AMPK signaling is dysregulated in tendinopathy, and Loss of AMPKα1 Leads to Age-Associated Cell, Matrix, and Mechanical Dysfunction in Mouse Achilles Tendon

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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, 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] Yet, if and how AMPK acts to regulate tendon homeostasis, ECM remodeling and cell-ECM interactions remains unknown. In this study, we tested the hypothesis that loss of AMPKα1 impairs cell-ECM interactions, ECM remodeling, cell survival, and mechanical function of mouse Achilles tendons.

METHODS: Human study procedures and protocols were approved by the Institutional Review Board (REC 11/S0704/7, HUM00196928). Bulk RNAseq was performed on tendinopathic Achilles tendons and healthy hamstring tendons obtained from patients (n=7 samples/group). All animal work was approved by the Institutional Animal Care & Use Committee. Wildtype (WT) and *Prkcaa1*$^{fl/fl}$;*Scx*Cre (*cKO)* mice) were generated for evaluation of Achilles tendon structure and function at 1-, 3-, and 9-months (1M, 3M, 9M). *Scx*Cre-negative floxed mice (WT) were used as controls (n $\geq$ 3/group). We evaluated ECM organization using quantitative polarized light microscopy (qPLI), Paraffin embedded histologic sections were prepared using standard techniques and stained with picrosirius red to enhance sample birefringence. ECM morphology was visualized by silver staining to highlight lesion areas in the tendon. Digital x-ray was used to identify the lesion areas as ectopic bone formation. Functional properties of tendon were assessed using uniaxial biomechanical testing, and tendon cross-sectional area (CSA) was measured using photogrammetry. To evaluate cellular senescence, we stained WT and *cKO* tail tendon fibroblasts with senescence-associated p16 and p21. Achilles and tail tendons fibroblasts (TFs) from WT and *cKO* mice were isolated and expanded in culture. To determine changes in cellular adhesion affinity 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 *cKO* TFs, between different substrates, or preferential adhesion of the strains for different substrates, we compared the fit of a range of Bayesian regression models using R.
Statistical analyses were performed using Prism GraphPad (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. The AMPK signaling pathway was driven by 7 differentially expressed genes (DEG), of which 6 were downregulated with tendinopathy (Fig. 1). We found tendons from *cKO* mice had no visible ECM differences at 1M compared to WT tendons but developed ECM degeneration, indicated by positive Silver-stained lesions, by 3M and further progressed to heterotopic ossification by 9M. Conversely, *cKO* tendons had increased tendon organization at 1 month only. *cKO* tendons had impaired mechanical function at all timepoints (Fig. 2 A, B). Additionally, *cKO* tendons had increased markers of senescence at 9M (Fig 3). 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 *cKO* strain was less adherent than the WT. Additionally, COL1, COL6, fibronectin and vitronectin were more permissive and COL3 was less permissive for adhesion (Fig 4). The interaction modeling (genotype and matrix interact) suggests there was a modest preference of *cKO* cell adhesion for Col4 and decreased preference for COL1 and laminin relative than what would be expected from the genotype:substrate effects by themselves (Fig 4).

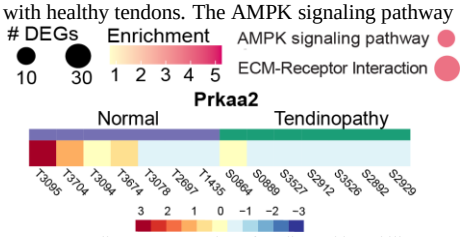

Figure 1: Bulk RNA sequencing of tendinopathic Achille's tendons and healthy hamstring tendons. Tendinopathic tendons are enriched for AMPK signaling pathway and ECM-receptor interactions. Prkaa2 is downregulated in tendinopathic tendons.

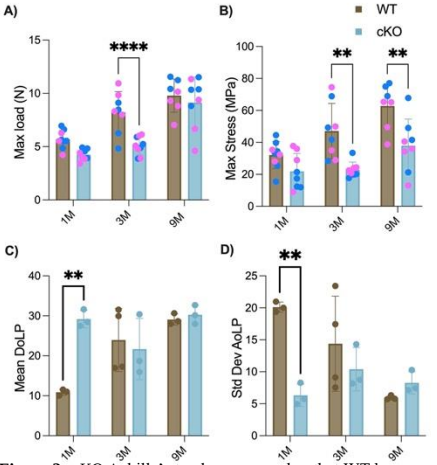

Figure 2: *cKO* Achille's tendons are weaker that WT but more aligned. A) Max load is significantly reduced at 3M for *cKO* tendons. B) Max stress is significantly reduced at 3M and 9M for *cKO* tendons. C) Mean DoLP is significantly increased in *cKO* tendons at 1 month. D) Std Dec AoLP I significantly decreased in *cKO* tendons at 1 month.** p<.01,**** p<.0001. Male = blue, Female = pink.

CONCLUSION: We found that AMPK signaling is dysregulated in tendinopathic patients and our findings support our hypothesis that AMPK is necessary for tendon homeostasis, cell survival, and tendon function. Furthermore, we observed that loss of AMPK disrupts tendon fibroblast function including adhesion of primary mouse tendon cells to specific ECM proteins. 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.

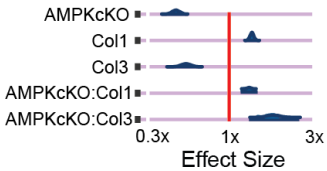

Figure 3: Posterior distributions with median and 80% confidence intervals of Bayesian regression baseline and interaction models. *cKO* strain was less adherent than the WT after 24hrs. COL1 Was more permissive for adhesion than COL3. cKO cells had an increased affinity COL3 relative to COL1.

REFERENCES: 1. Millar, et. al *Nat Rev Dis Primers* **7**, 1 (2021); 2. Fouda, et. al *Am J Transl Res* **9,** 4341-60 (2017); 3. Grieve, et. Al *Scand J Med Sci Sports* **22**, 55-63 (2012); 4. Zhou, S. *Sci Rep* **7**, 43245 (2017); 5. Feng, et.al *Aging* **12**, 1087-1103 (2020)