Supplementary Data

Figure S1. Validation of two-cell stages derived from cell division and quantification of phenotypes at different stages in the cyst-assay. (A) Non-enzymatic detachment yields a single cell suspension of MDCK cells. Cells were split with Trypsin and seeded at an appropriate density to become sub-confluent the next day. Cells were washed three times in PBS and incubated with cell dissociation buffer for 30 minutes. Light microscopy revealed an almost complete detachment. Cells were washed in PBS, counted, repeatedly pipetted, and embedded in DMEM with 2% Geltrex at a density of 5000 cells per well in an 8-well chamber coverslip. 20 random fields were acquired through light microscopy, predominantly single cells become apparent on the underlying 100% Geltrex base. The number of single cells and multi-cellular aggregates were quantified for each field. 24 hours post-embedding, cells were fixed, and 20 random fields were acquired through light microscopy. The number of structures per field was counted and compared to the number of structures directly after embedding. (B) Quantification of imaged cellular structures. Directly after embedding, 87.4% of the counted structures were single cells. 24 hours after embedding, the average number of structures per field was reduced from 11.6 directly after embedding to 10.8 after 24 hours, indicating that 6.5% of the resulting structures at 24 hours are the result of migratory adhesion events. Thus the estimated number of structures resulting from a single embedded cell is 81.0%. (C) Quantification of early stages of cell division (Prepro- to Metaphase). All observed structures displayed a GFP-Crb3 staining in the area of the forming mitotic spindles. All cells displayed an exclusively cytoplasmic GFP signal. (D) Quantification of telophases. Telophases in the

absence of DNA condensation were identified through α-tubulin-positive staining of the midzone microtubules. All imaged telophases displayed the here-described typical pattern of internalized GFP-Crb3a. 26.7% displayed an additional membrane-staining for GFP-Crb3a, possibly due to over-expression of the protein. The same amount of cells also displayed GFP-Crb3a staining at the tips of the midzone microtubules. (E) Phenotypes of two-cell stages. Most structures presented large lumina with a diameter of > 5 μm. We observed no multiple-lumen phenotype, but 15.0% of the two-cell stages displayed additional basolateral GFP membrane staining.

Figure S2. Endogenous Crb3a staining resembles GFP-Crb3a (single Z-sections). MDCKII cells were embedded in Geltrex as described, fixed and stained with the indicated antibodies. Datasets were acquired through confocal laser microscopy and three-dimensionally reconstructed. (A) A nascent epithelial lumen is present after first division of MDCKII cells. Crb3a antibody staining (green) resembles GFP-Crb3a staining (see Figure 2) and overlays with GP135 (red) in the cytoplasmic compartments and at the apical surface from the single-cell to the two-cell stage. (B) Endogenous Crb3a (green) colocalizes with Rab11 (red) in apical recycling endosomes.

Figure S3. Live cell imaging of lumen formation from MDCKII cell division. MDCKII cells were sequentially virally transduced with GFP-Crb3a (in pRevTRE) and mCherry-E-cadherin (in pQCXIP). (A) Double fluorescent cells were grown as a monolayer on transwell filters, DAPI stained and analyzed by confocal laser microscopy. (B) Double fluorescent cells retained the ability to form cysts. Cysts were prepared as previously

described. They were fixed at day 4, stained with DAPI and analyzed by epifluorescence microscopy. (C) Live-cell images (Z-stacks) were acquired every 15 minutes as previously described, starting 6 hours post embedding. The images were deconvolved and collapsed over the Z-plane.

Figure S4. Dividing Cytochalasin-treated cells retain the ability to take up and traffic apical membrane. GFP-Crb3a-expressing MDCKII cells were embedded in Geltrex as described, fixed after 36 hours, and stained with the indicated antibodies or Phalloidin, respectively. To inhibit actin polymerization, liquid medium was replaced with 5 μM Cytochalasin B-containing Geltrex medium, or medium containing an identical volume of DMSO (control), after cells had adhered. Images represent single Z-sections. Uninhibited cells reached predominantly the 4-cell stages at 36 hours post-embedding, whereas Cytochalasin-treated cells remain at a double nucleated stage as a result of impaired cytokinesis. Uptake of GFP-Crb3a and its transport to the spindle poles and to the area between the two daughter nuclei remain intact (see Figure 4D). GFP-Crb3a-positive structures overlay with Phalloidin and GP135 staining, but not E-cadherin and Rab11. Rab11 seems to surround these areas.

Figure S5. Two cell stages of GFP-Crb3a cells expressing either control vector, wild-type (WT) Flag-Rab11a, Flag-Rab11a S25N (DN), or Flag-Rab11a Q70L (CA) were stained and visualized for the presence of a single lumen (A). In GFP-Crb3a cells expressing control, WT Flag-Rab11a, and Flag-Rab11a Q70L a single, GFP-positive lumen can be seen between cells. GFP-Crb3a cells expressing Flag-Rab11a S25N do not

possess a single lumen and GFP-Crb3a is seen in cytosolic puncta. (B) Two cell stages of GFP-Crb3a cells expressing control, WT, CA, or DN Flag-Rab11a were counted and the numbers of two-cell stages with a single lumen were quantified. Numbers represent the sum of two independent experiments.

Figure S6. Colocalization of endogenous Pals1 and GFP-Crb3a. GFP-Crb3a-expressing MDCKII cells were embedded in Geltrex as previously described, fixed after 36 hours, and stained with the indicated antibodies. Images represent single Z-sections. Endogenous Pals1 staining resembles GFP-Pals1 staining (see Figure 6A).

Figure S7. Crb3 knockdown: Western blot and alternative knockdown. Crb3 shRNA clones and controls were grown in tissue culture dishes for three days, lysed in RIPA buffer, and analyzed by Western blot. (A) Western blot of Crb3 knockdown clones presented in Figure 7. (B) Western blot of Crb3 knockdown in MDCKII performed with the alternative hairpin sequence (C) Quantification of phenotypes from cyst-formation assay at two-cell stage, performed with alternative hairpin sequence. Lumina > 5 μm were classified as 'large', sizes below as 'small'. Results resemble data presented in Figure 7.

Figure S8. Par3-staining in two-cell stage grown from Crb3 SH clone 3 rescued with GFP-Crb3a FL. The image displays a collapsed Z-stack. As previously demonstrated in MDCKII wt cells, Par3 localized to the TJ, but not to the apical membrane domain in GFP-Crb3a FL rescued Crb3 SH-RNAi cells.

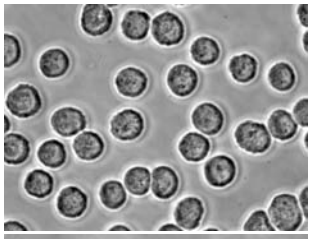
Movie S1. 3D reconstruction of a two-cell stage with a solitary lumen. GFP-Crb3a-expressing MDCKII cells were embedded into Geltrex matrix as previously described and fixed after 24 hours. The dataset was acquired through confocal laser microscopy and three-dimensionally reconstructed. Green represents GFP-Crb3a, red ZO-1, blue E-cadherin, and grey DAPI nuclear staining.

Movie S2. 3D reconstruction of a two-cell stage possessing a double lumen. GFP-Crb3a expressing MDCKII cells were embedded into Geltrex matrix as previously described. After cells had attached, the medium was removed and replaced by medium containing 2% Geltrex plus 50 μM myristoylated aPKCζ pseudosubstrate (Calbiochem). Cells were fixed after 24 hours. The dataset was acquired through confocal laser microscopy and three-dimensionally reconstructed. Green represents GFP-Crb3a, red ZO-1, blue E-cadherin, and grey DAPI nuclear staining.

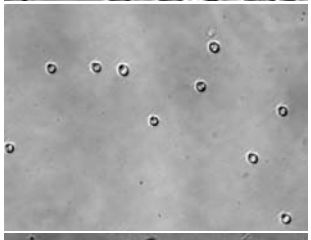
Figure S1

A

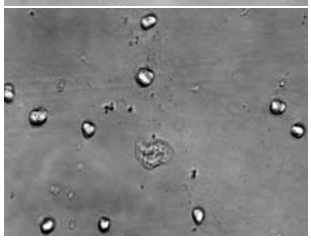
After 30 min. under cell dissociation buffer



20 minutes after embedding into Geltrex, 5000 cells per well (8-well chamber coverslip)



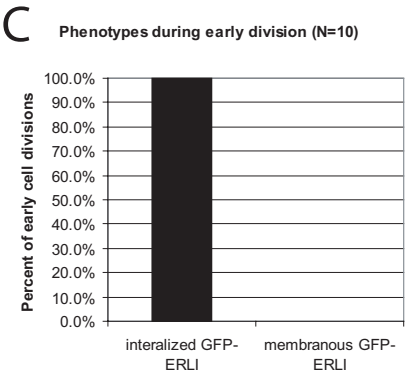
24 hours after embedding into Geltrex, 5000 cells per well (8-well chamber coverslip)



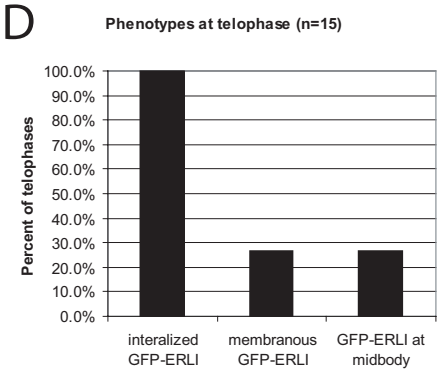
B

fields counted immedeately and 24 hrs. after embedding	20
structures after embedding (total)	231
single cells after embedding	202
multicellular structures after embedding	29
share of single cells after embedding	87.4%
average number of structures after embedding per field	11.6
structures at 24 hrs. after embedding (total)	216
average number of structures at 24h per field	10.8
total number of structures (after 24 hrs : after embedding)	93.5%
percent reduction (after 24 hrs : after embedding)	6.5%
number of structures resulting from single cells	81.0%

C



D



E

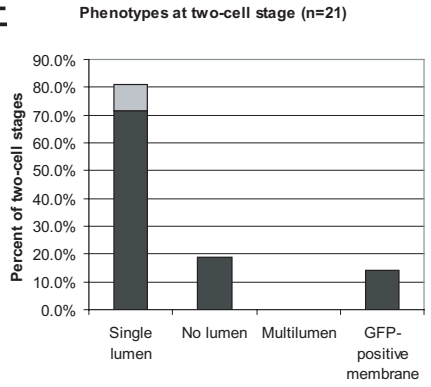


Figure S2

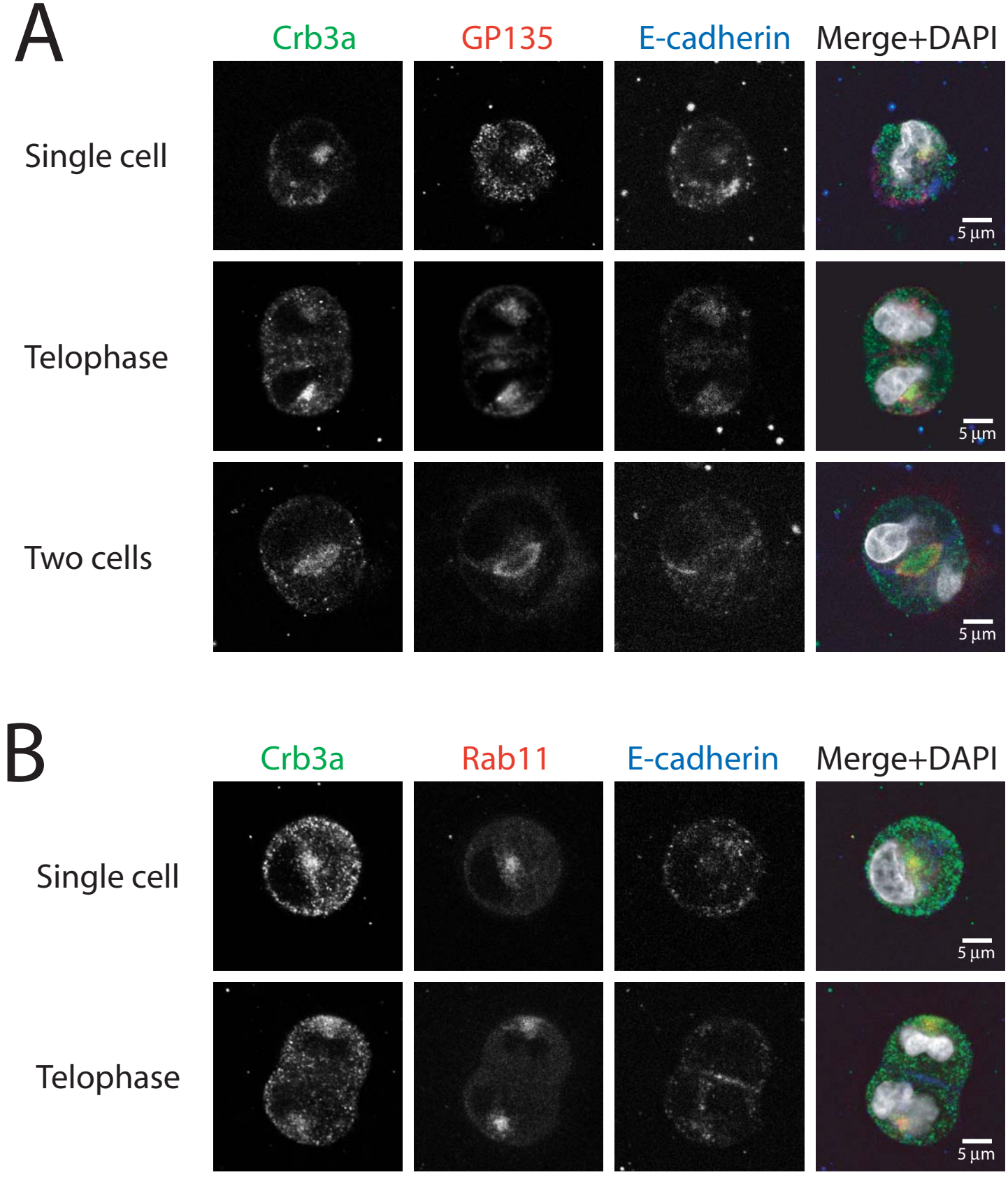

A
Crb3a
GP135
E-cadherin
Merge+DAPI
Single cell
Telophase
Two cells
5 µm
B
Crb3a
Rab11
E-cadherin
Merge+DAPI
Single cell
Telophase
5 µm

Figure S3

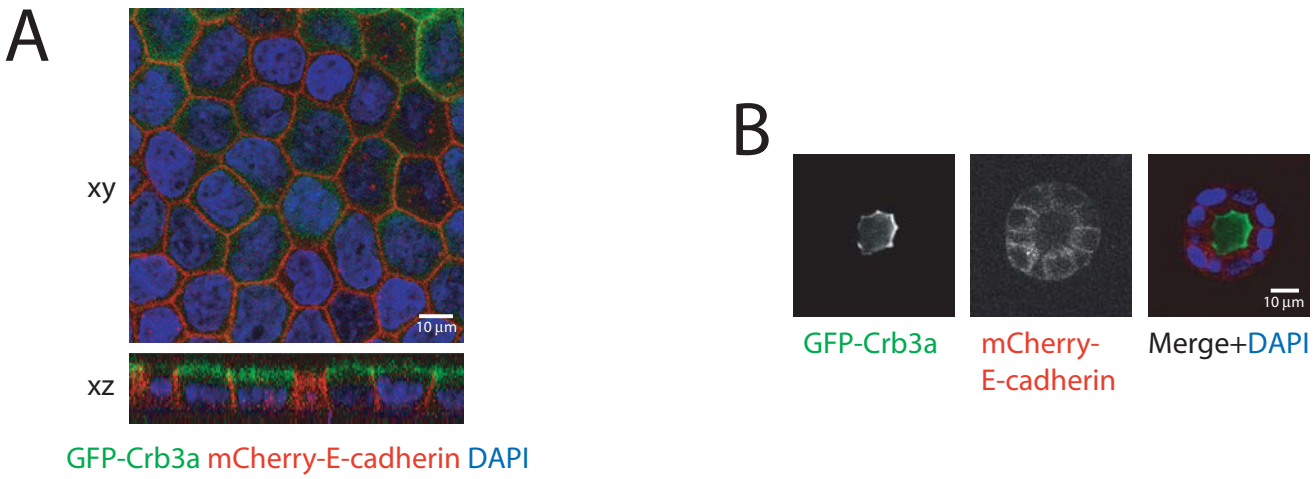

A
xy
xz
10 µm
GFP-Crb3a mCherry-E-cadherin DAPI
B
10 µm
GFP-Crb3a
mCherry-
E-cadherin
Merge+DAPI
C
Time (hours)
6:15
8:30
11:30
12:00
15:00
17:45
19:00
Brightfield
GFP-Crb3a
mCherry-
E-cadherin
Merge
Merge
+brightfield
5 µm

Figure S4

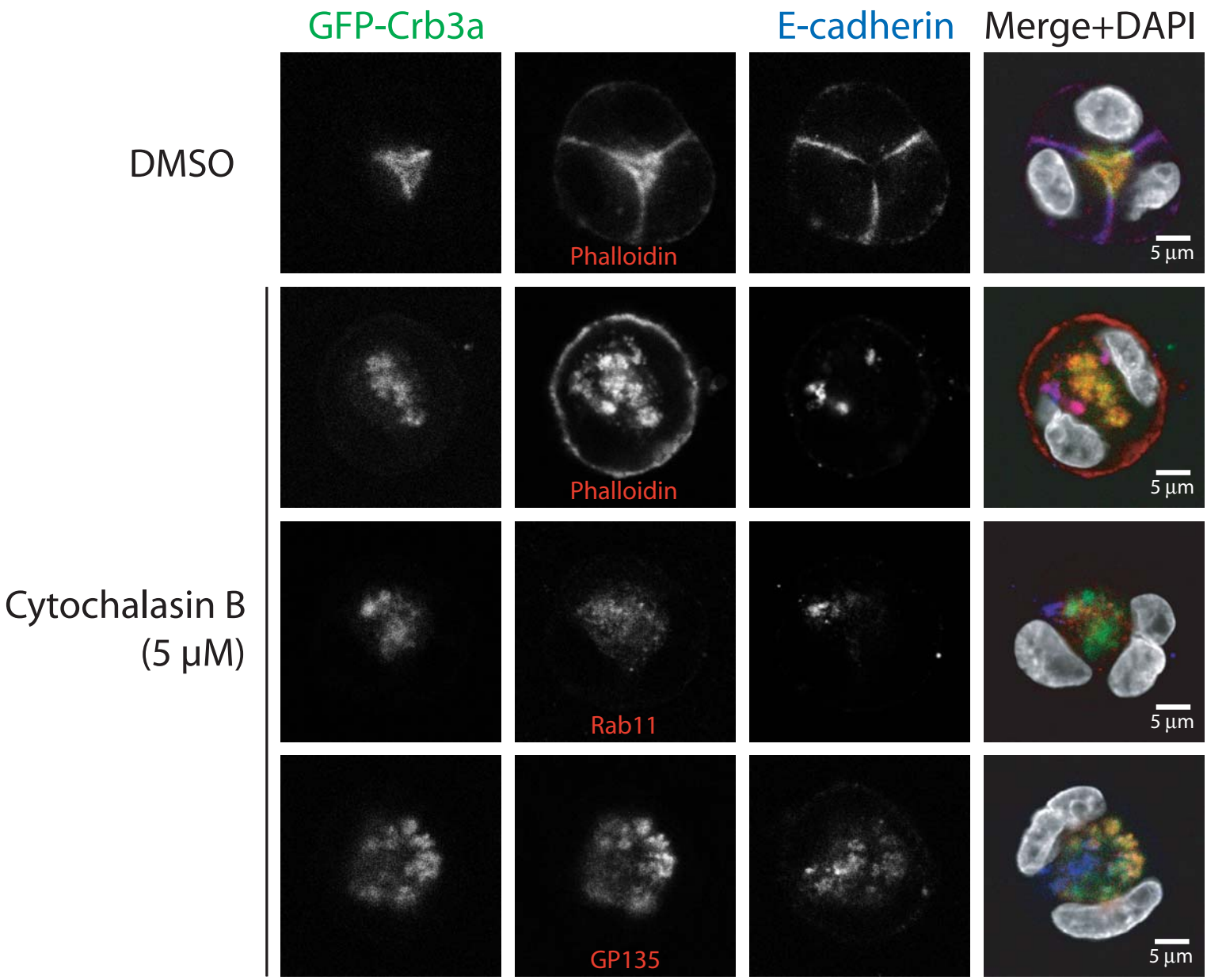
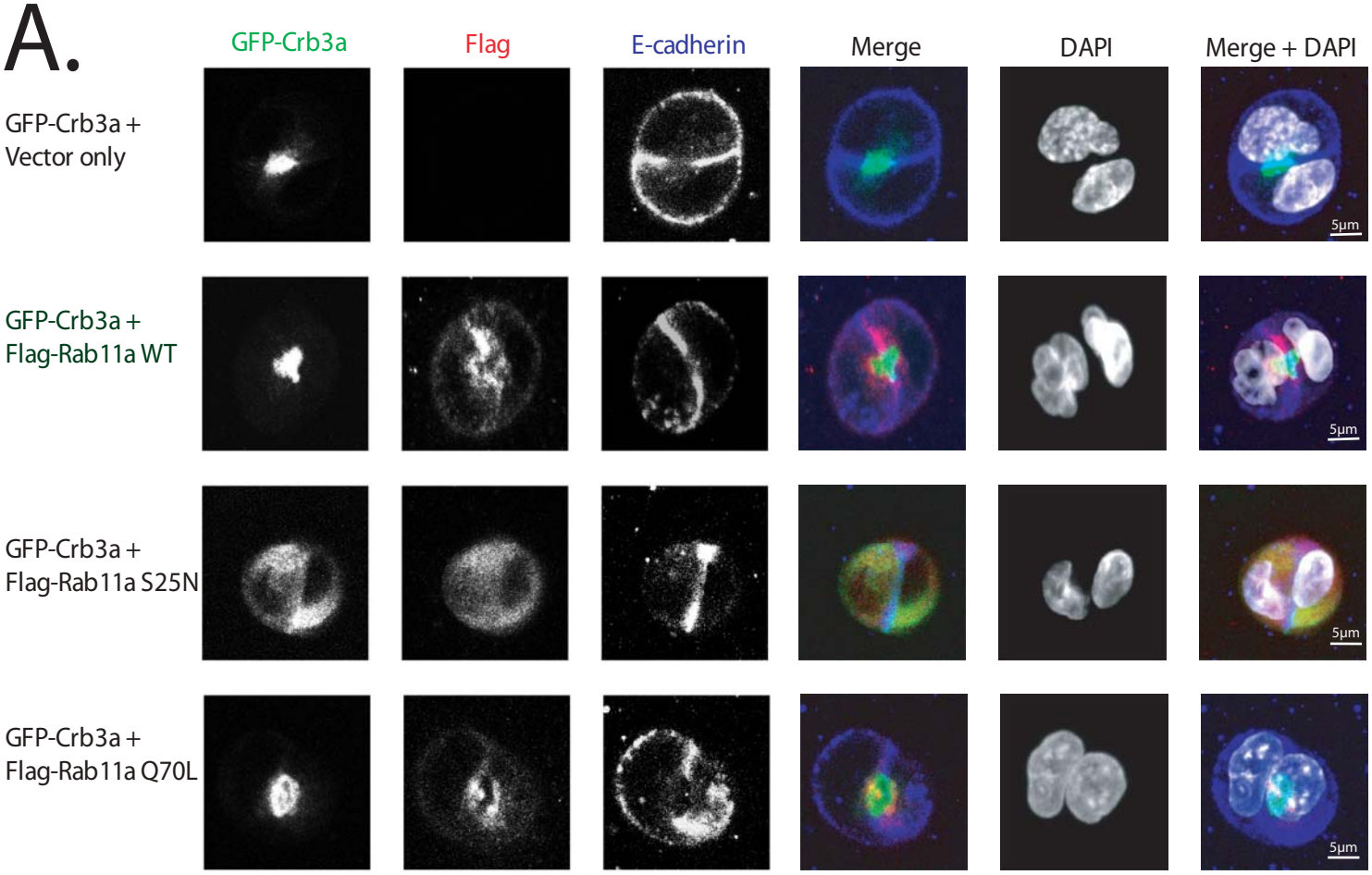

GFP-Crb3a
E-cadherin
Merge+DAPI
DMSO
Phalloidin
5 µm
Phalloidin
5 µm
Cytochalasin B
(5 µM)
Rab11
5 µm
GP135
5 µm

Figure S5

A.


GFP-Crb3a
Flag
E-cadherin
Merge
DAPI
Merge + DAPI
GFP-Crb3a + Vector only
GFP-Crb3a + Flag-Rab11a WT
GFP-Crb3a + Flag-Rab11a S25N
GFP-Crb3a + Flag-Rab11a Q70L
5μm

B.

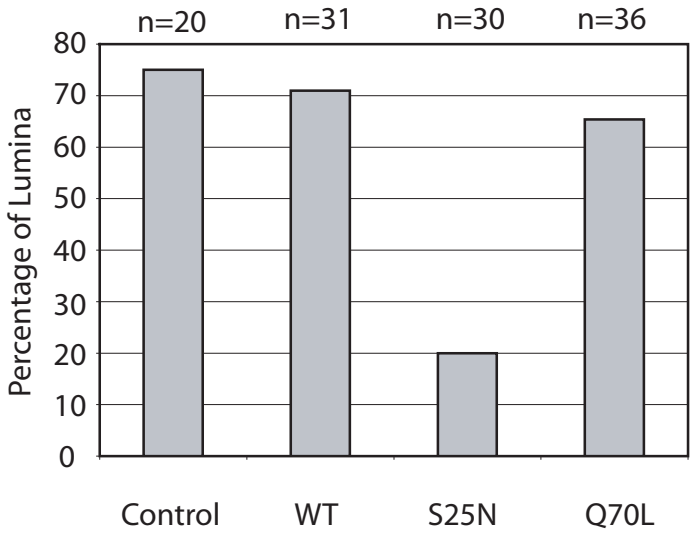

n=20
n=31
n=30
n=36
Percentage of Lumina
80
70
60
50
40
30
20
10
0
Control
WT
S25N
Q70L

Figure S6

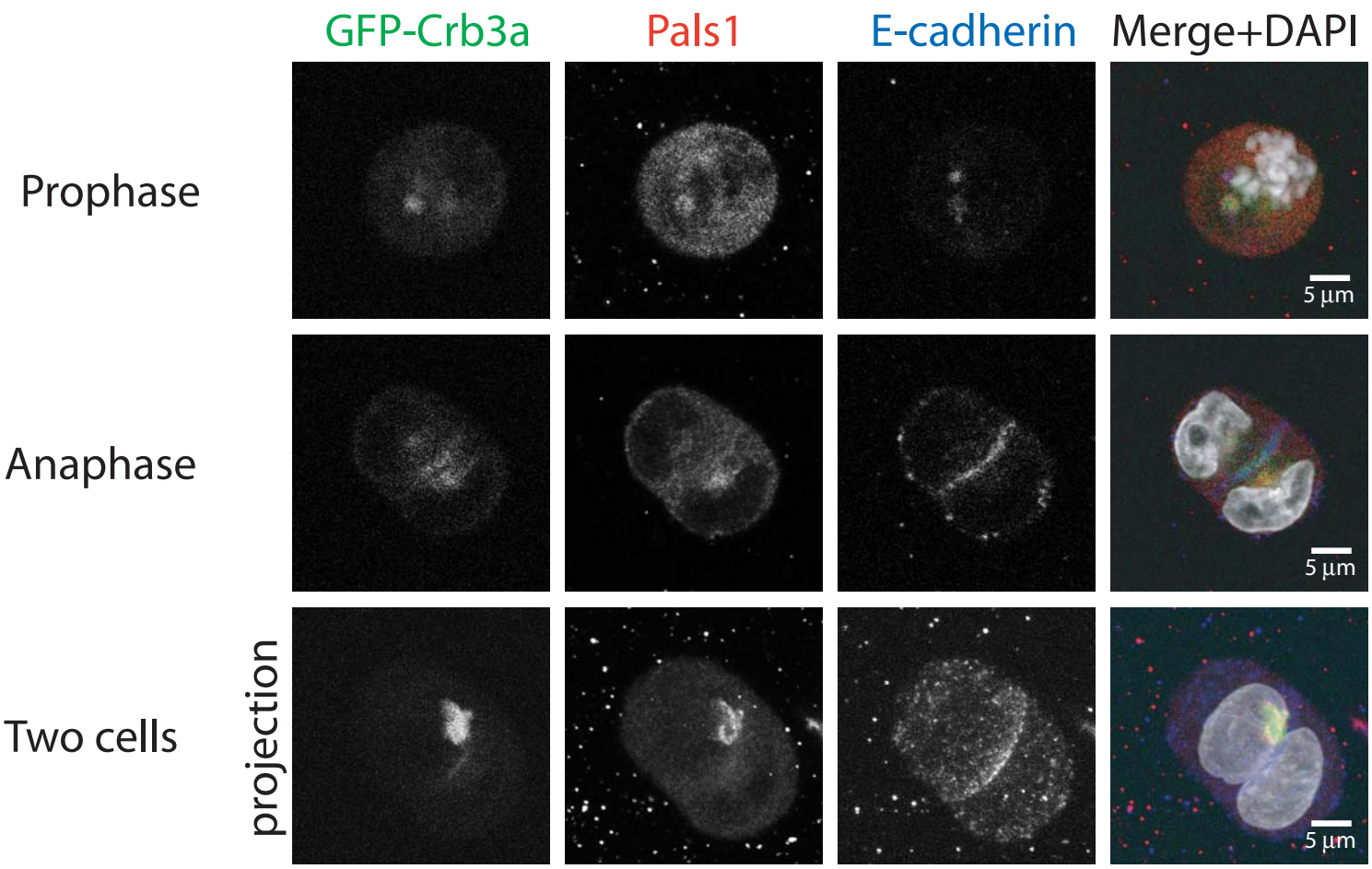
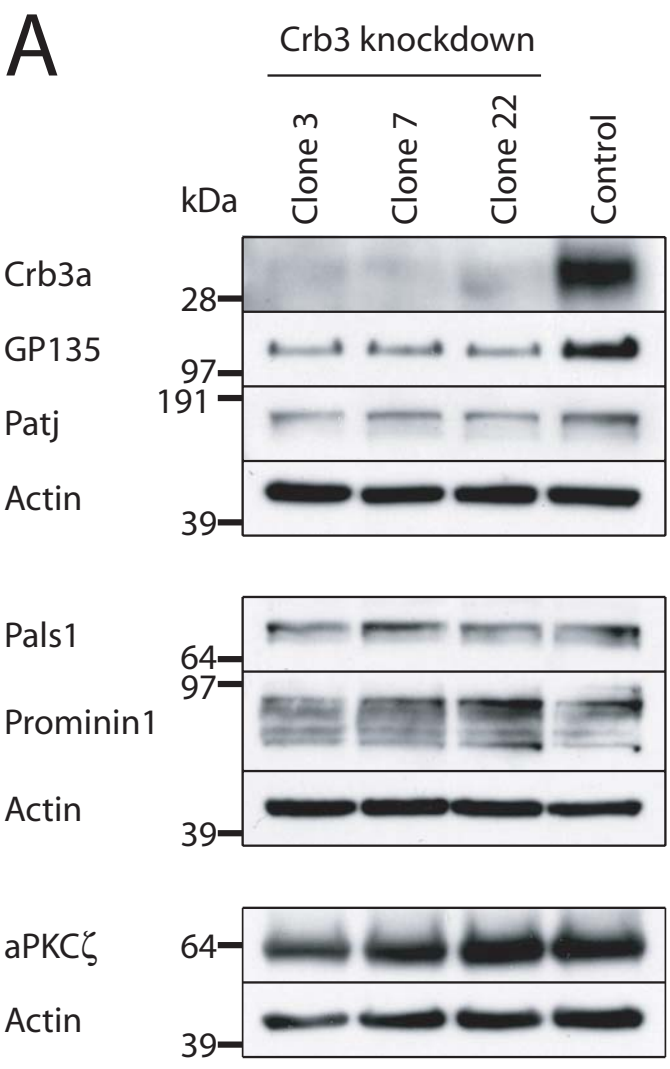
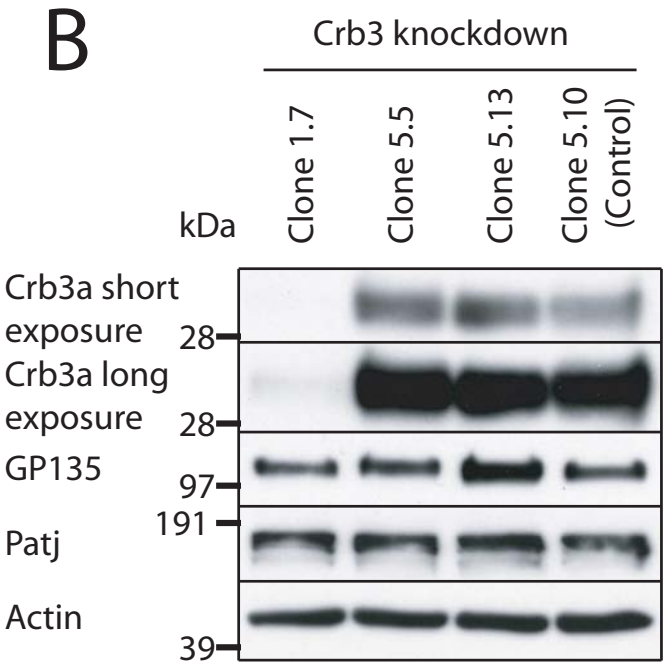

GFP-Crb3a
Pals1
E-cadherin
Merge+DAPI
Prophase
Anaphase
Two cells
projection
5 µm
5 µm
5 µm

Figure S7


A
Crb3 knockdown
Clone 3
Clone 7
Clone 22
Control
kDa
Crb3a
GP135
Patj
Actin
28
97
191
39
Pals1
Prominin1
Actin
64
97
39
aPKCζ
Actin
64
39


B
Crb3 knockdown
Clone 1.7
Clone 5.5
Clone 5.13
Clone 5.10 (Control)
kDa
Crb3a short exposure
Crb3a long exposure
GP135
Patj
Actin
28
28
97
191
39

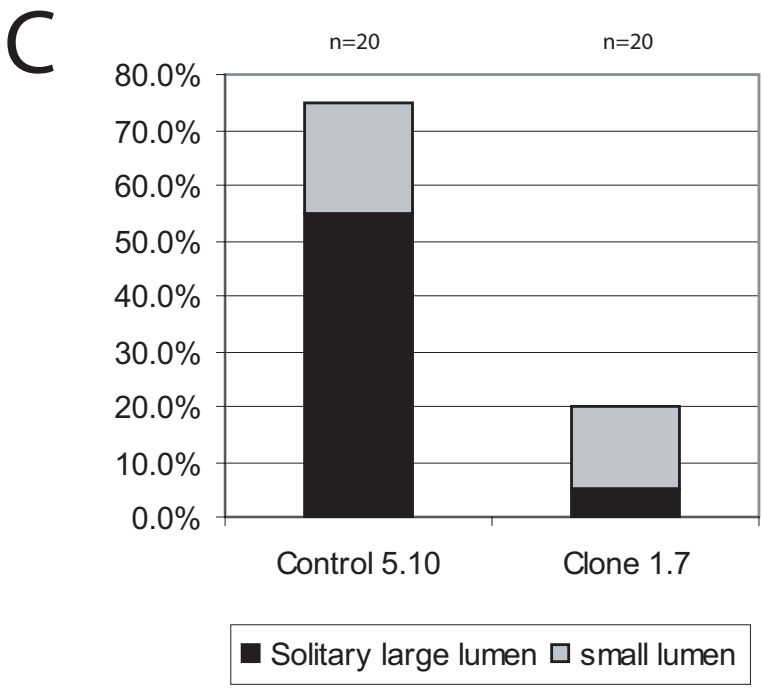
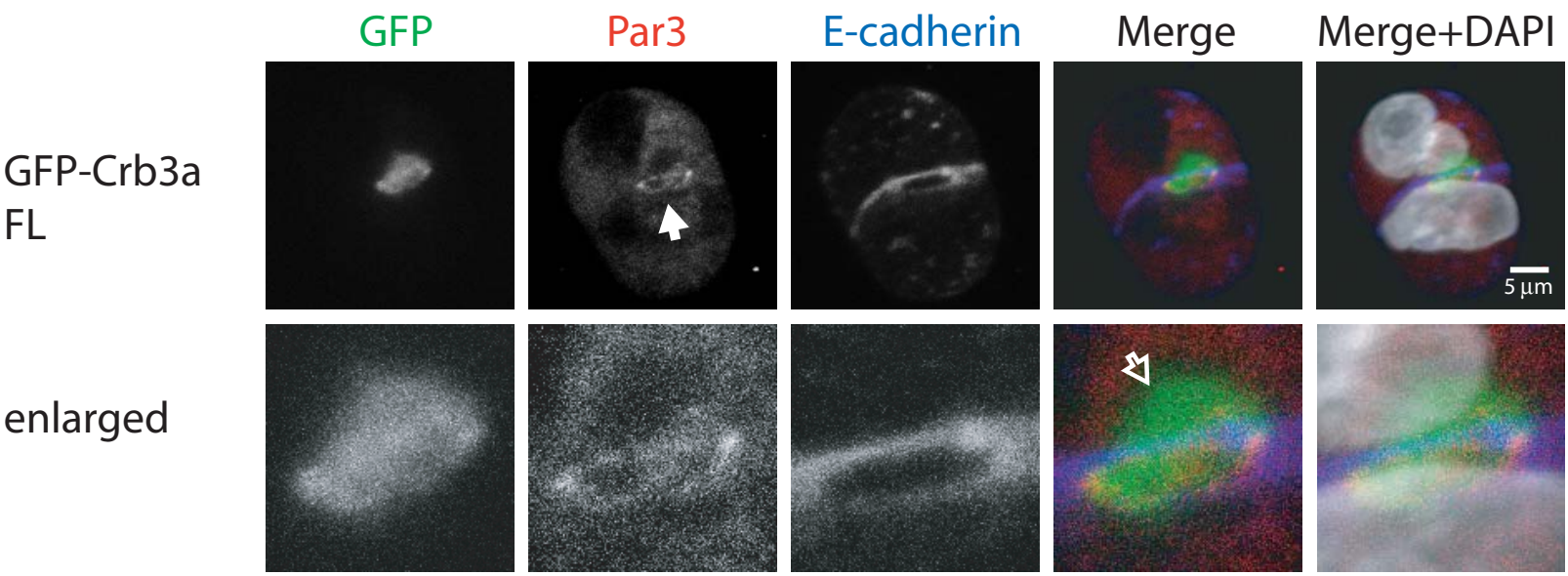

C
n=20
n=20
80.0%
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
Control 5.10
Clone 1.7
Solitary large lumen
small lumen

Figure S8


GFP
Par3
E-cadherin
Merge
Merge+DAPI
GFP-Crb3a FL
enlarged
5 µm