Coupling between endocytosis and sphingosine kinase 1 recruitment

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Genetic studies have suggested a functional link between cholesterol/sphingolipid metabolism and endocytic membrane traffic. Here we show that perturbing the cholesterol/sphingomyelin balance in the plasma membrane results in the massive formation of clusters of narrow endocytic tubular invaginations positive for N-BAR proteins. These tubules are intensely positive for sphingosine kinase 1 (SPHK1). SPHK1 is also targeted to physiologically occurring early endocytic intermediates, and is highly enriched in nerve terminals, which are cellular compartments specialized for exo/endocytosis. Membrane recruitment of SPHK1 involves a direct, curvature-sensitive interaction with the lipid bilayer mediated by a hydrophobic patch on the enzyme’s surface. The knockdown of SPHKs results in endocytic recycling defects, and a mutation that disrupts the hydrophobic patch of Caenorhabditis elegans SPHK fails to rescue the neurotransmission defects in loss-of-function mutants of this enzyme. Our studies support a role for sphingosine phosphorylation in endocytic membrane trafficking beyond the established function of sphingosine-1-phosphate in intercellular signalling.

Endocytosis is a highly regulated membrane remodelling process that critically depends on the coordination of endocytic factors, their interaction with membrane lipids and metabolic changes in the bilayer. In particular, a proper balance between sterols and sphingolipids within the plasma membrane is required to maintain plasma membrane homeostasis and to support endocytosis. Sterols, which are intercalated among the acyl chains of phospholipids, help modulate membrane rigidity. Sphingolipids, a class of lipids that use sphingoid bases as the backbone and that interact with sterols, also serve as structural components of membranes and help modulate their physical properties1,5.

The involvement of sterols and sphingolipids in normal endocytic membrane trafficking is supported by many lines of evidence. Pharmacological perturbations of cholesterol by sequestration with filipin, or extraction with methyl-β-cyclodextrin (MβCD), impair several endocytic pathways3–5. In yeast, genetic perturbations of the metabolism of ergosterol, the yeast sterol, impair endocytosis4. Cholesterol has also been implicated in synaptic vesicle recycling7,8, a process that critically relies on clathrin-mediated endocytosis.

Similarly, genetic studies revealed that sphingoid bases are required for endocytosis in yeast9, and mutations in enzymes of sphingolipid metabolism produce endocytic membrane traffic defects in metazoa. For instance, mutations in neutral ceramidase and in sphingosine kinase are responsible for defects in synaptic vesicle recycling at the neuromuscular junction in Drosophila melanogaster9 and Caenorhabditis elegans10,11, respectively. In addition, enzymes that regulate ceramide and sphingoid base homeostasis in flies are critical for the endocytic trafficking of rhodopsin and their absence results in the degeneration of photoreceptor cells12,13.

Beyond a generic impact of perturbations of cholesterol and sphingolipid metabolism on endocytosis, a more specific genetic connection emerged in yeast between these lipids and the endocytic N-BAR domain-containing proteins, RVS161 and RVS167. N-BAR domains are membrane-binding modules that can generate and sense bilayer curvature14–16. RVS161 and RVS167, and their metazoan homologues amphiphysin and endophilin, coordinate constriction of endocytic necks with other events required for the endocytic reaction17–21. Forward genetic screens for suppressors of rvs161 or...
rvs167 mutants identified mutations in enzymes of sphingolipid metabolism\textsuperscript{22,23}. Subsequently, large-scale epistatic miniarray profile studies extended these results by identifying additional enzymes of both sphingolipid and ergosterol metabolism that genetically interact with N-BAR proteins\textsuperscript{24}.

The goal of this study was to shed light on the mechanisms underlying the genetic link between sterol and sphingolipid metabolism and endocytic membrane traffic. We started by exploring the impact on N-BAR proteins of perturbing the cholesterol/sphingomyelin balance in the plasma membrane. These manipulations resulted in a robust redistribution of N-BAR proteins that correlates with the formation of massive narrow tubular plasma membrane invaginations. Strikingly, an enzyme of sphingolipid metabolism, SPHK1, was enriched on these membranes through a direct, curvature-sensitive interaction. We further observed that SPHK1 is enriched on physiologically occurring early endocytic intermediates and that defective SPHK function impairs endocytic recycling, pointing to a role for sphingosine phosphorylation in endocytosis.

RESULTS
Acute perturbation of cholesterol or sphingomyelin induces massive N-BAR protein-positive endocytic tubular invaginations

Acute cholesterol extraction from cells with MβCD results in the perturbation of clathrin-mediated endocytosis accompanied by formation of shallow clathrin-coated pits\textsuperscript{3,4}. To monitor the dynamics of this effect, we examined live cells expressing GFP-clathrin light chain (CLC) and endophilin-2–Ruby by total internal reflection fluorescence (TIRF) microscopy (Fig. 1a–c). Endophilin is an endocytic adaptor recruited at the necks of late stage endocytic clathrin-coated pits, where it coordinates acquisition of bilayer curvature (through its BAR domain) with the recruitment of dynamin and synaptojanin (through its SH3 domain)\textsuperscript{18–20}, two factors required for fission and uncoating respectively.

Within minutes of MβCD treatment, a slower dynamics of the GFP–CLC spots (coated pits) was observed, consistent with a delay in the maturation of the pits (Fig. 1b). A corresponding modest increase in pit number was also detected (Fig. 1b). Surprisingly, a pronounced change in the localization and dynamics of endophilin-2–Ruby was also noted. In control cells, endophilin-2–Ruby accumulated transiently at hotspots (Fig. 1c, left), many of which represented late stage clathrin-coated pits. Within minutes of MβCD addition, endophilin-2–Ruby relocated and clustered at multiple large foci that were up to 1 μm in size and did not coincide with clathrin spots (Fig. 1c, right). Similar results were observed with endophilin-2–GFP (Fig. 1d and Supplementary Video 1). These foci appeared and disappeared in a dynamic fashion within minutes (Fig. 1e,f), but the rate of their formation progressively decayed until no new ones formed. The N-BAR domain of endophilin 2 fused to GFP (endo2ΔSH3–GFP) had a similar response to MβCD (Supplementary Fig. 1a), indicating that the SH3 domain is dispensable for this localization. Confocal images of middle sections of MβCD-treated cells revealed that large endophilin foci occurred exclusively near the cortical region (Fig. 1g). Analysis of cellular free cholesterol at 5 min after MβCD addition, that is, when most of the endophilin foci had formed, showed an approximately 30% reduction (Fig. 1h), in agreement with previous reports\textsuperscript{25}.

These effects of MβCD were robustly observed in multiple cell lines (Supplementary Fig. 1b,c) and were not an artefact due to endophilin overexpression, because endogenous endophilin 2 immunoreactivity underwent a similar redistribution (Supplementary Fig. 1d). An N-BAR domain-containing protein similar to endophilin, amphiphysin 2–GFP, co-clustered with endophilin-2–Ruby (Supplementary Fig. 1e) and accumulated into similar foci when expressed in endophilin null cells (mouse embryonic fibroblasts (MEFs) from endophilin triple knockout mice\textsuperscript{20}; Supplementary Fig. 1f). Thus, endophilin marks the formation of structures induced by cholesterol depletion, but is not needed for their formation.

MβCD extracts fatty acids and amphipathic molecules from the cells in addition to cholesterol\textsuperscript{25}, and thus could cause effects independent of cholesterol. This possibility was excluded, as cholesterol add-back manipulations using cholesterol–MβCD complexes rescued the phenotype (Supplementary Fig. 1g). Moreover, a milder cholesterol extraction reagent, hydroxypropyl-β-cyclodextrin (HPβCD), resulted in a similar redistribution of endophilin (Supplementary Fig. 1h).

When the plasma membrane was prelabelled with the DiI lipid dye before MβCD treatment, the large endophilin foci were strongly positive for this dye (Fig. 1i), suggesting that they are sites of plasma membrane invaginations. Endophilin foci were also positive for chemical (TopFluor-PS; ref. 27) and genetically encoded (C2 domain of lactadherin–GFP; ref. 28) PtdSer probes (Supplementary Fig. 2a,b), consistent with the abundance of PtdSer in the plasma membrane. However, they were negative for makers of PtdIns(4,5)P\textsubscript{2} (the genetically encoded probe PH\textsuperscript{PLC\_2} and an anti-PtdIns(4,5)P\textsubscript{2} antibody; Supplementary Fig. 2c,d), a phosphoinositide typically rapidly removed from membranes on endocytosis, and for a marker of PtdIns(3)P (the genetically encoded probe FYVE\textsuperscript{Hrs}; Supplementary Fig. 2e), a signature phospholipid of early endosomes. Thus, they have the properties of very early endocytic intermediates.

The structure of the endophilin foci was investigated by electron microscopy. This analysis revealed in MβCD-treated cells clusters of densely packed intertwined narrow membrane tubules (Fig. 1j), which in electron microscopy tomograms seemed to originate from the plasma membrane (Supplementary Video 2). Their diameter was homogeneous (23.2 nm ± 4.7 nm; Fig. 1k) and in the range of typical endophilin- and amphiphysin-coated tubules\textsuperscript{29,30}. Importantly, anti-GFP immunogold electron microscopy labelling of endophilin-2–GFP-expressing cells revealed intense immunoreactivity on the tubular clusters, confirming their correspondence to the endophilin foci detected by immunofluorescence (Fig. 1l).

Formation of massive tubular plasma membrane invaginations should result in a decrease of the outer cell surface. Accordingly, scanning electron microscopy of MEFs revealed a reduction of plasma membrane ruffles and filopodia on MβCD treatment (Supplementary Fig. 2f), that is, as expected from a reduction of the surface-to-volume ratio. The footprint of the cells was also reduced (Fig. 1m and Supplementary Fig. 2g) and the time course of this reduction correlated with the increase of the area occupied by endophilin foci in fluorescence images (Fig. 1n).

As formation of the clusters of membrane tubules was not dependent on endophilin (see above), endophilin may be recruited to the tubules through its bilayer-curvature-sensing properties.
Figure 1  Acute perturbation of plasma membrane cholesterol induces massive endocytic tubular invaginations positive for N-BAR proteins. (a) TIRF images of a COS-7 cell expressing GFP–CLC before (control, left) and after (+MjCD, right) 10 mM MjCD treatment at 37°C. (b) Statistical analysis of tracked clathrin-coated pits before (Ctrl) and after (+MjCD) MjCD treatment (pooled results from three independent experiments). Mean lifetime: n = 158 (Ctrl) and 211 (+MjCD) pits. Mean fluorescence intensity: n = 330 (Ctrl) and 270 (+MjCD) pits. Error bars: standard error of the mean. ***P < 0.0001, Student’s t-test. Mean clathrin-coated pits number: n = 3 (Ctrl) and 3 (+MjCD) cells. (c) TIRF images of a COS-7 cell expressing GFP–CLC and endophilin-2–Ruby before (Control, left) and after (+MjCD, right) 10 mM MjCD treatment at 37°C. (d) Confocal images of HeLa cells expressing endophilin-2–GFP before (top) and after (bottom) MjCD treatment. (e,f) Selected frames from a time series of the endophilin-2–GFP fluorescence from the cell shown in d. (g) Confocal image of a middle section of a HeLa cell expressing endophilin-2–GFP after MjCD treatment. (h) Measurement of cellular free cholesterol before and after MjCD treatment. n = 6 dishes. Data are pooled from two independent experiments. Error bars: standard error of the mean. (i) DiI staining of a cell expressing endophilin-2–GFP and treated with MjCD. (j) Transmission electron micrograph of a tubular membrane cluster formed during MjCD treatment. (k) Histogram of tubule diameter. n = 61 tubules. (l) Anti-GFP immunogold labelling of a cell expressing endophilin-2–GFP and treated with MjCD. (m) Average change of the footprint of cells (green) and of the percentage areas (relative to the original footprints) occupied by endophilin 2 foci (red) during MjCD treatment. n = 7 cells. Data are pooled from 7 independent experiments. Error bars: standard error of the mean. Scale bars: 3 μm in a,c and e; 5 μm in i; 10 μm in d and g; 200 nm in j and l.
Alternatively, endophilin may participate in their formation, but this role may be overlapping, and thus not essential, with the role of other N-BAR proteins, such as amphiphysin (see above). Clathrin and dynamin were also dispensable for tubule formation, as endophilin foci occurred in cells where levels of clathrin heavy chain had been markedly reduced by RNA-mediated interference (RNAi) and in cells lacking dynamin (dynamin triple knockout MEFs (ref. 31); Supplementary Fig. 2h). In fact, endophilin foci formation in response to MβCD treatment was enhanced in cells lacking dynamin relative to control cells (Supplementary Fig. 2i), indicating that MβCD does not act by inhibiting dynamin-dependent membrane fission. Dynamin was concentrated at endophilin–GFP-positive foci through an SH3-dependent interaction (Supplementary Fig. 2j); such accumulation did not occur with the overexpression of endo2ΔSH3–GFP (Supplementary Fig. 2j)), yet did not prevent tubule formation.

Cholesterol and sphingomyelin interact within the plasma membrane, and help define its physical properties32. We explored whether perturbing this partnership by hydrolysing sphingomyelin, rather than by depleting cholesterol, also results in the tubular invaginations. Addition of purified bacterial sphingomyelinase (SMase) to endophilin–GFP-expressing cells produced a redistribution of GFP fluorescence similar to that produced by MβCD treatment and with a similar time course (Fig. 2a). Likewise, electron microscopy revealed also in these cells the presence of clusters of small tubules (Fig. 2b).

The sphingoid-base-modifying enzyme, SPHK1, is recruited to the tubular endocytic invaginations

In view of the strong genetic evidence linking sphingolipid metabolism to endocytic events, and in particular to the action of endocytic N-BAR proteins24 (Supplementary Fig. 3a), we explored the potential presence of sphingolipid-modulating enzymes on the tubular endocytic intermediates generated by the above-mentioned treatments that alter the cholesterol/sphingolipid balance.

We examined the localization of GFP fusions of several enzymes known to regulate sphingoid base levels (Fig. 3a and Supplementary Fig. 3b,c): neutral sphingomyelinase (SMPD3), neutral ceramidase (ASAH2), ceramide kinase (CerK), SPHK1, sphingosine-1-phosphate phosphatase 1 (SGPP1), sphingosine-1-phosphate lyase (SGPL1) and ceramide synthase 1 (CerS1). Strikingly, SPHK1, but not the other enzymes, was strongly enriched at the tubules induced by MβCD (Fig. 3b) or SMase treatment (Fig. 3c), where it co-localized with endophilin (Fig. 3f) or sphingomyelin reflects a coupling of SPHK1 function to early endocytic intermediates that alter the cholesterol/sphingolipid balance.

Live-cell imaging revealed that recruitment of SPHK1 lagged slightly behind formation of endophilin-positive foci (Fig. 3f,g). However, SPHK1 accumulated at tubule clusters in a manner independent of endophilin, as SPHK1 persisted at these foci after the loss of endophilin (Fig. 3f) and SPHK1 foci still formed in endophilin triple knockout cells (Supplementary Fig. 3e). Accordingly, no physical interaction between the two proteins was observed. Fluorescent sphingomyelin metabolites, BODIPY-TR-ceramide and BODIPY–sphingosine, accumulated at tubule clusters (Fig. 3h), raising the possibility that the corresponding endogenous metabolites may facilitate SPHK1 recruitment. SPHK1-positive foci eventually disassembled through the emergence from them of SPHK1-positive filament-like structures (most likely bundles of tubules; Fig. 3i).

Recruitment of SPHK1 to physiologically occurring endocytic intermediates

SPHK1 was shown to have a primarily cytosolic localization and to be recruited to the plasma membrane in response to pharmacological stimuli34,35. SPHK1 was also reported to be localized on endosomes30 and phagosomes37,38. Our results shown above, that is, the recruitment of SPHK1 to ‘exaggerated’ early endocytic intermediates induced by experimental perturbations, support the possibility that physiologically occurring early endocytic structures represent one of its sites of action. We revisited the subcellular targeting of SPHK1 under control conditions using SPHK1–GFP and observed an enrichment of this protein both on Rab5-positive early endosomes (Fig. 4a) and on even earlier endocytic intermediates. For example, SPHK1 was present at a subset (62%) of endocytic clathrin-coated pits (Fig. 4b,c), and at such pits the SPHK1–GFP signal peaked at late stages, when the clathrin signal had already started to decay (Fig. 4d,e). As the clathrin signal dims in parallel with the endophilin signal19,39, even at these physiologically occurring sites SPHK1 recruitment lagged behind the endophilin recruitment, as observed at MβCD-induced endophilin foci. In addition, SPHK1 was also recruited to macropinosomes induced by PDGF stimulation (Fig. 4f) and neighbouring membranes.

A function of SPHK1 in the early endocytic system was supported by loss-of-function experiments in HeLa cells. As shown in Fig. 4g, RNAi-induced knockdown of SPHK1 and SPHK2 (Supplementary Fig. 4) resulted in an accumulation of internalized transferrin relative to controls, both in a single-round assay (uptake and release of prebound transferrin) and in a multiple-rounds assay (uptake and release in the continued presence of transferrin), indicating a defect in recycling. An accumulation of internalized transferrin on SPHK1 knockdown was also previously observed in a genome-wide RNAi screen in HeLa cells40.

These observations suggest that the accumulation of SPHK1 on the tubular structures produced by depletion of cholesterol or sphingomyelin reflects a coupling of SPHK1 function to early endocytic events that occurs under physiological conditions.

SPHK1 directly binds to negatively charged membranes with a preference for high positive curvature

Previous studies suggested that both protein–protein41,42 and protein–lipid interactions43,44 contribute to the membrane recruitment of SPHK1, a cytosolic enzyme. Given the strong enrichment of SPHK1...
on narrow tubular membranes, we tested the possibility that such recruitment may be mediated by direct bilayer binding and positive-curvature-sensing properties.

Carboxy-terminally tagged human SPHK1 (either SPHK1–FLAG or SPHK1–GFP–FLAG) was purified to near homogeneity from extracts of transfected Expi293T cells by anti-FLAG affinity chromatography followed by size-exclusion chromatography (Fig. 5f and Supplementary Fig. 5a). The purified protein was enzymatically active as shown by an in vitro kinase assay involving sphingosine and [32P]ATP as substrates, which revealed an activity of approximately 106 pmol−1 mg−1 min−1 (Supplementary Fig. 5b), consistent with previous reports45. SPHK1–FLAG bound to liposomes containing negatively charged lipids (PS or an acidic lipids mixture), but not to liposomes containing only neutral lipids (Fig. 5a). Moreover, when SPHK1–GFP–FLAG was tested for binding to giant unilamellar vesicles (GUVs, labelled by a lipid dye) from which narrow tubules had been pulled, it became selectively enriched relative to lipids on the narrow tubules (Fig. 5b), although it did not change the membrane curvature (Supplementary Fig. 5c), suggesting that SPHK1 senses, but does not generate, high bilayer curvature (Fig. 5c) under the experimental conditions tested.

The recently published crystal structure of human SPHK1 (ref. 46) provided an opportunity to predict potential binding interfaces. SPHK1 is represented nearly exclusively by the kinase module, which is characterized by an amino-terminal ATP-binding lobe (blue in Fig. 5d, left) and a C-terminal substrate-recognition lobe (magenta in Fig. 5d, left). Inspection of surface hydrophobicity demonstrates an evolutionarily conserved hydrophobic patch in the substrate-recognition lobe (Fig. 5d, middle). The patch corresponds to one of the two helices that were proposed to function as a gate to control the flux of the substrate sphingosine into the interior of the protein (Fig. 5c). This may explain why the L194Q mutation (Fig. 5e) in this patch was previously shown to impair catalytic function41, although Leu 194 is not part of the catalytic site. This result had previously been attributed to misfolding of the mutant protein41, but we did not find evidence for this possibility based on circular dichroism spectroscopy of purified SPHK1L194Q (Fig. 5f,g). A plausible explanation is that the L194Q mutation may impair access to the substrate. As this hydrophobic patch is surrounded by some positively charged residues (Fig. 5d, right), it may represent a lipid bilayer-binding interface with hydrophobic residues partially penetrating the membrane. This interpretation was supported by the diffuse cytosolic distribution of SPHK1L194Q and of a SPHK1 mutant that harbours two other mutations in the same hydrophobic patch, SPHK1V268Q,L194Q (Fig. 5i). Both mutants folded correctly (Fig. 5g), but failed to bind to acidic liposomes in vitro (Fig. 5h) and were not recruited to the tubular membrane invaginations induced by cholesterol extraction in living cells (Fig. 5i).

**A SPHK1 mutant that does not localize to tubular invaginations is non-functional in vivo**

To determine whether the properties that mediate the recruitment of SPHK1 to the tubular invaginations have a physiological significance, we next investigated whether perturbation of the hydrophobic patch rescues the absence of SPHK1 in a functional assay. In neurons, SPHK1 is preferentially targeted to nerve terminals, a compartment highly specialized for the endocytic recycling of synaptic vesicles, as shown by immunocytochemistry47 and by the accumulation of SPHK1–GFP in presynaptic terminals of cortical neurons in vitro47 (and Fig. 6a), or of retinal photoreceptors in vivo (Fig. 6b). The single SPHK1 in C. elegans, cSPHK-1, is also concentrated at synaptic sites along axons, and its absence results in lower sensitivity to aldarcid, indicating a defect in neurotransmitter secretion10,48. Interestingly, cSPHK-1 is primarily localized at periactive zones of synapses, which are sites of high endocytic activity10. In cSPHK-1, Leu 194 of the hydrophobic patch is replaced by another hydrophobic amino acid, Val 268. Supporting a critical importance of the patch, cSPHK-1V268Q–GFP did not exhibit the same highly punctate localization as cSPHKWT–GFP in axons (Fig. 6c,d). Importantly, whereas cSPHK–1WT–GFP (Fig. 6e) and even human SPHK1 (Supplementary Fig. 6) rescued neurotransmission in cSPHK–1 mutant worms, cSPHK–1V268Q–GFP did not (Fig. 6e).

**DISCUSSION**

Our study shows that lowering cholesterol or sphingomyelin levels in the plasma membrane results in its marked remodelling, with
Figure 3 The sphingoid-base-modifying enzyme, SPHK1, is recruited to the tubular endocytic invaginations. (a) Sphingolipid metabolic pathway. SPTLC, serine palmitoyltransferase; SPHK, sphingosine kinase; SGPP, sphingosine-1-phosphate phosphatase; SGPL1, sphingosine-1-phosphate lyase 1; CerS, ceramide synthase; CDase, ceramidase; SMS, sphingomyelin synthase; SMase, sphingomyelinase; CerK, ceramide kinase. (b,c) GFP-tagged SPHK1 co-segregates into the clusters positive for Ruby-tagged endophilin 2 in cells treated with MβCD in b or treated with SMase in c. (d,e) Concentration of both SPHK1 and endophilin 2 on the endocytic tubular intermediates induced by MβCD treatment in a cell transfected with SPHK1–GFP alone in d or both SPHK1–HA and endophilin-2–GFP in e. Single or dual immunogold labelling with 15 nm gold particles for anti-GFP in c and e and 10 nm gold particles for anti-HA in e. (f) Selected frames from a time series of a cluster co-labelled for SPHK1–GFP and endophilin-2–Ruby (left). Fluorescence intensity of endophilin 2 (red) and SPHK1 (green) positive tubule clusters (n = 15 foci from 6 different cells) showing that recruitment of endophilin precedes SPHK1 (right). Pooled data from 6 independent experiments. Error bars: standard errors of the mean. (g) Selected frames from a time series of a tubule cluster co-labelled by SPHK1–GFP and dynamin 2–RFP (left). Fluorescence intensity of dynamin 2 (red) and SPHK1 (green) positive tubule clusters (n = 16 foci from 6 different cells) showing that recruitment of dynamin precedes SPHK1 (right). Pooled data from 6 independent experiments. Error bars: standard error of the mean. (h) Tubule clusters formed after MβCD treatment are positive for fluorescent ceramide (BODIPY-TR–ceramide, top) and fluorescent sphingosine (BODIPY–sphingosine, bottom). (i) Confocal images of a cell expressing SPHK1–GFP 2 min (top), 4 min (middle) and 10 min (bottom) after MβCD treatment. SPHK1 strongly labels filament-like structures originated from the clusters. Scale bars: 10 μm in b and c; 200 nm in d and e; 3 μm in h; and 5 μm in i.
the formation of massive tubular endocytic intermediates. These findings emphasize the critical importance of an appropriate cholesterol/sphingolipid balance in the control of plasma membrane integrity and cell surface area. N-BAR proteins and SPHK1 are highly enriched on the tubular endocytic structures formed under these conditions. Together with evidence for the presence of SPHK1 on physiologically occurring endocytic membranes, and for a defect of endocytic recycling in cells lacking SPHKs, these findings point to a link between sphingosine metabolism and early stages of endocytosis.

The reciprocal interactions of cholesterol and sphingomyelin help determine bilayer rigidity and generate microdomains that control protein localization and function\(^1\)\(^2\). We speculate that perturbing the balance of these two lipids affects the membrane either directly by changing its physical properties\(^49\)-\(^51\), or indirectly by modifying protein–lipid interactions. These changes, in turn, increase the propensity of the membrane to be deformed by the numerous proteins with curvature-generating properties that are present in the cytosol\(^15\),\(^16\),\(^32\). These include BAR domain-containing endocytic proteins, as demonstrated by the accumulation of amphiphysin and endophilin, which are normally selectively concentrated at the short necks of endocytic pits\(^17\)-\(^19\),\(^21\),\(^39\), on the tubules. Under physiological conditions, equilibrium between these factors probably plays an important role in regulating cell surface dynamics. Sequestration of N-BAR proteins on the tubules may contribute to the reported accumulation of shallow clathrin-coated endocytic pits in cholesterol-extracted cells\(^3\),\(^4\).

The massive recruitment of SPHK1 at the tubule clusters revealed a link between these ‘exaggerated’ endocytic intermediates and sphingolipid metabolism and helped us discover a physiologically occurring relation between endocytosis and SPHK1 recruitment. Membrane-curvature sensing\(^53\) of SPHK1 may play a role in its recruitment, as we have found that SPHK1 binds negatively charged lipid bilayers with curvature-sensing properties. However, the delay of its recruitment relative to endophilin suggests the importance of additional factors. One factor could be the accumulation of its substrate, sphingosine, in the membrane. The propensity of SPHK1 to bind the bilayer may facilitate its access to the substrate and allow it to remain membrane bound through multiple catalytic cycles.

Exogenous sphingomyelinase, which generates ceramide, probably has the dual effect of altering the physicochemical state of the plasma membrane and of producing an accumulation of sphingosine, a catabolic product of ceramide. Depletion of cholesterol may
Figure 5 SPHK1 directly binds to negatively charged membranes with a preference for high positive curvature. (a) Liposome flotation assay demonstrating that purified SPHK1 does not bind neutral liposomes (PE/PC/chol = 45:50:5), but does bind negatively charged liposomes that contain PS (PE/PC/PS/chol = 40:10:45:5) or a mixture of acidic phospholipids (mix; PE/PC/PS/PA/PtdIns(4)P/PtdIns(4,5)P2/chol = 25:10:30:10:10:10:5). Arrowhead indicates SPHK1–FLAG and arrow indicates fluorescent lipids. (b) Curvature-dependent sorting of SPHK1–GFP on an artificial lipid bilayer. SPHK1–GFP is enriched relative to a membrane lipid (red, Texas red–DHPE) on a membrane tether pulled from a low-curvature GUV. (c) Mean ratio between green (SPHK1–GFP) and red (Texas red–DHPE) fluorescence intensities from the cross-sectional area of the tether, normalized to the two fluorescence intensities on the GUVs, for varying membrane tension (and thus membrane curvature). n = 6 pulled tubules. Each tubule represents one independent experiment, and data from 6 measurements were pooled. Error bars: standard error of the mean. (d) Crystal structure of human SPHK1 (PDB ID 3VZB). Ribbon representation (left), surface hydrophobicity (middle) and electrostatic surface (right) are shown. In the ribbon representation, the N-terminal lobe is shown in blue and the C-terminal lobe in magenta. The bound substrate and ADP (superposed from PDB ID 3VZD) are also shown. (e) Zoomed-in view of the framed region in d, highlighting the three mutated, surface-exposed and hydrophobic residues, Leu 194, Phe 197 and Leu 198 (green), which are key components of the hydrophobic patch shown in the middle panel of d. (f) Coomassie blue-stained SDS–PAGE showing purified SPHK1–FLAG (WT), SPHK1L194Q–FLAG (L194Q) and SPHK1F197A/L198Q–FLAG (F197A/L198Q). (g) Circular dichroism spectrum of purified SPHK1–FLAG (WT), SPHK1L194Q–FLAG (L194Q) and SPHK1F197A/L198Q–FLAG (F197A/L198Q) showing correct folding of the mutant fusion proteins. (h) SPHK1L194Q–FLAG (L194Q) and SPHK1F197A/L198Q–FLAG (F197A/L198Q) do not bind either neutral liposomes (Ctrl) or negatively charged liposomes (PS and Mix). (i) Confocal images of cells co-expressing endophilin-2–Ruby (bottom) and either SPHK1WT–GFP, SPHK1L194Q–GFP or SPHK1F197A/L198Q–GFP as indicated, after MjICD treatment. Scale bars: 1 μm in b and 10 μm in i.
achieve the same effect indirectly. By destabilizing cholesterol– sphingomyelin microdomains, MIJCD may trigger internalization of excess sphingomyelin, thus activating the cascade that results in upregulated levels of sphingosine.

The metabolism of sphingomyelin requires a cooperation of enzymes located on intracellular membranes and at the cell surface. There is evidence, however, for a selective concentration of sphingomyelin at the plasma membrane, thus suggesting mechanisms to enrich it at this membrane and to remove it and its metabolites from internalized membranes. The building blocks of sphingomyelin are mainly synthesized in the ER and at the Golgi complex, but at least a pool of sphingomyelin is generated directly at the plasma membrane by a plasma membrane-localized sphingomyelin synthase (SMS2). Likewise, a neutral sphingomyelinase, which hydrolyses sphingomyelin to ceramide, and a neutral ceramidase, which hydrolyses ceramide to sphingosine, are also present at the cell surface. We speculate that endocytosis may correlate with the conversion of at least a pool of sphingomyelin to sphingosine, which can then flip across the membrane and be converted to sphingosine-1-phosphate by SPHKs. As endocytic recycling is affected in the absence of SPHKs, this metabolic cascade may not be simply a by-product of membrane traffic but also impact endocytic trafficking.

Sphingosine-1-phosphate can then translocate to other membranes, such as the ER, through the cytosol as it is moderately aqueous soluble. In the ER it can be dephosphorylated by sphingosine-1-phosphate phosphatase (SGPP1) and recycled for sphingomyelin synthesis, or irreversibly degraded by sphingosine-1-phosphate lyase (SGPL1) to provide precursors for phospholipid synthesis. A pool of sphingosine-1-phosphate may also translocate across membranes and function as an extracellular signalling molecule through the binding to a group of G-protein-coupled receptors (GPCRs). The signalling action of sphingosine-1-phosphate through GPCRs is, at present, the most established function of this metabolite.
in mammals. However, as these GPCRs are not expressed in lower organisms, where SPHK and sphingosine-1-phosphate are present, it is possible that the most fundamental role for sphingosine-1-phosphate may relate to membrane dynamics.

Methods

and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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Author contributions

H.S. and P.D.C. designed the experiments and wrote the manuscript; H.S. performed the experiments. Experimental work was also contributed by F.G. (electron microscopy), J.W. (Neuronal experiments), C.Z. (curvature sorting), I.M. (neuronal experiment), K.Y. (retina experiment) and X.W. (microscopy), Y.W. (electron microscopy), J.C. and D.S. (circular dichroism spectroscopy).

Competing financial interests

The authors declare no competing financial interests.

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ARTICLES

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METHODS

Cell culture. HeLa cells, COS-7 cells, Ptk cells and HEK293T cells were purchased from ATCC (Manassas). Primary fibroblasts were isolated from 18- to 20-day embryos by the 3T3 method. Endothelin 1, 2 and 3 triple knockout mouse fibroblasts (clone 1, 2 and 3) were obtained from the Novocorder (USA). The following plasmids were gifts: GFP-clathrin light chain (GFP-CLC) (J. Ken, Thomas Jefferson University Philadelphia, USA), PM–GFP (T. Meyer, Stanford University, USA), GFP-PH-FC (A. De Matteis, Telethon Institute of Genetics and Medicine, Italy) and GFP–2xFYVE (H. Stenmark, Oslo University, Norway). mRFP–clathrin light chain (RFP-CLC), endophilin-2–GFP, endophilin-2–Ruby, endophilin-2ΔSH3–GFP, GFP–ampiphysin II, dynamin 2–RFP and RFP– Rab5 were previously generated in our laboratory. C2–GFP was from Addgene (number 12765); mRFP–SH3 (human) plasmid was from GeneCopoeia (catalogue number EX-U1166-M29). Protein-coding cDNA clones corresponding to mouse SAH2 missing the N-terminal 19 amino acids (clone ID 40018929), mouse SGPL1 (clone ID 4987298) and human SPHK1 (clone ID 5213270) were transferred (without pre-incubation on ice) to pre-warmed (37 °C) DMEM always on ice. Coverslips were then transferred to pre-chilled DMEM and then further processed on ice as follows: rinses in DMEM; stripping of surface-bound transferrin with PBS and fixation with cold 4% formaldehyde in 0.1 M sodium phosphate buffer before permeabilisation with 100 μg ml \(^{-1}\) of digitonin. Samples were imaged with a Zeiss Axioskop 2 microscope using a Plan-Apochromatic ×40 objective and a Hamamatsu ORCA digital camera under the control of MetaMorph software (Molecular Devices) or by spinning-disc microscopy. Immunofluorescence staining was achieved using the anti-PtdIns(4,5)P \(_2\) antibody (clone SAP7F407, catalogue number ADI-V AM-PS003, Enzo Life Sciences) and anti-FLAG antibody (clone M2, catalogue number F3165, Sigma–Aldrich, dilution 1:1000 for western blot) and anti-Pdlns(4,5)P \(_2\) antibody (catalogue number Z-A405, mouse IgM in ascites clone 2C11, Echelon Biosciences, dilution 1:100 for immunofluorescence). Rabbit anti-endophilin 2 antibody (dilution 1:50 for immunofluorescence) were generated in our laboratory.

Other reagents. BODIPY–sphingosine was a gift from R. Bittman (Queens College of CUNY Flushing, USA). Reagents purchased from commercial sources: methyl-β-cyclodextrin, cholesterol–methyl-β-cyclodextrin (cholesterol-water soluble), 2-hydroxypropyl-β-cyclodextrin and sphingomyelins from Bacillus cereus (Sigma-Aldrich); Dil and BODIPY–TR–ceramide, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (TR-DHPE; Life Technologies); TopFluor–PS, brain sphingosine, egg PC, liver PE, cholesterol from sheep wool, brain PG, egg PA, brain PtdIns(4)P, brain PtdIns(4,5)P \(_2\), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycerol-3-phospho-L-serine (DOPS) and 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-\([\text{biotinyl}](\text{polyethylene glycol})-2000\) (Avanti Polar Lipids); and ATP, [γ-\(^32\)P]-ATP (PerkinElmer). Electron microscopy reagents were purchased from Electron Microscopy Sciences.

Immunofluorescence. Immunofluorescence was performed according to standard procedures. Samples were imaged with a Zeiss Axiosplan 2 microscope using a Plan-Apochromatic ×40 objective and a Hamamatsu ORCA II digital camera under the control of MetaMorph software (Molecular Devices) or by spinning-disc microscopy. Immunofluorescence staining using the anti-Pdlns(4,5)P \(_2\) antibody was performed according to ref. 68.

Transferrin internalization. Control and SPHK double knockdown cells were plated on 12 mm glass coverslips at a density of 50,000 cells per coverslip 1 day before assay. Cells were depleted of unlabelled transferrin by incubating them with serum-free DMEM for 1 h at 37 °C. The multiple rounds assay, cells were chilled with ice-cold DMEM for 10 min, labelled with 10 μg ml \(^{-1}\) Alexa Fluor 594–transferrin (Life Technologies) in DMEM for 1 h, and briefly rinsed with DMEM always on ice. Coverslips were then transferred to pre- warmed (37 °C) DMEM and incubated at 37 °C for different times (2.5, 5, 10, 20, 30 and 45 min) to allow uptake and release of transferrin. For the multiple-rounds uptake assay, cells were transferred (without pre-incubation on ice) to pre-warmed (37 °C) DMEM containing 10 μg ml \(^{-1}\) Alexa Fluor 594–transferrin and incubated at 37 °C for different times (2.5, 5, 10, 20, 30 and 45 min). After different times, cells were chilled with ice-cold DMEM for 10 min, labelled with 10 μg ml \(^{-1}\) Alexa Fluor 594–transferrin and incubated at 37 °C for different times (2.5, 5, 10, 20, 30 and 45 min). Next, cells were transferred to pre-chilled DMEM and then further processed on ice as follows: rinses in DMEM; stripping of surface-bound transferrin with phosphate-buffered saline (PBS) and fixation with cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 for 3 min on ice and further fixation at room temperature for 15 min. Coverslips were washed twice with PBS and mounted for analysis. Fluorescence images were acquired by spinning-disc confocal microscopy at 500 nm intervals in the z-axis (5 μm total depth). The resulting z-stack of images was collapsed to a single image by the average intensity projection method in ImageJ software (version 1.43u, NIH). After background correction, whole-cell fluorescence signals was quantified and analysed. For the single-round assay, n = 32, 34, 33, 36, 32 and 31 (control); n = 38, 35, 34, 33, 31 and 34 (DKD) were quantified for time points at 2.5, 5, 10, 20, 30, and 45 min, respectively. For the multiple-rounds assay, n = 36, 30, 35, 31, 32 and 37 (control); n = 33, 33, 30, 33, 35 and 31 (DKD) were quantified for time points at 2.5, 5, 10, 20, 30, and 45 min, respectively.

Cell transfection. Fibroblasts and hippocampal neuronal cultures were electroporated with Amazex Nucleofector using solution R (program A-24, and mouse neuron solution/program O-03 (Lona). HeLa and COS-7 cells were transfected with Lipofectamine 2000 (Life Technologies).

Live-cell imaging. Spinning-disc confocal microscopy and total internal reflection fluorescence microscopy were performed as described previously.

Antibodies. Antibodies were obtained from the following sources: rabbit–Bassoon antibody (clone SAPF7407, catalogue number ADI-V-PS003, Enzo Life Sciences, dilution 1:200 for immunofluorescence), anti-synaptobrevin 2 antibody (clone 69.1, catalogue number 104 211, Synaptic Systems, dilution 1:400 for immunofluorescence), anti-HA antibody (clone 3F10, catalogue number 11867423001, Roche, dilution 1:500 for immunoglobulin labelling), anti-GFP antibody (catalogue number A-11122, Life Technologies, dilution 1:100 for immunoglobulin labelling), anti-FLAG antibody (clone M2, catalogue number F3165, Sigma–Aldrich, dilution 1:1000 for western blot) and anti-Pdlns(4,5)P \(_2\) antibody (catalogue number Z-A405, mouse IgM in ascites clone 2C11, Echelon Biosciences, dilution 1:100 for immunofluorescence). Rabbit anti-endophilin 2 antibodies (dilution 1:50 for immunofluorescence) were generated in our laboratory.
appeared and disappeared either before or after the addition of MJcD-containing solutions (that is, with the entire lifetime in the control or in the MJcD condition) were used for quantification and analysed separately. Statistical significance was calculated by two-tailed Student’s t-test. For the analysis of the presence of SPHK1 at endocytic clathrin-coated pits, 53 clathrin-coated pits from 5 different live cells were counted.

Cholesterol measurement. Cells were plated in 6-well plates in sextuplicate the day before the assay. MJcD-treated (5 min) or sphingomyelinase-treated (10 min) cells and the corresponding coordinates on the grid were imaged using scanning-disc confocal microscopy while fixing cells with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Cells were then post-fixed in 1% OsO4, 1.5% K2Fe(CN)6, 0.1 M sodium cacodylate buffer, en bloc stained with 0.5% uranyl magnesium acetate, dehydrated, and embedded in Embed 812. Ultrathin sections were viewed with a Philips CM10 microscope at 80 kV and images were taken with a Morada 1,000 CCD camera (charge-coupled device) camera (Olympus).

For immunogold labelling for HA or GFP tags, cells were fixed with a mixture of 2% formaldehyde and 0.125% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for ultracytomy as described previously39. Ultrathin sections were immunolabelled with primary antibodies followed by protein A coupled to 10 or 15 nm gold particles.

For electron tomography, the plastic sections (200–250 nm) were placed in a Fischione 2020 single-tilt holder (PA), and examined in a Tecnai TF20 transmission electron microscope (Fei) operated at 200 kV (Yale Center for Cellular and Molecular Imaging, Yale University). The single-tilt series were collected at every 1° over a ±60° range using an Fei Eagle 4K×4K CCD camera. Images were aligned using gold particles, and the final tomograms were reconstructed using the R-weighted algorithm of the IMOD software27.

For scanning electron microscopy, control and MJcD-treated (5 min) cells were fixed with 4% formaldehyde, 2.5% glutaraldehyde. Samples were post-fixed in 2% osmium tetroxide in sodium cacodylate buffer and dehydrated through an ethanol series to 100%. Samples were dried using a Polaron critical point dryer using liquid carbon dioxide as the transitional fluid. Coverslips were glued to aluminium stubs, sputter coater. The samples were viewed and digital images acquired in an FEI ESEM (Electron Microscopy Science) at 1500× magnification.

Primary sequence analysis of sphingosine kinase and crystal structure visualization. Primary sequences corresponding to sphingosine kinases from different species were obtained from the UniProt database, and the sequences were aligned using the ClustalW server. The crystal structure of hSPHK1 (PDB 3VZB) was rendered in Pymol with ADP superposed from 3VZD (PDB ID) to show the active site. Hydrophobic surfaces are coloured on the basis of a normalized hydrophobicity scale using the ColorJ script1, and the electrostatic surface potential was calculated using APBS software64.

Circular dichroism spectrometer experiment. A Chiracircular dichroism spectrometer (Applied Photochemistry) was used to examine the conformation of purified SPHK1–FLAG, SPHK1L194Q–FLAG and SPHK1V268Q–FLAG at a concentration of 0.2 mg ml–1. Measurements were carried out in 20 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM TCEP, 10% glycerol at 20°C.

In vivo experiment in mouse retina. In vivo electrophoresis to transfect mouse retinal progenitors at P0 with plasmid DNA (pCAG–SPHK1–GFP) was performed as previously described2 in three mice. Retinae were collected one to two months after electroporation and dissected under a fluorescence microscope (Leica, MZFL III) to select GFP-positive area. No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not to allocation during experiments and outcome assessment. Animal care and use was carried out in accordance with our institutional guidelines.
Methods

Microscopy and behavioral analysis were performed as described previously. For analysis of neurotransmission, animals were tested for their sensitivity to aldicarb (Bayer CropScience), an inhibitor of acetylcholinesterase. The percentages were averaged at each time point per genotype and plotted graphically. For each experiment, the scorer was blinded to the genotype.

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Supplementary Figure 1  Acute perturbation of plasma membrane cholesterol induces massive endocytic tubular invaginations positive N-BAR proteins. 

**a.** Confocal image of a cell expressing GFP-tagged endophilin 2ΔSH3 after MβCD treatment. **b and c.** Formation of endophilin 2 foci in different cell lines upon MβCD treatment. MEF = mouse embryonic fibroblast. For each cell type, 50 cells expressing endophilin 2-GFP were counted and percentages of the cells that contain endophilin 2 clusters were plotted. Data represent a single experiment. **d.** Anti-endophilin 2 immunofluorescence staining of a control cell or a cell treated with MβCD for 5 min (+MβCD). **e.** Cell co-expressing amphiphysin 2-GFP and endophilin 2-Ruby after MβCD treatment. **f.** Confocal image of an endophilin TKO cell expressing amphiphysin 2-GFP before and after MβCD treatment. **g.** Confocal images of a WT cell expressing endophilin 2-Ruby (left), after MβCD treatment (middle) and supplemented with cholesterol after MβCD treatment (right). **h.** Confocal image of a cell expressing endophilin 2-GFP treated with HPβCD (an analogue of MβCD). Scale bar: 10 μm in a, b, d, f, and h; 5 μm in e and g.
Supplementary Figure 2 Characterization of endocytic tubular invaginations induced by perturbation of plasma membrane cholesterol. a and b. Double fluorescence images showing that endophilin 2 foci are positive for chemical (TopFluor-PS, a) and genetically encoded (C2 władz-GFP, b) PtdSer markers. c and d. Double fluorescence images showing that endophilin 2 foci are negative for the genetically encoded PI(4,5)P2 probe (GFP-PHPLCδ, c) and immunoreactivity recognized by an anti-PI(4,5)P2 antibody, d). e. Double fluorescence images showing that endophilin 2 foci are negative for the genetically encoded PI3P probe GFP-FYVEHrs. f. Scanning electron microscopy micrographs of a mouse fibroblast before (left) and after (right) MβCD treatment. Framed regions, which are shown at a higher magnification at the right of each field, highlight the disappearance of filopodia after treatment. g. Representative example of the change in the footprint of a cell labeled by PM-GFP before (white) and after MβCD treatment (gray). h and i. Representative images (h) and quantification (i) of endophilin 2 foci induced by MβCD in WT, clathrin heavy chain (CHC) knockdown (KD), and dynamin triple KO mouse fibroblasts. n = 37 (WT), 36 (CHC KD), and 38 (dyn TKO). Pooled data from three independent experiments. Error bars represent standard errors of the mean. [ns] not significant; [***] P < 0.001, Student’s t-test. j. Double fluorescence confocal images of a cell expressing dynamin 2-RFP and endophilin 2-GFP after MβCD treatment. k. Double fluorescence confocal images of a cell expressing dynamin 2-RFP and endophilin 2ΔSH3-GFP after MβCD treatment. Scale bar: 3 μm in a-e, j and k, and 10 μm in f, g and h. All pictures shown in the figure are from mouse fibroblasts.
Supplementary Figure 3 The sphingoid base modifying enzyme, sphingosine kinase 1 (SPHK1), is recruited to the endophilin 2 foci. a. A genetic interaction map in budding yeast\(^30\) revealed a genetic interaction between RVS161 and RVS167 and genes encoding enzymes involved in sphingolipid and ergosterol synthesis (top). Blue and yellow indicate negative and positive interaction, respectively. The interactions of RVS161 and RVS167 with enzymes regulating sphingoid base level are framed by a purple rectangle. The corresponding pathway and orthologous mammalian enzymes (black letters) are shown at the bottom. b. Localization of several sphingolipid metabolic enzymes (all transmembrane proteins with the exception of CerK) in mammalian cells as shown by confocal microscopy analysis of transfected GFP-fusion proteins. SMPD3: neutral sphingomyelinase 2 (plasma membrane); ASAH2: neutral ceramidase 2 (plasma membrane); CerK: ceramide kinase (plasma membrane, but also partially cytosolic); SGPP1: sphingosine-1-phosphate phosphatase 1 (ER); CerS1: ceramide synthase 1 (ER); SGPL1: sphingosine-1-phosphate lyase 1 (ER). c. Sphingolipid metabolic pathway where the enzymes analyzed in b is shown in red. d. Double fluorescence images of a cell expressing endophilin 2-GFP and SPHK2-FLAG following M\(\beta\)CD treatment, fixation and subsequent immunostaining with anti-FLAG antibody. e. Confocal image of an endophilin triple KO cell expressing SPHK1-GFP after M\(\beta\)CD treatment. Scale bar: 10 \(\mu\)m in b and e; and 5 \(\mu\)m in d.
**Supplementary Figure 4** SPHK1 and SPHK2 knockdown in HeLa cells. HeLa cells were transfected with control siRNA (ctrl) or siRNA directed against SPHK1 and SPHK2 (DKD). SPHK1 and SPHK2 mRNA levels were measured by real-time qPCR. n = 3 measurements. Error bar: standard error of the mean. Data are from one experiment, but are representative of three independent experiments.
**Supplementary Figure 5** *In vitro* assay of purified SPHK1. 

**a.** Coomassie-stained SDS-PAGE showing purified SPHK1-GFP-FLAG.

**b.** Sphingosine kinase assay. Autoradiography of a TLC plate showing that purified SPHK1 is catalytically active as it phosphorylates sphingosine to generate radiolabeled sphingosine-1-phosphate *in vitro*. As the two lanes shown in the image were from the same TLC plate but not adjacent to each other, a black splice mark was included to separate the two lanes. The full TLC plate from which the data are extracted is shown in Supplementary Figure 7.

**c.** The presence of SPHK1 on the membrane bilayer does not change the relationship between tubule radius and tension in the membrane tethering assay. \( n = 6 \) (with SPHK1) and 9 (without SPHK) pulled tubules. Each tubule represents an independent experiment, and data from independent experiments are averaged. Error bars represent standard error of the mean.
Supplementary Figure 6 Human SPHK1 rescues the synaptic transmission defect observed in SPHK-1 mutant worms. Time-course of the onset of paralysis of the indicated worm strains upon exposure to the acetylcholine esterase inhibitor aldicarb (1 mM). sphk-1; H.s. SPHK1 stands for sphk-1 null mutants expressing full length human SPHK1 cDNA in neurons. n = 3 plates. One transgenic line was assayed and 25 animals were examined from each plate. Average paralysis rate was determined by pooling data from the three assays. Error bars represent standard error of the mean.
**Supplementary Figure 7** Original TLC from which the data of Supplementary Figure 5b were extracted (lanes 2 and 7). Lane 3 to 6 are from a purified SPHK1 expressed in bacteria. The low catalytic activity of this preparation most likely reflects protein misfolding. For this reason, SPHK1 was expressed in and purified from mammalian cells, Expi293F cells (lanes 1 and 2). Lane 7 is the control where no protein was added in the reaction.
Supplementary Video Legends

Supplementary video 1 Spinning disk confocal movie of HeLa cells expressing endophilin 2-GFP upon MβCD treatment. Spinning disc confocal microscopy of HeLa cells expressing endophilin 2-GFP showing the formation and disappearance of the large endophilin foci during 10 mM MβCD treatment. The interval between frames is 4 s. Playback rate is 12 frames per second. Scale bar = 10 μm.

Supplementary video 2 Transmission electron microscopy tomography of a tubular membrane cluster generated by MβCD treatment. Mouse fibroblasts expressing endophilin 2-GFP were treated with 10 mM MβCD, and processed as described in the Methods section. A plastic section (250 nm) containing a tubular cluster was visualized by electron tomography.