Supporting Information

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SI Materials and Methods

Antibodies. The extracellular domain of p75 was detected with a mouse monoclonal antibody purified from hybridoma CRN-ME 20.4 (Babco) in our laboratory. Tight junctions were stained with a rat monoclonal anti-zonula occludens-1 protein (ZO-1) purified from R26.4C hybridoma cultures originally provided by B. Stevenson (Alberta University, Canada). Rabbit anti-μ1A antibody was provided by Alfonso González (Universidad Catolica de Chile, Santiago, Chile) and GAPDH was probed with a chicken polyclonal antibody (Sigma; GW22763). Alexa-conjugated secondary antibodies were purchased from Invitrogen and IRDye-conjugated antibodies were purchased from Li-Cor.

Plasmids and Mutagenesis. The plasmid encoding p75 without its cytoplasmic tail was generated by PCR with oligos forward (agtcAGATCTgccaccATGGGGGCAGGTGCCAC 3′) and reverse (AGCTGCGGCCGCggcgcgccgtcgactcaGATAGTCTGgaattcctGGATCCCGTAGGCCACAAGGC) using Pfu turbo polymerase (Agilent). SalI and EcoRI restriction sites were introduced in the reverse primer after p75's transmembrane domain to allow cloning of WT or mutant coxsackie and adenovirus receptor (CAR) tails (p75-CAR tails). Full-length and mutant CAR tails were prepared by PCR from the plasmid pCB7-CAR-GFP, previously published by our laboratory (1). Mutagenesis in both, CAR-GFP or p75 GFP, was performed using Quickchange IIA following the manufacturer's recommendations (Stratagene). For yeast two-hybrid (Y2H) experiments, CAR's cytoplasmic tail was cloned into pGBKT7 (Clontech) using SalI and EcoRI restriction sites.

Immunofluorescence Microscopy. The p75-CAR tail plasmids (10 μg/mL) were electroporated into fully polarized Madin-Darby canine kidney (MDCK) monolayers according to a previously published protocol (2). Cells were fixed for 15 min at room temperature in 4% freshly prepared paraformaldehyde in PBS and quenched with 50 mM NH_4Cl in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM). Cells were decorated with a monoclonal antibody to the ectodomain of p75, permeabilized with 0.075% saponin, and stained with an antibody to ZO-1. Nuclei of cells stained for p75 were stained with DAPI. Images were collected with a laser scanning confocal microscope (Leica TCS SP2). For the different MDCK cell lines expressing CAR-GFP constructs, cell were fixed with 4% (vol/vol) PFA and permeabilized with 0.075% saponin to stain for ZO-1. Images were collected with a Zeiss Axio Observer inverted microscope equipped with a Yokogawa Confocal Scanner Unit CSU-X1 and a Photometics Cool Snap XQ^2 Camera using a Zeiss Planapochromat 63×/1.46–0.60 oil-immersion objective.

Yeast Two-Hybrid Assays. Y2H assays were performed as reported previously (3). Briefly, the yeast strain AH109 was cotransformed with pGBKT7-GAL4-BD-cytoplasmic-tail of CAR and pACTII-AD-μ1B137-423, pACTII-AD-μ1A137-423, pACII-AD-μ3136-419, or pACII-AD-μ4139-453. Colonies expressing both constructs were selected by their ability to grow in DOB-2 medium (lacking tryptophan and leucine). After selection for 3 d in these plates, colonies were resuspended in water and the cell concentration was measured by OD_{600}. Equal amount of cells and volumes were dotted on dishes containing DOB-2 or DOB-3 medium (lacking tryptophan, leucine, and histidine), or diluted in media DOB-3 for experiments with CAR containing point mutations in its basolateral sorting signal. DOB-2 plates represent the viability controls, and DOB-3 plates determined the positive interaction pairs after incubation at 30 °C for 3 d. Plates were scanned using an Epson scanner. To determine the strength in the interaction of CAR with μ1A and μ1B, 3-AT (3-Amino-1,2,4-triazole; A8056-25G, Sigma) was added to DOB-3 media during plate preparation. For kinetics, OD_{600} was measured every 24 h in DOB-3 medium up to 7 d. Quantitative analysis of the interaction of μ1A and μ1B with CAR WT and mutants was performed in triplicate at high dilutions (OD_{600} ~0.001).

Steady-State Distribution of Membrane Proteins by Domain-Selective Biotinylation. Polarized MDCK monolayers, expressing pCB7-CAR-GFP (WT and mutants) confluent for 3.5 d were rinsed twice with ice-cold PBS/CM, followed by two successive 20-min incubations at 4 °C with 0.5 $mg{\cdot}mL^{-1}$ sulfo-NHS-LC-Biotin in PBS/CM, added to the apical (0.6 mL) or to the basolateral (0.3 mL) sides. Cells were rinsed twice with PBS/CM and incubated at 4 °C for 20 min with NH_4Cl in PBS/CM. Cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM KCl, 2 mM EDTA, 1% Na-deoxycholate, 0.1% SDS, 1% triton X-100, supplemented with 1 mM PMSF and 15 $\mu g{\cdot}mL^{-1}$ Leupeptin/Pepstatin/Antipain and 37 $\mu g{\cdot}mL^{-1}$ benzamidine-HCl for 30 min at 4 °C. Biotinylated proteins were retrieved from lysates cleared by centrifugation (18,000 × *g* for 10 min) with streptavidin-Sepharose and subjected to Western blot analysis.

SDS/PAGE and Western Blotting. Cell extracts were supplemented with half a volume of 3× Laemmli sample buffer, boiled for 10 min and subjected to SDS/PAGE followed by electrotransfer to nitrocellulose membrane (Invitrogene). Membranes were blocked with blocking buffer (Odyssey) for 45 min at room temperature and subsequently rocked overnight at 4 °C with optimized dilutions of indicated antibodies made in blocking buffer. Blots were washed with PBS followed by 45-min incubation at room temperature with fluorescence secondary antibody (Odyssey). Bands were scanned and analyzed using Odyssey scanner and software.

1. Diaz F, et al. (2009) Clathrin adaptor AP1B controls adenovirus infectivity of epithelial cells. *Proc Natl Acad Sci USA* 106:11143–11148.
2. Deora AA, Diaz F, Schreiner R, Rodriguez-Boulan E (2007) Efficient electroporation of DNA and protein into confluent and differentiated epithelial cells in culture. *Traffic* 8: 1304–1312.
3. Ohno H, et al. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* 269:1872–1875.

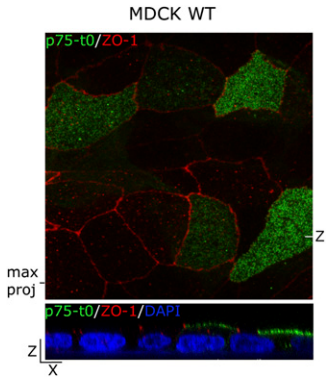

Fig. S1. Polarized distribution of tailless p75 in confluent MDCK monolayers. A cDNA encoding p75 lacking the entire cytoplasmic tail was electroporated into confluent MDCK monolayers and the expression of the protein studied by immunofluorescence under the confocal microscope. (Magnification: 63×.)

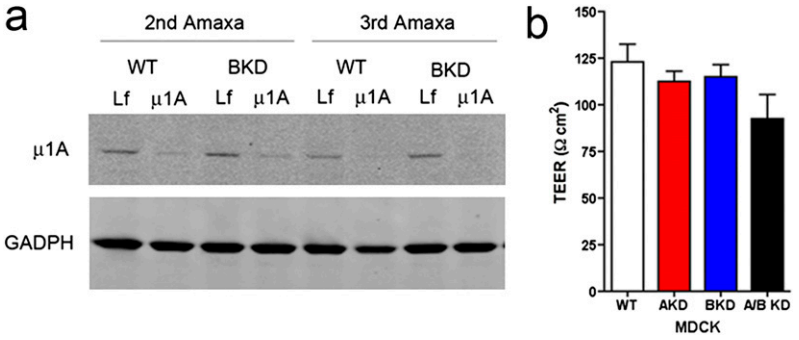

Fig. S2. AP-1A knock-down (KD) efficiency in WT and B-KD cells. (*A*) Western blot detection of µ1A protein levels in WT MDCK and B-KD cells after two or three cycles of Amaxa electroporation with a specific siRNA against µ1A or against Luciferase. GADPH was used as a loading control. (*B*) Transepithelial electrical resistance was measured in all four genotypes after 3.5 d plated on filters.

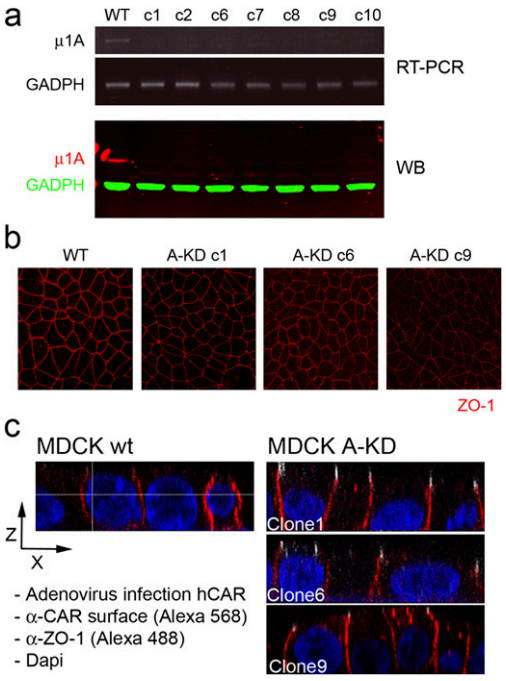

Fig. S3. Characterization of stable MDCK cell lines lacking µ1A. MDCK cells were infected with lentivirus expressing a specific shRNA against µ1A. Single clones were selected with puromycin and tested for their knock-down efficiency. (*A*) RT-PCR and Western blot showed decreased expression of µ1A compared with WT MDCK cells in various clones. (*B*) Three clones were labeled with ZO-1 (red) to show their ability to form tight junctions. (*C*) The distribution of CAR (red) in the basolateral membrane of polarized WT MDCK and A-KD clones 1, 6, and 9. DAPI was used to label the nuclei and ZO-1 to label the tight junctions. (Magnifications: *B*, 40×; *C*, 63×.)

Table S1. Statistical analysis of the steady-state of Na/K ATPase, and CAR WT and mutants using ANOVA followed by Bonferroni test for multiple comparisons

	WT	V321A	V321E	Y318A
CAR				
Mean	96.5	91.00	69.90	48.35
SEM	1.466	2.386	0.4359	6.576
Na/K ATPase				
Mean	97.53	95.37	97.37	98.54
SEM	1.268	0.8090	0.8570	0.2857
CAR	ANOVA	$P < 0.001$		
Bonferroni's test				
WT		n.s.	$P < 0.05$	$P < 0.001$
V321A			n.s.	$P < 0.001$
V321E				n.s.
Na/K ATPase	ANOVA	$P = 0.07$		

n.s., not significant.

Table S2. Statistical analysis of CAR WT and mutant biosynthetic delivery after 2 h of chase using ANOVA followed by Bonferroni test for multiple comparisons

	WT	Y318A	V321E
CAR AP			
Mean	13.34	14.39	13.68
SEM	2.130	3.325	3.331
CAR BL			
Mean	56.56	13.71	17.45
SEM	11.48	3.649	3.364
CAR AP	ANOVA	$P = 0.9632$	
CAR BL	ANOVA	$P = 0.0126$	
Bonferroni's test			
WT		$P < 0.05$	$P < 0.05$
Y318A			n.s.

n.s., not significant.

Table S3. Statistical analysis of CAR and Na/K ATPase steady-state in WT, A-KD, B-KD, and AB-KD MDCK cells using ANOVA followed by Bonferroni test for multiple comparisons

	WT	A-KD	B-KD	AB-KD
CAR				
Mean	95.92	98.37	68.93	57.15
SEM	1.581	1.484	0.6489	0.5500
Na/K ATPase				
Mean	99.42	99.35	97.60	78.45
SEM	0.1272	0.05000	0.7371	0.3500
CAR	ANOVA	$P < 0.0001$		
Bonferroni's test				
WT		n.s.	$P < 0.001$	$P < 0.001$
A-KD			$P < 0.001$	$P < 0.001$
B-KD				$P < 0.01$
Na/K ATPase	ANOVA	$P < 0.0001$		
Bonferroni's test				
WT		n.s.	n.s.	$P < 0.001$
A-KD			n.s.	$P < 0.001$
B-KD				$P < 0.001$

n.s., not significant.

Table S4. Statistical analysis of CAR biosynthetic delivery after 2 h of chase in WT, A-KD, B-KD, and AB-KD MDCK cells using ANOVA followed by Bonferroni test for multiple comparisons

	WT	A-KD	B-KD	AB-KD
CAR AP				
Mean	13.34	16.89	11.63	31.60
SEM	2.310	3.174	2.112	3.470
CAR BL				
Mean	50.47	52.96	38.16	41.61
SEM	11.65	2.859	2.159	4.150
CAR AP	ANOVA	$P = 0.0003$		
Bonferroni's test				
WT		n.s.	n.s.	$P < 0.001$
A-KD			n.s.	$P < 0.05$
B-KD				$P < 0.001$
CAR BL	ANOVA	$P = 0.5596$		

n.s., not significant.