Insufficiency of ciliary cholesterol in hereditary Zellweger syndrome

Tatsuo Miyamoto1,*,†, Kosuke Hosoba1,*, Takeshi Itabashi3, Atsuko H Iwane3, Silvia Natsuko Akutsu1, Hiroshi Ochiai2, Yumiko Saito2, Takashi Yamamoto2, 5 & Shinya Matsuura1,*,†

Abstract

Primary cilia are antenna-like organelles on the surface of most mammalian cells that receive sonic hedgehog (Shh) signaling in embryogenesis and carcinogenesis. Cellular cholesterol functions as a direct activator of a seven-transmembrane oncoprotein called Smoothened (Smo) and thereby induces Smo accumulation on the ciliary membrane where it transduces the Shh signal. However, how cholesterol is supplied to the ciliary membrane remains unclear. Here, we report that peroxisomes are essential for the transport of cholesterol into the ciliary membrane. Zellweger syndrome (ZS) is a peroxisome-deficient hereditary disorder with several ciliopathy-related features and cells from these patients showed a reduced cholesterol level in the ciliary membrane. Reverse genetics approaches revealed that the GTP exchange factor Rab8, the Rab GTPase Rab10, and the microtubule minus-end-directed kinesin KIF3C form a peroxisome-associated complex to control the movement of peroxisomes along microtubules, enabling communication between peroxisomes and ciliary pocket membranes. Our findings suggest that insufficient ciliary cholesterol levels may underlie ciliopathies.

Keywords cholesterol; ciliopathy; primary cilia; Zellweger syndrome

Subject Categories Membranes & Trafficking; Metabolism

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Introduction

Primary cilia are microtubule (MT)-based, nonmotile organelles located on the surface of most mammalian cells, which sense extracellular information to transduce signals required for embryonic development and adult tissue homeostasis (Nigg & Raff, 2009; Ishikawa & Marshall, 2011). Germline mutations of primary cilia-associated genes cause ciliopathies characterized by a range of clinical features, including polycystic kidney, polydactyly, retinitis pigmentosa, obesity, mental retardation, and neuronal and other developmental abnormalities (Baker & Beales, 2009). To date, at least 35 ciliopathies have been established. According to recent proteomic and human genetic analyses, more than 240 candidate ciliopathy-related genes are associated with ciliary structures and/or functions that cause known or unique ciliopathies if mutated in humans (Reiter & Leroux, 2017).

The vertebrate sonic hedgehog (Shh) signal is interpreted by primary cilia (Goetz & Anderson, 2010). In the absence of the Shh ligand, its receptor patched1 (Ptc1) localizes to primary cilia to silence downstream factors such as Gli transcription factors. During Shh stimulation, the ligand binds to Ptc1 to promote the ciliary exit of Ptc1 and the ciliary entry of Smoothened (Smo), thereby translocating the Gli transcription factors from the primary cilia to the nucleus to activate target gene expression (Corbit et al, 2005; Rohatgi et al, 2007). Insufficient Shh signal transduction in embryogenesis causes congenital malformations such as holoprosencephaly, cerebellar hypoplasia, and polydactyly overlapping with the clinical spectrum of ciliopathies (Ming et al, 1998; Muenke & Beachy, 2000; Varjosalo et al, 2006), while the hyperactivation after birth is involved in carcinogenesis including basal carcinoma and medulloblastoma (Teglund & Toftgard, 2010). With respect to the link between Shh signaling and ciliary membrane lipid composition, cellular cholesterol directly binds to Smo to orchestrate the Shh transcriptional program (Byrne et al, 2016; Huang et al, 2016, 2018; Luchetti et al, 2016; Myers et al, 2017; Xiao et al, 2017), suggesting that cholesterol might be essential for the functions of primary cilia. Indeed, germline mutations of the cholesterol biosynthetic enzyme DHC7 in Smith–Lemli–Opitz syndrome (SLOS, MIM: 270400) lead to congenital abnormalities including micrognathia, cleft palate, holoprosencephaly, syndactyly, polydactyly, and polycystic kidney (Fitzky et al, 1998; Wassif et al, 1998; Matsumoto et al, 2005;...
Nowaczyk & Irons, 2012), implying that a low level of cellular cholesterol might cause ciliopathies.

Mammalian cells synthesize cholesterol in the endoplasmic reticulum (ER) de novo and acquire it via receptor-mediated endocytosis of low-density lipoprotein (LDL; Simons & Ikonen, 2000). Cellular cholesterol is dynamically transported and unevenly distributed in the intracellular membranes (Ikonen, 2008). Only ~0.5–1% of total cellular cholesterol is present in the ER membrane (Lange et al., 1999), while its concentration is highest at ~60–80% in the plasma membrane (PM; Liscum & Faust, 1989). Cholesterol is also chemically modified in different organelles. For example, it is esterified in the ER for storage and converted to steroids and bile acids in mitochondria and peroxisomes (Vance & Vance, 1990; Ishihashi et al., 1996; Chang et al., 1997). Dynamic intracellular trafficking of cholesterol is thus important for its physiological roles. LDL-derived cholesterol trafficking is the best-known route of intracellular cholesterol transport in most mammalian cells (Brown & Goldstein, 1986). Upon the binding of plasma LDL to its receptor and subsequent internalization, LDL is delivered from early endosome to late endosome/lysosome, where LDL-derived cholesterol esters are hydrolyzed by lysosomal acid lipase to free cholesterol. Free cholesterol then egresses from late endosome/lysosome to be targeted into downstream organelles, including PM, ER, peroxisomes, and mitochondria (Ikonen, 2008; Chu et al., 2015). A series of studies of the hereditary neurodegenerative disorder Niemann–Pick type C (NPC) syndrome (MIM: 607623 and 607625) caused by germline mutation of the NPC1 or NPC2 gene have provided the most mechanistic knowledge on the egress of free cholesterol from late endosome/lysosome to other organelles (Carstea et al., 1997; Naurecki et al., 2000). NPC2 binds free cholesterol in the lysosomal lumen to deliver it to the membrane-bound NPC1 (Sleat et al., 2004). In the route of free cholesterol egress from late endosome/lysosome to the PM, one type of regulatory machinery involved is small GTPase Rabs-mediated vesicular trafficking along the actin filaments (Kanerva et al., 2013).

It has been reported that the cholesterol in the ciliary membranes is distinctively abundant compared with the level in the rest of the PM (Chailley et al., 1983; Chailley & Boivin-ULrich, 1985). In contrast, other studies have failed to detect the enrichment of cholesterol in the ciliary membranes (Breslow et al., 2013; Kinnebrew et al., 2019). In many cell types, the basal part of primary cilia is embedded within a membrane invagination called the ciliary pocket, which compartmentalizes the ciliary membrane from the rest of the PM (Molla-Herman et al., 2010; Benmerah, 2013). Although the cells of NPC patients exhibit lower formation of cilia and notable accumulation of cholesterol in the lysosomal lumen to severely reduce the cholesterol level in the PM (Formichi et al., 2018), most NPC patients do not show typical ciliopathy-related symptoms. These findings imply the existence of unknown trafficking routes of cellular cholesterol into the primary cilium membrane.

Peroxisomes are single-membrane-bounded organelles that perform various physiological functions, including α- and β-oxidation of fatty acids, amino acid synthesis, and metabolism of reactive oxygen species (ROS) and bile acids (Wanders, 2014). ZS is an autosomal recessive peroxisome biogenesis disorder (PBD) caused by germline mutations of the 13 known peroxisomal biogenesis factor (PEX) genes, which encode proteins called peroxins (Fujiki, 2016). Among these, PEX1 (~60%; MIM: 602136) encoding AAA++ ATPase for the assembly of peroxisomes is the most commonly defective (Portsteffen et al., 1997; Reuber et al., 1997). Patients with ZS show various clinical features from mild to early lethal, ranging from mild neurosensory defects to severe neonatal hyptonia and liver dysfunction (Argyriou et al., 2016; Waterham et al., 2016). They also often exhibit poly cystic kidney and retinitis pigmentosa (Luisiri et al., 1988; Folz & Trobe, 1991; FitzPatrick, 1996; Klouwer et al., 2015), suggesting a possible correlation of peroxisome gene mutations with ciliopathies. Recently, it was reported that peroxisomes contact lysosomes to control intracellular cholesterol trafficking and that the cells of patients with ZS show massive accumulation of cholesterol in the cytoplasm (Chu et al., 2015). Although poly cystic kidney and retinitis pigmentosa observed in ZS are not the typical defects in Shh pathway and cholesterol biosynthesis, ZS-related pathologic links between the ciliopathy-associated symptoms and the intracellular excess accumulation of cholesterol led us to explore how peroxisomes regulate the physiological roles of primary cilia via intracellular cholesterol.

In this study, we demonstrate that skin fibroblasts from ZS patients and PEX1- and PEX14-deficient hTERT-immortalized human retinal pigment epithelial (hTERT-RPE1) cells generated using CRISPR/Cas9 technology show a reduced level of cholesterol in the ciliary membranes and dampened Shh signal transduction. Using time-lapse imaging, we observed that peroxisomes as a carrier of cholesterol move along the microtubules to contact the ciliary pocket membranes. We also identified that the Rabin8–Rab10–KIFC3 complex is involved in the peroxisome-mediated trafficking of cholesterol to the primary cilia.

## Results

### Cholesterol in the ciliary membrane is insufficient in ZS

Patients with ZS often exhibit ciliopathy-associated features such as polycystic kidney and retinitis pigmentosa (Luisiri et al., 1988; Folz & Trobe, 1991; FitzPatrick, 1996; Klouwer et al., 2015). We thus speculated that ZS involves ciliary dysfunction. Primary skin fibroblasts from two unrelated ZS patients with mutation of either the PEX1 or the PEX26 gene were synchronized by serum starvation at the quiescent G0 phase and observed for the formation of primary cilia. They were ciliated as much as cells from a normal individual, total cholesterol levels in ZS, X-ALD and NPC patients’ SLOS patient’s cells compared with those in cells from a normal individual, total cholesterol levels in ZS, X-ALD and NPC patients’ cells and free cholesterol levels in X-ALD and NPC patients’ cells were significantly increased (Appendix Fig S1A and B). Since the involvement of cholesterol in cilium-dependent Shh signaling has been suggested, we then examined the localization of cholesterol in cilia in patient cells by staining with a cholesterol probe, Filipin III. In the ZS patients’ cells, there was a significant decrease in ciliary
cholesterol, like in the SLOS patient’s cells (Fig 1A and B). Interestingly, this level was not affected in cells from the X-ALD and NPC patients without conditions on the ciliomembrane-related disease spectrum (Fig 1A and B), implying that the supply of cholesterol to the ciliary membrane is independent of the well-known NPC1-mediated cholesterol trafficking route.

Next, we investigated whether peroxisomes are required for Shh signaling. Stimulating normal individual cells with the N-terminal signaling portion of Shh, Shh-N, effectively enhanced the ciliary localization of Smo (Fig 1C–E). To evaluate the Shh signal transduction, we monitored the elevated transcription of the Shh target gene Gli1 induced by the Smo agonist SAG (Hui & Angers, 2011; Garcia-Gonzalo et al., 2015; Blassberg et al., 2016; Appendix Fig S1C). These responses were impaired in the cells of SLOS and ZS patients, but not in those of X-ALD and NPC1 patients, indicating the role of peroxisomes in activating Shh signal transduction (Fig 1C–E). To investigate whether the dampened Shh signaling in the cells of ZS patients is attributable to the insufficient ciliary cholesterol, we analyzed the effects of exogenous cholesterol on the ciliary accumulation of Smo in patient cells stimulated with Shh-N. As expected, cholesterol depletion using methyl-β-cyclodextrin inhibited the ciliary accumulation of Smo even in the cells from a normal individual (Fig 1F and G), demonstrating that cellular cholesterol is required for the ciliary entry of Smo. Culturing the cells from SLOS and ZS patients in the presence of exogenous cholesterol (cholesterol/methyl-β-cyclodextrin complex), which is thought to directly provide cholesterol to the cellular membranes (Lopez et al., 2011), and a cholesterol biosynthesis inhibitor, pravastatin, restored the reduced Shh-N-induced ciliary accumulation of Smo to the level in the cells from a normal individual (Fig 1F and G). In contrast, the LDL-mediated compensation at an incorporated cholesterol amount similar to that with 100 μM cholesterol/methyl-β-cyclodextrin complex rescued the defect in Shh-N-induced ciliary Smo localization in SLOS patient-derived cells, but not perfectly in the cells from ZS patients (Fig 1F and G, Appendix Fig S1G). The cholesterol incorporation occurred with similar efficacy among the treated cells, suggesting that the difference of ciliary Smo localization might not be attributable to the variation of cholesterol incorporation (Appendix Fig S1G). These findings demonstrate that the defect in intracellular cholesterol trafficking causes the reduced level of ciliary cholesterol to perturb the Shh signal transduction in ZS.

CRISPR/Cas9-mediated disruption of PEX genes in human cultured cell line confirms ciliary dysfunction

It is problematic to compare primary fibroblasts derived from different human patients under different conditions at different times and to limit further cell biological analyses in the primary fibroblasts because of their extremely low efficacy of transgene introduction. In addition to the PEX1–PEX26 biochemical complex, other PEX gene products are known to form distinct complexes in the context of peroxisome formation (Fujiki, 2016). To confirm that peroxisomes per se are indeed involved in the ciliary function, we attempted to disrupt the PEX1 and PEX14 genes in human ciliated hTERT-RPE1 cell lines with a uniform genetic background using the nonhomologous end-joining (NHEJ)-mediated targeting method named ObLIGaRe (obligate ligation-gated recombination; Maresco et al., 2013; Royba et al., 2017). In this method, cotransfection of CRISPR/Cas9 and drug-resistant gene cassette vectors tagged with the CRISPR/Cas9 site located in the genome into hTERT-RPE1 cells enabled the generation of PEX1- and PEX14-knockout cell clones at more than 50% efficacy after drug selection (Appendix Tables S1 and S2). Western blotting and immunostaining analyses demonstrated no PEX1 and PEX14 products in the PEX1–/– and PEX14–/– hTERT-RPE1 cell clones (Fig 2A and B). Consistent with the observations of the fibroblasts derived from the ZS patients (Castro et al., 2018a), these clones also showed remarkably impaired peroxisome formation (Fig 2B and C). The PMP70 and ACOX1 (acyl-CoA oxidase 1)-double-positive peroxisomes were dramatically reduced in the PEX1–/– and PEX14–/– hTERT-RPE1 cell clones (Appendix Fig S2A), suggesting that they also had the defect in peroxisomal matrix protein import. Therefore, these knockout cell clones were successfully established as a model of ZS and used for subsequent studies.

The number and length of primary cilia were investigated in the serum-starved PEX1- and PEX14-knockout cells. They showed no significant alterations in ciliogenesis and melanin-concentrating hormone (MCH)-dependent ciliary shortening (Hamamoto et al., 2016) compared with wild-type (parental) cells (Fig 2D, Appendix Fig S2B and C). However, Filipin III staining analysis revealed that ciliary cholesterol was significantly insufficient in the PEX1–/– and PEX14–/– hTERT-RPE1 cells (Fig 2E and F). Since Filipin III can be affected by factors other than cholesterol level, we evaluated the ciliary cholesterol using fluorescently labeled Domain 4 (D4) of Perfringolysin O as another cholesterol probe (Ohn-Iwashita et al., 2004; Das et al., 2013) (Fig 2E and G, Appendix Fig S2D and E). In the parental hTERT-RPE1 cells, the signal intensity of AcGFP1-tagged D4 in the ciliary membrane was stronger than that in the rest of the plasma membrane (Appendix Fig S2D and E). Consistent with the Filipin III staining data, ciliary insufficiency and cytosolic accumulation of AcGFP1-tagged D4 in the PEX1–/– and PEX14–/– hTERT-RPE1 cells were observed (Fig 2E and G, Appendix Fig S2D and E), suggesting that peroxisomes are required for the enrichment of ciliary cholesterol and intracellular cholesterol trafficking. These mutant hTERT-RPE1 cells and the PEX14–/–/NIHT3T cells generated with the CRISPR-ObLIGaRe method also exhibited impaired ciliary accumulation of Smo following Shh-N stimulation (Fig 2H and I, Appendix Fig S3G–I, Appendix Table S1 and S2), while the Pex14 deficiency did not affect the ciliary localization of Ptc1 (Appendix Fig S3A and B). These findings suggested that the peroxisome-mediated control of the ciliary Smo localization is conserved across multiple cell lines. These ciliary phenotypes in the PEX14–/– hTERT-RPE1 cells were complementary under the exogenous PEX14 gene expression (Appendix Fig S3C–F), suggesting that the PEX genes are essential for the appropriate response to Shh ligands. Notably, exogenous cholesterol (cholesterol/methyl-β-cyclodextrin complex), but not LDL, effectively led to the restoration of Shh-N-mediated ciliary accumulation of Smo in these knockout cells (Fig 2H and I, Appendix Fig S1H), suggesting that peroxisomes are involved in the supply of cholesterol into the ciliary membrane.

Processing of the Shh ligand from the full-length precursor to mature Shh-N is also controlled by cholesterol. To test whether disruption of the PEX genes impairs the maturation of the Shh ligand, we monitored the states of Shh protein in the PEX1–/– and PEX14–/– hTERT-RPE1 cells using Western blotting analysis. They
Figure 1.
exhibited no significant change in Shh protein processing compared with wild-type hTERT-RPE1 cells (Fig 2A), suggesting that the dampened Shh signaling in ZS is attributable to the ability toreceive the Shh signal rather than the production of mature Shh ligand. These results confirm that the PEX genes play a role in the localization of ciliary cholesterol for transducing the Shh signals.

**Peroxisomes contact the ciliary pocket in a microtubule-dependent manner**

To explore how peroxisomes mediate the supply of cholesterol to the ciliary membrane, we first examined the subcellular distribution of cholesterol by performing Filipin III staining in serum-starved hTERT-RPE1 cells. Three-dimensional reconstitution analysis of confocal images demonstrated that Filippin III signals were located at the peripheral membrane regions of both the primary cilium and peroxisomes (Fig 3A), suggesting that peroxisomes might function as a carrier of cellular cholesterol to the ciliary membrane.

To test whether peroxisomes physically interact with primary cilium, we next observed the dynamics of peroxisomes by time-lapse confocal imaging of the ciliary membrane receptor MCHR1-GFP, the basal body (centrosome) marker DsRed2-PACT (pericentrin-AKAP450 centrosomal targeting domain), and the peroxisomal targeting molecule ECFP-SKL in serum-starved hTERT-RPE1 cells. Approximately 10 independent contacts between peroxisome and primary cilium occurred in 2 h, and the contacts continued for 10–20 min in the untreated hTERT-RPE1 cells containing approximately 150–200 peroxisomes labeled with ECFP-SKL (Appendix Fig S4, Movie EV1). To precisely determine the spatial interaction between peroxisomes and primary cilium, we stained endogenous peroxisomes and primary cilium with anti-PMP70 (peroxisomes), antinein (basal bodies), anti-ARL13B (cilary axonemes), and anti-phospho Ser473 Akt (cilary pockets) antibodies (Suzui et al, 2016). Some PMP70-positive spots partially overlapped with the phospho Ser473-Akt spot at the ciliary pocket in around 15% of the hTERT-RPE1 cell population (Fig 3B and C). 3D-structure illuminated microscopy (3D-SIM) also revealed that the PMP70-positive peroxisomes contacted the ciliary pocket labeled with anti-phospho Ser473 Akt antibody (Appendix Fig S5A and B). Cilium-bound PMP70-positive peroxisomes were not detected in the upper axonemal compartment from the distal appendages labeled with anti-ODF2 antibody (Appendix Fig S5C). Three-dimensional focused ion beam scanning electron microscopy (FIB-SEM) combined with correlative light and electron microscopy (CLEM) revealed that peroxisomes labeled with ECFP-SKL associated with the ciliary pocket (Fig 4). Treatment of hTERT-RPE1 cells with Colcemid and Ciliobrevin-D, a microtubule depolymerizer and dynein motor inhibitor, respectively, did not alter the total number of peroxisomes, but impaired the dynamic contact between peroxisomes and cilium (Fig 3B and Appendix Fig S4, Movie EV3 and EV4), implying that microtubules might be required for peroxisome dynamics. In contrast, Cytochalasin-D, an actin depolymerizer, did not affect the contact (Fig 3B and Appendix Fig S4, Movie EV2), suggesting that actin filaments are dispensable for peroxisome dynamics. Consistent with the time-lapse imaging data, an immunostaining analysis revealed that the hTERT-RPE1 cell population with a PMP70-positive signal merged with the phospho-Ser473 Akt signal at the ciliary pocket significantly decreased after the Colcemid and Ciliobrevin-D treatments, but not after the Cytochalasin-D treatment (Fig 3B). These results suggest that the dynamic contact between peroxisomes and primary cilia is dependent on microtubules.

To examine whether the microtubule-dependent dynamic contact between peroxisomes and cilia contributes to the supply of cholesterol into the ciliary membrane, we stained the Colcemid-, Cilobiervin-D-, or Cytochalasin-D-treated hTERT-RPE1 cells with Filipin III. The signals of the surrounding plasma membrane in the cytoskeletal inhibitor-treated cells were reduced compared with those of the untreated cells (Fig 3D). The signals at the ciliary axonemal membrane were also significantly reduced in the Colcemid- and Ciliobrevin-treated cells, whereas they were clearly detected in the untreated and Cytochalasin-D-treated cells (Fig 3D and E). These findings suggested the involvement of the microtubule-dependent dynamic contact between peroxisomes and ciliary pockets in the trafficking of cholesterol to the ciliary membrane.
Figure 2.
Since EHD1 and EHD3, which belong to the EPS15 homology domain family involved in endosomal membrane trafficking, localize to the ciliary pocket membrane (Lu et al., 2015), we investigated their role in the contact between peroxisomes and primary cilia. The peroxisomal signals of PMP70 overlapped with the AcGFP1-tagged EHD3 signal in the ciliary pocket of hTERT-RPE1 cells (Fig 3F), suggesting that EHD1 and EHD3 are involved in the contact between peroxisomes and primary cilia. To verify this, we investigated the biochemical interaction between EHD1 or EHD3 and PEX14. Immunoprecipitation analysis and GST pull-down analysis demonstrated that EHD1 and EHD3 physically interact with PEX14 (Fig 3G and H). Three-dimensional constituent analysis of confocal images also revealed that the cholesterol-containing peroxisomes localized at the ciliary pocket labeled with AcGFP1-tagged EHD3 in the hTERT-RPE1 cells (Fig 3I). Next, we generated EHD1+/− and EHD3+/− hTERT-RPE1 cell clones using the CRISPR-ObLiGaRe method (Appendix Fig S6A–D, Appendix Tables S1 and S2). They showed lower levels of cholesterol in the ciliary membranes than wild-type hTERT-RPE1 cells (Appendix Fig S6E and F, Appendix Fig S2F and G). Although the EHD1+/− and EHD3+/− hTERT-RPE1 cells showed the contact times between peroxisomes and cilia, they exhibited significantly shorter duration of contact compared with the parental hTERT-RPE1 cells (Appendix Fig S7A–C, Movie EV5–EV7). We thus concluded that EHD1 and EHD3 are involved in the contact between peroxisomes and primary cilia.

ORP3 controls the cholesterol trafficking at the peroxisome–primary cilium contact site

Since ORP1, an OSH/ORP (oxysterol binding homology protein/OSBP-related protein) protein family member involved in intracellular cholesterol trafficking, was detected in several sets of ciliary proteome data published previously (Fig 5A; Ishikawa et al., 2012; Kohli et al., 2017), we focused on the ORP3 molecule as a candidate lipid transfer protein underlying ciliary cholesterol trafficking. To test whether ORP3 is indeed involved in the supply of cholesterol into the primary cilia, we depleted the ORP3 gene in hTERT-RPE1 cells using the CRISPR-ObLiGaRe method (Fig 5B, Appendix Tables S1 and S2). The ORP3+/− hTERT-RPE1 cell clones exhibited a lower level of cholesterol in the ciliary membranes even with a more significant increase of intracellular cholesterol levels than those of the parental hTERT-RPE1 cells (Fig 5C and D, Appendix Fig S2F and G, Appendix Fig S7D and E). They did not exhibit altered dynamic contact numbers between peroxisomes and cilia, but there was significant shortening of the duration of contact compared with that of the parental hTERT-RPE1 cells (Appendix Fig S7F–H, Movies EV8 and EV9), implying that ORP3 might prolong the half-life of contact between peroxisomes and primary cilia to mediate ciliary cholesterol trafficking.

To precisely determine the localization of ORP3 in the primary cilia, we stained endogenous ORP3 in the GO-quiescent hTERT-RPE1 cells with anti-ORP3 antibody. The ORP3 signals were detected at the ciliary pocket and overlapped with the peroxisomal PMP70 signals of wild-type hTERT-RPE1 cells (Fig 5E). Consistent with the time-lapse imaging data, the PMP-70-positive signals merging with the phosphor-Ser473 Akt signal at the ciliary pocket significantly decreased in the ORP3+/− hTERT-RPE1 cells (Fig 5E and F), suggesting that the ORP3 molecule is a regulator of ciliary cholesterol trafficking at the peroxisome–primary cilium contact site. Analysis of the deletion mutant of ORP3 in the ORP3+/− hTERT-RPE1 cells revealed that the pleckstrin homology (PH) domain was required for the ciliary pocket localization for ciliary cholesterol trafficking (Fig 5H and I). Notably, the ORP3 mutants with deletion of the FFAT motif, which specifically interacts with the vesicle-associated ORP3, an OSH/ORP (oxysterol binding homology protein/OSBP-related protein) protein family member involved in intracellular cholesterol trafficking, was detected in several sets of ciliary

**Figure 2.** CRISPR/Cas9-mediated disruption of the PEX genes phenocopies the dysfunction of cholesterol trafficking to ciliary membranes.

A Western blot analysis showing the depletion of PEX1 and PEX14 and the normal processing of Shh protein in the PEX1−/− and PEX14−/− hTERT-RPE1 cell clones. GAPDH served as a loading control.

B Immunostaining with anti-PEX1 (red) or PEX14 (red), anti-PMP70 (green), anti-ninein (blue), and anti-acetylated-tubulin (white) in wild-type, PEX1−/−, and PEX14−/− hTERT-RPE1 cells in quiescent G0 phase. Arrowheads indicate primary cilia. Scale bar, 10 μm.

C Quantification of the number of PMP70-positive peroxisomes per cell from (B). Peroxisome formation in PEX1−/− and PEX14−/− hTERT-RPE1 cells was significantly impaired compared with that of the parental cells (mean ± s.d.: ***P < 0.001; one-way ANOVA with Tukey’s HSD, n = 3: 20–25 cells per experiment).

D Quantification of proportion of ciliated cells from (B). Ciliogenesis in PEX1−/− and PEX14−/− hTERT-RPE1 cells was not significantly altered compared with that of the parental cells (mean ± s.d.: one-way ANOVA with Tukey’s HSD, n = 3: 190–200 cells per experiment).

E Quiescent G0-phase wild-type, PEX1−/−, and PEX14−/− hTERT-RPE1 cells transfected with AcGFP1-tagged D4 as a cholesterol probe were immunostained with anti-pericentrin (white) and anti-acetylated-tubulin (blue) antibodies. Cholesterol was stained with Filipin III (red). Arrows and arrowheads indicate primary cilia and cytosolic accumulations of AcGFP1-tagged D4, respectively. Scale bar, 5 μm.

F Quantification of the Filipin III intensity at primary cilia from (E). PEX1−/− and PEX14−/− hTERT-RPE1 cells had significant reductions in the ciliary signal of Filipin III (***P < 0.001; one-way ANOVA with Tukey’s HSD, n = 3: 40–50 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

G Quantification of the AcGFP1-tagged D4 intensity at primary cilia from (E). The ciliary signal of AcGFP1-tagged D4 in PEX1−/− and PEX14−/− hTERT-RPE1 cells was significantly diminished (***P < 0.001; one-way ANOVA with Tukey’s HSD, n = 3: 25–30 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

H Quiescent G0-phase wild-type, PEX1−/−, and PEX14−/− hTERT-RPE1 cells were treated with or without 1.5% methyl-β-cyclodextrin for 45 min and then incubated with or without cholesterol (cholesterol/methyl-β-cyclodextrin complex) for 1 h. After removing exogenous cholesterol, they were stimulated with 50 nM Shh-N for 24 h in the presence of pravastatin, and then immunostained with anti-Smo (green), anti-acetylated-tubulin (blue), and anti-γ-tubulin (red) antibodies. For the alternative cholesterol complementation, LDL (0.06 mg/ml) was co-incubated with Shh-N and pravastatin for 24 h after methyl-β-cyclodextrin-mediated cholesterol depletion. Scale bar, 2.5 μm.

I Quantification of the Smo intensity at primary cilia in wild-type, PEX1−/−, and PEX14−/− hTERT-RPE1 cells from (H). PEX1−/− and PEX14−/− hTERT-RPE1 cells exhibited the dampened Shh-N ligand-induced ciliary accumulation of Smo. The complementation of exogenous cholesterol (cholesterol/methyl-β-cyclodextrin complex) restored the ciliary accumulation of Smo in both PEX-knockout cells, while the LDL complementation did not rescue the ciliary phenotypes efficiently (P < 0.05; ***: 0.01, **: 0.001; one-way ANOVA with Tukey’s HSD, n = 3: 90–100 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

Source data are available online for this figure.

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Figure 3.
Figure 3. Peroxisomes accompanied by cholesterol move along the microtubules to interact with the ciliary pocket structure.

A. Quiescent G0-phase hTERT-RPE1 cells were immunostained with anti-PEX14 (red), anti-ninein (white), and anti-acetylated-tubulin (blue) antibodies. Cholesterol was stained with Filipin III (green). Magnified images of the boxed regions showing peroxisome accompanied by cholesterol (arrows). Three-dimensional reconstitution of the same cell indicates that Filipin III stains the membrane regions of ciliary axonemes and peroxisomes. The scale bars indicate 2.5 μm and 1.25 μm in lower- and higher-magnified images, respectively.

B. Quiescent G0-phase wild-type hTERT-RPE1 cells treated with Cytochalasin-D (200 nM), colcemid (50 nM), or Ciliobrevin-D (10 μM) for 6 h were immunostained with anti-ARL13B (blue), anti-phospho-S473-Akt (red), anti-ninein (green), and anti-PMP70 (white) antibodies. Arrows indicate the peroxisomes interacting with the ciliary pocket. Scale bar, 5 μm.

C. Quantification of proportion of primary cilia interacting with peroxisomes from (B). Colcemid (50 nM) and Ciliobrevin-D (50 μM) significantly inhibited the spatial interaction between peroxisomes and primary cilia (mean ± s.d. **P < 0.001; one-way ANOVA with Tukey’s HSD, n = 3–45–50 cells per experiment).

D. Quiescent G0-phase wild-type hTERT-RPE1 cells were treated with Cytochalasin-D (200 nM), Colcemid (50 nM), or Ciliobrevin-D (10 μM) for 6 h and then immunostained with anti-acetylated-tubulin (blue) and anti-pericentrin (green) antibodies. Cholesterol was stained with Filipin III (green). Arrows indicate primary cilia. Scale bar, 2.5 μm.

E. Quantification of the Filipin III intensity at primary cilia from (D). Colcemid and Ciliobrevin-D interfered with the distribution of cholesterol in the ciliary membrane (**P < 0.001: one-way ANOVA with Tukey’s HSD, n = 3–40–50 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

F. hTERT-RPE1 cells were transfected with AcGFPI-tagged EHD3 and cultured without serum for 24 h before immunostaining with anti-GFP (green), anti-ARL13B (blue), anti-PMP70 (white), and anti-phospho-S473-Akt (red) antibodies. The scale bars represent 25 μm.

G. 3×FLAG-tagged EHD1 or EHD3 and AcGFPI-tagged PEX14 were coexpressed in HEK293T cells and then immunoprecipitated from whole-cell lysates using the anti-FLAG antibody. AcGFPI-tagged PEX14 and 3×FLAG-tagged EHD1 or EHD3 fragments in the IF fractions and inputs were detected by Western blotting.

H. Recombinant GST (0.2 μg) or GST fused to PEX14 proteins (1 μg) and 6×His-EHD3 protein (1 μg) were pulled down using glutathione-Sepharose beads. GST-tagged PEX14 and 6×His-tagged EHD3 proteins in the pull-down fractions and inputs were detected by western blotting.

I. 3D reconstitution of the quiescent G0-phase hTERT-RPE1 cells transfected with AcGFPI-tagged EHD3 (white) immunostained with anti-acetylated-tubulin (blue) and anti-PEX14 (red) antibodies. Cholesterol was stained with Filipin III (green). Cholesterol-containing peroxisome interacted with the ciliary pocket (arrow). The scale bars indicate 2 μm.

Source data are available online for this figure.

membrane protein-associated A/B (VAP-A/B), and the OSBP-homology domain (OH), localized to the ciliary pocket, but they did not restore the defect in ciliary cholesterol in the ORP3−/− hTERT-RPE1 cells (Fig 5H and I), suggesting that the FFAT motif and OHD of ORP3 were indispensable for cholesterol trafficking at the ciliary pocket. To investigate the physical communication between peroxisome and primary cilia, we examined the biochemical interaction between ORP3 and PEX14, EHD1, or EHD3. Immunoprecipitation analysis demonstrated that ORP3 physically binds to PEX14 and EHD1 (Fig 5G). Indeed, the interaction in which peroxisomes lingered at the AcGFPI-tagged EHD1-labeled ciliary pocket in ORP3−/− and EHD3−/− hTERT-RPE1 cells was significantly shortened (Appendix Fig S8, Movie EV10–EV12). These findings revealed that ORP3 mediates the formation of peroxisome–primary cilium contact to control the cholesterol trafficking into the ciliary membrane.

The Rabin8 and Rab10 axis is required for the peroxisome dynamics for ciliary cholesterol trafficking

Rabin8, the specific GTP exchange factor (GEF) for the Rab8 GTPase family, has been implicated in both ciliogenesis (Nachury et al., 2007) and cholesterol trafficking to the PM (Kanerva et al., 2013). Thus, we focused on the Rabin8 molecule as a candidate regulator of peroxisome-mediated cholesterol trafficking to the ciliary membrane. Specifically, we depleted the Rabin8 gene in hTERT-RPE1 cells using the CRISPR-ObLiGaRe method (Appendix Tables S1 and S2). Western blot analysis confirmed that there were no Rabin8 products in the Rabin8−/− hTERT-RPE1 cell clones (Appendix Fig S9A). Notably, ciliogenesis was not completely deficient in the Rabin8−/− hTERT-RPE1 cells (Appendix Fig S9B and C), implying that these knockout cell clones could be used for subsequent studies focusing on the dysfunction of ciliary cholesterol. As expected, depletion of the Rabin8 gene significantly diminished the ciliary cholesterol levels compared with that in the parental hTERT-RPE1 cells (Fig 6A and B, Appendix Fig S2F and G). The Rabin8−/− hTERT-RPE1 cells also showed defects in peroxisome dynamics and the subsequent contact between peroxisomes and primary cilia (Appendix Fig S9D–F, Movie EV13 and EV14, Fig 6C and D), suggesting that Rabin8 is involved in the peroxisome-mediated trafficking of cholesterol to the ciliary membrane. In contrast, ABCD1−/− and NPC1−/− hTERT-RPE1 cells generated by the CRISPR-ObLiGaRe method as a nonciliopathic but intracellular cholesterol accumulation disease model (X-ALD and NPC) did not alter the ciliary cholesterol level and dynamic contact between peroxisomes and primary cilia (Appendix Fig S10, Appendix Fig S2F and G, Movies EV15 and EV16).

Next, we sought to clarify whether the ciliary cholesterol insufficiency in the Rabin8−/− hTERT-RPE1 cells is a direct and specific result of losing the physical communication between peroxisomes and primary cilia using an inducible FRB-FKBP hetero-dimerization system. Application of the chemical inducer rapamycin enables coupling of the peroxisomes labeled with PEX3-GFP-FRB to the Bicaudal-D2 (BicD2) molecule, which is a subunit of the dynein motor complex involved in motile cilia minus-end-directed organelle transport, fused with a fluorescent protein tdTomato and FKBP, thereby targeting the peroxisomes to the ciliary pocket rapidly and efficiently (Fig 6E and F, Movie EV17). Rapamycin treatment in the Rabin8−/− hTERT-RPE1 cells co-expressing PEX3-GFP-FRB and tdTomato-BicD2-FKBP restored the reduced ciliary cholesterol to the level in the parental hTERT-RPE1 cells (Fig 6G and H), indicating that the peroxisome is a direct source of the ciliary membrane cholesterol. These results suggest that the peroxisome-associated phenotypes in Rabin8−/− hTERT-RPE1 cells are correlated with ciliary dysfunction. Consistent with insufficient ciliary cholesterol in the Rabin8−/− hTERT-RPE1 cells, they exhibited significantly impaired ciliary localization of Smo following the Shh-N stimulation (Appendix Fig S9G and H), suggesting that the Rabin8 gene is required for the Shh signal transduction. Notably, exogenous cholesterol (cholesterol/methyl-β-cyclodextrin complex), but not...
LDL, effectively restored the Shh-N-mediated accumulation of Smo in the \( \text{Rabin}^{8/-} \) hTERT-RPE1 cells (Appendix Fig S9G and H), implying that their ciliary dysfunction is attributable to insufficient ciliary cholesterol.

To identify the effector molecule of Rabin8 in the context of peroxisome-mediated ciliary cholesterol trafficking, we introduced Rab8 family members including Rab8A, Rab8B, and Rab10 and their constitutively active forms (Rab8A-Q67L, Rab8B-Q67L, and Rab10-Q68L; Nachury et al, 2007; Homma & Fukuda, 2016) tagged with AcGFP1 or GFP into the \( \text{Rabin}^{8/-} \) hTERT-RPE1 cells. We found that only AcGFP1-tagged Rab10-Q68L mutant effectively restored the accumulation of ciliary cholesterol to a level similar to that of AcGFP1-tagged Rabin8 in the \( \text{Rabin}^{8/-} \) hTERT-RPE1 cells (Fig 6I and J). Notably, it was reported that Rab10 localizes to peroxisomes in mammalian cells (Gronemeyer et al, 2013). Confocal images revealed that the Rabin8- and Rab10-bound peroxisomes localized near the ciliary pocket labeled with AcGFP1-tagged EHD3 in the hTERT-RPE1 cells (Appendix Fig S9I, Appendix Fig S11G). GST-pull-down assay also demonstrated that both Rabin8 and Rab10 directly bind to PEX14 (Appendix Fig S9J, Appendix Fig S11H). These findings suggest that Rab10 functions as a main effector of Rabin8 in the context of ciliary cholesterol trafficking. Disruption of the \( \text{Rab}^{10} \) gene in hTERT-RPE1 cells by the CRISPR-ObLiGaRe method also significantly decreased ciliary cholesterol and the dynamic contacts between peroxisomes and primary cilia (Appendix Tables S1 and S2, Appendix Fig S2F and G, Appendix Fig

**Figure 4.** Three-dimensional analysis of interaction between the peroxisome and the ciliary pocket.

A Maximum projection of z-stack sections of hTERT-RPE1 cells expressing MCHR1-GFP (blue), DsRed2-PACT (red), and ECFP-SKL (green) 48 h after serum starvation. The ECFP-SKL-positive cell with primary cilia, indicated by the red box, was analyzed by FIB-SEM. Top, differential interference contrast (DIC); bottom, merged image. Scale bar, 10 \( \mu \)m.

B The fluorescent image of primary cilia, enlarged the area boxed in red in panel (A). Arrow indicates the ECFP-SKL-positive peroxisome associated with primary cilium. Scale bar, 5 \( \mu \)m.

C Single FIB-SEM image (left) showing a cross section the ciliary pocket membrane, and 3D surface model reconstructed from segmented FIB-SEM images (right). Region of interest was coated by a protection layer of platinum before FIB milling. Plasma membrane (PM), yellow (surface view); cilia, green (surface view); ciliary pocket membrane (CPM), red (surface view 70% transmission); mitochondria (Mito), orange (surface view); peroxisome (PO), purple (surface view); basal body (BB), white (surface view). Scale bar, 1 \( \mu \)m.

D Enlarged 3D view of peroxisome interacting with ciliary pocket and mitochondria. Left, x-y view; right, oblique view. Scale bar, 1 \( \mu \)m.
Figure 5.
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Figure 5. ORP3 is involved in the peroxisome-mediated cholesterol supply into the ciliary membrane.

A Schematic of ORP3 structure. ORP3 contains an N-terminal pleckstrin homology (PH) domain that interacts with phospholipids, an FFAT motif that specifically interacts with VAP-A/B, and a highly conserved C-terminal OSBP-homology domain (OHD).

B Western blot analysis showing depletion of ORP3 in the ORP3−/− hTERT-RPE1 cell clones. GAPDH served as a loading control.

C ORP3−/− and ORP3+/+ hTERT-RPE1 cells incubated for 24 h without serum were immunostained with anti-pericentrin (red) and anti-acetylated-tubulin (blue) antibodies. Cholesterol was stained with Filipin III (green). Arrows indicate primary cilia. Scale bar, 5 μm.

D Quantification of the Filipin III intensity at primary cilia from (C). ORP3−/− hTERT-RPE1 cells exhibited a significant reduction of ciliary cholesterol (**P < 0.001: one-way ANOVA with Tukey HSD, n = 3: 40–50 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

E Three-dimensional reconstitution of the quiescent G0-Phase ORP3+/+ and ORP3−/− hTERT-RPE1 cells immunostained with anti-ARL13B (blue), anti-phospho-S473-Akt (red), anti-ORP3 (green), and anti-PMP70 (white) antibodies indicates that ORP3 at the ciliary pocket (arrow and arrowhead) mediates the membrane regions of ciliary pocket and peroxisomes (arrowhead). The scale bars indicate 5 μm.

F Quantification of proportion of primary cilia interacting with peroxisomes from (E). Depletion of ORP3 significantly interfered with the spatial interaction between peroxisomes and primary cilia (mean ± s.d.: ***P < 0.001: one-way ANOVA with Tukey’s HSD, n = 3: 45–50 cells per experiment).

G ORP3+/+ and ORP3−/− hTERT-RPE1 cells transfected with AcGFP1-tagged ORP3, Ph domain-deleted-ORP3 mutant (ΔPh), FFAT motif-deleted-ORP3 mutant (ΔFFAT), or OHD domain-deleted-ORP3 mutant (ΔOHD) and cultured without serum for 24 h before Filipin III (green)-mediated cholesterol staining and immunostaining with anti-GFP (red), anti-pericentrin (white), and anti-acetylated-tubulin (blue) antibodies. Arrows represent AcGFP1-tagged ORP3 or the mutants localized to the ciliary pocket.

H Quantification of the Filipin III intensity at primary cilia from (H). AcGFP1-tagged ORP3 deletion mutants did not restore the ciliary cholesterol insufficiency in the ORP3+/− hTERT-RPE1 cells (***P < 0.001: one-way ANOVA with Tukey’s HSD, n = 3: 40–50 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

Source data are available online for this figure.

Figure 6. Rab18 cooperates with Rab10 to control the peroxisome-mediated supply of cholesterol to the ciliary membrane.

A Rab10−/− and Rab10+/− hTERT-RPE1 cells incubated for 24 h without serum were immunostained with anti-pericentrin (red) and anti-acetylated-tubulin (blue) antibodies. Cholesterol was stained with Filipin III (green). Scale bar, 5 μm.

B Quantification of the Filipin III intensity at primary cilia from (A). Rab10−/− hTERT-RPE1 cells exhibited a significant reduction of ciliary cholesterol (**P < 0.001: one-way ANOVA with Tukey’s HSD, n = 3: 40–50 cells per experiment). In the boxplot, medians, 25th/75th percentile and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

C Quiescent G0-phase Rab18+/+ and Rab18−/− hTERT-RPE1 cells were immunostained with anti-ARL13B (blue), anti-phospho-S473-Akt (red), anti-ninein (green), and anti-PMP70 (white) antibodies. Arrow indicates the peroxisomes contacting the ciliary pocket. Scale bar, 5 μm.

D Quantification of (C) indicating that disruption of the Rab18 gene significantly inhibited the spatial interaction between peroxisomes and primary cilia (mean ± s.d.: ***P < 0.001: one-way ANOVA with Tukey’s HSD, n = 3: 45–50 cells per experiment).

E Schematic representation of the rapamycin-inducible peroxisome targeting to the ciliary pocket system.

F Quantification of (C) showing that AcGFP1-tagged Rab18 in and AcGFP1-tagged Rab10-D68L effectively restored the defect in ciliary enrichment of cholesterol in Rab18−/− hTERT-RPE1 cells (**P < 0.001: one-way ANOVA with Tukey’s HSD, n = 3: 35–40 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively.

S11A-F, Movie EV18 and EV19), indicating that Rab10 is required for peroxisome-mediated ciliary cholesterol trafficking. These findings suggest that the Rab8–Rab10 cascade controls the dynamics of peroxisomes for ciliary cholesterol trafficking.

The PEX14–Rab10–KIFC3 complex drives peroxisome dynamics along the microtubules for ciliary cholesterol trafficking

A C-terminal kinesin molecule, KIFC3, involved in microtubule minus-end-directed organelle transport (Appendix Fig S12A) was previously identified as a binding partner of PEX1 using a yeast two-hybrid screen (Dietrich et al., 2013). To study whether KIFC3 physically interacts with the peroxisomal membrane proteins, we investigated the biochemical interaction between KIFC3 and PEX14. Immunoprecipitation analysis with FLAG antibody in HEK293T cells transfected with 3×FLAG-tagged KIFC3 and AcGFP1-tagged PEX14 demonstrated the physical interaction between KIFC3 and PEX14 (Fig 7A), implying that peroxisomes associate with microtubules via KIFC3. The N-terminal region including the rod domain in KIFC3 is required for the interaction with PEX14 (Appendix Fig S12B). Next,
Figure 6.
Figure 7.
we tested the mutual physical interactions among KIFC3, PEX14, and Rab10 using immunoprecipitation analyses. KIFC3 also physically interacts with Rab10 in a manner dependent on kinesin motor domain (Fig 7B and Appendix Fig S12C), implying that KIFC3 might be a downstream effector of Rab10 for the peroxisome-mediated trafficking of ciliary cholesterol. Moreover, the physical interaction between Rab10 and PEX14 was also detected in immunoprecipitation analysis (Fig 7C), suggesting that PEX14, Rab10, and KIFC3 form a complex associated with peroxisomes. Peroxisomal fractionation of whole-cell lysates from PEX14+/− and PEX14−/− hTERT-RPE1 cells demonstrated that Rabin8, Rab10, and KIFC3 are concentrated in the PEX1- and PEX14-enriched fraction, suggesting that the Rab10–KIFC3 complex associates with peroxisomes (Fig 7D). To precisely determine the spatial relationship among peroxisomes, microtubules, and KIFC3, we stained them in serum-starved hTERT-RPE1 cells transfected with 3×-FLAG-tagged KIFC3. Three-dimensional reconstruction analysis of confocal images revealed that the KIFC3 signals were present at the interface between α-tubulin signal-marked microtubules and PEX14 signal-labeled peroxisomes (Fig 7E). Time-lapse imaging demonstrated that the KIFC3-bound peroxisomes moved to centrosomes in a motor domain-dependent manner (Appendix Fig S12D and E, Movies EV21 and EV23), implying that KIFC3 loads peroxisomes on the microtubule networks to control the microtuble minus-end-directed transport of peroxisomes. In addition, the deleted KIFC3-Rod domain mutant moved to centrosomes, but did not associate with peroxisomes, thereby inhibiting the peroxisome-to-centrosome contact (Appendix Fig S12D and E, Movie EV22). These results suggest that the PEX14–Rab10–KIFC3 complex regulates the microtubule-dependent dynamics of peroxisomes for the trafficking of ciliary cholesterol.

To clarify whether the KIFC3 motor activity contributes to the peroxisome-mediated supply of cholesterol to the ciliary membrane, we disrupted the KIFC3 gene in hTERT-RPE1 cells using the CRISPR-ObLiGaRe method (Appendix Tables S1 and S2). Western blot analysis demonstrated no KIFC3 products in the knockout cell clones (Fig 7F). Depletion of the KIFC3 gene significantly reduced the ciliary localization of cholesterol (median ± s.d.: ***P < 0.001; one-way ANOVA with Tukey’s HSD, n = 3: 40–50 cells per experiment) (Fig 7G). Rab10, and KIFC3 are concentrated in the parental hTERT-RPE1 cells transfected with ACGFP1, ACGFP1-tagged KIFC3, and rod domain-deleted KIFC3 mutant (ΔRab), or motor domain-deleted KIFC3 mutant (ΔMotor) and cultured without serum for 24 h before Filipin III (green)-mediated cholesterol staining and immunostaining with anti-GFP (red), anti-pericentrin (green), and anti-acetylated-tubulin (blue) antibodies. Arrows represent the peroxisomes contacting the ciliary pocket. Scale bar, 5 μm.** Quantification of (f) showing that disruption of the KIFC3 gene significantly interfered with the contact between peroxisomes and primary cilia (mean ± s.d.: ***P < 0.001; one-way ANOVA with Tukey’s HSD, n = 3: 45–50 cells per experiment). KIFC3−/− hTERT-RPE1 cells transfected with ACGFP1, ACGFP1-tagged KIFC3, or rod domain-deleted KIFC3 mutant (ΔRab), or motor domain-deleted KIFC3 mutant (ΔMotor) and cultured without serum for 24 h before Filipin III (green)-mediated cholesterol staining and immunostaining with anti-GFP (red), anti-pericentrin (green), and anti-acetylated-tubulin (blue) antibodies. Arrows represent ciliary localization of cholesterol. The scale bars indicate 5 μm. L Quantification of the minimum intensity at primary cilia from (k). ACGFP1-tagged KIFC3 deletion mutants did not restore the ciliary cholesterol insufficiency in the KIFC3−/− hTERT-RPE1 cells (mean ± s.d.: ***P < 0.001; one-way ANOVA with Tukey’s HSD, n = 3: 40–50 cells per experiment). In the boxplot, medians, 25th/75th percentile, and min/max were represented by the central lines, the box limits, and the whiskers/error bars, respectively. Source data are available online for this figure.
cholesterol are attached to microtubule networks via the PEX14–Rab10–KIFC3 complex and (ii) the Rabin8–Rab10–KIFC3 activity directs peroxisomes to the microtubule minus end, enabling the communication of peroxisomes with the ciliary pocket via ORP3, EHD1, and EHD3 for the supply of cholesterol to the ciliary membrane (Fig 8C).

Discussion

Primary cilia composed of a core of axonemal microtubules overlain by a membrane sense extracellular signals via ciliary membrane receptors to control cell proliferation and differentiation. For cilia to signal sensitively, both the protein and the lipid compositions of their
membranes must be compartmentalized from those of the PM (Garcia et al., 2018; Nachury, 2018). In mammalian cells, ciliary cholesteryl is essential for Shh signaling, as both Ptc1 and Smo directly bind to cholesterol as a form of regulation in the shuttling from the ciliary membrane to other membranes (Corbit et al., 2005; Rohatgi et al., 2007; Byrne et al., 2016; Luchetti et al., 2016; Myers et al., 2017). Although it has been established that cells from NPC patients show the severe insufficiency of cholesterol in the PM (Kwon et al., 2009), most patients do not exhibit conditions on the ciliopathy clinical spectrum, implying the existence of specific routes of cholesterol to primary cilia. However, the mechanisms by which cellular cholesteryl is trafficked into ciliary membranes are poorly understood. Here, we found that peroxisomes are necessary for the intracellular cholesterol trafficking into ciliary membranes, and that cells from ZS patients and PEX-knockout cell lines show defects in ciliary cholesteryl localization and Shh signal transduction (Figs 1 and 2). Our findings suggest that the severe decrease in the number of peroxisomes as a carrier of cholesterol to primary cilia contributes to ciliopathy-associated symptoms including polycystic kidney and retinitis pigmentosa in ZS. In contrast, the MCH signal-dependent ciliary shortening was not impaired in the PEX14-knockout cells (Appendix Fig S2B and C), implying that all ciliary signaling pathways are not controlled by ciliary cholesterol. It was reported that siRNA-mediated knockdown of the PEX1 or PEX3 genes impaired ciliogenesis in RPE1 cells (Abe et al., 2017). However, the PEX1- and PEX14-knockout RPE1 cells and the ZS patient cells did not show any impairment of ciliogenesis (Fig 2D, Appendix Fig S1B). In a previous study, a knockdown assay using two independent siRNAs against the PEX genes was carried out, but the genetic complementation assay was not performed (Abe et al., 2017), implying that the possibility of the off-target effect of siRNA on ciliogenesis could not be completely ruled out. Another possible explanation for the phenotypic discrepancy of ciliogenesis among the PEX-knockout and knockdown cells is genetic compensation induced by deleterious mutations but not transcriptional or translational knockdown (Rossi et al., 2015). Peroxisome biogenesis disorders (PBDs) caused by germline mutations of PEX genes have genetic and pathogenic heterogeneity. PBDs are categorized into ZS, neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD), and rhizomelic chondrodysplasia punctata (RCDP) according to the clinical features (Fujiki, 2016). In general, the syndromes other than ZS, which is the most severe across the lifespan, are not accompanied by polycystic kidney, a hallmark symptom of ciliopathy. It remains unclear which factors determine whether the ciliopathy-associated features appear in PBDs. Further comparative studies of all PBD patient cells or disease model cells/animals will be required to clarify this.

In addition to PBDs, congenital peroxisomal diseases include many single-enzyme deficiencies. Of these, X-ALD is a neurodegenerative but nonciliopathic disorder caused by germline mutations of the ABCD1 gene encoding an ATP-binding cassette (ABC) transporter, ABCD1, involved in the transport of long-chain and very long-chain fatty acids or their CoA derivatives into the peroxisomal matrix for lipid metabolism (Mosser et al., 1993). Since the issue of whether peroxisomal lipid metabolism is involved in ciliary functions is important for understanding the pathological mechanisms underlying ciliopathies, X-ALD is a suitable target disease for study. Consistent with a previous report (Chu et al., 2015), we here also observed the profound intracellular accumulation of cholesterol in patients’ cells (Appendix Fig S1F), suggesting that ABCD1 is involved in intracellular cholesterol trafficking. However, the patients’ cells and ABCD1-knockout cells showed a normal distribution of ciliary cholesterol and Shh signal response (Fig 1A–E, Appendix Fig S1C and Appendix Fig S10). Notably, disruption of the ABCD1 gene did not alter the number of peroxisomes (Chang et al., 1999), indicating that ABCD1 is dispensable for peroxisome biogenesis. These findings imply that peroxisomes are essential for ciliary cholesterol trafficking. Since many peroxisomal single-enzyme deficiencies have been reported (Wanders, 2014; Argyriou et al., 2016; Fujiki, 2016), it has remained unclear whether defects in peroxisomal metabolic activities contribute to ciliopathies, the clarification of which requires further studies.

How do peroxisomes communicate with primary cilia in the context of cholesterol trafficking? Here, we demonstrate that peroxisomes move along the microtubules to interact with primary cilia. It was previously reported that LDL-derived cholesterol after NPC1/NPC2-dependent egress is trafficked to the PM via a Rab8A–myosin-5B–actin-dependent membrane transport system (Kanerva et al., 2013). In contrast, our inhibitor analyses revealed that the peroxisomal dynamics to primary cilia is not dependent on actin filaments (Fig 3B and C, Appendix Fig S4), suggesting the peroxisome-mediated cholesterol trafficking to primary cilia is clearly distinct from the route of actin-mediated cholesterol trafficking to the PM. We also here used a reverse genetics screen of the candidate genes to indicate that the microtubule minus-end-directed movement of peroxisomes is driven by the Rab8–Rab10–KIFC3 complex. Since the ciliary membrane and the plasma membrane are contiguous in the cell, it is difficult to clarify whether the peroxisomes controlled by the Rab8–Rab10–KIFC3 complex deliver cholesterol into the ciliary membrane directly or indirectly through the other plasma membrane. Rapamycin-induced targeting of peroxisomes to the ciliary pocket enabled the delivery of ciliary cholesterol even in the Rab8−, Rab10−, and KIFC3-deficient cells (Figs 6G and H, and 8A
and B). Since our findings did not completely rule out the possibility that cholesterol could access the ciliary membrane from the plasma membrane and the cytoplasmic membrane, it is safe to conclude that the peroxisomal Rabin8–Rab10–KIFC3 axis is a molecular mechanism of trafficking cholesterol to the ciliary membrane. Besides our screening candidates, several types of molecular machinery for peroxisome dynamics have been reported. For example, it was recently demonstrated that human mitochondrial Rho GTPase-1 (Miro1) localizes to peroxisomes to cooperate with the adaptors proteins TRAK1 and TRAK2, thereby mediating kinesin- and dynein-driven transport of peroxisomes in both directions (Castro et al., 2018b; Okamoto et al., 2018). Moreover, findings have suggested the occurrence of dynein-based minus-end-directed transport of peroxisomes in mammalian cells (Schrader et al., 2000). Further screens are thus needed to clarify the entire mechanism underlying the peroxisome dynamics to primary cilia for cholesterol trafficking. Taken together, our findings suggest that the diversity of intracellular cholesterol trafficking routes might contribute to the clinical heterogeneity of ciliopathy-related symptoms in NPC and ZS characterized by severe intracellular cholesterol accumulation.

How is cholesterol transferred from peroxisomal membranes to ciliary membranes? Although a more mechanistic understanding remains elusive, we suggest that the ciliary pocket membrane proteins EHD1 and EHD3 are involved in this step. It was previously shown that EHD1 and EHD3 cooperate with a SNARE membrane fusion regulator, SNAP29, to promote the Rab11–Rab8 cascade-mediated ciliary membrane assembly in the early stage of cilia formation (Lu et al., 2015). In the context of peroxisome-mediated cholesterol trafficking to the ciliary membrane, EHD1 and EHD3 physically interact with a peroxisomal membrane protein, PEX14 (Fig 3F–H), implying that they mediate the heterophilic organelle–organelle interaction between peroxisomes and primary cilia. Our time-lapse imaging data demonstrate that peroxisomes contact the region of the ciliary pocket for around 10–20 min, and that they are freely released from primary cilia after this contact. Based on our results, we speculate that the cholesterol transfer from peroxisomes to primary cilia might be mediated by not membrane fusions but membrane contacts. Here, we observed that pAkt (Ser 473) and EHD1/3 localize to discrete domains in the pocket membrane (Fig 3F), implying that the ciliary pocket might have different biochemical and physiological compartments. After peroxisomes are transported to the base of the cilia via microtubules, there are two potential mechanisms of the peroxisome and ciliary pocket interaction for cholesterol trafficking. The first is that the peroxisomes directly communicate with the EHD-rich ciliary pocket membrane. The second is that the peroxisomes interact with the bottom of the pAkt (Ser473)-labeled pocket membrane and then become associated with the EHD region of the ciliary pocket membrane. To verify these possibilities, there is a need to develop real-time microscopy technology with higher spatiotemporal resolution.

It was previously shown that, in mammalian cells, the integral lysosomal membrane protein synaptotagmin VII (Syt7) binds to the lipid PI(4,5)P2 on the peroxisomal membrane to form a membrane contact site, thereby transporting cholesterol from lysosomes to peroxisomes (Chu et al., 2015). Recently, it was also reported that a tethering complex of VAP-A/B on the endoplasmic reticulum (ER) and ACBD5 (acyl-CoA binding protein S) or PEX16 on the peroxisomes forms a peroxisome-ER contact site in human cells for peroxisome biogenesis, the biosynthesis of plasmalogens, and cholesterol homeostasis (Costello et al., 2017; Hua et al., 2017). In this study, we identified the ORP3 protein as a lipid transfer protein for the ciliary cholesterol trafficking from peroxisomes to primary cilia (Fig 5). Many ORP3-positive punctate signals were detected not only at the ciliary pocket, but also at other cellular regions (Fig 5E and H). Previous reports demonstrated that ORP3 localizes to the PM, ER, and endosomes (Lehto et al., 2005; Santos et al., 2018), suggesting that these structures have potency to transfer cholesterol to primary cilia. In contrast, the FRB-FKBP system-mediated peroxisome targeting to the ciliary pocket did not restore the ciliary cholesterol insufficiency in ORP3−/− hTERT-RPE1 cells (Fig 8A and B), suggesting that ORP3 is required for at least the peroxisome-mediated cholesterol trafficking into the ciliary membrane.

Our data demonstrate that peroxisomes do not enter the axonemal compartment in primary cilia (Appendix Fig S4 and Appendix Fig S5C), implying that, after the peroxisome–ciliary pocket contact, cholesterol might be transported in the ciliary membrane surrounding the axonemal region by another trafficking system. It was recently reported that a kinesin-3 family member, KIF13B, establishes a lipid raft microdomain enriched in the protein caveolin 1 at the cilium transition zone (TZ), which is located between the basal body and cilium, to promote Shh signaling (Schou et al., 2017). Since cholesterol is an important component of lipid rafts formed in the PM (Lingwood & Simons, 2010), ciliary cholesterol might also contribute to the formation and physiological functions of the caveolin 1-enriched microdomain at the TZ. The LDL-mediated cholesterol compensation partially restored the ciliary phenotype in the ZS patient cells and ZS-modeled cells compared with the case in cholesterol-depleted conditions (Figs 1G and 2I), implying that unknown pathways for ciliary cholesterol trafficking might exist. Chu et al reported an excellent screening system to identify proteins critical for intracellular cholesterol trafficking using an antifungal antibiotic, amphotericin B, in which cells only survive when they have damaged intracellular cholesterol trafficking (Chu et al., 2015). In future, combined approaches of amphotericin B cell-based screening and genome-scale CRISPR/Cas9 knockout (GeCKO) libraries (Shalem et al, 2014) should enable us to uncover the molecular machinery behind intra-ciliary membrane cholesterol transport.

In conclusion, we demonstrate that the dynamics of peroxisomes along the microtubules via the PEX14–Rabin8–Rab10–KIFC3 complex are essential for the supply of cholesterol into ciliary membranes.

Materials and Methods

Cell cultures

Human primary skin fibroblasts from a normal individual (GM 22277; Coriell Institute), SLO patient (GM05788; Coriell Institute), ZS patients (GM16513 and GM17398; Coriell Institute), X-ALD patient (GM17819; Coriell Institute), and NPC patient (GM23151; Coriell Institute) were cultured in Dulbeco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies) supplemented with 20% fetal bovine serum (FBS) and 50 mg/ml gentamycin. Genetic information on the skin fibroblasts from each patient and the
The primary antibodies used were as follows: mouse anti-acetylated-tubulin monoclonal antibody mAb (T7451; Sigma-Aldrich); mouse anti-Smo mAb (sc-166685; Santa Cruz); rabbit anti-Arl13b polyclonal antibody pAb (598; MBL); mouse anti-phospho-S473 Akt mAb (#4051; Cell Signaling Technology); mouse anti-GFP mAb (11-814-460-001; Proteintech); rabbit anti-pericentrin pAb (A301-348A; Bethyl Laboratories); mouse anti-PMP70 mAb (SAB4200181; Sigma-Aldrich); mouse anti-ninein mAb (MABT29; Merck Millipore); mouse anti-CYPOR mAb (sc-25270; Sigma-Aldrich); rabbit anti-ODF2 pAb (HPA001874; Novus); rabbit anti-ACOX1 pAb (10957-1-AP; Proteintech); goat anti-Patched antibody pAb (17711-1-AP; Proteintech); rabbit anti-EHD1 pAb (24657-1-AP; Proteintech); rabbit anti-EHD3 pAb (25320-1-AP; Proteintech); rabbit anti-Shh pAb (ab53281-Abcam); mouse anti-ORP3 mAb (sc-398326; Santa Cruz); rabbit anti-ACOX1 pAb (10957-1-AP; Proteintech); goat anti-Patched 1 pAb (sc-6149; Santa Cruz); rabbit anti-OFR2 pAb (HPA001874; Sigma-Aldrich); and mouse anti-DYKDDDDK (FLAG) tag mAb (018-22381; Fujifilm Wako).

**Antibodies**

The primary antibodies used were as follows: mouse anti-acetylated-tubulin monoclonal antibody mAb (T7451; Sigma-Aldrich); mouse anti-Smo mAb (sc-166685; Santa Cruz); rabbit anti-Arl13b polyclonal antibody pAb (17711-1-AP; Proteintech); rabbit anti-PEX1 pAb (13669-1-AP; Proteintech); rabbit anti-PERCP14 pAb (10594-1-AP; Proteintech); rabbit anti-pericentrin pAb (A301-348A; Bethyl Laboratories); mouse anti-PMP70 mAb (SAB4200181; Sigma-Aldrich); mouse anti-ninein mAb (MABT29; Merck Millipore); mouse anti-CYPOR mAb (sc-25270; Sigma-Aldrich); rabbit anti-ODF2 pAb (HPA001874; Novus); mouse anti-γ-tubulin mAb (T6557; Sigma-Aldrich); mouse anti-CYPOR mAb (sc-25270; Sigma-Aldrich); rabbit anti-PMP70 mAb (SAB4200181; Sigma-Aldrich); mouse anti-ninein mAb (MABT29; Merck Millipore); mouse anti-CYPOR mAb (sc-25270; Sigma-Aldrich); rabbit anti-ODF2 pAb (HPA001874; Sigma-Aldrich); and mouse anti-DYKDDDDK (FLAG) tag mAb (018-22381; Fujifilm Wako).

**Plasmids**

AcGFP1-tagged human Pex14, Rabbin8, RabB8, Rab10, and KIFC3, AcGFP1-tagged D4-domain of perfringolysin O (Ø-toxin) from Clostridium perfringens, DiRed2-tagged PACT (the C-terminal region of human pericentrin 3097–3336 a.a. and Smo, and 3×FLAG-tagged human Pex14, Rab10, EHD1, EHD3, ORP3, and KIFC3 expression vectors for mammalian cells were constructed by PCR and standard cloning techniques. We also used site-directed mutagenesis to insert mutations into Rab8B and Rab10. ECFP-SKL (#54548), GFP-Rab8A (#24898), GFP-Rab2A-Q67L (#24900), and tdTomato-BicD2-FKBP (#64205) expression vectors were purchased from Addgene. The PEX3-GFP-FRB expression vector for mammalian cells was constructed from PEX3-GFP-Halo (#67764; Addgene) and Mito-mCherry-FRB (#59352; Addgene) using PCR and the Gibson assembly kit (New England Biolabs). GFP-MCHR1 was previously constructed as reported elsewhere (Hamamoto et al., 2016). All mutations were verified by automated sequencing. For the construction of an expression vector of both sgRNA targeting gene and spCas9, a pair of annealed oligodeoxynucleotides designed on the target sites addressed in the key resource table with overhangs of the BbsI restriction enzyme site were inserted into the pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (#42230; Addgene). The targeting plasmid vector contained a BbsI restriction enzyme site flanked with a CMV promoter-driven hsoTK-2A-

Gene targeting in hTERT-RPE1 cells using the CRISPR/ObLiGaRe method

A total of 2 × 10⁶ hTERT-RPE1 cells were seeded into one well of a six-well plate 24 h before lipofection. Then, 20 ng of the targeting vector and 600 ng of the pX330 plasmid vector for the target gene editing were transfected into the cells using LipoHunter LTX (ThermoFisher), in accordance with the manufacturer’s protocol. At 48 h after the transfection, the transfected cells were reseeded into 15-cm dishes and then subjected to selection using 2 mg/ml G418 (Nacalai Tesque). Eight to sixteen drug-resistant cell colonies were then picked up on days 14–18 after transfection. These colonies were divided into two aliquots: One was transferred into a well of a 96-well plate for cloning expansion, while the other was lysed and used for PCR and direct-sequence genotyping. As described previously (Royba et al., 2017), PCR genotyping to screen the hTERT-RPE1 cell clones was performed using extracted genomic DNA as a template and KOD-FX Neo DNA polymerase (Toyobo) with three types of primer pair: the first primer pair for detecting the target gene locus (Appendix Table S1), the second primer pair consisting of the forward primer in the target gene locus and Neo−reverse primer (5’-GCGGATCCGCGTTCAC-TAAACCAGC-3’) for detecting the forward insertion of the drug-resistant gene cassette into the target gene locus, and the third primer pair consisting of the reverse primer in the target gene locus and Neo−reverse primer for detecting the reversed insertion. PCR products were run on 2.0% agarose gel. The wild-type-sized PCR products amplified with the third primer pair were directly sequenced to determine the presence or absence of insertion or deletion mutations using 3130 Genetic Analyzer (Applied Biosystems). The efficiency of gene targeting using this method is dependent on the gene locus. In this study, the average efficiencies of gene knockout in the G418-resistant clones were 40–75%.

**Immunoprecipitation and Western blot analyses**

Cells were transfected with plasmid DNA for immunoprecipitation analysis and cultured in serum-free DMEM for 24 h. The cells were lysed in lysis buffer (0.5% Triton X-100, 150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5 mM PMSF, 2 mg/ml pepstatin A, 10 mg/ml leupeptin, 5 mg/ml aprotinin). The lysates were sheared with a 21-gauge needle, incubated on ice for 15 min, and clarified by centrifugation at 20,817 g for 15 min at 4°C. The supernatants were precleared with protein A/G-conjugated agarose beads (Santa Cruz) and incubated with anti-FLAG antibody covalently conjugated agarose beads (012-22781; Fujifilm Wako); the mixtures were then rotated for a further 16 h at 4°C. The agarose beads were washed three times with wash buffer (1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5 mM PMSF, 2 mg/ml pepstatin A, 10 mg/ml leupeptin, 5 mg/ml

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aprotinin) before elution with sample buffer. The immunoprecipitates were analyzed by 10% SDS–PAGE and transferred to PVDF membranes for Western blot analyses, as described previously (Miyamoto et al, 2015). Here, 15% IP fractions and 0.75% inputs were detected by Western blotting. For the validation of gene knock-out, 1.5% cell lysate was applied to each lane.

**In vitro binding analysis**

GST (0.2 µg, Abcam: ab81793) or GST fused to PEX14 proteins (1 µg, Abnova: H00005195-P01) was loaded to glutathione-Sepharose beads (GE Healthcare) and then incubated with 6×His-EHD3 (1 µg, ORIGENE: TP761227), 6×His-Rabin8 (1 µg, ORIGENE: TP760069), or 6×His-Rab10 (1 µg, NOVUS: NBP2-23392). The mixtures were then rotated for 2 h at 4°C in an *in vitro* binding buffer (50 mM Tris–HCl pH7.5, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.5 mM PMSF, 2 mg/ml pepstatin A, 10 mg/ml leupeptin, 5 mg/ml aprotinin). The glutathione-Sepharose beads were washed three times with the *in vitro* binding buffer minus glycerol before elution with sample buffer. Bound and input proteins were analyzed by SDS–PAGE and Western blotting. Here, 15% pull-down fractions and 0.75% inputs were detected by Western blotting.

**Immunofluorescence microscopy**

For detection of the protein epitopes, cells grown on coverslips (Matsunami Glass) were fixed in 4% paraformaldehyde at room temperature for 15 min or 100% methanol at −20°C for 10 min, permeabilized in 0.2% Triton X-100, briefly washed with PBS three times, blocked with 1% BSA in PBS for 30 min, and probed with primary antibodies. Antibody–antigen complexes were detected with Alexa Fluor 405-, Alexa Fluor 594-, Alexa Fluor 488-, or Alexa Fluor 647-conjugated goat or guinea pig secondary antibodies (ThermoFisher) by incubation for 30 min at room temperature. The cells were washed three times with PBS and then counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). For the detection of intracellular cholesterol, the serum-starved cells transfected with or without the AcGFP1-D4 expression vector were fixed in 4% paraformaldehyde at room temperature for 15 min, briefly washed with PBS three times, blocked with 1% BSA in PBS for 30 min, and incubated with 50 µg/ml Filipin III (Cayman Chemical) in Can Get Signal immunostain solution A (Toyobo) and each antibody for counterstaining at room temperature for 1 h. Antibody–antigen complexes were detected with Alexa Fluor 594-, Alexa Fluor 488-, or Alexa Fluor 647-conjugated goat secondary antibodies (ThermoFisher) by incubation for 3 min at room temperature. The cells were washed three times with PBS and then mounted with Prolong DAPI containing DAPI (ThermoFisher). At 24 h after transfection, the medium was replaced with serum-free DMEM and then cells were incubated for 24 h to induce ciliogenesis. Cells were monitored with a confocal microscope (LSM800; Carl Zeiss Microimaging Inc.) in a moisture chamber (Tokai Hit) at 37°C in humidified air with 5% CO2. Images through each color filter were simultaneously acquired. Z-stack images were captured at 5-min intervals for 2 h, and overlapping images and movies were created using ZEN software (Carl Zeiss Microimaging Inc.).

**Cholesterol depletion and rescue experiments**

To remove intracellular cholesterol, confluent cells were incubated in serum-free DMEM for 24 h, after which they were treated for 45 min with 1.5% methyl-β-cyclodextrin (Sigma-Aldrich) in DMEM. All subsequent incubations were performed in DMEM containing 40 µM pravastatin to attenuate cholesterol biosynthesis, with or without the indicated additives. For cholesterol complementation assays, cholesterol was delivered by incubating the cells for 1 h with 50 µM or 100 µM water-soluble methyl-β-cyclodextrin–cholesterol complex (Sigma-Aldrich) in DMEM supplemented with 40 µM pravastatin. Alternatively, cholesterol was compensated for by incubating the cells with 0.06 mg/ml LDL (Sigma-Aldrich) in DMEM supplemented with 40 µM pravastatin and the desired agents. The cells were then treated for 24 h with the desired agents and subsequently processed for immunofluorescence analyses.

**Shh pathway assay**

For qPCR assays, confluent skin fibroblasts in 96-well plates were cultured in serum-free DMEM for 24 h, after which they were incubated for 24 h in serum-free DMEM containing 1 µM SAG (EMD Millipore™ Calbiochem™). The cells were washed with ice-cold PBS, lysed, and then cDNAs were synthesized with reverse transcriptase from their extracted total RNA using Cells-to-CT Kit (ThermoFisher), in accordance with the manufacturer’s protocol. Expression was measured by TaqMan PCR analysis using *Gli1* and *β-actin* TaqMan probes (ThermoFisher) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad); the levels of *Gli1* expression were normalized to those of *β-actin*. The primers and Taqman probes were *Gli1* (Hs00171790_m1) and *β-actin* (Hs01060665_g1). All qPCR assays were performed in triplicate starting from three cell cultures, with error bars indicating SD. For evaluation of the ciliary localization of Smo, confluent cells grown on coverslips were
cultured in serum-free DMEM for 24 h, after which they were incubated for 24 h in serum-free DMEM with 50 nM Shh-N (EMD Millipore™, Calbiochem™). Shh-N-stimulated cells were immuno-fluorescently stained as described above. The primary antibodies used were as follows: mouse monoclonal anti-acetylated-tubulin IgG2b isotope (T7451, 1:500 dilution; Sigma-Aldrich), mouse monoclonal anti-Smo IgG2a isotope (sc-166685, 1:200 dilution; Santa Cruz), and mouse monoclonal anti-γ-tubulin monoclonal IgG1 isotope (T6557, 1:500 dilution; Sigma-Aldrich).

MCH-dependent ciliary shortening assay

MCHR1-EGFP expression vector (Hamamoto et al., 2016) was transfected to hTERT-RPE1 cells grown on coverslips (Matsunami Glass) using Lipofectamine LTX reagent (ThermoFisher). At 24 h after transfection, the medium was replaced with serum-free DMEM, and then, cells were incubated for 24 h to induce ciliogenesis. The ciliated cells were incubated in DMEM containing 1 μM MCH (Peptide Institute) for 6 h and then processed for the immunofluorescence analysis.

Fractionation of peroxisomes

Peroxisome fractions were isolated using a Peroxisome isolation kit (Sigma-Aldrich), in accordance with the manufacturer’s protocol. Cells were collected by centrifugation at 250 g for 5 min at 4°C and cell pellets were homogenized using a pestle in 1× Peroxisome Extraction Buffer (5 mM MOPS, pH 7.65, 1.25 M sucrose, 5 mM EDTA, 0.5% ethanol). After centrifugation at 25,000 g for 20 min at 4°C, the supernatants were removed and the pellets were suspended in 1× Peroxisome Extraction Buffer to obtain the Crude Peroxisome Fraction (CPF). OptiPrep Density Gradient Medium (60% solution of iodoxanol in water) was diluted to 22.5% and 27.5% iodixanol concentration using 1× Optiprep Dilution Buffer (5 mM MOPS, pH 8.0, 1 mM EDTA, 0.1% ethanol). Diluted OptiPrep Density Gradient Medium was applied to an ultracentrifuge tube, onto which CPF was layered. Tubes were centrifuged at 100,000 g for 1.5 h at 4°C, and the bottom layer was collected as the peroxisome fraction. Finally, 0.05% and 0.5% total cell lysates, 0.75% crude peroxisome fraction (CPF), 0.4% lysosome/mitochondria fraction (Lyso/Mit) fraction, and 0.5% peroxisome fraction (PEX) obtained by this procedure were applied and detected by Western blotting.

Rapamycin-induced targeting of peroxisomes to the ciliary pocket

PEX3-GFP-FRB and tdTomato-BicD2-FKBP expression vectors were co-transfected to hTERT-RPE1 cells grown on coverslips (Matsunami Glass) using Lipofectamine LTX reagent (ThermoFisher). At 24 h after transfection, the medium was replaced with serum-free DMEM, and then, cells were incubated for 24 h to induce ciliogenesis. The ciliated cells were incubated in DMEM containing 100 μM rapamycin (LC Laboratories) for 30 min and then processed for the Filipin III staining and immunofluorescence analyses.

Quantification of intracellular cholesterol levels

Total cellular cholesterol and free cholesterol were measured using a Total Cholesterol and Cholesteryl Ester Colorimetric/Fluorometric Assay Kit (BioVision), in accordance with the manufacturer’s protocol. Human primary skin fibroblasts cultured in DMEM containing 20% FBS were collected by centrifugation at 250 g and 83 pA, and subsequent SEM imaging of the block face was performed at 1.5 kV and 100 pA using the in-column BSE detector. Obtained serial images with the voxel size of 4 × 4 × 10 nm were reconstructed and manually segmented using the Amira software Ver.5.6 (Thermo Fisher Scientific).

Statistical analysis

All experiments were performed independently at least three times. The data are shown as mean ± s.d., at least carried out in triplicate. Differences between groups were evaluated for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc tests (Tukey’s HSD). The software of R version 3.4.4 was used. Values of P < 0.05 were considered to be statistically significant.

Data availability

Raw data and CRISPR/Cas9-mediated knockout cells associated with the figures will be made available on a reasonable request.

Expanded View for this article is available online.
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Author contributions
TM, KH, TI, AHI, SNA, and HO performed experiments and analyzed data. TM conceived the study. AHI, YS, TY, and SM supervised the study. TM, KH, TI, AHI, SNA, and HO performed experiments and analyzed data. TM, KH, TI, AHI, SNA, and HO wrote the paper. All authors interpreted data.

Conflict of interest
The authors declare that they have no conflict of interest.

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