YAP and TAZ mediate transcriptional feedback loops for tenocyte tensional homeostasis

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INTRODUCTION: Tendon fibroblasts (tenocytes) exist within a highly organized collagen matrix and experience both matrix-generated and cell-generated tension. Healthy tenocytes maintain tissue homeostasis through feedback loops, referred to as tensional homeostasis. Damage to the tendon matrix de-tensions the tenocytes, which induces matrix metalloproteinase (MMP) production that remodels the matrix. This remodeling leaves the matrix disorganized, with reduced mechanical properties, but enables cytoskeletal re-tensioning [1]. However, the mechanisms that maintain tenocyte cytoskeletal tensional homeostasis remain unknown. Damage-induced loss of matrix tension de-activates the mechanosensitive transcriptional regulators, Yes-associated Protein (YAP) and Transcriptional co-activator with PDZ-binding motif (TAZ), which are driven out of the nucleus. Previously, we demonstrated that tenocyte de-tensioning inactivates YAP/TAZ-mediated epigenetic repression of MMP transcription, resulting in progressive matrix degradation [2]. These data present a potential mechanism for matrix tensional homeostasis in tenocytes, which has yet to be evaluated *in vivo*. Because these mechanosignaling pathways mediate tendon development, inducible tenocyte-conditional knockout models will be needed to study tensional homeostasis in adult tendons. Here, we sought to identify mediators of cytoskeletal tensional homeostasis and to develop a conditional knockout mouse model for inducible ablation of YAP/TAZ signaling *in vivo*.

YAP and TAZ are transcriptional regulators that control gene expression through co-transcriptional regulation of DNA-binding transcription factors, such as TEAD1-4. Myosin-induced cytoskeletal tension induces YAP/TAZ nuclear localization for transcriptional co-activation. Upstream, the small GTPase enzyme, RhoA, initiates myosin-induced cytoskeletal tension when in its GTP-bound active state but is inactivated by GAPs. Here, we show that loss of tenocyte cytoskeletal tension and YAP/TAZ depletion reduces expression of the RhoA-inactivating GAP, DLC1. We hypothesize that reduction of RhoA-inactivating DLC1 allows RhoA to remain in the active state restoring cytoskeletal homeostasis. Further, we introduce a new mouse model for inducible activation of Cre-recombinase in tenocytes *in vivo*, toward an inducible conditional knockout model for the study of tensional homeostasis *in vivo*.

METHODS: *In vitro:* Human tendon cells from adult donor patellar and Achilles tendons were transfected with SMARTpool ONTARGETplus siRNA. One group of tenocytes was treated with siRNA to suppress YAP1 and WWTR1 (TAZ) expression. Another experimental group was treated with siRNA to suppress Tead1, Tead2, Tead3, and Tead4. Non-targeting siRNA was used as RNAi control. Cells were treated with either 20 μM Blebbistatin or DMSO for 6 hours. Transfection was performed using Lipofectamine RNAiMAX. 48 hours after transfection, cells were lysed and purified using Qiagen RNeasy Mini Kit. RNA was converted to cDNA with Applied Biosystems' HighCapacity cDNA Reverse Transcription Kit. Gene expression was evaluated using quantitative real-time PCR and Applied Biosystems' PowerTrack SYBR PCR Master Mix on a QuantStudio 6 Pro machine. Fold change values were obtained through the delta delta Ct method and compared by unpaired, two-tailed t-tests ($\alpha = 0.05$). *In vivo:* Mice with tamoxifen-inducible Cre-ERT2 protein controlled by the pro alpha 2(1) collagen gene and Ai9 TdTomato reporter were crossed to generate a Col1a2-CreERT2;Ai9 reporter mouse. Two Cre + mice were injected with 3 intraperitoneal tamoxifen injections dissolved in corn oil at 100 mg/kg dosage approximately 24 hours apart (Day 1 = First Injection Day). On Day 10, hindlimbs were harvested for histology, fixed in 4% PFA and cryo-embedded. This study was approved by IACUC. Sex disclosure: Both mice were female as n=1. We plan to expand sex to males and females in the future.

RESULTS SECTION: *In vitro:* YAP/TAZ depletion by siRNA significantly reduced YAP and TAZ mRNA expression. Depletion of all four TEAD genes (TEAD1-4) significantly reduced Tead1-4 mRNA expression. Both YAP/TAZ depletion and Tead depletion significantly downregulated downstream targets of YAP/TAZ-TEAD signaling: Cyr61 and Ctgf **(Fig. 1 A-B).** Further, treatment with the myosin inhibitor, blebbistatin, significantly downregulated Cyr61 and Ctgf mRNA expression within siCtrl, siY/T, and siTead tenocytes compared to those treated with DMSO **(Fig. 1 A-B).** Additionally, depletion of either YAP and TAZ or Tead 1-4 significantly downregulated expression of the RhoA-inactivating GAP, DLC1 **(Fig. 1 C)**. Finally, blebbistatin significantly downregulated DLC1 mRNA expression within siCtrl, siY/T, or siTead experimental groups. *In vivo:* Tamoxifen injections induced Col1a2-CreERT2 expression in tendon cells in adult female mouse patellar tendons, marked by TdTomato expression in cells in the patellar tendon **(Fig. 2)**.

DISCUSSION: Here, we used blebbistatin to model tenocyte response to cellular detensioning, such as occurs after tendon injury. Here, we show that cytoskeletal detensioning inactivates YAP/TAZ-TEAD signaling in human tenocytes. Importantly, cytoskeletal detensioning reduces expression of the RhoA-inactivating GAP, DLC1, through disruption of YAP/TAZ-TEAD transcription. As DLC1 represses RhoA signaling, we hypothesize that a reduction in DLC1 expression by cytoskeletal detensioning represents a feedback loop that restores RhoA signaling and myosin activation for restoration of cellular tensional homeostasis. We also introduce Col1a2-CreERT2 as an inducible mouse model for the study of tendon biology. With these tools, we are positioned to explore the effects and mechanisms of tenocyte tensional homeostasis *in vivo*.

SIGNIFICANCE: Understanding tenocyte tensional homeostasis at both cell and matrix levels will allow us to better prevent tendon scarring after injury and to promote tendon repair in tendinopathy patients via therapeutic intervention.

REFERENCES: [1] Lavagnino+ J Orthop Res. 2005 [2] Jones+ J PNAS. 2023

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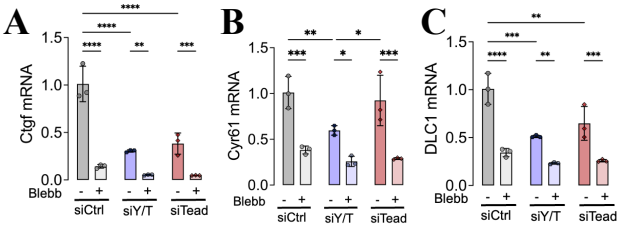

Figure 1. Human tendon fibroblasts transfected with siRNA targeting Yap and Taz (Wwtr) or Teads(1-4), treated with 20 μM Blebbistatin or DMSO for 6 hours **(***Utilization of 2 way ANOVA, Šídák's multiple comparisons test, * = p<0.05*

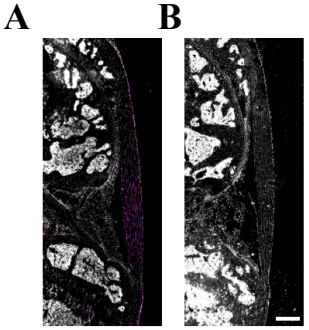

Figure 2. Female adult Col1a2 CreERT$^{+/-}$ Ai9 TdTomato$^{+/-}$ injected mice patellar tendons injected with tamoxifen in corn oil **(A)** or without injections **(B)** Hoescht shown in white. TdTomato shown in magenta. Scale bar = 200 μm