Primary cilia are specialized calcium signalling organelles

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Primary cilia are solitary, non-motile extensions of the centriole found on nearly all nucleated eukaryotic cells between cell divisions. Only ~200–300 nm in diameter and a few micrometres long, they are separated from the cytoplasm by the ciliary neck and basal body. Often called sensory cilia, they are thought to receive chemical and mechanical stimuli and initiate specific cellular signal transduction pathways. When activated by a ligand, hedgehog pathway proteins, such as GLI2 and smoothened (SMO), translocate from the cell into the cilium1,2. Mutations in primary ciliary proteins are associated with severe developmental defects3. The ionic conditions, permeability of the primary cilia membrane, and effectiveness of the diffusion barriers between the cilia and cell body are unknown. Here we show that cilia are a unique calcium compartment regulated by a heteromeric TRP channel, PKD1L1–PKD2L1, in mice and humans. In contrast to the hypothesis that polycystin (PKD) channels initiate ciliary calcium that are conducted into the cytoplasm4, we show that changes in ciliary calcium concentration occur without substantially altering global cytoplasmic calcium. PKD1L1–PKD2L1 acts as a ciliary calcium channel controlling ciliary calcium concentration and thereby modifying SMO-activated GLI2 translocation and GLI1 expression.

We generated a transgenic ARL13B–EGFP mouse (Ar13b-EGFP) in which primary and motile cilia show spectacular fluorescence labelling throughout the animal, developed a ratiometric Ca2+ sensor directed specifically to cilia, and patch-clamped individual cilia and measured the resting membrane potential and calcium-permeant conductance (Icilia; see ref. 5). The Ar13b-EGFP mice displayed ubiquitous green fluorescence only in primary and motile cilia but not in microvilli (Fig. 1, Extended Data Fig. 1 and Supplementary Video 1). After incubation of E14.5 embryos in clarifying ScaleA2 solution6 (Fig. 1a–c and Supplementary Data Fig. 1 and Supplementary Video 1). After incubation of E14.5 embryos in clarifying ScaleA2 solution6 (Fig. 1a–c and Supplementary Video 1), three-dimensional imaging of ARL13B–GFP reveals its exclusive localization to cilia. Staining of wild-type and Ar13b-EGFP retinal pigmented epithelial (mRPE) cells and mouse embryonic fibroblasts (MEFs) with ciliary markers confirmed that ARL13B–EGFP is localized exclusively to cilia without noticeable alteration of cilia morphology (Extended Data Fig. 2).

Primary cilia are not reliably loaded with Ca2+-sensitive dyes, forcing experimentalists to rely on changes in cytoplasmic calcium concentration ([Ca2+]cyto) as an indirect indicator of ciliary calcium concentration ([Ca2+]cilia). To ameliorate this issue, we generated a genetically encoded calcium sensor that is targeted to the cillum by fusing GCaMP3 (ref. 7) to the carboxy terminus of smoothened (SMO–GCaMP3), enabling monitoring of [Ca2+]cilia and [Ca2+]cyto simultaneously. As shown in Extended Data Fig. 3 and Supplementary Video 3, addition of the Ca2+ ionophore, ionomycin, increased fluorescence in both the cytoplasm and cillum of hRPE1 cells stably expressing SMO–GCaMP3, although variation in ionomycin incorporation precludes precise [Ca2+]cilia comparison between the two compartments.

To quantify [Ca2+]cilia we developed a ratiometric SMO–mCherry–GCaMP3 calcium sensor8 (Fig. 2a, b). In hRPE1 cells stably expressing SMO–mCherry–GCaMP3, the cilia-targeted GCaMP3 and mCherry fluorescence co-localized with the cilia-specific marker, acetylated tubulin (Extended Data Fig. 3e–h). To determine whether [Ca2+]cilia could be increased without affecting [Ca2+]cyto, we ruptured the cillum membrane at the tip of the cillum (circle; Extended Data Fig. 3i, j) with an intense 1–2 s laser pulse (405 nm), leading to a rapid increase in [Ca2+]cilia from the tip that travelled to the ciliary base (Supplementary Video 4). Peak [Ca2+]cilia propagated down the cillum at a rate of 4.6 ± 0.6 μm s−1, yielding an apparent diffusion constant (Dcilia) of 5.3 μm2 s−1 (similar to 5–10 μm2 s−1 for Dcyto in stellate cell dendrites3).

In order to closely monitor changes in [Ca2+]cyto at the cillum–cytoplasm junction, we loaded the calcium indicator Fluo-4 into hRPE1 cells stably expressing SMO–mCherry–GCaMP3. Ciliary membrane rupture increased [Ca2+]cilia and was detectable at the ciliary base after a ~40 s delay (Fig. 2c–e and Supplementary Video 5). More distant parts of the...
cytoplasmic $\text{Ca}^{2+}$ normalizes. Circles in indicate cytoplasm to cilium transition. Scale bars: 5 μm. The dissociation constant ($K_d$) for the ratiometric ciliary $\text{Ca}^{2+}$ sensor in situ was comparable to that in solution (625 nM versus 660 nM (ref. 7); Extended Data Fig. 4). In 2 mM extracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_{\text{ex}}$), resting $[\text{Ca}^{2+}]_{\text{cilia}}$ was 580 nM in hRPE1 cells (Fig. 3b). We also calibrated the ratiometric $\text{Ca}^{2+}$ sensor in the cytoplasm using cells that had not yet formed a cilium (SMO in the plasma membrane; Fig. 3a, bottom), and obtained a similar calibration curve and $K_d$ (550 nM), suggesting that the sensor reported $[\text{Ca}^{2+}]_{\text{cyto}}$ similarly in cilium and cytoplasm. The average ratio for the sensor in the cytoplasm of 0.28 ± 0.02, $n = 20$ cells) versus estimated $[\text{Ca}^{2+}]_{\text{cilia}}$. Resting $[\text{Ca}^{2+}]_{\text{cilia}} = 580$ nM. c, Plot of measured cytoplasmic fluorescence ratio (average = 0.28 ± 0.02) versus estimated $[\text{Ca}^{2+}]_{\text{cyto}}$. Resting $[\text{Ca}^{2+}]_{\text{cyto}}$ is 107 nM. d, By measuring current amplitudes in perforated patches (Methods), we estimated resting free $[\text{Ca}^{2+}]_{\text{cilia}}$, as 742 nM. Black circles indicate current at +100 mV; grey circles indicate current at −100 mV. e, Changes in cell (VM) and cilia (V_cilia) potentials in response to external $K^+$. f, Average potential of the cell body and cilia plotted as a function of external $K^+$. V_m differs from V_cilia at all $K^+$ other than $[K^+]_e = 145$ mM; $P < 0.05$. The measured resting membrane potential is −18 mV for the cilia and −54 mV for the cell (±s.e.m., $n = 5$ cells and 4 cilia). The grey dashed line is the $K^+$ Nernst potential.

Figure 3 | Resting cilium $[\text{Ca}^{2+}]$ is substantially higher than resting cytoplasmic $[\text{Ca}^{2+}]$. a, Live hRPE1 cell FGCaMP3/FmCherry ratios in 2 mM $[\text{Ca}^{2+}]_e$, in the cilium (top) and cytoplasm (bottom). Scale bar: 5 μm. b, Plot of measured cytoplasmic $[\text{Ca}^{2+}]$ elevation ($[\text{Ca}^{2+}]_c$, 550 nM), suggesting that the sensor reported $[\text{Ca}^{2+}]_{\text{cyto}}$ similarly in cilium and cytoplasm. The average ratio for the sensor in the cytoplasm of 0.28 ± 0.02, $n = 20$ cells) versus estimated $[\text{Ca}^{2+}]_{\text{cilia}}$. Resting $[\text{Ca}^{2+}]_{\text{cilia}} = 580$ nM. c, Plot of measured cytoplasmic $[\text{Ca}^{2+}]$ elevation ($[\text{Ca}^{2+}]_c$, 550 nM), suggesting that the sensor reported $[\text{Ca}^{2+}]_{\text{cyto}}$ similarly in cilium and cytoplasm. The average ratio for the sensor in the cytoplasm of 0.28 ± 0.02, $n = 20$ cells) versus estimated $[\text{Ca}^{2+}]_{\text{cilia}}$. Resting $[\text{Ca}^{2+}]_{\text{cilia}} = 580$ nM. d, By measuring current amplitudes in perforated patches (Methods), we estimated resting free $[\text{Ca}^{2+}]_{\text{cilia}}$, as 742 nM. Black circles indicate current at +100 mV; grey circles indicate current at −100 mV. e, Changes in cell (VM) and cilia (V_cilia) potentials in response to external $K^+$. f, Average potential of the cell body and cilia plotted as a function of external $K^+$. V_m differs from V_cilia at all $K^+$ other than $[K^+]_e = 145$ mM; $P < 0.05$. The measured resting membrane potential is −18 mV for the cilia and −54 mV for the cell (±s.e.m., $n = 5$ cells and 4 cilia). The grey dashed line is the $K^+$ Nernst potential.

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We next determined the cilia’s resting membrane potential \( (E_{\text{clu}}) \) by measuring changes in response to depolarizing concentrations of extracellular potassium \( (|K^+|_o) \) from hRPE1 SMO–EGFP cytoplasm and from its primary cilia. Consistently, \( E_{\text{clu}} \) was \( >30 \) mV more positive than the cytoplasm \( (E_{\text{clu}} = -18 \pm 1 \) mV; \( E_{\text{cyt}} = -54 \pm 2 \) mV, respectively; Fig. 3e) and significantly less \( |K^+|_o (70 \) mM versus 129 mM) was required to depolarize the ciliary membrane potential to 0 mV (Fig. 3f). In summary, the cilia is a functionally distinct cell compartment with respect to ions. This calcium compartment is maintained by a favourable influx/efflux ratio; the large number of calcium-permeant channels’ or other ion channels/transporters can easily maintain high \( [Ca^{2+}] \) in the small volume of the cilia, despite steady diffusion of \( Ca^{2+} \) into the cytoplasm as its base. An analogy is a water tower (\( Ca^{2+} \) within the cilia) connected to a large lake (cytoplasm) by a small pipe (basal body). Because ciliary \( [Ca^{2+}] \) is high \( (~600 \) nM) compared to the cytoplasm \( (~100 \) nM), calcium flows from ciliot to cytoplasm, but not cytoplasm to cilia. In addition, the approximately 30 mV gradient from cilia to cytoplasm further ensures asymmetry of \( Ca^{2+} \) between genotypes (Fig. 4g, h). Finally, we asked whether SAG stimulation for 24 h with 400 nM SAG. In contrast, GLI1 protein increased from 1.9 in wild-type MEFs stimulated for 24 h with 400 nM SAG. These data suggest that SAG initiates PKD2L1 recruitment to the cilium instead of activating GLI2 at the distal tip of the cilium (Fig. 4e, f), consistent with previous reports that these factors insulate cilia from the extracellular potassium \( ([K^+]_o) \). From its primary cilia. Consistently, cell type MEFs (Extended Data Fig. 6). These data suggest that SAG initiates recruitment of PKD2L1 channels into the cilium rather than activating the channel.

Our results indicate that primary cilia are functionally distinct from the cytoplasm. \( E_{\text{clu}} \) is encoded by a heteromeric PKD1L1–PKD2L1 channel and resting \( [Ca^{2+}]_{\text{clu}} \) is at least 0.4 \( \mu \)M higher than resting \( [Ca^{2+}]_{\text{cyt}} \) which regulates trafficking of hedgehog-mediated transcription factors in the cilium. PKD2L1 is increased by SHH pathway stimulation, probably by channel recruitment to the cilium. Interestingly, the IFT25–IFT27 complex seems to be specific for transporting SHH components and an IFT25 mutant MEF showed an impaired GLI2 ciliary trafficking phenotype\(^7\) similar to Pkd2l1 mutant MEFs. Because IFT25 has a unique "binding site" \( Ca^{2+} \) to optimize IFT25 function. \( Ca^{2+} \) is probably also adjusted by other PKD members or SMO receptor, leading to an upregulation of GLI1. SMH2 mice exhibit defects in SMO-mediated GLI1 activation. A intestinal malrotation in about 50% of mice (Fig. 4a), indicating a mild penetrance of the phenotype. Intestinal malformations are associated with sonic hedgehog (SHH) pathway defects during early development.\(^8\)

Treatment of MEFs with the SMO agonist SAG directly activates the SMO receptor, leading to an upregulation of GLI1 and IFT2L expression.\(^6\) As Pkd2l1 is transcribed and localizes to cilia in wild-type MEFs (Fig. 4b and Extended Data Fig. 5), we next asked whether the SHH pathway might be affected in Pkd2l1−/− mice by measuring the upregulation of GLI1 in response to stimulation with SAG. As shown in Fig. 4c, d, GLI1 protein increased from 1.9 ± 0.3 to 9.3 ± 0.7 AU (arbitrary units) in wild-type MEFs stimulated for 24 h with 400 nM SAG. In contrast, SAG upregulated GLI1 from 0.9 ± 0.04 to only 3.9 ± 0.6 in Pkd2l1−/− embroyonic MEFs. As shown in Fig. 4d, key members of the SHH pathway were not altered. However, ARL13b–EGFP\(^6\) MEFs accumulated significant amounts of GLI2 at the distal tip of the cilium (Fig. 4e, f), as is required for activation of GL2 and full activation of downstream transcription events.\(^18\) In Pkd2l1−/− × Arl13b–EGFP\(^\#\) mutant cells, GLI2 accumulation at the ciliary tip was reduced by ~50% compared to wild-type cells. Pkd2l1 is not required for cilial formation, as cilial length and the percentage of ciliated cells did not differ significantly between genotypes (Fig. 4g, h). Finally, we asked whether SAG stimulation itself regulates \( E_{\text{clu}} \) and thus ciliary \( [Ca^{2+}] \). Although there was no immediate effect on ciliary current stimulation with SAG, after 24 h \( E_{\text{clu}} \) amplitude and resting \( [Ca^{2+}]_{\text{clu}} \) increased in the cilium of wild-type MEFs (Extended Data Fig. 6). These data suggest that SAG initiates recruitment of PKD2L1 channels into the cilium rather than activating the channel.

Ca^{2+} binding site,\(^9\) the observed SHH defect of Pkd2l1 null mice is indicative that PKD2L1 may ‘tune’ \( Ca^{2+} \) to optimize IFT25 function. \( Ca^{2+} \) is probably also adjusted by other PKD members or even other as-yet unidentified ion pumps or transporters in the cilium during development, which may explain the mild phenotype of the Pkd2l1 mutant mouse compared to other SHH-deficient mutant mice (\( Jf t 2s^{−/−} \) and Shh−/− mice\(^{5,17} \)). An alternative, but not mutually exclusive, hypothesis is that acute \( E_{\text{clu}} \) regulation by G-protein–coupled receptors and growth factors dynamically regulates ciliary trafficking. Further studies are necessary to determine whether other intermediates in the SHH pathway, such as SUFU–SMO dissociation or GLI proteolytic processing, are \( Ca^{2+} \)-dependent. A second conclusion from this work is that the cilia funnels a small but steady \( Ca^{2+} \) load into the peri-ciliary cytoplasm.
METHODS SUMMARY

A ratimetric GCaMP3-based calcium sensor was expressed in cilia to measure ciliary calcium in IRPE1 cells. A transgenic mouse model expressing GFP in cilia was generated to measure ciliary calcium and membrane potential by patch-clamp recordings of cilia. Defects in SHH signalling of MEFs isolated from Pkd2l1−/− mutant mice were quantified by western blotting and ciliary localization of GLI1 and GLI2 proteins.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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METHODS
Molecular biology. The hArl13b clone was obtained from Open Biosystems and cloned into the pEGFP-N1 vector. Smo–EGFP, GCaMP2, and GCaMP3 were obtained from Addgene. GCaMP1.2 is an improved version of GCaMP3 (ref. 20). GCaMP3 was spliced in frame to the 3′ end of Smo. The ratiometric sensor Smo–mCherry–GCaMP3 was obtained by adding short glycine-serine linkers to the 5′ and 3′ ends of mCherry by PCR amplification. mCherry was cloned non-directionally into mSmo–GCaMP3 after linearization with AgeI. Correct orientation of mCherry was confirmed by sequencing. The same approach was used to add mCherry–GECO1.2 to hArl13b.

Transgenic and Pkd2l1 knockout animals. We injected both a human Arl13b–EGFP cDNA construct and a hArl13b–mCherry–GECO1.2 cDNA construct under the control of a chicken actin promoter (CAG) into the pronucleus of mouse C57BL/6 oocytes and obtained two independent founder lines for Arl13b–EGFP and four independent founder lines for Arl13b–mCherry–GECO1.2. Transgenic males and females were viable and fertile. The Sprague–Dawley line was used in all experiments. Individual male and female mice were genotyped by PCR. The age at which we observed cilia calcium oscillations in Pkd2l1−/− mice was as described previously13. P21 Pkd2l1−/− or wild-type littermate animals were obtained from Jackson Laboratories and have been described previously2. Mice were genotyped by PCR.

Immunohistochemistry. 15 μm formalin-fixed frozen tissue sections were permeabilized with 0.5% Triton X-100/PBS pH 7.4 for 15 min. Sections were blocked with 5% goat serum, 0.1% fish gelatin, 0.1% Triton X-100 and 0.05% Tween20. For primary mouse antibodies, endogenous mouse IgG was blocked by incubating sections with the unconjugated Fab fragment goat anti-mouse IgG for 1 h at room temperature. Sections were washed twice in PBS-T and primary antibodies were diluted in blocking solution and incubated overnight at 4 °C. Slides were washed twice in PBS-T and goat anti-rabbit/anti-mouse fluorescent-labelled secondary antibodies were applied at room temperature for 1 h together with Hoechst 33342 nuclear dye. Sections were washed twice in PBS-T, mounted in Prolong Gold Antifade (Life Technologies) and imaged with an Olympus FX1000; water immersion ×60, 1.2 N.A. objective. Images were further processed using ImageJ (NIH).

Electrophysiology. Unless otherwise stated, all experimental conditions and methods are described in ref. 5. For the experiments in Fig. 3d, intracellular free [Ca2+]i was calculated using the MaxChelator website (http://maxchelator.stanford.edu) and formulated by titrating CaCl2 in 10 mM BAPTA-Ca buffering conditions. The resting membrane potential measurements from the cell and cilia were made in current-clamp mode. Electrical access was measured in the perforated-patch configuration using amphotericin B. Electrodes contained (in mM): 95 K-aspartate, 50 K-gluconate, 30 KCl, 10 HEPS, 2 NaCl, 2 MgCl2, 5 EGTA, 100 mM free Ca2+. Extracellular solutions in Fig. 3e contained one of following ratios of NaCl/HEPS (in mM): 140/5, 135/10, 125/20, 75/70, 5/145; and 10 HEPS, 1 MgCl2 and 1.8 CaCl2. Intracellular [Ca2+]i was equilibrated with the known pipette [Ca2+]i in whole-cilia recordings. Note that increasing [Ca2+]i in the tip inhibited the dye diffusion. The dose–response curve was fitted to the Hill equation. The IC50 ([Ca2+]i) concentration at which current is inhibited 50% = 445 ± 18 mM and was used as a calibration curve. We estimated resting free [Ca2+]i by comparing this curve to the current amplitude measured in perforated patches (where intracellular calcium levels are supposed to be low) and considered this a significant. Cytoplasmic [Ca2+]i was raised by loading hRPE1 SMO–mCherry–GCaMP3 cells with o-nitrophenyl EGTA AM (NP-EGTA AM) for 30 min at room temperature. Calcium was uncaged in the cytoplasm near the base of the cilia with a SIM scanner coupled to a 405-nm laser. The region of interest (ROI) for photobleaching was chosen to cover the tip of the cilium and 

Three-dimensional reconstruction of cilia. E14.5 embryo was fixed in 4% PFA for 48 h and dissected. Tissue was treated with Scale solutions as described8. In brief, samples were incubated in ScaleA2 and ScaleB4 until they appeared transparent (3 weeks–3 months). Samples were mounted in 2% agarose in ScaleA2 on a Petri dish. Samples were imaged in ScaleA2 with an XLUMPLFL 25× water immersion, 1.2 NA. objective. Images were further processed using ImageJ (NIH) and Imaris software (Bitplane AG). The region of interest (ROI) for photobleaching was chosen to cover the tip of the cilium and 

Immunohistochemistry and confocal microscopy. Cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked by 10% goat serum in PBS. Cells were incubated with indicated antibody and secondary goat anti-rabbit or anti-mouse IgG (Life Technologies) and Hoechst 33342 (Life Technologies). Confocal images were obtained using an Olympus FX1000 with a 60× water immersion, 1.2 N.A. objective. Images were further processed using ImageJ (NIH). Differential interference contrast images (DIC) are shown to outline cells. 

Cell culture and generation of stable cell lines. hRPE1 cells were transfected using TransIT-LT1 (Mirus Bio). SMO–EGFP expressing hRPE1 cells have been described previously2. Stable cells were selected in DMEM/F12 (Life Technologies) containing 414 (400 μg ml−1) added 24 h after transfection. Stable cell lines expressing the protein were enriched by fluorescence-activated cell sorting (FACS) 2–4 weeks after initial selection. For SAG (Calbiochem) stimulation experiments, MEFs were serum-starved at 80% confluency in 5%–7% CO2 for 21 h in DMEM/0.2% FCS. MEF cells were stimulated and protein levels were analysed as described1. In brief, MEFs were stimulated with 400 nM SAG for 24 h at 37 °C. Cells were washed ×1 with PBS and lysed directly in 2× sample buffer (Life Technologies). Western blots were developed using chemiluminescence (Super Signal West Dura, Pierce Thermo) and a LAS-3000 imaging system (Fujiﬁlm). Detection of RNA levels was performed as described previously15.
Calibration of the ratiometric SMO–mCherry–GCaMP3 sensor. Standard solutions of various \([\text{Ca}^{2+}]\) concentrations were prepared ranging from \(-10\ \text{nM}\) to \(50\ \text{μM}\) by adjusting the ratio of EGTA and CaCl2 in each preparation to clamp-free \([\text{Ca}^{2+}]\) at the desired value. The solutions contained \(137\ \text{mM}\ \text{NaCl}, 5.4\ \text{mM}\ \text{KCl}, 10\ \text{mM}\ \text{HEPES}, 5\ \text{mM}\ \text{EGTA}\) and \(\text{CaCl}_2\) ranging from \(0\) to \(5.04\ \text{mM}\) (corresponding to \(50\ \text{μM}\) free \([\text{Ca}^{2+}]\)).

For imaging, hRPE1 cells were plated onto \(12\)-mm glass coverslips, serum-starved for 4–5 days to allow for cilia formation, and imaged in the various standard solutions following digitonin membrane permeabilization. Briefly, coverslips were washed twice in \(\text{Ca}^{2+}\)-free solution to remove residual \(\text{Ca}^{2+}\) and incubated in the \(\text{Ca}^{2+}\) standard solution. The samples were then imaged using an Olympus Fluoview FV1000 laser point-scanning confocal microscope (60× water immersion, 1.2 N.A. objective) with spectral detectors set up for optimal detection of GFP and mCherry fluorescence with sequential excitation with 488 nm and 543 nm lasers, respectively. The settings were adjusted for a GCaMP3 signal in the 1,000 sub-range and mCherry signal in the 2,000 sub-range of the 16-bit intensity range. Identical settings were used for all \(\text{Ca}^{2+}\) standard solutions. Subsequently, the cells were permeabilized on the microscope stage by addition of an identical volume (0.5 ml) of \(32\ \text{μM}\) digitonin dissolved in the same \(\text{Ca}^{2+}\) standard, resulting in a final concentration of \(\sim 16\ \text{μM}\) digitonin. Images were acquired for multiple fields of view after allowing permeabilization to occur for \(\sim 1\ \text{min}\). Cytoplasmic \([\text{Ca}^{2+}]\) was measured in RPE cells that had not formed a cilium and thus had significant levels of SMO–mCherry–GCaMP3 protein in the plasma membrane.

To obtain the standard calibration curve, the acquired images were processed with ImageJ in the following way. Briefly, after background subtraction, the images were thresholded in the mCherry channel to only take into account pixels with a minimum expression level of the sensor (a threshold of \(20\times\) background signal was generally used). Dividing the GCaMP3 fluorescence image by the mCherry channel intensity generated ratio images. ROIs exclusively located in cilia of multiple cells were selected, and the average ratios measured for multiple cells and coverslips were reported. The average ratios obtained were plotted as a function of free \([\text{Ca}^{2+}]\) and the resulting points were fitted with a sigmoid curve. Images in Fig. 3a–d were acquired using the same acquisition settings as in Extended Data Fig. 5.

For quantification of GLI2 at ciliary tips, MEFs isolated from \(\text{Arl13b EGFP}^{\text{tg}}\) and \(\text{Pkd2l1}^{-/-}\) \(\text{Arl13b EGFP}^{\text{tg}}\) mice were stained with anti-GLI2 antibody. Cilia were outlined in ImageJ based on the EGFP signal. Mean background signal in the GLI2 channel was determined using ImageJ, multiplied by 1.2 and subtracted from the image. Integrated fluorescence intensity was measured within the ciliary outline. GLI2 quantification was compiled from measurements of 40 cilia from MEFs each isolated from three wild-type and five \(\text{Pkd2l1}^{-/-}\) embryos.

Data analysis. Group data are presented as mean ± s.e.m. Statistical comparisons were made using unpaired t-tests (Origin 8). Statistical significance is denoted with asterisk (*\(P < 0.05\); **\(P < 0.01\)).

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Extended Data Figure 1 | ARL13B–EGFP identifies primary and motile cilia in transgenic mouse tissue. a–l, Frozen tissue sections were prepared from P0 intestine (a–c); E15 nasal cavity (d–f); 6-week-old hippocampus (g–i); P0 retina from Arl13b-EGFP<sup>tg</sup> mice (j–l). First column, Arl13b-EGFP<sup>tg</sup> (green) fluorescence; second column (red) labels villin in b, ACIII in e, h, or HMB-45 (human melanoma black antibody, retinal pigmented epithelial cells) in k. a–c, Intestine: EGFP fluorescence in a does not overlap with the anti-villin staining in b as shown in the merged image in c, indicating that ARL13B–EGFP is absent from microvilli (arrows). Scale bar: 100 μm. d–f, Nasal cavity: (d) several ARL13B–EGFP-positive cilia (arrows) that face the lumen of the turbinate co-localize with ACIII (e) as shown in the merged image (f). Scale bar: 100 μm. Inset shows magnification of nasal cavity surface. Scale bar of inset in f, 5 μm. g–i, CA1 region of hippocampus: (g) prominent cilia (arrows) overlap with ACIII staining (h) as shown in the merged image (i). Scale bar: 50 μm. Inset shows magnification of hippocampal cilia. Inset scale bar: 5 μm. Merged red and green channels were offset for clarity (inset). j–l, Retinal pigmented epithelium (RPE): (j) short cilia (arrow) are visible at the intersection between RPE (labelled with HMB-45 antibody) and (k) developing photoreceptor cells, as shown in the merged image (l). Scale bar: 10 μm. Inset shows magnification of RPE–photoreceptor interface. Inset scale bar: 2 μm.
Extended Data Figure 2 | ARL13B–EGFP labels a primary cilium in cultured MEF and RPE cells. a–l, The first column shows ARL13B–EGFP\(^{tg}\) (green) fluorescence; second column labels ACIII (b, e) or acetylated tubulin (h, k). Third column: merged red and green channels were offset for clarity. Primary MEFs of Arl13b-EGFP\(^{tg}\) (a–c) and wild-type (d–f) mice isolated from E14.5 embryos. ARL13B–EGFP co-localizes with ciliary ACIII in c. MEFs isolated from wild-type mice show no fluorescence in the cilium (488 nm excitation). g–i, Primary RPE cells isolated from P12 Arl13b-EGFP\(^{tg}\) mice. Cells were fixed and stained with antibody to acetylated tubulin. ARL13B–EGFP (g) exclusively localized to the primary cilium identified by antibody to acetylated tubulin (h, i). j–l, Stable cell line (hRPE1) expressing SMO–EGFP. After 2 days of serum starvation, SMO–EGFP labelled the primary cilium of hRPE1 cells (j) as indicated by acetylated tubulin labelling (k, l). Scale bars: c, f, i, l, 5 \(\mu\)m. m, n, To determine whether ARL13B–EGFP expression adversely affected ciliogenesis, ciliary length and per cent of cells with cilia were quantified from wild-type and ARL13B–EGFP-expressing MEFs stained by anti-acetylated tubulin. m, Ciliary length was similar in wild-type and ARL13B–EGFP-expressing MEFs (2.6 ± 0.5 \(\mu\)m versus 2.9 ± 0.8 \(\mu\)m, respectively, \(n = 200\)). n, The number of cells with cilia was also comparable between wild-type and ARL13B–EGFP-expressing MEFs (60.2% ± 5.1% versus 65.5% ± 7.3%; \(n = 120\)).
Extended Data Figure 3 | Ciliary \( \text{[Ca}^{2+}\text{]}_c \) changes in stably transfected RPE cell cilia. 

a–c, Live hRPE1 cells stably expressing SMO–GCaMP3 were treated with 5 \( \mu \text{M} \) ionomycin. Fluorescence increases were measured in the cilium and cytoplasm. Image in c is DIC. 

d, Changes in fluorescence (\( \Delta F/F \)) of the calcium sensor, GCaMP3, are plotted for both cytoplasm and cilium. 

e–h, hRPE1 cells expressing SMO–mCherry–GCaMP3 were stained with acetylated tubulin. GCaMP3 (e) and mCherry (f) fluorescence overlays with acetylated tubulin staining (g, h). Scale bar: 5 \( \mu \text{m} \); merged channels were offset for clarity. 

i, The tip of a cilium was ruptured with an intense 1-s laser pulse (405 nm, hRPE1 cell expressing SMO–mCherry–GCaMP3). Circle indicates area of rupture. Numbered arrowheads indicate positions where changes in fluorescence were measured. Asterisk indicates mCherry fluorescence outside of cilium, indicating that some SMO–mCherry–GCaMP3 is retained in the ER. 

j, Quantification of changes in fluorescence at the positions marked in i. Rupture of the ciliary membrane rapidly increases \( \text{[Ca}^{2+}\text{]}_c \) at the tip and travels along the cilium at 4.6 \( \pm \) 0.6 \( \mu \text{m/s} \) (\( n = 16 \)).
Extended Data Figure 4 | [Ca$^{2+}$]$_{i}$ calibration. a, Images of hRPE1 cells stably expressing SMO–mCherry–GCaMP3 were acquired after permeabilization with 15 μM digitonin in varying extracellular [Ca$^{2+}$]. b, Averages of several ratios ($n = 12–16; \pm$ s.d.) per concentration are plotted against free [Ca$^{2+}$], yielding the calibration curve for SMO–mCherry–GCaMP3: $K_d = 625$ nM.
Extended Data Figure 5 | Overexpressed and endogenous PKD2L1 localizes to the primary cilium. a–c, Rabbit anti-PKD2L1 (Thermo Scientific) recognizes overexpressed PKD2L1. HEK cells were transfected with hPKD2L1-IRES mCherry construct and stained with PKD2L1 antibody. PKD2L1 staining (a) is specific to cells that also express mCherry (b). c, Overlay. d–f, Overexpressed hPKD2L1 localizes to the primary cilium in mIMCD3 cells. mIMCD3 cells were transfected with HA-tagged hPKD2L1 and stained with an anti-HA antibody (d) and anti-acetylated tubulin antibody (e). HA immunoreactivity is visible both in the cytoplasm and in the cilium. f, Overlay. g, h, PKD2L1 antibody labels the primary cilium of mIMCD3 cells. g, Confluent mIMCD3 cells were stained with anti-PKD2L1 antibody used in a; and h, acetylated tubulin antibody to label cilia. PKD2L1 immunoreactivity is visible in the primary cilium. i, Overlay. j–o, Primary MEFs of Arl13b-EGFP$^{+/}$ (j–l) and Arl13b-EGFP$^{+/}$ × Pkd2l1$^{-/-}$ mice (m–o) isolated from E14.5 embryos were stained with anti-PKD2L1 antibody used in a. In Arl13b-EGFP$^{+/}$ MEFs, PKD2L1 immunoreactivity (j) co-localizes with ARL13B–EGFP signal (k) labelling the primary cilium. l, Overlay. m–o, PKD2L1 staining is absent in cilia of Arl13b-EGFP$^{+/}$ × Pkd2l1$^{-/-}$ mice. Scale bars: 10 μm.
Extended Data Figure 6 | $[\text{Ca}^{2+}]_\text{cilia}$ increases 24 h after SAG stimulation.

**a**, MEFs expressing ARL13B–mCherry–GECO1.2 were stained with acetylated tubulin. GECO1.2 and mCherry fluorescence overlaps with acetylated tubulin staining. Scale bar: 5 μm; merged channels were offset for clarity.

**b**, Ratio maps of MEFs isolated from ARL13B–mCherry–GECO1.2 mice stimulated with 0.05% DMSO (left) or 400 nM SAG (right) for 24 h. Scale bar: 5 μm.

**c**, Quantification of ciliary GECO1.2/mCherry ratios obtained for MEFs with and without SAG stimulation. Ratio increases from 0.4 ± 0.05 to 0.8 ± 0.2 after SAG stimulation (*P < 0.05; n = 20–30 cilia). **d**, Example ciliary current measured from MEFs treated with 500 nM SAG (SMO agonist) or with DMSO vehicle (0.05%) in culture for 24–36 h in control conditions and after activation with 10 μM calmidazolium (CMZ). **e**, Scatter and whisker (± s.d.) plots from cilia show total outward (+100 mV) and inward (−100 mV) current measured for both treatment groups. Averages are indicated by the thick horizontal lines and individual cillum current magnitudes are represented as circles. *P values resulting from Student’s t-test comparing treatment groups are indicated (*< 0.05; n = 11 cilia).