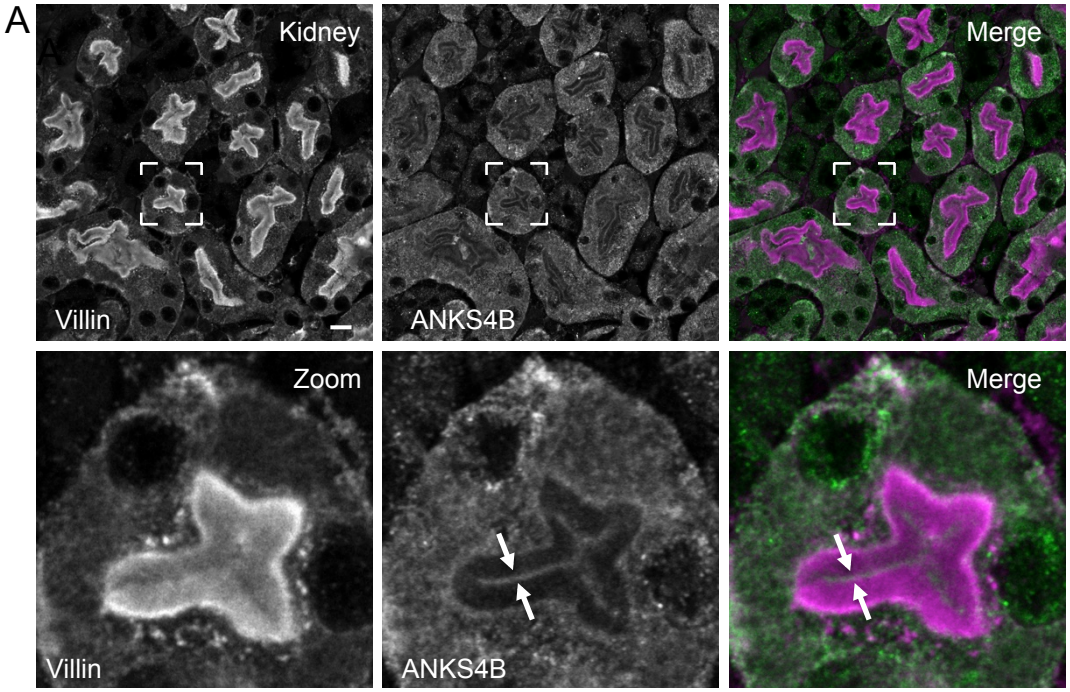
SUPPLEMENTARY FIGURE LEGENDS

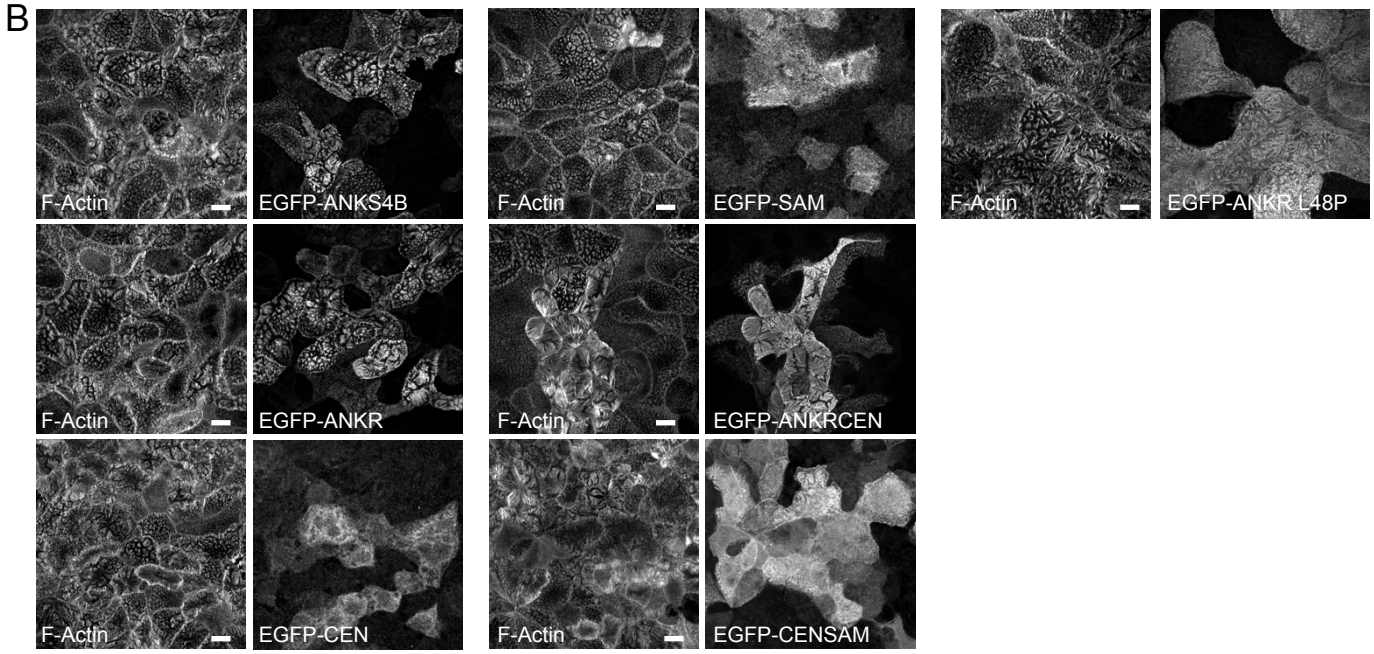
Figure S1. Endogenous ANKS4B localization in mouse kidney tissue and domain construct expression in CACO-2$_{BBE}$ cells, related to Figure 1 and Figure 2. (A) Confocal microscopy of mouse kidney tissue stained for ANKS4B (green) and villin (magenta). Boxed regions denote area in zoomed image panels. Arrows point to the distal tips of BB microvilli. Scale bar, 40 μm. (B) Single channel versions of confocal images presented in Fig. 2B-H. Scale bars, 10 μm. (C) Pull-downs of CDs of CDHR2 and CDHR5 fused to GST with the EGFP-tagged ANKR domain of ANKS4B. GST alone was used a control.

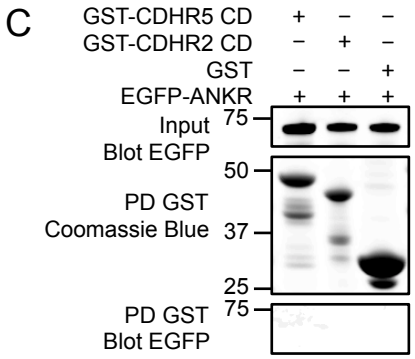
Figure S2. Knockdown and overexpression of ANKS4B in CACO-2$_{BBE}$ cells, related to Figure 3. (A) Additional SEM images of 20 DPC CACO-2$_{BBE}$ cells stably expressing either a scramble shRNA construct (*left*) or two independent shRNAs targeting ANKS4B (*middle* and *right*). Scale bars, 1 μm. (B) Confocal images of 12 DPC CACO-2$_{BBE}$ monolayers stably expressing EGFP-ANKS4B (*left*) or CDHR2-EGFP (*right*). Plus signs highlight EGFP-tagged construct expressing cells with robust microvillar clustering; minus signs indicate cells that are over-expressing the EGFP-tagged construct, but do not exhibit strong microvilli clustering.

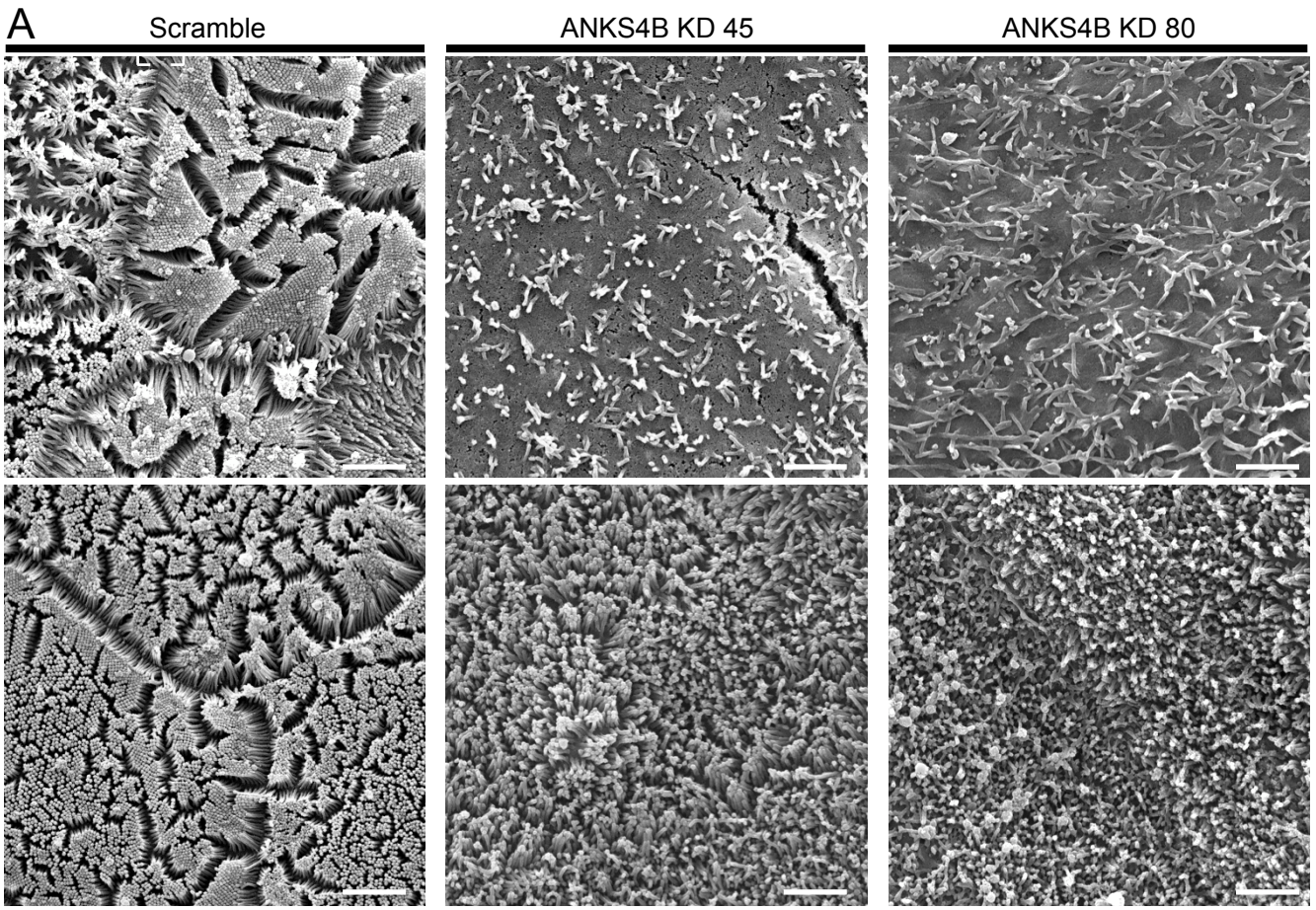
Figure S3. Characterization of the functional interactions between ANKS4B and other IMAC components, related to Figure 4 and Figure 5. (A) Single and merged channel, low magnification versions of confocal images presented in Fig. 4C. Tissue is stained for ANKS4B (green) and villin (magenta). Boxed regions denote zoomed areas presented in Fig 4C. Scale bars, 40 μm. (B) Confocal microscopy of USH1C KO mouse kidney tissue stained for ANKS4B (green) and villin (magenta). Boxed regions denote area in zoomed image panels. Arrows point to the distal tips of BB microvilli. Scale bars, 40 μm. (C) Single channel, low magnification versions of confocal images presented in Fig. 4F. Scale bars, 10 μm. (D) Pull-down analysis of ANKS4B sub-domains with the MYO7B SH3MF2 tail fragment. FLAG-tagged MYO7B SH3MF2 tail served as bait where as EGFP-tagged fragments of ANKS4B served as prey. (E) Pull-down analysis of MYO7B tail domain constructs with USH1C. FLAG-tagged MYO7B tail fragments served as bait where as myc-tagged USH1C served as prey. (F) Pull-down analysis of ANKS4B sub-domains with the full-length tail of MYO7B. FLAG-tagged MYO7B full-length tail served as bait where as EGFP-tagged fragments of ANKS4B served as prey.

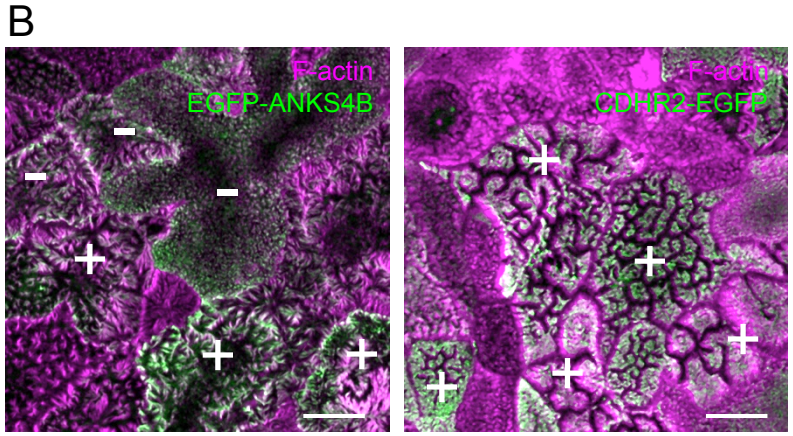
Figure S4. ANKS4B is required for proper localization of other IMAC components in CACO-2$_{BBE}$ cells, related to Figure 6. (A) Single channel versions of confocal images presented in Fig. 6A. Scale bars, 10 μm.

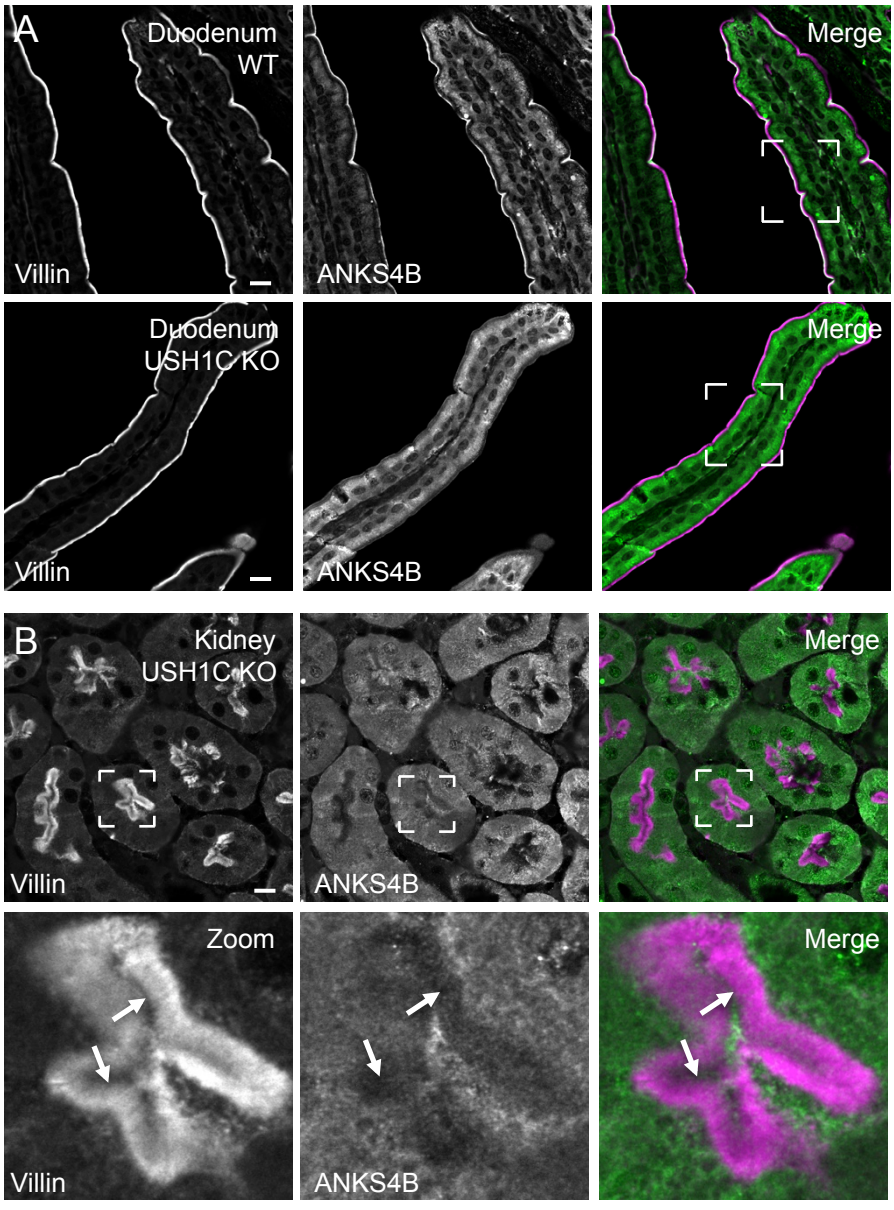

A
Kidney
Villin
ANKS4B
Merge
Zoom
Villin
ANKS4B
Merge

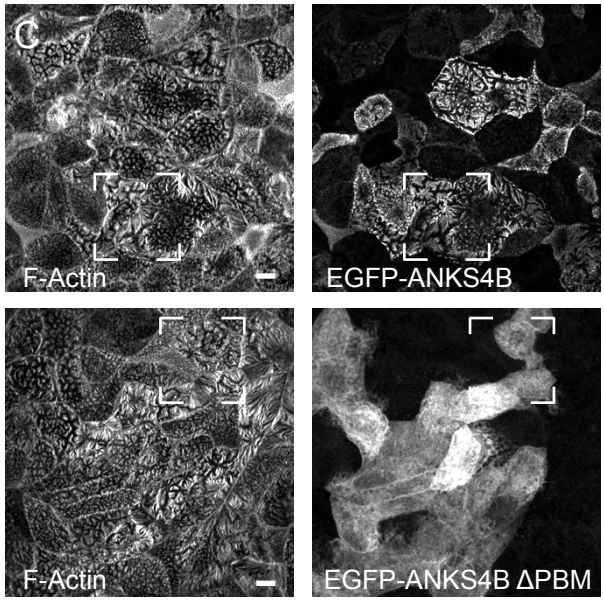

B
F-Actin
EGFP-ANKS4B
F-Actin
EGFP-SAM
F-Actin
EGFP-ANKR L48P
F-Actin
EGFP-ANKR
F-Actin
EGFP-ANKRCEN
F-Actin
EGFP-CEN
F-Actin
EGFP-CENSAM

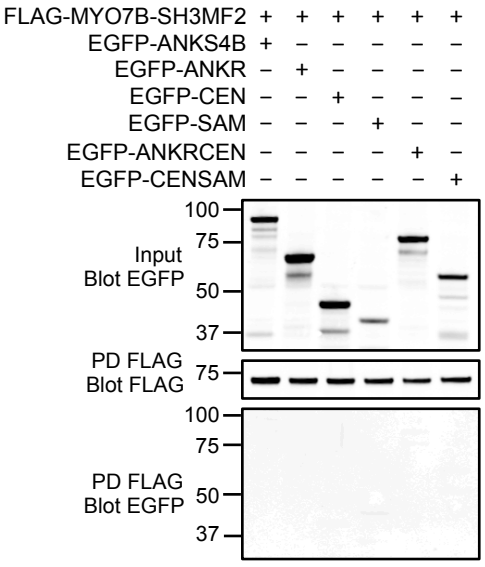

C
GST-CDHR5 CD + − −
GST-CDHR2 CD − + −
GST − − +
EGFP-ANKR + + +
Input
Blot EGFP
75
50
PD GST
Coomassie Blue
37
25
75
PD GST
Blot EGFP

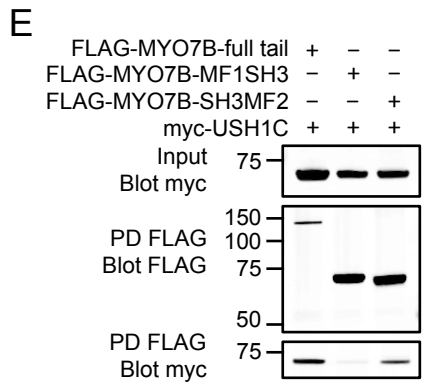

A
Scramble
ANKS4B KD 45
ANKS4B KD 80

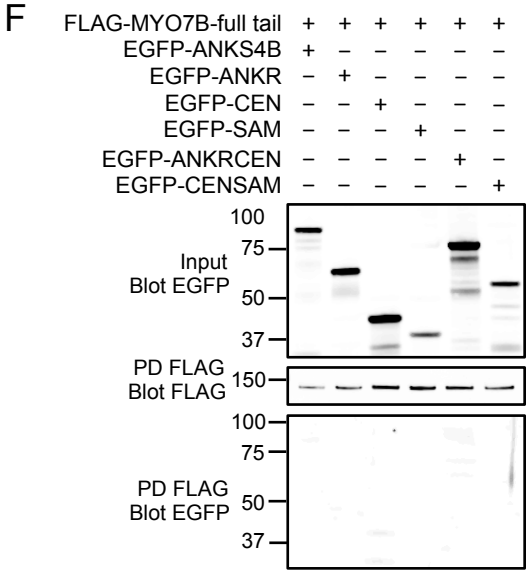

B
F-actin
EGFP-ANKS4B
F-actin
CDHR2-EGFP

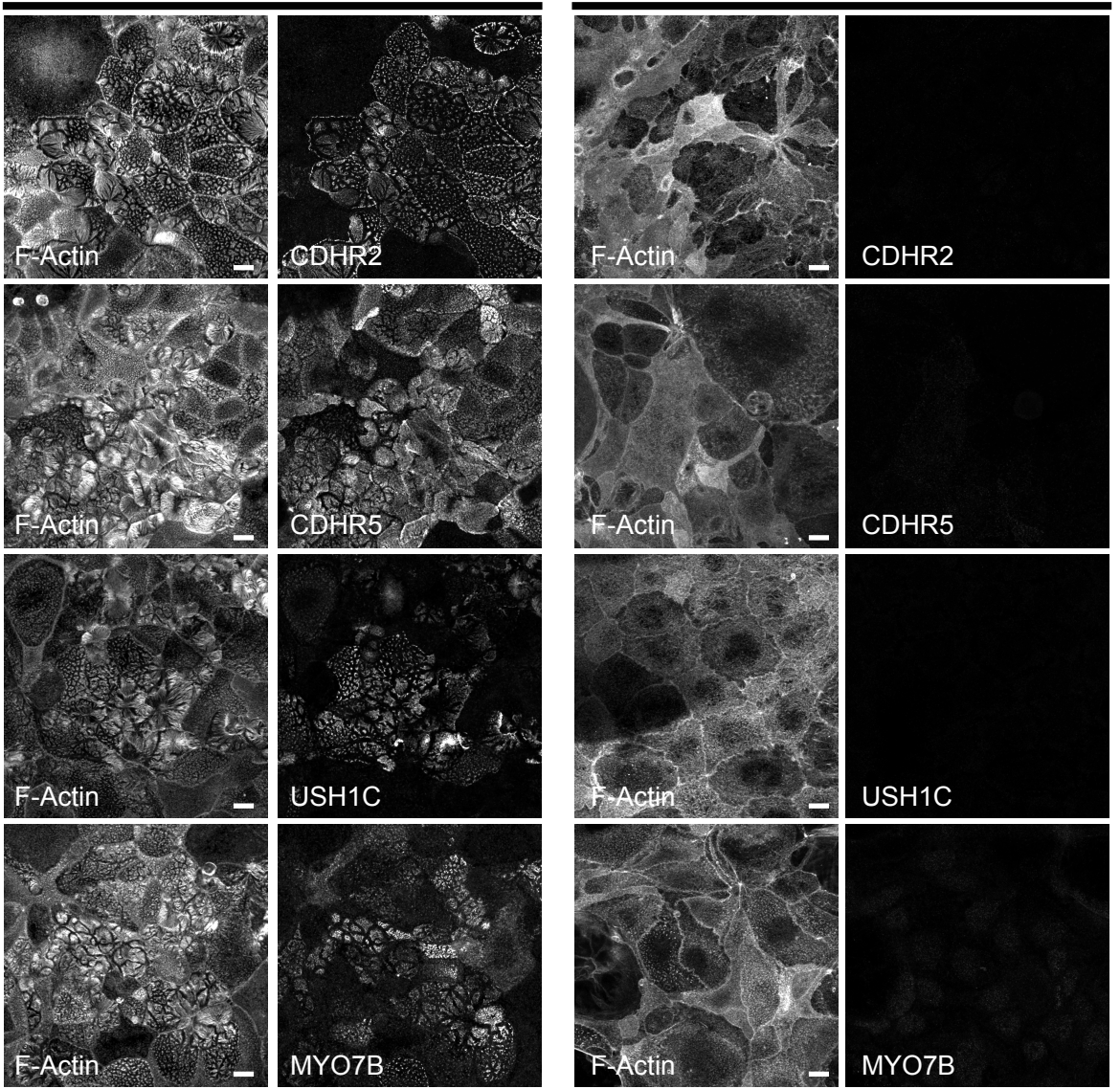

A
Duodenum
WT
Villin
ANKS4B
Merge
Duodenum
USH1C KO
Villin
ANKS4B
Merge
B
Kidney
USH1C KO
Villin
ANKS4B
Merge
Zoom
Villin
ANKS4B
Merge


C
F-Actin
EGFP-ANKS4B
F-Actin
EGFP-ANKS4B ΔPBM


D
FLAG-MYO7B-SH3MF2 + + + + + +
EGFP-ANKS4B + – – – – –
EGFP-ANKR – + – – – –
EGFP-CEN – – + – – –
EGFP-SAM – – – + – –
EGFP-ANKRCEN – – – – + –
EGFP-CENSAM – – – – – +
100
75
Input
Blot EGFP
50
37
PD FLAG
Blot FLAG
75
100
75
PD FLAG
Blot EGFP
50
37


E
FLAG-MYO7B-full tail + – –
FLAG-MYO7B-MF1SH3 – + –
FLAG-MYO7B-SH3MF2 – – +
myc-USH1C + + +
Input
Blot myc
75
PD FLAG
Blot FLAG
150
100
75
50
PD FLAG
Blot myc
75


F
FLAG-MYO7B-full tail + + + + + +
EGFP-ANKS4B + – – – – –
EGFP-ANKR – + – – – –
EGFP-CEN – – + – – –
EGFP-SAM – – – + – –
EGFP-ANKRCEN – – – – + –
EGFP-CENSAM – – – – – +
100
75
Input
Blot EGFP
50
37
PD FLAG
Blot FLAG
150
100
75
PD FLAG
Blot EGFP
50
37


Scramble
ANKS4B KD
F-Actin
CDHR2
F-Actin
CDHR2
F-Actin
CDHR5
F-Actin
CDHR5
F-Actin
USH1C
F-Actin
USH1C
F-Actin
MYO7B
F-Actin
MYO7B