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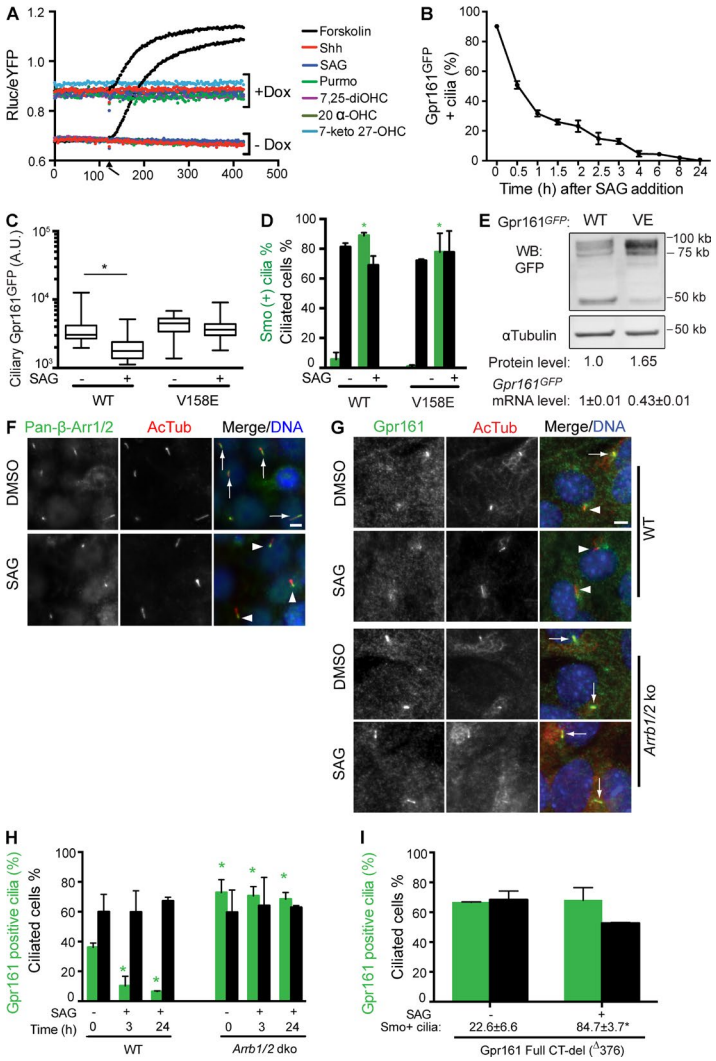

Figure S1. **Ciliary loss of Gpr161 depends on its signaling activity and direct binding to β-arrestins.** (A) Stable T-REx-293 cells expressing CAMYEL (conformation-based cAMP BRET biosensor) and inducibly coexpressing untagged mouse *Gpr161* were treated with 4 µg/ml doxycycline (Dox) for 12 h. The media was replaced with assay buffer containing 1 mM IBMX 45 min before the experiment. Readings were taken using 475- and 535-nm emission filters simultaneously after the addition of 5 µM coelenterazine-h. After tracking the baseline BRET ratio for 2 min, cells were treated with drugs (10 µM each) or 10 µM Forskolin using injectors (indicated by arrow). Oxysterols used were as follows: 7-keto-27-hydroxycholesterol, a naturally occurring oxysterol (Myers et al., 2013), 20α–hydroxycholesterol, a synthetic oxysterol (Nachtergaele et al., 2012), and 7α,25-dihydroxycholesterol, which is an agonist for Gpr183 (Hannedouche et al., 2011). The BRET ratios were subsequently quantified for 5 min. Mean values of two independent experiments are represented graphically. (B) Confluent NIH 3T3 Flp-In cells stably expressing GFP-tagged WT *Gpr161* were starved for a total of 24 h and treated with SAG (500 nM) for indicated time periods before fixation. Fixed cells were processed for immunofluorescence with anti-GFP and anti–acetylated tubulin antibodies, for detecting GFP-positive cilia. Data represents mean ± SD from two experiments. (C and D) Confluent NIH 3T3 Flp-In cells stably expressing GFP-tagged full-length *Gpr161* V158E mutant (VE) were starved ±1 µM SAG for 24 h, and Gpr161 (C) and Smo (D) levels in cilia were determined by immunofluorescence. For C, GFP ciliary intensities in cilia were quantified using ImageJ (n = 50 each; *, P < 0.01). For D, data represent mean ± SD from three separate fields from one experiment (*, P < 0.001 with respect to untreated cells in each cell line). (E) Confluent NIH 3T3 Flp-In cells stably expressing indicated constructs were starved for 30 h, and immunoblotted for indicated proteins (Materials and methods). Relative transcript levels for $Gpr161^{GFP}$ were determined in parallel by qRT-PCR (mean ± SD). (F) Confluent IMCD3 Flp-In cells were starved for 24 h and treated with ±250 nM SAG for the last 90 min. *β-arrestin* localization to cilia is shown after immunofluorescence with anti–pan-*β-arrestin* and acetylated tubulin (AcTub) antibodies. Bar, 5 µm. (G and H) Confluent WT and *β-arrestin1/2* double-knockout MEFs (*Arrb1/2*) were starved for 48 h, treated with 500 nM SAG for the indicated time points before fixation, and processed for immunofluorescence using anti-Gpr161 and anti-acetylated tubulin antibodies to quantify cilia positive for Gpr161 by microscopy. Data represent mean ± SD from three independent experiments. *, P < 0.001, with respect to untreated WT cells. (I) Confluent NIH 3T3 Flp-In cells stably expressing GFP-tagged $Gpr161^{\Delta 376}$ mutant were starved for 24 h ±1 µM SAG before fixation, and immunostained using anti-GFP/anti-Smo and anti–acetylated tubulin antibodies. Data represent mean ± SD from two independent experiments for Gpr161 IF, and three separate fields from one experiment for Smo IF. *, P < 0.001 with respect to untreated cells.

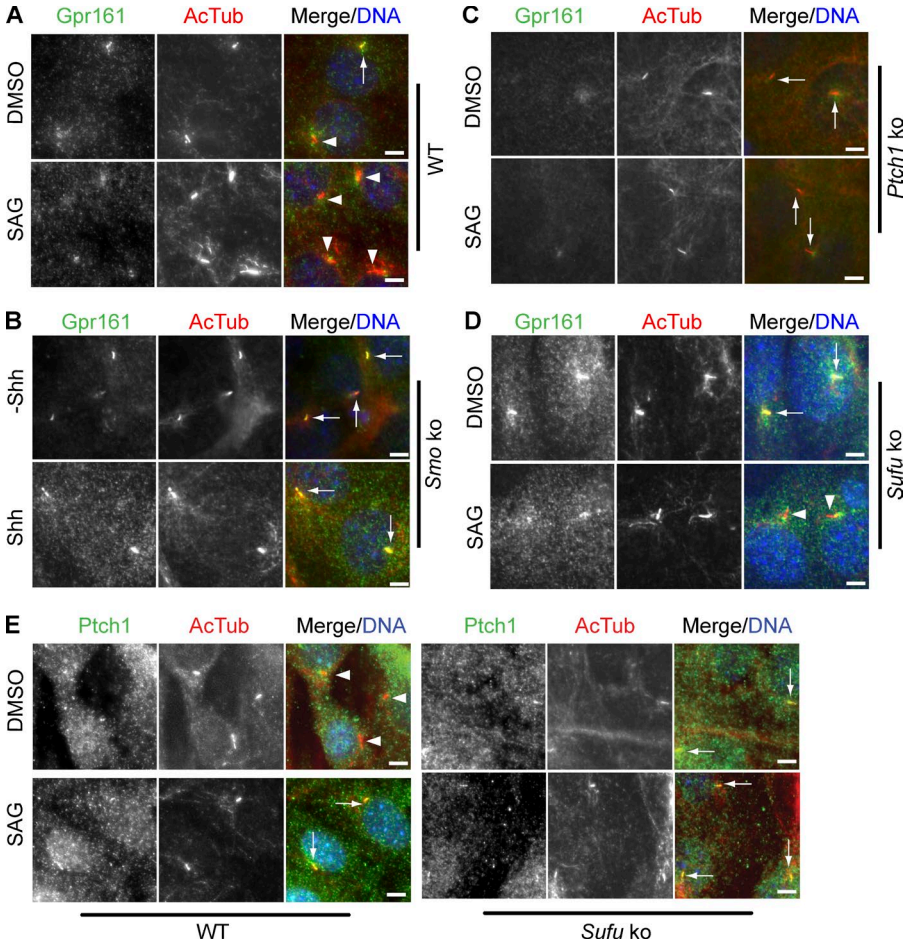

Figure S2. **Steady-state localization of Gpr161 in cilia is not controlled by Shh pathway activity.** (A–D) Confluent WT, *Smo*$^{-/-}$, *Ptch1*$^{-/-}$, or *Sufu*$^{-/-}$ MEFs were serum starved for 48 h, treated with 500 nM SAG or 300 ng/ml octyl-Shh for the last 24 h before fixation, and immunostained using anti-Gpr161 (green) and anti-acetylated tubulin (AcTub; red) antibodies. Bar, 5 µm. (E) Confluent WT and *Sufu*$^{-/-}$ MEFs were serum starved for 48 h, treated with 500 nM SAG for the last 24 h before fixation, and immunostained using anti-Ptch1 (green) and anti–acetylated tubulin (AcTub; red) antibodies. Bar, 5 µm.

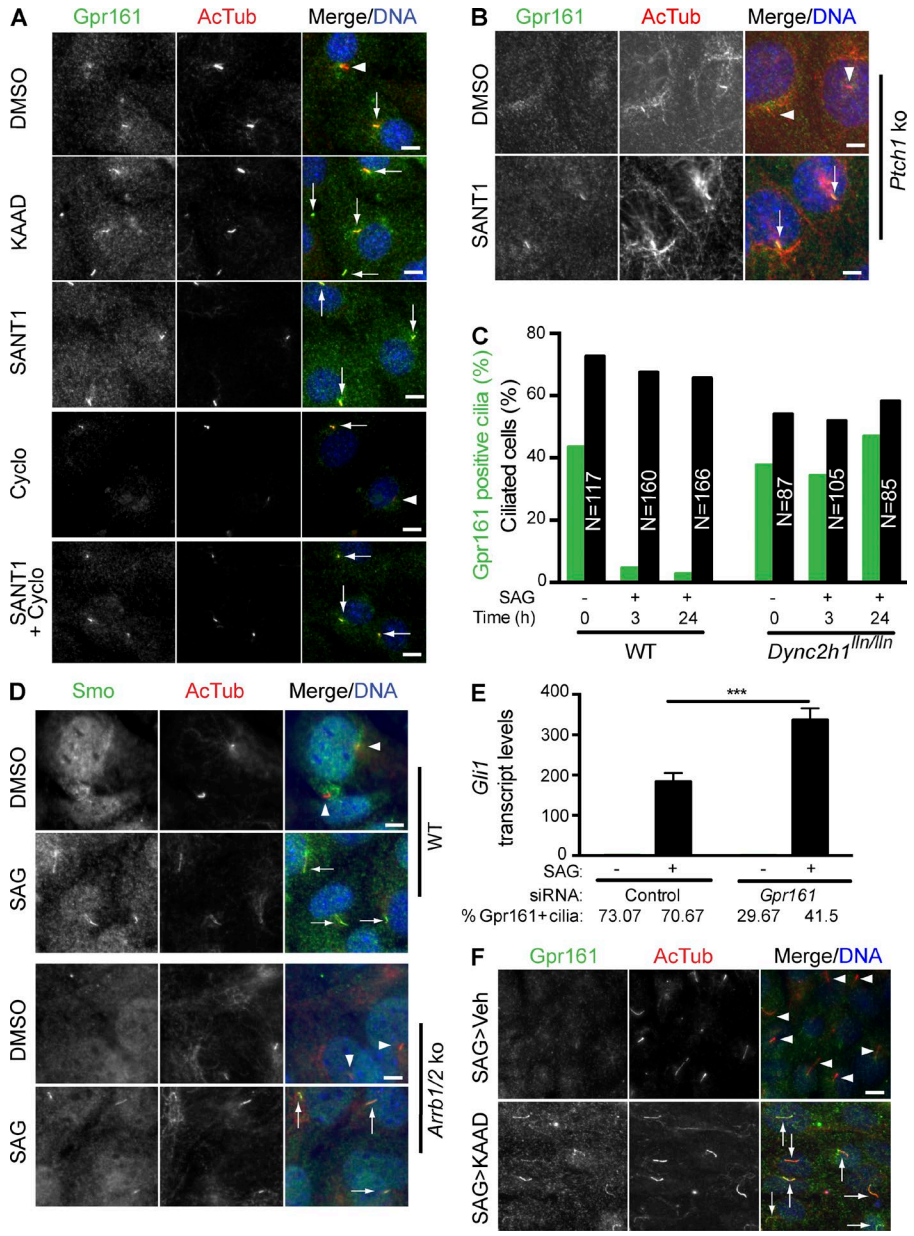

Figure S3. **Presence of activated Smo in cilia determines steady-state levels of Gpr161.** (A) Confluent WT MEFs were serum starved for 48 h, treated with drugs for last 24 h before fixation, and immunostained for Gpr161 (green) and acetylated tubulin (AcTub; red). Drugs used were as follows: 500 nM SAG, 1 µM KAAD-cyclopamine (KAAD), 1 µM GDC-0449 (GDC), 1 µM SANT1, and 5 µM cyclopamine (cyclo). Arrows and arrowheads indicate cilia positive and negative for Gpr161, respectively. Bar, 5 µm. (B) Confluent *Ptch1*$^{-/-}$ MEFs were serum starved for 48 h, treated ±1 µM SANT1 for the last 24 h before fixation, and immunostained using anti-Gpr161 (green) and anti–acetylated tubulin (AcTub; red) antibodies. Bar, 5 µm. (C) Confluent WT and *Dync2h1*$^{lln/lln}$ MEFs (Ocbina et al., 2011) were serum starved for 48 h, treated ±500 nM SAG for the indicated time periods before fixation, and immunostained using anti-Gpr161 (green) and anti–acetylated tubulin (red) antibodies to quantify Gpr161-positive cilia. N, total counted cilia from one experiment. (D) Confluent WT and *β-arrestin1/2* double-knockout MEFs (*Arrb1/2* ko) were serum starved for 48 h, treated ±1 µM SAG in starvation media for 36 h before fixation, and immunostained using anti-Smo (green) and anti–acetylated tubulin (AcTub; red) antibodies. Arrows and arrowheads indicate cilia positive and negative for Smo, respectively. (E) *β-arrestin1/2* double-knockout MEFs were transfected with siRNAs against *Gpr161* (Materials and methods). Confluent cells were starved for 24 h and further treated ±1 µM SAG for 30 h in starvation media. Total RNA was isolated, and *Gli1* expression levels were determined by quantitative real-time RT-PCR. Percentages of Gpr161-positive cilia upon RNAi suggests partial knockdown of the receptor in the primary MEFs. Data represent mean ± SEM from three independent experiments. ***, $P < 0.001$. (F) SAG-pretreated IMCD3 cells ± KAAD-cyclopamine, as described in Fig. 3 F, were immunostained to detect ciliary Gpr161 levels. Arrowheads and arrows indicate cilia negative and positive for Gpr161, respectively. Bar, 10 µm.

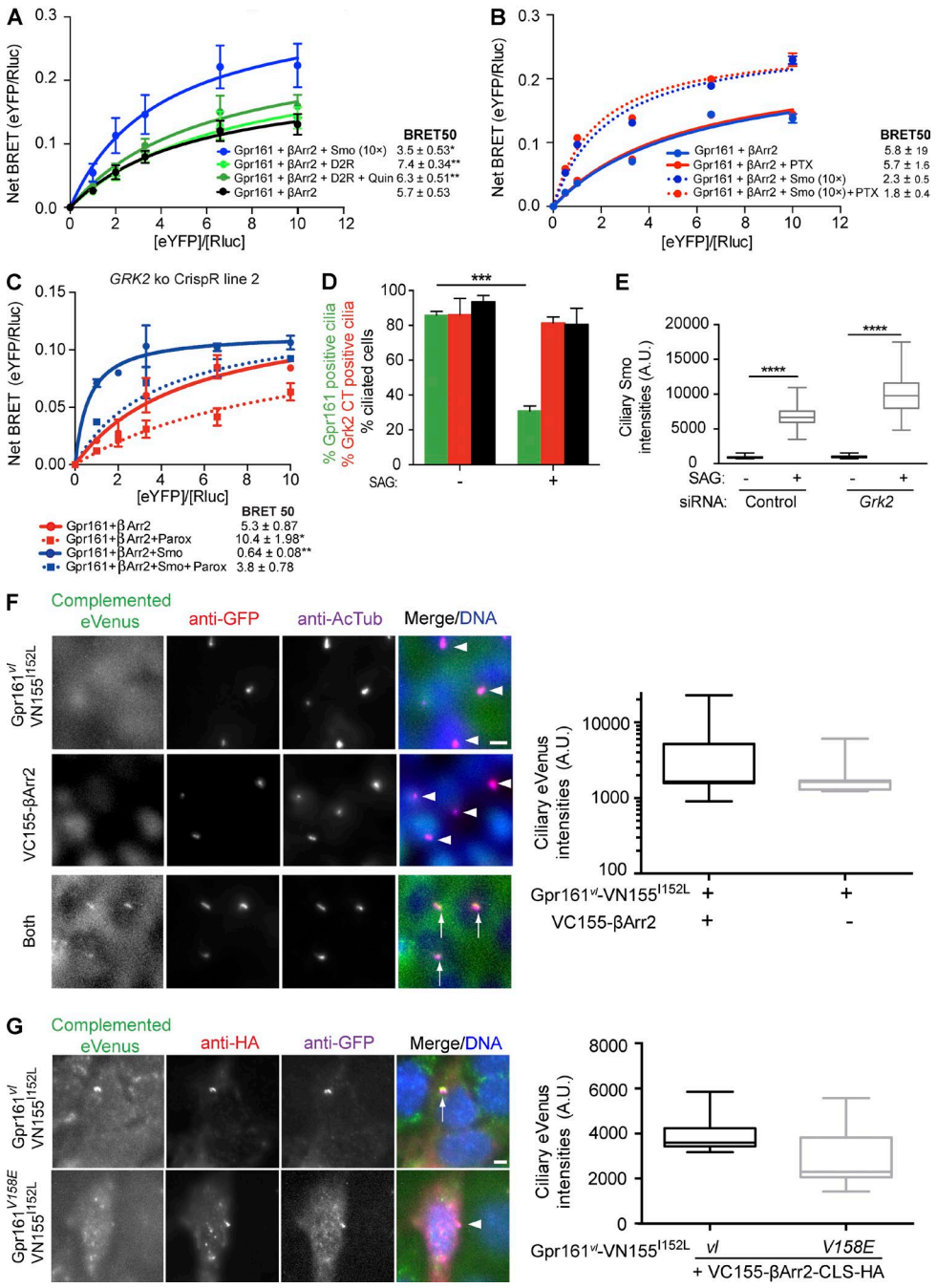

Figure S4. **Smo enhances recruitment of β-Arrestin to Gpr161.** (A and B) T-REx 293 cells were cotransfected with a constant amount of Rluc–β-arrestin1/2 (donor) and increasing amounts of Gpr161-eYFP (acceptor), ±1 µg/well of a six-well plate untagged human Smo (10×), ±1 µg/well of a six-well plate Flag-D2R. Flag-D2R-transfected cells were also finally treated with 1 µM of the D2R agonist quinpirole for 20 min (A). The cells were incubated with 800 ng/ml pertussis toxin for 12 h before the BRET assays (B). Cells were subjected to BRET analysis (Materials and methods). The titration curves are mean ± SEM from three independent experiments (A) and mean ± SD from one experiment performed in duplicate (B). *, $P < 0.05$; **, $P < 0.01$ for $BRET_{50}$ values with respect to only Gpr161/β-arrestin2 cotransfected cells in A. (C) BRET assays for Gpr161–β-arrestin2 interactions as in Fig. 5 C with *GRK2* knockout line 2 from two independent experiments (mean ± SD). *, $P < 0.05$; **, $P < 0.01$ with respect to Rluc–β-arrestin2 binding to Gpr161-eYFP. (D) IMCD3 cells transfected with ciliary-targeted Grk2 C-terminus fusion (CLS-mCherry-Grk2CT; Materials and methods) were starved ±500 nM SAG for 17 h and subjected to immunofluorescence for Gpr161 (green) and anti–acetylated tubulin (magenta). CLS-mCherry-Grk2CT positive (red) cilia were scored for Gpr161. Data represents mean ± SD from two experiments. ***, $P < 0.001$. (E) Pixel intensities of Smo in control or *Grk2* siRNA-treated IMCD3 cilia ± SAG treatment represented graphically. $n = 100$ each; ****, $P < 0.0001$ with respect to untreated cells in each background. (F) IMCD3 cells with stable expression of $Gpr161^{vl}VN155^{I1522L}$ were infected ± retroviruses expressing VC155-βArr2. After antibiotic selection, confluent cells were starved for 24 h before fixing and immunostained with anti-GFP (red) anti–acetylated tubulin (AcTub; magenta) antibodies. Intensities of eVenus complementation in the cilia (green channel) of GFP-positive cells (red channel) were quantified using ImageJ and represented graphically (right); $n = 21$ each. Bar, 5 µm. (G) IMCD3 cells were transfected with the indicated constructs (Materials and methods), starved for 17 h before fixing, and immunostained with anti-HA (red) anti-GFP (magenta) antibodies. Intensities of eVenus complementation in the cilia (green channel) of HA/GFP-positive cells were quantified using ImageJ and represented graphically (right); $n \geq 22$ each. Bar, 5 µm. $P < 0.001$ between groups. Arrows and arrowheads indicate cilia positive and negative for eVenus complementation, respectively (F and G).

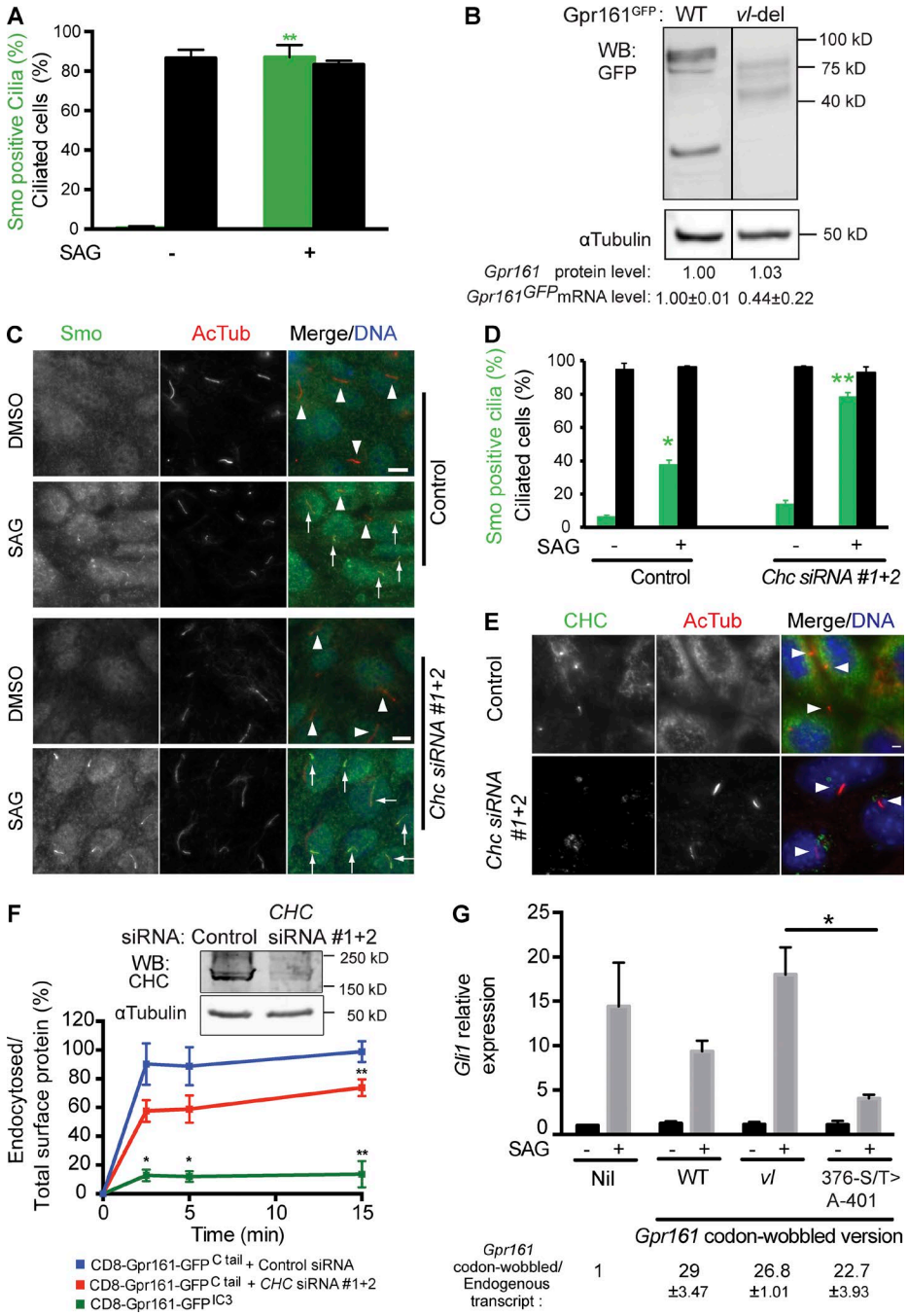

Figure S5. **Disappearance of Gpr161 from cilia is dependent on clathrin-mediated endocytosis.** (A) Confluent NIH 3T3 Flp-In cells stably expressing GFP-tagged *Gpr161*^{vl} mutant were treated ±1 μM SAG for 24 h in starvation media before fixation, and Smo levels were quantified by immunofluorescence. Data represent mean ± SD from three separate fields from one experiment. **, P < 0.01 with respect to untreated cells. (B) Confluent NIH 3T3 Flp-In cells stably transfected with indicated constructs were starved for 30 h and immunoblotted for indicated proteins (Materials and methods). Relative transcript levels for *Gpr161*^{GFP} were determined in parallel by quantitative real-time RT-PCR (mean ± SD). (C and D) IMCD3 Flp-In cells were transfected with predesigned On-target Plus siRNAs against clathrin heavy chain (*Chc*) for 72 h. Confluent cells were starved for 30 h, treated ±250 nM SAG for 5 h before fixing, and immunostained using anti-Smo (green) and anti-acetylated tubulin (AcTub; red) antibodies to quantify cilia positive for Smo. Arrows and arrowheads indicate cilia positive and negative for Smo, respectively. Bar, 10 μm. Data represent mean ± SD from three separate fields from one experiment. *, P < 0.001; **, P < 0.0001 with respect to DMSO-treated cells in each case. (E) IMCD3 Flp-In cells transfected with indicated predesigned siRNAs for 72 h were starved for the last 24 h, fixed, and immunostained for CHC. *CHC* siRNA-treated cells show specificity of the anti-CHC antibody. Arrowheads denote cilia. Bar, 5 μm. (F) ARPE-19 cells reverse transfected with siRNAs against *CHC* were transfected with plasmids expressing CD8α-tagged with Gpr161 C tail or third intracellular loop (IC3) fragments (Materials and methods) the next day. Cells were subjected to ELISA (Materials and methods). Endocytosed CD8α fusion proteins were expressed as a percentage of surface CD8 fusions. Inset shows immunoblot for *CHC*. Data represent mean ± SEM from three experiments. *, P < 0.05; **, P < 0.01 with respect to control siRNA-treated cells at respective time points. (G) Confluent NIH 3T3 cells stably expressing untagged codon-wobbled wild type or mutant *Gpr161* (Materials and methods) were treated ±SAG in starvation media for 30 h, followed by RNA isolation and quantification of *Gli1* levels by quantitative real-time RT-PCR. Data represent mean ± SEM from three separate stable lines in each background and one to four independent experiments for each line. Quantitative real-time RT-PCR confirmed *Gpr161* overexpression to be identical between the respective lines (Materials and methods). *, P < 0.05.

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