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Review Article

HTR6 and SSTR3 targeting to primary cilia

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Primary cilia are hair-like projections of the cell membrane supported by an inner microtubule scaffold, the axoneme, which polymerizes out of a membrane-docked centriole at the ciliary base. By working as specialized signaling compartments, primary cilia provide an optimal environment for many G protein-coupled receptors (GPCRs) and their effectors to efficiently transmit their signals to the rest of the cell. For this to occur, however, all necessary receptors and signal transducers must first accumulate at the ciliary membrane. Serotonin receptor 6 (HTR6) and Somatostatin receptor 3 (SSTR3) are two GPCRs whose signaling in brain neuronal cilia affects cognition and is implicated in psychiatric, neurodegenerative, and oncologic diseases. Over a decade ago, the third intracellular loops (IC3s) of HTR6 and SSTR3 were shown to contain ciliary localization sequences (CLSs) that, when grafted onto non-ciliary GPCRs, could drive their ciliary accumulation. Nevertheless, these CLSs were dispensable for ciliary targeting of HTR6 and SSTR3, suggesting the presence of additional CLSs, which we have recently identified in their C-terminal tails. Herein, we review the discovery and mapping of these CLSs, as well as the state of the art regarding how these CLSs may orchestrate ciliary accumulation of these GPCRs by controlling when and where they interact with the ciliary entry and exit machinery via adaptors such as TULP3, RABL2 and the BBSome.

The GPCR-cilia connection

About one thousand human genes encode G protein-coupled receptors (GPCRs), all with their well-known serpentine structure, seven transmembrane helices (TM1-7), an extracellular (or lumenal) N-terminus (NT), three extracellular (EC1-3) and three intracellular (IC1-3) loops, and a cytosolic C-terminus (CT) (Figure 1a). How this structure enables GPCRs to bind ligands, such as hormones or neuropeptides, leading to G protein activation and downstream signaling, has been reviewed elsewhere, as have their important pathophysiological and pharmacological roles [1,2].

Perhaps less well known is that many GPCRs (most, if olfactory receptors are taken into account) function inside sensory or primary cilia, hair-like cell surface protrusions with an inner microtubule shaft, or axoneme, emanating from a membrane-docked centriole, or basal body [3–7] (Figure 1b). By working as cell type-specific cellular antennae, primary cilia provide specialized signaling compartments where GPCRs and their effectors can transmit their signals more efficiently [3–7]. The GPCR-cilia connection is ancient, likely preceding the last bilaterian common ancestor [8]. Consistently, ciliary GPCRs are present all across the GPCR phylogenetic tree, whose roots lie near the last eukaryotic common ancestor (Figure 1c) [3,9,10].

Ciliary GPCR functions are wide-ranging and have been reviewed elsewhere [3,5]. Our vision and smell rely on them, as do multiple aspects of development, tissue homeostasis and disease. For instance, SMO and GPR161 are key mediators of Hedgehog (Hh) signaling, thereby controlling embryonic development, stem cell regulation and cancer [11]. Several ciliary GPCRs control food intake or adipogenesis, with their mutations causing obesity [12,13]. Others are important for kidney, thyroid, airway, endothelial and hepatic function, among others [3,5]. Clearly, though, most ciliary

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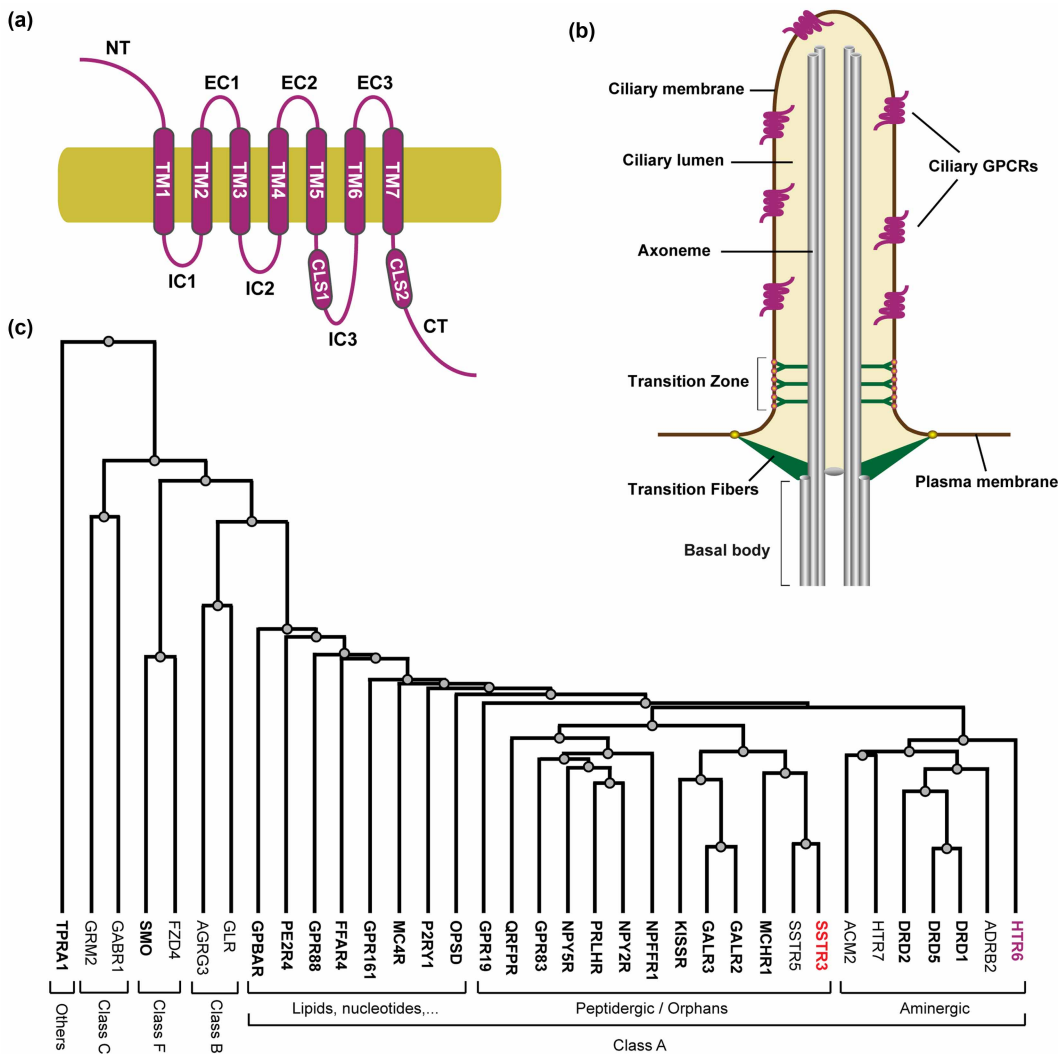

Figure 1. Primary cilia as GPCR signaling platforms.

(**a**) Schematic of a GPCR including its seven transmembrane helices (TM1–TM7), its extracellular or lumenal face (top) containing its amino-terminal region (NT) and three extracellular loops (EC1–EC3), and its cytosolic face including three intracellular loops (IC1–IC3) and carboxyl-terminal region (CT). Also shown are the approximate locations of the ciliary localization sequences (CLS1 and CLS2) identified in HTR6 and SSTR3. (**b**) Schematic of a primary cilium and its parts, including several GPCRs. Only two of the nine microtubule pairs/triplets and associated structures are depicted. (**c**) Phylogenetic tree including most known human non-olfactory ciliary GPCRs (in bold), as well as some non-ciliary ones for context. Tree was assembled at GPCRdb.org using UPGMA (unweighted pair group method with arithmetic mean) for distance calculations and no bootstrapping. Full-length human sequences of the canonical Uniprot isoforms were used in all cases. Protein names also as in Uniprot. GPCR classes according to GPCRdb are indicated below. All known ciliary GPCRs, except SMO and TPRA1 (GPR175), are in Class A, for which main types of ligands are also indicated (MC4R ligand is also peptidergic, despite clustering away from other peptidergic GPCRs). HTR6 and SSTR3 are highlighted in purple and red, respectively.

GPCRs function in neuronal cilia, responding to aminergic neurotransmitters like serotonin or dopamine, or to neuropeptides like melanocortin, kisspeptin, galanin, neuropeptide Y, melanin-concentrating hormone, or somatostatin, to name a few [3,5].

Serotonin, also known as 5-hydroxytryptamine or 5-HT, is a very ancient signaling molecule, modulating swimming and growth in unicellular ciliates like *Paramecium* and *Tetrahymena*, and playing multiple roles in

both vertebrates and invertebrates [14]. In humans, serotonin regulates gastrointestinal and cardiovascular function, among others, but is better known for its myriad effects on brain and behavior [14]. Mammals contain fourteen serotonin receptor genes (*HTR1A-B*, *HTR1D-F*, *HTR2A-C*, *HTR3*, *HTR4*, *HTR5A-B*, *HTR6*, *HTR7*), encoding seven receptor types (HTR1-7 or 5-HT_{1-7}). Except for HTR3, a ligand-gated ion channel, all serotonin receptors are GPCRs [14].

HTR6, also known as 5-HT_6 or 5-HTR_6, is expressed in brain, with highest levels in basal ganglia and cerebral cortex [15,16]. It is involved in many pathophysiological processes including, among others, learning, memory, reward-motivated behaviors, depression, anxiety, drug addiction, schizophrenia, epilepsy, eating disorders and Alzheimer's disease (AD) [15,17]. Unlike other serotonin receptors, HTR6 robustly localizes to cilia, both *in vivo* and *in vitro*, where it is often used as a ciliary membrane marker or ciliary targeting tool [18–22]. In some neurons, however, HTR6 is also found in dendrites, where it may function postsynaptically like other HTRs [15,23–25].

HTR6 signals through G_s proteins and adenylyl cyclase (AC) to promote cAMP synthesis, but it can also stimulate CDK5 and mTOR kinases, among others [15,26–29]. Through its multiple interactors and downstream effectors, HTR6 controls neuronal migration and morphogenesis, affecting axonal and dendritic morphology, as well as ciliary length, shape, and composition [15,24,27,28,30–33]. For an excellent review on HTR6's interactome, signaling networks and cellular and pathophysiological functions, see [15].

The relative contributions of ciliary and postsynaptic signaling to HTR6's multiple functions remain to be elucidated. However, given that cilia-localized HTR6 regulates neuronal morphology [15,24,33], it seems likely that HTR6 ciliary signaling has neuromodulatory roles, affecting configuration and output of neural circuits, thereby contributing to the establishment of long-term behavioral patterns, an evolutionarily conserved role of serotonin [34,35]. Accordingly, other ciliary GPCRs, like SSTR3, regulate the establishment and remodeling of synaptic connectivity [36–38].

Somatostatin (SST) is a peptide hormone with broad anti-secretory and anti-proliferative actions. In mammals, SST signals via five receptors, SSTR1-5, all G_i-coupled GPCRs that lower cAMP levels by inhibiting AC [39]. Of these, only SSTR3 is known to localize to cilia, which it does robustly, making SSTR3 another common ciliary marker and ciliary targeting module [3,40–43]. SSTR3 is expressed throughout the brain, and less intensely in gastrointestinal tract and testes [16]. Its unique anti-angiogenic and pro-apoptotic actions among SSTRs make SSTR3 a promising cancer drug target [39,44]. Furthermore, SSTR3 affects cognition, as revealed by the learning and memory impairment caused by conditional SSTR3 deletion in mouse hippocampus. Since hippocampal-specific cilia deletion causes the same effects, this suggests that SSTR3 acts through cilia to enhance cognition, and is consistent with the above-mentioned effects of SSTR3 on neuronal connectivity [3,36–38].

Ciliary GPCR signaling is also important in ciliopathies, inherited diseases caused by cilia dysfunction [45]. Two such ciliopathies, Bardet-Biedl syndrome (BBS) and Joubert syndrome (JS), are both associated with ciliary GPCR mistargeting and cognitive defects [3,45–49]. In JS mouse models, ciliary GPCR signaling regulates axon tract development and neuronal connections important for JS etiopathogenesis [36,37]. In BBS mouse models, brain cilia abnormally lack SSTR3 and other ciliary GPCRs, like MCHR1 or DRD1 [3,47,50]. Hence, cognitive impairment in BBS and JS is likely due, in part, to mistargeting of ciliary GPCRs like SSTR3 or HTR6.

A better molecular understanding of ciliary GPCR targeting may provide novel approaches for modulating GPCR signaling, with important biomedical implications. Herein, we will review recent advances in HTR6 and SSTR3 ciliary targeting and discuss their meaning in the wider context of ciliary GPCR trafficking. We will finish by pointing out some outstanding questions in the field, and possible approaches to answer them.

Ciliary localization sequences in HTR6

HTR6 and SSTR3 localization to neuronal cilia was described twenty years ago [23,25,43]. Thereafter, they became the first ciliary GPCRs known to contain ciliary localization sequences (CLSs) in their IC3s [51]. Since then, IC3 CLSs have been found in several other ciliary GPCRs, such as GPR161, MCHR1, MC4R, NPY2R or FFAR4 [3–5,12,52–54]. In contrast, other ciliary GPCRs, like Rhodopsin, SMO or the D1 dopamine receptor (DRD1), turned out to contain CT CLSs [50,55–58]. This suggested that ciliary GPCRs contain their CLSs in either IC3 or CT.

However, some data suggested things were not that simple. To discover the IC3 CLSs in HTR6 and SSTR3, Berbari et al. created HTR6-HTR7 and SSTR3-SSTR5 chimeras. Their main finding was that the non-ciliary

Biochemical Society Transactions (2021) **49** 79–91
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HTR7 and SSTR5 accumulated in cilia if their IC3s were replaced by those of HTR6 and SSTR3, respectively (Figure 2a) [51]. Hence, HTR6 and SSTR3 contained IC3 CLSs sufficient for ciliary targeting of non-ciliary GPCRs.

Nevertheless, in their discussion, Berbari et al. mentioned an intriguing finding: HTR6 and SSTR3 still accumulated in cilia when their IC3s were replaced by those of HTR7 and SSTR5, respectively. Therefore, the IC3 CLSs in HTR6 and SSTR3 were sufficient but dispensable for cilia localization. The most parsimonious explanation for this was that these GPCRs contained additional CLSs outside their IC3s (Figure 2a) [51]. More recently, similar chimera studies indicated that two other ciliary GPCRs, NPY2R, and GPR83, contain CLSs not only in IC3 but also in CT [53].

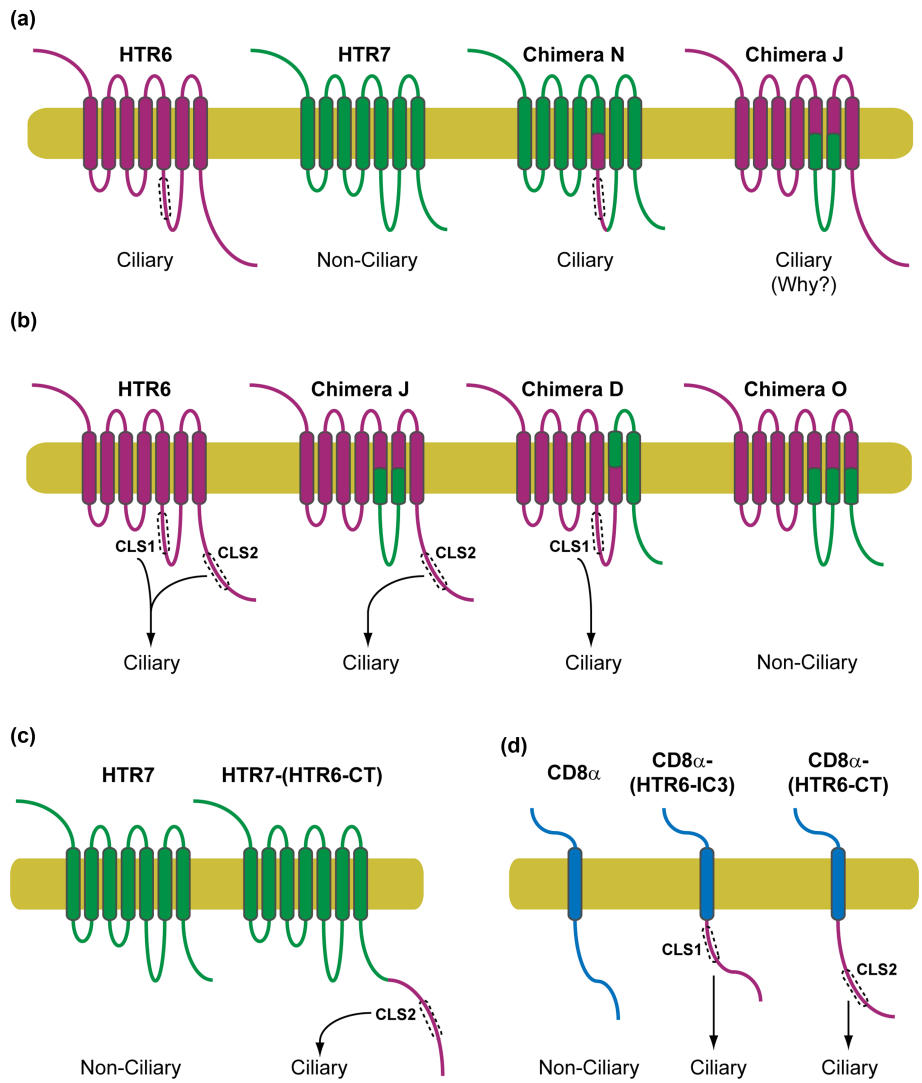

Figure 2. HTR6 contains redundant CLSs in IC3 and CT.

(**a**) Schematic of HTR6, HTR7 and their indicated chimeras. In Chimera N, the first half of HTR6-IC3 (containing CLS1) suffices for ciliary targeting, yet removal of that sequence in Chimera J does not disrupt cilia localization. This suggested HTR6 contained another CLS, leading to identification of CLS2. (**b**) Schematic of HTR6 and HTR6-HTR7 Chimeras J, D and O. The two CLSs in HTR6 are indicated. Chimeras J and D preserve one of the CLSs and still target to cilia, whereas Chimera O, lacking both CLSs, completely fails to localize to cilia. (**c**) Fusing the CLS2-containing HTR6-CT to the C-terminal end of HTR7 suffices to turn the latter into a ciliary receptor. (**d**) Replacing CD8α cytosolic tail by either CLS1-containing HTR6-IC3 or CLS2-containing HTR6-CT is sufficient to confer ciliary localization to CD8α.

To identify the missing CLSs in HTR6, we performed additional chimera studies with the HTR6/HTR7 pair [59]. This made it clear that HTR6 also contains CLSs in both IC3 and CT. Indeed, HTR6 ciliary localization is completely lost if both its IC3 and CT are replaced by those of HTR7 (Figure 2b) [59]. This chimera, however, readily reaches the plasma membrane (PM), showing that its folding and intracellular trafficking are not perturbed [59]. Thus, HTR6 ciliary targeting relies on two CLSs, one in IC3 (CLS1) and one in CT (CLS2) (Figure 2b).

We also showed that CLS1 and CLS2 act redundantly, each being sufficient for HTR6 ciliary targeting (Figure 2b). Furthermore, both CLS1 and CLS2 can target other transmembrane proteins to cilia, including not only HTR7 (Figure 2a,c), but also CD8α, a non-ciliary single-pass transmembrane protein (Figure 2d). In contrast, CLS2 fusion to soluble EGFP did not suffice for ciliary localization, indicating a requirement for membrane association [59].

We then mapped the key residues for CLS2 function (Figure 3a). HTR7-CT only aligns with the first half of HTR6-CT, which is twice as long. Replacing HTR6-CT's first half by the homologous HTR7-CT did not strongly decrease ciliary targeting. In contrast, deleting HTR6-CT's second half abolished ciliary localization. Finer deletion mapping, followed by alanine-scanning mutagenesis of individual residues, led to the identification of three critical residues for CLS2 function: the LPG motif (aa 400–402 of mouse HTR6, fully conserved in humans) (Figure 3a) [59].

We then switched our attention back to CLS1. In their study, Berbari and colleagues provided evidence that CLS1 function relies on the ATAGQ motif (aa 242–246 of mouse HTR6, not conserved in humans). More specifically, using the same chimera with which they proved that HTR6-IC3 targets HTR7 to cilia (Figure 2a, Chimera N), they showed that introducing the A242F + Q246F mutation into HTR6-IC3 prevents ciliary localization [51]. Although we fully confirmed this result, we also found that this mutation strongly reduced the ability of this chimera to traffic to the cell surface, instead accumulating intracellularly, most likely in the endoplasmic reticulum [59]. Thus, this mutant chimera failed to fold properly, explaining its inability to accumulate in cilia [59]. In contrast, other non-ciliary HTR6-HTR7 chimeras readily reached the plasma membrane (Figure 2b, Chimera O).

Interestingly, the A242F + Q246F mutation only caused intracellular retention when introduced to Chimera N, but not when introduced to wild type HTR6, whose ciliary targeting was completely unaffected by this mutation. The same was true for HTR6 lacking an intact CLS2 (HTR6-ΔCLS2): its ciliary targeting was not at all perturbed by the mutation. Likewise, deletion of the entire ATAGQ motif had no effect on ciliary targeting of HTR6-ΔCLS2. Therefore, the ATAGQ residues in HTR6-IC3 are not responsible for CLS1 function, as initially thought [59]. The actual key residues, which we identified, are mentioned below.

Parenthetically, it should be noted that the chimera-specific effects of A242F + Q246F are not entirely surprising: while the different parts of a native GPCR have coevolved for millions of years to work together as a whole, chimeras are human creations that have not undergone such selection, so their parts may not be finely tuned to each other, and their function may be more vulnerable to perturbations, as observed with this

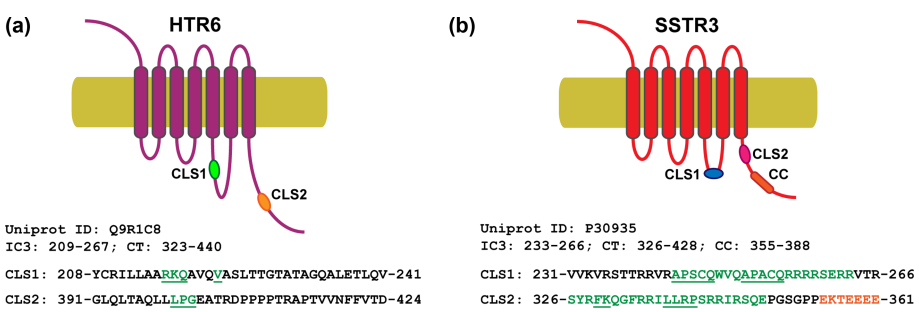

Figure 3. Ciliary localization sequences in HTR6 and SSTR3.

(**a**) Schematic of HTR6 and its CLSs. Uniprot database identifier, IC3 and CT residues, and the region of interest for each CLS are shown below. Key CLS residues are shown in green and underlined. (**b**) Schematic of SSTR3 with its CLSs and coiled coil (CC) region. Uniprot database identifier, IC3, CT and CC residues, and the region of interest for each CLS, are shown below. For SSTR3, CLS mapping did not reach single residue resolution. The regions needed for ciliary targeting are highlighted in green, with important motifs underlined. The beginning of CC is shown in vermilion.

Biochemical Society Transactions (2021) 49 79–91
https://doi.org/10.1042/BST20191005

mutation. Indeed, a fraction of the chimeras we and others generated in these kinds of studies were retained intracellularly, either completely or partially, in contrast to their parental GPCRs [51,53,59]. In the above case, the A242F + Q246F mutation caused a 7-fold reduction in the chimera's ability to reach the PM, more than enough to explain the observed 5-fold reduction in ciliary accumulation [59]. Thus, although chimeras are a powerful research tool for CLS studies, these potential caveats must be taken into consideration.

If not the ATAGQ motif, then what residues drive CLS1 function in HTR6? Our mapping, by introducing mutations in HTR6-ΔCLS2, identified four important residues in HTR6-IC3: the RKQ triplet (aa 216–218) and V222. The R216A + K217A + Q218A mutation completely abolished ciliary targeting, while V222A reduced it by half. Within the triplet, R216A also causes a 2-fold reduction, while the single K217A or Q218A mutations had no effect. We also looked at the R216A + K217A and R216A + Q218A mutations, both of which fully abolished targeting. Hence, R216 is the key residue within the triplet, but its function can be partially substituted by the K217-Q218 pair (Figure 3a) [59].

Altogether, we discovered HTR6 ciliary targeting relies on redundancy between CLS1 and CLS2, both of which we mapped in detail. Furthermore, even though we made our discoveries in the IMCD3 kidney epithelium cell line, we confirmed that HTR6 ciliary targeting in cultured neurons also depends on these same CLSs [59].

Ciliary localization sequences in SSTR3

As with HTR6, the Berbari et al. study also indicated that SSTR3-IC3 was sufficient but dispensable for cilia localization, suggesting the presence of CLSs outside IC3 [51]. We demonstrated this by showing that, as done for HTR6, both SSTR3-IC3 and SSTR3-CT suffice to drive non-ciliary CD8α to cilia (Figure 2d). Hence, SSTR3 also has CLSs in both IC3 (CLS1) and CT (CLS2) [59].

Mapping of SSTR3-CT (aa 326–428, Figure 3b) to identify residues key for CLS2 function showed that: (i) deletion of the juxtamembrane region (aa 326–348) completely abolishes ciliary targeting; (ii) deletion of the glutamate-rich coiled coil (aa 355–388) reduces CLS2 function 2-fold; and (iii) deletion of aa 389–428 has no effect [59]. Within the juxtamembrane region, we identified four mutations fully disrupting CLS2 function: (i) F329A + K330A, mutating a motif homologous to the previously reported SMO CLS [55]; (ii) ΔRILLRP (aa 335–340); (iii) L337A + L338A + P340A, mutating the LLxP motif reminiscent of Rhodopsin's VxP motif [56,57]; and (iv) ΔSRRIRSQE (aa 341–348) [59]. Despite interfering with ciliary accumulation, none of these mutations prevented PM targeting [59]. The precise roles of each residue within these motifs remain to be elucidated (Figure 3b).

As with HTR6, we also performed a systematic analysis of the SSTR3-IC3 residues involved in CLS1 function (Figure 3b). Previously, such function was shown to rely on its tandem AP[AS]CQ motifs within the APSCQWVQAPACQ stretch (aa 243-255) [51,60]. Specifically, a quadruple mutation turning A243, Q247, A251 and Q255 into phenylalanines was shown to block ciliary targeting of a chimera containing SSTR3-IC3 in a SSTR5 background [51]. When we introduced this mutation into mutants lacking CLS2 function (SSTR3-ΔCLS2, which still localize to cilia owing to CLS1 function), ciliary targeting was lost, even though PM targeting was preserved. This indicated that the quadruple mutation in the AP[AS]CQ motifs specifically disrupts CLS1 function.

Still, this might be due to dominant negative effects of the bulky phenylalanines. If so, adding the phenylalanines should have a stronger effect than just deleting the AP[AS]CQ motifs in SSTR3-ΔCLS2. This was indeed the case. In contrast to the quadruple mutation, deletion of these motifs (Δ243–255) only mildly reduced the percentage of positive cilia (≈100% in SSTR3-ΔCLS2 control; ≈80% with Δ243–255; ≈10% with the quadruple phenylalanine mutation). Thus, the phenylalanines do have dominant effects. However, upon closer examination, we saw that Δ243–255 also caused a 70% reduction in ciliary staining intensity relative to control, even if most of these cilia remained positive for the mutant. Therefore, after all, the tandem AP[AS]CQ motifs do have an important effect on CLS1 function (Figure 3b) [59]. Unlike combined deletion of both AP[AS]CQ motifs, separately deleting each motif had no effect on cilia localization, indicating redundancy. However, this redundancy only applies to mouse SSTR3, which was used for these studies, but not to human SSTR3, which has a single APSCQ motif [59].

Regarding the specific residues involved in the function of the tandem AP[AS]CQ motifs, the phenylalanine dominant effects suggest that the A-Q residues may not be as important as initially thought. Instead, the best candidates are the cysteines, whose mutation reduced BBSome binding and CD8α-(SSTR3-IC3) ciliary

targeting, effects not seen when the A-Q residues in both motifs were mutated to F-F [60]. Likewise, alanine mutation of the glutamines or prolines in the motifs did not affect BBSome binding [60]. Still, mutating these residue pairs (P-P, C-C, Q-Q) in SSTR3-ΔCLS2 would further clarify these issues.

We also addressed whether SSTR3-IC3 residues other than the AP[AS]CQ motifs affect CLS1 function (Figure 3b). Indeed, deleting the arginine-rich stretch RRRRSERR (aa 256–263) immediately following the AP[AS]CQ motifs did disrupt CLS1 function, albeit not completely (≈2-fold reduction in both positivity and intensity). However, this effect was at least partly due to interference with PM targeting [59]. Moreover, these residues are almost perfectly conserved in non-ciliary SSTR5 (RRRRSERK), suggesting that their main role is not as a CLS. Since all other SSTR3-IC3 residues had very subtle or no effects on ciliary targeting, this suggests that the AP[AS]CQ motifs are key to SSTR3 CLS1 function, as initially proposed (Figure 3b) [51]. As with HTR6, ciliary targeting of SSTR3 in cultured neurons obeyed the same principles as in IMCD3 cells [59].

Mechanisms of HTR6 and SSTR3 ciliary targeting

Intraflagellar transport (IFT) trains, consisting of IFT-A and IFT-B complexes coupled to microtubule motors, mediate anterograde (cilia tip-bound) and retrograde (cilia base-bound) intraciliary trafficking [6,7]. By associating with IFT trains via adapters, GPCRs can travel into and out of cilia. Accordingly, ciliary targeting of many GPCRs depends on known adapters connecting membrane cargo to IFT complexes [6,7,54,61–65]. Prominent among such adapters are TULP3, RABL2 and the BBSome complex, whose roles in HTR6/SSTR3 targeting we will now discuss.

Role of TULP3 in HTR6 and SSTR3 ciliary targeting

Tubby-like protein 3 (TULP3) is a ubiquitously expressed paralog of Tubby, a protein that functions like TULP3 but whose expression is eye and brain-specific [54]. TULP3 N-terminal region binds IFT-A, whereas its C-terminal Tubby homology domain binds phosphoinositides (PIPs) [66]. TULP3 is required for ciliary targeting of multiple Rhodopsin family GPCRs, including among others SSTR3, HTR6, GPR161 and MCHR1 [52,54,59,66]. Although TULP3 and these GPCRs do not coimmunoprecipitate, proximity biotinylation shows a close and specific association dependent on the Tubby domain and its PIP-binding ability. Interestingly, although this association is mediated by IC3 in GPR161 and MCHR1, it is CT-dependent in HTR6 and SSTR3 [54,59]. These and other data, such as accumulation of TULP3 and its cargoes inside cilia lacking INPP5E, a ciliary PI(4,5)P_2 5-phosphatase, suggest a model according to which TULP3 connects IFT trains to GPCRs at the PI(4,5)P_2-rich ciliary base, thereby enabling their movement across the transition zone and into the ciliary membrane, where low PI(4,5)P_2 levels induce cargo release from TULP3, allowing its return to the ciliary base for further rounds of transport (Figure 4a,b) [18,54,63,67].

To better understand how TULP3 controls HTR6 trafficking, we studied how CLS2 mutation affects TULP3 association to HTR6-CT. To our surprise, disrupting HTR6's CLS2 (by mutating or deleting the LPG motif) strongly enhanced this association, instead of weakening it, as seen by mutating the CLSs of other TULP3 cargoes, like GPR161, MCHR1 and Fibrocystin [54,59]. Moreover, we found that, although the LPG motif antagonizes TULP3 association, HTR6-CT residues both upstream and downstream of the LPG motif promote it. This might be explained by the LPG motif inducing HTR6 release once inside the cilium, but this hypothesis remains untested.

Another mystery is why the HTR6-CT residues needed for TULP3 binding are seemingly not required for ciliary targeting. Since our BioID2-mediated proximity biotinylation assays were performed with CD8α-(HTR6-CT)-BioID2 chimeras, it may be that HTR6 regions outside its CT also promote TULP3 association. HTR6-IC3 might contribute, as we found that TULP3 is strongly required for ciliary targeting of CD8α-(HTR6-IC3)-EYFP. However, our association assays with CD8α-(HTR6-IC3)-BioID2 suggested very weak or no TULP3 association [59]. Alternatively, other cytosol-facing regions of HTR6, like IC1 or IC2, might help recruit TULP3 when HTR6-CT cannot.

Given that TULP3 association is promoted by HTR6-CT regions both before and after the LPG motif, it is possible that concomitant mutations in both these regions are required to prevent TULP3 recruitment and thus block ciliary targeting. The main caveat of this hypothesis is that Δ373–389 completely abolished TULP3 association with HTR6-CT, and yet two separate deletions encompassing these residues (Δ371–378 and Δ379–391) had no detectable effect on ciliary targeting [59]. However, these separate deletions may not suffice to disrupt TULP3 recruitment, so more exhaustive analysis is needed to clarify these points.

Biochemical Society Transactions (2021) **49** 79–91
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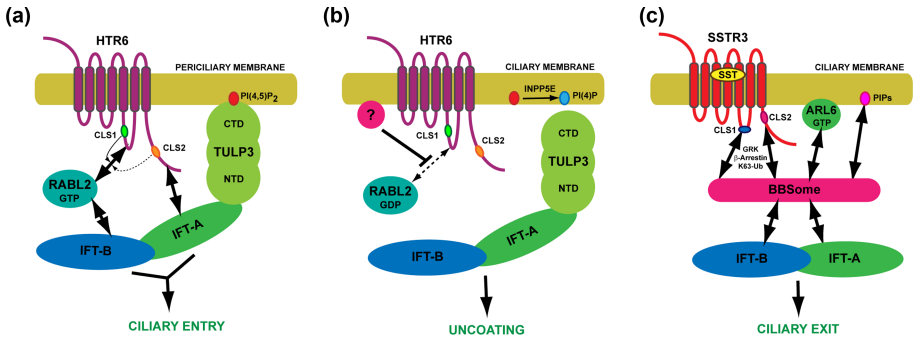

Figure 4. Mechanisms of HTR6 and SSTR3 ciliary targeting.

(**a**) Model of HTR6 ciliary entry. HTR6-CT recruits TULP3/IFT-A, whereas HTR6-IC3 promotes binding to RABL2, whose activation leads to IFT-B recruitment and initiation of IFT train movement into the cilium. (**b**) Model of HTR6 release from ciliary entry machinery once inside cilia. GTP hydrolysis in RABL2 triggers its dissociation from IFT-B, while PI(4,5)P2 depletion releases TULP3-IFT-A from membrane. This is associated with HTR6 release. (**c**) Model of SSTR3 ciliary exit. Ligand activation triggers G protein signaling, followed by GPCR kinase (GRK)-mediated phosphorylations, beta-arrestin recruitment and K63-linked ubiquitination in SSTR3 IC3 and CT. All this promotes binding of the active membrane-bound BBSome to SSTR3 IC3 and CT in a CLS-dependent manner, and efficient trafficking of these complexes in outbound IFT trains. See text for further details.

Role of RABL2 in HTR6 and SSTR3 ciliary targeting

RABL2 is a small GTPase whose GTP-bound form, resulting from RABL2's intrinsic guanine nucleotide exchange activity, is recruited by CEP19 to the distal mother centriole, where RABL2-GTP recruits its effector, the IFT-B holocomplex, with which RABL2-GTP is subsequently released into the cilium to initiate anterograde IFT, thus promoting ciliogenesis and ciliary protein entry [61,62,64]. Once inside cilia, GTP hydrolysis is required for RABL2 dissociation from IFT-B, and for BBSome-dependent ciliary exit [68]. Accordingly, RABL2-null mice display a BBS-like phenotype [61,64,68]. Unlike mice, humans have two nearly identical and functionally redundant RABL2 isoforms, RABL2A and RABL2B [61,64].

Through its effects on ciliary entry and exit, RABL2 also regulates ciliary GPCR trafficking. Ciliary levels of GPCRs, including HTR6, are reduced by RABL2 siRNAs, whereas overexpression of GTP-locked RABL2-Q80L increases ciliary GPCR accumulation [61,62,68]. Furthermore, HTR6 and other ciliary GPCRs interact with RABL2, regardless of GTP status, as opposed to IFT-B, which specifically interacts with GTP-bound RABL2 [61,62,64]. This suggests that GPCRs accompany RABL2 as it is recruited to the distal basal body, leading to GPCR loading into IFT trains entering cilia (Figure 4a). Inside the cilium, GTP hydrolysis would allow RABL2 to get off anterograde IFT trains and release its cargo GPCRs (Figure 4b). Subsequently, RABL2 would board BBSome-associated retrograde trains carrying GPCRs earmarked for ciliary exit, a process dependent on their K63-linked ubiquitination (Figure 4c) [69].

To test whether CLSs play a role in recruiting ciliary GPCRs to RABL2 complexes, we tested how HTR6 interacts with RABL2. Although RABL2 interacted with both HTR6-IC3 and HTR6-CT, HTR6-RABL2 binding was only perturbed by disruption of CLS1, but not of CLS2. Accordingly, RABL2 was required for ciliary targeting of CD8α-(HTR6-IC3)-EYFP, but only had a minor effect on CD8α-(HTR6-CT)-EYFP. Hence, at least for HTR6, CLS1 appears to be the key CLS connecting the GPCR to RABL2 [59].

We also tested whether TULP3 affects HTR6-RABL2 binding. Overexpression of wild type TULP3 had no effect on it, but a reduction was seen with the TULP3-KR mutant (K268A + R270A), which cannot bind PIPs and, as recently shown, cannot be acetylated at K268 [59,66,70]. This suggests RABL2 and TULP3 may cooperate in ciliary HTR6 targeting. For instance, these data could mean that, after trafficking complexes enter cilia by crossing the transition zone, the low $PI(4,5)P_2$ levels at the ciliary membrane induce TULP3 membrane disengagement, thereby allowing TULP3 to dissociate the HTR6-RABL2 complex. Nevertheless, although these findings point to a complex interplay between TULP3, RABL2 and HTR6, the nature of these connections, and whether they apply to other GPCRs like SSTR3, has barely begun to be explored.

Biochemical Society Transactions (2021) 49 79–91
https://doi.org/10.1042/BST20191005

Role of the BBSome in HTR6 and SSTR3 ciliary targeting

The BBSome is a multiprotein complex whose dysfunction underlies Bardet-Biedl syndrome in humans [6,7]. In BBSome mutant mice, SSTR3, MCHR1 and NPY2R fail to accumulate in brain neuronal cilia, whereas other cell types display ciliary overaccumulation of GPR161, SMO, DRD1 and other ciliary membrane proteins [6,47,48,53,71–74]. Although seemingly contradictory, it now seems both phenotypes are due to lack of BBSome-mediated ciliary exit, which, under certain conditions, may secondarily lead to GPCRs leaving cilia aboard ectovesicles (EVs) budding off the ciliary tip [73,75].

Signaling downstream of SSTR3 and other G_i-coupled GPCRs causes the BBSome to assemble into large, highly processive, retrograde IFT trains that traffic activated GPCRs from cilia tip to base, crossing the transition zone on their way out [73,76–78]. In BBSome mutants, failure of this retrieval mechanism is offset by overactivation of an alternative downregulation mechanism for active GPCRs: actin-dependent ectocytosis of ciliary EVs. In wild type cells, SSTR3 and GPR161 downregulation occurs via BBSome-dependent retrieval, whereas NPY2R preferentially uses ectocytosis. However, when β-arrestin or BBSome mutations prevent retrieval, all these GPCRs are released via EVs [73,75]. Thus, the BBSome controls ciliary GPCR levels by determining ciliary exit rates and mechanisms.

The BBSome, which shares homology with vesicle coats and adaptors, exists in a soluble autoinhibited state that is activated by the active form of a small GTPase, ARL6, leading to formation of planar membrane coats through interactions with both PIPs and transmembrane cargoes, like GPCRs [60,79–83]. Recent structural work sheds light on BBSome membrane recruitment and GPCR interactions [79–83]. When active, the BBSome uses a positively charged surface to interact with the negative membrane, while IFT-binding sites on the BBSome face away from membrane. Interestingly, a deep negatively charged cleft opens from the membrane side of the BBSome, extending perpendicularly from the plane of the membrane. This cleft is predicted to simultaneously accommodate both IC3 and CT of a GPCR [79,80]. Accordingly, virtually every ciliary GPCR analyzed, including SSTR3 and HTR6, directly interacts with the BBSome through both IC3 and CT, even if affinities vary considerably [82]. In CT, an important role is played by helix H8, an amphipathic helix that interacts with the inner leaflet of the lipid bilayer, and which is located right after TM7 [83]. In SMO, H8 is pulled away from the inner leaflet in order to interact with the BBSome cleft [83]. This interaction is probably conserved in other ciliary GPCRs, and in SSTR3 it involves the juxtamembrane CLS2 region [83].

BBSome binding has been most studied for SSTR3 [60,82]. We already mentioned how the BBSome binds the tandem AP[AS]CQ motifs of mouse SSTR3 in a cysteine-dependent manner [60]. More recently, BBSome binding to peptides covering the entire human SSTR3 sequence was systematically analyzed [82]. Remarkably, BBSome binding was strongest for two peptides: one including the single APSCQ motif of human SSTR3-IC3 (AGRRVWAPSCQRRRR), and another one in SSTR3-CT's juxtamembrane helix H8 region, largely matching our CLS2 mapping data, including FK and LLxP motifs [59,82].

The latter peptide (GFLSYRFKQGFRRVLLRPSRRVRS) was mutagenized to assess effects on BBSome-binding affinity. Mutation of the FK motif, and of similar aromatic-basic YR and FR motifs, all strongly reduce binding affinity, as does mutating the last three arginines to glutamates. Whether LLxP mutation affects BBSome binding was not tested [82]. It would also be interesting to test whether SSTR3-CT's glutamate-rich coiled coil (CC) antagonizes BBSome binding to SSTR3, which could explain why CC deletion reduces SSTR3 ciliary targeting two-fold (Figure 4c) [59].

Like SSTR3, BBSome binding and ciliary targeting of SMO also relies on aromatic-basic motifs in its juxtamembrane CT [55,82,83]. HTR6 juxtamembrane CT also binds the BBSome [82], and it contains a single FK motif, which we also mutated. Its mutation moderately impaired ciliary targeting, but much less so than disrupting the LPG motif (not shown). Whether the BBSome binds HTR6's LPG and RKQ motifs remains unknown.

If, as predicted, BBSome-GPCR interactions bury both IC3 and CT within a BBSome cleft, then such interactions would likely prevent binding of IC3-CT to other ciliary trafficking components, like IFT-A, which also binds directly to SSTR3-IC3 [76]. This raises the issue of how IC3-CT interactions are regulated by different signals over a GPCR's lifetime, and how these signal-dependent interactions determine the GPCR's behavior.

Under basal conditions, IC3-CT may connect to ciliary entry machinery like TULP3/IFT-A and RABL2/IFT-B (Figure 4a). Dissociation from this machinery would occur once inside cilia, possibly in response to the different PIP and small GTPase environment (Figure 4a,b). Upon GPCR activation, IC3-CT would help transduce signals to G proteins and other effectors, after which IC3-CT would be phosphorylated by GPCR kinases

Biochemical Society Transactions (2021) **49** 79–91
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(GRKs), leading to β-arrestin recruitment, K63-linked ubiquitination, and BBSome-mediated retrieval or, alternatively, to ciliary tip ectocytosis (Figure 4c) [63,69,77]. Thus, by orchestrating ciliary entry and exit in response to diverse inputs, GPCR CLSs are the conductors controlling time-dependent ciliary GPCR accumulation or depletion.

Perspectives

- The human genome encodes nearly one thousand GPCRs, the most common drug targets in the human clinic. Many GPCRs function inside primary cilia, specialized signaling organelles working as cellular antennae. HTR6 and SSTR3 are two ciliary GPCRs regulating multiple aspects of brain function, and whose pharmacological modulation holds much promise for the treatment of psychiatric, neurodegenerative, and oncologic diseases. Since ciliary localization of these GPCRs is key for their functions, a deeper understanding of their ciliary targeting mechanisms will likely have a positive impact on human health.

- Until recently, HTR6 and SSTR3 ciliary targeting was thought to depend on a single CLS in their IC3 loops. However, recent data shows their ciliary targeting is instead driven by two redundant CLSs: one in IC3 (CLS1), the other in CT (CLS2). Furthermore, CLS1 function of both GPCRs had been proposed to rely on Ax[AS]xQ motifs in IC3, yet it is now clear that this motif is not needed in HTR6, whereas SSTR3 does rely on tandem AP[AS]CQ motifs, with the cysteines being important. HTR6 CLS1 instead relies on a RKQxxxV motif promoting RABL2 association, while CLS2 requires an LPG motif antagonizing TULP3 binding. In SSTR3, CLS2 function requires its juxtamembrane residues and is modulated by its CC. The mechanisms of action of these CLSs are still poorly understood, but the emerging picture indicates that CLSs orchestrate ciliary targeting by affecting how and when the GPCRs interact with the machinery controlling ciliary entry, like TULP3/IFT-A and RABL2/IFT-B, and ciliary exit, like the BBSome.

- Future studies will establish whether other GPCRs also rely on redundant CLSs for ciliary targeting, and whether their CLSs resemble those of HTR6 and SSTR3 in form and function. As showcased by several recent BBSome structure-function studies [79–83], the ciliary GPCR targeting field is now entering a new and exciting phase in which CLS interactions with ciliary entry and exit machinery will be characterized in much greater structural and biochemical detail. HTR6 and SSTR3 are likely to feature prominently in such studies.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Author Contribution
F.R.G.G. and P.B. wrote the manuscript and prepared the figures.

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Abbreviations

AC, adenylyl cyclase; BBS, Bardet-Biedl syndrome; CC, coiled coil; CT, C-terminus; DRD1, D1 dopamine receptor; EVs, ectovesicles; GPCRs, G protein-coupled receptors; IC3s, third intracellular loops; IFT, intraflagellar transport; JS, Joubert syndrome; PIPs, phosphoinositides; PM, plasma membrane; SST, Somatostatin; TULP3, Tubby-like protein 3.

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