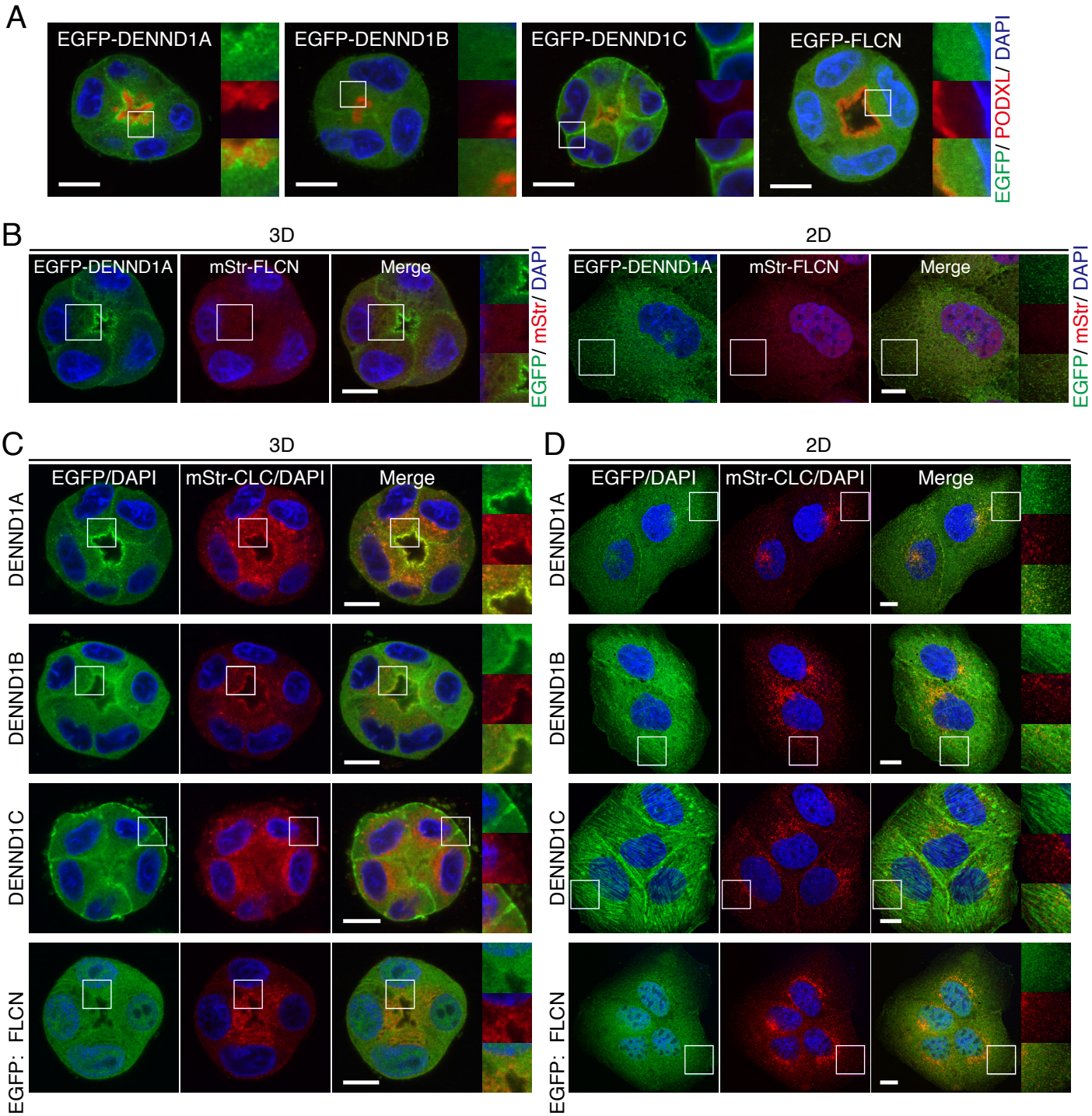
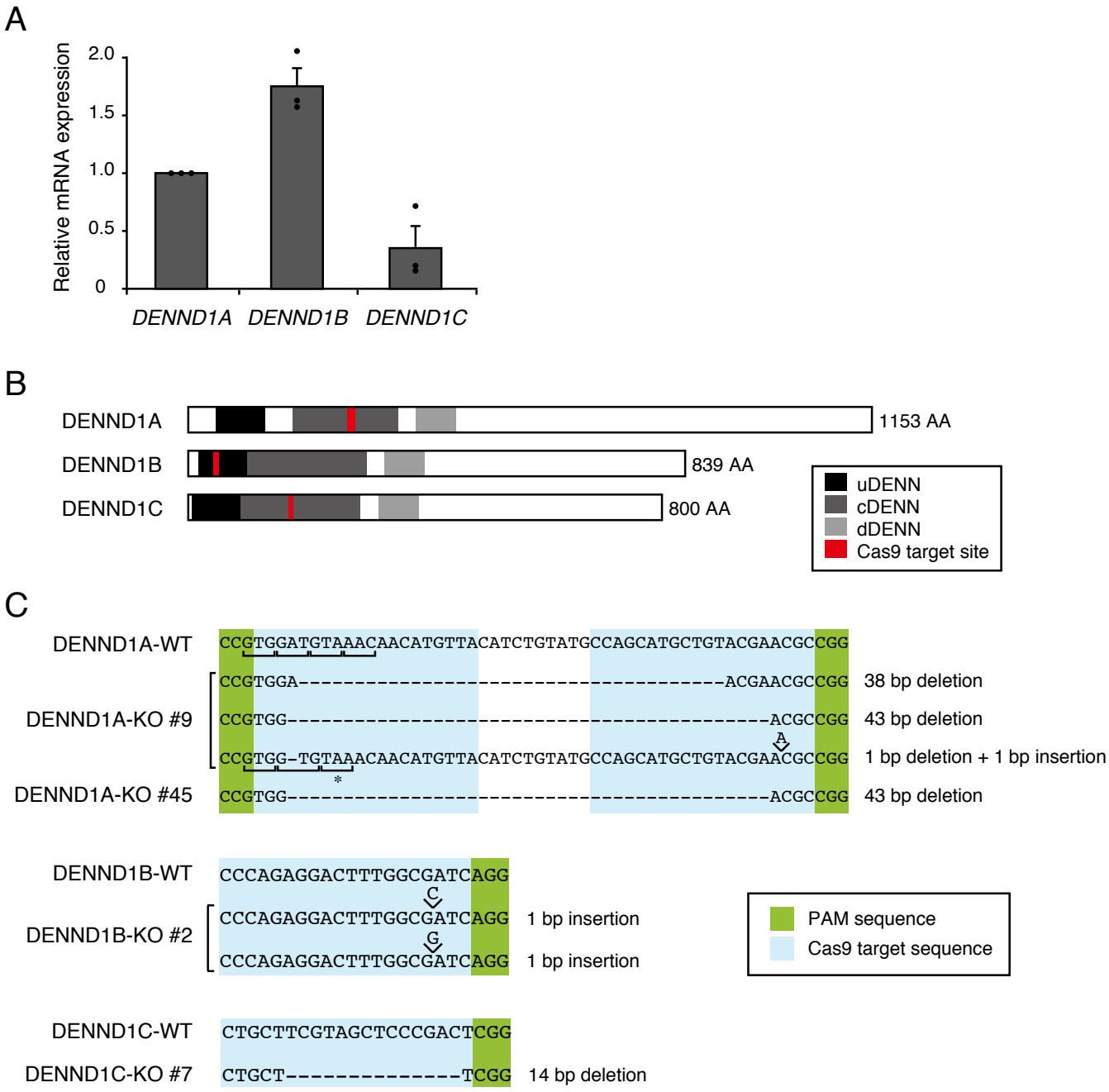
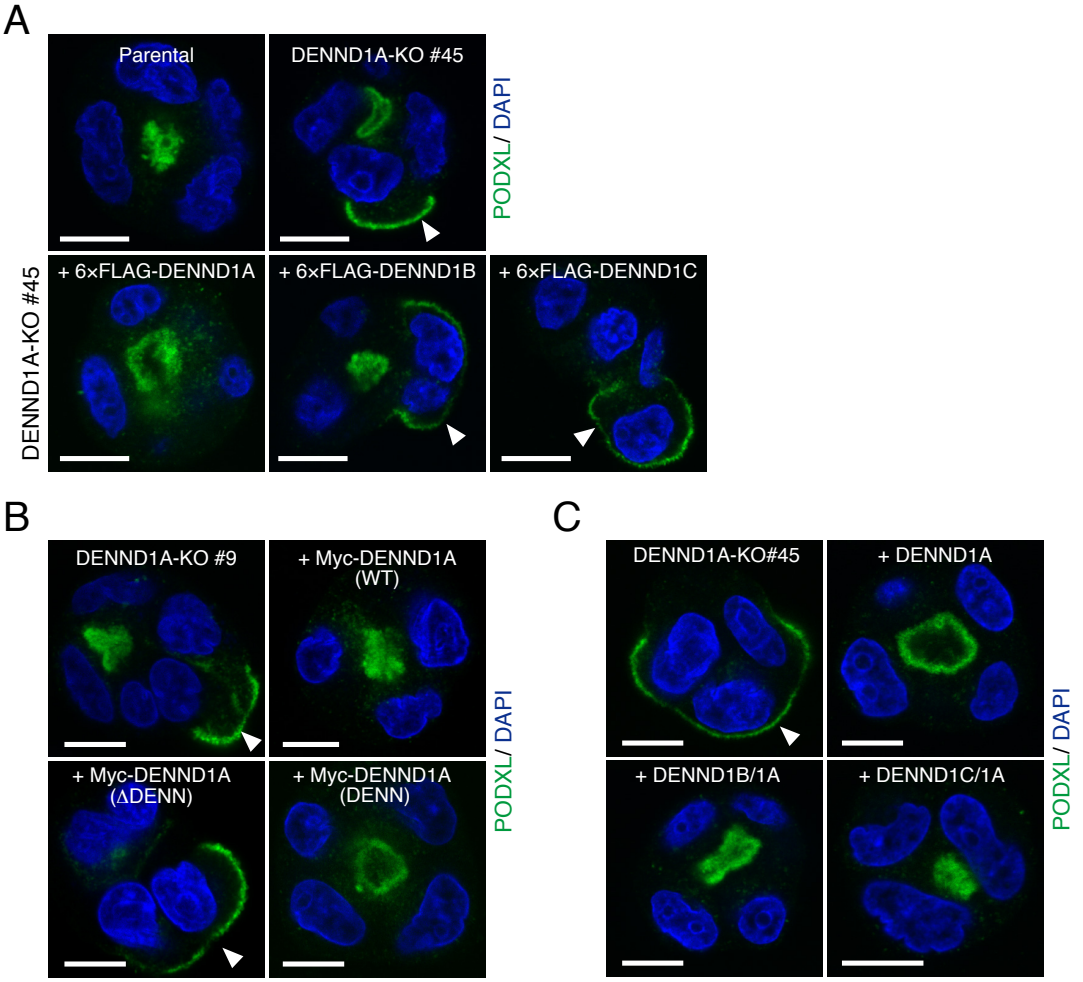


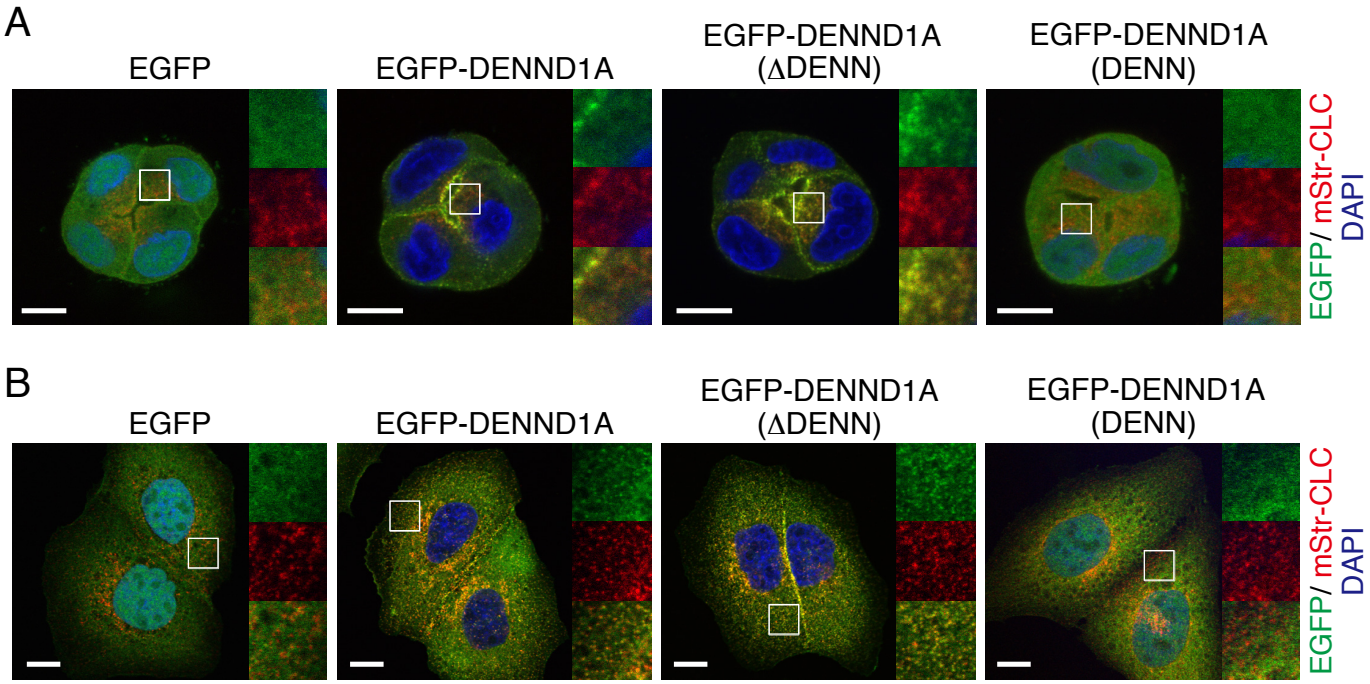
SUPPLEMENTAL FIGURE S1. Localization of DENND1 family proteins and FLCN in 2D and 3D MDCK II cell cultures. *A*, parental cells stably expressing EGFP-DENND1A, -DENND1B, -DENND1C, or -FLCN (*green*) were plated on Matrigel and fixed at 42 h after plating. The cells were stained for PODXL (*red*) and DAPI (*blue*). *B*, parental cells stably expressing EGFP-DENND1A (*green*) and mStr-FLCN (*red*) were plated on Matrigel and fixed at 42 h after plating (*3D*) or plated on a glass-bottom dish and fixed at 24 h after plating (*2D*). The cells were stained for DAPI (*blue*). *C* and *D*, parental cells stably expressing each EGFP-tagged protein (EGFP-DENND1A, EGFP-DENND1B, EGFP-DENND1C, or EGFP-FLCN: shown in *green*) and mStr-clathrin light chain (CLC) (*red*) were plated on Matrigel and fixed at 42 h after plating (*C*), or plated on glass-bottom dishes and fixed at 24 h after plating (*D*). The cells were stained for DAPI (*blue*). Scale bars, 10 μm.



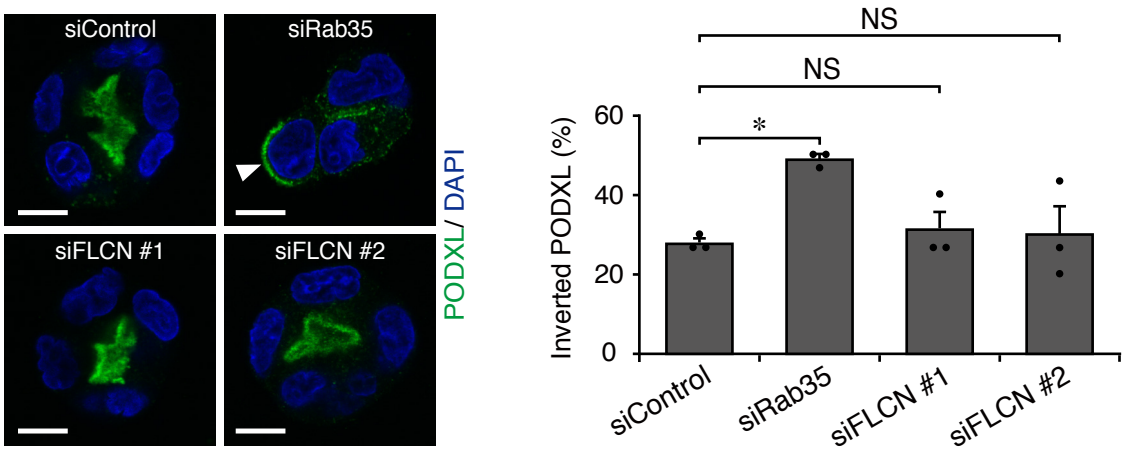
SUPPLEMENTAL FIGURE S2. Genomic information about DENND1A-KO, DENND1B-KO, and DENND1C-KO cells. *A*, relative mRNA levels of DENND1 family proteins in MDCK II cells as revealed using real time PCR analysis. The graph shows the means of each mRNA levels normalized to that of *DENND1A* and the SEM of three independent experiments. *B*, schematic representation of canine DENND1 family proteins. The Cas9 target sites (indicated by *red lines*) are located within the DENN domain. The NCBI accession numbers that were used were as flollows: DENND1A, XP_022279564.1; DENND1B, XP_022276545.1; and DENND1C, XP_013977397.1. *C*, genomic mutations in DENND1A-KO (#9 and #45), DENND1B-KO (#2), and DENND1C-KO (#7) cells. Genomic sequences around the Cas9 target sites of these cells are shown. The brackets below the sequence show the reading frames of the *DENND1A* gene. Although one out of three types of indels in the DENND1A-KO cells (#9) did not induce a frame shift in the DENND1A sequence (1 bp deletion + 1 bp insertion), a stop codon mutation (shown by the asterisk) occurred downstream of the Cas9 target site. PAM, protospacer adjacent motif.



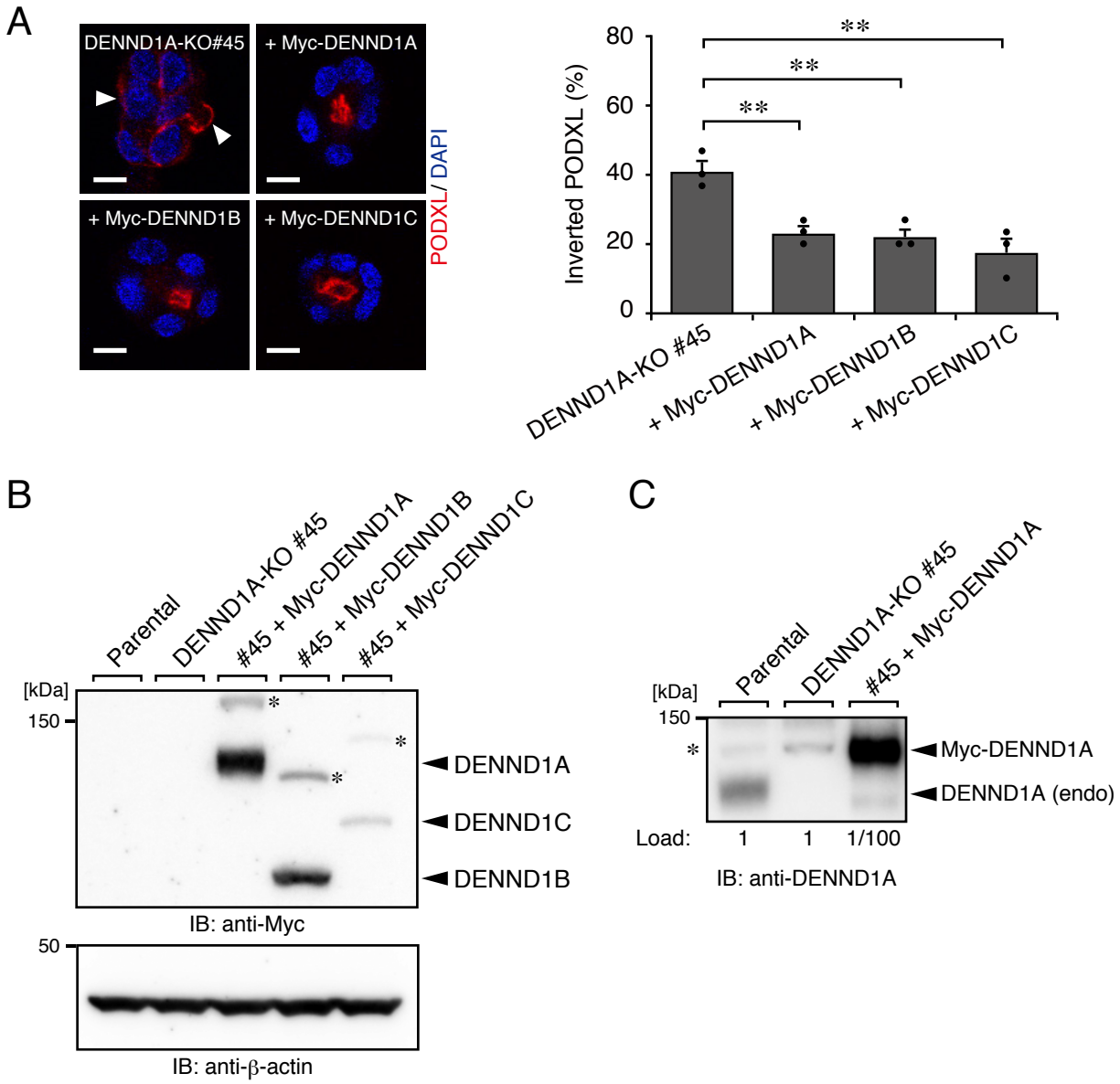
SUPPLEMENTAL FIGURE S3. Localization of PODXL in DENND1A-KO and its rescued cells. *A*, parental, DENND1A-KO (#45), and its rescued cells (+6×FLAG-tagged DENND1A, DENND1B, or DENND1C) were plated on Matrigel and fixed at 42 h after plating. The cells were stained for PODXL (*green*) and DAPI (*blue*). The arrowheads show PODXL localizing on the outer membrane (*see also* Fig. 3). *B*, DENND1A-KO (#9) and its rescued cells (+Myc-DENND1A [WT, ΔDENN or DENN]) were plated on Matrigel and fixed at 42 h after plating. The cells were stained for PODXL (*green*) and DAPI (*blue*). The arrowheads show PODXL localizing on the outer membrane (*see also* Fig. 4). *C*, DENND1A-KO (#45) and its rescued cells (+6×FLAG- tagged DENND1A, DENND1B/1A, or DENND1C/1A) were plated on Matrigel and fixed at 42 h after plating. The cells were stained for PODXL (*green*) and DAPI (*blue*). The arrowhead shows PODXL localizing on the outer membrane (*see also* Fig. 4). Scale bars, 10 μm.



SUPPLEMENTAL FIGURE S4. Localization of the DENND1A-ΔDENN and -DENN mutants in 2D and 3D MDCK II cell cultures. *A* and *B*, localization of DENND1A and its mutants (ΔDENN and DENN) in 3D cysts (*A*) and 2D cultured cells (*B*). DENND1A-KO (#9) cells stably expressing each EGFP-tagged protein (EGFP alone, EGFP-DENND1A, EGFP-DENND1A-ΔDENN, or EGFP-DENND1A-DENN: shown in *green*) and mStr-tagged clathrin light-chain (CLC) (*red*) were plated on Matrigel and fixed at 42 h after plating (*A*), or plated on glass-bottom dishes and fixed at 24 h after plating (*B*). The cells were stained with DAPI (*blue*). Scale bars, 10 μm.



SUPPLEMENTAL FIGURE S5. FLCN is not involved in PODXL trafficking in 3D cysts. Quantification of the percentage of inverted 3D cysts of FLCN-KD MDCK II cells. Parental cells that had been treated with control siRNA, siRNA against Rab35 (*siRab35*), or FLCN (#1/#2) (*siFLCN*) were plated on Matrigel and fixed at 42 h after plating, followed by counting of the inverted cysts (30 cysts per condition). Scale bars, 10 µm. The graph shows the means and SEM of three independent experiments. *, $p < 0.05$; NS, not significant (Dunnett's test).



SUPPLEMENTAL FIGURE S6. Forced overexpression of DENND1B or DENND1C in DENND1A-KO cells can rescue the DENND1A-KO phenotype. *A*, DENND1A-KO (#45), and its rescued cells (+Myc-tagged DENND1A, DENND1B, or DENND1C; overexpression) were plated on Matrigel and fixed at 42 h after plating. The cells were stained for PODXL (*red*) and DAPI (*blue*), followed by counting of the inverted PODXL (30 cysts per condition). Scale bars, 10 μm. The graph shows the means and SEM of three independent experiments. **, p <0.01 (Dunnett's test). *B*, lysates of the cells used in *A* and parental cells were analyzed by immunoblotting with anti-Myc and anti-β-actin antibodies. The additional higher bands (asterisks) presumably result from post-translational modifications or an SDS-insensitive dimer. *C*, lysates of parental, DENND1A-KO (#45), and its rescued cells (+ Myc-DENND1A) used in *B* were analyzed by immunoblotting with anti-DENND1A antibody. The same amount of the lysates of the parental and DENND1A-KO cells was loaded in *B* and *C*. The 100-fold diluted sample of the rescued cells (+ Myc-DENND1A) was loaded in *C*. As judged from the band intensity, the amount of Myc-DENND1A/B/C was estimated to be more than 30 times greater than that of endogenous (*endo*) DENND1A. The asterisk indicates a non-specific band of the primary antibody.

Table S1. A list of the oligonucleotides used in this study

Oligonucleotides	Sequence	Source
siRNAs		
Control siRNA	CGUACGCGGAAUACUUCGA	Nippon Gene
human *Rab35* siRNA	AGCGGUGGCUUCACGAAAU	Nippon Gene
canine *FLCN* siRNA #1	ACACGGCCUUCACACCAUU	Nippon Gene
canine *FLCN* siRNA #2	GCCACACUUUCUUCAUCAA	Nippon Gene
Real-time PCR primers		
DENND1A-qPCR-fw	GGTGAAGGTTTCAGTGACG	eurofins
DENND1A-qPCR-rv	CAGTCTTCATAGCCGGGT	eurofins
DENND1B-qPCR-fw	AAACGAAGATCCGGTGGT	eurofins
DENND1B-qPCR-rv	TTCAATGTCTGTCAGTACAAAGGT	eurofins
DENND1C-qPCR-fw	CCCTGAGTCCCATCTCCTAA	eurofins
DENND1C-qPCR-rv	TGGGAGGTCCGGGTAATA	eurofins
sgRNA primers		
DENND1A-sgRNA-1-fw	CACCGCCAGCATGCTGTACGAACGC	eurofins
DENND1A-sgRNA-1-rv	AAACGCGTTCGTACAGCATGCTGGC	eurofins
DENND1A-sgRNA-2-fw	CACCGTAACATGTTGTTTACATCCA	eurofins
DENND1A-sgRNA-2-rv	AAACTGGATGTAAACAACATGTTAC	eurofins
DENND1B-sgRNA-fw	CACCGCCCAGAGGACTTTGGCGATC	eurofins
DENND1B-sgRNA-rv	AAACGATCGCCAAAGTCCTCTGGGC	eurofins
DENND1C-sgRNA-fw	CACCGCTGCTTCGTAGCTCCCGACT	eurofins
DENND1C-sgRNA-rv	AAACAGTCGGGAGCTACGAAGCAGC	eurofins
Genomic PCR primers		
DENND1A-genome-fw	TTATAATAAGAACAAACAAAATAAGCCACT	eurofins
DENND1A-genome-rv	ACTGTAAAGAAACACGAATAATTAATGAAA	eurofins
DENND1B-genome-fw	TGAGTTGTGAAAAGAAATAATGAGTTTTA	eurofins
DENND1B-genome-rv	AATATACATGTAAGCAATTCAGACATCAC	eurofins
DENND1C-genome-fw	AGAATCCCCAGGCCTGACCTAAGACCCTCA	eurofins
DENND1C-genome-rv	CCTCCTCCTAGCGATGCAGCTAGGATTTGC	eurofins
Primers used for plasmid construction		
D1A-ΔDENN (for 1B/1A)-fw	AAGCTTAATTCCGGTGAAGGTTTCAG	eurofins

D1A-ΔDENN (for 1C/1A)-fw	CTCGAGCTTCTCAATTCCGGTGAAGG	eurofins
DENND1A-rv	TCACTCAAAGGTCTCCCACT	Hokkaido System Science
DENND1B-fw	AGATCTATGGACTGCAGGACCAAGGC	Hokkaido System Science
DENND1B-DENN-rv	ACCCCTTCCTGCATTAAGCTTTGCCA	eurofins
DENND1C-fw	AGATCTATGGAATCCAGAGCTGAAGG	Hokkaido System Science
DENND1C-DENN-rv	TTGTTGAGCTTCTCGAGCCGGGCTTC	eurofins

qPCR, quantitative PCR; sgRNA, single guide RNA; fw, forward; rv, reverse.