INTRODUCTION: Mechanical loading is a regulatory stimulus that modulates bone shape and density. In the cellular process of mechanotransduction, mechanical forces serve as critical physical signals that cells sense and result in biochemical changes involved in bone maintenance such as increased bone formation. The primary cilium is a single immobile organelle that projects from the apical membrane of each cell and has been implicated in mechanosensing in orthopaedic tissues such as cartilage, tendon, and recently by our lab, among others, in bone [1]. In a previous study, we demonstrated that adenyl cyclase 6 (AC6) localizes to primary cilium in MLO-Y4 osteocyte-like cells. Furthermore, the loss of primary cilium inhibited fluid shear stress-induced increases in COX-2 gene expression in MLO-Y4 cells. The knockdown of AC6 produced a similar effect under fluid flow, suggesting that primary cilium mediated mechanotransduction is dependent on AC6 [2, AC6 is an enzyme that regulates cyclic AMP (cAMP), a second messenger. Here, our goal was to determine the role of AC6 in loading-induced bone remodeling in vivo.

METHODS: Animals: Transgenic mice on a C57BL/6 background with a targeted deletion of AC6 were crossed with wildtype (WT) mice. Global knockout (KO) and WT offspring generated by heterozygous breeding pairs were used for all studies. The procedures performed in this study were in accordance with Columbia University Institutional Animal Care and Use Committee guidelines.

MicroCT Analysis: Right ulnae and tibiae from 16-week-old mice were isolated and cleaned of soft tissues. Samples were imaged using micro-computed tomography (Scanco vivACT 40, Bassersdorf, Switzerland). Cortical bone analyses were performed on ulna and tibia mid-diaphyses. Trabecular bone analyses were performed on proximal tibiae.

In Vivo Axial Ulnar Loading: An axial load of 3 N was applied to the right forearms of 16-week-old mice on day 1-3 while under isoflurane anesthesia. The regime consisted of 120 cycles/day with a 2 Hz sine wave using an electromagnetic loading system with feedback control (EnduraTEC, Bone, Eden Prairie, MN). Left forearms served as non-loaded internal controls. Mice were subcutaneously injected with Calcine (10 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) on day 5 and Alizarin Red (70 mg/kg body weight; Sigma-Aldrich) on day 9. All animals were euthanized on day 12.

Dynamic Histomorphometry: Left and right ulnae were collected, cleaned of soft tissue, and stored in 70% ethanol for preservation. The ulnae were dehydrated in graded ethanol, infiltrated in methyl methacrylate (Sigma-Aldrich, St. Louis, MO), and embedded in methyl methacrylate and benzoyl peroxide. Transverse sections of the ulnar midshaft were separated using a diamond saw (Isomet, Buehler, Lake Bluff, IL), then imaged on a laser scanning confocal microscope (Leica TCS SP5, Buffalo Grove, IL). Dynamic histomorphometric measurements were completed using Image J. Relative (r) measurements of rMS/BS, rMAR, rBFR/BS, were calculated by subtracting the values from the left ulna from the right ulna to show differences due to mechanical loading.

Statistical Analysis: Data are presented as mean ± SEM. MicroCT analyses between AC6 WT and KO samples were tested for significance using unpaired t-tests. Dynamic histomorphometry analyses between loaded and unloaded ulnae were tested for significance using paired t-tests. rMS/BS, rMAR, and rBFR/BS between AC6 KO and WT mice were tested for significance using unpaired t-tests. For all tests, α= 0.05.

RESULTS: Body weight, crown-to-rump length, tibia and ulna lengths, and trabecular and cortical bone architecture were not significantly different between AC6 KO and WT mice (n=7-10). The axial compressive ulnar loading elicited a significant increase in rMS/BS, rMAR, and rBFR/BS in both WT (n=27) and AC6 KO (n=14) animals (Fig. 1). However, mice lacking AC6 had significantly less loading-induced bone formation in comparison to WT mice (32%, 30%, and 47% less rMS/BS, rMAR, and rBFR/BS, respectively). These results demonstrate that the loss of AC6 impairs the response of bone to mechanical loading.

DISCUSSION: Collectively, our data demonstrate that AC6 plays a role in regulating loading-induced bone formation in vivo. While AC6 does not appear critical for normal skeletal development, the deletion of AC6 inhibits the adaptation of bone to mechanical loads. Our study indicates the involvement of AC6 in bone adaptation and suggests modulation of AC6 activity as a potential mechanism of mechanotransduction in bone. Furthermore, AC6 localizes to the primary cilium and is inhibited by intracellular calcium, suggesting that it may act as a point of integration of the cAMP and calcium signaling systems and supports the role of the primary cilium as an intracellular signaling nexus. Determining the mechanical response of the primary cilium under loading and how these physical signals regulate AC6 will further demonstrate the contribution of primary cilium-mediated mechanotransduction.

SIGNIFICANCE: While mechanical loading regulates bone remodeling, the molecular mechanisms through which bone cells sense and respond to these loads are currently unknown. In this study, we demonstrate that loading-induced bone formation involves AC6 in vivo which enhances our understanding of primary cilium-mediated mechanotransduction in bone and may ultimately lead to better treatments to prevent bone loss.

Figure 1. Effects of AC6 deletion on loading-induced bone formation in vivo. There is a significant decrease in rMS/BS, rMAR, and rBFR/BS in AC6 KO mice compared to control mice. (* p<0.05)

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