Supplemental Table 1:
Junctional localization of proteins after calcium switch in ZO-1 KD cells expressing EGFP or EGFP-tagged ZO-1 mutants

Occludin Junctional Fraction

time (h)	1	3	12
EGFP	0.42±0.01*	0.52±0.02*	0.47±0.05
EGFP-FL-ZO-1	0.62±0.05#	0.64±0.03#	0.56±0.04
EGFP-ZO-1$^{\Delta ABR}$	0.58±0.03#	0.63±0.01#	0.50±0.02
EGFP-ZO-1$^{\Delta U5\text{-}GuK}$	0.44±0.03*	0.39±0.02*#	0.37±0.02*#

PATJ Junctional Fraction

time (h)	1	3	12
EGFP	0.25±0.01*	0.32±0.03*	0.37±0.04*
EGFP-FL-ZO-1	0.47±0.02#	0.50±0.03#	0.49±0.02#
EGFP-ZO-1$^{\Delta ABR}$	0.41±0.02#	0.48±0.03#	0.53±0.03#
EGFP-ZO-1$^{\Delta U5\text{-}GuK}$	0.29±0.03*	0.31±0.02*	0.24±0.01*#

E-cadherin Junctional Fraction

time (h)	1	3	12
EGFP	0.78±0.01	0.84±0.01	0.88±0.01
EGFP-FL-ZO-1	0.82±0.04	0.88±0.02	0.88±0.01
EGFP-ZO-1$^{\Delta ABR}$	0.84±0.02#	0.86±0.01	0.88±0.02
EGFP-ZO-1$^{\Delta U5\text{-}GuK}$	0.84±0.02#	0.90±0.02#	0.87±0.02

***, $p<0.05$ vs. EGFP-FL-ZO-1 at given time point**
#, $p<0.05$ vs. EGFP at given time point

Supplemental Table 2:
Junctional localization of proteins after calcium switch in cells lacking U5-GuK binding partners

ZO-1 Junctional Fraction

time (h)	1	3	12
WT	0.65±0.02	0.69±0.02	0.67±0.02
occludin KD #1	0.57±0.03*	0.63±0.02*	0.60±0.03*
occludin KD #2	0.64±0.03	0.66±0.02	0.66±0.01
shroom2 KD #1	0.61±0.02	0.68±0.02	0.71±0.02
shroom2 KD #2	0.66±0.02	0.72±0.02	0.73±0.02*

PATJ Junctional Fraction

time (h)	1	3	12
WT	0.37±0.02	0.42±0.02	0.48±0.03
occludin KD #1	0.32±0.02*	0.45±0.01	0.43±0.03
occludin KD #2	0.41±0.03	0.46±0.01	0.46±0.01
shroom2 KD #1	0.35±0.01	0.40±0.03	0.45±0.02
shroom2 KD #2	0.37±0.02	0.43±0.02	0.43±0.01

E-cadherin Junctional Fraction

time (h)	1	3	12
WT	0.75±0.03	0.68±0.02	0.66±0.02
occludin KD #1	0.74±0.03	0.69±0.02	0.64±0.01
occludin KD #2	0.76±0.01	0.70±0.01	0.72±0.01*
shroom2 KD #1	0.71±0.03	0.70±0.03	0.66±0.02
shroom2 KD #2	0.80±0.01	0.74±0.03	0.66±0.01

***, p<0.05 vs. WT at given time point**

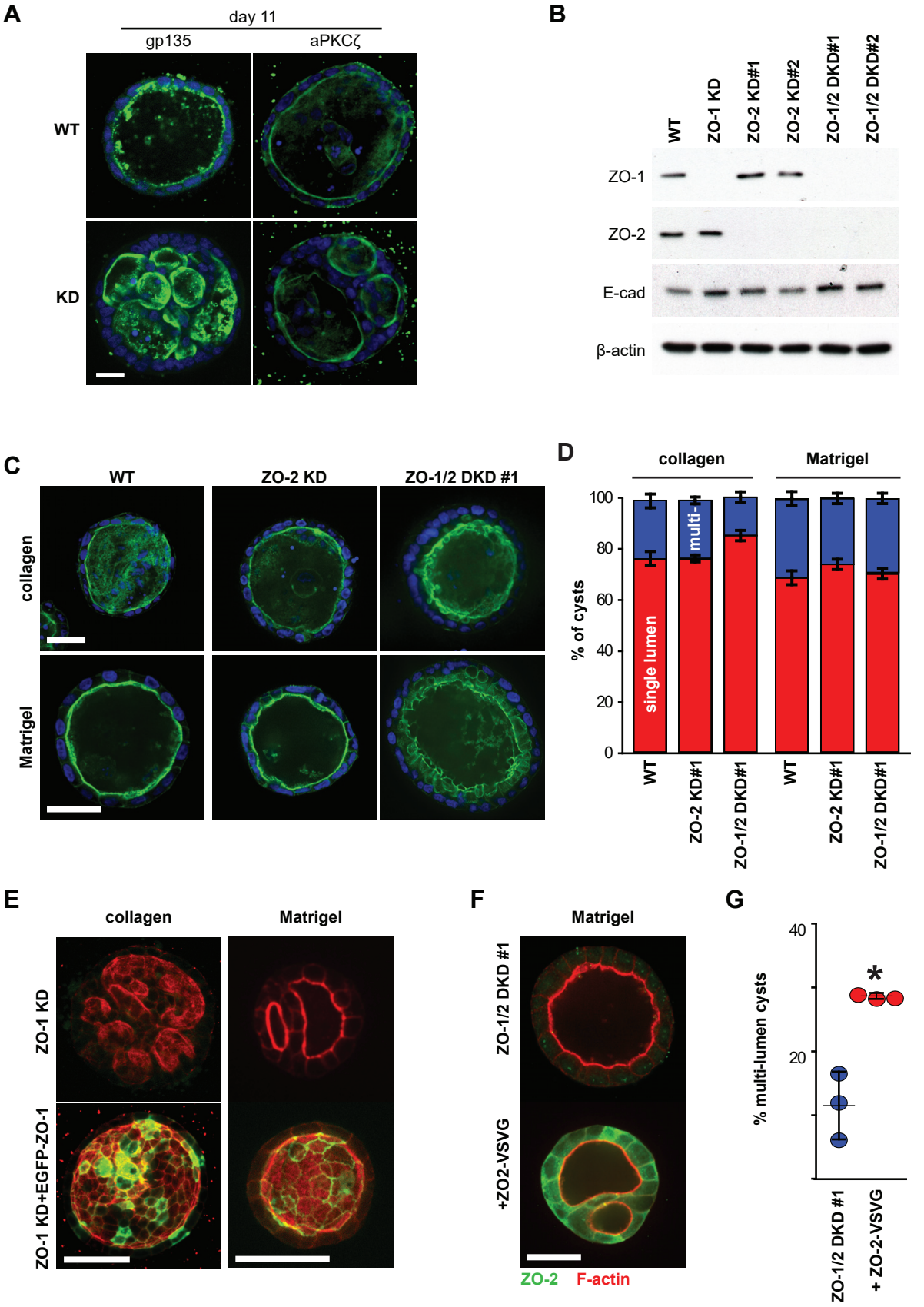
Supplemental Table 3:

Antibodies used:

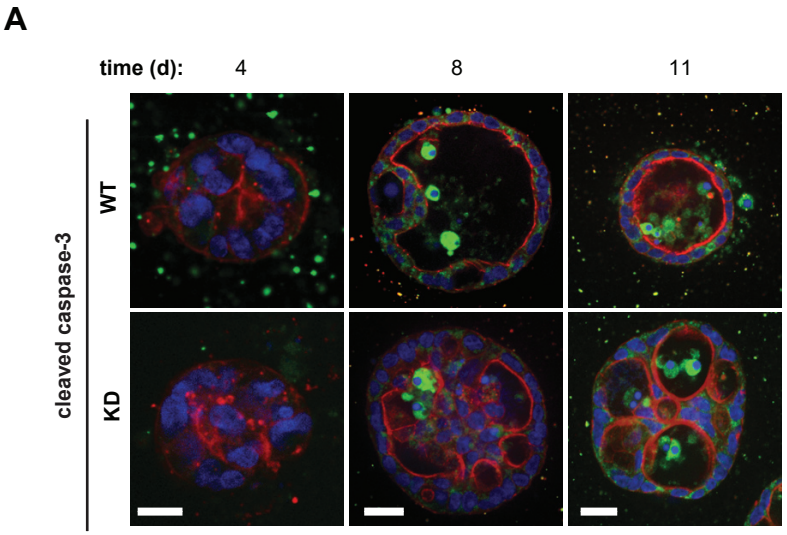
Antigen	Species	Source	Dilution (IF)	Dilution (WB)
ZO-1	rat	clone R40.76 (culture supernatant, Dan Goodenough, Harvard Medical School)	1:2	1:5
ZO-1	mouse	Invitrogen (33-9100)	1:100	1:1,000
occludin	mouse	Invitrogen (33-1500)	1:100	1:1,000
PATJ	rabbit	Ben Margolis, Univ. Michigan (UM356)	1:100	1:1,000
E-cadherin	rabbit	Cell Signaling (clone 24E10, 3195S)	1:100	1:10,000
E-cadherin	mouse	BD Biosciences (clone 34, 610404)	1:100	N/A
PKC-zeta	rabbit	Sigma-Aldrich (P0713)	1:100	1:1,000
β-actin	mouse	Sigma-Aldrich (clone AC-15, A1978)	N/A	1:10,000
GFP	mouse	Fitch monoclonal antibody core, Univ. Chicago (clone F56-6A.1.2.3)	N/A	1:1,000
Ki67	mouse	Vector Labs (clone SP6, A1978)	1:100	N/A
Cleaved caspase-3	rabbit	Cell Signaling (9664)	1:100	N/A
Shroom2	rabbit	Jeff Hildebrand, Univ. Pittsburgh (UPT115)	1:100	1:1,000
α-catenin	rabbit	Cell Signaling (3236)	1:100	1:1,000
NuMA	rabbit	Abcam (36999)	1:200	N/A
aPKC-lambda	mouse	BD (610207)	1:250	N/A
ZO-2	rabbit	ThermoFisher (38-9100)	1:250	N/A
VSVG	mouse	Sigma (V5507)	1:100	N/A
α-catenin	rabbit	ThermoFisher (71-1200)	1:200	N/A

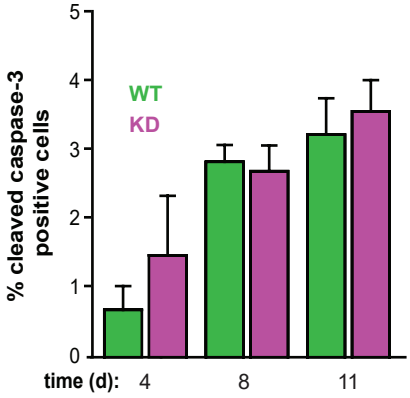
Supplemental Figure 1: The multiple lumen phenotype is specific to ZO-1 loss. A) WT and ZO-1 KD cysts were grown to maturity in collagen and stained for either gp135 (green, left) or aPKCζ (green, right) and nuclei (blue). Representative images are shown. Bar = 10μm. Images are representative of at least 3 independent experiments for each condition, all with similar results.

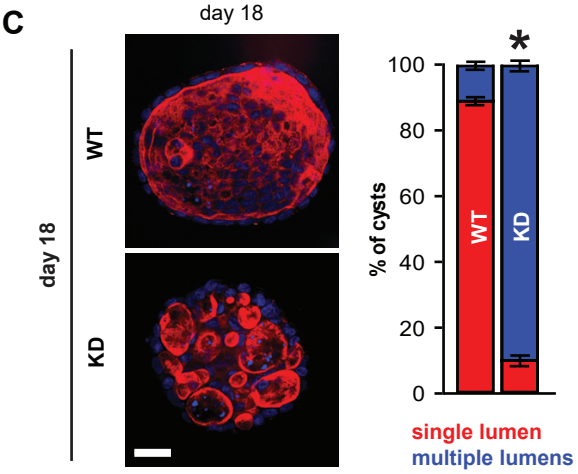
A previous study reported that ZO-1/ZO-2 double knockdown (DKD) cells are capable of forming single lumen cysts in collagen gels, thereby suggesting that ZO-1 is dispensable for single lumen formation (Fanning et al., 2012). To directly address this potential discrepancy, we tested ZO-1/ZO-2 DKD cells as well as in ZO-2 KD cells in both collagen and Matrigel substrates. B) Western blot for ZO-1 and ZO-2 protein expression in confluent WT, ZO-1 KD, ZO-2 KD, and ZO-1/ZO-2 DKD monolayers. E-cadherin and β-actin were used as loading controls. C) Representative micrographs of mature WT, ZO-2 KD, and ZO-1/ZO-2 KD grown in collagen and Matrigel stained for F-actin (green) and nuclei (blue). In contrast to ZO-1 KD cells, single lumen cysts formed in both ZO-2 KD and ZO-1/ZO-2 DKD cells. Bars = 50μm. D) Lumen phenotype of mature WT, ZO-2 KD, and ZO-1/ZO-2 DKD cysts grown in collagen or Matrigel was qualified as multi-lumen (blue) or single lumen (red). Values are mean±s.e.m. of >100 cysts from 3 independent experiments per condition. E) EGFP-ZO-1 expression in ZO-1 KD cells restored single lumen formation. Representative images of mature ZO-1 KD cysts and ZO-1 KD cysts expressing EGFP-ZO-1 (shown in green) grown in either collagen or Matrigel. Cysts were stained for F-actin (red). Bars = 50μm. Data are representative of at least 3 independent experiments for each condition, all with similar results. F) ZO-2 expression in ZO-1/ZO-2 DKD cells increased numbers of multi-lumen cysts. ZO-2-VSVG was expressed in ZO-1/ZO-2 DKD cells. ZO-1/ZO-2 DKD cells with and without ZO-2-VSVG expression were grown to maturity in Matrigel and stained for ZO-2 (green) and F-actin (red). Representative images are shown. Bar = 25μm. G) Lumen phenotype was scored as single or multiple lumens, and the percent of multiple-lumen cysts is shown for ZO-1/ZO-2 DKD cells with (red dots) and without (blue dots) ZO-2-VSVG expression. Individual data points are independent experiments and represent >100 cysts scored for each cell type. Brackets represent mean±s.e.m of 3 independent experiments. *, $p<0.05$ by two-tailed t-test.

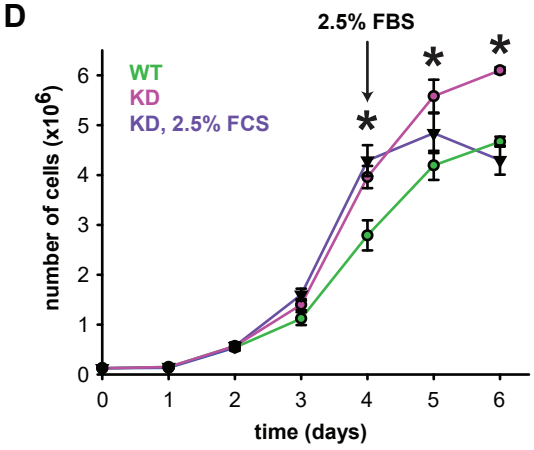

A
day 11
gp135
aPKCζ
WT
KD
B
WT
ZO-1 KD
ZO-2 KD#1
ZO-2 KD#2
ZO-1/2 DKD#1
ZO-1/2 DKD#2
ZO-1
ZO-2
E-cad
β-actin
C
WT
ZO-2 KD
ZO-1/2 DKD #1
collagen
Matrigel
D
collagen
Matrigel
% of cysts
multi-
single lumen
WT
ZO-2 KD#1
ZO-1/2 DKD#1
E
collagen
Matrigel
ZO-1 KD
ZO-1 KD+EGFP-ZO-1
F
Matrigel
ZO-1/2 DKD #1
+ZO2-VSVG
ZO-2
F-actin
G
% multi-lumen cysts
ZO-1/2 DKD #1
+ ZO-2-VSVG

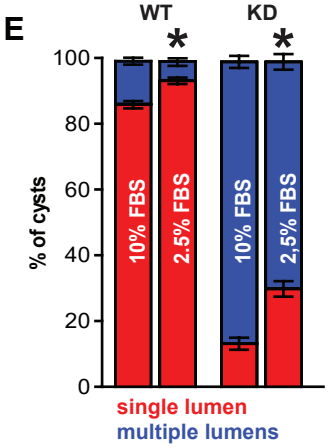
Supplemental Figure 2: ZO-1 is responsible for diverse morphogenetic processes. A) Cysts were grown in collagen and stained for cleaved caspase 3 (green), F-actin (red), and nuclei (blue) at indicated times. Bar = 10μm. Images are representative of 3 independent experiments for each condition, all with similar results. B) Thirty cysts of each genotype were scored for cleaved caspase-3 staining. Mean±s.e.m from 3 independent experiments. C) WT and ZO-1 KD cells were grown in collagen gels for 18 days and stained for F-actin (red) and nuclei (blue). Bar = 10μm. Cysts were classified either single lumen (red) or multi-lumen (blue). Mean±s.e.m. of >100 cysts from each of 3 independent experiments. *, $p<0.05$ by two-tailed t-test comparing percent multi-lumen of WT vs. KD cysts. D) Growth curves of WT and KD monolayers. Reduced serum (2.5%) media was used from day 4 through 6, as indicated. Mean±s.e.m. of triplicate samples from an experiment that is representative of 3 independent experiments, all with similar results. *, $p<0.05$ by two-tailed t-test between WT vs. KD. E) Cysts were grown in collagen gels and fed with media containing either normal (10%) or reduced (2.5%) serum from days 4 through 11. Lumen phenotype was scored as either single (red) or multi-lumen (blue). Mean±s.e.m. of >100 cysts in each of 3 independent experiments. *, $p<0.05$ by two-tailed t-test.



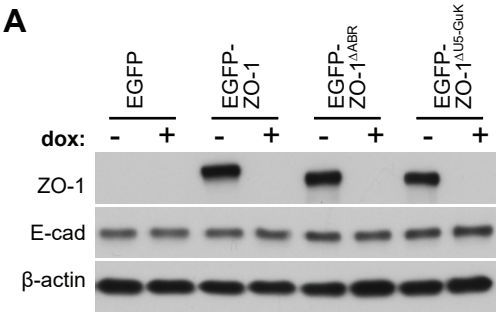


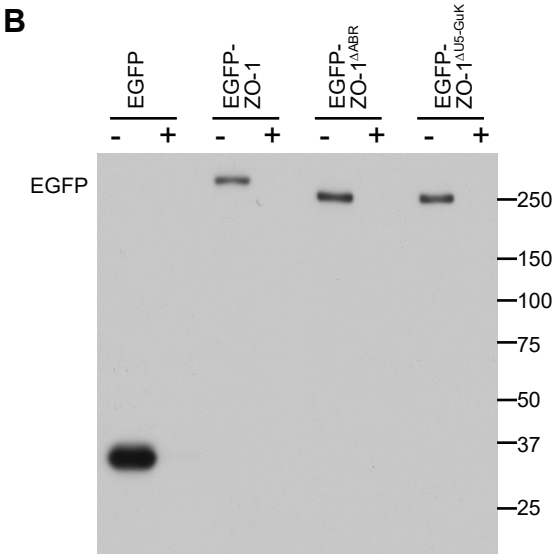


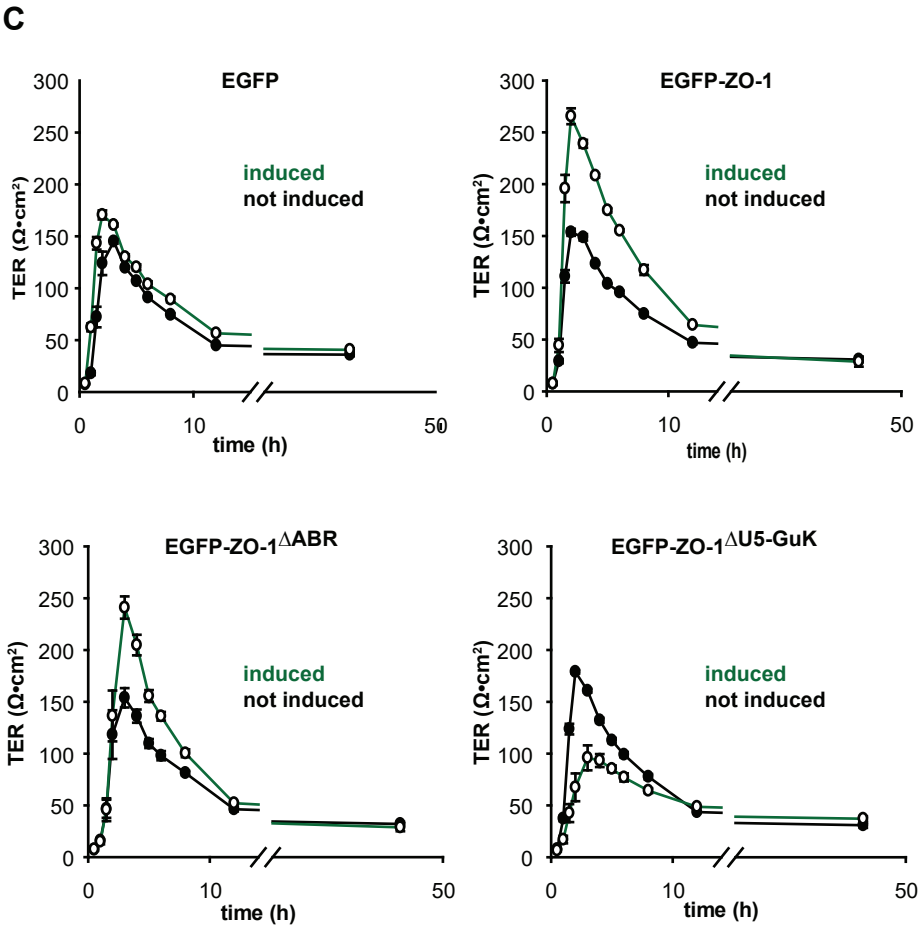


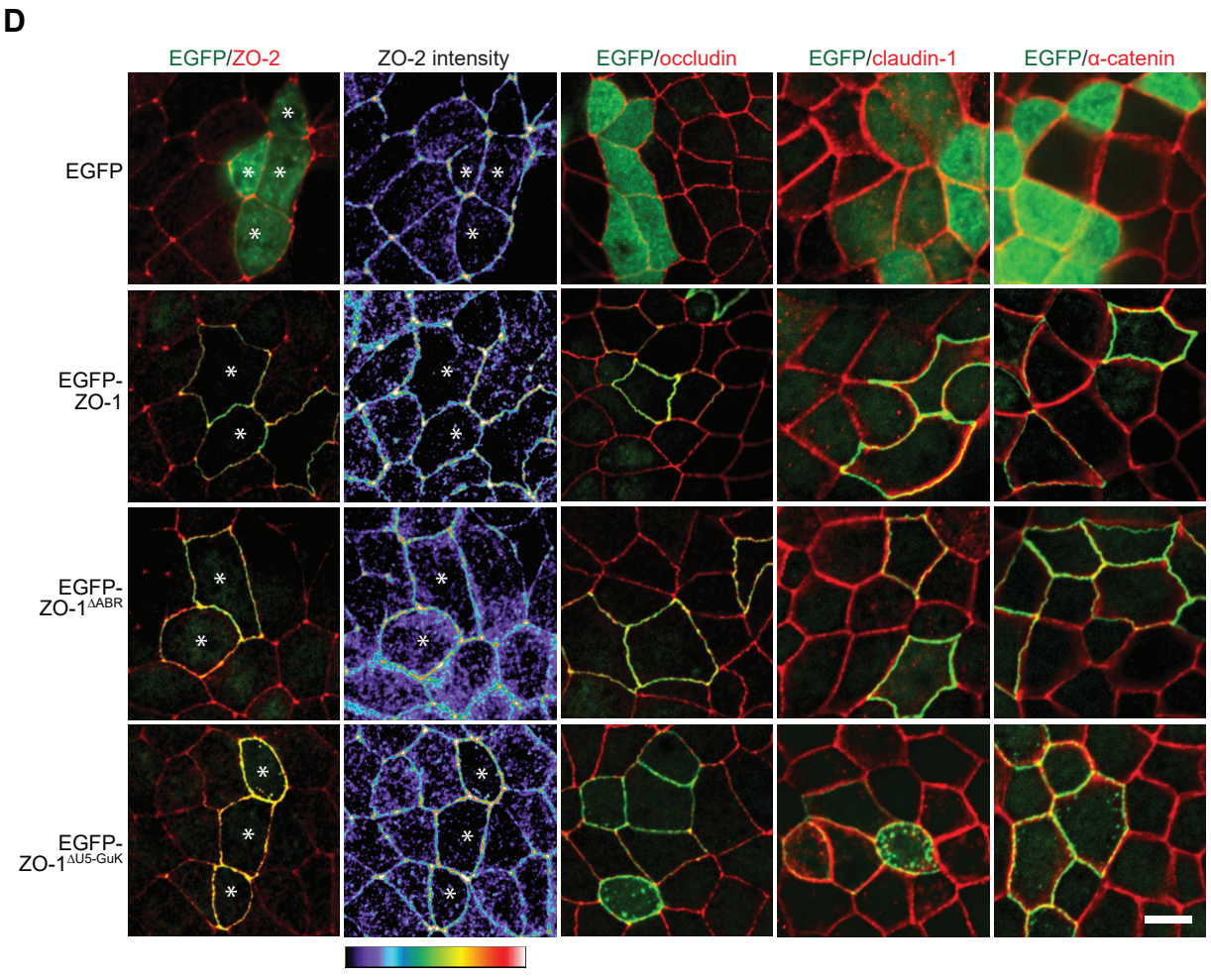


Supplemental Figure 3: Multiple ZO-1 domains are necessary for epithelial morphogenesis and barrier assembly. A) Inducible expression of EGFP, EGFP-ZO-1, EGFP-ZO-1$^{\Delta ABR}$, or EGFP-ZO-1$^{\Delta U5\text{-}GuK}$ in ZO-1 KD cells was confirmed by western blot. Lysates from confluent monolayers cultured in media with or without doxycycline were probed for ZO-1 to confirm inducible expression of ZO-1 proteins. E-cadherin and β-actin were used as loading controls. Data are representative of at least 3 independent experiments for each condition, all with similar results. B) Expression of EGFP epitope was confirmed by immunoblotting the same lysates as in panel (A) for EGFP. C) TER of monolayers with inducible expression of EGFP-ZO-1 mutants was monitored at indicated times after calcium repletion. Monolayers were cultured in media –dox (green lines) to induce expression or +dox (black lines) to repress expression during barrier development. Mean±s.e.m. are representative of 3 independent experiments, each performed in triplicate. D) EGFP, EGFP-ZO-1, EGFP-ZO-1$^{\Delta ABR}$, or EGFP-ZO-1$^{\Delta U5\text{-}GuK}$ expression was induced in a small proportion of cells within a ZO-1 KD monolayer. Mature monolayers were stained for ZO-2, occludin, claudin-1, and α-catenin (shown in red) 4 days after plating. ZO-2 recruitment to the tight junction was markedly enhanced by expression of EGFP-ZO-1$^{\Delta U5\text{-}GuK}$, but not other EGFP-tagged proteins (see pseudocolor intensity images).

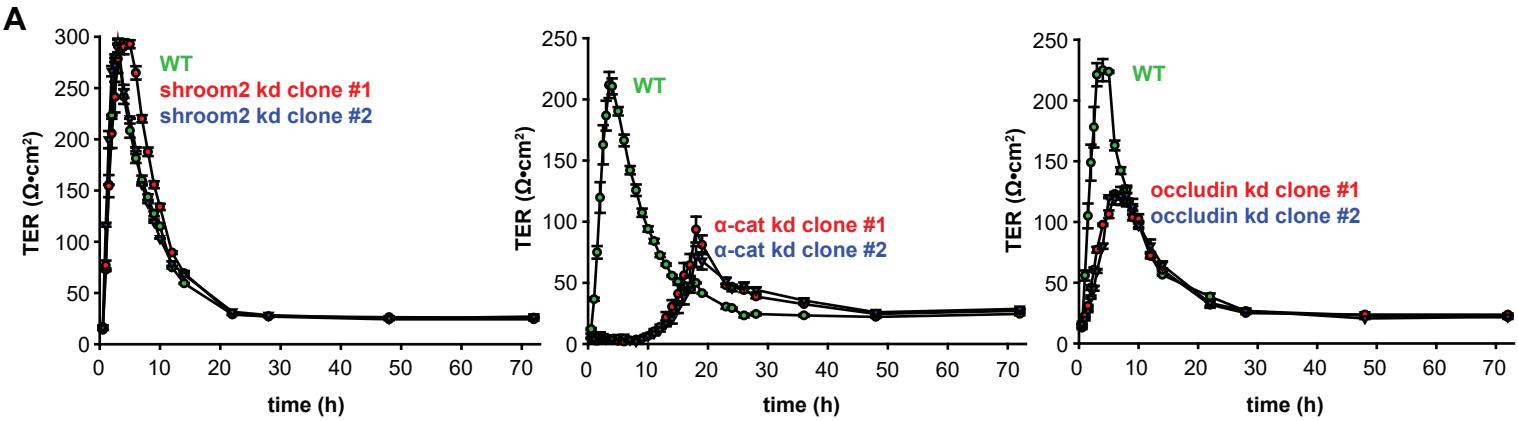


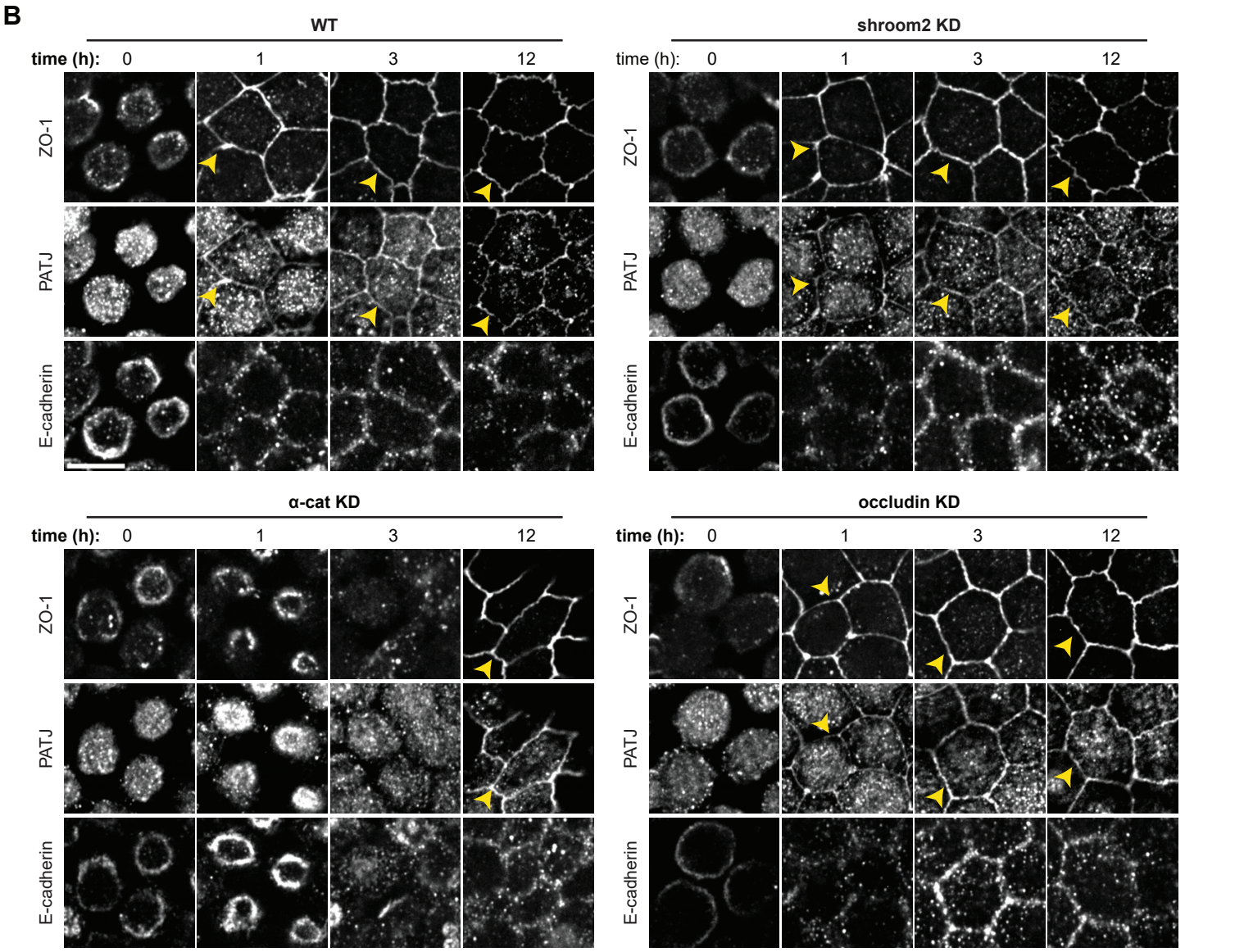






Supplemental Figure 4: ZO-1 U5-GuK binding partners orchestrate functional and structural barrier formation. A) TER of WT and 2 independent clones of shroom2 KD, α-catenin KD, and occludin KD monolayers at indicated times after calcium switch. Data for each line are displayed as mean±s.e.m. and are representative of 3 independent experiments performed in triplicate (for each line). One representative trace of WT and one clone of each KD cell line were combined to create the traces in Figure 7B. B) WT, shroom2 KD, α-catenin KD, and occludin KD monolayers were immunostained for ZO-1, PATJ, and E-cadherin at indicated times after calcium repletion. Grayscale images are shown. Yellow arrowheads mark areas of PATJ or ZO-1 recruitment to tight junctions. Fluorescence intensities are scaled identically for a given antigen. Merged images are shown in Figure 7C. Images are representative of at least 3 independent experiments for each condition, all with similar results. Bar = 10μm.





Supplemental Movie: Mitotic spindle orientation is disturbed in ZO-1 KD cells. Movie shows live imaging of cysts grown from WT (control) and ZO-1 KD (ZO-1 kd) cells expressing Lifeact-sfGFP (green) and H2B-mCherry (red). Arrows indicated mitotic events (movie pauses at these points).