Identifying Nuclear Membrane Proteins that Facilitate Chromosomal Mechanotransduction

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Technical Project Team Members
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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Identifying Nuclear Membrane Proteins that Facilitate Chromosomal Mechanotransduction

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Abstract

Idiopathic Pulmonary Fibrosis (IPF) is an end-stage lung disease that is mediated by force interactions within the lung epithelium. The current standard of care aims to reduce the symptoms of the disease through non-curative drug treatments or by lung transplantation. Our team aims to determine an additional upstream target that would reduce the progression of fibrosis. We hypothesize that LRP-130, CAPZ-α, and MATR3 play a role in force mechanotransduction and ultimately IPF. To determine the proteins involved in the mechanosensitive signaling pathway, a force immunoprecipitation technique is used to pull down the proteins involved. These proteins are then analyzed through proteomics techniques such as western blotting and immunofluorescence. A knockdown study was performed to determine the individual role the proteins play in YAP/TAZ nuclear translocation. LRP-130, CAPZ-α, and MATR3 have been established as potential proteins in the pathway and have been identified in samples subject to force. The localization of these proteins is linked to the nuclear membrane. Additional knockdown studies will be performed to determine the extent to the protein's effect on YAP/TAZ nuclear translocation. Overall, we have observed that these proteins play a role in regulating the cellular response to force mechanotransduction. This work is significant to the future of IPF treatments because these proteins serve as potential targets for curative therapies.

Keywords: Proteomics, Early Stage Mechanotransduction, Idiopathic Pulmonary Fibrosis

Introduction

Background of Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a chronic disease of the lungs, which leads to irreversible fibrotic remodeling and loss of pulmonary function. With only a 2-3 year median survival rate after diagnosis, IPF is a devastating and terminal diagnosis.[1] Over the past decade, the understanding of IPF has increased dramatically. Although IPF was once thought to worsen as a result of constant inflammation, it is now understood that IPF is worsened due to abnormalities in the wound healing cascade and biochemical responses to mechanical stimuli. This shift in understanding has led to increased research into extracellular matrix proteins and pro-collagen factors that lead to pulmonary fibroproliferation.[2] Once the faulty locations of the fibrotic mediator's pathway are found, the resulting treatments could prevent the onset of fibrosis.

IPF and the Mechanosignaling Pathway

Mechanotransduction has been implicated in the progressive nature of IPF. When cells sense extracellular forces, they are able to convert these mechanical stimuli into biochemical signals through mechanosignaling pathways. These pathways are important for wound healing and closure to prevent infection. In non-regenerative tissues, such as cardiac and pulmonary tissues, healthy cells are replaced by fibroblasts as a result of the mechanosignaling pathway. While this fibroproliferation and scar formation maintains mechanical integrity, pulmonary function is compromised. Indeed, with IPF, there is hyper activation of this pathway. This hyperactivation is a result of the respiratory cycle, in which there is continual introduction of mechanical stresses and strains to pulmonary tissue.[3] Responding to these mechanical stimuli, cells activate the mechanosignaling pathway and scar remodeling processes.

Current Understanding of the Mechanosignaling Pathway

The mechanical signaling pathway is a complex system involving both inter- and intracellular signaling. Currently, researchers believe that the mechanical signaling pathway begins at the integrin that senses force. This signal then travels from the extracellular matrix (ECM) to the cytoskeleton, cytoskeleton to nucleoskeleton, and ultimately chromatin for transcription of genes. From the ECM to the cytoskeleton, cell surface receptors like fibrinogen and fibronectin recognize ECM molecules and bind via integrins. Integrins are transmembrane proteins that can both extracellularly bind to ECM and intracellularly bind to actin through cross-linking proteins. These cross-linking proteins create the mechanical connections of the ECM to the cytoskeleton that allows for initial force transduction. Cytoskeletal cross-linkers also mechanically connect the other cytoskeletal proteins to one another.[4]

The Linker of Nucleoskeleton and Cytoskeleton (LINC) Complex was recently discovered to play a key role in mechanotransduction. Just as integrins connect the ECM to the cytoskeleton, the LINC complex connects the cytoskeleton to the nucleoskeleton. The nuclear membrane consists of an outer nuclear membrane (ONM), inner nuclear membrane

(INM), and nuclear lamina (NL). Each layer has distinct proteins. The ONM consists of nesprins or Klarsicht ANC-1 Syne Homology (KASH) domain proteins. Nesprins connect with cytoskeletal proteins. The INM contains Sun-domain proteins, which connect with proteins in the nucleoplasm. The KASH-domain and Sun-domain proteins connect in the perinuclear space.[5] It is still unclear the specific proteins involved from the LINC Complex to chromatin.

Novel Force Immunoprecipitation Assay

The current, accepted mechanosignaling pathway is weakly linked and vague. While momentum has been afforded through elucidation of the LINC Complex, there are still gaps in the network. The in situ predictions via Cytoscape will be tested through a novel force immunoprecipitation assay. Through cross-linking magnetic beads to cells, a force will be applied to recapitulate the mechanosignaling pathway in vitro. The presence of predicted proteins can then be tested using proteomic analysis. While this method has been used previously, it has not been performed in relation to mechanotransduction. Further defining this model will encourage drug development targeting the novel signaling pathway.

Novel Protein Targets

Although there are two FDA approved drugs on the market for IPF treatment, they only slow the progression of fibrosis. These drugs, Nintedanib and Pirfenidone, target downstream proteins in fibroblast activity and development. Nintenidab inhibits tyrosine kinases which are involved in expression of profibrotic mediators including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF). Pirfenidone inhibits the small molecules like VEGF, procollagen I and FGF.[6] This inhibition of growth factors reduces fibrotic activity.[7]

While both Nintedanib and Pirfenidone slow down the progressive fibrosis, they do not stop disease progression. However, they have provided momentum to study profibrotic mediators as targets for fibrotic development. This project aims to elucidate proteins that occur earlier in fibrosis development. Due to mechanotransduction involvement in initial fibrotic remodeling and wound healing, stopping its early pathways would prevent the fibrosis that still occurs with Nintedanib and Pirfenidone.

Materials and Methods

Cells and Reagents

All cells were maintained in a standard cell culture incubator at 37°C and 5% CO_2. Primary human lung fibroblasts (ATCC CCL-210) were isolated and cultured in high glucose DMEM (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin. Cell flasks were cultured to 80% confluence on T-175 culture flasks and maintained at a low number passage to ensure efficient activity.

Force Immunoprecipitation Assay

The force immunoprecipitation protocol was adapted from Fiore et al. to collect protein clusters at an early stage of mechanotransduction.[8] CCL-210 cells were subsequently plated at 2.5 million cells on 100x20 mm Corning tissue culture dishes for 24 hours in serum-containing media. The media was changed to Opti-MEM Reduced Serum Media (Thermo Fisher Scientific) for 24 hours. Magnetic Dynabeads beads (Invitrogen) were washed with 0.1 M Na-phosphate buffer, pH 7.4 and conjugated with fibronectin at a concentration of 50ug/mL. After a 24-hour incubation, the fibronectin-conjugated beads were blocked with 0.5% BSA for one hour to control for non-specific binding. Fibronectin-coated beads were added to tissue culture plates containing CCL-210 cells in Opti-MEM and incubated in the tissue culture incubator at 37°C and 5% CO_2 for one hour. A circular magnet that applied 10 pN of force was placed on top of the force positive tissue culture plate for five minutes. Cells were immediately collected from all samples with a cell scraper and lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100, and 25 mM NaF and supplemented with 1× cOmplete Protease Inhibitor and PhosSTOP phosphatase inhibitor cocktails. Lysates were separated by centrifugation for 15 min at 15,000 rpm at 4°C. The supernatant was then removed and the beads were eluted from the protein cluster in 3X SDS_sample buffer and boiled in a heat block at 97.5°C for five minutes. The remaining protein supernatant was collected and resuspended.

Mass Spectrometry

Following cell lysis, protein clusters are crosslinked with DSSO, eluted from the beads in 3X SDS_sample buffer with glucose, and boiled in a heat block at 97.5°C for five minutes. Liquid chromatography-mass spectrometry was performed in the University of Virginia core facility.

Western Blotting

Protein expression levels were quantified using western blotting. Eluted protein samples and loading dye were combined at a 4:1 ratio and boiled in a heat block at 97.5°C for five minutes. 40 µl of each sample was loaded into a gradient gel (4-20%) gel and run at 70 V for 15 minutes. The gel was then run at 120 V for an additional 60 minutes. The proteins were transferred to a PDGF membrane using the semi-dry transfer system. The membrane was blocked with 5% dry milk for 30 minutes. The following primary antibodies were incubated with the membranes for 24 hours: CAPZ-α (1/1000; sc-376134, santa cruz), LRP-130 (1/1000; 1H19L11. ThermoFisher), MATR3 (1:1000; M2419-24J, USBiological), Paxillin (1:1000; ab32084 abcam), Histone-3 (1:1500; #9715 cell signaling), and GAPDH (1:1000; #2118 cell signaling). The membrane was washed with TBS and 0.1% Tween-20. The secondary antibodies were incubated with the membrane for 1 hour in RT. Images were taken with Amersham Imager 600.

Immunostaining and Confocal Microscopy

Cells were added to chamber slides and cultured overnight. Samples were washed twice with cold PBS and fixed with acetone-methanol 1:1 reagent. Cells were blocked with 5% milk and incubated with the following primary antibodies for 24 hours: CAPZ-α (1:100; sc-376134, santa cruz), LRP-130 (1:50; ab97505, abcam), and MATR3(1:50; M2419-24J, USBiological). The slides were incubated with the secondary antibodies for 1 hour in RT, keep in dark. Cells were mounted

onto glass coverslips stained with DAPI and imaged in the Perkin fluorescence confocal microscope system.

YAP/TAZ Nuclear Translocation

Force immunoprecipitation precipitation assay was performed as previously described. After magnetic force is applied, cell collection occurred at three time points post force application for force positive samples: 30 minutes, 1 hour, and 3 hours. Cells were incubated with a hypotonic buffer to break the cell membrane. The cytosolic portion was collected after centrifugation at 14000x rpm for 1 minute, and the nuclear portion was incubated with a cold nuclear extraction buffer to break the nuclear membrane. Then, the nuclear portion was centrifuged and collected. Protein concentrations were measured using a spectrophotometer. YAP1 protein levels were quantified using the western blot protocol as previously described for both nuclear and cytosolic extracts.

Quantification and ImageJ Analysis

Mass spectrometry data was localized to various cellular compartments using Scaffold. Protein expression levels in western blots were quantified with ImageJ. Intensity plots were obtained for each lane. The peaks for these plots represent the relative protein densities. The area of these plots was obtained using the ImageJ Wand tool after highlighting the desired peak. Each density value was then normalized to the loading control and a control sample. The normalized values represented the changes in protein expression after force immunoprecipitation assay. Immunofluorescence images were analyzed in ImageJ. Z-stack images were compressed to show individual channel images and merged images were layered to visualize channel overlap.

Results

Proteins are isolated and identified via mass spectrometry

Using the mass spectrometry data and the online application Scaffold, 1555 proteins were characterized as part of the force-induced protein cluster (Supplemental Figure 1). Particular attention was given to the nuclear membrane proteins because the nuclear membrane and into the nucleus has not previously been as well characterized as the actin cytoskeleton. Within the nuclear membrane proteins, only two proteins appeared in both the inner and outer nuclear membrane data sets: Emerin and LRP-130. Emerin has been shown to be a mechanosensitive transmembrane protein that plays a role in nuclear shape and expression of mechanosensitive genes.[9] In comparison, LRP-130 has only been studied on mitochondrial membranes,[10] thus, we hypothesized that LRP-130 exists as a nuclear transmembrane protein and plays a role in mechanotransduction signaling. Particular attention was paid to two other proteins, CAPZ-α and MATR3, in the mass spectrometry data set due to their potential indirect and direct interactions with LRP-130.

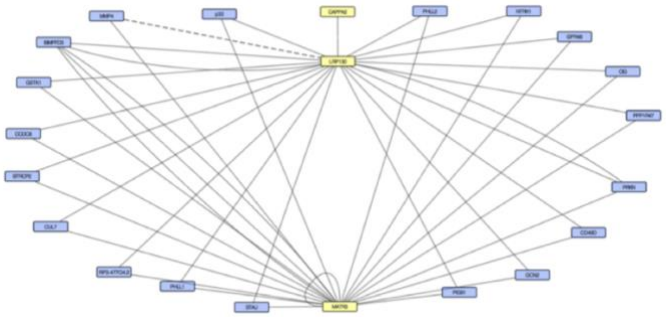


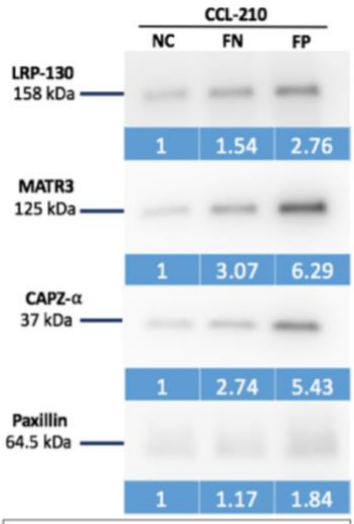
Fig. 1. Cytoscape analysis visualizes relevant proteins involved in early stage mechanotransduction. LRP-130, CAPZ-α, and MATR3 are identified as relevant proteins to the signaling pathway and their direct and indirect interactions are mapped. The proteins of interest are highlighted in yellow and the known indirect interactions between LRP-130 and MATR3 are shown in blue.

Protein interactions are visualized in Cytoscape

Through Cytoscape, using the BioGrid database, the interactions between CAPZ-α and LRP-130 were mapped and predicted (Figure 1). Hein et al. had previously shown interactions between LRP-130 and CAPZ-α outside the scope of mechanotransduction.[11] We hypothesized that LRP-130 and CAPZ-α interact, thus connecting the actin cytoskeleton to the nuclear membrane. The interactions between LRP-130 and MATR3 were also mapped and predicted (Figure 1). Since MATR3 has high protein interaction in the nucleus and is upstream from interactions with chromatin proteins, we also hypothesized that an interaction with MATR3 could trigger a chromatin interaction.

LRP-130, CAPZ-a, and MATR3 are identified in force negative and force positive samples

After force immunoprecipitation, the proteins were identified using western blot analysis. Using ImageJ, intensity values were obtained. These values were then normalized to both the negative control as well as a loading control Paxilin. The negative control functions to determine the non-specific binding to the beads associated with the lysis buffer used after magnetic force application and cell collection. A shown in Figure 2, CAPZ-α, LRP-130 and MATR3 increased 26.1%,



Values Normalized to Paxillin Control			
	MART3	LRP130	CAPZ-α
Negative Control (NC	1	1	1
Force Negative (FN)	2.61	1.31	2.33
Force Positive (FP)	3.40	1.49	2.93

Fig. 2. Western blotting confirms existence of LRP-130, CAPZ-, and MATR3 in protein cluster. Relevant proteins are blotted in both samples with an increased fold change in all force positive samples.

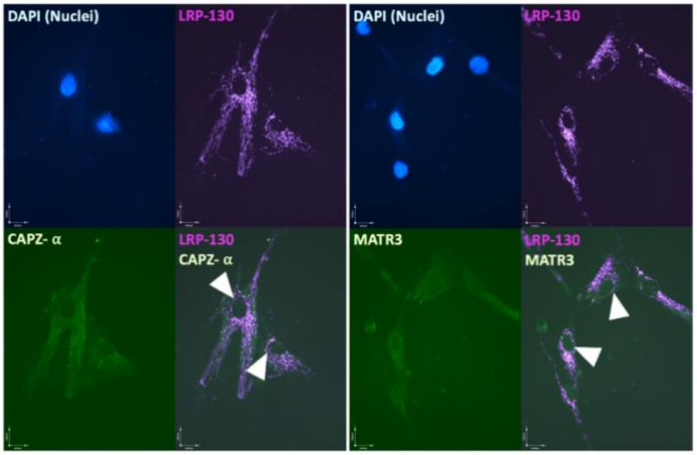


Fig. 3. Immunofluorescence imaging shows localization of relevant proteins. Proteins of interest were stained to show specific localization in a cell, not subject to force, to confirm mass spectrometry localization categories. White arrow show overlap of proteins on the nuclear membrane and scale bars are 18 μm.

14.0% and 30.4% in intensity after force application, respectively. Therefore, these data imply that there is an upregulation of these proteins following force application.

Localization of LRP-130, CAPZ-a, and MATR3 is confirmed to the nuclear membrane

The localization of the proteins of interest was analyzed through immunofluorescence (Figure 3). LRP-130 and CAPZ-a localized to the outer nuclear membrane, while LRP-130 and MATR3 localized to the inner nuclear membrane. Specific localization is highlighted by white arrows. LRP-130 can be seen throughout the entire cell due to its additional localization to the mitochondria, thus particular attention should be drawn to the localization of LRP-130 on the nuclear membrane.

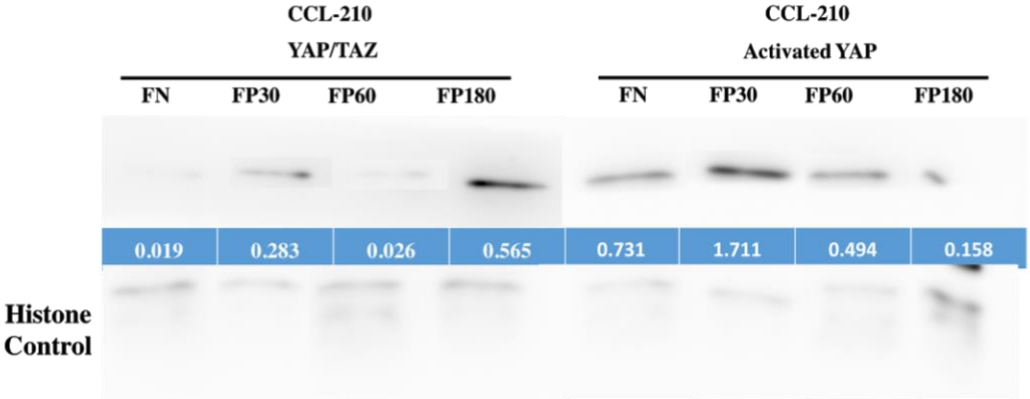
YAP/TAZ nuclear translocation is triggered by force immunoprecipitation

To determine the effect of the magnetic force on the mechanotransduction pathway, cytosolic and nuclear YAP/TAZ was quantified to detect the baseline changes in nuclear translocation of YAP/TAZ prior to performing a knockdown study on the proteins. Four samples were tested: Force Negative (FN), Force Positive - 30 minutes (FP30), Force Positive - 60 minutes (FP60), and Force Positive - 180 minutes (FP180). Different time points were collected after applying force to determine which time point showed the strongest signal in the least amount of time. This time point was then used in subsequent siRNA knockdown studies to maintain efficiency. Figure 4 shows YAP/TAZ nuclear translocation of CCL-210 cells in a western blot as well as staining for activated YAP. Histone H3 was used for a nuclear fraction loading control because it has a molecular weight of 17 kDa in contrast to the 70 and 50 kDa YAP/TAZ complex, and it maintains constant expression in the nucleus. GAPDH was used for a cytosolic fraction loading control because it also has a lower molecular weight of 36 kDa and is a constantly-expressing glycolytic enzyme.[12,13] The fold changes between samples were calculated via ImageJ and can be seen in Figure 1. Data for nuclear proteins was first normalized to histone as a total protein control, followed by the sample without force application as a negative control. According to the data, YAP/TAZ nuclear expression levels oscillated upon force application. This data is consistent with current literature regarding the biphasic nature of YAP/TAZ nucleo-cytoplasmic shuttling.[14] The canonical HIPPO pathway has been expanded to suggest that YAP and TAZ are dynamically shuttled between the nucleus and cytoplasm, and that YAP activation requires the initial nuclear exit and re-entry.[14] These results support that the novel force application assay induces nuclear YAP/TAZ translocation and that the expression levels are consistent with the normal mechanosignaling pathway. This baseline data suggests that YAP/TAZ nuclear translocation can serve as an indicator for activation of the mechanosignaling pathway. In addition, preliminary data (Supplemental Figure 2) compares the nuclear and cytosolic activated YAP1, confirming the results in Figure 4.

Discussion

Novel force immunoprecipitation assay allows identification of proteins in the mechanosignaling pathway

In this paper, we have shown the ability of our force precipitation assay to effectively isolate proteins involved in the force mechanotransduction signaling pathway from integrin to DNA. In previous mechanotransduction studies, the effect of force on cell signaling was



Values Normalized to Histone Control		
	YAP/TAZ	Activated YAP
Force Negative (FN)	1	1
Force Positive 30 minutes (FP30)	17.94	2.34
Force Positive 60 minutes (FP60)	1.69	0.68
Force Positive 180 minutes (FP180)	35.82	0.22

Fig. 4. Western blot of nuclear YAP/TAZ transcription factors. Nuclear proteins were pulled down from the magnetic precipitation samples. A western blot was performed to confirm the existence of YAP/TAZ and activated YAP1 in the nucleus at various time points. Densitometry values were normalized to the force negative sample to remove background translocation and then normalized to the histone control.

studied through altering the stiffness of substrates to determine the impact of stiffness of cell signaling and gene expression.[15] In these studies, cells must be cultured for several hours to attach to the substrate and induce the force signaling pathways. This timeline restricts the ability of these techniques to measure the immediate effect of force on early stage mechanotransduction signaling. Through the force immunoprecipitation assay, early mechanotransduction signaling is analyzed by immediately lysing the cells after force application to maintain the state of the protein cluster as the proteins are recruited and interacting. Further analysis of the protein cluster through mass spectrometry, western blot, and immunofluorescence allows identification of both protein identities and protein localization through the compartments of the cell. In this study, we have used this assay and the subsequent proteomics techniques to identify CAPZ-α, LRP-130 and MATR3 as proteins that can be isolated by force immunoprecipitation. Further research is needed to confirm that these proteins play a specific role in regulating mechanosensitive cell signaling.

Limitations of the force immunoprecipitation assay

While the force immunoprecipitation assay provides a unique technique to cluster the proteins involved in early stage mechanotransduction, the assay also maintains limitations that lead to difficulty collecting meaningful data. In both the Force Negative and Force Positive samples, the magnetic beads are attached to the cells via fibronectin. Due to the minimal weight of the beads, the mechanosignaling pathway is triggered in both the Force Positive and Force Negative samples, regardless of the fact that only the Force Positive samples receives signaling through magnetic force. This limitation was confirmed through the similar mass spectrometry results between samples (data not shown), as well as the visible blotting of all three proteins in the Force Negative sample in Figure 2. Thus, we determined that it is necessary to quantify the amount of each specific protein in the two samples. Preliminary studies utilized a urea precipitation to elute the proteins from the beads to obtain an accurate protein concentration.

Furthermore, since the protein cluster contains protein from the integrin to the DNA, it is possible to isolate the DNA from the end of the protein cluster as well. While DNA is also pulled down through the force immunoprecipitation process it is tedious and difficult to adequately isolate a significant concentration of DNA for sequencing. This limitation leads to difficulty in comparing DNA expression changes for knockdown studies and further suggests the need to compare changes in known mechanosensitive proteins like we have suggested with the YAP/TAZ complex.

Implications for Future Studies

Our initial studies have hypothesized that CAPZ-α, LRP-130 and MATR3 impact cell signaling and gene expression when cells are subject to a significant force. To confirm the role of these proteins in mechanotransduction, a knockdown study must be performed. This study can be performed through either analysis of gene expression changes through RNA sequencing or through determining the impact of these proteins on known mechanosensitive factors. In this study, we opted to focus on the YAP/TAZ complex, as it is well known that these transcription factors play a significant role in changes in gene expression due to force. YAP/TAZ is known to translocate into the nucleus when cells are subject to force,[16] therefore, if the mechanosensitive pathway is disrupted prior to YAP/TAZ signaling, both nuclear translocation and changes in gene expression would also decrease. Thus, further research aims to show that a knockdown of our identified proteins could show decreased YAP/TAZ nuclear translocation.

Additional future work could continue to elucidate the direct and indirect interactions of the identified proteins. A proximity ligation assay could confirm the localization of these proteins within 40 nm and further visualization through STORM microscopy could visualize direct interactions between these proteins. By determining these interactions, the mechanism to which the mechanosignaling pathway transmits signals from integrin to DNA can be further understood.

This work impacts the field of mechanobiology as it suggests novel techniques to understand early stage mechanotransduction. Due to the fact that IPF is driven by mechanosignaling, the proteins identified by these techniques have potential to play a role in the gene expression changes associated with IPF. By understanding the important proteins for IPF signaling, viral vectors could be developed to target and knockdown these proteins to provide a curative treatment to the progression of IPF.

End Matter

Author Contributions and Notes

A.H., V.H., and C.Y. designed research, A.H. and V.H. performed research, A.H. and V.H. analyzed data, and A.H. and V.H. wrote the paper. The authors declare no conflict of interest.

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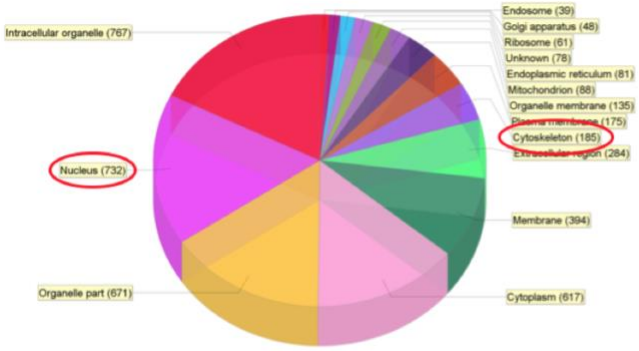
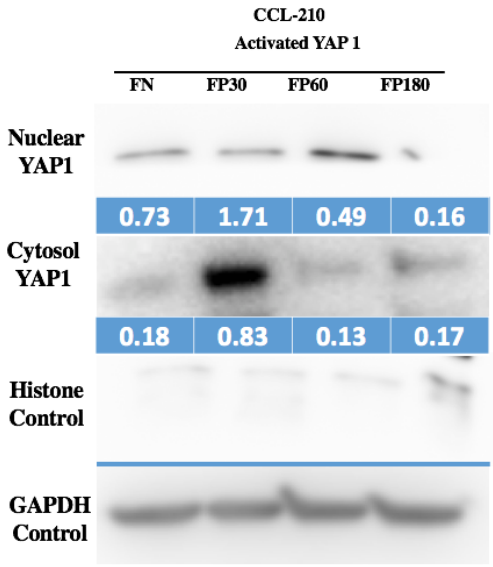
Supplemental Figures



Supplemental Fig. 1. Mass spectroscopy protein localization categories. Particular attention is paid to the cytoskeletal and nuclear proteins as initial predictions begin with these data sets.



Final Normalized Values			
	Nucleus	Cytosol	Fraction
Force Negative (FN)	1	1	--
Force Positive 30 minutes (FP30)	0.68	4.63	0.128
Force Positive 60 minutes (FP60)	2.34	0.73	0.762
Force Positive 180 minutes (FP180)	0.22	0.93	0.191

Supplemental Fig. 2. Western blot of activated YAP1 nuclear translocation. Nuclear proteins were pulled down from the magnetic precipitation samples. and separated from cytosolic proteins. A western blot was performed to confirm the existence of activated YAP1 at various time points in the nucleus and cytosol. Densitometry values were normalized to the histone and GAPDH controls and then normalized to the force negative sample to remove background translocation. The fraction of activated YAP1 in the nucleus was calculated based on the amount of YAP1 in the nucleus over the total amount of YAP1 in the cell.