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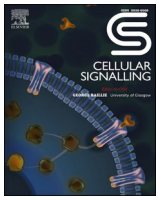

Adaptor protein complex 1 facilitates ciliary localization of serotonin receptor type 6

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ABSTRACT

The primary cilium, an immotile protrusion of vertebrate cells, detects chemical and mechanical stimuli in the extracellular milieu and transduces them into the cell body, thereby contributing to cellular development and homeostasis. In the mammalian brain, serotonin receptor type 6 (Htr6) and other specific G protein-coupled receptors (GPCRs) localize preferentially to primary cilia and function in ciliary chemical detection; however, the molecular mechanism by which a subset of GPCRs is transported to primary cilia has not been fully elucidated. In the present study, we demonstrate that a region in the fourth intracellular domain of Htr6 (Htr6 i4) is sufficient for ciliary localization. In yeast, the interaction of this region with the C-terminal region of γ1-Adaptin, a subunit of adaptor protein complex 1 (AP-1), was identified. The interaction between Htr6 and γ1-Adaptin was confirmed by immunoprecipitation analysis using HEK293T cells. The preference for ciliary localization of Htr6 and Htr6 i4 was significantly decreased by ablation of *γ1-Adaptin* in hTERT RPE-1 cells, which was rescued by exogenous expression of γ1-Adaptin. Furthermore, Htr6 and Htr6 i4 showed reduced localization to primary cilia in *γ1-Adaptin*-depleted cultured hippocampal neurons compared with control neurons. These results indicate that the ciliary localization of Htr6 is facilitated by AP-1-mediated membrane trafficking.

1. Introduction

Almost all vertebrate cells have a tiny immotile primary cilium that singly protrudes like an antenna into the environment surrounding the cell and transduces chemical and mechanical sensory stimuli to the cell body. These cilia-mediated signaling processes contribute to cellular development and homeostasis [1]. In most regions of the mammalian brain, each neuron has a solitary primary cilium, although some neuronal subtypes lack a primary cilium [2] and some possess 2–3 primary cilia [3]. Neuronal primary cilia are believed to be a sensory device; however, their functions remain largely unknown [4,5]. Specific G protein-coupled receptors (GPCRs), including serotonin receptor type 6 (Htr6) and somatostatin receptor type 3 (Sstr3), localize preferentially to primary cilia together with adenylyl cyclase 3 (AC3) and detect their ligands at the ciliary membrane [6]. Intriguingly, it has recently been shown that serotonin transmission occurs at synapses between serotonergic axons and Htr6-expressing primary cilia of hippocampal CA1 pyramidal neurons, thereby modulating chromatin accessibility in the postsynaptic neurons [7].

Transport of transmembrane proteins into the ciliary membrane is believed to be mediated by multiple pathways [8]. Several amino acid motifs in GPCRs have been proposed to play a critical role in targeting receptors to the ciliary membrane. These ciliary localization sequences (CLSs) have been mapped in the fourth intracellular domain (i4) of Smoothened (Smo), a GPCR that mediates hedgehog signaling in vertebrate cilia [9], and in the third intracellular domain (i3) of Htr6, Sstr3, melanin-concentrating hormone receptor type 1 (Mchr1) [10], neuropeptide Y receptor type 2 [11], and Gpr161, an orphan receptor that regulates hedgehog signaling [12]. Furthermore, Tubby-like protein 3 (Tulp3), a member of the tubby family proteins, participates in ciliary delivery of multiple GPCRs [13]. Tulp3 physically interacts with i3 of Mchr1 and Gpr161, on the basis of the presence of the CLS within their i3, at the periciliary plasma membrane before subsequent delivery of these GPCRs into the ciliary compartment in an IFT-A complex-

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dependent manner [13,14]. Moreover, Rab-like 2 (Rabl2), an atypical small GTPase, physically interacts with Htr6 and Gpr161 and promotes their entry into primary cilia [15]. Intriguingly, Barbeito et al. identified redundant CLSs in both i3 and i4 of Htr6 and Sstr3 using IMCD3 cells and cultured neurons and demonstrated a complicated mechanism by which CLSs in i3 and i4 of Htr6 contribute to its entry into primary cilia through modulating interactions with Tulp3 and Rabl2 [16]. These studies provide insights into the molecular machinery that underlies the delivery of a subset of GPCRs into primary cilia, which occurs at the base of primary cilia. However, it remains unclear how specific GPCRs are destined to localize preferentially to primary cilia.

Among ciliary GPCRs, Htr6 has a high preference for ciliary localization; it localizes almost exclusively to primary cilia of neurons in various mouse brain regions, including the hippocampus, cerebral cortex, and striatum [17]. Moreover, Htr6 is involved in neurite extension [18] and is associated with psychiatric diseases, such as schizophrenia [19] and bipolar disorder [20].

In this study, we identified γ1-Adaptin, a subunit of adaptor protein complex 1 (AP-1), as an interacting partner of Htr6. Furthermore, we examined the involvement of γ1-Adaptin in the preferential localization of Htr6 to primary cilia in hTERT RPE-1 (RPE1) cells, a human retinal pigment epithelial cell line, and in cultured rat hippocampal neurons.

2. Materials and methods

2.1. Animals

Wistar rats were purchased from Japan SLC (Hamamatsu, Japan). All experimental procedures were approved by the Animal Experiment Committee of the University of Osaka. All animal experiments were conducted under the Guidelines and Regulations on Animal Experimentation at the University of Osaka.

2.2. Antibodies and reagents

All antibodies used in this study are listed in Table S1.

2.3. Plasmids

Mouse *Htr6*, *Htr7*, and *γ1-Adaptin* cDNAs and human *γ1-Adaptin* and *CD8α* cDNAs were obtained by RT-PCR using total RNA from a C57BL/6 J mouse brain and from an adult human hippocampus (BioChain, Newark, CA, USA), respectively, PrimeScript II reverse transcriptase (Takara Bio, Kusatsu, Japan), and PrimeSTAR Max DNA polymerase (Takara Bio). Full-length or partial cDNAs were inserted into pGBKT7 (Takara Bio), pcDNA3.1(+) (Thermo Fisher Scientific, Waltham, MA, USA), pEF-BOS-EX [21], or IRES-tdTomato-NLS×3 [22] with or without an upstream or downstream sequence encoding Myc-tag or Flag-tag. Htr6 deletion mutations were generated by inverse PCR using a KOD Plus Mutagenesis Kit (TOYOBO, Osaka, Japan). The cDNA sequences were verified by Sanger sequencing. The backbone vector and inserted cDNA information of the plasmids used in this study is summarized in Table S2.

2.4. Culture of cell lines and transfection

RPE1 cells (ATCC, CRL-4000, Manassas, VA, USA) were cultured in DMEM/Ham's F-12 medium (Nacalai Tesque, Kyoto, Japan) containing 10 % fetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM GlutaMAX (Thermo Fisher Scientific), and 0.01 mg/mL hygromycin B (Merck, Darmstadt, Germany). HEK293T cells (ATCC, CRL-3216) were cultured in DMEM (Nacalai Tesque) containing 10 % FBS and 2 mM GlutaMAX. For immunofluorescence staining, RPE1 cells were seeded on four-well chamber slides at a density of 7.5×10^4 cells/well and cultured for 48 h. Then, cells were starved for 24 h in medium containing 0.5 % FBS for ciliation before fixation. RPE1 cells were transfected with the indicated plasmids 24 h after seeding, using ViaFect Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. For immunoblotting, RPE1 cells were cultured on 60-mm dishes for 48 h, starved for 24 h, and then harvested. For immunoprecipitation analysis, HEK293T cells were seeded on 60-mm dishes at a density of 7.5×10^5 cells/dish, transfected with the indicated plasmids 24 h after seeding, using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and harvested 24 h after transfection.

2.5. Yeast two-hybrid screening

Yeast two-hybrid screening was performed using Matchmaker Gold Yeast Two-Hybrid System (Takara Bio) according to the manufacturer's instructions. To identify interacting partners of the C-terminus of Htr6, a cDNA encoding the i4-(M+CT) fragment of Htr6 (aa 359–440, see Fig. 2A) was cloned into the pGBKT7 vector as the bait. The Y2HGold yeast strain transformed with the bait plasmid was mated with the Y187 yeast strain pre-transformed with a normalized universal mouse cDNA library cloned into the prey vector, pGADT7-RecAB (Mate & Plate Library-Universal Mouse, Takara Bio). The mated yeast were grown on Dropout media supplemented with X-α-Gal and Aureobasidin A (Takara Bio) to select for colonies with activated reporter genes owing to an interaction between the Bait and Prey.

To confirm that the interaction between the Bait/i4-EC and Prey in yeast is genuine, the Y2HGold yeast were co-transformed with plasmids encoding Bait/i4-EC and Prey, and then grown on Dropout media supplemented with X-α-Gal and Aureobasidin A. p53 and SV40 large T-antigen (T-antigen) served as controls. p53, Bait, and i4-EC were cloned into the pGBKT7 vector. T-antigen and Prey were cloned into the pGADT7 and pGADT7-RecAB vectors, respectively.

2.6. Generation of γ1-Adaptin-KO RPE1 cell lines using CRISPR/Cas9

γ1-Adaptin-KO RPE1 cell lines were generated as described previously [22]. Briefly, two single-guide RNA (sgRNA) sequences were cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 vector (Addgene plasmid # 62988), a gift from Feng Zhang [23]. The sgRNA sequences were as follows: #1: GGAGGACTACAGATGTGTGG and #2: GGGTTAGCACTTTGTACCCT targeting exons 6 and 4 of the human *γ1-Adaptin* gene (*AP1G1*), respectively. RPE1 cells transfected with PX459 carrying each sgRNA sequence were selected in medium containing puromycin (15 μg/mL) for 72 h. Cells were then cultured in puromycin-free medium until cell colonies were formed. The DNA sequences of the targeted genomic regions in isolated and expanded colonies were analyzed. Two independent cell lines (#1 and #2 from sgRNA#1 and sgRNA#2, respectively) harboring biallelic indel mutations were selected for further experiments.

2.7. Culture of hippocampal neurons and electroporation

Hippocampal neurons were cultured as described previously [24]. Briefly, embryonic hippocampi were dissected from embryonic-day-18 Wistar rat pups. The tissues were digested with papain solution containing 0.667 mg/mL papain (Worthington, Lakewood, NJ, USA), 1 mg/mL bovine serum albumin (BSA, Merck), 0.012 mg/mL DNase I (Merck), 1.8 mg/mL D-(+)-glucose (Merck), and phosphate buffered saline (PBS), and gently triturated with a fire-polished Pasteur pipette to produce a single neuron suspension. Neurons were plated onto glass coverslips coated with poly-D-lysine hydrobromide (Merck) located in 24-well plates at a density of 50,000 cells/well. Neurons were maintained in Neurobasal medium (Thermo Fisher Scientific) containing 2 % B-27 supplement (Thermo Fisher Scientific) and 2 mM GlutaMAX. Every 3 days half of the culture medium was replaced with fresh medium.

At 10 days in vitro (DIV10), hippocampal neurons were simultaneously transfected with the Dicer-Substrate Short Interfering RNA

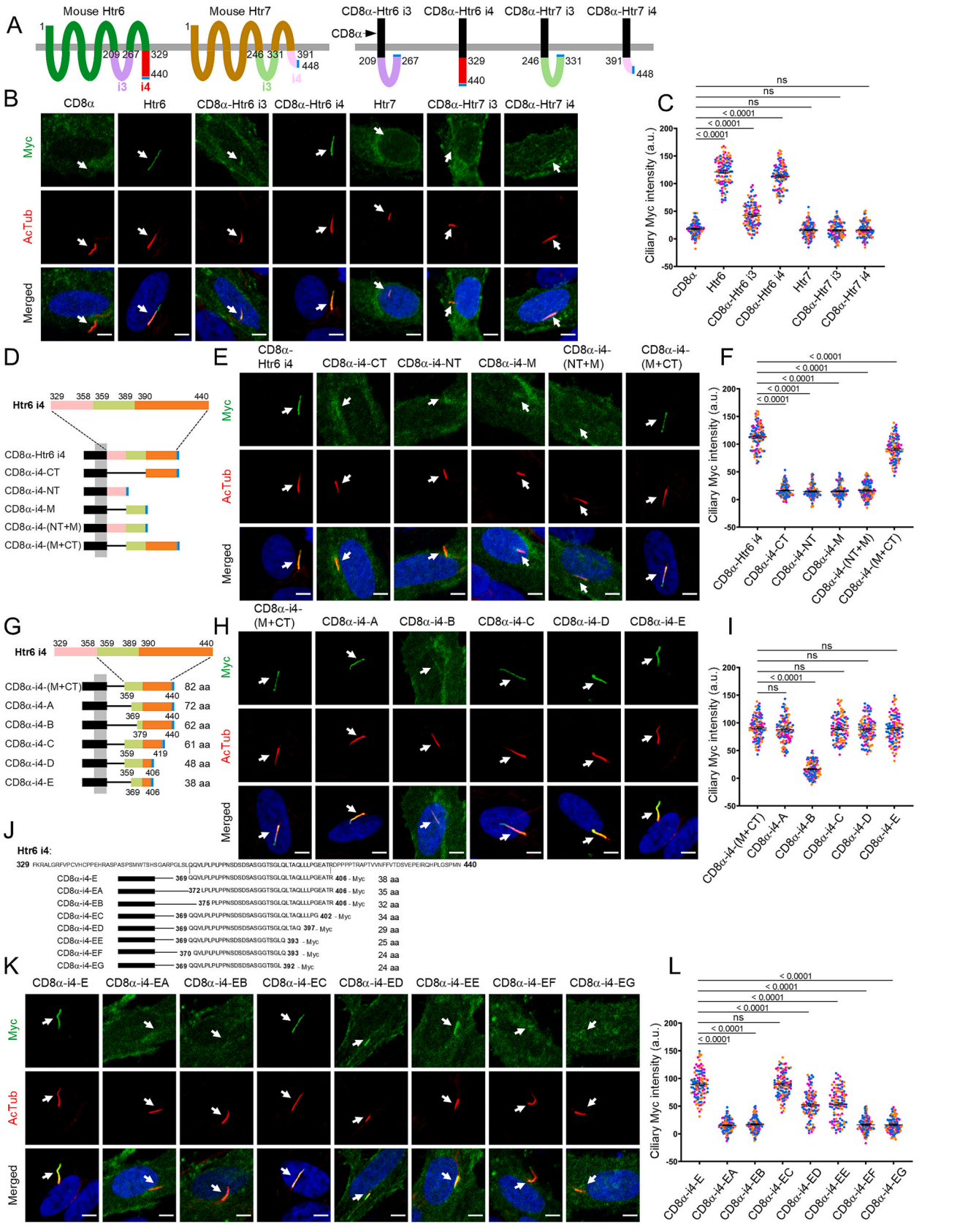

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Fig. 1. Identification of the intracellular region of Htr6 that contributes to its ciliary localization in RPE1 cells.
(A) *Left*: Schematic diagrams of mouse Htr6 highlighting its third (Htr6 i3) and fourth (Htr6 i4) intracellular domains and of mouse Htr7 highlighting its third (Htr7 i3) and fourth (Htr7 i4) intracellular domains. *Right*: Schematic diagrams of human CD8α amino acids (aa) 1–206 fused with Htr6 i3, Htr6 i4, Htr7 i3, or Htr7 i4. The Htr6, Htr7, and CD8α chimeras were tagged with Myc (blue rectangles) at their C-terminus. The gray line indicates the plasma membrane and above and below the gray line indicate the extracellular and intracellular spaces, respectively.
(B) Htr6, Htr7, and CD8α chimeras tagged with Myc in (A) were expressed in RPE1 cells and their localization was analyzed by immunostaining using an anti-Myc antibody. Primary cilia were stained with an anti-acetylated α-tubulin (AcTub) antibody. In merged images, nuclei stained with DAPI (blue) are shown.
(C) The intensity of Myc signals at cilia was measured and is shown in scatter plots. Differently colored dots denote three independent experiments ($n = 35 \times 3$). Horizontal lines and error bars represent means and SEM, respectively, of the three experiments.
(D–F) Htr6 i4 was divided into the three indicated parts. CD8α aa 1–206 (black rectangles) was fused with the indicated deletion mutants containing one or two parts of Htr6 i4. CD8α chimeras were tagged with Myc (blue rectangles) at their C-terminus (D). Localization of these chimeras expressed in RPE1 cells was analyzed by immunostaining (E), and the intensity of Myc signals at cilia was analyzed ($n = 35 \times 3$) (F) as described in (C).
(G–I) N- and C-terminal residues of i4-(M+CT) were deleted to generate the indicated mutants. CD8α aa 1–206 (black rectangles) was fused with these mutants. CD8α chimeras were tagged with Myc (blue rectangles) at their C-terminus (G). Localization of these chimeras expressed in RPE1 cells was analyzed by immunostaining (H), and the intensity of Myc signals at cilia was analyzed (n = 35 × 3) (I) as described in (C).
(J–L) N- and C-terminal residues of i4-E were deleted to generate the indicated mutants. CD8α aa 1–206 (black rectangles) was fused with these mutants. CD8α chimeras were tagged with Myc at their C-terminus (J). Localization of these chimeras expressed in RPE1 cells was analyzed by immunostaining (K), and the intensity of Myc signals at cilia was analyzed (n = 35 × 3) (L) as described in (C).
Arrows indicate primary cilia and scale bars represent 5 μm in (B), (E), (H), and (K). a.u., arbitrary unit; ns, not significant in (C), (F), (I), and (L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(DsiRNA) against the rat *γ1-Adaptin* gene (*Ap1g1*, TriFECTa Kit, Integrated DNA Technologies, Coralville, IA, USA) and the expression plasmid encoding the indicated receptor or the CD8α chimera by electroporation using a NEPA21 Electroporator (NEPAGENE, Ichikawa, Japan) according to the manufacturer's protocol. The DsiRNA and the plasmid were dissolved in Opti-MEM (Thermo Fisher Scientific) at concentrations of 50 nM and 0.5 μg/μL, respectively. Neurons were harvested or fixed at DIV13 for immunoblotting or for immunofluorescence staining, respectively.

2.8. Immunoblotting and immunoprecipitation

Harvested cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5, Nippon Gene, Tokyo, Japan), 1 mM dithiothreitol (Nacalai Tesque), 1 mM ethylenediaminetetraacetic acid (pH 8.0, Nacalai Tesque), 100 mM NaCl, 1 % Nonidet P-40 substitute (Fujifilm Wako Pure Chemical Co., Osaka, Japan), and a protease inhibitor cocktail (Roche, Mannheim, Germany) on ice for 1 h, and then centrifuged at 17,700 ×*g* for 15 min. The supernatants were denatured in sodium dodecyl sulfate (SDS) sample buffer at 100 °C for 5 min, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membranes (Immobilon-P, Merck). After blocking with PBS containing 1 % skim milk (Morinaga, Tokyo, Japan), membranes were incubated with primary antibodies diluted in PBS containing 1 % skim milk at 4 °C overnight. After washing with PBS containing 0.1 % polyoxyethylene sorbitan monolaurate (Tween 20, Nacalai Tesque), membranes were incubated with secondary antibodies at room temperature (RT) for 1 h. Bands were visualized by chemiluminescence using an ECL Western Blotting Detection Reagent (Cytiva, Tokyo, Japan) and X-ray films (Fujifilm, Tokyo, Japan). The supernatants for immunoblotting using a rabbit anti-Flag antibody were not denatured to reduce aggregation of GPCRs in the samples [25].

For immunoprecipitation, harvested cells transfected with the indicated cDNA constructs were incubated in the lysis buffer and centrifuged. The supernatants were incubated with mouse anti-Flag antibody-bound Dynabeads Protein G (Thermo Fisher Scientific) at 4 °C for 2 h. The beads were then washed five times with the lysis buffer. The immunoprecipitates were denatured (only samples for anti-Myc antibody blotting), separated by SDS-PAGE, and then subjected to immunoblotting using a rabbit anti-Myc antibody or a rabbit anti-Flag antibody.

2.9. Immunofluorescence staining

Immunofluorescence staining was performed as described previously [26] with some modifications. Briefly, RPE1 cells and primary hippocampal neurons were fixed with 4 % paraformaldehyde in phosphate buffer (Nacalai Tesque) at RT for 10 min. Neurons were post-fixed with methanol at −20 °C for 15 min. After permeabilization and blocking with blocking buffer containing 10 mM PBS, 0.2 % Triton X-100 (Merck), 10 mg/mL BSA, 2 % goat serum (Thermo Fisher Scientific), and 0.02 % sodium azide (Merck) for RPE1 cells or 10 mM PBS, 0.1 % Triton X-100, 10 mg/mL BSA, 5 % goat serum, and 0.02 % sodium azide for neurons, cells were incubated with primary antibodies at 4 °C overnight, and subsequently with secondary antibodies at RT for 1 h. Antibodies were diluted in the blocking buffer without Triton X-100. Stained samples were mounted using Vectashield mounting medium with or without DAPI (Vector Laboratories, Burlingame, CA, USA).

2.10. Microscopy

Immunostained cells were observed using a confocal laser microscope (Nikon C2, Nikon, Tokyo, Japan). *Z*-stack images were captured and analyzed using ImageJ software (NIH, Bethesda, MD, USA). Signal intensity at the cilium was quantified in a region of interest (ROI) constructed by drawing a line along the signal of acetylated α-tubulin (AcTub) or adenylyl cyclase 3 (AC3), subtracted by the signal intensity at a duplicated background ROI set to a nearby region in the cell body, and normalized to the cilium length.

2.11. Statistical analysis

Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test using GraphPad Prism software (La Jolla, CA, USA). For comparison in Fig. 7B,D and Fig. S5B–C, data were analyzed by Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Identification of the intracellular region contributing to the Htr6 ciliary localization in RPE1 cells

Htr7 shares homology with Htr6; however, Htr7 localizes to cilia to a much lesser extent than Htr6 in IMCD3 cells [10,16]. Therefore, in this study, we used Htr7 as a negative control GPCR for localization to primary cilia.

To determine the intracellular region contributing to the ciliary localization of Htr6, RPE1 cells were transfected with expression plasmids encoding the following proteins: Htr6, Htr7, the single transmembrane protein CD8α, and chimeric proteins in which the i3 or i4 domains of Htr6 and Htr7 were fused with the extracellular and transmembrane regions of CD8α (Fig. 1A). All these proteins were tagged with Myc at their C-terminus to enable detection of their location by

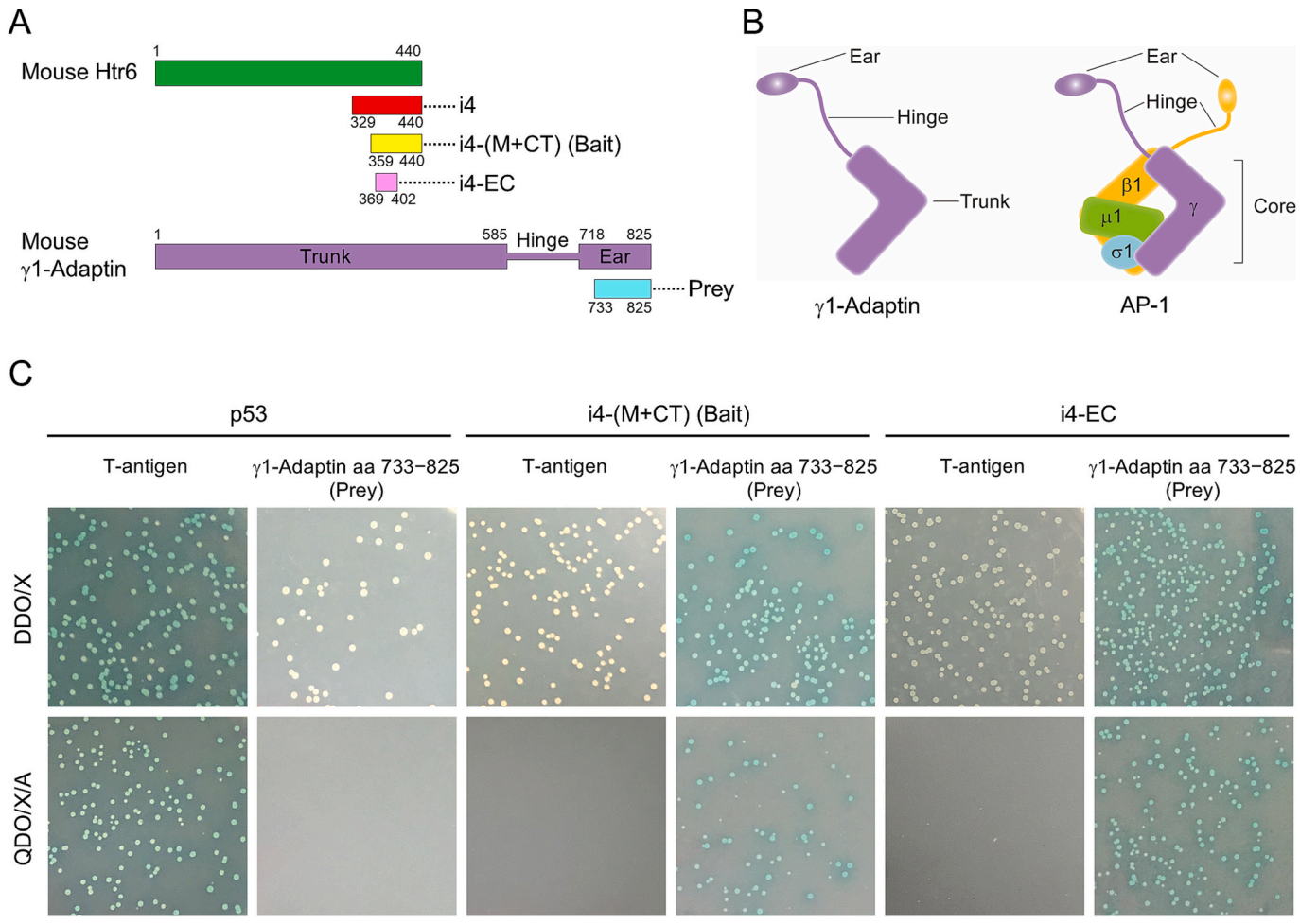

Fig. 2. γ1-Adaptin interacts with Htr6 in yeast.
(A) *Top*: the mouse Htr6 protein and the location of its fragments, Htr6 i4, Htr6 i4-(M+CT) used as the bait in the yeast two-hybrid screening, and Htr6 i4-EC. *Bottom*: Schematic representation of the domain organization of the mouse γ1-Adaptin protein. Like other large subunits of AP complexes, γ1-Adaptin consists of "trunk", "hinge", and "ear" domains. The location of the γ1-Adaptin prey region (aa 733–825) identified to interact with the bait, Htr6 i4-(M+CT), by the screening is also shown.
(B) Diagrams of γ1-Adaptin and AP-1. AP-1 is formed from γ-Adaptin (γ1 or γ2), β1-Adaptin, μ1, and σ1 subunits. The "core" domain of AP-1 consists of subunits μ1 and σ1 and the "trunk" domains of γ-Adaptin and β1-Adaptin.
(C) Yeast co-transformed with a combination of Bait and Prey and of i4-EC and Prey as well as of p53 (DNA-BD control) and SV40 large T-antigen (T-antigen, AD control) formed blue colonies not only on the DDO/X medium (Double dropout: SD/−Leu/−Trp supplemented with X-α-Gal) but also on the QDO/X/A medium (Quadruple dropout: SD/−Ade/−His/−Leu/−Trp supplemented with X-α-Gal and Aureobasidin A). Yeast co-transformed with a combination of p53 and Prey, of Bait and T-antigen, and of i4-EC and T-antigen formed white colonies on the DDO/X medium and no colonies on the QDO/X/A medium.

immunofluorescence analysis using an antibody against Myc (Fig. 1B). Primary cilia were marked by an antibody against acetylated α-tubulin (AcTub). Quantification of Myc signal intensity at cilia revealed that Htr6, CD8α-Htr6 i3, and CD8α-Htr6 i4 displayed more preferential localization to primary cilia than CD8α (Fig. 1C). However, Htr7 and its two chimeric proteins revealed a similar extent of ciliary localization as CD8α. Intriguingly, CD8α-Htr6 i3 localized to cilia less than Htr6 and CD8α-Htr6 i4, indicating that the i4 domain of Htr6 is sufficient for ciliary localization of the receptor, while its i3 domain contributes to the localization to a lesser extent than its i4 domain in RPE1 cells. This result prompted us to identify the minimum region sufficient for ciliary localization within the i4 domain of Htr6. RPE1 cells were transfected with different CD8α-fused and Myc-tagged fragments of the Htr6 i4 domain to examine their preference for ciliary localization (Fig. 1D). Among these chimeras, CD8α-i4-(M+CT) showed robust localization to cilia, although its ciliary localization was less potent than that of CD8α-Htr6 i4 (Fig. 1E–F). When RPE1 cells were transfected with CD8α-fused fragments of the i4-(M+CT) region (Fig. 1G), CD8α-i4-B localized to cilia much less intensely than CD8α-i4-(M+CT), while the ciliary localization of all other chimeras was comparable with that of CD8α-i4-(M+CT) (Fig. 1H–I). This finding indicates that amino acids (aa) 369–378 are required for ciliary localization of the i4 domain. Additionally, within i4-E, which is the smallest among these cilia-localized fragments, several fragments were constructed (Fig. 1J), and the localization of their CD8α-fused chimeras was analyzed. CD8α-i4-EC localized to cilia to a similar extent as CD8α-i4-E, while the ciliary localization of all the other chimeras was less intense than CD8α-i4-E (Fig. 1K–L). These results indicate that i4-EC is the minimal region sufficient for the ciliary localization of Htr6 i4.

3.2. γ1-Adaptin interacts with Htr6 in yeast

We speculated that the ciliary localization of Htr6 is facilitated by key molecules that interact with i4-EC or the region spanning i4-EC and neighboring residues within Htr6 i4. Therefore, we aimed to identify the interacting partners of i4-(M+CT). To this end, yeast two-hybrid screening of a mouse cDNA library was conducted using i4-(M+CT) as bait. Among several prey clones obtained from the screening, we focused

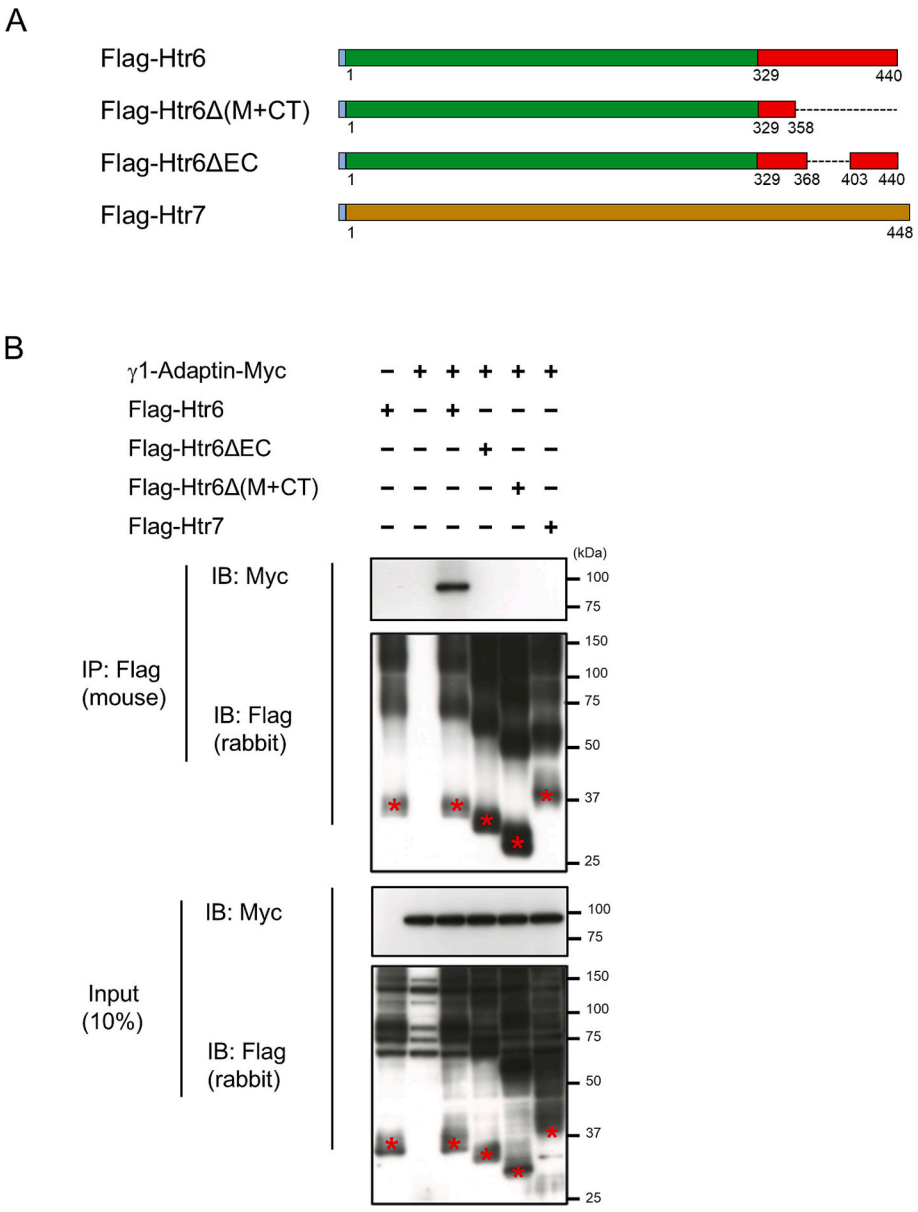

Fig. 3. Htr6 interacts with γ1-Adaptin in mammalian cells.
(A) Htr6 containing its i4 domain (red rectangle), Htr6 deletion mutants Δ(M+CT), lacking the i4-(M+CT) bait region, and ΔEC, lacking the i4-EC region, and Htr7 were tagged with a Flag tag (purple rectangle) at their N-terminus and used in immunoprecipitation analysis. Dashed lines indicate deleted regions.
(B) For immunoprecipitation, γ1-Adaptin tagged with Myc at its C-terminus was co-expressed with each of the proteins in (A) in HEK293T cells. Cell lysates were immunoprecipitated (IP) by a mouse anti-Flag antibody. The resultant immunoprepicitates were analyzed by immunoblotting (IB) using an anti-Myc antibody and a rabbit anti-Flag antibody. Ten percent of the lysate used for immunoprecipitation was analyzed as an input control. The bands denoted by red asterisks correspond to monomer proteins, while the bands with higher molecular weights are likely to represent polymerized proteins because GPCRs tend to aggregate during cell lysis [25]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the C-terminal 733–825 aa region of γ1-Adaptin, a subunit of AP-1 [27,28] (Fig. 2A–B). This prey region is within the "ear" domain of γ1-Adaptin (Fig. 2A). Growth of co-transformed yeast colonies on Dropout media confirmed that the interaction between the bait and the γ1-Adaptin prey region in yeast was genuine (Fig. 2C). Furthermore, this assay demonstrated in yeast an interaction between i4-EC, the minimal region sufficient for ciliary localization of Htr6 i4 (Fig. 1J–L, Fig. 2A), and the γ1-Adaptin prey region (Fig. 2C).

3.3. Htr6 interacts with γ1-Adaptin in mammalian cells

Next, we investigated by immunoprecipitation whether Htr6 and γ1-Adaptin interact with each other in mammalian cells. C-terminally Myc-tagged γ1-Adaptin was exogenously co-expressed with N-terminally Flag-tagged Htr6, its deletion mutants, or Htr7 in HEK293T cells (Fig. 3A–B). Anti-Flag immunoprecipitates were subjected to immunoblotting using an antibody against Myc or Flag (Fig. 3B). γ1-Adaptin co-immunoprecipitated with Htr6, but not with its deletion mutant lacking i4-(M+CT) or i4-EC, nor with Htr7, indicating that Htr6 specifically interacts with γ1-Adaptin through the i4-EC region in its i4 domain in mammalian cells.

3.4. γ1-Adaptin facilitates Htr6 ciliary localization in RPE1 cells

To examine the involvement of γ1-Adaptin in the ciliary localization of Htr6, the gene encoding γ1-Adaptin was ablated in RPE1 cells using

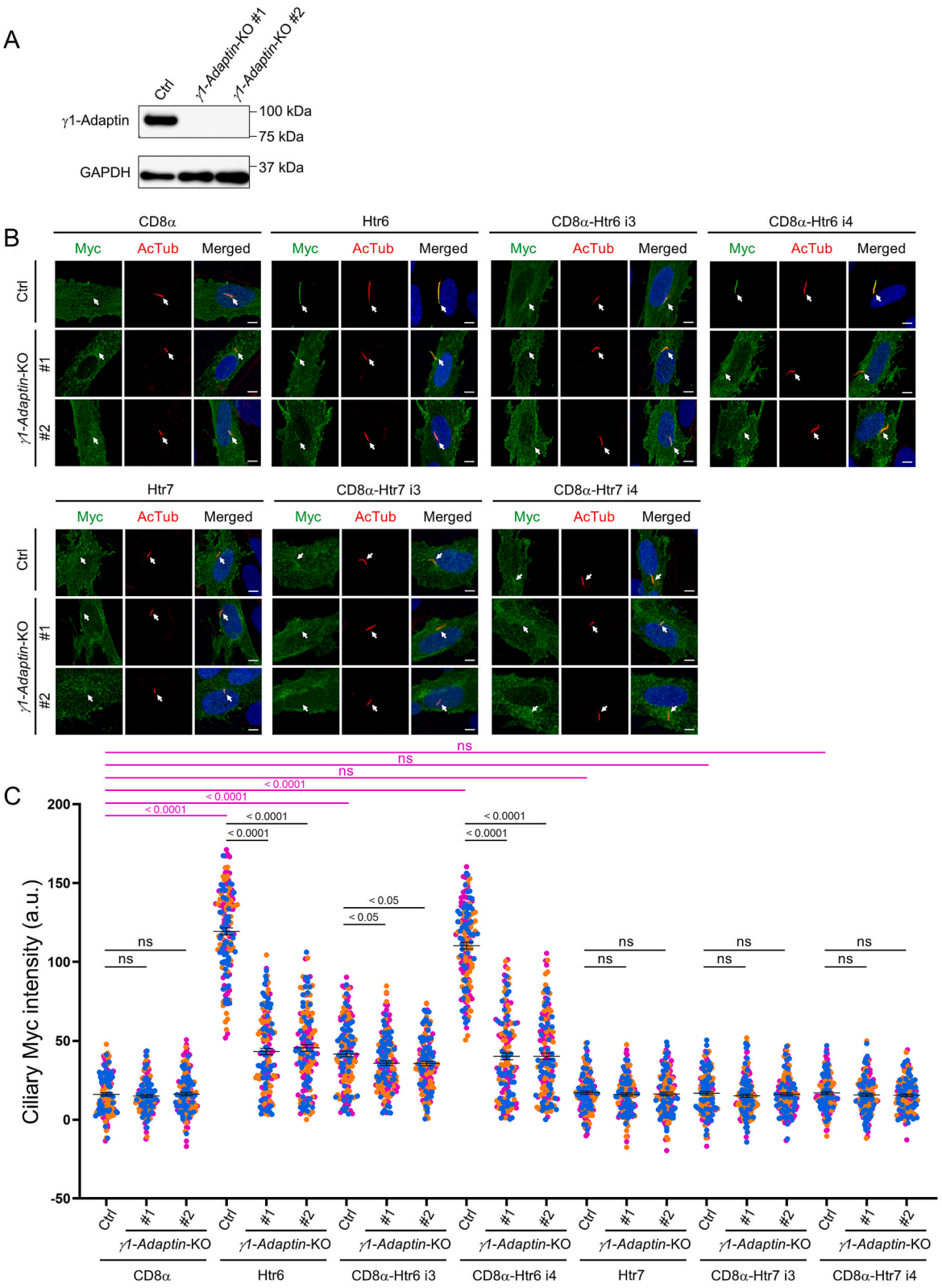

Fig. 4. γ1-Adaptin facilitates ciliary localization of Htr6 in RPE1 cells.
(A) Establishment of *γ1-Adaptin*-knockout (KO) RPE1 cells using CRISPR/Cas9. Immunoblotting using an anti-γ1-Adaptin antibody confirmed the absence of the γ1-Adaptin protein in *γ1-Adaptin*-KO cell lines #1 and #2. GAPDH served as a loading control.
(B) Htr6, Htr7, and the indicated CD8α chimeras tagged with Myc were expressed in control (Ctrl) RPE1 and *γ1-Adaptin*-KO cells. Their localization was analyzed by immunostaining using an anti-Myc antibody. Primary cilia were stained with an anti-AcTub antibody. In merged images, nuclei stained with DAPI (blue) are shown. Arrows indicate primary cilia and scale bars represent 5 μm.
(C) The intensity of Myc signals at cilia was measured and is shown in scatter plots ($n = 50 \times 3$). Symbols are used as described in Fig. 1C. a.u., arbitrary unit; ns, not significant.

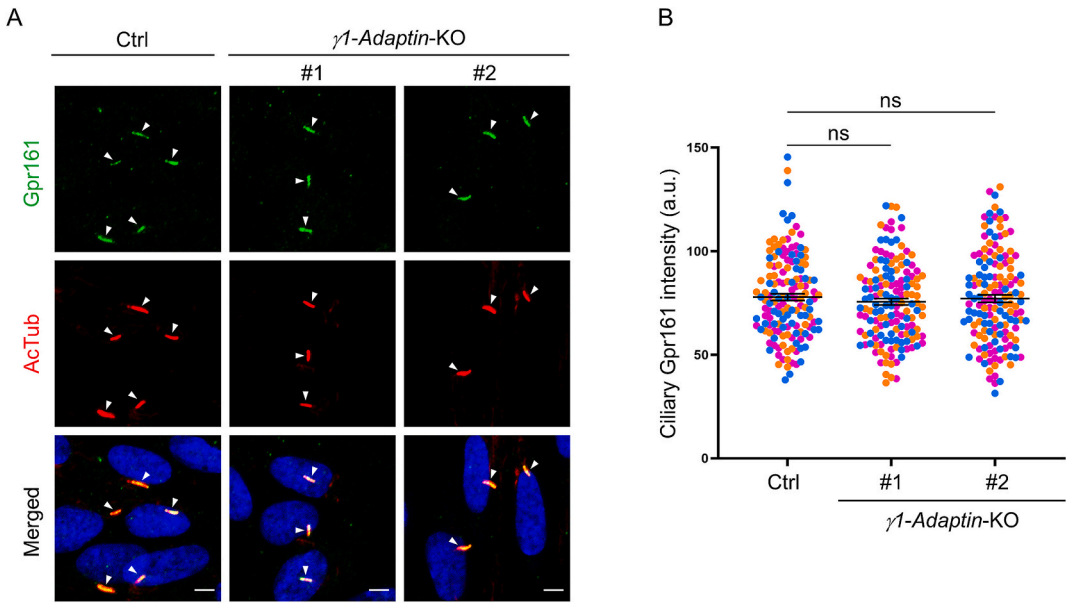

Fig. 5. Absence of γ1-Adaptin does not affect the ciliary localization of Gpr161 in RPE1 cells.
(A) Localization of the endogenous Gpr161 protein was analyzed by immunostaining using an anti-Gpr161 antibody in control (Ctrl) RPE1 and *γ1-Adaptin*-KO cell lines. Primary cilia were stained with an anti-AcTub antibody. In merged images, nuclei stained with DAPI (blue) are shown. Arrowheads indicate primary cilia and scale bars represent 5 μm.
(B) The intensity of Gpr161 signals at cilia was measured and is shown in scatter plots (n = 50 × 3). Symbols are used as described in Fig. 1C. a.u., arbitrary unit; ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CRISPR/Cas9. Two independent *γ1-Adaptin*-knockout (KO) cell lines (#1 and #2) were established using two different sgRNAs targeting exon 6 and exon 4, respectively, of the *γ1-Adaptin* gene. Lack of the γ1-Adaptin protein in the *γ1-Adaptin*-KO cell lines was confirmed by immunoblotting using an antibody against γ1-Adaptin (Fig. 4A). These KO cell lines displayed less ciliation and formed shorter cilia compared with the control cells (Fig. S1A–C). When C-terminally Myc-tagged Htr6 and CD8α-Htr6 i4 were exogenously expressed, their localization to primary cilia was dramatically reduced in KO cell lines compared with that in control cells (Fig. 4B–C). Myc-tagged CD8α-Htr6 i3 also displayed weaker ciliary localization in KO cells than in control cells (Fig. 4B–C). In contrast, ciliary localization of Myc-tagged Htr7, CD8α-Htr7 i3, and i4 was comparable between control cells and KO cells (Fig. 4B–C). These results indicate that γ1-Adaptin facilitates ciliary localization of Htr6, but not of Htr7, and that this facilitation is mostly ascribed to the effect of γ1-Adaptin on the i4 domain of Htr6. The compromised ciliary localization of Myc-tagged Htr6 and CD8α-Htr6 i4 in the KO cells was rescued by exogenous co-expression of human γ1-Adaptin (Fig. S2A–D), confirming the specific effect of γ1-Adaptin on their ciliary localization and excluding the off-target effects of CRISPR/Cas9.

Ciliary localization of Htr6 and CD8α-Htr6 i4 was downregulated but not completely lost in *γ1-Adaptin*-KO cells (Fig. 4C). This remaining ciliary localization in the KO cells might result from the participation of AP-1 containing another γ-Adaptin, γ2-Adaptin [29], on the basis of moderate similarity of the amino acid sequence between the "ear" domain of γ1-Adaptin containing the prey region and the same domain of γ2-Adaptin (Fig. S3A–B). However, this idea seems unlikely because immunofluorescence staining revealed an absence of the σ1A subunit of AP-1 in *γ1-Adaptin*-KO cells (Fig. S4), indicating complete lack of AP-1 formation by ablation of *γ1-Adaptin* and unavailability of γ2-Adaptin for AP-1 formation in RPE1 cells.

To assess whether the ciliary localization of transmembrane proteins is generally facilitated by γ1-Adaptin, the localization of Gpr161, a ciliary orphan GPCR expressed endogenously in RPE1 cells [12], was examined in *γ1-Adaptin*-KO cells and control cells. Immunofluorescence staining revealed that the ciliary localization of Gpr161 was not compromised by ablation of *γ1-Adaptin* in RPE1 cells (Fig. 5A–B).

3.5. *γ1-Adaptin facilitates Htr6 ciliary localization in cultured hippocampal neurons*

Next, to evaluate the involvement of γ1-Adaptin in the ciliary localization of Htr6 in neurons, primary neurons from rat embryonic hippocampi were transfected simultaneously with DsiRNA against rat *γ1-Adaptin* and an expression plasmid encoding Myc-tagged Htr6, CD8α-Htr6 i3 or i4, or Htr7 by electroporation at DIV10. Immunoblotting confirmed a profound reduction in the γ1-Adaptin protein level in neurons transfected with DsiRNA against *γ1-Adaptin* (Fig. 6A). Delivery of both control and *γ1-Adaptin*-targeted DsiRNAs resulted in production of a protein immunoreactive to the anti-γ1-Adaptin antibody with a lower molecular weight (indicated by arrowhead) than that of γ1-Adaptin (Fig. 6A). It appears that this extra protein was generated by the electroporation procedure, however we could not clarify the nature of this protein and the mechanism for its production. Similar to *γ1-Adaptin*-KO RPE1 cells, *γ1-Adaptin*-deficient neurons displayed less ciliation and formed shorter cilia compared with the control neurons (Fig. S5A–C). Silencing *γ1-Adaptin* expression led to reduced ciliary localization of Htr6 and CD8α-Htr6 i4, but it did not affect CD8α-Htr6 i3 or Htr7 localization, in neurons fixed at DIV13 (Fig. 6B–C), indicating that the ciliary localization of Htr6 is facilitated by γ1-Adaptin also in neurons via its effect on the i4 domain of Htr6.

We then analyzed the localization of endogenous Gpr161 and Sstr3 proteins, whose ciliary localization in primary neurons was previously shown [13,30], in cultured hippocampal neurons transfected with control or *γ1-Adaptin*-targeted DsiRNA at DIV10. Ciliary localization of Gpr161 and Sstr3, unlike that of Htr6, was unaltered by silencing of *γ1-Adaptin* in neurons fixed at DIV13 (Fig. 7A–D).

4. Discussion

Our analysis of CD8α-Htr6 i3/i4 localization revealed that Htr6 i3 has a weaker preference for ciliary localization than Htr6 i4 in RPE1 cells. However, Barbeito et al. demonstrated that Htr6 i3 participates in Htr6 ciliary localization to a comparable extent as Htr6 i4 in IMCD3 cells, a murine renal collecting duct cell line [16]. This discrepancy may

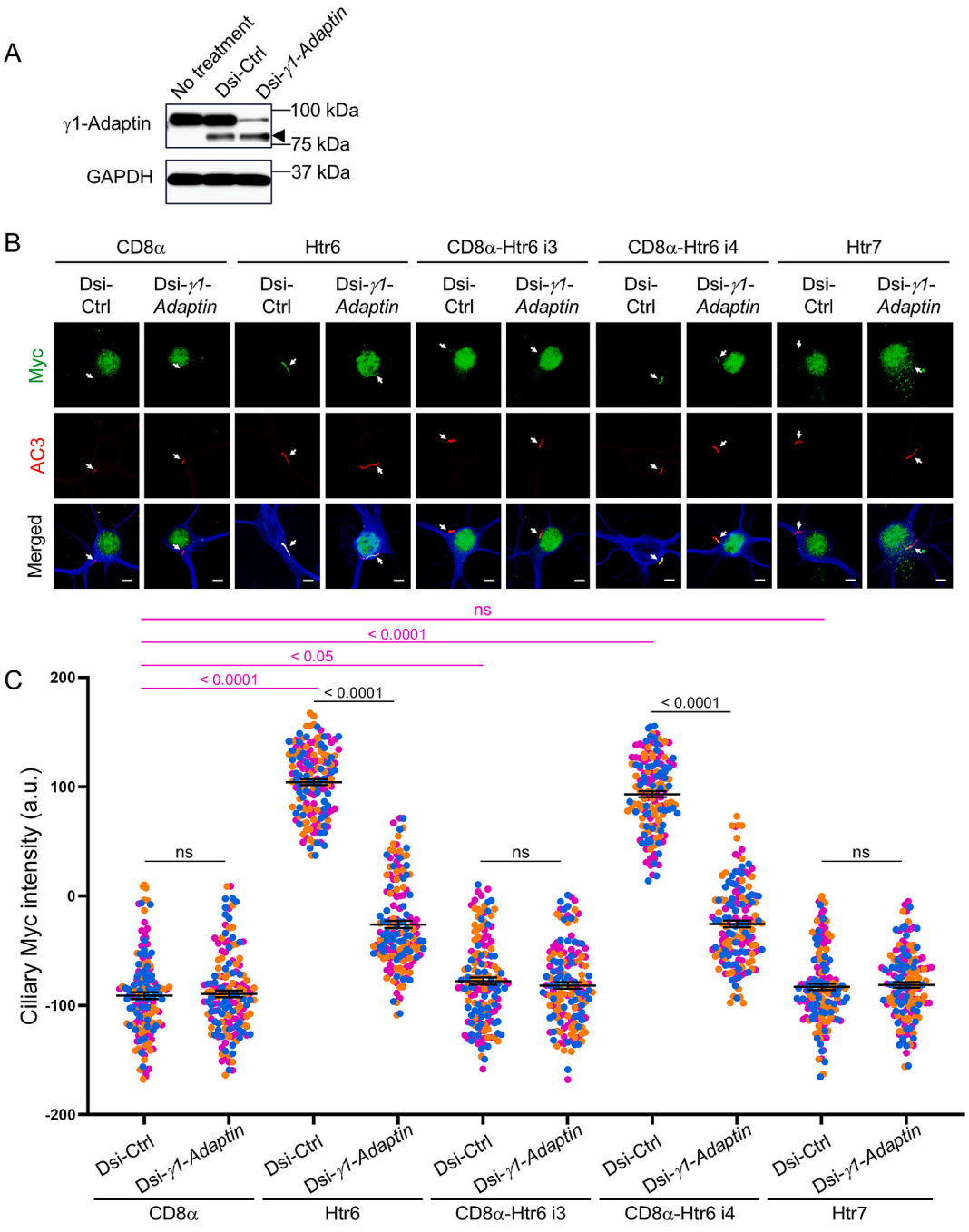

Fig. 6. γ1-Adaptin facilitates ciliary localization of Htr6 in cultured hippocampal neurons.
(A) Cultured hippocampal neurons were transfected with control DsiRNA (Dsi-Ctrl) or DsiRNA against *γ1-Adaptin* (Dsi-*γ1-Adaptin*) by electroporation at DIV10. Immunoblotting using an anti-γ1-Adaptin antibody confirmed the reduction in the γ1-Adaptin protein level in neurons at DIV13 after treatment with Dsi-*γ1-Adaptin*. The arrowhead indicates a band with a lower molecular weight than that of γ1-Adaptin (see Results section). GAPDH served as a loading control.
(B) Hippocampal neurons were transfected with Myc-tagged CD8α, Htr6, CD8α-Htr6 i3, CD8α-Htr6 i4, or Htr7 simultaneously with Dsi-Ctrl or Dsi-*γ1-Adaptin* by electroporation at DIV10. Neurons were fixed at DIV13, and localization of Myc-tagged proteins was analyzed by immunostaining using an anti-Myc antibody. Primary cilia were stained with an anti-adenylyl cyclase 3 (AC3) antibody. In merged images, MAP2 staining (blue) is shown. Arrows indicate primary cilia and scale bars represent 5 μm.
(C) The intensity of Myc signals at cilia was measured and is shown in scatter plots ($n = 50 \times 3$). Symbols are used as described in Fig. 1C. a.u., arbitrary unit; ns, not significant.

be ascribed to the following factors. First, different cell lines were used for the experiments. Second, different tags were fused to the proteins of interest; Barbeito et al. fused enhanced GFP or YFP, 239-aa fluorescent tags, to the C-terminus of the proteins [16], which have a higher possibility of influencing the native localization of untagged proteins than the 10-aa Myc-tag used in the present study. Third, different analysis methods were used; the frequency of cilia localization was mainly examined by Barbeito et al. [16], whereas the intensity of cilia localization was investigated in the present study. Our results demonstrated aa 369–402 of Htr6 to be the minimal region sufficient for ciliary localization of Htr6 i4. This is consistent with the finding that aa 400–402 of Htr6 are the three critical residues for the cilia targeting

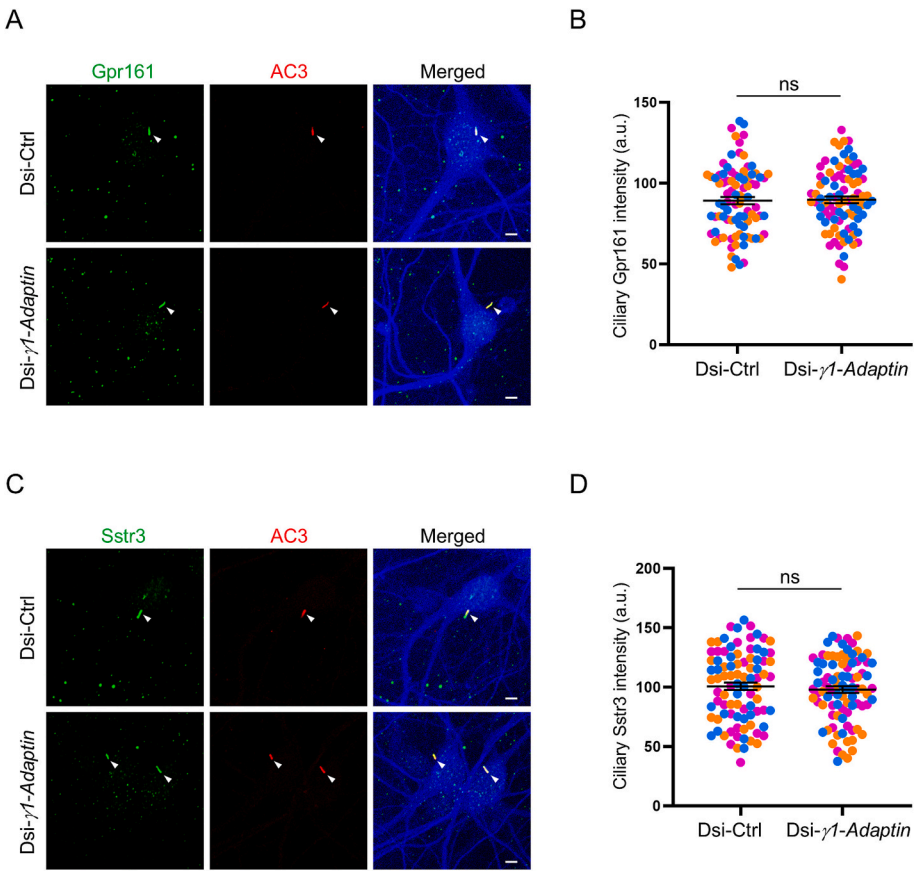

Fig. 7. Ciliary localization of Gpr161 and Sstr3 is unaltered by silencing of *γ1-Adaptin* in cultured hippocampal neurons.
(A–D) Cultured hippocampal neurons were transfected with control DsiRNA (Dsi-Ctrl) or DsiRNA against *γ1-Adaptin* (Dsi-*γ1-Adaptin*) by electroporation at DIV10. After fixation at DIV13, the neurons were immunostained for Gpr161 (A) or Sstr3 (C) and for AC3, a neuronal cilia marker. In merged images, MAP2 staining (blue) is shown. Arrowheads indicate primary cilia and scale bars represent 5 μm. The intensity of Gpr161 (B) and Sstr3 (D) signals at cilia was measured and is shown in scatter plots ($n = 30 \times 3$). Symbols are used as described in Fig. 1C. a.u., arbitrary unit; ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

property of Htr6 i4 [16].

In eukaryotic cells, adaptor protein (AP) complexes play a vital role in the transport of cargo transmembrane proteins from one membrane domain to another [27,31]. AP complexes attach to the donor membrane and assemble cargo proteins into vesicular carriers by budding, which are conveyed to the acceptor membrane [27,31]. Each of the five AP complexes, AP-1–5, comprises two large subunits, one medium subunit, and one small subunit. The medium subunit, the small subunit, and the N-terminal "trunk" domains of the two large subunits form the "core" domain of the AP complexes [27]. The C-terminal "ear" domain and the intermediate "hinge" domain of the large subunits interact with cytosolic regulatory proteins and, in the case of AP-1, AP-2, and AP-3, the vesicle coat protein clathrin, respectively [27]. The two large subunits of AP-1 are β1-Adaptin and γ-Adaptin, which has two isoforms: γ1-Adaptin or γ2-Adaptin [29]. The interaction of AP-1 with the cytosolic domains of cargo transmembrane proteins is mediated either by the medium subunit μ1 or by the combination of the "trunk" domain of γ-Adaptin and the small subunit σ1 [27] (Fig. 2B).

While AP-1 plays a role in the transport of cargo proteins between the trans-Golgi network (TGN) and endosomes via clathrin-coated vesicles, it also participates in polarized sorting of cargo proteins to the epithelial basolateral domain and the neuronal somato-dendritic domain [27,32,33]. For example, in hippocampal neurons, the AP-1 complex is involved in somato-dendritic localization of transmembrane glutamate receptors, where the specific amino acid motif in the cytosolic domain of the cargo receptors is recognized by the μ1A subunit of AP-1 [34]. While additional AP-1-dependent transport pathways and cargo proteins are being discovered [32], the present study identified an unexpected role of AP-1 in Htr6 transport to the ciliary membrane, which is a type of polarized sorting. In this study, the "ear" domain of γ1-Adaptin, which interacts with cytosolic regulatory proteins [27], was found to interact with a 34-aa motif in the i4 domain of Htr6. It is unclear whether this non-canonical interaction of the γ1-Adaptin "ear" domain with the cargo protein Htr6 is associated with transport to primary cilia. In this context, it is noteworthy that endocytosis of the transmembrane cargo protein HM1.24 is mediated by interaction of its cytosolic domain with the "ear" domain of α-Adaptin, a large subunit of the AP-2 complex [35], indicating that the "ear" domains of the large subunits in AP complexes interact with cargo proteins as well as with regulatory proteins.

Ablation of *γ1-Adaptin* in RPE1 cells and silencing of *γ1-Adaptin* in cultured hippocampal neurons impaired the preferential localization of Htr6 and CD8α-fused Htr6 i4 to primary cilia. A previous study by Kaplan et al. revealed that the AP-1 complex, in cooperation with a small GTPase Rab8, participates in cilia formation and in transmembrane protein transport into cilia in *Caenorhabditis elegans* sensory neurons [36]. They also examined the involvement of AP-1 in cilia assembly and in transmembrane protein traffic to cilia in mammalian cells using RPE1 cells; treatment of RPE1 cells with siRNA against *γ1-Adaptin* resulted in profound reduction in the σ1A subunit of AP-1, indicating impaired formation of AP-1 [36]. These AP-1-depleted RPE1 cells displayed normal ciliation, but they formed longer cilia than control cells [36]. However, we identified reduced ciliation as well as formation of shorter cilia in *γ1-Adaptin*-KO RPE1 cells, compared with control cells (Fig. S1). This inconsistency may result from residual γ1-Adaptin after gene

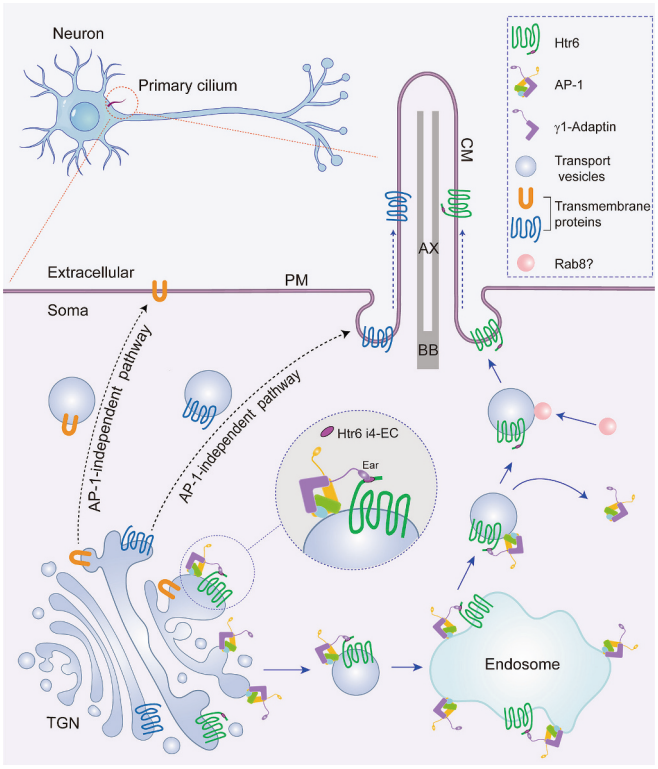

Fig. 8. A model of preferential Htr6 localization to primary cilia in neurons via an AP-1-dependent pathway.
Htr6 in the TGN is recognized by AP-1 and transferred to an endosome via a clathrin-coated vesicle. Htr6 in the endosome is then loaded again into a clathrin-coated vesicle by AP-1. After the Htr6-containing vesicle releases AP-1 and clathrin, the vesicle associates with a regulatory factor, such as Rab8. Finally, the vesicle reaches the base of a primary cilium, where the vesicle membrane is fused to the plasma membrane, leading to the localization of Htr6 at the ciliary membrane. Additionally, transmembrane proteins can be transported from the TGN to the plasma membrane via an AP-1-independent pathway, wherein ciliary proteins, such as Smo and Gpr161, are trafficked to the ciliary membrane. PM, plasma membrane; BB, basal body; CM, ciliary membrane; AX, axoneme; TGN, trans-Golgi network.

silencing. Based on the indispensability of γ1-Adaptin for AP-1 formation in RPE1 cells noted above [36] and indicated by our current finding (Fig. S4), it is likely that impairment of enriched ciliary localization of Htr6 and CD8α-Htr6 i4 in *γ1-Adaptin*-KO RPE1 cells found in the present study is ascribed not to a loss of interaction of the AP-1 complex with Htr6 via the γ1-Adaptin "ear" domain, but to lack of AP-1 formation. Kaplan et al. demonstrated that AP-1-depleted RPE1 cells and AP-1-depleted MDCK cells had normal ciliary localization of Smo and of CNGA2, a CNG channel, respectively [36]. In our study, the endogenous Gpr161 protein, a ciliary orphan GPCR that regulates hedgehog signaling in a manner opposite to that of Smo [12], displayed comparable ciliary localization in *γ1-Adaptin*-KO RPE1 cells and control cells (Fig. 5). In addition, ciliary localization of Gpr161 and Sstr3 was unaltered by silencing of *γ1-Adaptin* in cultured hippocampal neurons (Fig. 7). Furthermore, pairwise comparisons of amino acid sequences between Htr6 and all GPCRs known to localize to neuronal cilia [6], as well as a BLAST search, could not find any GPCRs with a motif similar to Htr6 i4-EC, suggesting that Htr6 is a unique GPCR whose ciliary localization is facilitated by γ1-Adaptin-containing AP-1 in mammalian cells. Taken together, transport of transmembrane proteins into primary cilia is unlikely to be generally dependent on AP-1, whereas a subset of transmembrane proteins, which includes Htr6 in mammalian cells and the ciliary proteins in nematode sensory neurons [36], may rely on a conserved role of AP-1 for ciliary transport.

Based on our present findings and previous studies [32,36], we propose a model for the preferential localization of Htr6 to primary cilia in neurons (Fig. 8). First, Htr6 situated in the TGN is recognized by AP-1 and conveyed to the endosome via a clathrin-coated vesicle. Second, Htr6 at the endosome is loaded again into a clathrin-coated vesicle by AP-1. Third, the Htr6-containing vesicle releases AP-1 and clathrin and is then associated with a regulatory factor, such as Rab8, which mediates transport to cilia in vertebrates and *C. elegans* [36,37]. After reaching the base of primary cilia, the vesicle membrane fuses with the plasma membrane to place Htr6 at the ciliary membrane. However, transmembrane proteins can also be delivered from the TGN to the plasma membrane via an AP-1-independent pathway, wherein ciliary proteins, such as Smo and Gpr161, are transported to the ciliary membrane.

Of note, Golgi-localized, γ-adaptin ear domain homology, ADP ribosylation factor (Arf)-binding proteins (GGAs), a family of monomeric clathrin adaptor proteins, have the C-terminal GAE domain homologous to the γ-Adaptin "ear" domain, which interacts with regulatory proteins [38–40]. GGAs also have the N-terminal VHS domain recognizing cargoes as well as the intermediate GAT and hinge domains interacting with Arf GTPases and clathrin, respectively [40]. While GGAs cooperate with the tetrameric AP-1 complex in TGN-endosome transport [40,41], they can also organize the formation of clathrin-coated vesicles in AP-1-depleted cells [42]. Therefore, if the GGA GAE domain, like the γ1-Adaptin "ear" domain, interacts with Htr6 i4, GGAs might play a role in clathrin-mediated transport of Htr6 and CD8α-Htr6 i4 from the TGN to the endosome independently of AP-1 in *γ1-Adaptin*-KO cells, underlying reduced but remaining ciliary enrichment of Htr6 and CD8α-Htr6 i4 despite ablation of *γ1-Adaptin* and consequent disruption of AP-1 formation (Fig. 4, Fig. S4).

AP-1 is associated with multiple human developmental disorders [32,43]. Given our present results, it is possible that these AP-1-associated developmental disorders may be caused, at least in part, by impaired ciliary localization of Htr6 in the developing brain of affected children.

In conclusion, our findings demonstrate that Htr6 is detected by AP-1 through an interaction between the "ear" domain of γ1-Adaptin, a component of AP-1, and the i4 domain of Htr6, and indicate that AP-1 facilitates ciliary localization of Htr6 in RPE1 cells and in cultured neurons. Further studies are needed to clarify the entire molecular mechanism of Htr6 ciliary transport, especially after the AP-1-mediated phase.

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CRediT authorship contribution statement

Yuanyuan Qin: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Ko Miyoshi:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Zhuoma Yinsheng:** Investigation. **Yuuki Fujiwara:** Supervision. **Takeshi Yoshimura:** Supervision. **Taiichi Katayama:** Supervision.

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Declaration of competing interest

The authors declare no competing interests.

Data availability

The data underlying this article are available from the corresponding

author upon reasonable request.

References

[1] K.I. Hilgendorf, B.R. Myers, J.F. Reiter, Emerging mechanistic understanding of cilia function in cellular signalling, Nat. Rev. Mol. Cell Biol. 25 (7) (2024) 555–573, https://doi.org/10.1038/s41580-023-00698-5.

[2] C.M. Ott, S. Constable, T.M. Nguyen, K. White, W.A. Lee, J. Lippincott-Schwartz, S. Mukhopadhyay, Permanent deconstruction of intracellular primary cilia in differentiating granule cell neurons, J. Cell Biol. 223 (10) (2024), https://doi.org/10.1083/jcb.202404038.

[3] A.I. Koemeter-Cox, T.W. Sherwood, J.A. Green, R.A. Steiner, N.F. Berbari, B. K. Yoder, A.S. Kauffman, P.C. Monsma, A. Brown, C.C. Askwith, K. Mykytyn, Primary cilia enhance kisspeptin receptor signaling on gonadotropin-releasing hormone neurons, Proc. Natl. Acad. Sci. USA 111 (28) (2014) 10335–10340, https://doi.org/10.1073/pnas.1403286111.

[4] N. Jurisch-Yaksi, D. Wachten, J. Gopalakrishnan, The neuronal cilium - a highly diverse and dynamic organelle involved in sensory detection and neuromodulation, Trends Neurosci. 47 (5) (2024) 383–394, https://doi.org/10.1016/j.tins.2024.03.004.

[5] P. Volos, K. Fujise, N.M. Rafiq, Roles for primary cilia in synapses and neurological disorders, Trends Cell Biol. 35 (1) (2025) 6–10, https://doi.org/10.1016/j.tcb.2024.10.014.

[6] K.I. Hilgendorf, C.T. Johnson, P.K. Jackson, The primary cilium as a cellular receiver: organizing ciliary GPCR signaling, Curr. Opin. Cell Biol. 39 (2016) 84–92, https://doi.org/10.1016/j.ceb.2016.02.008.

[7] S.H. Sheu, S. Upadhyayula, V. Dupuy, S. Pang, F. Deng, J. Wan, D. Walpita, H. A. Pasolli, J. Houser, S. Sanchez-Martinez, S.E. Brauchi, S. Banala, M. Freeman, C. S. Xu, T. Kirchhausen, H.F. Hess, L. Lavis, Y. Li, S. Chaumont-Dubel, D.E. Clapham, A serotonergic axon-cilium synapse drives nuclear signaling to alter chromatin accessibility, Cell 185 (18) (2022) 3390–3407 e18, https://doi.org/10.1016/j.cell.2022.07.026.

[8] S. Mukhopadhyay, H.B. Badgandi, S.H. Hwang, B. Somatilaka, I.S. Shimada, K. Pal, Trafficking to the primary cilium membrane, Mol. Biol. Cell 28 (2) (2017) 233–239, https://doi.org/10.1091/mbc.E16-07-0505.

[9] K.C. Corbit, P. Aanstad, V. Singla, A.R. Norman, D.Y. Stainier, J.F. Reiter, Vertebrate smoothened functions at the primary cilium, Nature 437 (7061) (2005) 1018–1021, https://doi.org/10.1038/nature04117.

[10] N.F. Berbari, A.D. Johnson, J.S. Lewis, C.C. Askwith, K. Mykytyn, Identification of ciliary localization sequences within the third intracellular loop of G protein-coupled receptors, Mol. Biol. Cell 19 (4) (2008) 1540–1547, https://doi.org/10.1091/mbc.e07-09-0942.

[11] A.V. Loktev, P.K. Jackson, Neuropeptide Y family receptors traffic via the Bardet-Biedl syndrome pathway to signal in neuronal primary cilia, Cell Rep. 5 (5) (2013) 1316–1329, https://doi.org/10.1016/j.celrep.2013.11.011.

[12] S. Mukhopadhyay, X. Wen, N. Ratti, A. Loktev, L. Rangell, S.J. Scales, P.K. Jackson, The ciliary G-protein-coupled receptor Gpr161 negatively regulates the Sonic hedgehog pathway via cAMP signaling, Cell 152 (1–2) (2013) 210–223, https://doi.org/10.1016/j.cell.2012.12.026.

[13] H.B. Badgandi, S.H. Hwang, I.S. Shimada, E. Loriot, S. Mukhopadhyay, Tubby family proteins are adapters for ciliary trafficking of integral membrane proteins, J. Cell Biol. 216 (3) (2017) 743–760, https://doi.org/10.1083/jcb.201607095.

[14] S. Mukhopadhyay, X. Wen, B. Chih, C.D. Nelson, W.S. Lane, S.J. Scales, P. K. Jackson, TULP3 bridges the IFT-A complex and membrane phosphoinositides to promote trafficking of G protein-coupled receptors into primary cilia, Genes Dev. 24 (19) (2010) 2180–2193, https://doi.org/10.1101/gad.1966210.

[15] I. Dateyama, Y. Sugihara, S. Chiba, R. Ota, R. Nakagawa, T. Kobayashi, H. Itoh, RABL2 positively controls localization of GPCRs in mammalian primary cilia, J. Cell Sci. 132 (2) (2019), https://doi.org/10.1242/jcs.224428.

[16] P. Barbeito, Y. Tachibana, R. Martin-Morales, P. Moreno, K. Mykytyn, T. Kobayashi, F.R. Garcia-Gonzalo, HTR6 and SSTR3 ciliary targeting relies on both IC3 loops and C-terminal tails, Life Sci. Alliance 4 (3) (2021), https://doi.org/10.26508/lsa.202000746.

[17] V. Dupuy, M. Prieur, A. Pizzoccaro, C. Margarido, E. Valjent, J. Bockaert, T. Bouschet, P. Marin, S. Chaumont-Dubel, Spatiotemporal dynamics of 5-HT(6) receptor ciliary localization during mouse brain development, Neurobiol. Dis. 176 (2023) 105949, https://doi.org/10.1016/j.nbd.2022.105949.

[18] F. Duhr, P. Deleris, F. Raynaud, M. Seveno, S. Morisset-Lopez, C. Mannoury la Cour, M.J. Millan, J. Bockaert, P. Marin, S. Chaumont-Dubel, Cdk5 induces constitutive activation of 5-HT6 receptors to promote neurite growth, Nat. Chem. Biol. 10 (7) (2014) 590–597, https://doi.org/10.1038/nchembio.1547.

[19] J. Meffre, S. Chaumont-Dubel, C. Mannoury la Cour, F. Loiseau, D.J. Watson, A. Dekeyne, M. Seveno, J.M. Rivet, F. Gaven, P. Deleris, D. Herve, K.C. Fone, J. Bockaert, M.J. Millan, P. Marin, 5-HT(6) receptor recruitment of mTOR as a mechanism for perturbed cognition in schizophrenia, EMBO Mol. Med. 4 (10) (2012) 1043–1056, https://doi.org/10.1002/emmm.201201410.

[20] N. Mullins, A.J. Forstner, K.S. O'Connell, B. Coombes, J.R.I. Coleman, Z. Qiao, T. D. Als, T.B. Bigdeli, S. Borte, J. Bryois, et al., Genome-wide association study of more than 40,000 bipolar disorder cases provides new insights into the underlying biology, Nat. Genet. 53 (6) (2021) 817–829, https://doi.org/10.1038/s41588-021-00857-4.

[21] S. Mizushima, S. Nagata, pEF-BOS, a powerful mammalian expression vector, Nucleic Acids Res. 18 (17) (1990) 5322, https://doi.org/10.1093/nar/18.17.5322.

[22] S. Han, K. Miyoshi, S. Shikada, G. Amano, Y. Wang, T. Yoshimura, T. Katayama, TULP3 is required for localization of membrane-associated proteins ARL13B and INPP5E to primary cilia, Biochem. Biophys. Res. Commun. 509 (1) (2019) 227–234, https://doi.org/10.1016/j.bbrc.2018.12.109.

[23] F.A. Ran, P.D. Hsu, J. Wright, V. Agarwala, D.A. Scott, F. Zhang, Genome engineering using the CRISPR-Cas9 system, Nat. Protoc. 8 (11) (2013) 2281–2308, https://doi.org/10.1038/nprot.2013.143.

[24] G. Amano, S. Matsuzaki, Y. Mori, K. Miyoshi, S. Han, S. Shikada, H. Takamura, T. Yoshimura, T. Katayama, SCYL1 arginine methylation by PRMT1 is essential for neurite outgrowth via Golgi morphogenesis, Mol. Biol. Cell 31 (18) (2020) 1963–1973, https://doi.org/10.1091/mbc.E20-02-0100.

[25] K. Pal, H. Badgandi, S. Mukhopadhyay, Studying G protein-coupled receptors: immunoblotting, immunoprecipitation, phosphorylation, surface labeling, and cross-linking protocols, Methods Cell Biol. 127 (2015) 303–322, https://doi.org/10.1016/bs.mcb.2014.12.003.

[26] Z. Yinsheng, K. Miyoshi, Y. Qin, Y. Fujiwara, T. Yoshimura, T. Katayama, TMEM67 is required for the gating function of the transition zone that controls entry of membrane-associated proteins ARL13B and INPP5E into primary cilia, Biochem. Biophys. Res. Commun. 636 (Pt 1) (2022) 162–169, https://doi.org/10.1016/j.bbrc.2022.10.078.

[27] S.Y. Park, X. Guo, Adaptor protein complexes and intracellular transport, Biosci. Rep. 34 (4) (2014), https://doi.org/10.1042/BSR20140069.

[28] L.A. Tavares, E.M. da Silva, M.E. da Silva-Januario, Y.C. Januario, J.V. de Cavalho, E.S. Czernisz, G.A. Mardones, L.L. daSilva, CD4 downregulation by the HIV-1 protein Nef reveals distinct roles for the gamma1 and gamma2 subunits of the AP-1 complex in protein trafficking, J. Cell Sci. 130 (2) (2017) 429–443, https://doi.org/10.1242/jcs.192104.

[29] H. Takatsu, M. Sakurai, H.W. Shin, K. Murakami, K. Nakayama, Identification and characterization of novel clathrin adaptor-related proteins, J. Biol. Chem. 273 (38) (1998) 24693–24700, https://doi.org/10.1074/jbc.273.38.24693.

[30] N.F. Berbari, G.A. Bishop, C.C. Askwith, J.S. Lewis, K. Mykytyn, Hippocampal neurons possess primary cilia in culture, J. Neurosci. Res. 85 (5) (2007) 1095–1100, https://doi.org/10.1002/jnr.21209.

[31] M. Boehm, J.S. Bonifacino, Adaptins: the final recount, Mol. Biol. Cell 12 (10) (2001) 2907–2920, https://doi.org/10.1091/mbc.12.10.2907.

[32] M.C. Duncan, New directions for the clathrin adaptor AP-1 in cell biology and human disease, Curr. Opin. Cell Biol. 76 (2022) 102079, https://doi.org/10.1016/j.ceb.2022.102079.

[33] J.S. Bonifacino, Adaptor proteins involved in polarized sorting, J. Cell Biol. 204 (1) (2014) 7–17, https://doi.org/10.1083/jcb.201310021.

[34] G.G. Farias, L. Cuitino, X. Guo, X. Ren, M. Jarnik, R. Mattera, J.S. Bonifacino, Signal-mediated, AP-1/clathrin-dependent sorting of transmembrane receptors to the somatodendritic domain of hippocampal neurons, Neuron 75 (5) (2012) 810–823, https://doi.org/10.1016/j.neuron.2012.07.007.

[35] N. Masuyama, T. Kuronita, R. Tanaka, T. Muto, Y. Hirota, A. Takigawa, H. Fujita, Y. Aso, J. Amano, Y. Tanaka, HM1.24 is internalized from lipid rafts by clathrin-mediated endocytosis through interaction with alpha-adaptin, J. Biol. Chem. 284 (23) (2009) 15927–15941, https://doi.org/10.1074/jbc.M109.005124.

[36] O.I. Kaplan, A. Molla-Herman, S. Cevik, R. Ghossoub, K. Kida, Y. Kimura, P. Jenkins, J.R. Martens, M. Setou, A. Benmerah, O.E. Blacque, The AP-1 clathrin adaptor facilitates cilium formation and functions with RAB-8 in C. Elegans ciliary membrane transport, J. Cell Sci. 123 (Pt 22) (2010) 3966–3977, https://doi.org/10.1242/jcs.073908.

[37] Y.S. Lim, C.E. Chua, B.L. Tang, Rabs and other small GTPases in ciliary transport, Biol. Cell. 103 (5) (2011) 209–221, https://doi.org/10.1042/BC20100150.

[38] A.L. Boman, C. Zhang, X. Zhu, R.A. Kahn, A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi, Mol. Biol. Cell 11 (4) (2000) 1241–1255, https://doi.org/10.1091/mbc.11.4.1241.

[39] H. Takatsu, K. Yoshino, K. Nakayama, Adaptor gamma ear homology domain conserved in gamma-adaptin and GGA proteins that interact with gamma-synergin, Biochem. Biophys. Res. Commun. 271 (3) (2000) 719–725, https://doi.org/10.1006/bbrc.2000.2700.

[40] T. Uemura, S. Waguri, Emerging roles of Golgi/endosome-localizing monomeric clathrin adaptors GGAs, Anat. Sci. Int. 95 (1) (2020) 12–21, https://doi.org/10.1007/s12565-019-00505-2.

[41] B. Doray, P. Ghosh, J. Griffith, H.J. Geuze, S. Kornfeld, Cooperation of GGAs and AP-1 in packaging MPRs at the trans-Golgi network, Science 297 (5587) (2002) 1700–1703, https://doi.org/10.1126/science.1075327.

[42] P. Navarro Negredo, J.R. Edgar, A.G. Wrobel, N.R. Zaccai, R. Antrobus, D.J. Owen, M.S. Robinson, Contribution of the clathrin adaptor AP-1 subunit μ1 to acidic cluster protein sorting, J. Cell Biol. 216 (9) (2017) 2927–2943, https://doi.org/10.1083/jcb.201602058.

[43] M.A. Usmani, Z.M. Ahmed, P. Magini, V.M. Pienkowski, K.J. Rasmussen, R. Hernan, F. Rasheed, M. Hussain, M. Shahzad, B.C. Lanpher, et al., De novo and bi-allelic variants in AP1G1 cause neurodevelopmental disorder with developmental delay, intellectual disability, and epilepsy, Am. J. Hum. Genet. 108 (7) (2021) 1330–1341, https://doi.org/10.1016/j.ajhg.2021.05.007.