ER-lysosome contacts enable cholesterol sensing by mTORC1 and drive aberrant growth signalling in Niemann-Pick type C

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Cholesterol activates the master growth regulator, mTORC1 kinase, by promoting its recruitment to the surface of lysosomes by the Rag guanosine triphosphatases (GTPases). The mechanisms that regulate lysosomal cholesterol content to enable mTORC1 signalling are unknown. Here, we show that oxysterol binding protein (OSBP) and its anchors at the endoplasmic reticulum (ER), VAPA and VAPB, deliver cholesterol across ER-lysosome contacts to activate mTORC1. In cells lacking OSBP, but not other VAP-interacting cholesterol carriers, the recruitment of mTORC1 by the Rag GTPases is inhibited owing to impaired transport of cholesterol to lysosomes. By contrast, OSBP-mediated cholesterol trafficking drives constitutive mTORC1 activation in a disease model caused by the loss of the lysosomal cholesterol transporter, Niemann-Pick C1 (NPC1). Chemical and genetic inactivation of OSBP suppresses aberrant mTORC1 signalling and restores autophagic function in cellular models of Niemann-Pick type C (NPC). Thus, ER-lysosome contacts are signalling hubs that enable cholesterol sensing by mTORC1, and targeting the sterol-transfer activity of these signalling hubs could be beneficial in patients with NPC.

The exchange of contents and signals between organelles is key to the execution of cellular programs for growth and homeostasis, and failure of this communication can cause disease. A form of organelle communication involves exchange of cholesterol and other lipids by specialized carriers that are located at physical contacts between the ER and other membranes1–3. Cholesterol was recently identified as an essential activator for the master growth regulator, mTORC1 kinase. Cholesterol promotes mTORC1 recruitment from the cytosol to the lysosomal membrane, where mTORC1 triggers downstream programs for biomass production and suppression of catabolism4–7. However, the mechanisms that deliver cholesterol to the lysosomal membrane to enable mTORC1 activation are unknown. More generally, whether and how interorganelle contacts govern cell-wide programs for growth and quality control are not understood.

Under low cholesterol, mTORC1 cannot interact with its lysosomal scaffold, the Rag GTPases, and remains inactive in the cytosol. By contrast, stimulating cells with cholesterol triggers rapid Rag-GTPase-dependent translocation of mTORC1 to the lysosomal surface and activation of its kinase function1. Experiments in cells and reconstituted systems suggest that the Rag GTPases specifically sense the cholesterol content of the lysosomal limiting membrane1. This cholesterol pool regulates the Rags, at least in part, through SLC38A9, which is a multi-pass amino acid permease that is also required for the activation of mTORC1 by amino acids2–5.

The cellular origins of the cholesterol pool that activates mTORC1 are unclear. Exogenous cholesterol carried by low-density lipoprotein (LDL) is trafficked to the lysosomal lumen, and from there it is exported to acceptor membranes by a mechanism that requires the putative cholesterol carrier, NPC1 (refs. 11–13). Genetic inactivation of NPC1 in humans leads to substantial accumulation of cholesterol within the lysosome, compromising its functionality and triggering NPC—a fatal metabolic and neurodegenerative disease14. LDL stimulates Rag- and SLC38A9-dependent activation of mTORC1 and, in cells lacking NPC1, mTORC1 is hyperactive and cannot be switched off by cholesterol depletion, although the mechanistic basis for this constitutive activation remains unclear.

Following its NPC1-dependent export from the lysosome, cholesterol can be detected in several acceptor compartments, including the ER, Golgi and plasma membrane, but whether these compartments represent separate routes or stations in a common export pathway is unclear15–18. Cholesterol can also be transferred back from the ER to several acceptor organelles, including the lysosome, through specialized carriers that reside at membrane contacts19,20. The points at which cholesterol is made available for mTORC1 activation along these routes are unclear.

An important class of cholesterol carriers are the oxysterol binding protein (OSBP)-related proteins (ORPs)19–21. ORPs contain, at their C terminus, large hydrophobic cavities that shield cholesterol molecules from the polar cytosolic environment and can also accommodate phospholipids17,18. The founding member of this family, OSBP, localizes at contacts between the ER and trans-Golgi, where it is thought to transfer ER-derived cholesterol to the Golgi in exchange for phosphatidylinositol 4-phosphate (PtdIns4P)19–21. OSBP was recently proposed to function at contacts between the ER and endolysosomes22,23. In concert with its binding partners on the ER, VAPA and VAPB (VAPA/B), OSBP regulates the PtdIns4P content of endolysosomes, which—in turn—affects their actin-dependent motility22. Whether OSBP also controls cholesterol levels on the lysosomal limiting membrane, and how the transport activity of OSBP across ER–lysosome contacts affects the activation of mTORC1 are unknown.

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Here we find that, unique among several sterol carriers associated with the lysosome, OSBP establishes a lysosomal cholesterol pool that is essential for Rag GTPase-dependent mTORC1 activation. In cells lacking NPC1, unopposed ER-to-lysosome transport by OSBP drives aberrant build-up of cholesterol on the lysosomal limiting membrane. Genetic and chemical inhibition of OSBP reverses cholesterol accumulation on the limiting membrane of NPC1-null lysosomes, suppresses aberrant mTORC1 signalling and restores defective autophagy, a major driver in the pathogenesis of NPC. Thus, ER–lysosome contacts emerge as signalling nodes that coordinateially regulate mTORC1 signalling and autophagy, and their manipulation by OSBP inhibitors could be beneficial in NPC and mTORC1-driven diseases.

Results
OSBP mediates cholesterol build-up on the limiting membrane of NPC1-null lysosomes. In cells lacking NPC1, mTORC1 is constitutively active and refractory to inhibition by cholesterol-depleting agents. This result seems paradoxical because, in the absence of NPC1, cholesterol should be trapped within the lysosomal interior and unable to reach its limiting membrane10–12 where mTORC1 activation occurs. A possible resolution of this paradox is that, in NPC1-null lysosomes, not only the interior but also the limiting membrane could be cholesterol-enriched, a possibility that is consistent with the altered trafficking pattern of NPC1-null lysosomes13–15. However, owing to limitations of current biochemical fractionation and cholesterol-imaging approaches, it has not been possible to univocally determine the cholesterol status of the limiting membrane of NPC1-null lysosomes. Therefore, we used a recently established cholesterol biosensor, mCherry-tagged D4H* (derived from the Clostridium Perfringens theta-toxin, *; improved version of the original D4H by introducing Y413A and A463W mutations, see Methods), which binds to membranes at which cholesterol exceeds 10% molar content17 (Supplementary Fig. 1a). Recombinant D4H*–mCherry was delivered to semi-permeabilized cells using a liquid-nitrogen pulse that breached the plasma membrane but left the lysosomal membrane intact (Fig. 1a), as shown by retention of LysoSensor staining (Supplementary Fig. 1b).

In non-permeabilized HEK293T cells, D4H*–mCherry bound to the only outer leaflet of the plasma membrane, consistent with its inability to cross membrane bilayers (Supplementary Fig. 1c, top). In semi-permeabilized cells with intact NPC1 function, including human fibroblasts, mouse embryonic fibroblasts (MEFs) and HEK293T cells, D4H*–mCherry did not bind to the cytoplasmic leaflet of lysosomes or other endomembranes, indicating that their cholesterol content is below 10% (Fig. 1b, Supplementary Fig. 1c,d). By contrast, D4H*–mCherry showed strong lysosomal accumulation in fibroblasts from patients carrying a pathogenic NPC1 mutation (NPC1Δ1264del; Fig. 1b), in Npc1−/− mouse-derived MEFs18,20 (Supplementary Fig. 1d), in HEK293T cells deleted for NPC1 using CRISPR–Cas9 (ref. 17; Fig. 1c) and in control human fibroblasts treated with the chemical NPC1 inhibitor, U18666A30 (Supplementary Fig. 1e,f). Thus, not only the lumen but also the limiting membranes of NPC1-null lysosomes show considerable cholesterol accumulation.

As LDL-derived cholesterol cannot escape from the lumen of NPC1-null lysosomes11–13, we hypothesized that the peripheral cholesterol build-up revealed by D4H*–mCherry might be transferred across interorganelle contacts. To identify targets that could mediate cholesterol transfer to lysosomes, we carried out a proteomics-based analysis of immunopurified lysosomes19,34,35. This analysis revealed several peptides from three ORP-family carriers: OSBP, ORP8 and ORP11 (ref. 17; Fig. 1d, Supplementary Table 1). We confirmed lysosomal association of endogenous OSBP, ORP11 and ORP8 by immunoblotting of the immunopurified lysosomal samples (Fig. 1e).

Notably, knockdown of OSBP—but not ORP11, ORP8 or ORP5 (which did not score as lysosome-associated)—largely reversed D4H*–mCherry accumulation on the lysosomal surface of NPC1-null human fibroblasts and HEK293T cells (Fig. 1f–h, Supplementary Fig. 1g). By contrast, OSBP depletion did not correct luminal cholesterol accumulation of NPC1-null lysosomes, as shown by unchanged cholesterol staining using filipin (Fig. 1g, Supplementary Fig. 1g). We confirmed this result using the natural product OSW-1, which inhibits OSBP (along with closely related OSBP2) with nanomolar potency16,30. Treatment with OSW-1 caused the D4H*–mCherry signal, but not the filipin signal, to disappear from lysosomes of NPC1-null human fibroblasts, MEFs and HEK293T cells (Supplementary Fig. 2a–f).

Thus, unique among the ORPs, OSBP seems to be responsible for the accumulation of cholesterol on the limiting membrane but not within the lumen of NPC1-null lysosomes.

OSBP localizes to ER–lysosome contacts and controls lysosomal cholesterol levels in wild-type cells. OSBP was shown to mainly reside at ER–trans-Golgi-network contacts32,34. However, in HEK293A cells, we also detected stably expressed green fluorescent protein (GFP)-tagged OSBP around LAMP2-positive lysosomes (Supplementary Fig. 3a). GFP–OSBP also co-localized with endogenous mTOR and with the p18 subunit of the Ragulator/LAMTOR complex, which anchors the Rag GTPases and mTORC1 to the lysosomal membrane16,17 (Supplementary Fig. 3b).

Association of OSBP with LAMP2-, mTOR- or p18-positive vesicles was abolished by deletion of its plekstrin homology (PH) domain, which anchors OSBP to the Golgi or endolysosomal membrane by binding to PtdIns4P17,27,28 (Supplementary Fig. 3a,b). As OSBP transfers PtdIns4P from Golgi and endolysosomes towards the ER, it is thought to weaken its own association with these organelles38. Consistent with this, a transport-defective OSBP mutant (ΔELS/A) was more strongly clustered on LAMP2-positive structures than wild-type OSBP (Supplementary Fig. 3a). Similarly, inhibiting OSBP with OSW-1 resulted in strong clustering of GFP–OSBP to lysosomes (Supplementary Fig. 3c). Staining HEK293A cells with an antibody that recognizes endogenous OSBP showed partial co-localization with LAMP2 that was enhanced by short hairpin RNA (shRNA)-mediated depletion of VAPA/B, as previously shown19 (Supplementary Fig. 3d).

Next, we investigated whether OSBP regulates limiting-membrane cholesterol of not only NPC1-null but also wild-type lysosomes. As D4H*–mCherry did not bind to lysosomes with intact NPC1 function, we performed lipidomic analysis of immunopurified lysosomes (Supplementary Fig. 4a). Inhibiting OSBP before immunopurification caused a 30% reduction in cholesterol content, suggesting that a significant fraction of the lysosomal cholesterol pool is ER-derived and transferred by OSBP (Fig. 2a). Whole-cell lipidomics showed that, although the total cholesterol content of OSBP-depleted cells was unchanged, a larger fraction was esterified to oleic acid than in control cells30,34 (Supplementary Fig. 4b,c). Moreover, filipin staining showed strong accumulation of cholesterol in the ER of cells treated with OSBP-targeting shRNA or with the OSBP inhibitor OSW-1 (Supplementary Fig. 4d,e).

To independently visualize OSBP-dependent sterol transport to the lysosomal limiting membrane, we pulse–chased cells with a fluorescent cholesterol analogue, TopFluor-cholesterol. In DMSO-treated cells, TopFluor-cholesterol initially appeared at the plasma membrane, and began accumulating in lysosomes by 30 min (Supplementary Fig. 5a,b). By contrast, OSW-1 treatment delayed clearance of TopFluor-cholesterol from the plasma membrane and caused it to acquire an ER-like distribution, never building up in lysosomes to a noticeable degree (Supplementary Fig. 5a,b). Although off-target inhibition of other ORPs cannot be ruled out entirely, the most likely explanation for this result is that OSBP mediates a key
step in a pathway that moves cholesterol from the plasma membrane to the lysosomal limiting membrane through the ER.

**OSBP is essential for mTORC1 activation by cholesterol.** As lysosomal membrane cholesterol drives mTORC1 activation, we tested the requirement for OSBP in mTORC1 lysosomal recruitment and kinase activation. In HEK293T cells, we found that OSBP was required for cholesterol-induced translocation of mTOR to LAMP2-positive structures, whereas ORP8, ORP11 or ORP5 were dispensable (Fig. 2b,c, Supplementary Fig. 6a). Consistent with the loss of lysosomal localization, depleting OSBP strongly reduced cholesterol-dependent mTORC1 activation, as shown by loss of phosphorylation of canonical substrates S6-kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1). By contrast, knockdown of ORP8, ORP11 and ORP5 had a smaller or no effect on the kinase activity of mTORC1 (Fig. 2d).

Inhibiting OSBP activity with OSW-1 also suppressed cholesterol-induced mTORC1 lysosomal recruitment and activation in a dose-dependent manner (Fig. 2e, Supplementary Fig. 6b,c). Even under complete medium conditions, OSBP ablation strongly reduced lysosomal mTORC1 recruitment (Supplementary Fig. 6d,e) and signalling (Supplementary Fig. 6f). By contrast, OSBP depletion affected acute mTORC1 activation by amino acids only modestly, suggesting that OSBP is not directly involved in amino acid sensing (Supplementary Fig. 6g).

**Fig. 1** | Cholesterol accumulates at the limiting membrane of NPC1-deficient lysosomes in an OSBP-dependent manner. **a,** In situ labelling assay for detecting cholesterol at the lysosomal membrane. **b,** Cholesterol is deposited at the lysosomal membrane in human NPC1 patient-derived fibroblasts. Control (NPC1WT) and NPC1 (NPC1mut) fibroblasts were fixed, breached with a liquid N2 pulse, subjected to cholesterol labelling by GST–D4H*–mCherry and filipin, and stained for LAMP2. Scale bars, 10 µm. **c,** NPC1 deletion by CRISPR–Cas9 genome editing in cells results in cholesterol accumulation at the lysosomal membrane. NPC1-deleted HEK293T cells expressing Flag–GFP–Tmem192, either naive or reconstituted with Flag-tagged NPC1, were processed for cholesterol labelling. Scale bars, 10 µm. Insets: three examples per genotype. **d,** Proteomic analysis of affinity-purified lysosomes. Lysosomes were immunopurified by anti-Flag M2 beads (or anti-haemagglutinin (HA) magnetic beads) from HEK293T cells expressing LAMP1–mRFP–2×Flag or Tmem192–mRFP–3×HA and analysed by mass spectrometry. Unique peptide counts for identified ORPs are shown; n = 2 independent experiments, 5 biologically independent samples in total (Supplementary Table 1). mRFP, monomeric red fluorescent protein. **e,** Pull-down of lysosomes revealing the lysosomal association of ORPs. Lysosomes were purified by anti-HA beads and immunoblotted for the indicated proteins. IP, immunoprecipitation.

**f,** Quantification of co-localization of D4H*–mCherry with filipin-labelled cholesterol deposits in NPC1-null cells depleted of ORPs. The box plots show the minimum, first quartile, median, third quartile and maximum; 10 fields of view per genotype; n represents the number of cells; shRNA targeting luciferase (shLuc) (n = 13), shOR8 (n = 12), shORP11 (n = 12), shOSBP (n = 11), shORP5 (n = 10); statistical analysis was performed using ANOVA with Dunnett’s multiple comparison test; **** adjusted P = 0.0001 compared with NPC1WT-shLuc (Supplementary Fig. 1g). **g,** Concomitant depletion of OSBP in NPC1-null cells reduces lysosomal membrane cholesterol levels. Cells were depleted of OSBP using doxycycline-induced shRNA and processed for cholesterol labelling. Scale bars, 10 µm. Insets: three examples per genotype. For **h,** the box plots are as described in f; 10 fields of view per group; n represents the number of cells: −Dox (n = 64), +Dox (n = 72); statistical analysis was performed using two-tailed unpaired t-tests; **** P = 1.66578 × 10−13 compared with the −Dox group. Insets in b,c and g represent regions of the respective panels magnified by 3.2 times. Experiments in b, c and g were performed three times; experiments in e, g and h were performed twice.
OSBP depletion had no effect on the lysosomal localization of RagC and p18/LAMTOR1 (Fig. 3a, Supplementary Fig. 6h). OSBP loss therefore impairs the ability of the Regulator–Rag complex to recruit mTORC1, but not its integrity. Consistent with this interpretation, stable expression of GFP–RagB 

OSBP mediates ER-to-lysosome cholesterol transfer that enables mTORC1 activation. To determine whether and how the cholesterol-transport function of OSBP participates in mTORC1 regulation, we reconstituted OSBP-depleted cells with isoforms that lack key functions (Fig. 4a, Supplementary Fig. 7a). OSBP attachment to the lysosome (by deleting or mutating the PH domain) or to the ER (by mutating the phenylalanines in an acidic tract (FFAT) motif that mediates the interaction of OSBP with anchoring and lipid-transporting functions of OSBP. **Fig. 2 | OSBP is required for cholesterol-dependent lysosomal recruitment and activation of mTORC1.** a. OSBP inhibition by OSW-1 treatment reduces the lysosomal cholesterol content. HEK293T cells expressing FlagmRFP–3xHA were treated with DMSO or 20 nM OSW-1 for 8 h. Lysosomes were purified and analysed by mass spectrometry. Data are mean ± s.d. of n = 4 biologically independent samples per treatment. Statistical analysis was performed using a two-tailed unpaired t-test; ****P = 1.61895 × 10^{-4} compared with DMSO-treated cells. Immunoblots are provided in Supplementary Fig. 4a, b. OSBP is specifically required for lysosomal recruitment of mTORC1 by cholesterol. Cells depleted of OSBP were subjected to cholesterol depletion and restimulation, where indicated, followed by immunofluorescence for endogenous mTOR and LAMP2. Representative images are shown. Scale bars, 10 μm. Insets in b represent regions of the respective panels magnified by 3.2 times. c. Quantification of co-localization of mTOR with LAMP2-positive lysosomes in cells expressing the indicated shRNAs. Data are mean ± s.d.; 10 fields of view per genotype or condition; n represents the number of cells: shLuc – cholesterol (Chol) (n = 182), shLuc + Chol (n = 181), shORP8 – Chol (n = 104), shORP8 + Chol (n = 91), shORP11 – Chol (n = 157), shORP11 + Chol (n = 156), shOSBP – Chol (n = 176), shOSBP + Chol (n = 182), shORP5 – Chol (n = 160), shORP5 + Chol (n = 166); statistical analysis was performed using ANOVA with Dunnett’s multiple comparison test; ****adjusted P = 0.0001 compared with shLuc + Chol. Representative images are provided in Supplementary Fig. 6a. d, ORPs affect the activation of mTORC1 by cholesterol. Cells were sterol-depleted using methyl-β-cyclodextrin (MCD, 0.5% w/v) for 4 h and restimulated for 2 h using 50 μM cholesterol complexed with 0.1% MCD (MCD:cholesterol). Cell lysates were analysed for the levels of the indicated proteins and for the phosphorylation status of S6K1 (T389), 4E-BP1 (S65). Fold changes of protein phosphorylation are indicated. e, OSW-1 inhibits sterol-induced mTORC1 signalling in a dose-dependent manner. Cells were subjected to cholesterol starvation and restimulation in the presence of DMSO or OSW-1 at the indicated concentrations and immunoblotted for the indicated proteins. The experiments in b and d were performed three times; the experiments in a and e were performed twice. Unprocessed blots in Supplementary Fig. 9. Statistics source data are provided in Supplementary Table 2.
As OSBP resides at both ER–lysosome and ER–Golgi contacts, we rigorously tested whether OSBP regulates mTORC1 through lipid transport from ER to lysosomes and not elsewhere. We reconstructed OSBP-depleted cells with OSBP isoforms in which the PH domain is replaced with organelle-specific targeting signals, while retaining attachment to the ER by VAP (Supplementary Fig. 7d). Only OSBP fused to a lysosomal targeting sequence (the 39 N-terminal amino acids of p18) rescued mTORC1 activation by cholesterol, whereas OSBP isoforms targeted to mitochondria, ER and, most importantly, Golgi failed to do so (Fig. 4d). Rescue of mTORC1 signalling by lysosome-targeted OSBP required its lipid-transport activity, as mTORC1 signalling was abolished by mutations in the ORD that disrupt either cholesterol or PtdIns4P binding (Fig. 4e).

The OSBP transport cycle involves exchange of cholesterol with PtdIns4P\(^{20,34}\). Consistent with this, OSBP depletion caused strong accumulation of PtdIns4P on lysosomes, as shown using a genetically encoded PtdIns4P probe, GFP–P4M\(^{20}\) (Fig. 5a).
To rule out a role of lysosomal PtdIns4P build-up in mTORC1 inhibition, we fused the catalytic domain of Sac1 phosphatase, which hydrolyses PtdIns4P to phosphatidylinositol, to the lyso-Sac1 targeting sequence of p18 (refs. 5,37,44; lyso-Sac1; Fig. 5b). In OSBP-depleted cells, lyso-Sac1 completely reversed lysosomal build-up of PtdIns4P caused by OSBP inactivation, whereas a catalytically inactive lyso-Sac1 mutant (C389S) failed to do so (Fig. 5c). However, neither the catalytically active nor the inactive lyso-Sac1 restored mTORC1 activation in OSBP-depleted cells (Fig. 5d). Thus, lysosomal PtdIns4P accumulation is unlikely to contribute to the mTORC1 inhibition triggered by inactivation of OSBP.

Finally, consistent with OSBP functioning at ER–lysosome contacts, shRNA-mediated depletion of VAPA/B suppressed lysosomal mTORC1 localization (Fig. 6a,b) and signalling (Fig. 6c). Although other FFAT-containing proteins, including STARD3 and ORP1L16,45, also transfer cholesterol across ER–lysosome contacts, shRNA-mediated depletion of VAPA/B suppressed lysosomal mTORC1 localization (Fig. 6a,b) and signalling (Fig. 6c). Although other FFAT-containing proteins, including STARD3 and ORP1L16,45, also transfer cholesterol across ER–lysosome contacts, they were not reverse accumulation of cholesterol at the lysosomal surface in OSBP-depleted cells and cells that were re-engineered as indicated were subjected to cholesterol starvation and restimulation, followed by immunofluorescence for endogenous mTOR and LAMP2. Scale bars, 10 µm. Insets in b represent regions of the respective panels magnified by 3.2 times.

Quantification of co-localization of mTOR with LAMP2-positive lysosomes in cells reconstituted with different truncations as indicated. Data are mean ± s.d.; 10 fields of view per genotype or condition; n represents the number of cells: shOSBP – Chol (n=173), shOSBP + Chol (n=170), rescued WT – Chol (n=185), rescued WT + Chol (n=154), rescued ΔN – Chol (n=179), rescued ΔN + Chol (n=131), rescued ΔPH – Chol (n=169), rescued ΔPH + Chol (n=168), rescued PH-FFAT – Chol (n=188), rescued PH-FFAT + Chol (n=179), rescued ORD – Chol (n=185), rescued ORD + Chol (n=168); statistical analysis was performed using ANOVA with Dunnett’s multiple comparison test; ****adjusted P=0.0001 compared with rescued WT + Chol. Linking OSBP to the lysosome but not to other organelles restores the activation of mTORC1 by cholesterol. Cells were depleted of OSBP and reconstituted with PH-domain-deleted isoforms that carry organelle-targeting sequences and were cholesterol starved and restimulated. Cell lysates were analysed for the indicated proteins and phosphorylated proteins. Experiments in d and e were repeated independently twice. Unprocessed blots are provided in Supplementary Fig. 9. Statistics source data are provided in Supplementary Table 2.
NPC1-null cells, as shown by unchanged D4H*-mCherry staining (Fig. 6f,g). Thus, OSBP and VAPs form a cholesterol-transporting system at ER–lysosome contacts that is uniquely involved in mTORC1 activation by cholesterol.

NPC1 regulates the number of ER–lysosome contacts and their sterol-transporting activity. Our D4H*-mCherry results strongly suggest that ER-to-lysosome cholesterol transport is increased in NPC1-null cells. Consistent with this, ER–lysosome contacts appeared enlarged in NPC1-null cells. In control human fibroblasts co-expressing the lysosomal and ER markers GFP–Tmem192 and mCherry–Sec61b, respectively, physical contacts between lysosomes and the ER could be readily visualized14,46 (Supplementary Fig. 8a). In NPC1-null fibroblasts, the ER domains in direct contact with lysosomes appeared significantly enlarged and were more persistent than those in control cells (Supplementary Fig. 8a). Further investigation using electron microscopy (EM) showed numerous ER tubules located close to the characteristically enlarged NPC1-null lysosomes, recognizable by the accumulation of undigested membranes and electron-dense material within their lumen14 (Supplementary Fig. 8b). As membrane contacts cannot be univocally identified by EM, we bolstered these observations with a

proximity ligation assay (PLA) for VAPA and LAMP2, which yields a fluorescent signal when the two membrane markers come within 10–30 nm of each other. VAPA–LAMP2 PLA confirmed that ER–lysosome contacts are significantly increased in NPC1-null compared with wild-type cells (Fig. 7a).

To determine the mechanistic basis for enhanced ER-to-lysosome cholesterol transport, we carried out OSBP–VAP co-immunoprecipitation experiments and found that the OSBP–VAP interaction is stronger in NPC1-null than in wild-type cells. Interestingly, in wild-type cells, the OSBP–VAP interaction is promoted by cholesterol, whereas it is constitutively high in NPC1-deleted cells (Fig. 7b). Cholesterol may therefore promote its own transport to the lysosome by increasing OSBP–VAP complex formation, whereas NPC1 inhibits this process leading to OSBP-dependent accumulation of cholesterol on the limiting membranes of NPC1-null lysosomes.

OSBP inactivation corrects aberrant mTORC1 signalling and defective autophagy in NPC1-null cells. We previously observed that, in NPC1-null cells, mTORC1 signalling is increased and resistant to cholesterol-depleting agents. On the basis of the D4H*-mCherry data and the increased formation of OSBP–VAP

**Fig. 5 | Excess lysosomal PtdIns4P after OSBP inhibition does not cause mTORC1 inhibition.** a. Depletion of OSBP results in accumulation of PtdIns4P at lysosomes. HEK293A cells stably expressing the PtdIns4P-specific probe, GFP–P4M, along with LAMP1–mRFP were depleted of OSBP by doxycycline-induced shRNA. Cells were plated on glass-bottom dishes, allowed to attach overnight and imaged live on a spinning-disk confocal microscope. Representative microscopy images are shown. Scale bars, 10 μm. Insets in a represent regions of the respective panels magnified by 3.2 times. b. Confirmation of lysosomal localization of the lysosome-targeted Sac1 catalytic-domain (Sac1cat) truncations in HEK293A cells. Schematic diagram showing that the Sac1 catalytic domain is targeted to the lysosome to hydrolyse its target PtdIns4P (left). PI, phosphatidylinositol. Right, representative microscopy images are shown. Scale bars, 10 μm. c. Ectopic expression of a lysosome-targeted Sac1 catalytic-domain truncations (Sac1cat) in HEK293T cells co-expressing mCherry–P4M and either catalytically active (WT) or inactive (CS) forms of lyso-Sac1 were depleted of OSBP and subjected to live-cell imaging. Representative confocal microscopy images are shown. Scale bars, 10 μm. d. Lysosomal build-up of PtdIns4P caused by OSBP depletion is not responsible for mTORC1 inhibition. Control cells and cells expressing catalytically active and inactive lyso-Sac1 were depleted of OSBP, followed by cholesterol starvation and restimulation, lysis and immunoblotting for the indicated proteins and phosphorylated proteins. Unprocessed blots are provided in Supplementary Fig. 9. Experiments in a–d were repeated independently twice.
Fig. 6 | VAPA and VAPB, the ER anchors for OSBP, are essential for cholesterol-dependent mTORC1 activation. a, Depletion of VAPA and VAPB abolishes the lysosomal recruitment of mTORC1 by cholesterol. HEK293T cells were depleted of VAPA and VAPB using shRNA and evaluated for mTOR lysosomal localization. Scale bars, 10 µm. Insets in a represent regions of the respective panels magnified by 3.2 times. b, Quantification of co-localization between mTOR- and LAMP2-positive lysosomes in control cells and cells depleted of VAPA and VAPB. Data are mean ± s.d.; 10 fields of view per genotype or condition; n represents the number of cells: shLuc – Chol (n = 124), shLuc + Chol (n = 74), shVAPA and shVAPB – Chol (n = 178), shVAPA and shVAPB + Chol (n = 163); statistical analysis was performed using ANOVA with Dunnett’s multiple comparison test; ****adjusted P = 0.0001 compared with shLuc + Chol. c, VAPA and VAPB are necessary for cholesterol-dependent mTORC1 activation. HEK293T cells were depleted of VAPA and VAPB, either individually or in combination, and evaluated for sterol-induced mTORC1 signalling using immunoblotting. d, e, STARD3 and ORP1L are not required for cholesterol-dependent mTORC1 activation by immunoblotting. f, Depletion of STARD3 or ORP1L has no effects on both peripheral and internal pools of lysosomal cholesterol in NPC1-deficient cells. NPC1-deficient fibroblasts obtained from patients with NPC, in which STARD3 or OSBP was depleted using shRNA or in which ORP1L was depleted using CRISPR–Cas9 genome editing, were subjected to cholesterol labelling by GST–D4H*-mCherry and filipin, and stained for endogenous LAMP2. Scale bar, 10 µm. Insets in f represent regions of the respective panels magnified by 3.2 times. g, Quantification of co-localization of D4H*-mCherry with filipin-labelled cholesterol deposits in NPC1-null cells that were silenced for STARD3, OSBP and ORP1L, respectively. The box plots show the minimum, first quartile, median, third quartile and maximum; 10 fields of view per genotype; n represents the number of cells: shLuc (n = 12), shSTARD3 (n = 10), shOSBP (n = 10), shORP1L (n = 11); statistical analysis was performed using ANOVA with Dunnett’s multiple comparison test; ****adjusted P = 0.0001 compared with NPC1mut-shLuc. Experiments in c–e were performed independently twice with similar results. The unprocessed blots are provided in Supplementary Fig. 9. Statistics source data are provided in Supplementary Table 2.
OSBP inhibition abrogates aberrant mTORC1 signalling in NPC1-deficient cells. a, Increased ER–lysosome contacts in NPC1 fibroblasts (left). Cells were fixed, permeabilized, labelled with VAPA or LAMP2 antibodies, or in combination as indicated, and analysed using PLA. Scale bars, 10 μm. Right, quantification of PLA signals. The box plots show the minimum, first quartile, median, third quartile and maximum; 60 fields of view per group; n represents the number of cells: NPC1WT (n = 60) and NPC1mut (n = 60). Statistical analysis was performed using two-tailed unpaired t-tests; ****P = 2.02047 × 10–19 compared with NPC1WT. b, Cholesterol regulates OSBP–VAP interaction in an NPC1-dependent manner. Control and NPC1-null cells expressing the Flag–GFP–Tmem192, Flag–GFP–OSBPWT and Flag–GFP–OSBP F359A/F360A were starved and refed with cholesterol, lysed and processed for Flag immunoprecipitation. Both immunoprecipitation and input samples were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting for the indicated proteins. The experiments were repeated twice with similar results. c, Depletion of OSBP abolishes constitutive mTORC1 signalling in NPC1-null cells. NPC1-deleted HEK293T cells, either naive or reconstituted (sgNPC1ResWT) with stably expressed Flag-tagged NPC1, were depleted of OSBP and analysed for sterol-induced mTORC1 signalling by immunoblotting. The experiments were repeated twice with similar results. 

— Amelie D. Staeber, *et al.*

**Fig. 7 | OSBP inhibition abrogates aberrant mTORC1 signalling in NPC1-deficient cells.** a, Increased ER–lysosome contacts in NPC1 fibroblasts (left). Cells were fixed, permeabilized, labelled with VAPA or LAMP2 antibodies, or in combination as indicated, and analysed using PLA. Scale bars, 10 μm. Right, quantification of PLA signals. The box plots show the minimum, first quartile, median, third quartile and maximum; 60 fields of view per group; n represents the number of cells: NPC1WT (n = 60) and NPC1mut (n = 60). Statistical analysis was performed using two-tailed unpaired t-tests; ****P = 2.02047 × 10–19 compared with NPC1WT. b, Cholesterol regulates OSBP–VAP interaction in an NPC1-dependent manner. Control and NPC1-null cells expressing the Flag–GFP–Tmem192, Flag–GFP–OSBPWT and Flag–GFP–OSBP F359A/F360A were starved and refed with cholesterol, lysed and processed for Flag immunoprecipitation. Both immunoprecipitation and input samples were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting for the indicated proteins. The experiments were repeated twice with similar results. c, Depletion of OSBP abolishes constitutive mTORC1 signalling in NPC1-null cells. NPC1-deleted HEK293T cells, either naive or reconstituted (sgNPC1ResWT) with stably expressed Flag-tagged NPC1, were depleted of OSBP and analysed for sterol-induced mTORC1 signalling by immunoblotting. The experiments were repeated twice with similar results. d, e, OSBP depletion abrogates constitutive mTORC1 lysosomal localization in NPC1-null cells. NPC1-deleted HEK293T cells, either naive or reconstituted with stably expressed Flag-tagged NPC1, were depleted of OSBP and evaluated for sterol-dependent mTORC1 lysosomal localization. Scale bars, 10 μm. Inset d represent regions of the respective panels magnified by 3.2 times. For e, data are mean ± s.d.; 10 fields of view per genotype or condition; n represents the number of cells: sgNPC1ResWT-shLuc – Chol (n = 166), sgNPC1ResWT-shLuc + Chol (n = 110), sgNPC1ResWT-shOSBP – Chol (n = 181), sgNPC1ResWT-shOSBP + Chol (n = 132), sgNPC1-shLuc – Chol (n = 127), sgNPC1-shLuc + Chol (n = 142), sgNPC1-shOSBP – Chol (n = 147) and sgNPC1-shOSBP + Chol (n = 131); statistical analysis was performed using ANOVA with Dunnett’s multiple comparison test; ****P = 0.0001. The unprocessed blots are provided in Supplementary Fig. 9. Statistics source data are provided in Supplementary Table 2.

complexes (Fig. 7a,b, Supplementary Fig. 8a,b), we hypothesized that constitutive mTORC1 activation may be driven by OSBP-dependent accumulation of cholesterol on the limiting membrane of NPC1-null lysosomes. Consistent with this model, knockdown of OSBP resulted in the complete inactivation of mTORC1, irrespective of NPC1 status (Fig. 7c). Moreover, OSBP depletion suppressed constitutive lysosomal mTORC1 localization in NPC1-deleted cells (Fig. 7d,e).

A major downstream consequence of aberrant activation of mTORC1 is suppression of autophagy. Indeed, as in other neurodegenerative disorders, autophagic flux is decreased in NPC and it may drive the defective cellular quality control that contributes to
NPC1mut cells. Statistics source data are provided in Supplementary Table 2.

Fig. 8 | OSBP inhibition restores autophagic function in NPC1-deficient cells. a, OSW-1 increases autophagic flux in both Npc1<sup>+/+</sup> and Npc1<sup>−/−</sup> MEFs expressing the GFP-LC3-RFP-LC3ΔG reporter. The normalized GFP/RFP fluorescence ratio is expressed as fold change over DMSO-treated Npc1<sup>+/+</sup> cells. Data are mean ± s.d.; n represents number of biologically independent samples: Npc1<sup>+/+</sup> DMSO (n = 7), Npc1<sup>+/+</sup> OSW-1 (n = 7), Npc1<sup>+/+</sup> BafA1 (n = 7), Npc1<sup>−/−</sup> Torin1 (n = 7), Npc1<sup>−/−</sup> DMSO (n = 6), Npc1<sup>−/−</sup> OSW-1 (n = 6), Npc1<sup>−/−</sup> BafA1 (n = 6) and Npc1<sup>−/−</sup> Torin1 (n = 6); statistical analysis was performed using ANOVA with Dunnett’s multiple comparison test; **** adjusted P = 0.0001 as indicated. OSW-1 treatment abolishes p62 accumulation in NPC1 fibroblasts. Cells were subjected to the indicated treatments and immunoblotted for p62 and LC3B. The experiments were repeated twice.

c, OSBP depletion reduces p62 accumulation in NPC1-deficient cells obtained from patients with NPC. Cells were depleted of OSBP and analysed by immunoblotting; n represents number of biologically independent samples per genotype. Fold changes of LC3B-II to actin in NPC1<sup>+/+;</sup> NPC1<sup>−/−;</sup> OSW-1 –+ –– ++ ––+.

f, Across ER–lysosome contacts, OSBP–VAP generates a peripheral pool of lysosomal cholesterol that recruits and activates mTORC1 through the Rag GTPases. By contrast, NPC1 suppresses mTORC1 by limiting the levels of peripheral cholesterol. The steady-state levels of mTORC1 activation are therefore balanced by the opposite transport route of cholesterol. In cells lacking NPC1, cholesterol accumulates both in the lysosomal lumen and by unopposed ER-to-lysosome transport via OSBP on the lysosomal membrane. In turn, excess lysosomal-membrane cholesterol drives constitutively increased mTORC1 signalling and inhibits autophagy. PM, plasma membrane.
lysosomal limiting membrane for subsequent export, or directly cholesterol from NPC2, NPC1 may facilitate its insertion into the dependent manner, decreasing its levels to those of control cells prevented cholesterol that is liberated from LDL from accumulating verified that autophagic flux is reduced in

Npc1

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bolic and neurodegeneration research14,56. After accepting luminal uxes, the regulatory action of these junctions on mTORC1 signalling circuit that coordinately regulates cholesterol levels and mTORC1 activation at the lysosomal membrane (Fig. 8f). In concert with its activation at the lysosomal membrane (Fig. 1b,c), consistent with the immunoblotting results, intracellular p62 aggregates were largely cleared by treatment with OSW-1 and Torin1, and this effect was reversed by treatment with BafA1 (Fig. 8d,e). Together, these data demonstrate that inhibiting aberrant OSBP-driven mTORC1 signalling in NPC cells is sufficient to restore delivery of autophagic cargo to the lysosome and its subsequent degradation.

Discussion
Here we identify OSBP as the key component of a transport circuit that coordinate regulates cholesterol levels and mTORC1 activation at the lysosomal membrane (Fig. 8f). In concert with its ER membrane anchors, VAPA/B, OSBP delivers cholesterol in the ER-to-lysosome direction in exchange for PtdIns4P23,28. Cholesterol deposited on the lysosome by OSBP may directly interact with the mTORC1 scaffolding machinery that includes SLC38A9, Regulator and vacuolar H+-ATPase, leading to Rag GTPase activation and mTORC1 recruitment5,29,31 (Fig. 8f). Notably, OSBP was unique among VAP-interacting proteins in directly controlling cholesterol transport in response to physiological stimuli.

In yeast, intracellular transport of ergosterol has been implicated in mTORC1 regulation by nitrogen, as well as its inactivation under stress5,33. Through the transport action of OSBP, ER–lysosome contacts in metazoan cells emerge as signalling nodes in which information about cholesterol availability is integrated and translated into mTORC1-driven growth programs. As the majority of endolysosomes contact the ER over time scales of several minutes, the regulatory action of these junctions on mTORC1 signalling is expected to be pervasive and to couple the pace of cell growth to the levels of intracellular cholesterol12–15.

Our results provide evidence of how NPC1 regulates lysosomal cholesterol levels, a problem with important implications for metabolic and neurodegeneration research14,56. After accepting luminal cholesterol from NPC2, NPC1 may facilitate its insertion into the lysosomal limiting membrane for subsequent export, or directly hand it over to acceptors that remain unidentified12,13,37. How does cholesterol handling by NPC1 lead to mTORC1 inhibition? Recent evidence indicates that LDL-derived cholesterol is rapidly removed from the lysosome and transported to the plasma membrane in an NPC1-dependent manner24. Rapid NPC1-dependent export may prevent cholesterol that is liberated from LDL from accumulating at the lysosomal surface and interacting with the mTORC1 scaffolding complex. Subsequently, OSBP–VAP may return cholesterol to the lysosomal surface across ER–lysosome contacts in a controlled manner. Consistent with this model, exogenously delivered TopFluor-cholesterol requires OSBP to exit the ER and reach the lysosomal limiting membrane.

Alternatively, NPC1 may promote the lateral segregation of lysosomal-membrane cholesterol into domains analogous to the ones reported on the yeast vacuole, and which may not be accessible to the mTORC1 scaffolding complex29,30. Ultimately, mTORC1 activity results from the balance between the sterol-transport activities of OSBP–VAP and NPC1. Although we were unable to detect a direct interaction between OSBP and NPC1 in co-immunoprecipitation studies, we did find that NPC1 status affects the interaction between OSBP and VAPA/B. NPC1 may therefore exert feedback regulation on OSBP, possibly through its cholesterol transport activity. Further studies are required to shed light on the mechanistic basis for this regulation. More broadly, these data support the idea that ER–lysosome contacts are bona fide signalling structures that can modulate the rate of cholesterol transport in response to physiological stimuli. Moreover, given that neither NPC1 nor OSBP are required for acute mTORC1 response to amino acids’ (Supplementary Fig. 6g), ER–lysosome contacts are probably dedicated to the regulation of mTORC1 by cholesterol inputs.

By modulating the cholesterol content of the lysosomal limiting membrane, OSBP has a profound influence on the initiation and execution of autophagy. This was especially evident in cellular models of NPC, in which OSBP inhibition was sufficient to restore autophagic flux, leading to clearance of the canonical autophagic substrate, p62. Previous studies in cells obtained from patients have uncovered defects in both autophagosome–lysosome fusion and in the subsequent breakdown of autophagic substrates32,47,48,61. These defects can be explained, at least in part, by the observed accumulation of cholesterol on the lysosomal limiting membrane (Fig. 1b,c), which could lead to mistrafficking of lysosomes and compromise their ability to fuse with other organelles29,32,33. In the context of NPC, OSBP inhibition restores autophagic function, probably by reducing cholesterol build-up at the lysosomal membrane, which has the dual effect of enhancing autophagy initiation (by mTORC1 inhibition) and, potentially, restoring lysosomal trafficking and fusion with autophagosomes.

The availability of nanomolar OSBP inhibitors, such as OSW-133, suggests that chemical modulation of OSBP could be a promising strategy to correct lysosomal function and to restore proteostasis in NPC, as well as in diseases driven by excess mTORC1 signalling.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41556-019-0391-5.

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this disease29,47,48. Using a cleavable, dual-colour LC3 reporter44, we verified that autophagic flux is reduced in Npc1−/− MEFs compared with wild-type MEFs (Fig. 8a). Notably, OSBP inhibition using OSW-1 increased autophagic flux both in Npc1−/− and Npc1+/− cells with potency that was similar to that of the catalytic mTOR inhibitor, Torin1 (Fig. 8a).

NPC1-defective MEFs and fibroblasts obtained from patients showed pronounced accumulation of the autophagic adapter p62 (also known as SQSTM1), indicating disrupted autophagic degradation35. OSW-1 resolved p62 accumulation in a dose-dependent manner, decreasing its levels to those of control cells (Supplementary Fig. 8c,d), as did shRNA-mediated OSBP knockdown (Fig. 8c). OSW-1-induced clearance of p62 was prevented by the vacuolar H+-ATPase inhibitor, bafilomycin A1 (BafA1; Fig. 8b, Supplementary Fig. 8e). Importantly, mTORC1 inhibition by Torin1 was as effective as OSW-1 in clearing p62, and their combined treatment provided no additional benefit, suggesting that both compound acts through a mechanism converging on mTORC1 (Fig. 8b, Supplementary Fig. 8f).

Immunofluorescence for p62 in human patient fibroblasts showed considerable accumulation of intracellular aggregates in NPC1−/− cells that partially overlapped with LAMP2-positive lysosomes (Fig. 8d,e). Consistent with the immunoblotting results, intracellular p62 aggregates were largely cleared by treatment with OSW-1 and Torin1, and this effect was reversed by treatment with BafA1 (Fig. 8d,e). Together, these data demonstrate that inhibiting aberrant OSBP-driven mTORC1 signalling in NPC cells is sufficient to restore delivery of autophagic cargo to the lysosome and its subsequent degradation.
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Author contributions
C.-Y.L. and R.Z. conceived the study. C.-Y.L., O.B.D., H.R.S. and R.Z. designed experiments. C.-Y.L., H.R.S., O.B.D. and J.Z. performed experiments. X.J., C.A.B. and J.L.C. performed mass spectrometry measurements. D.K.N. and D.S.O. provided advice on experimental design and data analysis. C.-Y.L. and R.Z. wrote the manuscript. All of the authors reviewed and edited the manuscript.

Competing interests
R.Z. is a co-founder, consultant and stockholder of Frontier Medicines Corporation. D.K.N. is a co-founder, stockholder and scientific adviser for Artris Therapeutics and Frontier Medicines Corporation.

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15% fetal bovine serum. All of the cell lines were maintained at 37 °C and 5% CO2.

and 2 mM l-glutamine. Human NPC1WT (GM23151) and NPC1mut fibroblasts

0.5% lipid-depleted serum (LDS) for 2 h and, where indicated, were stimulated7

immunoblot analysis of ORP1L expression.

supernatants generated from lentiCRISPRv2 and selected with puromycin for 2 d.

Cholesterol starvation and stimulation.

0.5% MCD supplemented with

TRCN0000072243. Where indicated, a doxycycline-inducible shRNA system using

The pLKO.1 lentiviral vector (The RNAi

108, F359A, F360A, H522A/H523A, AELSK (deletion of residues 430–433) and K736A) were

Giantin C-terminal 3131–3259 amino acids to the C terminus of

acids was inserted into the N terminus of OSBP

targeted OSBP truncations, the coding sequence of p18 N-terminal 1–39 amino

ELSK (deletion of residues 430–433) and K736A) were

imaged using a spinning-disk confocal system built on a Nikon Eclipse Ti microscope with Andor Zyla-4.5 sCMOS camera.

Co-localization analysis. For quantification of cells with the phenotype of interest, 20–25 non-overlapping whole-field images were randomly acquired throughout the slice of each sample. All of the RAW images were exported from the Andor iQ3 imaging software v.3.3.1 (Andor Technology, Oxford Instruments). Fiji v1.5.1j (ImageJ, NIH) was used to quantify the fluorescence intensity (https://fiji.sc).

To determine the co-localization of mTOR, OSBP or p62 with LAMP-4 fluorescent reporter (either GFP-PAM or mCherry-PAM) were seeded on 35 mm glass-bottom dishes (MitraTek, P35GC-1.5-14-C), allowed to attach overnight and imaged using a spinning-disk microscope. Before imaging, cells were transferred to imaging buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.2 mM MgCl2, 25 mM HEPES and 5 mM d-glucose with pH adjusted to 7.4 with NaOH.

Lysosome immunoprecipitation. Lysosomes carrying Tmem192–mRFP–3HA were purified as described previously16,17. Confluent HEK293T cells stably expressing Tmem192–mRFP–3HA in a 15 cm culture dish were scraped in chilled PBS and collected by centrifugation at 300g for 10 min at 4 °C. Cells were resuspended, lysed using 2× Cell Lysis reagent (Bio-Rad, 63105), and loaded into ultracentrifugation chamber according to the manufacturer’s instructions. Target cells were plated in 6-well plates with 8 µg/mL polybrene (Millipore, TR-1003-G) and incubated with virus-containing medium for 24 h. After removal of virus, cells were supplemented with fresh medium containing 2 µg/mL puromycin (Calbiochem, 54011). Protein expression was confirmed by immunoblotting and fluorescence microscopy.

To generate ORP1L knockout cells, guide RNA, a sequence targeting human OSBPL1A exon 2 (AATCCATCTACCTTGGCTCA), was cloned into the lentivirus vector (Addgene, 52961). HEK293T cells were infected with viral supernatants generated from lentivirus vector and selected with puromycin for 2 d. Clonal lines were subsequently performed by limiting dilution, and validated by immunoblot analysis of ORP1L expression.

Cholesterol starvation and stimulation.

HEK293T cells in culture dishes were rinsed with serum-free media and incubated in 0.5% MCD supplemented with 0.5% lipid-depleted serum (LDS) for 2 h and, where indicated, were stimulated

with DMEM supplemented with 0.1% MCD and 0.5% LDS and 50 µM cholesterol for 2 h. LDS was prepared as previously described9.

Cell lysis and immunoprecipitation. Cells were rinsed with cold PBS and lysed in lysis buffer (1% Triton X-100, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 4 mM EDTA, 40 mM HEPES, pH 7.4, and 1 EDTA-free protease inhibitor tablet per 50 ml). Cell lysates were cleared by centrifugation in a microcentrifuge at 17,000g for 10 min at 4 °C. Cell lysate samples were prepared by addition of 5x sample buffer, heated at 70 °C for 10 min, resolved by either 10% or 12% SDS–PAGE gels, and analysed by immunoblotting.

For Flag immunoprecipitations, 25 µl of a well-mixed 50% slurry of anti-Flag M2 Affinity Gel (Sigma, A2220) was added to each lysate (1 mg/mL) and incubated at 4 °C in a shaker for 2 h. The immunoprecipitates were washed five times, twice with lysis buffer and three times with lysis buffer with 50 µM NaCl. Immunoprecipitated proteins were denatured by adding 50 µl of 2× urea sample buffer and heating to 37 °C for 15 min, resolved by SDS–PAGE and analysed by immunoblotting.

Immunofluorescence. HEK293T or H1299 cells were seeded on fibronectin-coated glass coverslips in 6-well plates (35 mm diameter well per 3000–5000 cells per well and allowed to attach overnight. The next day, cells were subjected to steroid depletion and restimulation, where indicated, and fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. The coverslips were rinsed twice with PBS and cells were permeabilized with 0.1% (w/v) saponin in PBS for 10 min. After the slides were washed with PBS, the slides were incubated with primary antibodies in 5% normal donkey serum (Jackson Immunoresearch, 017-000-121) for 1 h at room temperature, rinsed four times with PBS and incubated with fluorophore-conjugated secondary antibodies derived from goat or donkey (Life Technologies, diluted 1:400 in 5% normal donkey serum, PBS) for 45 min at room temperature in the dark. After washing four times with PBS and one time with deionized water, the coverslips were mounted in glass coverslips using Fluoromount-G (SouthernBiotech, 0100-01) and imaged using a spinning disk confocal system built on a Nikon Eclipse Ti microscope with Andor Zyla-4.5 sCMOS camera.

Live-cell imaging of PtdIns4P distribution.

Cells expressing the PtdIns4P fluorescent reporter (either GFP-PAM or mCherry-PAM) were seeded on 35 mm glass-bottom dishes (MitraTek, P35GC-1.5-14-C), allowed to attach overnight and imaged using a spinning disk microscope. Before imaging, cells were transferred to imaging buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.2 mM MgCl2, 25 mM HEPES and 5 mM d-glucose with pH adjusted to 7.4 with NaOH.
sample was prepared by pooling the aliquots of the study samples and was used to monitor the instrument stability. The QC was injected six times in the beginning to stabilize the instrument and was injected every four study samples to monitor the instrument performance. The data were accepted if the coefficient variance of cholesterol in the QC sample was <15%. The data were reported as the peak area ratio of cholesterol to d7-cholesterol.

Whole-cell lipidome analysis. HEK293T cells cultured in 6-well plates were depleted of OSBP by doxycycline-induced shRNA, washed twice with PBS, collected by scraping and collected by bench-top centrifugation at 1,000g at 4°C, and cell pellets were flash-frozen and stored at −80°C until metabolome extraction. Lipid metabolites were extracted in 4 ml of a 2:1:1 mixture of chloroform:methanol:Tris buffer with inclusion of internal standards C12:0, 1-dodecylglycerol (10 nmol) and pentadecanoic acid (10 nmol). Organic and aqueous layers were separated by centrifugation at 1,000g for 5 min, and the organic layer was collected. The aqueous layer was acidified (for metabolites such as LPA) by adding 0.1% formic acid, followed by the addition of 2 ml of chloroform. The mixture was vortexed and the organic layers were combined, dried down under liquid nitrogen and dissolved in 120 μl of chloroform, of which 10 μl was analysed by both single-reaction monitoring (SRM)-based liquid chromatography with tandem mass spectrometry (LC–MS/MS) or untargeted LC–MS. LC separation was achieved using a Luna reverse-phase C5 column (50 mm x 4.6 mm with 5 μm diameter particles, Phenomenex). Mobile phase A was composed of a 95:5 ratio of water:methanol, and mobile phase B consisted of 2-propanol, methanol, and water at a ratio of 60:35:5. Solvent modifiers 0.1% formic acid with 5 mM ammonium formate and 0.1% ammonium hydroxide were used to assist ion formation as well as to improve the LC resolution in both positive and negative ionization modes, respectively. The flow rate for each run started at 0.1 ml min⁻¹ for 5 min, to alleviate backpressure associated with injection of chloroform. The gradient started at 0% B and increased linearly to 100% B over the course of 45 min with a flow rate of 0.4 ml min⁻¹, followed by an isocratic gradient of 100% B for 17 min at 0.5 ml min⁻¹ before equilibrating for 8 min at 0% B with a flow rate of 0.5 ml min⁻¹.

Mass spectrometry analysis was performed with an electrospray ionization (ESI) source using an Agilent 6430 QQQ LC–MS/MS. The capillary voltage was set to 3.0 kV, and the fragmentor voltage was set to 100 V. The drying gas temperature was 350°C, the drying gas flow rate was 10 l min⁻¹, and the fragmentor voltage was set to 100 V. The drying gas temperature was 50°C, and the backpressure associated with injection of chloroform. The gradient started at 0% B, and increased linearly to 100% B over the course of 45 min with a flow rate of 0.4 ml min⁻¹, followed by an isocratic gradient of 100% B for 17 min at 0.5 ml min⁻¹ before equilibrating for 8 min at 0% B with a flow rate of 0.5 ml min⁻¹.

Statistics and reproducibility. Data were expressed as mean ± s.d. where indicated in the figure legends. Statistical analyses were performed using unpaired two-tailed Student's t-tests or one-way ANOVA for group comparisons, where indicated, using GraphPad Prism v.7.0a (GraphPad Software). For box plots, the upper and lower edges of the box indicate the first and third quartiles (25th and 75th percentiles) of the data, the middle line indicates the median and the whiskers extend to the minimum and maximum. The levels of statistical significance are indicated by asterisks, and the exact P values are indicated in each figure legend along with the statistical tests. Experiments in Fig. 8c and Supplementary Figs. 4c and 6b were performed once. All of the other experiments reported here were performed at least two to three times independently and all attempts at replication were successful with similar results.

Data availability
Mass spectrometry data that support the findings of this study are provided in Supplementary Table 1 and have been deposited in ProteomeXchange with accession number PXD014733. Statistics source data for Figs. 1–4 and 6–8 and Supplementary Figs. 1–6 are provided in Supplementary Table 2. All data supporting the findings of this study are available from the corresponding author on reasonable request.

References
Goldstein, J. L., Basu, S. K. & Brown, M. S. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. Methods Enzymol. 98, 241–260 (1983).
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐ | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ | A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐ | The statistical test(s) used AND whether they are one- or two-sided
☐ | Only common tests should be described solely by name; describe more complex techniques in the Methods section.
☐ | A description of all covariates tested
☐ | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐ | A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐ | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted Give P values as exact values whenever possible.
☐ | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ | Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

RAW Images were acquired with the Andor IQ3 Imaging Software version 3.3.1 (Andor Technology, Oxford Instruments) on a spinning disk confocal system built upon a Nikon Eclipse Ti microscope with Andor Zyla-4.5 sCMOS camera.

Data analysis

Acquired images were processed using the Fiji Software version 1.51j (images, NIH) to quantify the fluorescence intensity as detailed in Methods. Data of lysosomal cholesterol measured by mass spectrometry were processed using Xcalibur™ Software version 4.0 (Thermo Fisher Scientific). Statistical analysis was performed using GraphPad Prism version 7.0a (GraphPad Software, Inc.).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Mass spectrometry data are provided in Supplementary Table 1 in Excel format and have been deposited in ProteomeXchange with the primary accession code [ ] .

Statistics source data for Figs. 1–4, 6–8 and Supplementary Figs. 1–6 are provided in Supplementary Table 2 and unprocessed images of all blots are provided in Supplementary Figure 9.

All other data supporting the findings of this study are available from the corresponding author on reasonable request.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For immunofluorescence experiments, sample size was selected based on commonly adopted standards in the field, resulting in statistically meaningful comparisons.

Data exclusions

No data were excluded from the analysis.

Replication

Experiments reported here were performed at least two times and all attempts at replication were successful with similar results except experiments in Fig. 8c, Suppl. Fig 4c and 6h, which were performed once.

Randomization

No randomization was applicable. All the samples were prepared with known composition.

Blinding

For the whole-cell and organelle lipidomics, the investigators who performed the mass spectrometry analysis were blinded to the identities of the samples.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | Antibodies           |
|     | Eukaryotic cell lines|
|     | Palaeontology        |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data        |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChIP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging|

Antibodies

Antibodies used

Please see Antibodies Information in Supplementary Table 3.

Validation

We included the validation information for each primary antibodies in Supplementary Table 3. We further verified the critical antibodies used in this study via immunoblotting, including those against OSBP, ORIP8, ORIP11, ORIP5, VAPA, VAPB, NPC1, ORIP1L, STARD3, to ensure that there was no signal of immunobots for the antibody in cells lacking the expression of the protein of interest via lentiviral shRNA or CRISPR/Cas9 genome editing. In the case of ‘knockdown and rescue’ experiments where indicated, OSBP was stably expressed with a double tag [FLAG-GFP], resulting in a shift of molecular weight that suffices to reveal the relative expression of the rescued proteins and the knockdown efficiency of the endogenous counterparts, as examined in immunobots using antibodies that recognize the native proteins and the tagged proteins, respectively.

Eukaryotic cell lines

Policy information about cell lines

HEK-293T cells were obtained from ATCC [https://www.atcc.org/]. HEK-293A cells were acquired from the Cell Culture Facility, UC Berkeley. HEK-293 NPC1-null cells were generated in-house via CRISPR/Cas9 genome editing. HEK-2937 NPRL3-null and KPTN-null lines were obtained from the laboratory of David Sabatini. Npc1+/− and Npc1−/− MEFs were a gift of Sovan Sarkar. Human NPC1 control (GM23151) and NPC1 mutant-i1061T fibroblasts (GM03123) were obtained from Coriell Cell Repositories in full compliance with the Office for Human Research Protections, Department of Health and Human Services (DHHS) regulations. The statement of research intent and the signed MTA were submitted and approved by the Coriell Institutional Review Board for use of patient cells in this study. This study did not involve human research participants.
**Authentication**

HEK293T was verified by ATCC (https://www.atcc.org/en/Products/All/CRL-3216.aspx) via Short Tandem Repeat (STR) profiling. NPC1 mutant-1061T fibroblasts (GM03123) and NPC1 control fibroblasts (GM23151) were confirmed by Coriell Cell Repositories via assay of sphingomyelin phosphodiesterase enzyme activity. HEK-293A, HEK-293T genome-edited cells and Npc1 MEFs have been reported in the literature and were not subjected to cell-line authentication tests in the laboratory.

**Mycoplasma contamination**

All cell lines used in this study were tested negative for mycoplasma contamination.

**Commonly misidentified lines**

(See ICLAC register)

No ICLAC cell lines were used in this study.