cells with $>25\%$ increase in fluorescence. (C) Untreated and BAPTA AM ester-loaded Ocy454 cells were subjected to 4 dynes/cm² FSS with Ca²⁺-containing or Ca²⁺-free flow buffer, respectively. Immunoblotting was performed for phosphorylated (p) CaMKII, total CaMKII, sclerostin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ($n = 3$ independent experiments). The sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown. (D) Control and KN-93-treated Ocy454 cells were subjected to 4 dynes/cm² FSS and immunoblotted for sclerostin and GAPDH ($n = 3$ independent experiments). The sclerostin/GAPDH ratios are indicated. (E) Ocy454 cells transfected with green fluorescent protein (GFP) control or CaMKII T286A constructs were subjected to 4 dynes/cm² FSS and immunoblotted for sclerostin and GAPDH ($n = 3$ independent experiments). The sclerostin/GAPDH ratios are shown. Graphs depict means $\pm$ SEM. ** $P < 0.001$, *** $P < 0.0001$ compared to control by Kruskal-Wallis test. ns, not significantly different.

Fig. 2. An intact MT network is required for FSS-induced Ca^{2+} influx, CaMKII phosphorylation, and decreased sclerostin abundance

(A) Ocy454 cells stained for α -tubulin (red), phalloidin (actin, green), and 4',6-diamidino-2-phenylindole (DAPI) (nuclei, blue). Red arrows in inset depict α -tubulin in osteocyte cell process and primary cilia. Scale bars, 10 μm . $n = 3$ independent experiments. (B) Murine femurs stained with SiR-tubulin. White arrows indicate MTs in the osteocyte cell processes in situ. Scale bar, 20 μm . $n = 3$ mice. (C and D) Ca^{2+} response of Ocy454 cells treated with colchicine and subjected to 4 (C) or 16 (D) dynes/cm² FSS. Trace indicates average Fluo-4 fluorescence changes over time (>200 cells per treatment, $n = 3$ independent experiments). “% Cells responding” indicates number of cells with >25% increase in fluorescence; “Peak (ΔF/F)” indicates peak magnitude of Ca^{2+} response. The Ca^{2+} data for control 4 dynes/cm² FSS are the same trace shown in Fig. 1B, because these were run in parallel with colchicine interventions. (E) Ocy454 cells treated with colchicine were subjected to 4 dynes/cm² FSS and immunoblotted for the indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown ($n = 3$ independent experiments). Images are from a single exposure of a contiguous membrane. Dotted lines indicate the removal of irrelevant lanes. (F) Immunostaining for α -tubulin in control and colchicine-treated Ocy454 cells. Scale bars, 10 μm . $n = 3$ independent experiments. Graphs depict means $\pm$ SEM. ** $P < 0.001$, *** $P < 0.0001$ compared to control by two-tailed Mann-Whitney test (C and D) or Kruskal-Wallis test (E).

Fig. 3. Taxol blunts the FSS-induced Ca^{2+} response, phosphorylation of CaMKII, and decrease in sclerostin abundance, effects that are overcome by increased FSS

(A and B) Ca^{2+} response of Fluo-4-loaded Ocy454 cells treated with Taxol and subjected to 4 (A) or 16 (B) dynes/cm² FSS. Trace indicates average Fluo-4 fluorescence changes over time (>200 cells per treatment, $n = 3$ independent experiments). “% Cells responding” indicates number of cells with >25% increase in fluorescence; “Peak ($\Delta F/F$)” indicates peak magnitude of Ca^{2+} response. The Ca^{2+} data for the controls at 4 and 16 dynes/cm² FSS are the same traces as in Figs. 1B and 2D, because these controls were run in parallel with the Taxol interventions. (C) Control and Taxol-treated Ocy454 cells were subjected to FSS and immunoblotted for the indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are indicated ($n = 3$ independent experiments). (D) Immunostaining for α -tubulin in control and Taxol-treated Ocy454 cells. Scale bars, 10 μm . $n = 3$ independent experiments. Graphs depict means $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ compared to control by two-tailed Mann-Whitney test (A and B) or Kruskal-Wallis test (C).

Fig. 4. Loss of deetyrosinated tubulin, which is found within mechanically sensitive areas of osteocytes and is increased by Taxol, abrogates FSS-induced mechanosignaling
(A) Ocy454 cells immunostained for α -tubulin (red), deetyrosinated (deTyr)-tubulin (green), and DAPI (blue). Osteocyte cell process and primary cilia are indicated by the red arrow and red arrowheads. Scale bars, 20 μ m. $n = 3$ independent experiments. **(B)** Murine long bone sections immunostained for deTyr-tubulin. Red arrows indicate deTyr-tubulin in the osteocyte cell processes in situ. Scale bar, 50 μ m. $n = 3$ mice. **(C)** Ocy454 cells and ex vivo murine long bone were treated with Taxol and immunoblotted for indicated proteins. DeTyr-tubulin/ α -tubulin ratios are indicated ($n = 3$ independent experiments). The image is from a single exposure of a contiguous membrane. Dotted lines indicate the removal of irrelevant lanes. **(D)** Immunostaining for α -tubulin (red), deTyr-tubulin (green), and DAPI (blue) in control and Taxol-treated Ocy454 cells. Scale bars, 10 μ m. $n = 3$ independent experiments. **(E and F)** Ca²⁺ response of Ocy454 cells treated with parthenolide (PTL) and subjected to 4 (E) or 16 (F) dynes/cm² FSS. Trace indicates average Fluo-4 fluorescence changes over time (>200 cells per treatment, $n = 3$ independent experiments). “% Cells responding” indicates number of cells with >25% increase in fluorescence; “Peak ($\Delta F/F$)” indicates peak magnitude of Ca²⁺ response. The Ca²⁺ data for the controls at 4 and 16 dynes/cm² FSS are the same traces as in Figs. 1B and 2D, respectively, because these controls were run in parallel with the PTL interventions. **(G)** Control and PTL-treated Ocy454 cells were subjected to FSS and immunoblotted for indicated proteins. Sclerostin/GAPDH and p-

CaMKII/total CaMKII ratios are shown ($n = 3$ independent experiments). (**H**) Control and PTL-treated Ocy454 cells were immunostained for α -tubulin (red), deTyr-tubulin (green), and DAPI (blue). $n = 3$ independent experiments. Scale bars, 10 μm . Graphs depict means $\pm$ SEM. ** $P < 0.001$, *** $P < 0.0001$ compared to control by Mann-Whitney test (C, E, and F) or Kruskal-Wallis test (G).

Fig. 5. Combination treatment with PTL and Taxol restores mechanosignaling and alters MT-dependent cytoskeletal stiffness

(A) Ca²⁺ response of Fluo-4 loaded Ocy454 cells treated with combination of PTL and Taxol and subjected to 4 dynes/cm² FSS. Trace indicates average Fluo-4 fluorescence changes over time (>200 cells per treatment, *n* = 3 independent experiments). “% Cells responding” indicates number of cells with >25% increase in fluorescence; “Peak (Δ*F*/*F*)” indicates peak magnitude of Ca²⁺ response. (B) Control Ocy454 cells and cells treated with combination of PTL and Taxol were subjected to FSS and immunoblotted for indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown (*n* = 3 independent experiments). (C) Control Ocy454 cells or Ocy454 cells treated with a combination of Taxol and PTL (PTL/Taxol) were immunostained for α-tubulin (red), deTyr-tubulin (green), and DAPI (blue). Scale bars, 10 μm. *n* = 4 independent experiments. Graphs depict means ± SEM. ***P* < 0.001, ****P* < 0.0001 compared to control by two-tailed Mann-Whitney test (A) or Kruskal-Wallis test (B). (D) Atomic force microscopy nanoindentation of control Ocy454 cells or cells treated with Taxol, PTL, or PTL/Taxol. Box edges denote 25th and 75th percentile, whiskers denote 10th and 90th percentile, and white lines indicate mean. Data are from three independent experiments, with number of cells per group indicated. (E) Protein extracts from control Ocy454 cells or Ocy454 cells treated with PTL, Taxol, or PTL/Taxol were probed for deTyr-tubulin and α-tubulin. The deTyr-tubulin to α-tubulin ratio (means ± SEM) is shown. (D and E) Statistical significance determined using one-way analysis of variance (ANOVA) with Holm-Sidak’s multiple comparison test. “***” denotes statistical significance between all groups. Exact *P* values for each comparison are shown in fig. S3.

Fig. 6. TRPV4 is necessary and sufficient for the osteocyte FSS-induced Ca^{2+} response, CaMKII phosphorylation, and decrease in sclerostin

(A) Ocy454 cells and sections of murine long bones immunostained with α -tubulin (red), TRPV4 (green), and DAPI (blue). Scale bars, 100 μ m. $n = 3$ independent experiments, $n = 3$ mice. (B) Immunoblotting of Ocy454 whole-cell lysates and murine long bone extracts for TRPV4 and GAPDH. $n = 3$ independent experiments. (C and D) Ca^{2+} response of Ocy454 cells in the presence or absence (control) of the TRPV4 antagonist (Antag.) GSK2193874 (C) or transfected with control or TRPV4 siRNA (D) and subjected to 4 dynes/cm² FSS. Trace indicates average Fluo-4 fluorescence changes over time (>200 cells per treatment, $n = 3$ independent experiments). “% Cells responding” indicates number of cells with $>25\%$ increase in fluorescence; “Peak ($\Delta F/F$)” indicates peak magnitude of Ca^{2+} response. (E) Ocy454 cells were treated with or without (control) the TRPV4 antagonist GSK2193874, subjected to 4 dynes/cm² FSS, and immunoblotted for the indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown ($n = 3$ independent experiments). (F) Ocy454 cells transfected with control or TRPV4 siRNA were subjected to 4 dynes/cm² FSS and immunoblotted for the indicated proteins. Sclerostin/GAPDH, p-CaMKII/total CaMKII, and TRPV4/GAPDH ratios are shown ($n = 3$ independent experiments). Image is from a single exposure of a contiguous membrane. Dotted lines indicate the removal of irrelevant lanes. (G) Ocy454 cells treated with or without (control) the TRPV4 agonist GSK-1016790A and immunoblotted for indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown ($n = 3$ independent experiments). Graphs depict

means $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ compared to control by two-tailed Mann-Whitney test (C, D, and G) or Kruskal-Wallis test (E and F).

Fig. 7. ROS is required for the FSS-induced Ca²⁺ response, CaMKII phosphorylation, and decrease in sclerostin

(A) Ca²⁺ response in Ocy454 cells treated with α-NAC and subjected to 4 dynes/cm² FSS. Trace indicates average Fluo-4 fluorescence changes over time (>200 cells per treatment, $n = 3$ independent experiments). “% Cells responding” indicates number of cells with >25% increase in fluorescence; “Peak (ΔF/F)” indicates peak magnitude of Ca²⁺ response. The Ca²⁺ data for the control at 4 dynes/cm² FSS are the same trace as in Fig. 1B, because these controls were run in parallel with the NAC interventions. (B) Ocy454 cells treated with or without (control) NAC were subjected to FSS and immunoblotted for indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown ($n = 3$ independent experiments). (C) Ocy454 cells were treated with H₂O₂ and immunoblotted for the indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown ($n = 3$ independent experiments). Graphs depict means ± SEM. *** $P < 0.0001$ compared to control by two-tailed Mann-Whitney test (A and C) or Kruskal-Wallis test (B). (D) Ca²⁺ and ROS response in Ocy454 cells simultaneously loaded with the Ca²⁺ indicator Fluo-4 and the ROS indicator CellROX and subjected to 4 dynes/cm² FSS. Ca²⁺ and ROS traces are aggregated from >200 cells per treatment over $n = 3$ independent experiments. Graphs depict means ± SEM. Statistical significance was determined using one-way ANOVA with Holm-Sidak’s multiple comparison test. ** $P < 0.001$, *** $P < 0.0001$ compared to control (line depicts statistical significance between indicated groups).

Fig. 8. NOX2 generates ROS in response to FSS and is required for FSS-induced Ca^{2+} response, CaMKII phosphorylation, and decrease in sclerostin

(A) Immunoblotting of Ocy454 whole-cell lysates for NOX2 and α -tubulin. $n = 3$ independent experiments. (B) ROS response in Ocy454 cells loaded with the ROS indicator CellROX and subjected to 4 dynes/cm² FSS. ROS traces are aggregated data from >200 cells per treatment from $n = 3$ independent experiments. Graphs depict means $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Holm-Sidak's multiple comparison test. *** $P < 0.0001$. (C) Ca^{2+} response of Ocy454 cells treated with GP91ds-TAT and subjected to 4 dynes/cm² FSS. Ca^{2+} traces are aggregated from >200 cells per treatment from $n = 3$ independent experiments. The Ca^{2+} data for the control at 4 dynes/cm² FSS are the same trace as in Fig. 5A, because these controls were run in parallel with the GP91ds-TAT interventions. (D) Ocy454 cells treated with or without (control) GP91ds-TAT were subjected to FSS and immunoblotted for the indicated proteins. Sclerostin/GAPDH and p-CaMKII/total CaMKII ratios are shown ($n = 3$ independent experiments). Graphs depict means $\pm$ SEM. ** $P < 0.001$, *** $P < 0.0001$ compared to control by two-tailed Mann-Whitney test (C) or Kruskal-Wallis test (D). (E) Representation of MT-dependent mechanotransduction pathway showing the interventions used to alter osteocyte mechanoresponse (top). Proposed model of deTyr-tubulin and cytoskeletal stiffness regulation of osteocyte response to mechanical stimuli (bottom), in which cytoskeletal stiffness tunes the mechanoresponsive range of an osteocyte. This responsive range can be influenced not only by the cytoskeletal stiffness but also by altering the amount of FSS applied to the cell.