# AMPK signaling is dysregulated in tendinopathy, altering ECM-specific cell adhesion and matrix organization

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**DISCLOSURES**: None.

**INTRODUCTION**: Tendinopathy, a disorder that results in pain, swelling, and impaired tendon function, is a clinical problem that affects ~3.5 million people in the US.[1] Tendinopathy is caused by failure of tendon to self-repair and is characterized by degenerative extracellular matrix (ECM), decreased cell viability, and poor biomechanical function.[2] AMP-activated protein kinase (AMPK), an energy stress sensor that maintains intracellular metabolism, homeostasis and autophagy, has recently been identified as a potential regulator of ECM remodeling in musculoskeletal tissues.[3,4] For example, cartilage-specific loss of both *Prkcaa1* and *Prkcaa2*, genes that encode AMPKα1 & 2, respectively, resulted in ECM degeneration, increased matrix metalloproteinase (MMP) expression, and cell death.[4] Conversely, activation of AMPK via metformin prevented ECM degeneration, decreased MMP expression, and decreased cellular senescence in a mouse osteoarthritis model.[5] Additionally, loss of AMPK promotes β1-integrin activity, the formation of centrally located active β1-integrin and cell spreading[6]. We have recently shown that *in vivo* loss of AMPKα1 in tendon fibroblasts (TFs) utilizing a *Prkcaa1*$^{fl/fl}$;*Scx*Cre (AMPKcKO*)* mouse model results in decreased cell viability, accelerated age-dependent ECM degeneration, and impaired biomechanical properties. While our preliminary data strongly supports the necessity of AMPK for maintenance of tendon homeostasis, it remains unknown how AMPK drives cell attachment and matrix interaction in TFs. In this study we tested the hypothesis that AMPK signaling is downregulated in tendinopathy and furthermore loss of AMPK regulates cell matrix interactions and ECM organization.

**METHODS**: Human study procedures and protocols were approved by Institutional Review Boards (REC 11/S0704/7, HUM00196928). Bulk RNAseq was performed on tendinopathic Achilles tendons and healthy hamstring tendons obtained from patients (n=7 samples/group, age=15-70 years). We used DESeq2 in R/Bioconductor[7] to determine the differentially expressed genes (DEG) in the tendinopathic tendon with healthy tendons as baseline. p-values were adjusted for multiple testing using the Benjamini and Hochberg method, and significance was set at p-adjusted (p-adj) <0.05. All animal work was approved by the IACUC. Achilles and tail TFS from *Prkcaa1*$^{fl/fl}$ (WT) and AMPKcKO mice (mixed genetic background) were isolated and expanded in culture. P1 TFs were plated on ECM array slides (36 conditions x 9 technical replicates per condition, n = 3/genotype) and cultured for 24 hours. Cells were stained with Hoechst, fixed, imaged using fluorescence microscopy, and segmented & counted in FIJI/ImageJ using the StarDist plugin. To test if there is differential adhesion between the WT and AMPKcKO TFs, between different substrates, or preferential adhesion of the strains for different substrates, we compared the fit of a range of Bayesian regression models. ECM organization of WT and AMPKcKO Achilles tendons were determined using quantitative polarized light microscopy (qPLI). Paraffin embedded histologic sections were prepared using standard techniques and stained with picrosirius red to enhance sample birefringence. Degree of Linear Polarization (DoLP) and Angle of Linear Polarization (AoLP) images were acquired using a polarization camera (Thorlabs) and a circularly polarizing lens (Edmund Optics). The mean DoLP and standard deviation of the AoLP were compared between groups using a two-way ANOVA with post-hoc Sidak's multiple comparisons to identify specific differences (Genotype, Time; GraphPad Prism v10).

**RESULTS**: We found 83 and 252 genes to be up and downregulated with tendinopathy, respectively. We identified enrichment of AMPK signaling, metabolism, and focal adhesion pathways in the tendinopathic samples compared with healthy tendons (Fig 1a). AMPK signaling pathway was driven by 7 differentially expressed genes (DEG), of which 6 were downregulated with tendinopathy (Fig 1b). Using ECM arrays, we found the negative binomial response models fit better than the Poisson response models, suggesting either shared spot level variation, substantial growth dynamics, and/or synergistic adhesion through cell-to-cell interaction. For the baseline models, where genotype and matrix were not allowed to interact, we found that the AMPKcKO strain was less adherent than the WT and that COL1, COL6, fibronectin and vitronectin were more permissive and COL3 to be less permissive for adhesion (data not shown). The interaction modeling (genotype and matrix interact) suggests there was a modest preference of AMPKcKO cell adhesion for Col4 and decreased preference for COL1 and laminin relative than what would be expected from the strain:substrate effects by themselves (Fig 2). Loss of AMPK increased tendon organization at 1 month only (Fig 3).

**DISCUSSION**: We found that AMPK signaling is dysregulated in tendinopathic patients. Furthermore, we observed that loss of AMPK disrupts tendon fibroblast function including adhesion of primary mouse tendon cells to specific ECM proteins but increases matrix organization earlier in life. Future work will define metabolic and transcriptional changes in AMPKcKO tendon cells as well as ECM remodeling. Our long-term goal is to identify targets of AMPK-dependent ECM remodeling for therapeutic intervention of tendon disease.

**SIGNIFICANCE/CLINICAL RELEVANCE**: Tendinopathy has few non-surgical treatment options.[1] Elucidating metabolic targets for druggable therapy will improve current clinical limitations.

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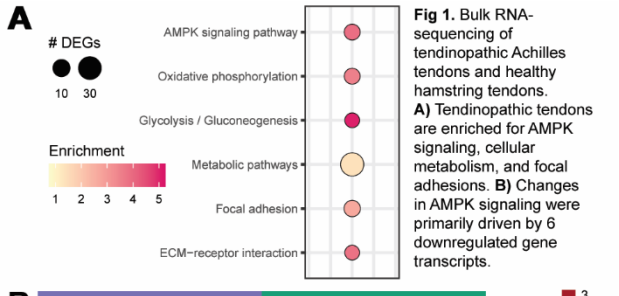


**Fig 1.** Bulk RNA-sequencing of tendinopathic Achilles tendons and healthy hamstring tendons. **A)** Tendinopathic tendons are enriched for AMPK signaling, cellular metabolism, and focal adhesions. **B)** Changes in AMPK signaling were primarily driven by 6 downregulated gene transcripts.

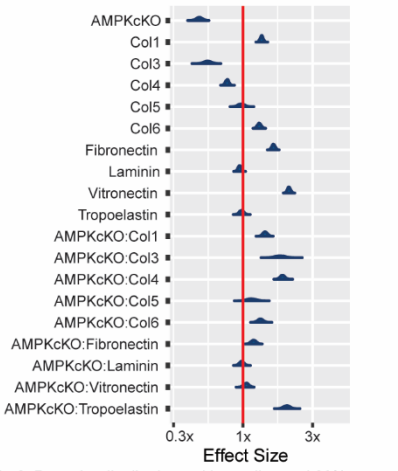


**Fig 2.** Posterior distributions with median and 80% confidence intervals of Bayesian regression interaction model. Distributions greater than 1x effect size (red line) indicate more cells adhered after 24 hours as compared to global median. Less than 1x indicate fewer cells adhered.

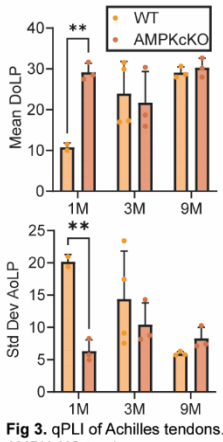


**Fig 3.** qPLI of Achilles tendons. AMPKcKO tendons were more organized (increased DoLP, decreased Std Dev AoLP) at 1 month (1M) only. **p<0.01