

## Hippo Signaling Modulates the Mechanoresponses of NFκB in Chondrocytes via Protein Kinase C

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**Introduction:** Knee osteoarthritis (KOA) is a chronic disease affecting 30% of individuals over age 60 [1]. Excessive mechanical loading contributes to compression induced remodeling, which is hypothesized to play a central role in both incident and progressive KOA [2]. Chondrocytes interpret strain and other external environmental factors through mechanotransduction pathways, including the Hippo-YAP/TAZ pathway [3]. Studies have suggested that the Hippo-YAP/TAZ pathway plays a central role in the pathogenesis of KOA [4,5]. We have demonstrated that this pathway is activated via chondrocyte mechanical compression that can be inhibited via the LATS1/2 inhibitor [6]. Protein Kinase C (PKC) acts as a regulator of other cell lines yielding an inflammatory response tightly regulated by mechanical loading [7]. NFκB plays a crucial role in OA-associated events, including synovial inflammation and chondrocyte catabolism [8]. However, the regulatory role of Protein Kinase C (PKC) and Nuclear factor-kappa B (NFκB) remain uncertain [9,10]. We explored their roles in activating the Hippo-YAP/TAZ pathway by inducing chondrocyte inflammation through compressive loading.

**Methods:** PKC inhibitor study: The C28/I2 human chondrocyte cell line was employed. One million chondrocytes were inoculated into a 3-dimensional (3D) 2% agarose disc ("disc"). Discs were cultured in DMEM with 10% FBS and 1% antibiotic for 24 hours and then divided into 3 groups: control, compression, and compression + PKC inhibitor (AEB-071, 5 or 10 μM). The inhibitor was supplemented 18 hours before compression with a 4 hour 20% deformation static compressive load using a compression bioreactor to model excessive loading. Western blot analysis: Either cell-agarose constructs or chondrocytes (monolayer culture) were lysed with 1xSDS PAGE sample buffer. 10 μL protein samples were loaded and separated on 10% SDS PAGE gels, and then were transferred to PVDF membranes and blotted with respective antibodies. Gene expression evaluation: LATS1/2 or YAP gene was deleted in chondrocytes using CRISPR/Cas9 technology and cells were subjected to static compression. RNA extraction was performed using the Trizol reagent protocol by Invitrogen and cDNA synthesis with the QuantiBio qScript cDNA kit. qPCR was performed using the PerfeCta SYBR Green Supermix and protocol by QuantaBio. Comparisons between two groups were done using a student's two tailed t test. Significance was defined as  $p < 0.05$  in all cases.

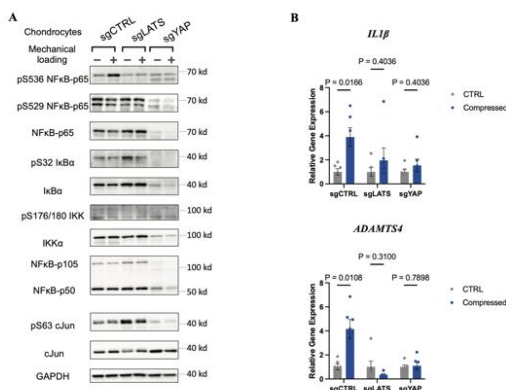
**Results:** Mechanical overloading increased expression of KOA-associated genes, IL-1β and ADAMTS-4 ( $n = 6$ ,  $p = 0.0055$ ,  $p = 0.0036$ ), and activate NFκB and Hippo signaling without triggering cell death. Deleting either LATS or YAP abolishes NFκB p65 phosphorylation at Serine 536 (Fig. 1A). Additionally, LATS1/2 and YAP knockout cells showed decreased expression of the NFκB target genes IL-1β and ADAMTS-4 ( $p = 0.0106$ ,  $p = 0.0108$ ) (Fig. 1B). PKC was activated by mechanical compression in a Hippo-dependent manner and required for NFκB activation. PKC activity was detected under chondrocyte mechanical loading (Fig. 2A). The PKC inhibitor AEB-071 blocked NFκB, inhibiting chondrocyte response to mechanical compression (Fig. 2B). AEB-071 also reduced LATS1/2 activity (Fig. 2C).

**Discussion:** We showed that either full inactivation of Hippo signaling or complete inactivation of YAP blocks NFκB p65 S536 phosphorylation and target gene expression, suggesting that the full cycle of YAP phosphorylation-dephosphorylation by the Hippo kinase cascade is essential for chondrocyte mechanoresponses. Our hypothesis is further supported by the mechanoresponses of PKC, consistent with other mechanotransduction pathways [8]. Based on our results, mechanical transduction induces PKC subsequently activating the Hippo pathway, followed by activation of NFκB to upregulate inflammatory genes (Figure 2D). A PKC inhibitor was shown to block this pathway, providing insight to the interplay between PKC and Hippo. This interplay introduces PKC as a new target for KOA therapeutic intervention by shutting down the Hippo pathway and suppressing NFκB activation.

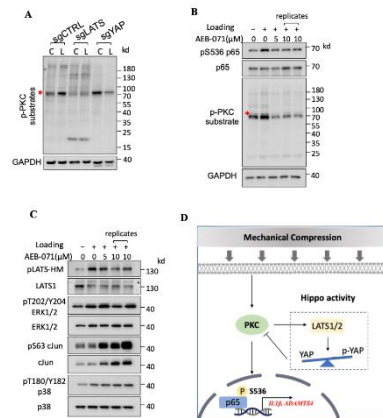
**Significance/Clinical Relevance:** Our results improve our understanding of the Hippo mechanotransduction pathway. Moving forward, we are exploring the efficacy of a PKC inhibitor in an *in vivo* model to mitigate or prevent post-traumatic KOA.

**References:** [1] Klinedinst et al., Nurse Res, 2022. [2] Kim et al., J Biomech, 1995. [3] Eckstein et al., J Anat 2006. [4] Deng et al., Nat Commun, 2018. [5] Sun et al., Mol Med, 2021. [6] Warburton et al. ORS conference, 2023. [7] Abe et al., J Mol Cell Cardiol, 2007. [8] Rigoglou and Papavassiliou Int J Biochem Cell Biol, 2013. [9] Deng et al., Nat Commun 2018. [10] Vantai et al., Development, 2020.

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**Figure 1.** Hippo pathway is required for mechanical loading-induced NFκB activation. Therefore, without the Hippo pathway, chondrocytes are unable to respond to mechanical compression via NFκB activation. Note the decreased NFκB expression with LATS and YAP knockout and subsequent decreased IL-1β and ADAMTS-4 gene expression.



**Figure 2.** PKC is activated by mechanical compression in a Hippo-dependent manner and required for NFκB p65 phosphorylation at Serine 536. Note the decreased in LATS1 activity with PKC inhibition (C) and decreased PKC substrates with LATS and YAP knockout after compressive loading (A). Figure 2D depicts the proposed mechanism of action for the Hippo chondrocyte pathway.