Phosphorylation of GMFγ by c-Abl coordinates lamellipodial and focal adhesion dynamics to regulate airway smooth muscle cell migration

Brennan D. Gerlach, Kate Tubbesing, Guoning Liao, Alyssa C. Rezey, Ruping Wang, Margarida Barroso and Dale D. Tang

ONLINE DATA SUPPLEMENT

EXTENDED SUPPLEMENTAL METHODS

Cell Culture

Human airway smooth muscle cells (HASMCs) were prepared from bronchi and adjacent tracheas of control subjects (died from non-asthmatic causes) obtained from the International Institute for Advanced Medicine (50). Non-asthmatic and asthmatic human airway smooth muscle cells were also obtained from Dr. Reynold A. Panettieri of Rutgers University (29, 33). Human tissues were non-transplantable and consented for research. This study was approved by the Albany Medical College Committee on Research Involving Human Subjects. Briefly, muscle tissues were incubated for 20 min with dissociation solution [130 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM Hepes, 0.25 mM EDTA, 10 mM D-glucose, 10 mM taurine, pH 7, 4.5 mg collagenase (type I), 10 mg papain (type IV), 1 mg/ml BSA and 1 mM dithiothreitol]. All enzymes were purchased from Sigma-Aldrich. The tissues were then washed with Hepes-buffered saline solution (composition in mM: 10 Hepes, 130 NaCl, 5 KCl, 10 glucose, 1 $CaCl_2$, 1 $MgCl_2$, 0.25 EDTA, 10 taurine, pH 7). The cell suspension was mixed with Ham's F12 medium (ThermoFisher) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). Cells were cultured at 37°C in the presence of 5% CO_2 in the same medium. The medium was changed every 3–4 days until cells reached confluence, and confluent cells were passaged with trypsin/EDTA solution. Cell lines from five control subjects were available for the experiments. In some cases, duplicated experiments were performed for a cell line from a donor. For immunostaining experiments, cells were plated at 8,000 cells/ml onto collagen I coated coverslips (10 µg/ml) for 60 minutes. All live-cell experiments were performed using phenol-red free DMEM/F12 with 20mM HEPES, 4mM L-glutamine and contained 10% FBS on 35mm No. 0 collagen I coated glass bottom dishes (MatTek).

Reagents and Transfection

mTagRFP-T-Lifeact-7 (Addgene plasmid # 54586) was a gift from Michael Davidson (Florida State University, Tallahassee, FL). Human GMFγ DNA (NCBI Accession Number, NM_004877.2) was synthesized by Life Technologies and subcloned into bacterial vectors pEGFP. Quickchange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to generate Y104F mutant (phenylalanine substitution at Tyr-104). The 5'-primer was 5'-GCC GGA ACA ACA GAT GAT GTT CGC AGG GAG TAA AAA CAG G-3'. The 3'-primer was 5'-CCT GTT TTT ACT CCC TGC GAA CAT CAT CTG TTG TTC CGG C-3'. To generate Y104D mutant (aspartic acid substitution at Tyr-104). The 5'-primer was 5'-GCC GGA ACA ACA GAT GAT GGA TGC AGG GAG TAA AAA CAG-3'. The 3'-primer was 5'-CTG TTT TTA CTC CCT GCA TCC ATC ATC TGT TGT TCC GGC-3'.

Transfections for migration assay were performed with 0.5 µg DNA using FuGene HD Transfection Reagent (Promega Corporation Madison, WI). Cells were transfected overnight, and the media was changed to 10% FBS/F12 before running the migration assay. Transfections of cells for fixed and live-cell confocal microscopy were performed with Neon electroporation and Neon reagents (ThermoFisher MPK10025) with pulse width/number optimized for this cell line. Cells were transfected and allowed to adhere overnight, and the media was changed to 10% FBS/F12. All transfected cell experiments were carried out 16hrs post-transfection.

Lentiviral particles containing shRNA specific for GMFγ or non-targeting control shRNA were purchased from Santa Cruz Biotechnology. HASM cells were infected with control shRNA lentivirus (Cat#sc-108080) or GMF-γ shRNA lentivirus (Cat#sc-97348-V) for 12hrs. They were then cultured for 3-4 days. Positive clones expressing shRNAs were selected by puromycin. Immunoblot analysis was used to determine the expression levels of GMF-γ in these cells. GMF-γ KD cells and cells expressing control shRNA were stable at least five passages after initial infection. In addition, our laboratory previously characterized stable c-Abl KD cells (27, 28, 30).

Primary antibodies used for immunofluorescence (IF) and western blot (WB): anti-goat total GMFγ (E-13) (1:25_IF, 1:50_WB, Santa Cruz Biotech Lot# B0915, Cat# sc-168016), anti-rabbit N-WASP Y256 (1:25_IF, 1:100_WB, EMD Millipore Lot# 2795491, 2838736, Cat# AB1966), anti-mouse Arp2 (E-12) (1:25_IF Santa Cruz Biotech Lot# B0612, Cat# sc-166103), anti-mouse vinculin (1:25_IF Invitrogen, ThermoFisher Lot# RA 2147394, Cat# MA1-34629), anti-rabbit vinculin (1:25_IF, 1:100_WB Sigma Aldrich), anti-mouse zyxin (1:25_IF Santa Cruz Biotech Lot# B1317, Cat# sc-293448). The anti-rabbit phospho-GMFγ (Tyr-104) antibody was custom made by Thermo Scientific (Pierce). The sequence of the peptide for generating phospho-GMF-γ antibody was CKPEQQMMY(P)AGSKNRLVQTA (NCBI Accession Number, NM_004877.2). Secondary antibodies were all purchased from Invitrogen (ThermoFisher), which includes Alexa 405, 488, 555, 546, and 647 at a concentration of 1:100.

Western Blot Analysis

Cells lysed with 2x SDS sample buffer composed of 1.5% dithiothreitol, 2% SDS, 80mM Tris-HCl (pH 6.8), 10% glycerol and 0.01% bromophenol blue were boiled for 5 mins and separated onto SDS PAGE, then electro-transferred to nitrocellulose paper. Membranes were blocked using 2% bovine serum albumin (BSA) in PBS for 1hr and then probed with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibody (Fisher Scientific). Proteins were visualized with Amersham enhanced chemiluminescence (ECL) select Western Blotting Detection Reagent (GE Healthcare Lot# 9774291 Cat# 45000999PM) using the Amersham Imager 600 (GE Healthcare). The levels of total protein or phosphoprotein were quantified by scanning densitometry of immunoblots (IQTL software by GE Healthcare).

NHASMC were grown to 100% confluence in 60mm cell-culture dishes and then subjected to blebbistatin (-/-Blebbistatin Sigma Aldrich Catalog #B0560-1MG, Lot #SLBK5210V) treatment 20uM in 1ml of Serum-free Ham's F12 media for 15 minutes. After 15 minutes cells were harvested on ice and washed quickly twice with 1x PBS, then 150ul of 2x SDS sample buffer with 1x Halt Protease and Phosphatase Inhibitor single-use cocktail, EDTA free (ThermoScientific Product #78443 Lot #SJ256802) was added, cells scraped, boiled for 5 mins and separated onto SDS PAGE, then electro-transferred to nitrocellulose paper. Membranes were blocked using 2% BSA in PBS for 1hr and then probed with c-Abl tyr-412 (1:500 Santa Cruz Biotech sc-101626, Lot #K0911), total c-Abl (1:500 Santa Cruz Biotech sc-23, Lot #I2116), GAPDH (6C5) (1:4000 Santa Cruz Biotech sc-32233, Lot #K0315), pMLC (1:500 Santa Cruz Biotech sc-19849-R, Lot #B2411), MLC antibody (1:1000 custom made).

Co-Immunoprecipitation

Cells were plated in 100mm cell culture dishes and allowed to grow to 90-100% confluence. Media was changed to SFM overnight. Media was removed and Lysis buffer (10% Triton X 100, 0.5M EDTA, 1M HEPES pH 7.6, 20% SDS, 100x Halt protease/phosphatase single-use Inhibitor cocktail Cat# 78442 Lot# RH235957) was added and incubated at 4°C for 10 minutes. Cells were scraped into 1.5ml tubes and rotated for 1hr at 4°C. Centrifugation at 13.0rpm (x1000g) for 20 minutes and removal of the supernatant was placed in new 1.5ml tube. Addition of Protein A/G Plus-agarose (Santa Cruz Biotech Lot# B0116 Cat# sc-2003) rotated for 30 minutes at 4°C. Centrifuge for 5 minutes and removal of supernatant and placed in new 1.5ml tube. Subsequently, the addition of primary antibody or normal IgG as control were added to supernatant and rotated overnight at 4°C. Following incubation, Protein A/G with agarose was added and rotated for 3 hours at 4°C then centrifuged for 15 minutes at 13,000 rpm (x1000g) and removal of supernatant. Pellets were resuspended in IP Wash Buffer (5M NaCl, 1M Tris pH 7.6, 10% Triton x100, and H_2O), centrifuged and the supernatants discarded 4 times. Pellets were saved and mixed with 2x SDS buffer, then boiled for 5 minutes and spun down. Co-IP samples were loaded and separated on an SDS PAGE gel, where they were electro-transferred onto nitrocellulose paper and immunoblotted with the correct primary antibodies. Visualization of co-IP was carried out using Amersham ECL (GE Healthcare) and imaged using the Amersham Imager 600 (GE Healthcare).

Immunofluorescence Microscopy

Cells were plated onto collagen I coated coverslips for 60mins, then fixed using 4% paraformaldehyde for 15minutes at room temperature and then permeabilized with 0.2% Triton X 100 in PBS. Coverslips were washed 3x for 5minutes with 1x PBS in between each step. Coverslips were blocked using 2% BSA/PBST for 30minutes then primary antibodies were added with 2% BSA/PBST and incubated for 1hr each. Secondary antibodies were added at a concentration of 1:200 for 1hr each. Coverslips were fixed onto slides using Prolong Diamond mounting medium (ThermoFisher).

Imaging for fixed-cells and live-cells was conducted on a Zeiss LSM 880 NLO confocal microscope with Fast Airyscan module (Carl Zeiss Microscopy Jena, Germany) equipped with 63x oil 1.4 numerical aperture (NA) objective lens and collected through a 32-channel GaAsP detector as 0.2 Airy units per channel (Huff, J. 2016). Cells were imaged using Argon405 (488 nm) laser and BP 420-480/BP 495-620 filter for GFP; DPSS 561-10 (561 nm) laser and BP 495-550/LP 570 filter for RFP, and HeNe633 (633nm) laser and BP 570-620/LP 645 filter for far-red. Z-stack collecting was under Nyquist sampling and with the fast airyscan SR settings. Z-stacks were acquired at an average of 11 slices with 0.17µm distance between each slice. Live-cell imaging of GMFγ-GFP tagged mutants, Life-Act-RFP, and paxillin-mcherry constructs utilized the Fast-Airyscan module on the Zeiss LSM 880 confocal microscope. Z-stack live-images were taken at an average of 6 slices with 0.17µm distance between slices for 10 or 20 minutes at a frame rate of 10 seconds. Microscope software used is the Zen Black 2 edition to process images for the Airyscan. The microscope has an incubation chamber set to 30-37°C and has 5% CO_2 for live-cell imaging. Time-lapse microscopy was achieved by using a Leica A600 microscope with a 6-well incubator chamber hooked up to 5% CO_2. Epifluorescence imaging was utilized for cells that were transfected with fluorescent protein constructs. Only cells expressing specific GFP fluorescent constructs were imaged and used for analysis. Epifluorescence was achieved by using a GFP filter cube. Multi-position marking function was used to designate specific x and y coordinates for the time-lapse capture of images every 10 minutes for a 16-hour experimental period.

Image Analysis

Spots to Surfaces Analysis

Imaris 9.1.2 (Bitplane, Oxford Instruments) software was used to generate and analyze the distance between spots and surfaces using a distance transformation. The surface module uses an algorithm to create 3D-reconstructed objects based upon fluorescent intensity, quality of rendering, and then a threshold was applied for focal adhesion areas ≥ 0.5μm. Once surfaces were created, MatLab algorithms were used to measure the total number and area of each surface. If the image contained two surfaces (vinculin and zyxin), a mask of each surface was created. Any voxel outside of the masked surface is signified as having an intensity value greater than 0.00. This allows separation of channels inside regions within a masked surface that is contacting both vinculin and zyxin or just vinculin alone. A separate algorithm was used to create spots based upon the fluorescent intensity and quality for either GMFγ or N-WASP pY256 channels. Once spots were created, a plugin was used to measure the total number of spots for specific channels. Also, a separate plugin was used to threshold on spots closest to surfaces another form of distance transformation (Figure 4F). From there a percentage of the number of spots closest to surface to a total number of spots could be generated for analysis (See Figures S2 and S3).

Focal Adhesion Dynamics Analysis

Imaris 9.1.2 (Bitplane, Oxford Instruments) software was used to generate and analyze Paxillin 3D-rendered surfaces over time. Paxillin surfaces were rendered based on fluorescent intensity, quality, and area. A Brownian algorithm was utilized to track the movement of paxillin surfaces over time. Paxillin surfaces were filtered based on an area >1um and track duration (minimum of 300seconds). Each paxillin surface was individually checked in vantage plot module to make sure the rendering was accurate over the entire time course. All paxillin surfaces were input into a vantage plot for each individual cell and analyzed for time changes in the area, bounding box length, intensity fluorescent sum, and volume. Vantage plots were saved as Microsoft Excel spreadsheets (Microsoft Office 365 package). Spreadsheets were sorted and filtered by Time and TrackID. Graphs of individual paxillin surfaces were created based off area over time (300second duration). Each cell (n=10 cells per WT, Y104F, Y104D-GMFγ) contained an average of 250 individual paxillin surfaces that were selected based on threshold criteria and were individually graphed. Representative cells with 1 paxillin ROI's per GMFγ expression were presented in the main text.

Filament Tracer Analysis

Imaris 9.1.2 (Bitplane, Oxford Instruments) software was used to generate and analyze actin fiber organization. Filament Tracer has been utilized to characterize neuron and dendrite morphology. Here we adapted this software package to analyze the actin cytoskeleton morphology and percent actin fibers contacting vinculin. Actin fibers were rendered using fluorescent intensity, the diameter of the largest fiber and quality. Vinculin objects were rendered using the surface module based on fluorescent intensity, surface area equivalent to >1μm, and quality. A mask was applied

to the rendered actin fibers and a new channel was created. Next a new surface module was used to render the masked channel of actin fibers. MatLab plugin “surface-surface colocalization” was applied to vinculin and actin fiber surfaces. This plugin performs a distance transformation algorithm to identify contacting objects rendered as surfaces. A surface contacting another surface has an output of 0.00, whereas a surface not contacting another surface has an output of >1.00 based on proximity. Next statistics were performed to identify the percent actin fibers contacting vinculin surfaces: (number of surfaces with an output of 0.00)/ (total number of surfaces) for each treatment (n=6 cells per WT, Y104F, Y104D-GMFγ).

Asters were identified by fluorescent intensity and having at least 5 actin fibers radially-projecting from the center of the aster and were marked as beginning points. Actin fibers that were branching in morphology were identified by having <5 branch points per fiber. Rendered actin fibers are shown in cyan and beginning points “actin asters” are shown in yellow. Imaris software “Filament Statistics” was applied to the rendered actin fibers and beginning point “asters” and branch points were graphed (n=10 cells per WT, Y104F, Y104D-GMFγ).

Protrusion/Retraction Analysis

The ImageJ plugin ADAPT (automated detection and analysis of protrusions), which utilizes the fluorescent intensities at the cell border to measure changes in protrusion/retraction position and velocity over time, was used to analyze Zeiss LSM 880 confocal live-cell movies (44). The input of the spatial resolution, frames per minute, thresholding method (Triangle), smoothing filter radius, erosion iterations, spatial filter radius, temporal filter radius, cortex depth, visualization line thickness, and minimum/maximum velocity parameters will generate protrusion boundaries based on changes in fluorescence intensity. Output analysis generates values of protrusion trajectory, protrusion/retraction velocity, and protrusion/retraction events.

Statistical Analysis

All data were analyzed using GraphPad Prism version 6.00 software (Windows, GraphPad Software, La Jolla, CA). A two-tailed one-way ANOVA followed by Tukey’s multiple comparisons test was used for comparing Time-lapse microscopy parameters, lamellipodial protrusion/retraction velocity and events, comparing vinculin and zyxin area and number, all filament tracer experiments, and comparing the percent of GMFγ inside or outside adhesions (significance was determined by a $p<0.05$). A two-tailed Student’s T-test was used to determine a significance of $p<0.05$ for knockdown cell focal adhesion parameters, protein level expression, and “wound” closure rates. A two-tailed Student’s T-test was used to determine a significance of $p<0.05$ for blebbistatin-treated cells. Number of independent experiments is indicated within figure legends. Box and whisker plots and bar graphs were used to represent data shown with ±SEM.

Supplemental Figures

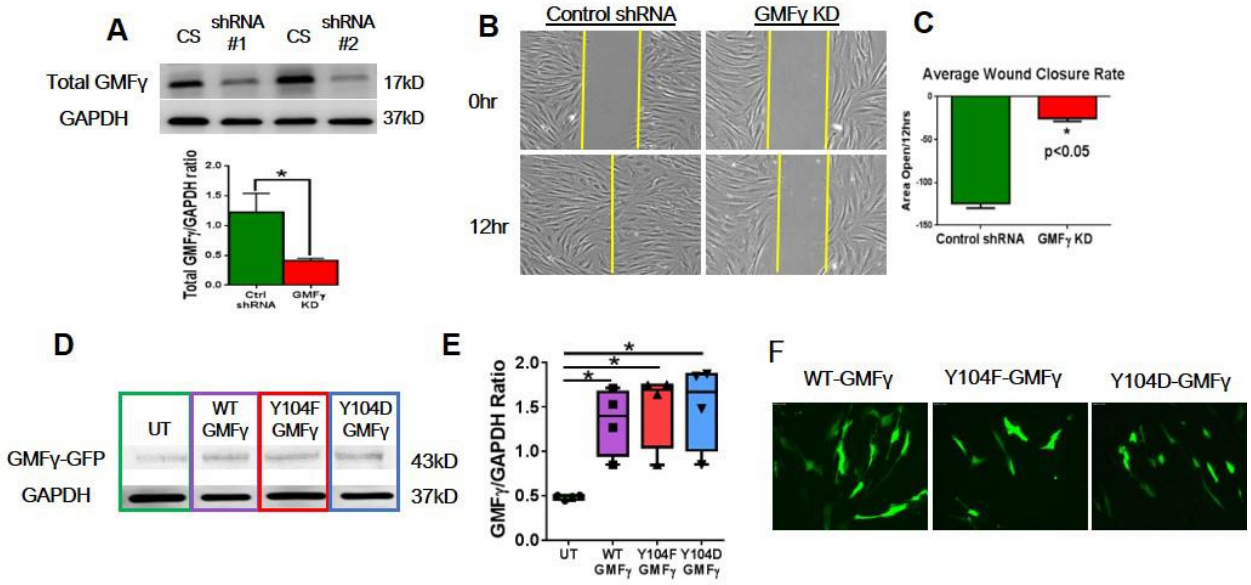

Figure E1: Knockdown of GMFγ inhibits directional migration. (A) NHASMC were infected with Control shRNA lentiviral particles or two separate pools of GMFγ shRNA lentiviral particles. Cells were selected using Puromycin over the course of 7 days post infection. Cells were harvested with 1x SDS buffer and ran on SDS-PAGE gel then electro-transferred onto nitrocellulose paper and immunoblotted for GMFγ and GAPDH to confirm successful knockdown n=4 independent experiments. **(B)** Stable-selected knockdown cells or control shRNA expressing cells were plated on collagen I coated 6-well dishes and grown to confluence. Once confluent each 6-well dish was scratched with a 1µl pipette tip and multi-positions were collected immediately on a Leica A600 microscope equipped with 5% CO_2 and 37°C incubation chamber. Images were collected every 10 minutes over the course of 12hrs. Images shown are beginning and end-points at time 0 and 12hrs post-scratch. Yellow lines outline the border of cells. N=12 independent experiments. **(C)** Quantification of the area between the scratch over the 12hr time-course was completed using NIH ImageJ software. Student's T-test was used to compare Control shRNA to GMFγ shRNA expressing cells, significance was *p<0.05 from n=12 independent experiments. **(D)** GMFγ shRNA expressing cells were transfected with 0.5µg eGFP-tagged WT, Y104F-, Y104D-GMFγ plasmids using FuGene HD Transfection Reagent. After 16hrs post-transfection, cells were harvested for Western Blot. Nitrocellulose paper was immunoblotted for GMFγ and GAPDH and imaged using an Amersham 600 imager. N=4 independent experiments. **(E)** The levels of total protein were quantified by scanning densitometry of immunoblots (IQTL software by GE Healthcare), One-way ANOVA with Tukey's posthoc test was used to determine significance at *p<0.05 from n=4 independent experiments. **(F)** Epifluorescent imaging of GFP-expressing WT, Y104F-, Y104D-GMFγ knockdown cells. Images were taken at 10x using a Leica A600 microscope from n=4 independent experiments.

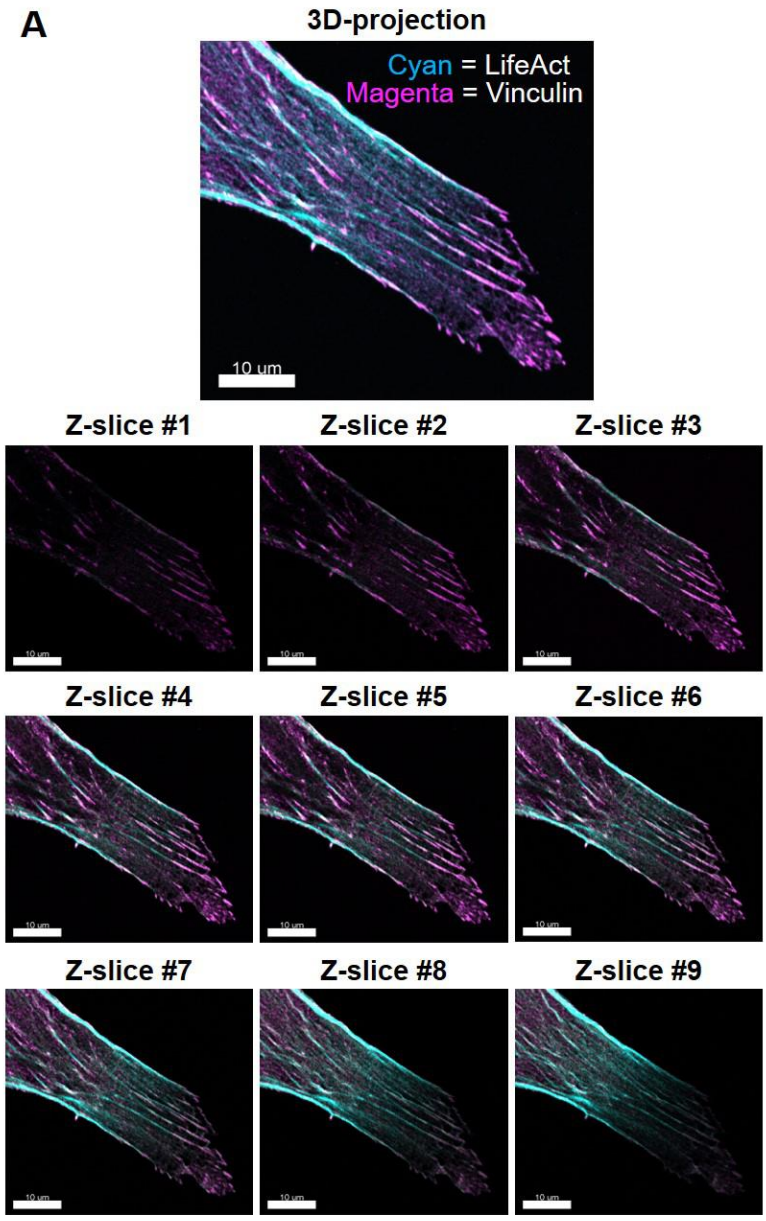

Figure E2: Focal adhesions within Human airway smooth muscle cells span multiple Airyscan confocal z-slices. (A). Human airway smooth muscles cells electroporated with LifeAct-RFP plasmid were then directly plated onto collagen-I coated glass MatTek dishes and allowed to adhere overnight. Following day cells were fixed with 4% PFA and immunostained for Vinculin. Images were taken on a Zeiss LSM 880 confocal with Fast Airyscan module. N=20 individual cells. Imaris software pseudo-colored the LifeAct-RFP channel to cyan for visualization purposes only. Note how vinculin-associated focal adhesions span multiple z-slices in human airway smooth muscle cells.

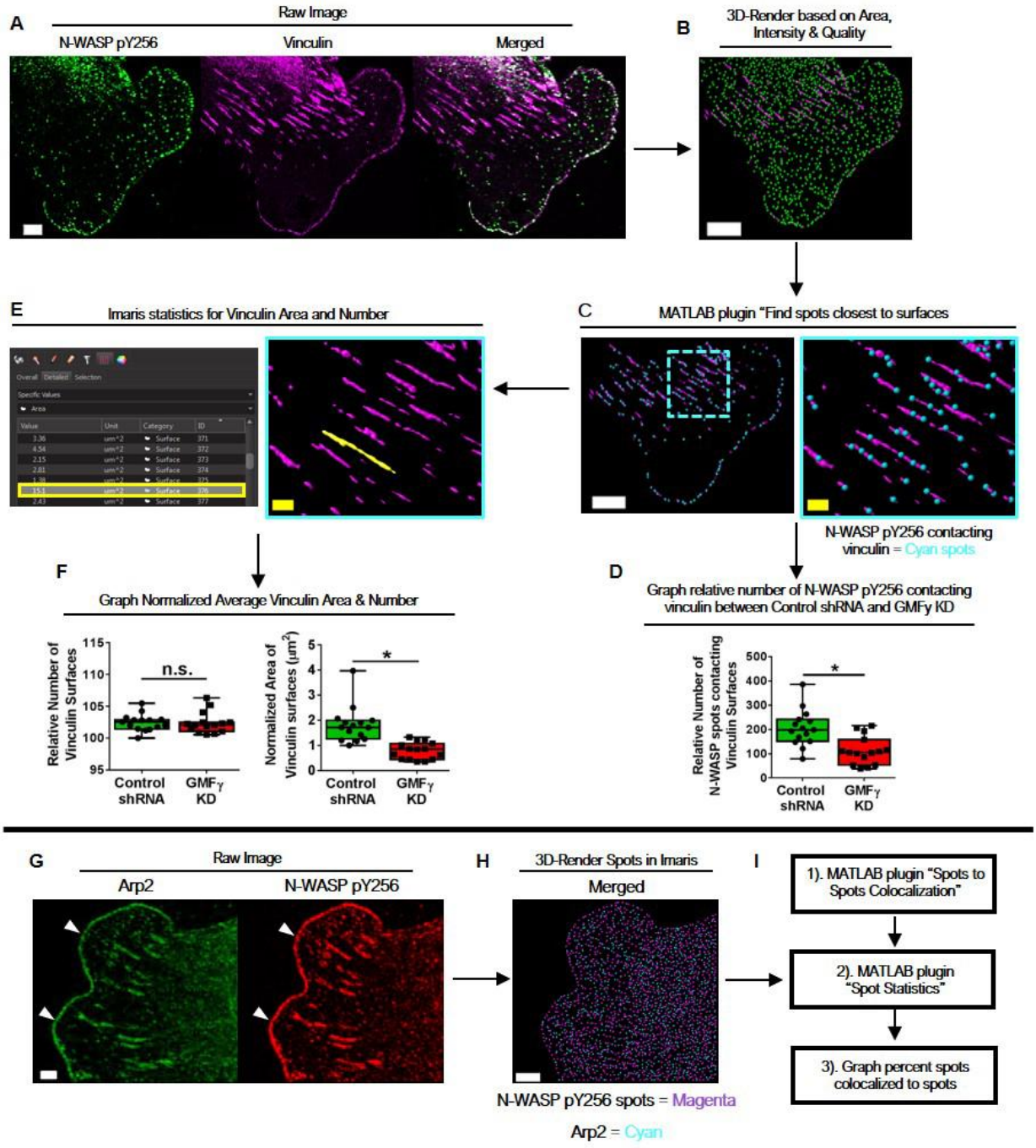
A
Raw Image
N-WASP pY256
Vinculin
Merged
B
3D-Render based on Area, Intensity & Quality
C
MATLAB plugin "Find spots closest to surfaces
N-WASP pY256 contacting vinculin = Cyan spots
D
Graph relative number of N-WASP pY256 contacting vinculin between Control shRNA and GMFγ KD
Relative Number of N-WASP spots contacting Vinculin Surfaces
*
Control shRNA
GMFγ KD
E
Imaris statistics for Vinculin Area and Number
F
Graph Normalized Average Vinculin Area & Number
Relative Number of Vinculin Surfaces
n.s.
Normalized Area of Vinculin surfaces (μm²)
*
Control shRNA
GMFγ KD
G
Raw Image
Arp2
N-WASP pY256
H
3D-Render Spots in Imaris
Merged
N-WASP pY256 spots = Magenta
Arp2 = Cyan
I
1). MATLAB plugin "Spots to Spots Colocalization"
2). MATLAB plugin "Spot Statistics"
3). Graph percent spots colocalized to spots

Figure E3: Imaris image analysis protocol for knockdown of GMFγ and its effects on focal adhesion dimension and N-WASP localization. (A) Raw 3D-projection immunofluorescent image taken using Fast Airyscan module on a Zeiss LSM880 confocal microscope. **(B)** Input image into Imaris software. Utilize surface module to 3D-reconstruct vinculin into surfaces based on fluorescent intensity, area, and quality. Utilize spot module to 3D-reconstruct punctate N-WASP (pY256) into spots based on fluorescent intensity, number of voxels, and quality. **(C)** MatLab plugin "Find spots close to surfaces" utilizes a distance transformation algorithm to identify objects based on their proximity in 3D-orientation. An output of 0.00 means the objects are contacting and any object >0 is not contacting any objects. **(D)** A relative number of objects contacting a surface can be generated based on this distance transformation. **(E)** Any 3D-reconstructed surface will have multiple statistics associated with itself. For example, the yellow highlighted surface has a surface area of 15μm^2 and an object ID of 376. **(F)** Using the Imaris statistics an average area and number of vinculin surfaces can be graphed. **(G)** Arp2 and N-WASP (pY256) were immunostained and imaged using the Fast Airyscan module on a Zeiss LSM880 confocal microscope. **(H)** Imaris software was used to 3D-reconstruct Arp2 and N-WASP (pY256) spots using fluorescent intensity, number of voxels, and quality filters. **(i)** MatLab plugin "Spots to Spots Colocalization" was used to determine how many Arp2 and N-WASP (pY256) spots were colocalized based on the distance transformation. N=10 individual cells.

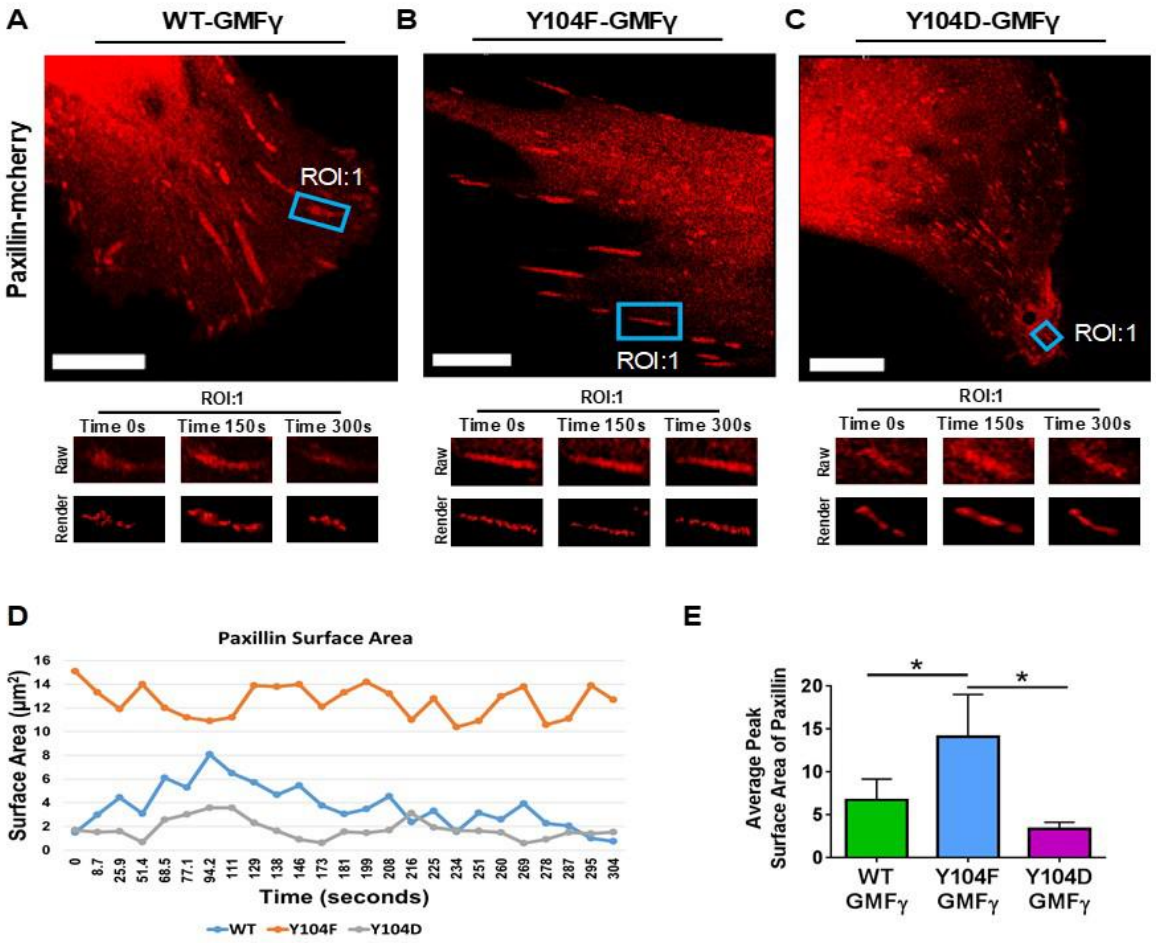

Figure E4: GMFγ phosphorylation at Y104 impacts focal adhesion dynamics in live cells. (A-C) GMFγ knockdown cells were transfected with WT, Y104F, or Y104D-GMFγ and paxillin-mcherry plasmids. Imaris 9.1.2 software was utilized to render paxillin surfaces based on fluorescent intensity, quality, area, and track duration (threshold minimum of 300 seconds). Brownian motion algorithm was used to track changes in the area over time (*Methods*). Representative paxillin regions of interest (ROI's) were selected and shown as raw or rendered images over 300secs (Rendered Paxillin: WT- GMFγ n=1,489, Y104F-GMFγ n=2,012, Y104D-GMFγ n=2,387) from n=10 individual cells per expression plasmid.**(D)** Line graphs represent the changes in paxillin area during track duration. **(E)** Graph of the average peak surface area for each reconstructed paxillin. One-way ANOVA with Tukey's posthoc test, significance *p<0.05.

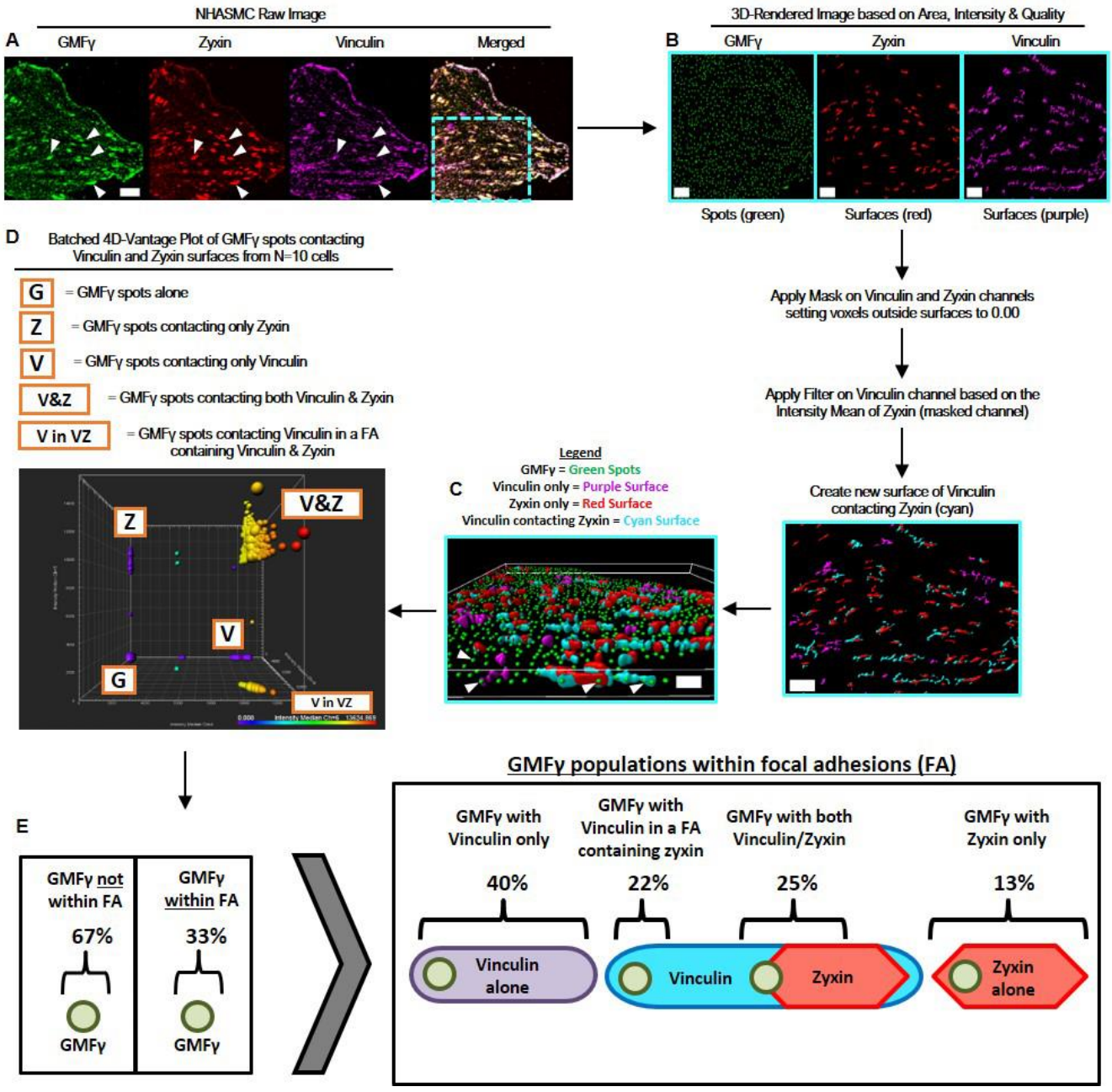
A
NHASMC Raw Image
GMFγ
Zyxin
Vinculin
Merged
B
3D-Rendered Image based on Area, Intensity & Quality
GMFγ
Zyxin
Vinculin
Spots (green)
Surfaces (red)
Surfaces (purple)
Apply Mask on Vinculin and Zyxin channels setting voxels outside surfaces to 0.00
Apply Filter on Vinculin channel based on the Intensity Mean of Zyxin (masked channel)
Create new surface of Vinculin contacting Zyxin (cyan)
C
Legend
GMFγ = Green Spots
Vinculin only = Purple Surface
Zyxin only = Red Surface
Vinculin contacting Zyxin = Cyan Surface
D
Batched 4D-Vantage Plot of GMFγ spots contacting Vinculin and Zyxin surfaces from N=10 cells
G = GMFγ spots alone
Z = GMFγ spots contacting only Zyxin
V = GMFγ spots contacting only Vinculin
V&Z = GMFγ spots contacting both Vinculin & Zyxin
V in VZ = GMFγ spots contacting Vinculin in a FA containing Vinculin & Zyxin
Z
V&Z
V
G
V in VZ
E
GMFγ not within FA
67%
GMFγ
GMFγ within FA
33%
GMFγ
GMFγ populations within focal adhesions (FA)
GMFγ with Vinculin only
40%
Vinculin alone
GMFγ with Vinculin in a FA containing zyxin
22%
Vinculin
GMFγ with both Vinculin/Zyxin
25%
Zyxin
GMFγ with Zyxin only
13%
Zyxin alone

Figure E5: Imaris image analysis protocol for GMFγ mutant expression and its effects on focal adhesion dimension and GMFγ localization. (A) Raw 3D-projection immunofluorescent image taken using Fast Airyscan module on a Zeiss LSM880 confocal microscope. **(B)** Input image into Imaris software. Utilize surface module to 3D-reconstruct zyxin and vinculin into surfaces based on fluorescent intensity, area, and quality. Utilize spot module to 3D-reconstruct punctate GMFγ into spots based on fluorescent intensity, number of voxels, and quality. Apply a mask on vinculin and zyxin channels setting voxels outside surfaces to 0.00. Apply filter on vinculin channel based on the fluorescent intensity mean of zyxin (masked channel). Create new surface of vinculin contacting zyxin surfaces (cyan). **(C)** Representative image displaying GMFγ spots (green), vinculin only (purple), zyxin only (red), and vinculin surfaces contacting zyxin surfaces (cyan). **(D)** Create a "Collection module" in Imaris, which will group all surfaces and spots from each N=10 cells rendered from the raw image together into one complete file. Next open collection as a Vantage Plot module within Imaris. Design a graph to plot GMFγ spots contacting zyxin and vinculin surfaces. Vantage Plot displays 5 separate GMFγ populations based on their proximity to vinculin and zyxin. **(E)** Imaris statistics can be extracted from the vantage plot and percentages of the total population outside and inside focal adhesions can be graphed. In addition, further analysis can determine the percent of GMFγ contacting vinculin only, GMFγ contacting vinculin in a focal adhesion containing zyxin, GMFγ contacting both vinculin and zyxin, and GMFγ contacting zyxin only.

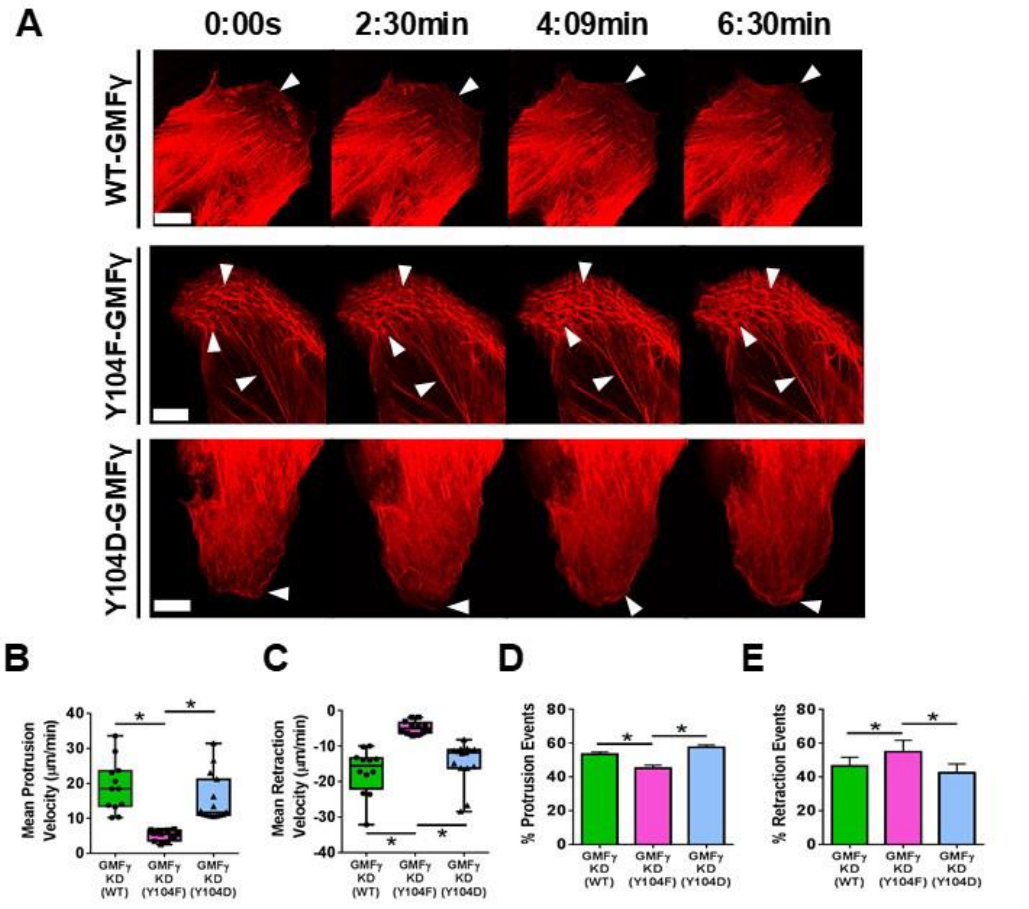

Figure E6: GMFγ phosphorylation at Y104 modulates lamellipodial dynamics. **(A)** GMFγ knockdown cells expressing WT, Y104F, or Y104D-GMFγ were transfected with LifeAct-RFP to monitor changes in lamellipodial dynamics. Live-cell imaging was performed on Zeiss LSM880 confocal with Fast Airyscan module. Images were taken every 10 seconds for a duration of 10 minutes, white scale bar = 10μm. Arrowheads point to actin fibers and leading edge. **(B-E)** To quantify protrusion retraction dynamics we utilized the ADAPT ImageJ plugin (Methods, Barry 2015). The mean protrusion and retraction velocity, as well as percent protrusion and retraction events (WT = 5153, Y104F = 5990, Y104D = 4460), were calculated using the ADAPT plugin from 12 individual cells per expression plasmid. A one-way ANOVA with a Tukey's post hoc test to compare between groups was utilized for statistical analysis, p-value significance was *$p<0.05$.

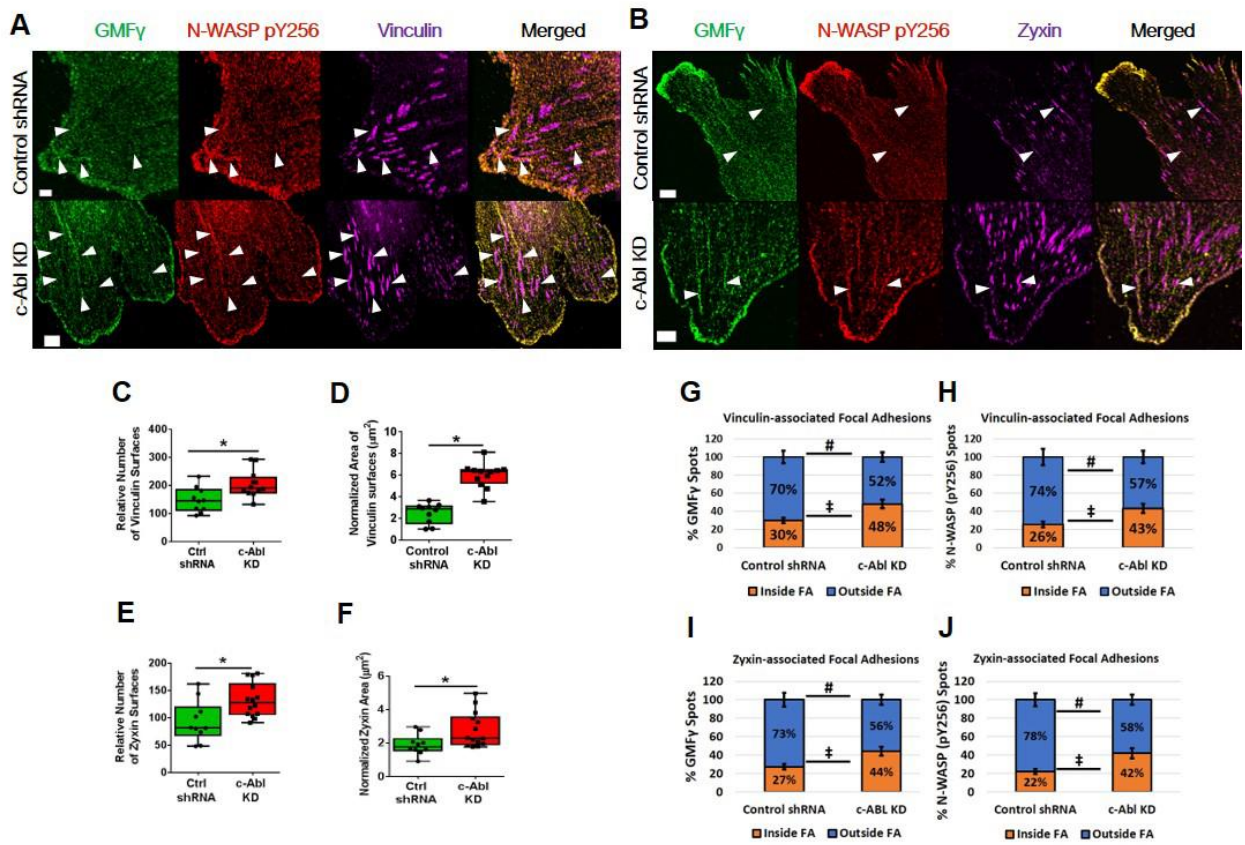

Figure E7: c-Abl knockdown increases GMFγ and N-WASP (pY256) recruitment to vinculin and zyxin-associated focal adhesions. (A,B) Immunofluorescent z-slice image taken using Fast Airyscan module on a Zeiss LSM880 confocal microscope. Arrowheads point to focal adhesions. Scale bar = 5μm. **(C-F)** Input image into Imaris software. Utilize surface module to 3D-reconstruct vinculin (Control shRNA n=1,474, c-Abl KD n=2,250) and zyxin (Control shRNA n=933, c-Abl KD n=1,844) from n=10 individual cells into surfaces based on fluorescent intensity, area, and quality. Use Imaris statistics to acquire relative number per cell and surface area of vinculin and zyxin surfaces as previously described in Supplementary Figure 2. **(G-J)** Utilize spot module to 3D-reconstruct punctate GMFγ (n=23,210) and N-WASP (pY256) (n=27,084) into spots based on fluorescent intensity, number of voxels, and quality. Utilize MatLab "Spot to Surface" distance transformation algorithm to acquire percent GMFγ and N-WASP (pY256) inside or outside of vinculin or zyxin-associated focal adhesions. Statistical Analysis Student's T-test with significance indicated by *p<0.05, #p<0.05 for outside FA, ‡p<0.05 for inside FA from n=10 individual cells per treatment.

Movie E1: Phosphorylation state of GMFγ regulates human airway smooth muscle migration. Knockdown of GMFγ and expression of the non-phosphorylated Y104F-GMFγ decreased human airway smooth muscle migration, whereas expression of both the wild-type and phospho-mimetic Y104D-GMFγ enhanced migration.

Movie E2: GMFγ phosphorylation at Y104 impacts focal adhesion turnover and size. Non-phosphorylated Y104F-GMFγ enhances focal adhesion size, while decreasing focal adhesion turnover. Expression of phospho-mimetic Y104D-GMFγ decreased focal adhesion size and enhanced its turnover.

Movie E3: GMFγ phosphorylation at Y104 modulates lamellipodial dynamics. Non-phosphorylated Y104F-GMFγ decreased lamellipodial dynamics, while the expression of both wild-type and phosphorylated Y104D-GMFγ promoted lamellipodial dynamics.