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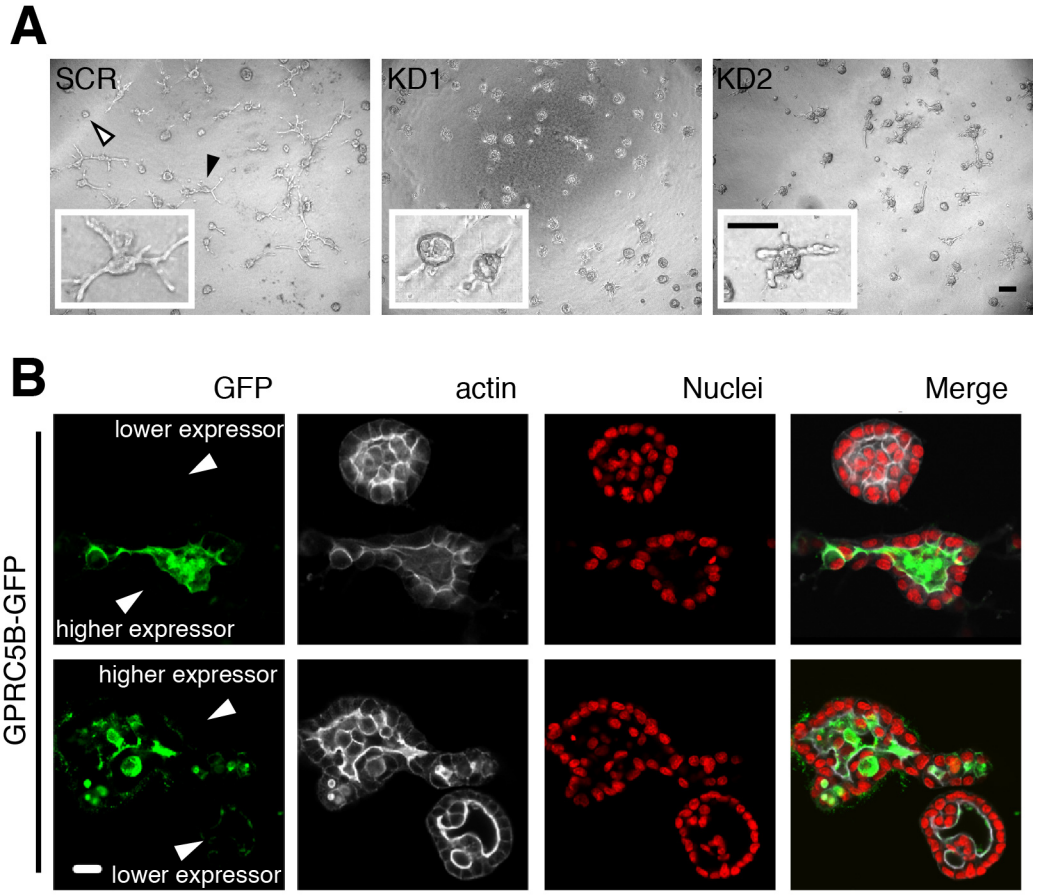
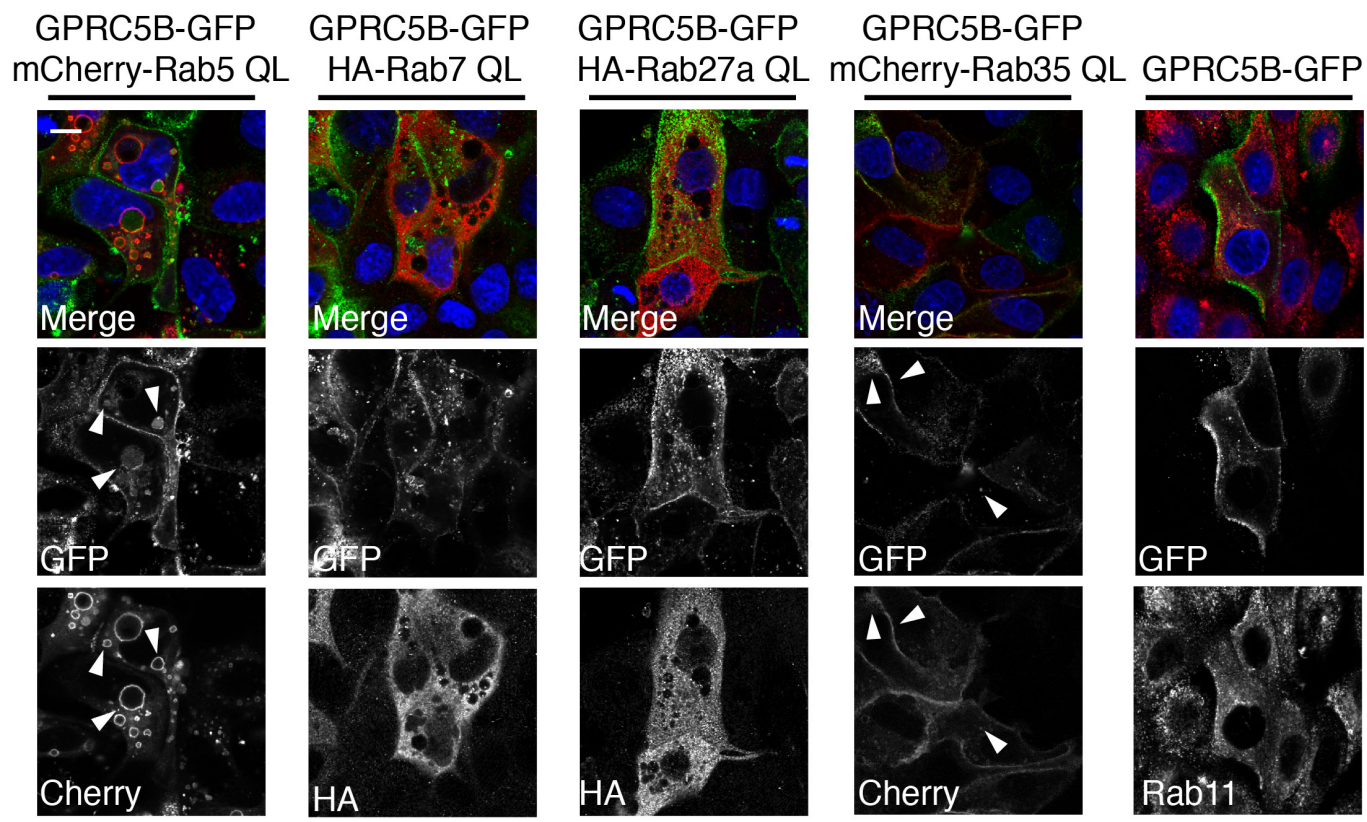

A
SCR
KD1
KD2
B
GFP
actin
Nuclei
Merge
GPRC5B-GFP
lower expressor
higher expressor
higher expressor
lower expressor
C
***
% Outward growth normalized to WT
100
80
1
0.5
0
GPRC5B-GFP
GFP
CD82

Figure S1: HGF-induced GPRC5B positively contributes to outward growth in ECM microenvironments.

(A) Representative phase contrast images of in vitro tubule formation assay in collagen I with control scrambled shRNA (SCR) and GPRC5B knockdown cells. Filled arrowhead indicates tubule growth from a cyst, whereas open arrowhead indicates a cyst with no tubule growth. Scale bar, 100 μm.

(B) Representative confocal images showing HGF-induced outward growth by GPRC5B-GFP in Matrigel. MDCK cells stably expressing GPRC5B-GFP with varying degree of expression overlaid on thin Matrigel coating were grown in culture media with 2% Matrigel for 4 days. Cysts were then stimulated with 12.5 ng/ml HGF in culture media with 2% Matrigel for 48 hrs. The figure contains two rows of panels. Each panel shows a field containing two cysts. One cyst in each field expresses a low level of GPRC5B-GFP, while the other cyst in each field expresses a high level of GPRC5B-GFP. Note that the cyst with lower expression of GPRC5B-GFP exhibits proliferating cells in the lumen in response to HGF (inward growth), while the cyst with higher GPRC5B-GFP expression shows outward growth. Scale bar, 20 μm

(C) Expression of CD82, a tetraspanin localized to exosomes, exhibits no outward growth induced by HGF in Matrigel. Outward growth upon HGF was calculated as described in Figure 1. $n \geq 3$; mean $\pm$ SEM.



r_p 0.099± 0.017 | 0.131 ± 0.019 | 0.205 ± 0.032 | 0.326 ± 0.059 | 0.121 ± 0.020

Figure S2: GPRC5B is apically released on exosomes through a Rab35-dependent pathway.
Representative confocal images of MDCK cells expressing indicated constructs. Arrowheads in cells expressing GPRC5B-GFP and mCherry-Rab5 QL (constitutively active) indicate intraluminal GPRC5B-GFP in enlarged endosomes. Note that the intracellular GPRCB-GFP is trapped in enlarged Rab5 QL-positive endosomes. Arrowheads in cells expressing mCherry-Rab35 QL (constitutively active) indicate its colocalization with GPRC5B-GFP. Pearson's colocalization coefficients indicated below the bottom panels were calculated to quantify colocalization between GPRC5B-GFP and the indicated Rab proteins, using Huygens software with deconvoluted images. Values are means ± SEM for three independent experiments ($n \geq 10$). Scale bar, 10 μm.

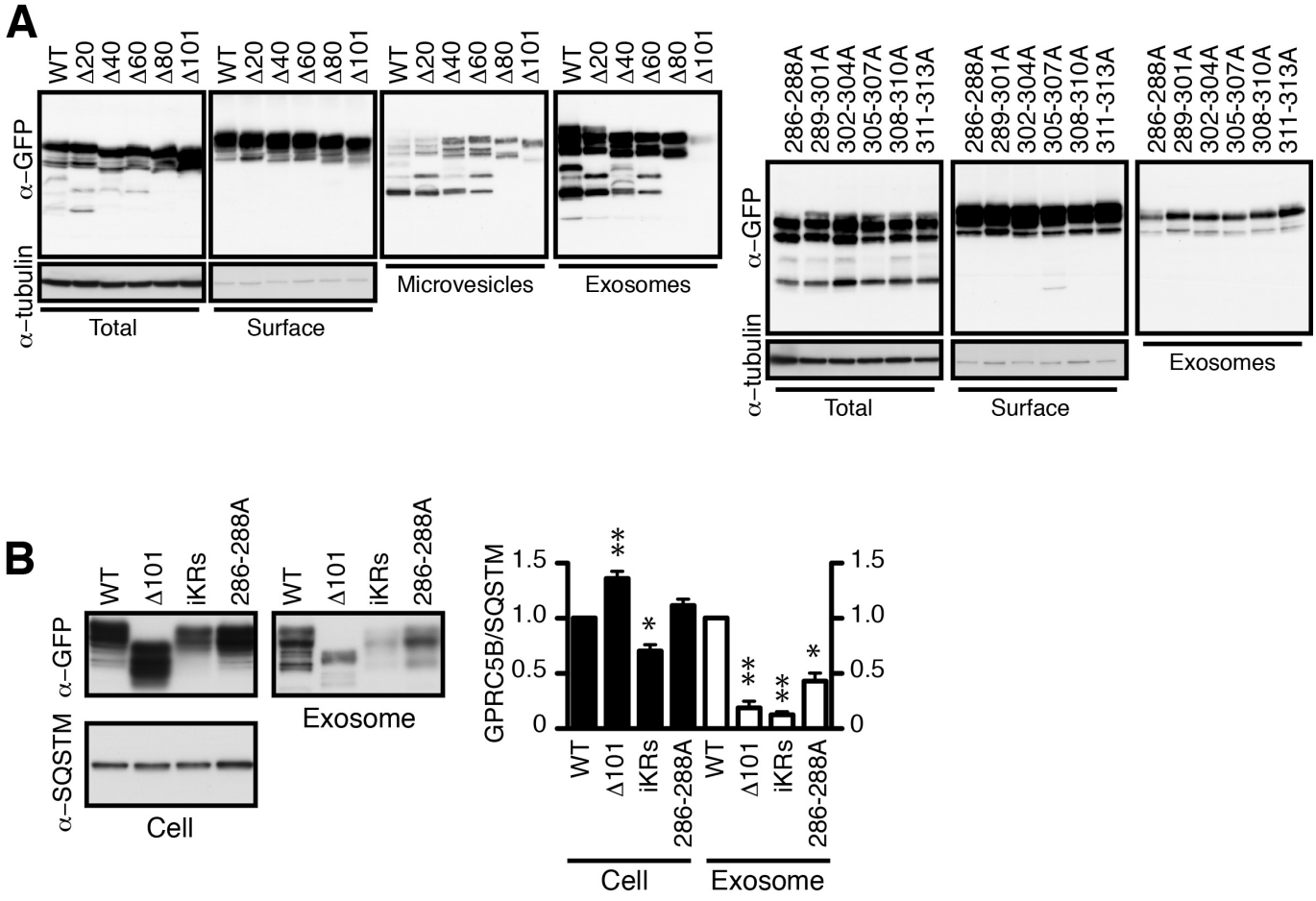

A
WT
Δ20
Δ40
Δ60
Δ80
Δ101
α–GFP
α–tubulin
Total
Surface
Microvesicles
Exosomes
286-288A
289-301A
302-304A
305-307A
308-310A
311-313A
B
WT
Δ101
iKRs
286-288A
α–GFP
α–SQSTM
Cell
Exosome
GPRC5B/SQSTM
1.5
1.0
0.5
0
**
*

Figure S3: The juxtamembrane residues of the C-terminal tail determine the transfer of GPRC5B from the limiting membrane to the lumen of late endosomes, thereby leading to its exosomal release.
(A) Representative immunoblots showing the expression of a series of C-terminal truncation (Δ20, Δ40, Δ60, Δ80, Δ101) and alanine scanning mutants (286-288A, 289-301A, 302-304A, 305-307A, 308-310A, 311-313A) of GPRC5B-GFP. Total cell, cell surface, microvesicle, and exosome fractions are shown. Tubulin was used as loading control.
(B) Representative immunoblots (left) and quantification (right) showing the indicated GPRC5B-GFP constructs (WT, 101, iKRs, 286-288A) in total cell and exosome fractions. Sequestosome1 (SQSTM) was used as loading control. Deletion of C-terminal tail (Δ101) or substitution of all intracellular lysine residues to arginine (iKRs) blocks exosomal release to the same extent as 286-288A. Note iKRs reduces total cell level, unlike other mutations tested.

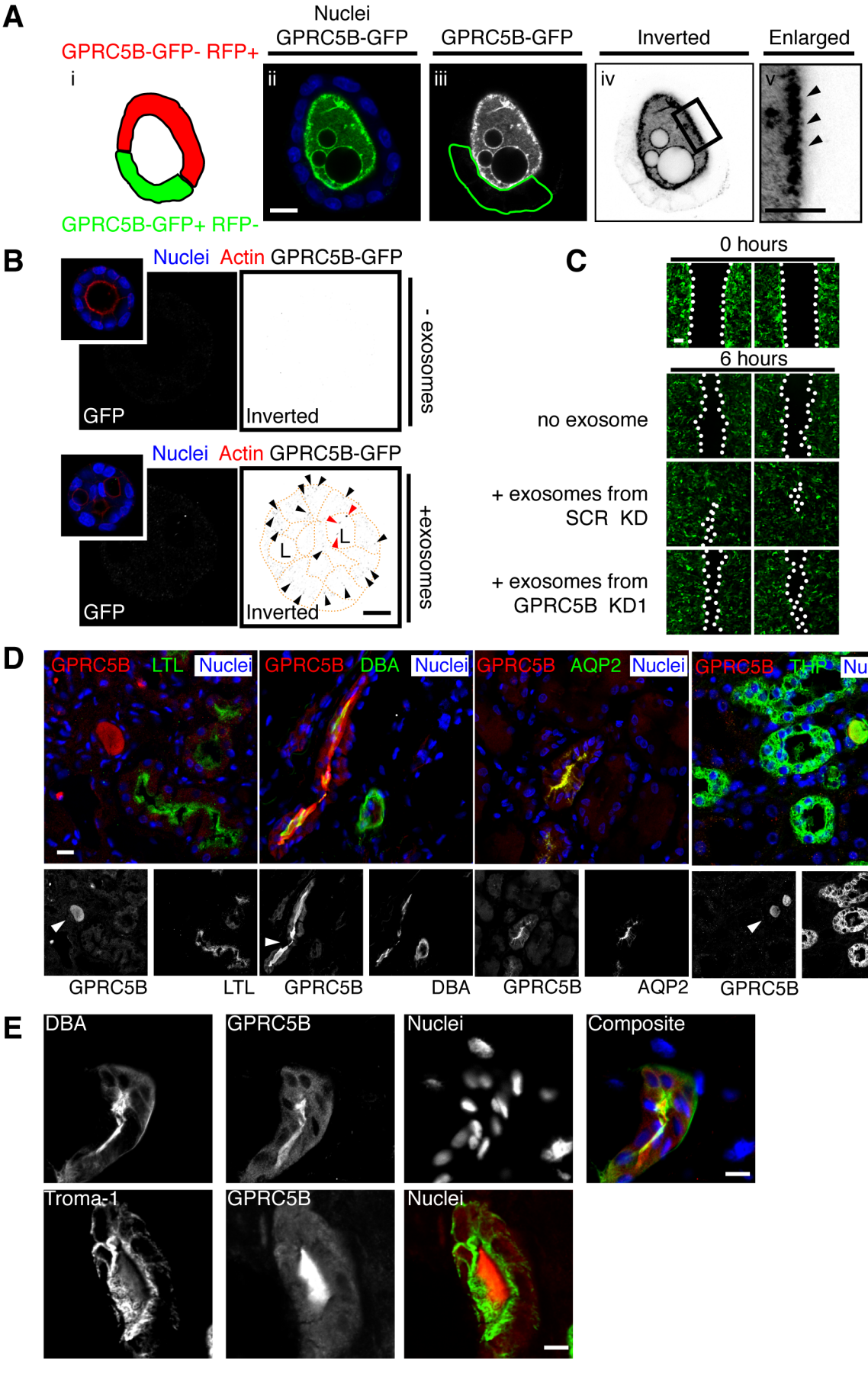

A
Nuclei
GPRC5B-GFP
GPRC5B-GFP
Inverted
Enlarged
GPRC5B-GFP- RFP+
i
ii
iii
iv
v
GPRC5B-GFP+ RFP-
B
Nuclei Actin GPRC5B-GFP
GFP
Inverted
- exosomes
Nuclei Actin GPRC5B-GFP
GFP
Inverted
L
L
+exosomes
C
0 hours
6 hours
no exosome
+ exosomes from SCR KD
+ exosomes from GPRC5B KD1
D
GPRC5B LTL Nuclei
GPRC5B DBA Nuclei
GPRC5B AQP2 Nuclei
GPRC5B THP Nuclei
GPRC5B
LTL
GPRC5B
DBA
GPRC5B
AQP2
GPRC5B
THP
E
DBA
GPRC5B
Nuclei
Composite
Troma-1
GPRC5B
Nuclei

Figure S4: Exosomal release of GPRC5B augments outward growth from ECM microenvironments.
(A) Representative confocal images showing a mosaic cyst with MDCK cells stably expressing either GPRC5B-GFP or RFP. In panel iii, the GPRC5B-GFP expressing cells are outlined by a hand-drawn green line. Box outlined in black in panel iv marks RFP-expressing target cells that are not contact with cells expressing GPRC5B-GFP, as determined by confocal sectioning (not shown). Panels iv and v are inverted (Black to white) to better visualize the GPRC5B-GFP that is at or near the surface of RFP-positive cells. Arrowheads in enlarged image indicate GPRC5B-GFP puncta at or near the surface of RFP-positive cells. Scale bar, 20 μm.
(B) Exosomes were purified from conditioned medium collected from MDCK GPCR5B-GFP cells grown as a monolayer on plastic dishes. These exosomes were added to the exterior of cysts grown from WT MDCK cells and representative confocal images are shown. To improve visualization, inverted images are shown at right. The cells in a cyst are outlined with brown- dashed lines to aid in visualization of intracellular GPRC5B-GFP puncta. Black arrowheads indicate intracellular GPRC5B-GFP puncta whereas red arrowheads indicate GPRC5B-GFP in the lumen (L). Scale bar, 20 μm.
(C) Representative images of wound migration assay. MRC5 cells were grown in serum free medium and the resultant conditioned medium was ultracentrifuged to remove any exosomes. This MRC5 conditioned medium was then added to MDCK cells grown as monolayers and expressing either shRNA for GPCR5B (GPCR5B KD) or a scrambled control (SCR KD). The conditioned medium from these MDCK cells was then subjected to ultracentrifugation to purify exosomes. The exosomes, either GPCR5B KD or SCR KD, were then added to a second set of cultures of WT MDCK cells grown as monolayers; these cultures had a silicone barrier to prevent growth in a gap region. The silicone barrier was removed immediately before addition of the purified exosomes. Migration was measured as the area of the gap that was covered by migrating cells over 6 hrs. Edges of migrating monolayer are marked with dotted lines. Scale bar, 100 μm.
(D) Representative endogenous GPRC5B localization in human nephron segments. Sections of kidneys stained with the proximal tubule-specific marker, *Lotus tetragolonobus* lectin (LTL), the collecting duct-specific marker, *Dolichos biflorus* agglutinin (DBA) and Aquaporin 2 (AQP2), and the loop of Henle marker, Tamm Horsfall protein (THP). GPRC5B rarely appears in the loop of Henle. Arrowheads indicate luminal localization of endogenous GPRC5B. Scale bar, 20 μm.
(E) Representative endogenous GPRC5B localization in collecting duct. Higher magnification of kidney sections stained with *Dolichos biflorus* agglutinin (DBA) and anti-Troma-1. Scale bar, 10 μm.

Supplemental Experimental Procedures

Cell culture. Madin-Darby canine kidney (MDCK II) cells were maintained in Minimum Essential Medium Eagle with 5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Antibodies and Reagents. Primary antibodies used were mouse anti-podocalyxin (gift of G. Ojakian); rat anti-ZO1 (DSHB); mouse anti-cytochrome C (BD Biosciences, 556433); rat anti-HA (Roche, 1867423), mouse anti-GFP (Roche, 11814460001); rat anti-RFP (Allele, ACT-CM-MRRFP10); rabbit anti-phospho histone (EMD Millipore, B00383); rat anti-tubulin (Abcam, ab6160), goat anti-GFP (Abcam, ab6673); rabbit anti-GFP (Invitrogen, A11122), rabbit anti-Rab11 (Invitrogen, 715300); goat anti-aquaporin 2 (Santa Cruz Biotech, sc9882); goat anti-Tamm Horsfall protein (R&D systems, AF5144); rat anti-Troma-1 (Developmental Studies Hybridoma Bank). Biotinylated lectins used were *Lotus tetragolonobus lectin* (LTL, B1325) and *Dolichos biflorus* agglutinin (DBA, B1035) from Vector Labs. We were unable to find antibodies against endogenous GPRC5B that worked in immunoblots of total cell lysates from human and MDCK II cell lines because of multiple non-specific bands, but anti-GPRC5B (Sigma) worked with purified recombinant GPRC5B and GPRC5B in human urine exosomes for immunoblotting. Secondary antibodies were Alexa Fluor 555 anti-mouse, Alexa Fluor 555 anti-rabbit, Alexa Fluor 647 anti-mouse, Alexa Fluor 647 anti-rabbit, Alexa Fluor 488 anti-rabbit, Alexa Fluor 488 anti-goat, Alexa Fluor 488-streptavidin (Invitrogen); HRP-anti-mouse, HRP-anti-rabbit (Jackson). Nuclei were stained with Hoechst 33342 (Invitrogen). Reagents were rhHGF (a kind gift from the late Dr. Ralph Schwall, Genentech); growth factor-reduced Matrigel (BD Biosciences); bovine collagen type I (Sigma); U0126 (EMD Millipore).

Plasmids. Full-length canine GPRC5B cDNA was cloned from cultured MDCK II cells. GPRC5B ORF was constructed into pEGFP-N1 (Clontech) through XhoI and BamHI. Site-specific mutations were generated using QuickChange Lightning Muti Site-directed mutagenesis kit (Stratagene) or gene synthesis (GenScript). All constructs were verified by DNA sequencing. Plasmids kindly provided were: mCherry-fused Rab5 Q79L (Dr. Mark Von Zastrow, UCSF); HA-tagged Rab7 Q86L, Triple HA-tagged Rab27A Q78L; Rab35 WT, Rab35 QL, Rab35 SN (Dr. Peter McPherson, McGill Univ). Rab27A Q79L ORF was constructed into pQCXIZ (addgene) for retroviral expression. Rab35 ORFs were constructed into pmApple-N1. For a reduced expression of Rab35, due to high toxicity when highly overexpressed, Rab35 WT, Rab35 QL, and Rab35 SN ORF were constructed into pRetroX-Tight-Hyg.

RNAi. The lentiviral vector, pLKO.1-puro for producing shRNA, was constructed according to the pLKO.1 protocol (Addgene) using iRNAi target selection program (Mekentosj.com). All target sequences were submitted to BLAST with query limit of txid 9615 (*Canis lupus familiaris*) to verify target specificity. All cloned shRNAs were verified by DNA sequencing. For lentivirus production, pLKO.1-puro plasmids were co-transfected with ViraPower packaging mix (Invitrogen) into HEK293-FT cells according to manufacturer's protocols. All viral supernatants were centrifuged to remove cell debris, snap frozen in liquid nitrogen, and stored at -80°C for future use. Knockdown was determined by either quantitative reverse transcription-PCR (qRT-PCR) or immunoblotting.

Transfection and retroviral transduction. For transient transfection, MDCK cells were transfected with Lipofectamine 2000 or nucleofected with Amaxa cell line Nucleofector kit L, according to the manufacturer's instructions. For retroviral transduction, pQCXIZ expression plasmids and pRetroX-Tight-Hyg were transfected to 293 GPG packaging cells (kindly provided by Dr. Orion Weiner, UCSF). 48 hours after transfection, viral supernatants were collected daily over 5 days.

Tubulogenesis assay. In vitro tubule formation assays were previously described [1]. Briefly, For HGF-induced tubule formation assay, 4 day-old cysts grown in 2% Matrigel matrix were simulated for 48 hours or 72 hours with 12.5 ng/ul of recombinant HGF, together with collagen I as supporting matrix. For outward growth in suppressive ECM microenvironment, 2% Matrigel matrix was used as supporting matrix. For co-culture based tubule formation, MDCK monolayer was grown on thick collagen I plug in a Transwell filter with 0.4 μm pore diameter. To induce tubulogenesis, a suspension of MRC-5 in collagen mixture was plated in the basolateral compartment, immediately followed by gelation. Seven days after co-culture, the number of tubules was counted.

Exosome preparation from tissue culture cells or urine. For exosomes from cultured MDCK cells, 16-19 hours before preparation, growth medium was replaced by exosome-depleted medium, and conditioned medium was collected and centrifuged at 300 × *g* for 10 min and at 17,000 × *g* for 15 min, sequentially. The resulting supernatant was centrifuged at 100,000 × *g* for 1 hr 15 min. The pellets were resuspended with 2x Laemmli sample buffer supplemented with DTT. All centrifugation steps were done at 4°C. Urinary exosome preparation was performed as previously described with minor modification [2, 3]. 1 ml of frozen urine samples were thawed on platform vortex for 20 min and then centrifuged at 2,000 × g for 10 min. Next, the supernatant was centrifuged further at 17,000 × *g* for 15 min. To rescue trapped exosomes by THP polymers, the pellet were resuspended in 250 ul of 10 mM triethanolamine (pH 7.6)/ 250 mM sucrose/ 500 mM DTT and incubated at 37°C for 10 min. The supernatant and resuspended pellet were centrifuged at 200,000 × g for 1 hr. All centrifugation steps in urinary exosome preparation were done at 25°C.

Exosome transfer. 48 hours before conditioned medium collection, 80% confluent MDCK cells were refreshed with 6ml of serum-free medium per 10cm dish. The collected medium was centrifuged at 300 x *g* for 10 min and at 17,000 x *g* for 15 min sequentially. 4 days-old cysts grown in Matrigel matrix were then simulated with the supernatant supplemented with the final concentration of 2% Matrigel and 12.5 ng/ml HGF for 48 hours. For exosome-depleted conditioned medium, the resulting supernatant from 17,000 x g spin was further centrifuged at 100,000 × *g* for 1 hr 15 min to remove exosomes. For Figure 4f, 24 hours before conditioned medium collection, 50% confluent MRC5 fibroblasts were refreshed with serum-free medium. The collected medium was centrifuged at 300 x *g* for 10 min and at 17,000 x g for 15min sequentially. The resulting supernatant from 17,000 x g spin was further centrifuged at 100,000 x *g* for 1 hour 15 min to remove exosomes (referred to as exosome-depleted, MRC5-conditioned medium (CM)). 80% confluent MDCK cells were then stimulated with diluted exosome-depleted MRC5 CM. After 24 hours stimulation, the collected medium was centrifuged at 2,000 x *g* for 30 min. Exosomes were prepared with the resulting supernatants using total exosome isolation reagent (Invitrogen).

Migration assay. 7 x 10^5 MDCK cells expressing GFP were plated into the ibidi culture insert (ibidi). 24 hours after plating, inserts were removed, and indicated exosomes were added to monolayers. Cell migration was imaged after 6 hour incubation at 37°C and 5% CO_2.

Immunofluorescence and microscopy. For immunofluorescence, cells were washed twice with phosphate buffered saline (PBS) containing 1mM $CaCl_2$ and 0.5mM $MgCl_2$, fixed for 20 min with 4% paraformaldehyde in PBS, and blocked and permeabilized with 0.7% fish skin gelatin/ 0.025% saponin/ 0.01% sodium azide in phosphate buffered saline (PFS) for at least 2 hrs. The indicated primary antibodies were incubated in blocking buffer at 4°C overnight. After extensive washing with blocking buffer, samples were incubated with fluorescent-conjugated secondary antibodies in PFS. Finally, samples were washed with PBS and then mounted using ProLong Gold Antifade (Invitrogen) in case mounting is needed. For confocal microscopy, digital images were taken with a krypton-argon laser (488nm and 564nm), a helium-neo laser (633nm), and a two photon laser coupled to a LSM 510 META NLO microscope (Zeiss) using Plan-Apochromat 63x 1.4NA oil lens, C-Apochromat 63x 1.2NA water lens, Plan-Neofluar 25x 0.80NA lens, or Plan-Neofluar 10x 0.3NA dry lens. For counting tubule length, digital images were taken from the center of culture chamber well with DinoXcope microscope (Dino-Lite). The distance between leading edge of tubule and cyst wall was marked with freehand selection tool, and was then measured using ImageJ with Multi Measure plugin.

Distribution of GPRC5B from limiting membrane to lntraluminal endosome membrane. Enlarged endosomes using mCherry-fused rab5 Q79L expression was imaged. For each optical section, mCherry signal was used to define the limiting endosomal membrane. Using ImageJ, all GFP pixel intensities over the area defined by the mCherry signal, including the limiting membrane, were measured. The distribution of GPRC5B-GFP pixel intensities in and at limiting membrane was plotted as percent coefficient of variation (C_V).

Immuohistohemistry. Paraffin-embedded adult kidney sections (Zyagen) were deparaffinized with Histo-Clear II (National Diagnostics) and rehydrated. Heat-induced antigen retrieval was then performed

with 10 mM sodium citrate (pH 6.0)/ 0.05% Tween-20. Sections were permeabilized with 0.05% Triton-X 100 and blocked with either 10% goat or donkey sera (Sigma). Antibody labeling and mounting steps were followed as described in immunofluorescence. All double immunofluorescence was performed with sequential antibody labeling.

Cell surface biotinylation. Cell surface biotinylation was performed as previously described [4]. Briefly, 0.8 mM membrane impermeable EZ-link Sulfo-NHS-SS biotin (pH 9.0) was used for labeling cell surface proteins and biotinylated proteins were subsequently captured using NeutrAvidin Agarose (Thermo Scientific). Bound proteins were eluted with 2x sample buffer supplemented with DTT after extensive washing.

Immunoblotting. For total fraction, cells were lysed in 2x Laemmli sample buffer supplemented with DTT and incubated at 37°C for 20 min. The resulting lysates were separated by SDS-PAGE, and transferred electrophoretically to PVDF. The blots were probed with the indicated primary antibodies, followed by either Alexa Fluor 680-conjugated secondary antibodies for near-infrared fluorescence detection (LI-COR Odyssey) or HRP-conjugated secondary antibodies for chemiluminescent detection. Unsaturated signals on the blot were captured and band intensities were quantified with ImageJ (NIH).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Total RNA of MDCK II cells were extracted using Trizol (Invitrogen), and further cleaned up with RNeasy Miniprep kit (Qiagen). qRT-PCR was performed with MX3005P or MX4000 qPCR systems (Stratagene) using one step Brilliant II SYBR Green QRT-pCR master mix kit (Stratagene). For each gene, qRT-PCR was run in at least triplicate. qRT-PCR data were normalized and standardized with MxPro software (Stratagene).

Statistics. All data were from at least three independent experiments. Values are mean ± SE. Significance was calculated using a Student's t-test and labeled as *$p < 0.05$, ** < 0.001, *** < 0.0001 in figures.

Supplemental References

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