Regulated trafficking of G protein-coupled receptors (GPCRs) controls cilium-based signaling pathways. β-Arrestin, a molecular sensor of activated GPCRs, and the BBSome, a complex of Bardet–Biedl syndrome (BBS) proteins, are required for the signal-dependent exit of ciliary GPCRs, but the functional interplay between β-arrestin and the BBSome remains elusive. Here we find that, upon activation, ciliary GPCRs become tagged with ubiquitin chains comprising K63 linkages (UbK63) in a β-arrestin-dependent manner before BBSome-mediated exit. Removal of ubiquitin acceptor residues from the somatostatin receptor 3 (SSTR3) and from the orphan GPCR GPR161 demonstrates that ubiquitination of ciliary GPCRs is required for their regulated exit from cilia. Furthermore, targeting a UbK63-specific deubiquitinase to cilia blocks the exit of GPR161, SSTR3, and Smoothened (SMO) from cilia. Finally, ubiquitinated proteins accumulate in cilium of mammalian photoreceptors and Chlamydomonas cells when BBSome function is compromised. We conclude that Ub chains mark GPCRs and other unwanted ciliary proteins for recognition by the ciliary exit machinery.

Introduction

The regulated trafficking of signaling receptors in and out of cilia is a central regulatory mechanism of many cilium-based signaling pathways (Nachury and Mick, 2019; Anvarian et al., 2019; Mykytyn and Askwith, 2017; Gigante and Caspary, 2020). For example, upon activation of the Hedgehog signaling pathway, the Hedgehog receptor Patched 1 and the G protein-coupled receptor (GPCR) GPR161 disappear from cilia, while the GPCR Smoothened (SMO) accumulates in cilia (Rohatgi et al., 2007; Corbit et al., 2005; Mukhopadhyay et al., 2013). In all three cases, regulated exit from cilia plays a major role in the on-demand redistribution of signaling molecules (Nachury and Mick, 2019). Signal-dependent exit is likely to be a general characteristic of ciliary GPCRs as the somatostatin receptor 3 (SSTR3), the dopamine receptor 1 (D1R), the melanocortin concentrating hormone receptor 1 (MCHRI), and the neuropeptide receptor 2 (NPY2R) all disappear from cilia upon exposure to agonist (Domire et al., 2011; Løkter and Jackson, 2013; Nager et al., 2017; Green et al., 2015). A major question is how GPCRs are selected for removal from cilia in an activity-dependent manner.

The major trafficking entities mediating signal-dependent exit from cilia are the BBSome and β-arrestin 2. The BBSome is an evolutionarily conserved complex of eight Bardet–Biedl syndrome (BBS) proteins that directly recognizes intracellular determinants in GPCRs and ferries them out of cilia (Nachury, 2018; Wingfield et al., 2018). The BBSome associates with the intraflagellar transport (IFT) machinery and has been proposed to act as adaptor between the motor-driven IFT trains and the membrane proteins that are to be removed from cilia. Because the BBSome is not known to have the ability to discriminate active from inactive GPCRs, there must exist another layer of regulation that commits activated GPCRs for exit. β-Arrestin 2 is a well-established molecular sensor of the activation state of GPCRs that is required for the signal-dependent exit of GPR161 and SSTR3 (Pal et al., 2016; Green et al., 2015; Nager et al., 2017). No association of β-arrestin 2 with ciliary trafficking complexes has been reported to date, and it remains unclear how β-arrestin 2 relays information regarding the state of activation of ciliary GPCRs to the ciliary exit machinery.

An emerging player in ciliary exit is ubiquitination (Shearer and Saunders, 2016). Ubiquitin (Ub), a 76-amino acid polypeptide, becomes conjugated to acceptor lysine residues on substrate proteins by Ub ligases and tags substrates for degradation or other regulatory fates (Yau and Rape, 2016; Swatek and Komander, 2016). A role for Ub in promoting ciliary exit is suggested by multiple lines of evidence. First, interfering with Patched1 ubiquitination blocks its signal-dependent exit from cilia (Kim et al., 2015; Yue et al., 2014). Second, fusing Ub to PKD-2, the olfactory receptor ODR-10, or the transient receptor potential channel OSM-9 at their cytoplasmic end results in the...
disappearance of these proteins from cilia (Hu et al., 2007; Xu et al., 2015). The accumulation of these Ub fusions inside cilia when BBSome function is compromised suggests that the BBSome might sort ubiquitinated signaling receptors out of cilia, in line with a reported association of BBSome with ubiquitinated proteins in trypanosomes (Langousis et al., 2016). Interestingly, TRIM32 is a Ub ligase mutated in BBS patients (Chiang et al., 2006). Third, the Ub ligase Cbl is recruited to cilia upon activation of the ciliary tyrosine kinase receptor platelet-derived growth factor receptor aa (PDGFRaa), and Cbl is required for termination of PDGFRaa signaling (Schmid et al., 2018). Last, a dramatic rise in ubiquitination of the ciliary proteome is determined (Liew et al., 2014; Ye et al., 2018). As in previous studies (Ye et al., 2018; Nager et al., 2017), all GPCRs were expressed at near-endogenous levels by driving expression with extremely weak promoters. The fluorescent protein mNeon-Green (NG) fused to the cytoplasm-facing C terminus of GPCRs allowed direct visualization. A biotinylation acceptor peptide (AP) fused to the extracellular N terminus biotinylated by an ER-localized biotin ligase (BirA-ER) enabled pulse labeling of surface-exposed molecules and denaturing purifications. When SSTR3 was expressed in Arl6−/− IMCD3 cells, addition of its agonist somatostatin (sst) led to a drastic increase in the ciliary levels of Ub observed via immunostaining with the well-characterized FK2 (Fig. 1, A and B) and FK1 (Fig. S1, A and B) monoclonal antibodies (Fujimuro et al., 1994; Emmerich and Cohen, 2015; Haglund et al., 2003). All further experiments were conducted with the FK2 antibody. Arl6−/− cells accumulated Ub signals inside cilia in the absence of SSTR3 expression (Fig. 1, A and B), demonstrating that some endogenous proteins become ubiquitinated inside the cilia of Arl6−/− IMCD3 cells. No Ub signal was detected inside cilia when BBSome function was intact (Fig. 1, A and B; and Fig. S1, A–D), indicating that the BBSome efficiently removes ubiquitinated proteins from cilia. Because the sst-dependent increase in ciliary Ub signal depends upon SSTR3 expression (Fig. 1, A and B), these results suggest that either SSTR3 itself or a downstream effector of SSTR3 becomes ubiquitinated inside cilia upon activation.

To determine whether SSTR3 becomes ubiquitinated in response to sst, we biochemically isolated ApSSTR3NG under denaturing conditions via the biotinylated AP tag and probed for Ub via an HA tag on transfected HA-Ub. SSTR3 migrated as a broad band—as typical for a glycosylated protein—centered around 100 kD (Fig. 1C). The amount of SSTR3 recovered did not change appreciably between WT and Arl6−/− cells or upon stimulation with sst (Fig. 1C). Remarkably, while only very faint Ub signals were detected associated with SSTR3 in WT cells, a Ub smear extending from 100 kD upward was detected in the SSTR3 pull-downs from Arl6−/− cells. Furthermore, stimulation with sst resulted in a modest but reproducible increase in SSTR3 ubiquitination in Arl6−/− cells (Fig. 1D). We conclude that the BBSome/Arl6 system is required for the degradation of ubiquitinated SSTR3 and that SSTR3 becomes ubiquitinated in response to stimulation with sst.

We next sought to test whether signaling receptors endogenous to IMCD3 cells are ubiquitinated inside cilia in an activity-dependent manner. One signaling pathway that is natively expressed in IMCD3 cells is the Hedgehog pathway. Prior experiments have detected normal trafficking dynamics of SMO and GPR161 in IMCD3 cells (Ye et al., 2018; Mukhopadhyay et al., 2013; see Fig. 4D). As GPR161 and SMO both accumulate in cilia of Arl6−/− cells without hedgehog (Hh) pathway stimulation (Liew et al., 2014; Zhang et al., 2011), the Ub signal detected in Arl6−/− cilia may correspond to Ub conjugated to SMO or GPR161. We detected a significant elevation of the ciliary Ub levels in Arl6−/− cells when treated with the SMO agonist SAG (Fig. 1E and F). Because SMO ubiquitination was previously shown to decrease upon its activation (Xia et al., 2012; Jiang et al., 2019), it is likely that the elevated ciliary Ub signal detected in SAG-treated cells is associated with GPR161. As activation of SMO triggers exit of GPR161 but this exit is frustrated when BBSome function goes awry, these data suggest that GPR161 becomes ubiquitinated before its BBSome-dependent exit. Collectively, these data suggest that GPCRs are ubiquitinated in a regulated fashion inside cilia and subsequently retrieved into the cell by the BBSome.
To test the functional importance of GPCR ubiquitination in BBSome-mediated exit from cilia, we removed all ubiquitination sites from SSTR3 and GPR161 by mutating all cytoplasm-exposed lysine residues to arginine (cK0 variants). Exit kinetics were precisely monitored by real-time tracking of individual cilia in live cells. In both cases, the cK0 variant underwent markedly slower signal-dependent exit from cilia than the WT allele (Fig. 2, A and B; and Fig. S2, A and B). Measurement of exit rates revealed that exit of SSTR3cK0 was approximately twofold slower than SSTR3 and that exit of GPR161cK0 was ∼2.5-fold slower than GPR161. The residual exit rates of the cK0 mutants indicate the existence of alternative mechanisms of exit that complement GPCR ubiquitination or bypass BBSome-dependent retrieval (see Discussion).

To rule out that the observed ciliary exit defect of the cK0 mutants was an indirect consequence of defective endocytosis, for example, because of clogging of the ciliary exit path, we directly assessed endocytosis of SSTR3 and SSTR3cK0. The surface-exposed pool of A5SSTR3 was pulse labeled with fluorescently labeled monovalent streptavidin (mSA), and cells were...
Figure 2. Ubiquitination of ciliary GPCRs is required for signal-dependent exit but not for endocytosis. (A) IMCD3-[pEF1α−/−SSTR3] cells stably expressed the BirA-ER to enable the biotinylation of α5SSTR3. SSTR3 was either the WT allele or a variant where all five cytoplasm-facing lysine residues (listed in Materials and methods) were mutated to arginine (cK0). Ciliary α5SSTR3 was pulse labeled by addition of Alexa Fluor 647-conjugated mSA (mSA647) to the medium for 5–10 min before addition of sst. Far red fluorescence was tracked in individual cilia at 10-min capture intervals. For each individual cilium, fluorescence intensities were normalized to the value at time (t) = 0. A comparison of the ciliary levels of SSTR3 at t = 0 is shown in Fig. S2 A. Data were plotted and fitted to a line. Error bars, 95% CI. n = 18–22 cilia. (B) GPR161 fused to three tandem repeats of NG at its C terminus was expressed under the control of the ultra-weak pCrys in IMCD3 cells. GPR161 was either the WT allele or a variant where all 18 cytoplasm-facing lysine residues (listed in Materials and methods) were mutated to arginine (cK0). IMCD3-[pCrys-GPR161NG] cells were treated with SAG for 2 h. During the course of the experiment, NG fluorescence was tracked in individual cilia at 10-min capture intervals. Fluorescence data were acquired and analyzed as in A. A comparison of the ciliary levels of GPR161 at t = 0 is shown in Fig. S2 B. Error bars, 95% CI. n = 18–22 cilia.

10–19 cilia. (C) IMCD3-[α5SSTR3; BirA-ER] cells expressing either WT or cK0 SSTR3 were pulse-labeled by addition of Alexa Fluor 647-conjugated mSA (mSA647) to the medium for 5 min before addition of sst and were imaged by far red fluorescence immediately after addition of sst (t0) and 10 min later (t10). The contrast level was adjusted to reveal plasma membrane–localized and internalized α5SSTR3, causing the cilia-localized α5SSTR3 signal to reach saturation. Scale bar, 5 μm. (D) Internalized α5SSTR3 foci were counted immediately after addition of sst (t0) and 10 min later (t10). n = 34 cells.

Stimulated with sst. The faint hazy signal corresponding to the plasma membrane pool of SSTR3 disappeared after 10 min in the presence of sst in a similar fashion for both WT and cK0 variants (Fig. 2 C). Concurrently, the WT and cK0 variants appeared in cytoplasmic foci corresponding to endocytic vesicles (Fig. 2 C). Counting cytoplasmic foci revealed that endocytosis proceeded normally irrespective of the ubiquitination competence of SSTR3 (Fig. 2 D). SSTR3 thus behaves similarly to nearly every GPCR tested to date, in that it does not require ubiquitination for its signal-dependent internalization. Indeed, while ubiquitination of plasma membrane proteins is a major driver of internalization in yeast, ubiquitination of signaling receptors is generally dispensable for signal-dependent internalization in mammalian cells (Dores and Trejo, 2019; Piper et al., 2014; Skierska et al., 2017).

These results strongly support a direct role for GPCR ubiquitination in promoting signal-dependent exit from cilia.

Ciliary UbK63 linkages are required for GPCR exit

We next sought to characterize the type of Ub conjugates that are attached to ciliary proteins. The FK1 and FK2 antibodies used in our immunofluorescent studies recognize Ub either as single adduct or in chain but not free Ub (Fujimuro et al., 1994; Emmerich and Cohen, 2018). The availability of antibodies specific for UbK48 and UbK63 linkages (Newton et al., 2008) enabled us to determine the types of Ub conjugates that are attached to ciliary GPCRs. While we found no detectable signal for UbK48 in cilia, the signal for UbK63 linkages mirrored the ciliary response observed with the FK1 and FK2 antibody in IMCD3-[SSTR3] cells subjected to agonist treatment (Fig. 3, A and B; and Fig. S1, C and D). Given the high specificity and selectivity of the K48 and K63 linkage antibodies, these data strongly suggest that UbK63 chains are added onto SSTR3 inside cilia upon activation.

To confirm that K63 of Ub is the main linkage used for the elongation of Ub chains on SSTR3, we transfected variants of Ub with lysines mutated to arginines into Arl6−/− cells stably expressing SSTR3. Signal-dependent ubiquitination of SSTR3 was abrogated when all seven lysines of Ub were mutated to arginines (Fig. 3, C and D). However, when K63 was the only lysine left intact on Ub, signal–dependent ubiquitination of SSTR3 was restored to the same extent as with WT Ub (Fig. 3, C and D). We conclude that the Ub chains assembled onto SSTR3 in response to SSTR3 activation inside cilia comprise K63 linkages. While UbK48 linkages were not detected inside cilia, it remains possible that mixed chains containing K63 linkages as well as other linkages besides K48 are assembled onto SSTR3 inside cilia.

To determine the functional importance of UbK63 linkages in tagging GPCRs for exit from cilia, we sought to specifically interfere with UbK63 linkages inside cilia (Fig. 4 A). Structural and biochemical studies have demonstrated that the deubiquitinase AMSH (associated molecule with the SH3 domain of STAM)
possesses a near-absolute specificity for UbK63 linkages and
does not cleave other Ub chain linkages or Ub-substrates link-
ages (Sato et al., 2008; McCullough et al., 2004). AMSH nor-
mally functions on the surface of late endosomes in concert with
the endosomal sorting complex required for transport (ESCRT)
protein STAM (McCullough et al., 2006). AMSH was previously
fused to the epidermal growth factor receptor (EGFR) to dem-
strate that UbK63 chains assembled on EGFR are dispensable
for internalization but required for sorting into lysosomes
(Huang et al., 2013). We targeted AMSH to cilia using the well-
validated ciliary targeting signal from the ciliopathy protein
NPHP3 (Nakata et al., 2012; Wright et al., 2011) and analyzed the
validated ciliary targeting signal from the ciliopathy protein
(huang et al., 2013). we targeted amsh to cilia using the well-
validated ciliary targeting signal from the ciliopathy protein
(nphp3; birA-ER) cells were transfected with the HA-tagged
ubiquitination. the signals of HA-ub conjugated to SSTR3 in the
streptavidin eluates were measured. the experiment shown in C
was captured on streptavidin resin. Eluates were probed for HA
by immunoblotting and for biotin via streptavidin-HRP. Two
major biotinylated proteins endogenous to cells are marked by
asterisks. Whole cell lysates were probed for Arl6 and, as a
loading control, actin. A nonspecific band cross-reacting with the
anti-Arl6 antibody is marked with a dot. WT IMCD3 cells were
processed in parallel as a control. (D) Quantitation of SSTR3 ubiquitination. The signals of HA-ub conjugated to SSTR3 in the
streptavidin eluates were measured. the experiment shown in C
was repeated four times, and for each Ub variant, ubiquitin
levels were normalized to the value at t = 0 of sst stimula-
tion and plotted as gray circles. the horizontal blue lines rep-
resent mean values. asterisks indicate ANOVA significance
value. ****, P < 0.0001. No ciliary signal is detected with the UbK48 linkage-specific antibody. (C) Arl6+/- IMCD3-[
[SSTR3; BirA-ER] cells were transfected with the HA-tagged
ubiquitination. the signals of HA-ub conjugated to SSTR3 in the
streptavidin eluates were measured. the experiment shown in C
was repeated four times, and for each Ub variant, Ub-SSTR3
levels were normalized to the value at t = 0 of sst stimula-
tion and plotted as gray circles. the horizontal blue lines rep-
resent mean values. asterisks indicate ANOVA significance
value. ***, P < 0.001; **, P < 0.01. acTub, acetylated tubulin; ns,
not significant, RU, relative unit.

Figure 3. Signal-dependent accumulation of K63-linked Ub
linkages inside cilia of Bbs mutant cells. (A) IMCD3 cells of the
indicated genotypes expressing [SSTR3NG] were treated with
sst-14 for 2 h. Cells were fixed and stained for acTub (magenta)
and with antibodies specific for the lysine 63 (UbK63)
or lysine 48 (UbK48) Ub chain linkages (yellow). SSTR3 (cyan)
was imaged through the intrinsic fluorescence of NG. Channels
are shifted in the insets to facilitate visualization of overlapping
ciliary signals. Scale bar, 5 µm (main panel), 2 µm (inset).
(B) The fluorescence intensities of the UbK48 and UbK63
channels in the cilia are represented as violin plots. a 14-fold
increase in ciliary Ub abundance is detected with the
UbK63 linkage-specific antibody. Asterisks indicate ANOVA sig-
nificance value. ****, P < 0.0001. No ciliary signal is detected
with the UbK48 linkage-specific antibody. (C) Arl6+/- IMCD3-
[SSTR3; BirA-ER] cells were transfected with the HA-tagged
ubiquitination. the signals of HA-ub conjugated to SSTR3 in the
streptavidin eluates were measured. the experiment shown in C
was repeated four times, and for each Ub variant, Ub-SSTR3
levels were normalized to the value at t = 0 of sst stimula-
tion and plotted as gray circles. the horizontal blue lines rep-
resent mean values. asterisks indicate ANOVA significance
value. ***, P < 0.001; **, P < 0.01. acTub, acetylated tubulin; ns,
not significant, RU, relative unit.

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specific signaling stimulus, are required for GPCR removal from cilia.

**β-Arrestin 2 mediates signal-dependent ubiquitination of ciliary GPCRs**

As β-arrestin 2 is required for signal-dependent exit of GPR161 and SSTR3 from cilia (Green et al., 2015; Pal et al., 2016; Ye et al., 2018), we sought to determine the functional relationship between β-arrestin 2 and the BBSome. As previously described (Green et al., 2015), β-arrestin 2 is initially undetectable inside cilia and becomes recruited to cilia within minutes of SSTR3 agonist addition (Fig. 5, A–C). The ciliary β-arrestin 2 signal reaches a plateau after ~10 min, and the t½ for ciliary accumulation is <5 min (Fig. 5 C). Meanwhile, the t½ for BBSome recruitment to the tip of cilia was 10 min, and the first signs of detectable SSTR3 exit were seen between 10 and 15 min (Fig. 5 C). These kinetics suggested that β-arrestin 2 is first recruited to activated ciliary SSTR3 before the BBSome ferries activated SSTR3 out of cilia. When ARL6 was depleted, the basal levels of ciliary β-arrestin 2 were elevated but the kinetics of β-arrestin 2 accumulation in cilia upon SSTR3 activation were indistinguishable from control-depleted cells (Fig. 5, A and B). These data suggest that a small fraction of SSTR3 that is tonically active fails to exit cilia in ARL6-depleted cells and recruits β-arrestin 2. From these data, we conclude that β-arrestin 2 functions either upstream of, or in parallel with, the BBSome in signal-dependent retrieval of GPCRs. Similarly, the kinetics of GPR161 exit and β-arrestin 2 ciliary recruitment upon activation of the Hh pathway indicated that β-arrestin 2 reaches its maximal ciliary level before the first signs of GPR161 exit become evident (Fig. 5 D).
We previously proposed that β-arrestin 2 may bridge activated GPCRs to the BBSome (Ye et al., 2018; Nachury, 2018). Yet we have thus far failed to detect a biochemical interaction between β-arrestin 2 and the BBSome. Furthermore, the continuous distribution patterns of β-arrestin 2 and SSTR3 inside cilia are similar to one another and distinct from the discontinuous pattern of the BBSome (Fig. 5 A), arguing against a direct association of β-arrestin 2 with BBSome/IFT trains inside cilia. We considered the alternative hypothesis that β-arrestin 2 functions upstream of the BBSome. Given that β-arrestin 2 functions in the signal-dependent targeting of GPCRs from the plasma membrane to the lysosome by recruiting Ub ligases to activated GPCRs (Henry et al., 2012; Bhandari et al., 2007; Shenoy et al., 2008; Martin et al., 2003), we posited that β-arrestin 2 (β-arrestin 2) may bridge activated GPCRs inside cilia to the BBSome/IFT trains inside cilia. We have thus far failed to detect a biochemical interaction between β-arrestin 2 and the BBSome (Ye et al., 2018; Nachury, 2018). Yet we have thus far failed to detect a biochemical interaction between β-arrestin 2 and the BBSome. Furthermore, the continuous distribution patterns of β-arrestin 2 and SSTR3 inside cilia are similar to one another and distinct from the discontinuous pattern of the BBSome (Fig. 5 A), arguing against a direct association of β-arrestin 2 with BBSome/IFT trains inside cilia. We considered the alternative hypothesis that β-arrestin 2 functions upstream of the BBSome. Given that β-arrestin 2 functions in the signal-dependent targeting of GPCRs from the plasma membrane to the lysosome by recruiting Ub ligases to activated GPCRs (Henry et al., 2012; Bhandari et al., 2007; Shenoy et al., 2008; Martin et al., 2003), we posited that β-arrestin 2 recognizes activated GPCRs inside cilia to direct their ubiquitination and subsequent selection by the BBSome for removal from cilia. A central prediction of this model is that β-arrestin 2 is required for the ubiquitination of ciliary GPCRs in response to stimulation. To test this prediction, we deleted the β-arrestin 2 gene Arrb2 in Arl6 knockout IMCD3 cells (Nager et al., 2017). While the signal-dependent exit of SSTR3 from cilia failed in both Arl6−/− and Arl6−/−/Arrb2−/− cells (Fig. 1, A and B; and Fig. 6, A and B), Ub staining in cilia and by inference signal-dependent ubiquitination of SSTR3 was only observed in Arl6−/− cells (Fig. 6, A and B). In Arl6−/−/Arrb2−/− cells, the signal-dependent increase in ciliary Ub signal was no longer observed (Fig. 6, A and B). Similarly, the increase in ciliary Ub signal seen in Arl6−/− cells treated with the Hedgehog pathway agonist SAG was no longer observed in the absence of β-arrestin 2 (Fig. 6, C and D). These data indicate that, in the absence of BBSome function, ciliary GPCRs become ubiquitinated in response to activation in a β-arrestin 2-dependent manner. We sought to confirm our imaging-based finding with a biochemical analysis of SSTR3 ubiquitination.

While SSTR3 ubiquitination was readily detected in Arl6−/− cells and measurably increased upon stimulation with sst, the deletion of β-arrestin 2 in Arl6−/− cells drastically reduced SSTR3 ubiquitination levels, and no sst-dependent increase of SSTR3 ubiquitination was detected in Arl6−/−/Arrb2−/− cells (Fig. 6, E and F).

Together, these data suggest the following order of action: GPCR activation, β-arrestin 2 engagement, Ub ligase recruitment, assembly of UbK63 chains on the GPCR, and finally selection of ubiquitinated GPCRs for BBSome-mediated retrieval (Fig. 7 E).

Constitutively retrieved BBSome cargoes are ubiquitinated before exit

Besides removing GPCRs from cilia in a regulated fashion, the BBSome also clears cilia of unwanted proteins that accidentally enter cilia. In the single-cell flagellated organism C. reinhardtii, mutations in the BBSome subunit Bbs4 cause a constitutive accumulation of several proteins including phospholipase D in cilia (Lechtreck et al., 2009). When stained for Ub, Bbs4−C. reinhardtii showed a twofold enrichment in ciliary signal compared with WT (Fig. 7, A and B). Isolation of cilia revealed the accumulation of Ub conjugates above 80 kD in Bbs4 mutant C. reinhardtii cilia (Fig. 7 C). These results are consistent with the hypothesis that ciliary proteins subject to constitutive retrieval are ubiquitinated before their removal from cilia by the BBSome.

In the mammalian retina, proteomics studies have found >100 proteins accumulating in the outer segment (the equivalent of the cillum) of Bsh photoreceptors compared with WT photoreceptors (Datta et al., 2015). When stained sections of mouse retina for Ub, we found a drastic accumulation of Ub signal in the outer segment of Bsh4−/− photoreceptors compared with WT photoreceptors (Fig. 7 D). We note that the nature of Ub
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Ub (Langousis et al., 2016), although direct biochemical evidence trypanosomes suggest that the BBSome may directly recognize proteins for BBSome-mediated removal from cilia. Prior findings in linkages mark activated GPCRs and likely other unwanted pro-

cargoes. These results suggest that a variety of proteins that accidentally flow into the photoreceptors outer segments, possibly caught on the tremendous flux of rhodopsin trafficking to the outer segment (Baker et al., 2008), are recognized as foreign to the cilium, ubiquitinated on site, and selected for removal by the BBSome (Fig. 7 E).

**Discussion**

**Recognition of Ub chains by the ciliary exit machinery**

Our results indicate that Ub chains built at least in part with K63 linkages mark activated GPCRs and likely other unwanted proteins for BBSome-mediated removal from cilia. Prior findings in trypanosomes suggest that the BBSome may directly recognize Ub (Langousis et al., 2016), although direct biochemical evidence and information regarding Ub chain specificity is still missing. It is also known that the BBSome directly recognizes cytoplasmic determinants in the GPCRs it ferries out of cilia (Jin et al., 2010; Klink et al., 2017; Ye et al., 2018; See et al., 2011; Domire et al., 2011). We propose that the summation of weak molecular interactions between the BBSome and Ub chains on the one hand and the BBSome and GPCRs cytoplasmic loops on the other hand increases the BBSome–cargo interaction to enable sorting of ubiquitinated GPCRs out of cilia. In support of this bivalent recognition of ubiquitinated cargoes by the BBSome, the affinity of the BBSome for cytoplasmic determinants on GPCRs is in the micromolar range (Klink et al., 2017; Ye et al., 2018; Seo et al., 2011; Domire et al., 2011). We propose that the summation of weak molecular interactions between the BBSome and Ub chains on the one hand and the BBSome and GPCRs cytoplasmic loops on the other hand increases the BBSome–cargo interaction to enable sorting of ubiquitinated GPCRs out of cilia. In support of this bivalent recognition of ubiquitinated cargoes by the BBSome, the affinity of the BBSome for cytoplasmic determinants on GPCRs is in the micromolar range (Klink et al., 2017), and affinities of Ub-binding proteins for Ub typically reside in the sub-millimolar range (Husnjak and Dikic, 2012). The nature of the biochemical entity that directly recognizes Ub, and in particular UbK63 linkages, remains to be determined: while the BBSome was pulled down on Ub-agarose resin from trypanosome lysates (Langousis et al., 2016), no known Ub-binding domain is found in the BBSome polypeptides. Furthermore, the IFT-A subunit IFT-139 associates with ubiquitinated α-tubulin in ciliary extracts from C. rheinaudtii, suggesting a possible recognition of Ub by IFT139 (Wang et al., 2019). Again, the absence of a known

![Figure 6](https://doi.org/10.1083/jcb.202003020)
Ub-binding domain in the sequence of IFT139 presents a challenge to this hypothesis.

Requirements for UbK63 linkages in ciliary exit and sorting to the lysosome

Wang et al. (2019) found that α-tubulin is modified by UbK63 chains and not UbK48 chains upon ciliary disassembly. The Ub ligases that have been associated with ciliary clearance of Patched 1 and of PDGFRαα specifically assemble UbK63 chains (Kim et al., 2015; Yue et al., 2014; Schmid et al., 2018). The suppression of GPR161, SSTR3, and SMO exit by cilia-AMSH (Fig. 4) demonstrates that UbK63 chains are recognized inside cilia to direct trafficking out of cilia. The milder exit defect observed when acceptor lysines are mutated on ciliary GPCRs (Fig. 2) than when cilia-AMSH is expressed (Fig. 4) suggests that UbK63 linkages added onto other ciliary proteins besides GPCRs may participate in ciliary exit. A role for ubiquitination of the ciliary transport machinery in exit is in line with the functional importance of ubiquitination of the endolysosomal machinery in GPCR trafficking (Dores and Trejo, 2019). β-Arrestin ubiquitination promotes internalization of the β2-adrenergic receptor (β2AR; Shenoy et al., 2001, 2007; Shenoy and Lefkowitz, 2003), ubiquitination of the ESCRT components Hrs and STAM participates in sorting of the chemokine receptor CXCR4 into the multivesicular body (MVB; Malik and Marchese, 2010; Marchese et al., 2003), and ubiquitination of the ESCRT-III recruitment factor ALIX enhances MVB sorting of the protease-activated receptor 1 (Dores et al., 2015).

The requirement for UbK63 chains in signal-dependent exit from the cilium contrasts with the more limited role of Ub and UbK63 chains in signal-dependent endocytosis of signaling receptors at the plasma membrane in mammals. Although ubiquitination is a major driver of endocytosis in yeast (Stringer and Piper, 2011; Piper et al., 2014), foundational studies on EGFR trafficking found that signal-dependent receptor ubiquitination is not essential for internalization because it is redundant with other internalization mechanisms (Goh et al., 2010; Huang et al., 2013; Fortian et al., 2015). Studies of an EGFR-AMSH fusion found that UbK63 chains attached onto EGFR become increasingly necessary as EGFR progresses along the degradative route, and a strict requirement for K63-linked Ub chains is observed at the terminal step of EGFR sorting into the lumen of MVB (Huang et al., 2013). A strict requirement for receptor ubiquitination in degradative sorting but not in internalization can be generalized to nearly all GPCRs (Skieterska et al., 2017), namely β2AR (Shenoy et al., 2001), CXCR4 (Marchese and Benovic, 2001), µ opioid receptor (Hislop et al., 2011), κ opioid receptor (Li et al., 2008), δ opioid receptor (Henry et al., 2011), neurokinin-1 receptor (Cottrell et al., 2006), protease-activated receptor 2 (Jacob et al., 2005), and vasopressin receptor 2 (Martin et al., 2003).
Similarly, we find that SSTR3 endocytosis proceeds normally in the absence of SSTR3 ubiquitination (Fig. 2, C and D). Studying the importance of UbK63 linkages in mammalian cells via genomics remains challenging because of the multiple genes encoding Ub, but in yeast strains expressing UbK63R as the sole source of Ub, the Gap1 permease is internalized normally and yet fails to get sorted into the MVB (Lauwers et al., 2009).

The parallels between sorting at the late endosome and ciliary trafficking extend to β-arrestin–directed ubiquitination. While β-arrestin functions inside cilia to direct the addition of Ub onto activated SSTR3 and GPR161 (Fig. 5 and Fig. 6), β-arrestin–mediated ubiquitination selectively affects sorting at the level of endosomes rather that at the plasma membrane for activated CXCR4 (Bhandari et al., 2007), β2-AR (Shenoy et al., 2008), and vasopressin receptor 2 (Martin et al., 2003), and β-arrestin–mediated ubiquitination of CXCR4 was shown to take place on endosomes (Malik and Marchese, 2010). In this context, the recognition of K63-linked Ub chain inside cilia may befit the endosomal origin of primary cilia (Sorokin, 1962; Westlake et al., 2011; Mitchell, 2017).

The parallels between endosomal and ciliary trafficking lead us to speculate that some ubiquitinated proteins might be accidentally sorted to cilia instead of late endosomes. These proteins would then need to be retrieved from cilia by the BBSome. Thus, the Ub signal detected in photoreceptor outer segment and in C. rheinardtii flagella might result from the accidental import of ubiquitinated proteins into cilia rather than in situ ubiquitination of unwanted proteins inside cilia.

Potential coupling between BBSome-mediated exit and degradative sorting

An interesting consideration is the relationship between ciliary exit and endolysosomal sorting. It has been proposed that endocytosis of signaling receptors is intimately coupled to their exit from cilia (Pedersen et al., 2016). However, single molecule imaging of GPR161 exiting cilia suggests that activated GPR161 diffuses into the plasma membrane after it exits from the ciliary compartment (Ye et al., 2018). Prior findings that worm mutants for BBSome or the ESCRT machinery both fail to remove ciliary proteins fused to Ub from cilia (Hu et al., 2007; Xu et al., 2015) suggest the provocative possibility that ciliary exit may be tightly coupled to degradative sorting. Nonetheless, the hypothesis that interfering with UbK63 chain formation on ciliary GPCRs indirectly blocks ciliary exit because of a primary defect in endocytosis can be rejected because ubiquitination is not a major determinant of SSTR3 endocytosis and because cilia-AMSH potently and specifically blocks the signal-dependent exit of ciliary GPCRs.

In this context, the contrast between the dramatic increase in ubiquitinated SSTR3 levels in Arl6−/− cells compared with WT cells even in the absence of stimulation (Fig. 1, C and D) and the modest increase in ciliary Ub levels in Arl6−/− cells compared with WT cells in the absence of stimulation (Fig. 1, A and B) suggest that a considerable amount of ubiquitinated SSTR3 accumulates outside of cilia in Arl6−/− cells. One interpretation consistent with a direct coupling between ciliary exit and degradation is that a small fraction of ubiquitinated SSTR3 escapes cilia in Arl6−/− cells but fails to reach the correct degradative route. In this interpretation, the BBSome would deliver its cargos to the ESCRT machinery for routing into the lysosomal degradative pathway.

Ubiquitination and control of the ciliary proteome

A role for ubiquitination in selecting unwanted proteins for removal from cilia may unify the functions of the BBSome in signal-dependent retrieval of GPCRs and the constitutive clearance of proteins that accidentally enter cilia. In both of these cases, unwanted ciliary proteins need to be recognized as “nonself” by a ciliary surveillance machinery. For GPCRs, β-arrestin orchestrates the recognition of activated GPCRs as unwanted by directing their ubiquitination. A fascinating question for future investigations is how the >100 proteins that accumulate in outer segments of Bbs photoreceptors are recognized as nonself and tagged with Ub.

Materials and methods

Cell culture

The mouse IMCD3 cell lines used in the study were generated from a parental IMCD3-FlpIn cell line (gift from P.K. Jackson, Stanford University, Stanford, CA). IMCD3-FlpIn cells were cultured in DMEM/F12 (11330–057; Gibco) supplemented with 10% FBS (100–106; Gemini Bio-products), 100 U/ml penicillin-streptomycin (400–109; Gemini Bio-products), and 2 mM L-glutamine (400–106; Gemini Bio-products). The RPE1-hTERT cell line (CRL-4000; ATCC) was cultured in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, and 0.26% sodium bicarbonate (25080; Gibco).

Ciliation was induced by serum starvation in media containing 0.2% FBS for 16–24 h.

Plasmid construction and generation of stable cell lines

Stable isogenic IMCD3 cell lines were generated using the Flp-In system (Thermo Fisher Scientific). Low-expression promoters and additional expression cassettes were introduced into pEF5/FRT (flippase recognition target) plasmid as described (Nager et al., 2017; Ye et al., 2018). To reduce expression levels, the EF1α promoter was attenuated by mutating the TATA-box (pEF1α) or replaced with a minimal chicken lens δ-crystallin promoter (pCrys).

Coding sequences were amplified from plasmids encoding mouse GPR161 (BC028163; Mammalian Gene Collection; GE Healthcare), mouse SSTR3 (gift from Kirk Mykytyn, Ohio State University, Columbus, OH), and human BBS5 (gift from V. Sheffield, University of Iowa, Iowa City, IA), BirA-ER (gift from Alice Ting, Stanford University), β-Arrestin 2 (a gift from Mark Scott, Institut Cochin, Paris, France) was stably expressed from a cytomegalovirus promoter-based plasmid (pEGFP-N). SSTR3 and SMO expression were driven by pEF1α, GPR161 expression by pCrys, and BBS5 expression by pEF1α. Coding sequences were fused to GFP, NG (Shaner et al., 2013), or an acceptor peptide for the biotin ligase BirA (AP; Howarth and Ting, 2008). Multiple rounds of site-directed mutagenesis were performed to generate

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SSTR3-cKO, a variant with all the cytoplasm-facing lysine residues (K233, K330, K356, K407, and K421) mutated to arginine. GPR161-cKO, a variant with 18 cytoplasm-exposed lysine residues (K83, K84, K93, K247, K250, K269, K296, K358, K362, K455, K469, K473, K481, K486, K497, K504, and K548) mutated to arginine, was gene synthesized (GenScript). Cilia-AMSH was generated by mutating the catalytic domain of mouse AMSH (gift from David Komander, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia; plasmid no. 66712; Addgene; Michel et al., 2018) with NPHPS1–200 and GFP to create NPHPS1–200–GFP-AMSH [243–424]. A catalytically dead variant of AMSH was generated by mutating the water-activating Glu280 residue to Ala (Sato et al., 2008; Huang et al., 2013).

Imaging and microscopy

Cells were imaged either on a DeltaVision system (Applied Precision) equipped with a PlanApo 60×/1.40 objective lens (Olympus), CoolSNAP HQ2 camera (Photometrics), and solid-state illumination module (InsightSSi), or on a confocal LSM 700 (Zeiss) microscope equipped with 40× Plan-Apochromat 1.3 DIC oil objective. Z stacks were acquired at 0.5-µm intervals (LSM 700; Fig. 1, Fig. 3, Fig. 7, and Fig. S1) or 0.2-µm intervals (DeltaVision; Fig. 4, Fig. 6, Fig. S3, and Fig. S4).

For fixed imaging, 60,000 cells were seeded on acid-washed coverglasses (12 mm no. 1.5; 12-545-81; Thermo Fisher Scientific). Cells were grown for 24 h and then starved for 16–24 h in 0.2% FBS media before experimental treatment. After treatment, cells were fixed with 4% PFA in PBS for 15 min at 37°C and extracted in –20°C methanol for 5 min. Cells were then permeabilized in immunofluorescence (IF) buffer (PBS supplemented with 0.1% Triton X-100, 5% normal donkey serum [017–000–121; Jackson ImmunoResearch Laboratories], and 3% BSA [BP1605-100; Thermo Fisher Scientific]), incubated at room temperature for 1 h with primary antibodies diluted in IF buffer, washed three times in IF buffer, incubated with secondary antibodies (Jackson ImmunoResearch Laboratories), diluted in IF buffer for 30 min, and washed three times with IF buffer. DNA was stained with Hoechst 33258 (H1398; Molecular Probes), cells were washed twice more with PBS, and coverglasses were mounted on slides using fluoromount-G (F7984–25; Electron Microscopy Sciences).

For live-cell imaging, 300,000 cells were seeded on acid-washed 25-mm coverglasses (Electron Microscopy Sciences). After 24 h of growth, cells were serum starved for 16 h and transferred to the DeltaVision stage for imaging at 37°C within an environmental chamber that encloses the microscope and the stage. Cells were imaged in DMEM/F12 media, Hepes, no phenol red (11039–021; Gibco). To measure SSTR3 exit, cells expressing 4xSSTR3 were first washed three times with PBS and then pulse labeled with 2 µg/ml mSA647 for 5 min at 37°C. Cells were then imaged after addition of sst (for SSTR3) or SAG (for GPR161) for 2 h with an acquisition every 10 min. Each acquisition consisted of a three-plane Z-stack with each plane separated by 0.5 µm. Transmittance was set to 5% and exposure time to 500 ms.

WT or Bbs4+/− mice (Mykytyn et al., 2004) eyes were enucleated at P21. Whole eyes were fixed in 4% PFA/PBS overnight at 4°C and then washed three times with PBS. The eyes were infiltrated in 30% surose/PBS at 4°C overnight. Eyes were placed in O.C.T. compound embedding medium (4583; Tissue-Tek), frozen on dry ice, and stored at −80°C. 14-µm sections were cut on a cryostat (CM 1850; Leica). Sections were processed for immunofluorescence as follows. Sections were blocked in blocking solution (50 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Triton X-100, 3% normal donkey serum, and 0.1% BSA) for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C, followed by three washes in wash solution (50 mM Tris, pH 7.4, 100 mM NaCl, and 0.1% Triton X-100). After rinsing in wash solution, sections were incubated with 200 nM SAG and 10 µM sst-14. Somatostatin 14 stocks were made in DMEM/F12 media, Hepes, no phenol red (11039–021; Gibco). SAG was dissolved in DMSO (276855; Sigma-Aldrich).
secondary antibodies for 1 h at room temperature. DNA was counterstained using Hoechst 33258 dye, and sections washed twice more with PBS and mounted on slides using Fluoromount-G. Sections were imaged on a Confocal LSM 700 (Zeiss) microscope.

**Image analysis**

Files were imported from the Deltavision or LSM700 workstations into ImageJ/Fiji (National Institutes of Health) for analysis. For the quantification of ciliary Ub signal in fixed cells, maximum intensity projections were used. The ciliary Ub intensity $F_{Ub\text{-}cilia}$ was measured using the following equation:

$$F_{Ub\text{-}cilia} = F_{Ub\text{-}cilia\text{-}measured} - F_{background}.$$  

$F_{Ub\text{-}cilia\text{-}measured}$ is the total ciliary Ub fluorescence detected, and $F_{background}$ is the background Ub fluorescence measured in the adjacent area. For all measurements, the fluorescence integrated density was used. $F_{Ub\text{-}cilia}$ were plotted as violin plots using the PlotsOfData web tool [https://huygens.science.uva.nl/PlotsOfData/]. Each violin represents the distribution of data, which includes all the data points. Median and interquartile range are marked by solid and dotted lines, respectively. No gamma adjustment was applied during figure preparation; all the representative micrographs for respective panels are displayed within the same dynamic range. For some representative micrographs, the most in-focus plane was used rather than the maximum intensity projection (Fig. 3 and Fig. 6, A and C).

To measure SSTR3 exit, the integrated density of Alexa Fluor 647 fluorescence in the maximum intensity projection was measured for each time point. The cilia-adjacent fluorescence was subtracted as the background, and a mathematical photobleaching correction was applied:

$$F_{cilia} = \left( F_{mSA647\text{-}measured} / F_{mSA647\text{-}1} \right) + \left[ \left( 1 - e^{-\lambda} \right) * (n - 1) \right],$$

where $\lambda$ is the photobleaching decay constant, $n$ is the number of images taken, $F_{mSA647\text{-}measured}$ is the integrated mSA647 fluorescence measured for image $n$, $F_{mSA647\text{-}1}$ is the measurement for the first time point, and $F_{cilia}$ is the reported fluorescence. In this equation, $F_{cilia}$ is reported in relative fluorescence units. AP\text{GPR161}NG exit was followed similarly with the difference that NG fluorescence intensity was measured. For SSTR3 as well as GPR61, photobleaching-corrected data ($F_{cilia}$) for each condition were linearly fitted ($F_{cilia} = m \times time + c$) and plotted in Excel.

Endocytosed SSTR3 foci were revealed by contrast enhancement and counted with the ImageJ particle analysis tool.

**C. rheinardtii culture, isolation of flagella, and immunofluorescence**

**C. rheinardtii** WT-g1 (nmt, ag11, mating type [mt+]; gift from George B. Witman, University of Massachusetts Medical School, Worcester, MA) and Bbs4- CC-4377 px5-1/bbs4-1::NIT1 agt1 mt+ (Chlamydomonas Resource Center) strains were cultured asynchronously under light in Tris-acetate-phosphate media (Gorman and Levine, 1965) for 72 h. Flagellar fractions were prepared from 2 liters of cultures of WT or Bbs4 cells as described (Craigie et al., 2013). Briefly, cells were harvested by centrifugation for 5 min at 2,000 rpm (1,100 g) at room temperature. Cells were resuspended in 10 mM Hepes, pH 7.4, and centrifuged for 5 min at 2,000 rpm (1,100 g). Next, the cell pellet was gently resuspended in ice-cold HMDS (10 mM Hepes, pH 7.4, 5 mM MgSO4, 1 mM DTT, and 4% [wt/vol] sucrose). Deflagellation was achieved by addition of 5 mM dibucaine to each tube of cells and by pipetting up and down ~10 times using a 10-ml plastic serological pipette. The suspension was spun for 5 min at 1,800 g at 4°C. The supernatant containing flagella was underlaid with 9 ml ice-cold HMDS-25% sucrose (10 mM Hepes, pH 7.4, 5 mM MgSO4, 1 mM DTT, and 25% [wt/vol] sucrose), followed by a centrifugation step for 10 min at 2,800 rpm (2,400 g), 4°C. The supernatant above the sucrose interface was collected using a 25-ml serological pipette, and flagella were pelleted by centrifugation for 20 min at 30,000 g (16,000 rpm), 4°C. The flagellar pellet was resuspended in 100 µl HMDS buffer. Protein concentrations were measured by Bradford assays, and 25 µg were resolved by SDS-PAGE for immunoblotting.

**C. rheinardtii immunofluorescence** was performed as follows. Cells were fixed with 4% PFA in MT buffer (30 mM Hepes, pH 7, 5 mM EGTA, 5 mM MgSO4, and 25 mM KCl) for 20 min in suspension. The cells were centrifuged at 1,000 rpm for 5 min, resuspended in 100 µl of fixative, and transferred onto slides coated with 1 mg/ml poly-L-lysines. After 5 min, the unadhered cells were washed off by rinsing with PBS. Cells were permeabilized in 0.5% Triton X-100 for 20 min followed by blocking for 1 h in blocking buffer (3% fish skin gelatin, 1% BSA, and 0.1% Tween 20 in PBS) at room temperature. Cells were incubated at 4°C overnight with primary antibodies diluted in blocking buffer, washed five times in PBS, and incubated with secondary antibodies for 2 h at room temperature. After five washes in PBS, coverglasses were mounted on slides using Fluoromount-G. Cells were imaged on the LSM700 confocal microscope.

**Biochemical analysis of SSTR3 ubiquitination**

IMCD3 cells stably expressing pEFtak-AP\text{SSTR3NG} and BirA-ER were transiently transfected with HA-tagged Ub plasmids. Cells were reverse transfected using XtremeGene9 and plated onto 15-cm plates. Cells were moved to medium containing 0.2% serum and scraping cells off on ice into 1 ml of ice-cold radioimmunoprecipitation assay buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 100 mM iodoacetamide, and 10 mM EDTA) supplemented with protease inhibitors (1 mM N-acetylbenzylbenzenesulfonyl fluoride hydrochloride, 0.8 mM aprotinin, 15 mM E-64, 10 mg/ml bestatin, 10 mg/ml pepstatin A, and 10 mg/ml leupeptin). After gently rocking at 4°C for 5 min, lysates were clarified by spinning in a tabletop Eppendorf centrifuge at 15,000 rpm and 4°C for 20 min. Each clarified lysate was incubated with 50 µl streptavidin Sepharose beads (GE Healthcare) for 1 h at 4°C to capture biotinylated SSTR3. Beads were washed four times with 500 µl radioimmunoprecipitation assay buffer, and bound proteins were eluted by heating the beads in 1× lauryl dodecyl sulfate sample loading buffer containing 2 mM biotin at 70°C for 10 min. After SDS-PAGE and transfer, polyvinylidene fluoride
membranes were probed with anti-HA antibody and streptavidin-HRP.

Signals were acquired with a ChemiDoc imager (Bio-Rad) and analyzed with ImageLab 6.0.1 (Bio-Rad). The integrated densities of the SSTR3 bands in the streptavidin-HRP blot were normalized to the integrated densities of the endogenous biotinylated proteins to correct for variations in the recovery of biotinylated proteins on streptavidin Sepharose. The resulting value is SSTR3bac. Second, the integrated densities of ubiquitinated SSTR3 were collected by measuring the area from 100 kD to the top of the gel on the HA blot. The values were background-corrected by subtracting the value of the equivalent area in the IMCD3 control lane. The resulting value is Ub-SSTR3raw. The relative amount of SSTR3 that is ubiquitinated, Ub-SSTR3upac = Ub-SSTR3raw/SSTR3bio, was plotted on the graphs using Plot-0Data (Postma and Goedhart, 2019).

Online supplemental material

Fig. S1 shows staining of WT and Arl6−/− IMCD3 cells expressing AP−SSTR3Ning with the FKI anti-Ub antibody and with the UbK63 antibody. Fig. S2 presents the ciliary levels of the cytoplasmic lysine mutants of SSTR3 and GPR161. Fig. S3 shows a micrograph representative of the NPHP3CTS-AMSH expression levels. Fig. S4 contains data supporting the removal of ciliary UbK63 chains by NPHP3CTS-AMSH.

Acknowledgments

We thank Drs. Kirk Mykytyn, Val Sheffield, Alice Ting, and Mark Scott for the gifts of cDNAs; Kathryn Anderson, Vishva Dixit (Genentech, San Francisco, CA), and Felice Dunn (University of California, San Francisco [UCSF], San Francisco, CA) for the gifts of antibodies; Val Sheffield for the gift of the Bbs4−/− mouse; George B. Wittman and the Chlamydomonas Resource Center for the gift of Chlamydomonas strains; Yen-Ming Kuo for help with cryosections of mouse retina and core support; Dhiya Kumar and Tyler Picariello for the assistance with the Chlamydomonas cultures; Nicholas Morante (UCSF) and Jeremy Reiter (UCSF) for providing Bbs4−/− mouse eyes; Fan Ye (UCSF) for generating some of the cell lines used in the study; Irene Ojeda Naharros for help with single cilia tracking; the Nachury and von Zastrow laboratories for helpful discussions; and the Ogden and Caspary laboratories for comments on the preprint.

This work was funded by the National Institute of General Medical Sciences (R01-GM099933 to M.V. Nachury). This work was made possible, in part, by a National Eye Institute Core Grant for Vision Research (EY002162) and by the Research to Prevent Blindness Unrestricted Grant (M.V. Nachury).

The authors declare no competing financial interests.

Author contributions: S.R. Shinde conducted all experimental work except for the experiments in Fig. 5, which were conducted by A.R. Nager. M.V. Nachury supervised research. S.R. Shinde and M.V. Nachury wrote the manuscript.

Submitted: 4 March 2020
Revised: 29 September 2020
Accepted: 21 October 2020

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Figure S1. **Signal-dependent ubiquitination of ciliary GPCRs.** (A) IMCD3 cells of the indicated genotypes expressing αSSTR3NG were treated with sst for 2 h. Cells were fixed and stained for Ub with the FK1 monoclonal antibody (Ub [FK1], yellow) and acetylated tubulin (acTub, magenta). αSSTR3NG (cyan) was imaged through the intrinsic fluorescence of NG. Channels are shifted in the insets to facilitate visualization of overlapping ciliary signals. Scale bars, 5 µm (main panel), 2 µm (inset). (B) Violin plots of the fluorescence intensity of the Ub channel in the cilium in each condition are shown. A threefold increase in ciliary Ub signal is observed upon addition of sst to SSTR3-expressing cells. Asterisks indicate ANOVA significance value. ****, P ≤ 0.0001. (C) IMCD3 cells of the indicated genotypes expressing αSSTR3NG were treated with sst for 2 h. Cells were fixed and stained for acTub (magenta) and UbK63 (yellow). αSSTR3NG (cyan) was imaged through the intrinsic fluorescence of NG. Channels are shifted in the insets to facilitate visualization of overlapping ciliary signals. Scale bars, 5 µm (main panel), 2 µm (inset). (D) The fluorescence intensity of the UbK63 channel in the cilium is represented as violin plots. Asterisks indicate ANOVA significance value. ****, P ≤ 0.0001. au, arbitrary units.
Figure S2. **Fluorescence intensities of WT and cK0 GPCR variants at t = 0.** (A) Ciliary SSTR3 was pulse labeled by addition of Alexa Fluor 647–conjugated mSA (mSA647) to the medium for 5–10 min before addition of sst. Violin plots of the ciliary fluorescence intensities for WT and cK0 SSTR3 imaged in the far-red channel at t = 0 are shown. n = 18–22 cilia. (B) Violin plots of the fluorescence intensities of NG fluorescence corresponding to WT and cK0 GPR161NG at t = 0 are shown. n = 10–19 cilia. RFU, relative fluorescence unit.
Figure S3. Expression levels of ciliary AMSH. IMCD3-\([\text{pEF}1\alpha_{\Delta}\text{AP5STR3}; \text{pEF}1\alpha\text{-BirA-ER}]\) cells were transfected with the plasmid expressing NPHP3\text{CTS}, AMSH as in Fig. 4, before fixation and staining for acetylated tubulin (acTub, magenta) and DNA (blue). The NPHP3\text{CTS} fusions were visualized through the intrinsic fluorescence of GFP (cyan). A representative micrograph is shown. A cell expressing modest levels of cilia-AMSH is indicated by an arrow and possesses normal ciliary staining of acetylated tubulin. A cell expressing high levels of cilia-AMSH indicated by an arrowhead displays abnormal ciliary levels of acetylated tubulin. Cells with high ciliary levels of NPHP3\text{CTS}, AMSH were excluded from the analysis, and only cells with modest ciliary levels of NPHP3\text{CTS}, AMSH were included in the experiment. Scale bars, 5 µm (main panel), 1 µm (inset).
Figure S4. **Cilia-targeted AMSH removes UbK63 chains from ciliary substrates.** (A) IMCD3-[pEF1αΔ-APSSTR3; pEF1α-BirA-ER] cells were transfected with control or Arl6 siRNAs. Cell lysates were immunoblotted for Arl6 or actin. A nonspecific band cross-reacting with the anti-Arl6 antibody is marked with a dot.

(B) IMCD3-(APSSTR3; BirA-ER) cells were transfected with siRNA targeting Arl6 and plasmids expressing NPHP3⁴CTS-AMSH or NPHP3⁴CTS-AMSH⁺. Surface-exposed APSSTR3 was pulse-labeled with mSA647 for 5–10 min, and cells were then treated with sst for 2 h before fixation and staining for UbK63 (yellow). The NPHP3⁴CTS-AMSH fusions were visualized through the intrinsic fluorescence of GFP (cyan), APSSTR3 was visualized via mSA647 (magenta), and DNA is blue. Channels are shifted in the insets to facilitate visualization of overlapping ciliary signals. Scale bars, 5 µm (main panel), 2 µm (inset).

(C) The fluorescence intensities of the UbK63 signal inside cilia are represented as violin plots. Asterisks indicate Mann–Whitney test significance value. ****, P ≤ 0.0001. au, arbitrary units; si, short interfering; MW, molecular weight.