

Supplemental Information

CRB3 and NF2 orchestrate cytoskeletal dynamics to control epithelial barrier assembly

Shuling Fan^{1^}, Saranyaraajan Varadarajan^{1^}, Vicky Garcia-Hernandez¹, Sven Flemming¹, Arturo Raya-Sandino¹, Ben Margolis², Charles A. Parkos^{1*}, Asma Nusrat^{1*}

¹Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

²Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

^ SF and SV contributed equally to this work.

*Corresponding authors

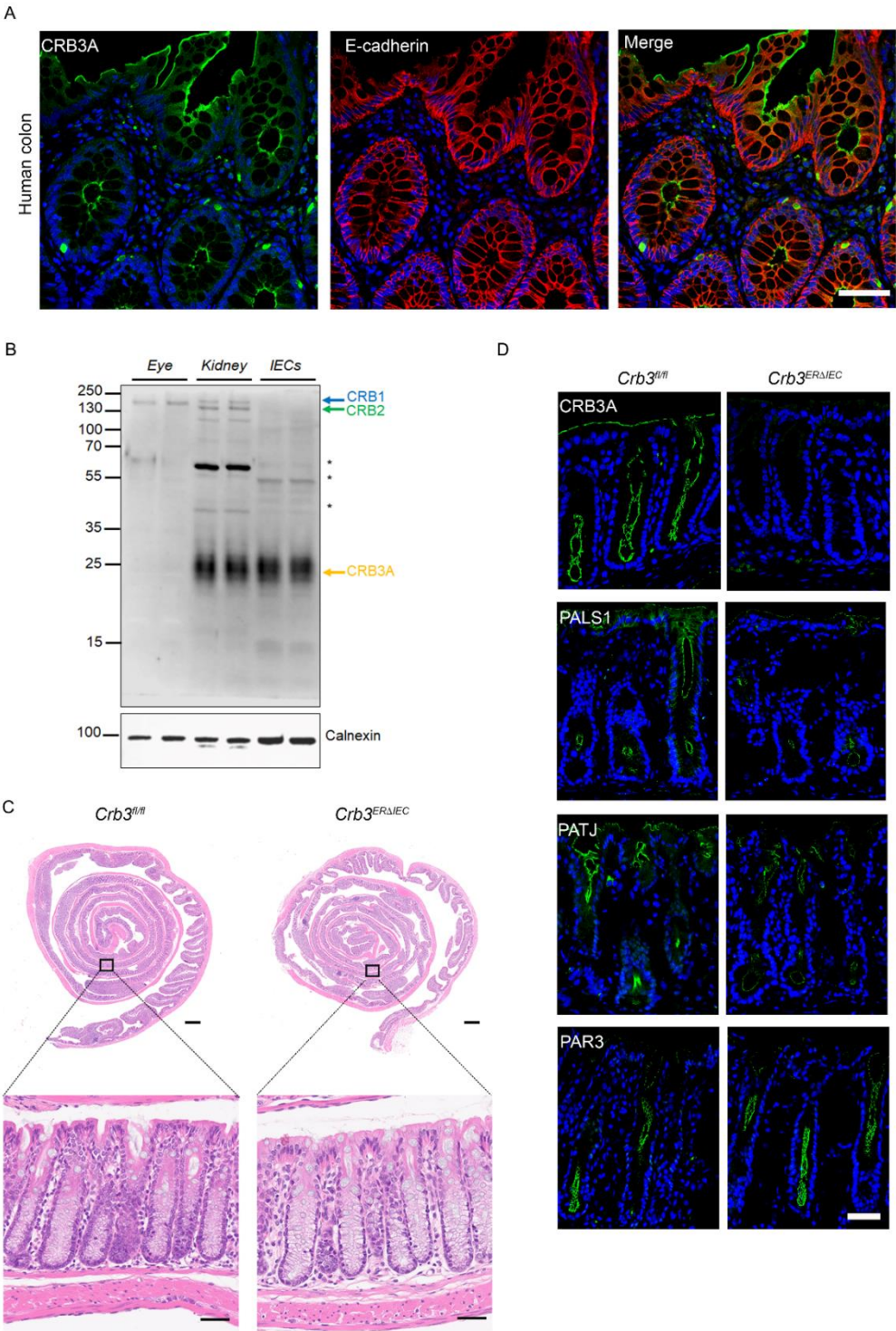
Correspondence:

Asma Nusrat
University of Michigan Medical School
Department of Pathology
4057 BSRB, 109 Zina Pitcher Place,
Ann Arbor, MI 48109, USA.
Phone: (734) 764-5712
Email: anusrat@umich.edu

Charles A Parkos
University of Michigan Medical School
Department of Pathology
NCRC, Building 35, Rm 30-1537
2800 Plymouth Road,
Ann Arbor, MI 48109, USA.
Phone: (734) 763-6384
Email: cparkos@med.umich.edu

Supplemental figures

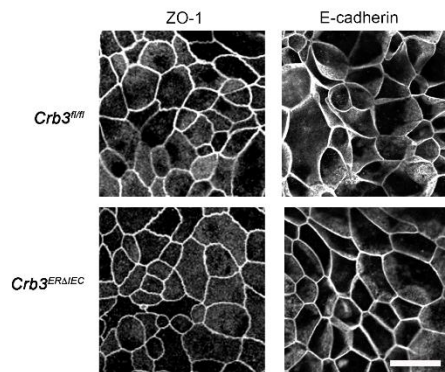
Supplemental figure 1



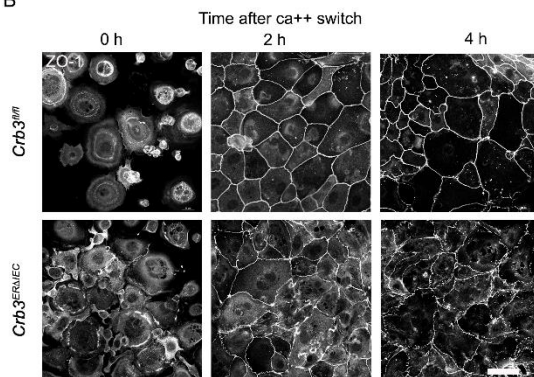
Supplemental Figure 1: Expression of CRB3 binding partners and histology of colonic mucosa. (a) Colonic tissue sections of human healthy donor were immunostained for CRB3A (green), E-cadherin (red), and nuclei/ (DAPI, blue). Scale bar = 50 μ m; n=2 biological replicates; (b) Immunoblotting of CRB3A and Calnexin (loading control) in tissue from murine eye, kidney and IECs. Blue arrow indicates CRB1, green arrow indicates CRB2, and red arrow indicates CRB3A. Asterisks indicate non-specific bands; n=1 experiment, 2 biological replicates; (c) Hematoxylin and eosin (H&E) stained Swiss roll colonic tissue sections of tamoxifen-treated *Crb3^{fl/fl}* and *Crb3^{ERΔIEC}* mice shows comparable tissue architecture; Scale bar = 500 μ m. Black box highlights an area that is enlarged in the bottom panel. Scale bar = 50 μ m; n=2 biological replicates; (d) Immunofluorescence labeling of colonic tissue sections of tamoxifen-treated *Crb3^{fl/fl}* and *Crb3^{ERΔIEC}* mice showing CRB3A, PALS1, PATJ, PAR3, and nuclei (DAPI, blue). Scale bar = 50 μ m. n=3 independent experiments; 2 technical replicates each.

Supplemental figure 2

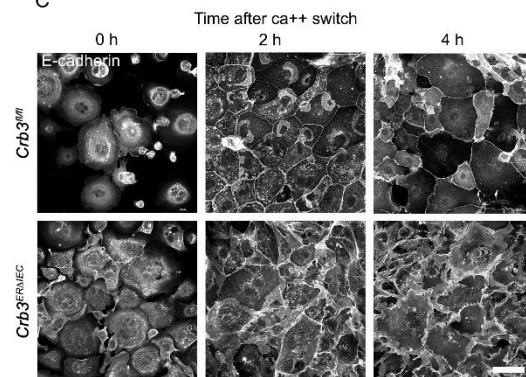
A



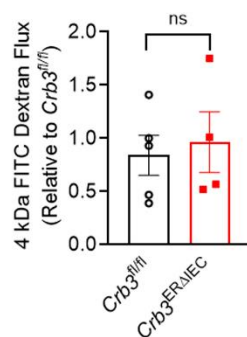
B



C



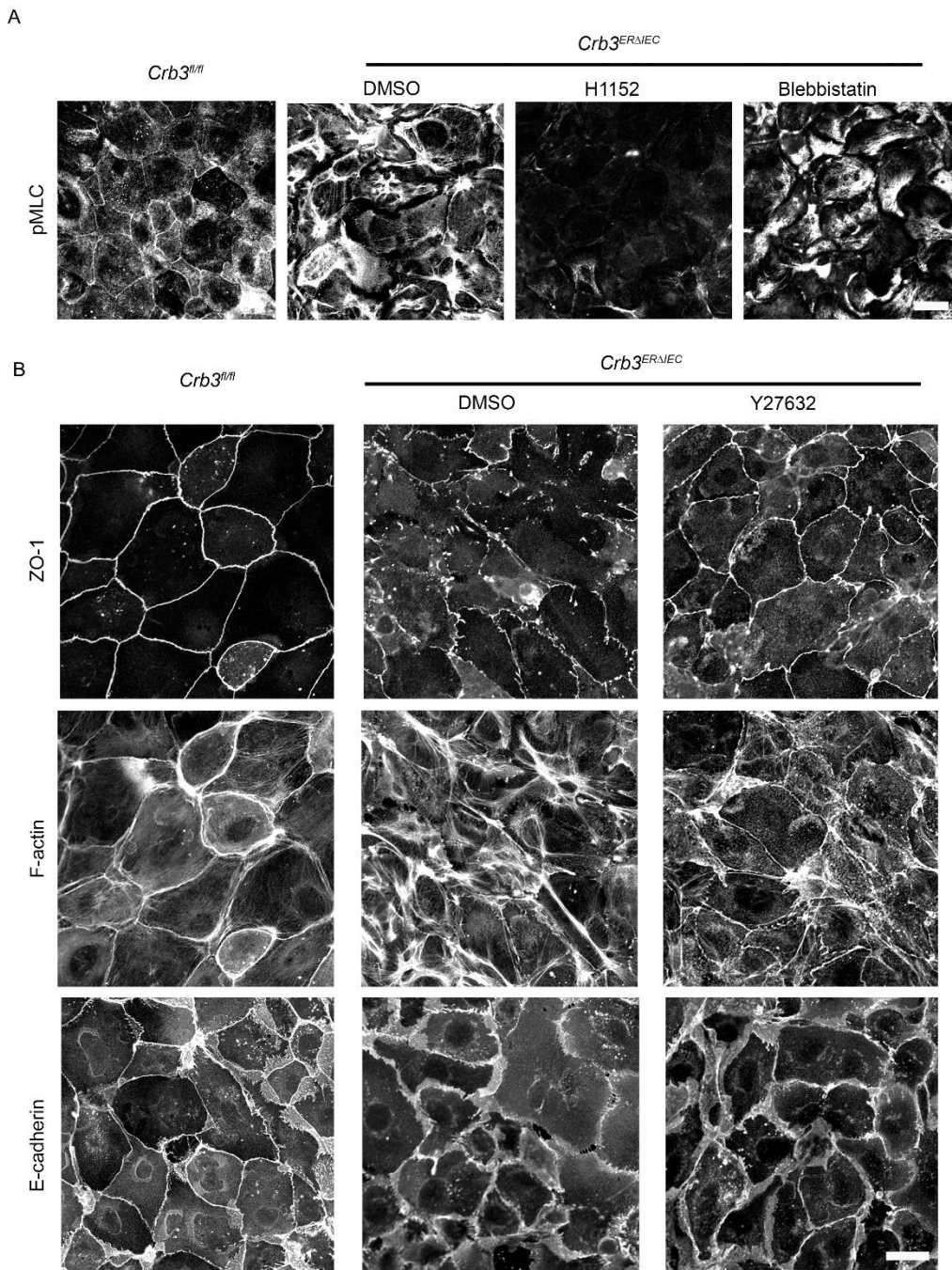
D



Supplemental Figure 2: CRB3 is not critical for barrier function and AJC morphology in stable epithelial cell-cell junctions. (a) Immunofluorescence labeling and confocal images of apical junctional proteins, ZO-1 and E-cadherin, in confluent and polarized colonoids from *Crb3^{fl/fl}* and *Crb3^{ERΔIEC}* mice grown on transwells filters. Scale bar = 25 μ m; n=3 independent experiments; 2 technical replicates each; (b-c) Immunofluorescence labeling and confocal images of apical junctional proteins, ZO-1 (b) and E-cadherin (c) in confluent and polarized colonoids from *Crb3^{fl/fl}*

and *Crb3*^{ERΔIEC} mice at 0, 2 and 4h following calcium switch. Scale bar = 25 μm; n=2 independent experiments; **(d)** Intestinal permeability measured by paracellular flux of 4KDa FITC-Dextran in serum in *Crb3*^{fl/fl} and *Crb3*^{ERΔIEC} mice at 2 hours post-surgery in mice. Data are mean ± SD. n=1 experiment with five technical replicates. p value calculated using two tailed student's t-test.

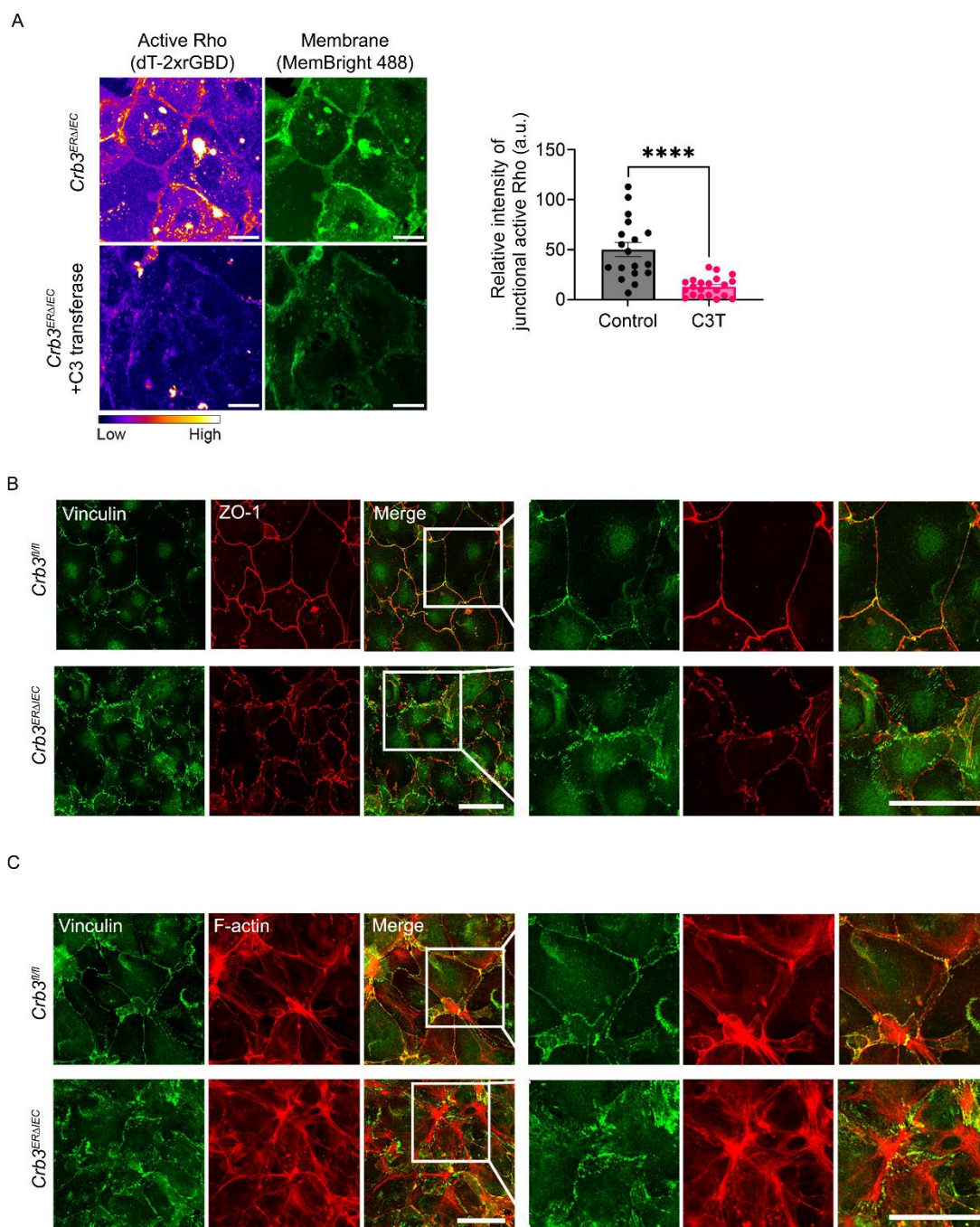
Supplemental figure 3



Supplemental Figure 3: Inhibition of ROCK or pMLC activity rescues the general architecture of perijunctional F-actin ring and AJC in CRB3 KO IEC. (a) Immunofluorescence labeling of colonoids derived from *Crb3^{ERΔIEC}* mice treated with vehicle (DMSO), ROCKII inhibitor (H1152, 10 μ M), and Non-Muscle Myosin II ATPase Inhibitor (Blebbistatin, 50 μ M) showing pMLC^{T18/S19} staining; Scale bar = 25 μ M. n=3 independent experiments; 2 technical replicates each;

(b) Representative confocal images of perijunctional F-actin (Phalloidin), and AJC proteins (ZO-1 and E-cadherin) in *Crb3^{fl/fl}* colonoids and *Crb3^{ERΔIEC}* colonoids treated with ROCK II inhibitor (Y27632, 50 μM) or vehicle (DMSO, 0.1%) for 30 minutes. Scale bar = 25 μM. n=3 independent experiments; 2 technical replicates each.

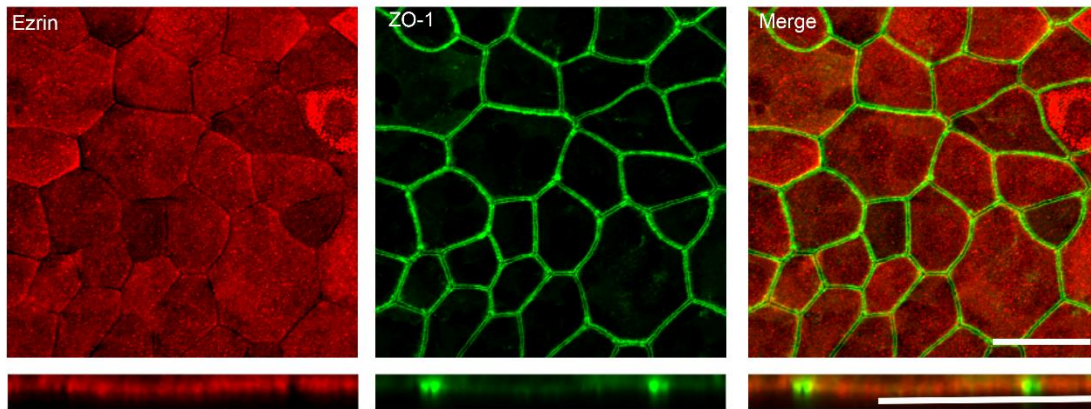
Supplemental Figure 4



Supplemental Figure 4: (a) Representative image of sub-confluent *Crb3^{ERΔIEC}* colonoids expressing pLenti-dTomato-2xrGBD (pseudo colored as FIRE LUT) and stained with MemBright 488 plasma membrane dye (green), treated acutely with vehicle (water) and active Rho inhibitor (C3 transferase, 2μg/ml). Images shown are sum of Z-projections. Scale bar = 20 μm; (Right) Graph showing the relative intensity of active RhoA at cell-cell junctions normalized to the

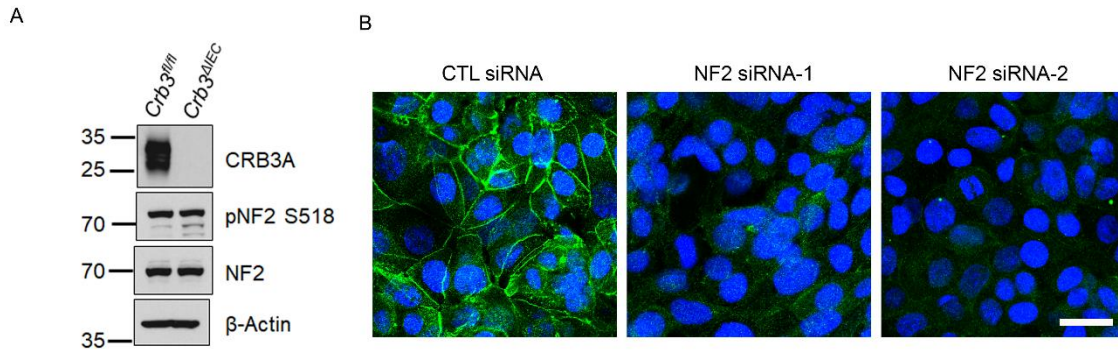
background signal of every junction. Graphs are mean $\pm$ SEM. Each dot represents an independent junction from 2 independent experiments **** $p < 0.0001$ by Mann-Whitney test. $n=18$ junctions for control ($Crb3^{ER\Delta IEC}$); $n=21$ junctions for C3 transferase ($C3T+Crb3^{ER\Delta IEC}$); **(b-c)** Representative confocal images of subconfluent colonoids derived from $Crb3^{fl/fl}$ and $Crb3^{ER\Delta IEC}$ mice immunostained for **(b)** Vinculin (rabbit anti-Vinculin, green) and ZO-1 (red); **(c)** Vinculin (mouse anti-Vinculin, green) and F-actin (red). White box represents zoomed in region shown on the right. $n=3$ independent experiments; 2 technical replicates each.

Supplemental Figure 5

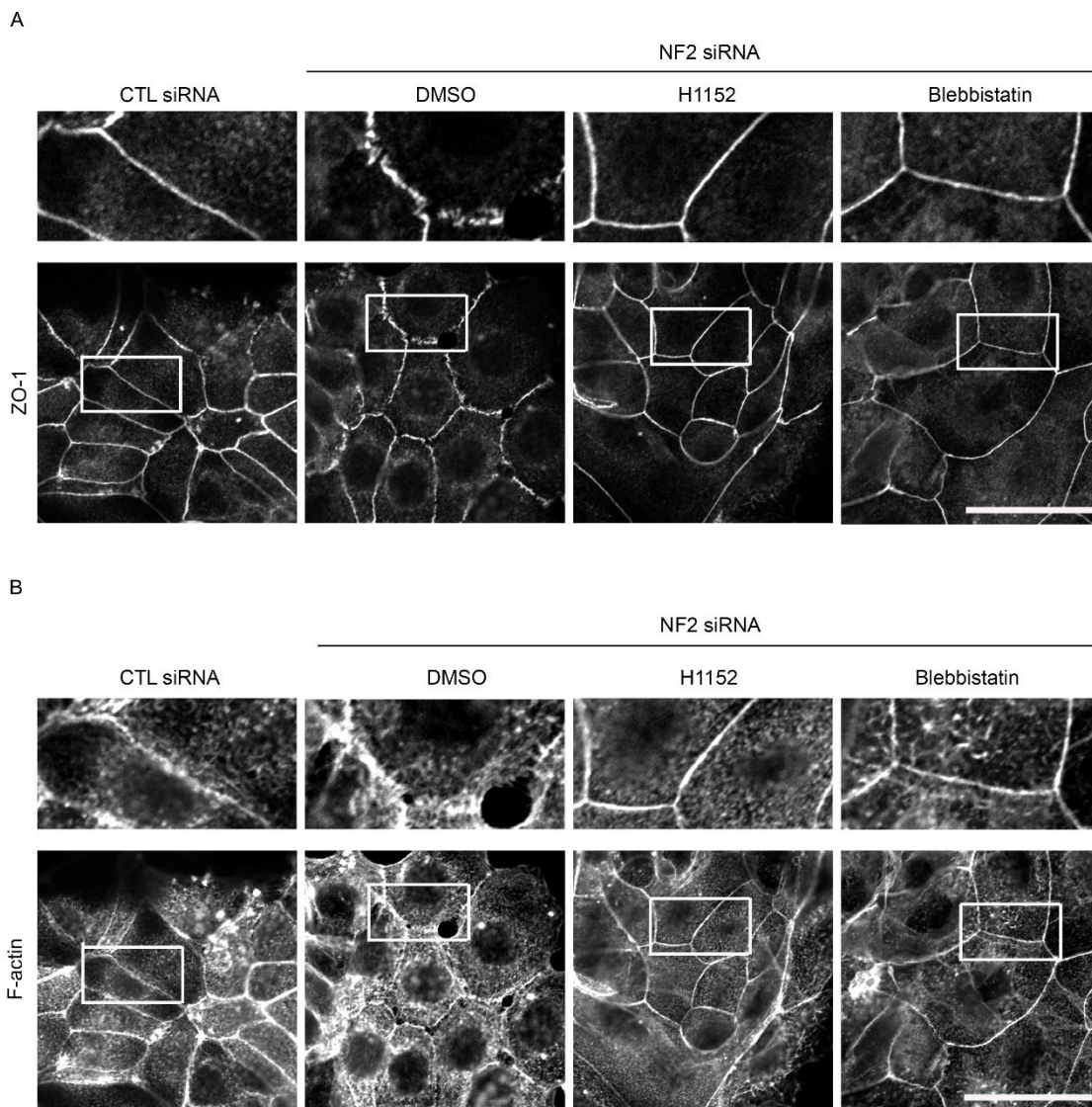


Supplemental Figure 5: Ezrin localizes to apical membrane. Immunofluorescence labeling of Ezrin (red) and ZO-1 (green) in confluent primary mouse colonoids grown on permeable support. Bottom panel: Z-projection shows that Ezrin and ZO-1 do not co-localize at TJ. Scale bar = 25 μm . n=2 independent experiments; 2 technical replicates each.

Supplemental figure 6



Supplemental Figure 6: (a) NF2 expression is independent of CRB3. Immunoblotting of CRB3A, NF2, pNF2S518 and β-actin (loading control) in colonoids derived from *Crb3^{fl/fl}* and *Crb3^{ERΔIEC}* mice. n=3 independent experiments; **(b) Efficiency of NF2 knockdown in model IECs.** Immunofluorescence labeling of SK CO-15 IECs transfected with control (scramble) or two independent NF2 siRNA showing NF2 (green) and DAPI (nuclei, blue). Scale bar = 25 μm. n=3 independent experiments.



Supplemental Figure 7: Inhibition of ROCK or pMLC activity rescues the general architecture of perijunctional F-actin ring and AJC in NF2 KD IEC. (a-b) Representative confocal images of TJ proteins ZO-1 (**a**) and perijunctional F-actin (Phalloidin, **b**) in SK CO-15 treated with NF2 siRNA or CTL siRNA for 72 hours, and treated with ROCK II inhibitor (H1152, 10 μ M), Myosin II Inhibitor (Blebbistatin, 50 μ M) or vehicle (DMSO, 0.1%) for 30 minutes. White box shows the region enlarged in the panel above. Scale bar = 25 μ m. n=3 independent experiments; 2 technical replicates each.

Supplemental table 1

Specificity	species	Suppliers	Catalog number	Dilution
CRB3a	rabbit	In-house	N/A	WB 1:4000; IF 1:500
β-actin	mouse	Sigma Aldrich	A5441	WB 1:5000
Merlin	rabbit	Cell Signaling Technology	12888	IF 1:100
Merlin	rabbit	Cell Signaling Technology	6995	WB 1:1000; IP 1:100
p-Merlin S518	rabbit	Cell Signaling Technology	9163	IP 1:100
Myc-tag	mouse	Cell Signaling Technology	2276	IP 1:100
Myc-tag	rabbit	Cell Signaling Technology	2278	WB 1:2000
PALS1	rabbit	In-house	N/A	WB 1:1000; IF 1:200
PARD3(PAR3)	rabbit	Novus	NBP1-88861	WB 1:1000; IF 1:100
PATJ	rabbit	In-house	N/A	IF 1:100
PATJ/INADL	rabbit	LifeSpan Bioscience	LS-C410011	WB 1:1000
p-MLCT18/S19	rabbit	Cell Signaling Technology	3674	IF 1:250
p-MLC S19	rabbit	Cell Signaling Technology	3671	IF 1:250
ZO-1	mouse	ThermoFisher	33-9100	WB 1:1000; IF 1:250
ZO-1	rabbit	ThermoFisher	40-2200	IF 1:250
E-cadherin	goat	R & D System	AF648	WB 1:2000; IF 1:500
Ezrin	mouse	ThermoFisher	35-7300	WB 1:1000
β-catenin	mouse	BD Bioscience	610153	WB 1:1000; IF 1:500
Alexa Fluor™ 488 Phalloidin		ThermoFisher	A12379	IF 1:400
Alexa Fluor™ 555 Phalloidin		ThermoFisher	A34055	IF 1:400
Vinculin	mouse	ThermoFisher	14-9777-82	IF 1:100
Vinculin	rabbit	ThermoFisher	700062	IF 1:100
Calnexin	rabbit	Abcam	ab22595	WB 1:4000