Supplemental Materials

Molecular Biology of the Cell

Ray et al.

Figure S1. Western analysis to confirm protein KD, morphometric and time-lapse analysis to show regulation of cleft initiation and progression. E13 SMG explants were grown for specific time points +/- siRNAs and subjected to Western Analysis. (A) Under 48 hours of incubation, LIMK 1 siRNA shows 41% knockdown and LIMK 2 siRNA exhibits 51% KD. (B) 24 hours incubation with p25 siRNA results in an 54% reduction in protein and (C) cofilin siRNA shows 40% KD in E13 intact SMGs. (n=3, ANOVA) (D) Brightfield images were quantified after 24 hr of incubation with BMS-5, LIMK 1+2 siRNA and cofilin siRNA. LIMK inhibition and KD and cofilin KD exhibit a significantly reduced number of stable initiated clefts relative to negative control SMGs. (n= 6, ANOVA) (E) Cofilin KD glands show slow progression (Late stage delayed clefts - red arrowhead) and lack of termination in 20 hours (white arrows). (Scale, 50 μm) * $P<0.05$, ** $P< 0.01$, ***$P<0.001$

Figure S2. Effect of LIMK inhibitor BMS-5 and Taxol on E13 SMG. (A) BMS-5-treated glands exhibit reduced levels of phosphorylated cofilin relative to vehicle control-treated gland, when normalized to SYBR green staining intensity. (n= 6) (B) LIMK inhibition by BMS-5 changes epithelial cell shapes by decreasing perimeter (C) and area of cells (D) as shown by E-cadherin staining in green and FN in red. (n= 250, Scale, 20 μm) (E) Taxol treatment induces over stabilization of microtubules and hence increases β-tubulin staining intensity around the epithelial cellular periphery. (F) BMS-5 did not affect apoptosis in E13 glands, as compared to Brefeldin A (BFA). (n= 3) (G) No significant reduction in phospho-paxillin at Tyr 118 was observed with BMS-5 treatment as seen with immunostaining of E13 epithelial rudiments and (H) western analysis of intact E13 SMGs. (n= 3) (I) A minor reduction in phospho-paxillin (Y118) was observed in LIMK and p25 KD glands using immunocytochemistry in E13 epithelial rudiments. (Scale, 20 μm, Paired T-test) ***$P<0.001$

Figure S3. Washout of BMS-5 after 72 hours restores branching, cofilin KD reduces focal adhesion assembly and exogenous FN stimulates cofilin phosphorylation and FAK activation. (A) E13 SMGs showed significant structural recovery through increased branching following BMS-5 washout after 24 hours, relative to BMS-5-treated glands. (Scale, 200 μm) (B) Glands subjected to BMS-5 washout exhibit increased levels of phosphorylated cofilin relative to BMS-5 treated glands. (C) Western blot and densitometric analysis shows increased phosphorylated cofilin in BMS-5 washout explants relative to BMS-5 treated glands. (n= 3,

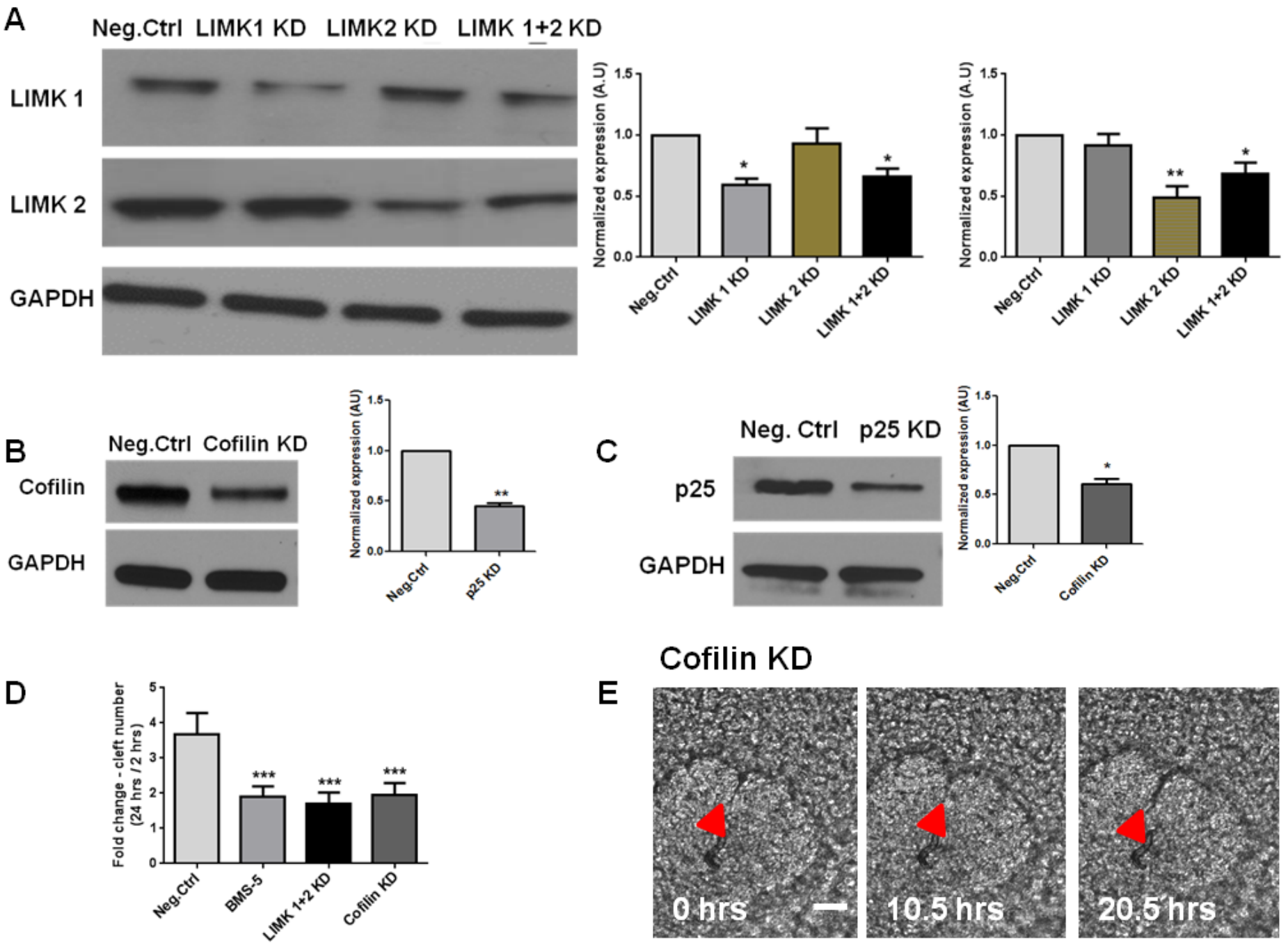
ANOVA) (D) Glands subjected to 72 hours of treatment with BMS-5 shows loss of branched architecture and reduced F-actin relative to vehicle-treated control glands. Glands subjected to 48 hour BMS-5 washout show recovery of branching by formation of acinar units with cortical localization of F-actin. (Scale, 20 μm). (E,F) SMGs treated with cofilin siRNA show reduced focal adhesion activation in the basal periphery of the epithelium as compared to glands treated with negative control siRNAs. (Scale, 20 μm) (G) Exogenously added plasma FN stimulates FAK phosphorylation (Y397) and cofilin phosphorylation (Ser3) in intact E13 SMGs. (n= 3, Paired T-test) * $P<0.05$, ** $P< 0.01$, ***$P<0.001$

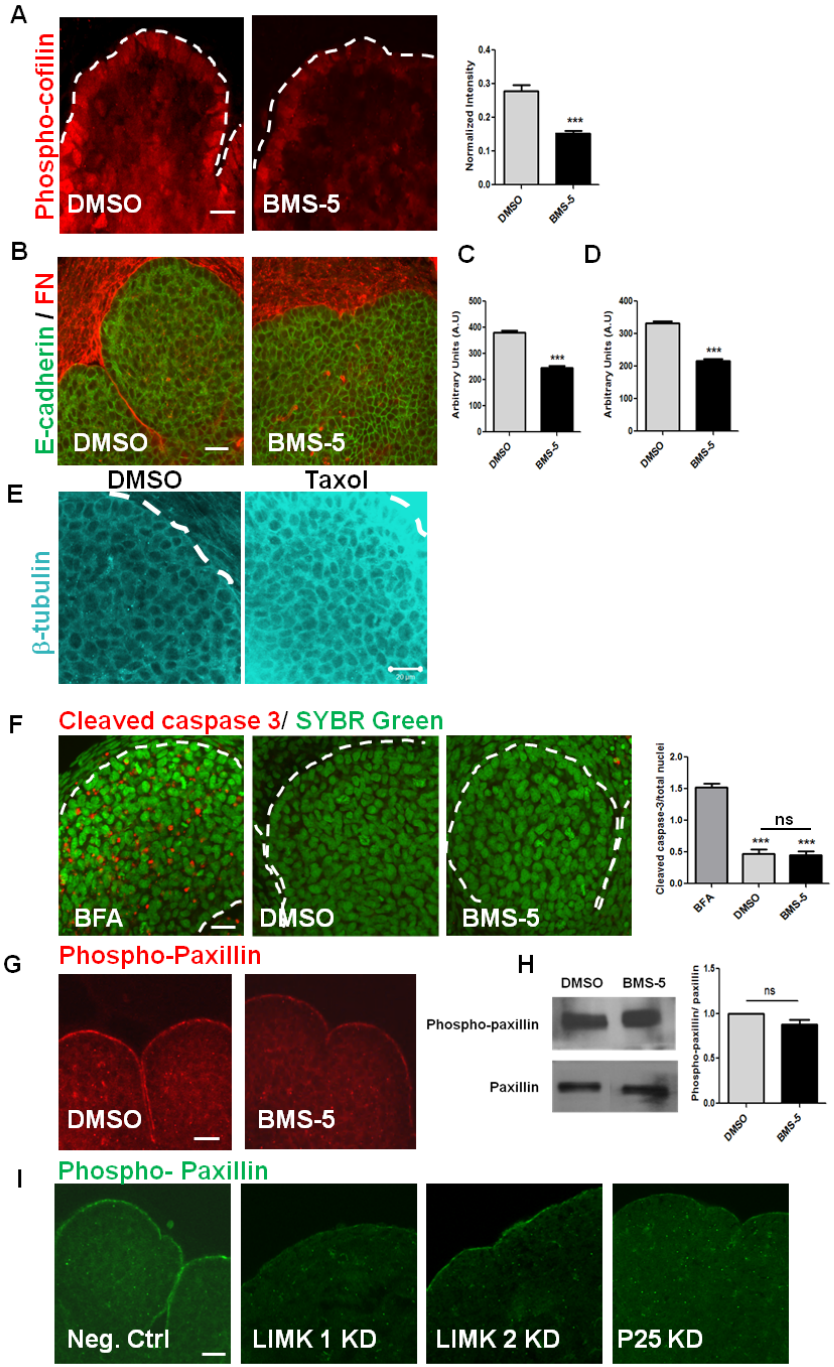
Figure S4. FN deposition is affected with LIMK 1/2 and p25 KD. (A) Mesenchyme-free rudiments pre-treated with Alexa-647 labeled purified human plasma FN show reduced basal deposition and aberrant ectopic deposition in the interior of the epithelium upon downregulation of LIMK and p25. (Scale, 20 μm) (B) Intact glands were immunostained with E-cadherin (red) and FN (cyan) upon LIMK and p25 KD. 2X zoomed images indicate the aberrant interiorly deposited FN likely to be extracellular. (Scale, 10 μm)

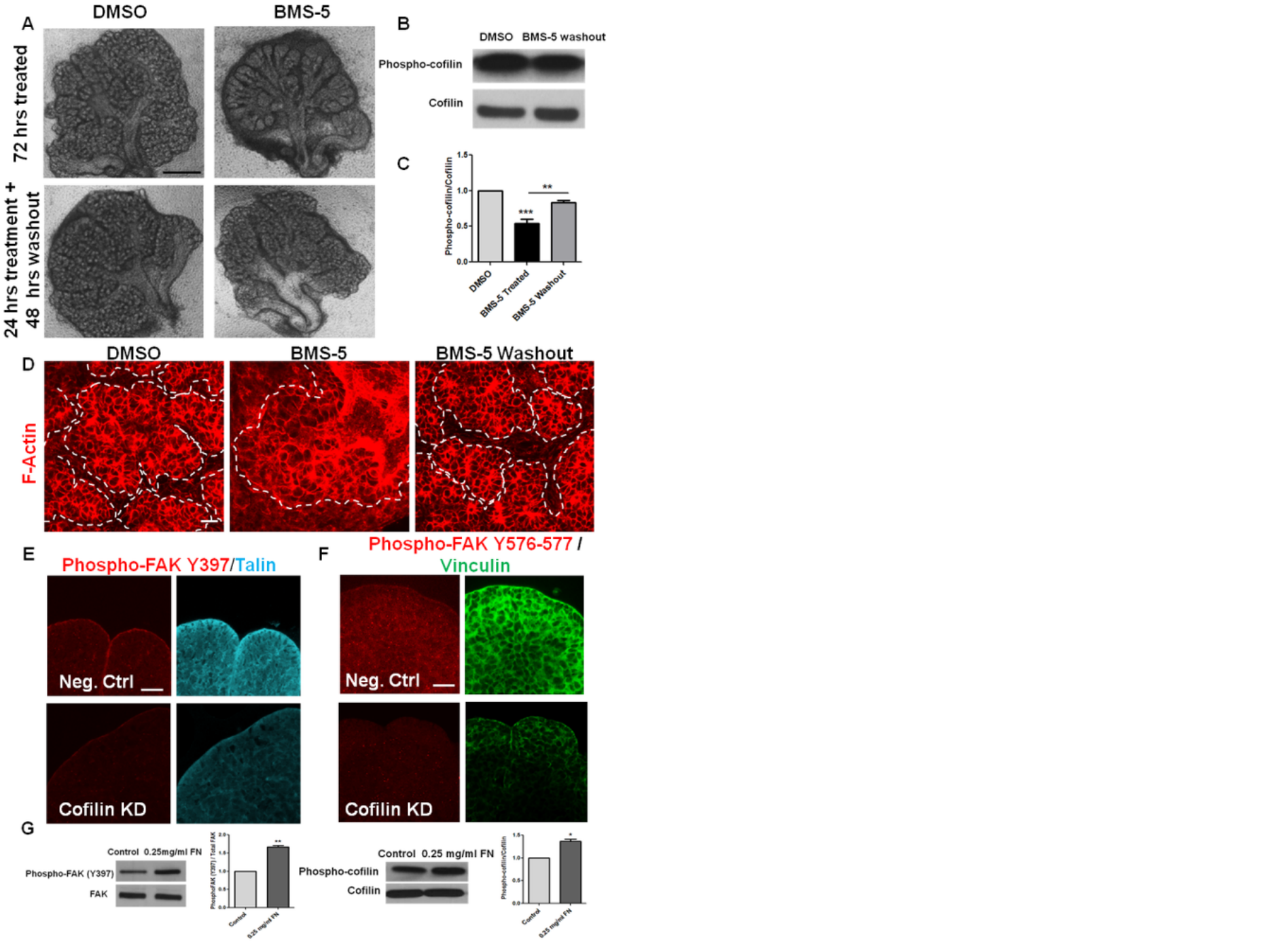
Figure S5. LIMK-mediated inhibition of branching morphogenesis is rescued by knockdown of cofilin and p25. (A, B) In the presence of LIMK inhibition and KD, cofilin siRNA and a combination of cofilin and p25 siRNAs, respectively were used. Western Blot and densitometric analysis reveals cofilin KD to be 34% and p25 KD to be 37%. (n=3, *$P< 0.05$, ***$P<0.001$, ANOVA) (B) The effect of LIMK knockdown on F-actin formation and FAK phosphorylation at Y397 was restored using p25 and cofilin siRNA in the presence of LIMK 2 siRNA. (Scale, 20 μm)

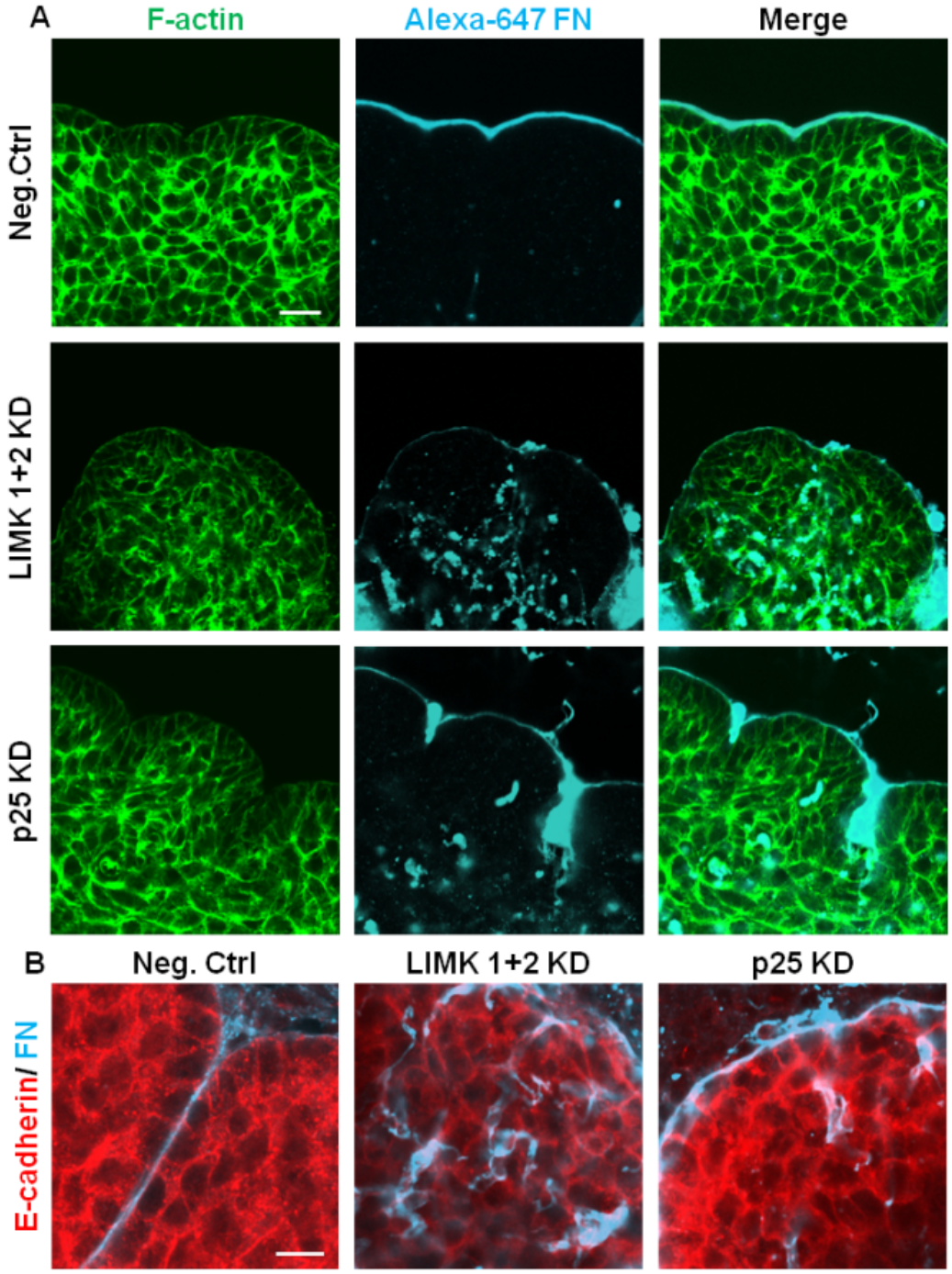
Figure S6. Rescue of LIMK inhibition restores microtubule dynamics and hence FN deposition and assembly in the cleft regions of rescued glands. (A) E13 organ explants treated with LIMK 2 siRNA and immunostained with β-tubulin shows increased staining around the cellular periphery. β-tubulin distribution in the epithelium of the LIMK 2 rescued glands were found to resemble that of the negative-control siRNA treated glands. (Scale, 20 μm) (B) LIMK inhibition by BMS-5 displays a decrease in assembled FN (L8 staining, red) around the epithelial periphery relative to negative control-treated SMGs. Reduced deposition of FN (Alexa-647 purified human plasma FN cyan) is observed in the interior of the epithelium in BMS-5 treated

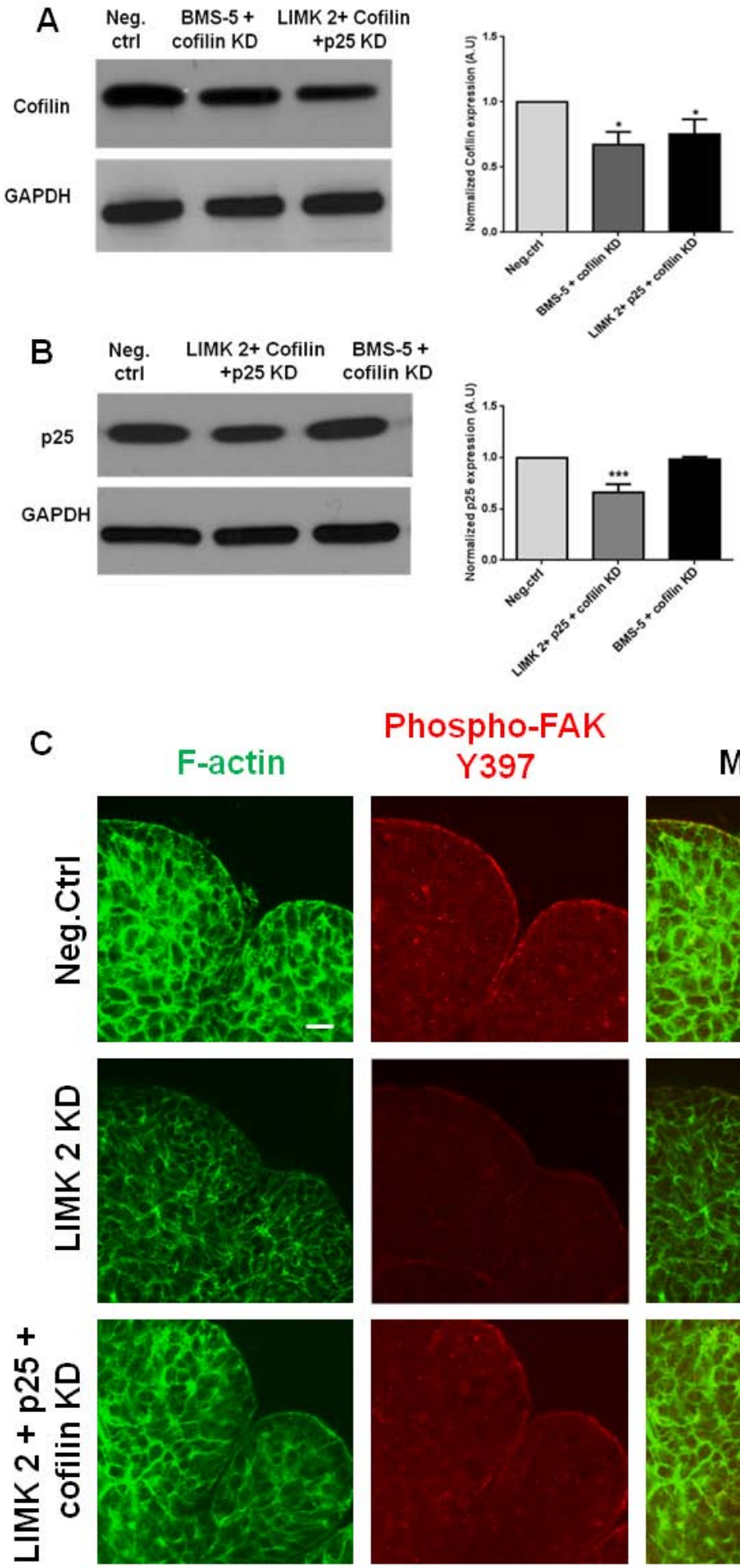
glands. These defects in deposition and assembly of FN are rescued by restoring the actin dynamics in the SMGs. (Scale, 20 μm).

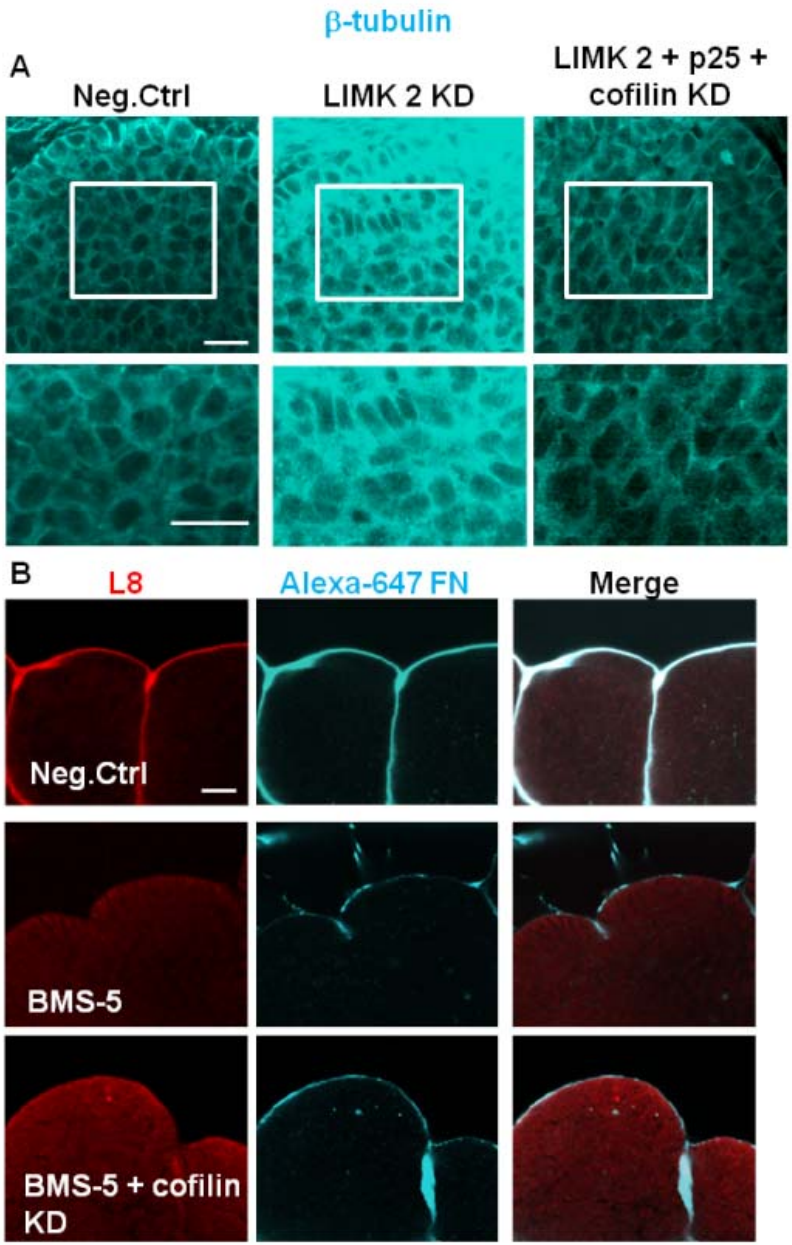

A
Neg.Ctrl
LIMK1 KD
LIMK2 KD
LIMK 1+2 KD
LIMK 1
LIMK 2
GAPDH
Normalized expression (A.U)
Neg.Ctrl
LIMK 1 KD
LIMK 2 KD
LIMK 1+2 KD
B
Neg.Ctrl
Cofilin KD
Cofilin
GAPDH
Normalized expression (AU)
Neg.Ctrl
p25 KD
C
Neg. Ctrl
p25 KD
p25
GAPDH
Normalized expression (AU)
Neg.Ctrl
Cofilin KD
D
Fold change - cleft number (24 hrs / 2 hrs)
Neg.Ctrl
BMS-5
LIMK 1+2 KD
Cofilin KD
E
Cofilin KD
0 hrs
10.5 hrs
20.5 hrs


A
Phospho-cofilin
DMSO
BMS-5
Normalized Intensity
***
DMSO
BMS-5
B
E-cadherin / FN
DMSO
BMS-5
C
Arbitrary Units (A.U)
***
DMSO
BMS-5
D
Arbitrary Units (A.U)
***
DMSO
BMS-5
E
DMSO
Taxol
β-tubulin
F
Cleaved caspase 3/ SYBR Green
BFA
DMSO
BMS-5
Cleaved caspase-3/total nuclei
ns
***
***
BFA
DMSO
BMS-5
G
Phospho-Paxillin
DMSO
BMS-5
H
DMSO
BMS-5
Phospho-paxillin
Paxillin
Phospho-paxillin/ paxillin
ns
DMSO
BMS-5
I
Phospho- Paxillin
Neg. Ctrl
LIMK 1 KD
LIMK 2 KD
P25 KD


A
DMSO
BMS-5
72 hrs treated
24 hrs treatment +
48 hrs washout
B
DMSO BMS-5 washout
Phospho-cofilin
Cofilin
C
Phospho-cofilin/Cofilin
1.5
1.0
0.5
0.0
**
***
DMSO
BMS-5 Treated
BMS-5 Washout
D
DMSO
BMS-5
BMS-5 Washout
F-Actin
E
Phospho-FAK Y397/Talin
Neg. Ctrl
Cofilin KD
F
Phospho-FAK Y576-577 /
Vinculin
Neg. Ctrl
Cofilin KD
G
Control 0.25mg/ml FN
Phospho-FAK (Y397)
FAK
Control
0.25 mg/ml FN
Phospho-cofilin
Cofilin


A
F-actin
Alexa-647 FN
Merge
Neg.Ctrl
LIMK 1+2 KD
p25 KD
B
Neg. Ctrl
LIMK 1+2 KD
p25 KD
E-cadherin/ FN


A
Neg. ctrl
BMS-5 + cofilin KD
LIMK 2+ Cofilin +p25 KD
Cofilin
GAPDH
Normalized Cofilin expression (A.U)
1.5
1.0
0.5
0.0
*
*
Neg.ctrl
BMS-5 + cofilin KD
LIMK 2+ p25 + cofilin KD
B
Neg. ctrl
LIMK 2+ Cofilin +p25 KD
BMS-5 + cofilin KD
p25
GAPDH
Normalized p25 expression (A.U)
1.5
1.0
0.5
0.0
***
Neg.ctrl
LIMK 2+ p25 + cofilin KD
BMS-5 + cofilin KD
C
F-actin
Phospho-FAK Y397
Merge
Neg.Ctrl
LIMK 2 KD
LIMK 2 + p25 + cofilin KD


β-tubulin
A
Neg.Ctrl
LIMK 2 KD
LIMK 2 + p25 +
cofilin KD
B
L8
Alexa-647 FN
Merge
Neg.Ctrl
BMS-5
BMS-5 + cofilin
KD