Sterols lower energetic barriers of membrane bending and fission necessary for efficient clathrin mediated endocytosis

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SUMMARY

As the principal internalization mechanism in mammalian cells, clathrin-mediated endocytosis (CME) is critical for cellular signal transduction, receptor recycling, and membrane homeostasis. Acute depletion of cholesterol disrupts CME, motivating analysis of CME dynamics in the context of disrupted cholesterol synthesis, sterol specificity, mechanisms involved, and relevance to disease pathology. Using genome-edited cell lines, we demonstrate that inhibition of post-squalene cholesterol biosynthesis as observed in inborn errors of cholesterol metabolism, results in striking immobilization of CME and impaired transferrin uptake. Imaging of membrane bending dynamics and CME pit ultrastructure revealed prolonged clathrin pit lifetimes and accumulation of shallow clathrin-coated structures that scaled with diminishing sterol abundance. Moreover, fibroblasts derived from Smith-Lemli-Opitz syndrome subjects displayed reduced CME function. We conclude that sterols lower the energetic costs of membrane bending during pit formation and vesicular scission during CME and suggest reduced CME contributes to cellular phenotypes observed within disorders of cholesterol metabolism.

INTRODUCTION

Lipid composition is critical to the biophysical properties of membrane bilayers, permitting maintenance of essential cellular functions such as membrane trafficking and signal transduction (Kusumi et al., 2012; Raghunathan and Kenworthy, 2018). Cholesterol has long been recognized as a major regulator of lipid organization. Its unique structure influences lipid packing, protein localization and interactions, and global membrane properties including permeability, fluidity, and rigidity (Maxfield and Tabas, 2005). Synthesized *de novo* in the endoplasmic reticulum or derived from circulating low-density lipoproteins, unesterified cholesterol is distributed heterogeneously amongst various cell membranes, being predominately enriched at the plasma membrane (PM) where it accounts for up to 45% of the total lipids present (Das et al., 2013) and 60-90% of total cellular cholesterol (de Duve 1971; Lange et al., 1989). The importance of cholesterol composition to PM structure and function is reflected in the tight regulation of intracellular transport and highly coordinated synthetic and efflux mechanisms maintaining physiologic cholesterol levels (Luo et al., 2019; Mitsche et al., 2015; Nohturfft et al., 1998; Sun et al., 2005; Tontonoz and Mangelsdorf, 2003; Vedhachalam et al., 2007).

Membrane properties including rigidity and tension are especially pertinent to highly dynamic membrane remodeling processes, such as endocytosis (Hassinger et al., 2017). As the major pathway regulating receptor-mediated signaling, clathrin-mediated endocytosis (CME) mediates nutrient uptake, signal transduction, receptor recycling and desensitization, membrane homeostasis, and developmental regulation (Kaksonen and Roux, 2018). Evidence for cholesterol's role in CME has been demonstrated by acute stripping of membrane cholesterol using methyl-beta-cyclodextrin (M β CD) (Rodal et al., 1999; Subtil et al., 1999). Although M β CD is a relatively non-specific means of cholesterol extraction contingent upon the carrier's

hydrophobic cavity (Zidovetzki and Levitan, 2007), these classic experiments suggest a strong defect in clathrin-coated pit budding and internalization upon M β CD treatment.

Amidst the extensive CME machinery described to date, the influence of cholesterol on plasma membrane architecture may be overlooked as an active component within assembly, curvature generation, and scission of clathrin-coated pits. CME is initiated by the recruitment of clathrin triskelia by adapter and accessory proteins to sites of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) enriched PM (Cocucci et al., 2012). This assembly process may be sensitive to alterations of lipid packing or shielding of charges secondary to cholesterol availability (Hirama et al., 2017). Progression of the PM from flat to highly curved clathrin-coated vesicles also requires overcoming stiffness of the membrane and tension generated by lateral membrane recruitment and opposed by membrane-cytoskeletal adhesions (Bucher et al., 2018; Scott et al., 2018). Recruitment of epsin NH₂-terminal homology (ENTH), AP180 N-terminal homology (ANTH) and later Bin/Amphiphysin/Rvs (BAR) domain containing proteins likely contribute to the energy required for membrane bending (Liu et al., 2009; McMahon and Gallop, 2005; Miller et al., 2015). By interacting with these curvature effectors and stabilizing positive PM fluctuations, the clathrin lattice is thought to act as a Brownian ratchet promoting curvature formation (Liu et al., 2009). This remodeling process creates energetically unfavorable membrane stress which may be alleviated by cholesterol through rapid transbilayer redistribution (Bruckner et al., 2009). During vesicular scission, an energetically costly constriction requiring steep membrane curvature must be achieved. The GTPase dynamin catalyzes this event by polymerizing into a helical coat and constricting the endocytic pit neck by GTP hydrolysis and removal of dynamin subunits. However, previous work has demonstrated constriction alone is insufficient for scission to occur (Danino et al., 2004; Roux et al., 2006). Considering the severity of membrane bending at these sites, the mechanical properties of the PM immediately adjacent to the dynamin coat, including tension and bending rigidity, are thought to influence scission (Morlot et

al., 2012). Bursts of actin polymerization also aid in overcoming resistance to membrane bending by providing additional force leading to vesicle internalization and scission (Collins et al., 2011; Grassart et al., 2014), especially in areas of high membrane tension (Boulant et al., 2011).

Human diseases associated with disruption of cholesterol homeostasis may provide insight into the lipid-specific requirements of CME, and conversely, find application from an understanding of cellular trafficking within these disease states. The best characterized of these orphan diseases, Smith-Lemli-Opitz syndrome (SLOS), is caused by genetic mutations within the post-squalene cholesterol synthesis enzyme 3 β -hydroxy-steroid- Δ^7 -reductase (DHCR7) (Wassif et al., 1998) and presents clinically with multiple congenital malformations including distinctive facial features, cardiac defects, genitourinary abnormalities, syndactyly, gastrointestinal intolerance, and profound CNS dysfunction (Smith et al., 1964). Clinical severity generally correlates inversely with residual enzymatic activity; however, significant phenotypic variability exists even amongst similar pathogenic variants (Cunniff et al., 1997; Wassif et al., 2005; Waterham and Hennekam, 2012), which ranges on a wide continuum from mild cognitive impairment to embryonic lethality (Tint et al., 1995). The relative contribution of cholesterol depletion and/or accumulation of sterol precursors such as 7-dehydrocholesterol (7DHC) to disease pathogenesis also remains an open question (Wassif et al., 2017). To date, membrane defects within SLOS have primarily been attributed to disruption of lipid rafts (Gou-Fabregas et al., 2016; Keller et al., 2004). As a theory to explain membrane heterogeneity, the propensity for cholesterol to tightly pack sphingomyelin and less saturated phospholipids underlies the basis for liquid-ordered (L_o) and liquid-disordered (L_d) phase separation. As such, cholesterol depletion is often considered evidence for lipid raft involvement. Nevertheless, consideration of global effects on plasma membrane architecture is warranted, which may perturb processes that are themselves independent of lipid rafts (Kwik et al., 2003; Sengupta et al., 2007; van

Rheenen et al., 2005). While sterols may influence developmental pathways through a variety of mechanisms, one hypothesis suggests that SLOS pathogenesis manifests in a highly temporal and tissue specific context, driven by changes in signal transduction pathways (Tulenکو et al., 2006). Thus, defining the effects of disrupted sterol synthesis on the major pathway regulating receptor-mediated signaling may also provide insight into the molecular and cellular basis for the physiologic manifestations within these rare diseases.

In this study, we demonstrate that multiple stages of membrane remodeling during CME progression are sensitive to sterol-dependent membrane properties. Clathrin pit ultrastructure with cholesterol depletion exhibited asymmetric distorted structures, consistent with aberrant membrane bending trajectories, suggesting that clathrin lattice assembly competes with the elevated free energy costs of bending. Using sterol substitution strategies, we further demonstrate that a small, C3 polar headgroup and B-ring conformation constitute structural requirements for supporting efficient CME. Finally, we present evidence for disruption of CME dynamics within SLOS and conclude that sterol abundance lowers the energetic barrier for curvature generation during initiation of membrane bending and formation of the vesicle neck during clathrin-mediated endocytosis.

RESULTS

Clathrin-mediated endocytosis is inhibited by altered cholesterol homeostasis

To test the hypothesis that sterol homeostasis contributes to efficient CME, we evaluated CME during acute cholesterol depletion and perturbations of cholesterol synthesis in HEK293T cells. Endogenous cholesterol biosynthesis was induced by culturing cells in media containing 7.5% lipoprotein-deficient fetal bovine serum (LPDS). We used multiple targeting strategies to delineate the effects of cholesterol depletion versus terminal cholesterol precursor accumulation within the competing Bloch and Kandutsch-Russell (KR) pathways (**Figure 1A**). AY9944

dihydrochloride, a potent inhibitor of DHCR7 (Δ^7 -sterol reductase) (Fernandez et al., 2005; Gaoua et al., 1999; Rahier and Taton, 1996), has been used extensively to recapitulate the biochemical hallmarks of SLOS both *in vitro* and *in vivo* (Francis et al., 2016; Kolf-Clauw et al., 1996). U18666A, a potent noncompetitive inhibitor of DHCR24, was used to inhibit the final step in the Bloch pathway at nanomolar concentrations where it does not perturb cholesterol trafficking (Cenedella, 2009; Lu et al., 2015). Comparison of AY9944 (KR targeting) with U18666A (Bloch targeting) also provided access to structure-activity relationships. Specifically, DHCR7 mediated reduction of the C(7-8) double bond confers the linear cholesterol ring structure (Serfis et al., 2001), such that treatment with AY9944 leads to accumulation of non-planar sterols. Conversely, inhibition of DHCR24 leads to accumulation of sterols with more rigidity as it prevents the DHCR24 mediated reduction of C(24-25) (Chen and Tripp, 2012). The HMG-CoA reductase inhibitor Atorvastatin was used to inhibit synthesis of cholesterol and other biosynthetic intermediates, while M β CD was used to acutely deplete sterols from the plasma membrane (Rodal et al., 1999; Subtil et al., 1999). In this study, sterol content in HEK293T cells relative to LPDS controls was raised ~25% when cultured in FBS and reduced by ~50% with Atorvastatin, AY9944, or M β CD. AY9944 treatment effectively led to the predicted accumulation of 7-dehydrocholesterol and zymostenol, while U18666A induced a mild accumulation of desmosterol (**Figure 1B**).

To monitor the effects of cholesterol homeostasis on CME, we used CRISPR/Cas9 gene editing to fluorescently tag clathrin light chain A (CLTA) in HEK293T using our previously described approach (**Figure S1A**) (Anderson et al., 2018; Scott et al., 2018). A pooled population of genome-edited cells was enriched by FACS to create a homogenous population of CLTA^{mTq2+} cells, thereby mitigating potential off-target effects that could arise in single cell clones (**Figure S1B, C**). Live-cell confocal microscopy taken at midsection planes revealed dynamic PM budding and rapid trafficking of clathrin-mTq2⁺ vesicles in LPDS cultured cells. All sterol

depleted conditions, exemplified by AY9944 treatment, lead to striking immobilization of clathrin vesicles at the PM despite ongoing intracellular transport (**Video 1, panels 1-2**). Trafficking deficits induced by AY9944 treatment were efficiently rescued by addition of cholesterol loaded methyl- β -cyclodextrin (M β CD-Chol) as observed by rapid redistribution of clathrin-mTq2⁺ traffic from the PM, indicating that plasma membrane cholesterol alone is sufficient and essential for clathrin trafficking (**Video 1, panel 3**). This effect was rapid, restoring CME within minutes of M β CD-Chol addition and rendering mTq2⁺-clathrin traffic indistinguishable from LPDS controls within 1 h (**Video 1, panel 3**). To exclude potential secondary effects from the lipophilic properties of the cholesterol synthesis inhibitors, small molecules were administered in the presence of exogenous cholesterol, thereby minimizing endogenous synthesis (Wassif et al., 2005). Under these conditions, the cholesterol synthesis inhibitors did not produce clathrin trafficking deficits (**Figure 1C**).

To determine the extent of functional CME impairment, we used a high-content, non-biased quantitation imaging assay to analyze the internalization of fluorescently-conjugated transferrin (Tfn) across sterol depleted conditions. The degree of sterol depletion achieved with these inhibitors (**Figure 1B**), predicted the reduction in CME. Specifically, treatment with either AY9944, Atorvastatin, or M β CD reduced both sterol abundance and Tfn internalization by approximately 50%. U18666A had a smaller effect (~20% reduction) on both sterols and CME (**Figure 1D**). Confocal optical sections taken at midplane also confirmed the inhibition of Tfn internalization (**Figure 1E**), as seen by the loss of Tfn-555 positive vesicles in the cytoplasm and localization to the PM. Although Tfn internalization was impaired, high Tfn-555 signal from the PM suggests that the Tfn receptor also accumulated on the PM. Because CME was impaired regardless of the step of cholesterol synthesis inhibition or time of cholesterol depletion, we speculated that cholesterol depletion or decreased total sterol content was the major mediator of this phenomenon, rather than accumulation of specific sterol precursors.

Parallel comparison of sterol profiles by GC/MS and Tfn uptake as a function of inhibitor concentration demonstrated that CME activity was dependent on total sterol content (**Figure 1F, G**).

Recovery of CME traffic was also predicted by sterol abundance and availability. Addition of M β CD-Chol following sterol depletion with AY9944 rapidly restored both mTq2⁺-clathrin traffic and transferrin internalization (**Figure 2B, top**). In contrast, clathrin-mTq2⁺ vesicle trafficking required 6-12 h to normalize following addition of FBS, indicating that lipoprotein-derived (LDL) cholesterol uptake and redistribution was much slower (**Figure 2B, middle**), likely due to depressed CME-dependent LDL uptake (Davis et al., 1986). Clathrin-mTq2⁺ vesicle trafficking also did not recover upon removal of AY9944 and addition of LPDS within 12 h, indicating the rate of CME rescue by endogenous cholesterol synthesis is much slower than either direct or LDL-dependent cholesterol uptake (**Figure 2B, bottom**). Feedback inhibition of 7DHC on the synthetic pathway may also limit sterol biosynthesis (Honda et al., 1998). Cellular sterol profiles corresponded with the resumption of CME (**Figure 2C**). Notably, incubation of AY9944 depleted cells with M β CD-Chol corrected cellular sterol profiles more completely than availability of LDL-derived cholesteryl esters over a 12 h period (**Figure 2C**), suggesting greater efficacy and bioavailability of cholesterol supplementation independent of endocytic traffic. These rescue assays demonstrate a definitive requirement for an optimal concentration of cellular cholesterol to facilitate clathrin vesicle trafficking.

7-dehydrocholesterol can compensate for cholesterol in facilitating CME

As SLOS leads to a significant accumulation of 7DHC (Irons et al., 1993; Tint et al., 1994), we wanted to directly address the ability of 7DHC to compensate for the activity of cholesterol with regards to supporting CME. To this aim, rescue of aberrant clathrin trafficking following AY9944 treatment was evaluated by loading of cholesterol or 7DHC directly to cellular membranes using

M β CD as a water-soluble carrier. Incubation with M β CD-7DHC efficiently rescued total sterol levels back to cholesterol-replete conditions with 7DHC being the predominate form (~80% of total sterols) (**Figure S2A**). Addition of uncomplexed M β CD to cells already depleted of sterols did not appreciably further reduce sterol levels, which may reflect diminished “free” cholesterol available for M β CD exchange. Restoration of total sterol levels by either M β CD-Chol or M β CD-7DHC resulted in resumed clathrin-mTq2⁺ trafficking (**Video 2**) and robust Tfn internalization (**Figure S2B, C**). This finding suggests that loss of planarity within the sterol ring, due to the additional double bond in 7DHC (Serfis et al., 2001), does not impede its endocytic function at the membrane. Although 7DHC has been suggested to impact other aspects of PM structure including bending rigidity (Gondre-Lewis et al., 2006) and lipid packing (Tulenko et al., 2006), our results demonstrate 7DHC can compensate for cholesterol in sustaining CME activity. Thus, CME appears to be sensitive to total sterol content but robust to sterol identity.

Altered sterol homeostasis inhibits clathrin-coated pit dynamics and membrane bending

Cholesterol's multifaceted influence on PM properties likely contributes to lowering the energetic cost of membrane remodeling (Bruckner et al., 2009). Because EM studies following M β CD treatment indicate arrest at preliminary stages of pit bending (Rodal et al., 1999; Subtil et al., 1999), sterol depletion may inhibit membrane curvature generation and recruitment of CME proteins. To determine how cholesterol and sterols influence membrane bending during clathrin assembly, we used polarized total internal reflection fluorescence microscopy (polTIRFM) of DiI, which allows imaging of PM topography (Aguet et al., 2013; Anantharam et al., 2010; Scott et al., 2018). Imaging of membrane bending in polTIRFM is achieved by selective excitation of DiI-labeled PM within the vertical (P polarization) or horizontal (S polarization) plane with respect to the coverslip and quantified by the ratio of P/S fluorescence (Anantharam et al., 2010). We previously demonstrated that this approach can accurately measure changes in membrane topography during membrane bending during CME by correlative light and electron microscopy

(CLEM) and atomic force microscopy (CLAFM) (Scott et al., 2018). The membrane bending dynamics of individual endocytic events were recorded in SK-MEL-2 cells endogenously expressing clathrin-mTq2⁺ and dynamin-eGFP⁺ with Dil labeled membranes (**Video 3**). CME dynamics of the SK-MEL-2 cell line have been well characterized by live-cell TIRFM and polTIRFM (Doyon et al., 2011; Scott et al., 2018).

Upon disruption of cholesterol homeostasis, total sterol content relative to LPDS culture was found to be 147 ± 24% (FBS), 81 ± 7% (Atorvastatin), 82 ± 11% (AY9944), or 34 ± 9% (MβCD) in SK-MEL-2 treated cells (data not shown). Under all sterol deplete conditions (AY9944, Atorvastatin, and MβCD), the total number of unique, distinct clathrin assembly spots per cell area over time was significantly lower relative to cells grown in complete media or LPDS (**Figure 3C**). Both moderate and severe sterol depletion led to an increase in the number of persistent clathrin tracks (>400 s) (**Figure 3D**) and average clathrin lifetimes per cell (**Figure 3F**). Accumulation of clathrin-mTq2⁺ spots with sterol depletion indicated that the reduction in clathrin tracks was not due to lack of clathrin assembly, but arrested CME (**Video 4, Figure 3A**). Clathrin-mTq2⁺ tracks were further categorized according to detection of dynamin and P/S signal. Although dynamin may contribute to early stages of clathrin-coated pit maturation (Aguet et al., 2013; Srinivasan et al., 2018), it is most abundant during membrane fission. Dynamin-eGFP⁺ was visualized as a burst of accumulation immediately prior to the completion of endocytosis and the disappearance of the clathrin-coated vesicle (**Figure 3B**). Using dynamin recruitment as a marker of completed CME events, we found that the number of productive CME events per cell area was dramatically reduced during sterol depletion (**Figure 3E**). When CME events did occur, they exhibited longer lifetimes compared with FBS or LPDS controls (**Figure 3G, Figure S3**). Following AY9944 treatment, the average lifespan of CME events which could be observed was increased by 40 s (95% CI [89,148]) compared to LPDS controls (95% CI [66, 97]). Moreover, CME lifetimes in AY9944 treated cells are underestimated by this

data due to temporal limitations in video tracking of persistent CME tracks. A detailed breakdown of clathrin lifetimes under control or sterol depleted conditions is shown in **Figure S4**.

While both moderate (AY9944 and Atorvastatin) and severe (M β CD) cholesterol depletion led to diminished rates of CME, we observed treatment-specific impacts on membrane curvature dynamics. Cells displaying ~20% reduction in total sterol content following AY9944 or Atorvastatin treatment exhibited clathrin-mTq2⁺ spots associated with strong P/S signals, indicating the presence of highly curved pits which stalled prior to acquiring bursts of dynamin (**Figure 3A**). DMN2-eGFP⁺ localization was delayed in both AY9944 and Atorvastatin treated cells compared to LPDS controls, suggesting clathrin events inefficiently recruited dynamin and many did not become productive CME events. As illustrated by kymographs of long-lived clathrin tracks failing to undergo scission (**Figure S5**), fluctuations in P/S membrane bending along clathrin tracks were noted. While weak DNM2^{eGFP+} signal was associated with many curved clathrin structures, robust dynamin signatures could also be seen dissociating without a loss of clathrin signal. No differences in dynamin lifetimes were observed in association with productive CME events (data not shown). These findings suggest moderate disruption of cholesterol synthesis allowed membrane bending during the early stages of CME, but precluded neck formation and the recruitment of dynamin. In contrast, cells displaying ~65% reduction in total sterols following M β CD treatment exhibited diminished P/S signals with little to no dynamin recruitment (**Figure 3A, H**), indicating that severe sterol depletion inhibits CME at the stage of initial membrane bending. This result is consistent with previous studies describing flat clathrin-coated structures following M β CD mediated sterol depletion, consistent with an inability of clathrin-coated pits to invaginate (Rodal et al., 1999). Altogether, these findings suggest a homeostatic level of cholesterol is required at multiple stages of PM bending for the CME pathway to proceed normally.

Sterol depletion inhibits curvature generation and neck formation during CME, producing asymmetric pit structure

In order to understand the effects of sterol depletion on membrane curvature during CME, we preformed platinum replica EM of unroofed SK-MEL-2 cells to quantitatively assess ultrastructure (Sochacki et al., 2017; Sochacki et al., 2014). We classified clathrin structures as flat, shallow, domed, or spherical to quantitatively assess curvature status (**Figure 4B**). In cholesterol replete conditions (FBS or LPDS culture), flat and spherical clathrin-coated pits were most commonly observed with fewer shallow or domed structures. This observation was consistent with rapid progression of CME pits from flat to spherical as previously described in SK-MEL-2 cells cultured in FBS (Scott et al., 2018). In contrast, moderate sterol depletion with either Atorvastatin or AY9944 increased the relative frequency of shallow and domed structures, predominately at the expense of spherical pits (**Figure 4B**). The increased frequency of shallow/domed structures explains the high P/S signal observed on clathrin spots by polTIRFM with AY9944 and Atorvastatin treatment (**Figure 3A**). Combined with the fluctuations observed in the P/S signals (**Figure S5**), these findings suggest that the structural transition from shallow to domed morphology is metastable, potentially due to the energetics of clathrin-coat lattice reorganization competing with elevated free energy costs of membrane bending upon sterol depletion. Consistent with polTIRFM studies, severe sterol depletion by M β CD shifted the relative frequency of coated pit curvature to predominately flat or shallow coated pits, reinforcing the concept that sterol depletion from the PM dramatically increases the energetic cost of membrane bending (**Figure 4A**). Absolute values of the categorized clathrin structures per cell are summarized in **Figure S6**. In support of intrinsic membrane properties altering the energetics of membrane bending, caveolae under cholesterol deplete conditions also exhibited a flattened, disassembled morphology (**Figure 4F**) and were decreased in number at the plasma membrane (**Figure 4G**). These findings provide direct structural evidence that sterol

abundance influences the progression of CME events by altering membrane bending dynamics at multiple stages of curvature generation.

Sterol depletion also resulted in loss of structural symmetry within pits. While most clathrin structures were flat in M β CD treated cells, the few observable spherical pits were asymmetric and distorted (**Figure 4D**), suggesting aberrant energetics of membrane bending. Upon sterol depletion with both AY9944 and Atorvastatin, distorted coated pits exhibiting elongated necks without a narrow base were also observed (**Figure 4D inset**) and the quantified asymmetry scaled with the severity of cholesterol depletion such that M β CD >> AY9944 ~ Atorvastatin (**Figure 4D**). Aside from the numerous flat clathrin structures within M β CD treatment which were beginning to converge (**Figure 4E, panel 1**), no significant differences in the average size of clathrin structures were observed across treatments within the shallow, domed, and spherical subsets of curvature classification (**Figure 4E, panels 2-4**). This suggests that once energetic barriers were crossed, general pit dimensions were constant. Notably, the area of the PM covered by clathrin increased dramatically from approximately 1.5% in FBS and LPDS controls to 3.0%, 4.0%, and 8.7% when treated with Atorvastatin, AY9944, or M β CD, respectively (**Figure 4C**). A possible explanation for the loss of clathrin pit symmetry upon sterol depletion may be due to the accumulation of stalled pits on the PM resulting in asymmetric membrane bending trajectories during the remodeling of large flat clathrin lattices into vesicles (Avinoam et al., 2015; Scott et al., 2018). This data demonstrates that perturbation of cholesterol homeostasis alters the process of clathrin assembly and the trajectory of membrane bending leading to an accumulation of normal and distorted clathrin-coated pits on the PM.

Phase separation predicts the sterol structural requirements for supporting CME

Considering that membrane bending was impacted at both early and late stages of pit formation congruent upon total sterol abundance, the ability of a structurally dissimilar sterol (7DHC) to

support CME, and the relative abundance of cholesterol within the PM, we wanted to further define the biophysical properties of sterols necessary for facilitating curvature and CME. Biophysical studies in model membranes have suggested rapid transbilayer redistribution (flip-flop) of cholesterol relieves stress associated with membrane remodeling by alleviating the molecular disparities between the inner and outer PM leaflets during bending (Bruckner et al., 2009; Lange et al., 1981). In contrast to other lipid components of the PM, cholesterol transbilayer flip-flop occurs within milliseconds (Choubey et al., 2013; Hamilton, 2003; Steck et al., 2002), a timescale sufficiently fast to support local membrane remodeling during an endocytic event, which typically takes 80-100 s in the case of CME (Taylor et al., 2011). Additionally, cholesterol and select sterols have the capacity to partition bilayers into liquid-ordered (L_o) or liquid-disordered (L_d) phases due to preferential tight packing of sterols and lipid components. Phase separation is postulated to underlie membrane shape transitions by minimizing line tension between domain boundaries (e.g. driving progressive curvature generation to neck structures) (Jülicher and Lipowsky, 1993, 1996) and is implicated in membrane curvature and budding behavior in artificial membranes (Bacia et al., 2005).

To clarify the sterol structural requirements necessary to support CME, we used M β CD to deliver structurally diverse sterols and tested their ability to rescue CME function following AY9944 inhibition. As summarized in **Figure 5A, Table S2**, sterols were chosen based on differences in ring structure (lathosterol, 7DHC), aliphatic tail structure (desmosterol), and predicted ability to phase separate or support transbilayer movement of sterols. Cholestenone, which substitutes the 3 β -hydroxyl with a ketone group as typical of steroid hormones, likely diminishes polar interactions at the membrane-water interface to promote more rapid interleaflet exchange (Róg et al., 2008). Conversely, increased polarity (4 β -hydroxycholesterol) or charge (cholesterol sulfate) would impede sterol exchange between membrane leaflets and do not support phase separation (Bacia et al., 2005; Wenz and Barrantes, 2003). Incubation with

desmosterol, 7DHC, lathosterol or 4 β -hydroxycholesterol M β CD-sterol complexes efficiently rescued total sterol levels to LPDS levels (**Figure 5B**). M β CD-cholesterol sulfate and cholestenone complexes only allowed for sterol exchange (total cellular sterol content remained near AY9944 levels). Sterols previously demonstrated to allow for sterol flipping and supporting phase separation (desmosterol, 7DHC) rescued Tfn internalization (**Figure 5C, D**). However, Tfn internalization was not rescued by sterols which do not support phase separation or allow membrane flip-flop (4 β -hydroxycholesterol, galactosyl cholesterol) (**Figure 5C, D**). This suggests that sterol structures supporting phase separation confer similar requirements necessary to support CME.

Integrity of the actin cytoskeleton does not significantly modulate CME sterol dependence

During CME, the polymerization energy of the clathrin lattice is opposed by membrane bending energy (Saleem et al., 2015). While the actin cytoskeleton is not absolutely required for CME in mammalian cells, it becomes increasingly important as membrane tension increases (Batchelder and Yarar, 2010; Boulant et al., 2011). Actin polymerization is thought to provide additional force during the transition of domed to spherical shaped pits in preparation for the vesicle scission phase (Grassart et al., 2014) (Hassinger et al., 2017; Skruzny et al., 2012). Inhibition of CME dynamics within mitotic cells has been observed where rigid cortical actin and increased membrane tension dominate (Kaur et al., 2014). Moreover, cytoskeletal alterations which occur following cholesterol depletion may enhance membrane-cytoskeletal adhesions, resulting in either sequestration of actin away from CME or elevated membrane tension (Ayee and Levitan, 2016; Khatibzadeh et al., 2012). To determine whether inhibition of CME by sterol depletion is mediated through changes to the actin cytoskeleton or specific to the membrane, we depolymerized actin with latrunculin B pre-treatment during inhibition by AY9944 alone or following recovery of CME activity by sterol repletion (**Figure S7C**). We did not find evidence of

significant latrunculin B sensitivity in either restoring CME activity in sterol depleted cells or inhibiting M β CD-cholesterol rescue (**Figure S7E**). These data suggest the influence of sterols on CME is largely independent of actin remodeling but directly dependent on changes to the physical properties of the PM.

Patient fibroblasts containing mutations within cholesterol synthesis genes exhibit functional CME deficits

To determine if CME activity was reduced within the context of a human disease characterized by sterol disruption, Tfn uptake assays were performed on fibroblasts derived from SLOS subjects. Three cell lines were selected to be representative of the SLOS phenotypic spectrum (**Table S3**). Compared to an unaffected control, Tfn uptake was greatly reduced in SLOS samples upon culture within LPDS conditions (**Figure 6A, D**), responding similarly to AY9944 treated control fibroblasts. While CME activity was similarly inhibited in all three SLOS fibroblasts when cultured in LPDS conditions, CME function correlated to the total sterol levels of each cell line as determined by GC/MS (**Figure 6B**). Notably, even under cholesterol replete conditions, fibroblasts from the most biochemically severe SLOS cell lines trended towards diminished Tfn internalization, suggesting that exogenous lipid supplementation and lipoprotein is unable to fully rescue CME function (**Figure 6C**). These data demonstrate a direct link between sterol homeostasis and CME within SLOS subjects, while suggesting functional deficits in CME may have clinical impact within this patient population.

DISCUSSION

Here, we present evidence for the role of sterols in aiding membrane curvature generation during CME by direct observation of alterations in membrane-bending dynamics. Ultrastructure analysis and polTIRFM of clathrin-coated pits under increasing degrees of cholesterol depletion reveal domed and shallow structures respectively, supporting the model that redistribution of

sterols impacts the energetics of membrane bending during both initiation of curvature and formation of the endocytic neck. We demonstrate that total sterol levels correlate with CME productivity in multiple cell types, consider the structural requirements of sterols to support CME, and find functional CME impairment in Smith-Lemli-Opitz patient samples, exhibiting *DHCR7* mutations and reduced sterol levels.

As the single most abundant lipid within the PM, the presence of cholesterol is critical to the biophysical properties of the bilayer. Our findings suggest that cholesterol is relevant to multiple stages of PM bending during an endocytic event, consistent with a structural role in alleviating energetic stress associated with curvature formation. Rescue of trafficking deficits using sterol substitution strategies suggest that the structural requirements for CME mirror the sterol requirements for supporting L_o domain formation, or minimally lipid packing capacity (Megha et al., 2006; Wenz and Barrantes, 2003). This is in agreement with a report by Kim, et.al. which concludes that the presence of the 3β -hydroxyl and ability of a sterol to support ordered domain formation is necessary for endocytosis (Kim et al., 2017). In our hands, desmosterol behaved most similarly to cholesterol while the strongly raft promoting sterols, 7DHC and lathosterol, compensated for cholesterol to a lesser degree. While this observation does not imply stable PM rafts nor exclude regulation of membrane protein effectors, it is notable given a large body of work implicating membrane phase separation within curved membranes (Bacia et al., 2005; Bruckner et al., 2009; Hilgemann et al., 2020; Huttner and Zimmerberg, 2001; Jülicher and Lipowsky, 1993, 1996; Pandit et al., 2004). Budding theory originally proposed by Jülicher and Lipowsky (Jülicher and Lipowsky, 1993, 1996) predicts that the boundaries between L_o and L_d domains tend to be minimized by “line tension”, such that induction of curvature progresses to budding of either domain. This behavior has recently been reviewed as a driving force in other clathrin-independent endocytic pathways (Hilgemann et al., 2020) and during CME (Frey and Schwarz, 2020). Immediate stress relaxation due to fast sterol transbilayer flip-flop is an

underlying assumption within this model (Jülicher and Lipowsky, 1996) and, moreover, rapid sterol flip-flop explains how sterol structure determines curvature and budding phenomena within GUVs undergoing osmotic deflation (Bacia et al., 2005). During CME progression, a high energetic cost is associated with the initiation of membrane bending and is problematic for vesicle neck formation (Lentz et al., 2002). *In vitro* studies in lipid monolayers demonstrate that lipid distribution becomes non-uniform following bending with cholesterol concentrating to regions of high curvature (Wang et al., 2007). Interestingly, the effects of cholesterol on membrane rigidity (Henriksen et al., 2004; Song and Waugh, 1993) would appear contradictory to its observed effect in facilitating membrane curvature. Biophysical studies in artificial membranes suggest cholesterol flip-flop may relax tension in L_o domains despite their increased stiffness (Bacia et al., 2005; Bruckner et al., 2009), explaining why lipid rafts may be preferential sites for membrane budding and endocytosis (Conner and Schmid, 2003; Huttner and Zimmerberg, 2001). Our findings suggest that sterol-mediated stress relaxation behavior previously described within model membranes (Bacia et al., 2005; Bruckner et al., 2009) may translate to membrane remodeling *in vivo*. While it's possible the relationship between sterol abundance and PM curvature involves a previously unrecognized and complex lipid-sensing mechanism, it seems parsimonious to consider that cholesterol passively facilitates membrane remodeling through rapid transbilayer flip-flop, relieving membrane stress between the inner and outer PM leaflets during pit formation to support high curvature (**Figure 7**).

In addition to modifying membrane bilayer architecture directly, sterol homeostasis may also impact CME through secondary mechanisms. Membrane curvature itself may influence the intrinsic activity of enzymes involved in membrane traffic (Bozelli et al., 2018). Furthermore, recruitment of curvature-sensing structural proteins may be affected, including ENTH/ANTH and BAR domain proteins which further promote PM curvature through hydrophobic insertion into the membrane or scaffolding mechanisms (Haucke and Kozlov, 2018). Cholesterol's influence

on lateral packing and ordering of the local lipid environment may also disrupt PI(4,5)P₂ organization (Kwik et al., 2003), which may affect association of PH domain containing CME proteins. Packing of cargo is likely less affected by sterol depletion, as previous studies have demonstrated that the concentration of transferrin receptor (TfR) increased in accordance with coated pit surface area (Rodal et al., 1999). Though not required for CME in mammalian cells, actin assembles during the final stages of CME where it is thought to aid in the transition of domed to spherical shaped pits and vesicle scission under conditions where membrane tension is increased (Boulant et al., 2011; Hassinger et al., 2017; Skruzny et al., 2012). While disruption of actin integrity did not appreciably change CME responses to disruption of sterol synthesis, acute cholesterol depletion with MβCD is known to lead to stabilization of the actin cytoskeleton and increase membrane-cytoskeleton adhesions through micropipette aspiration and optical tweezer membrane tether experiments (Khatibzadeh et al., 2012). Given that the balance between endocytosis/exocytosis and the cytoskeleton are highly coordinated through membrane tension (Diz-Munoz et al., 2013), a holistic understanding of membrane surface dynamics in response to cholesterol depletion may lead to a more cohesive understanding of cellular responses and those observed within SLOS (Jiang et al., 2010).

As our findings favor a biophysical rather than a biochemical explanation for cholesterol's role in facilitating membrane curvature, we would predict the findings presented here for CME may extend to other endocytic and membrane remodeling processes. Indeed, several other endocytic pathways, including caveolar, clathrin-independent, macropinocytosis, and clathrin- and caveolae/caveolin1-independent endocytosis, are known to be sensitive to cholesterol depletion (Doherty and McMahon, 2009). Within our own TEM analysis, we observed flattened and disassembled caveolar structures, consistent with reports that caveolin-1 may both sense and induce membrane curvature by cholesterol clustering (Krishna and Sengupta, 2019). Cholesterol also appears to be necessary for fusion pore kinetics during exocytosis as observed

in platelets (Ge et al., 2010), chromaffin cells (Wang et al., 2010), and neurons (Najafinobar et al., 2016). In the limited studies where endocytosis and exocytosis have been measured simultaneously, sterol depletion appears to principally impair endocytosis (Subtil et al., 1999; Yue and Xu, 2015). Furthermore, intracellular recycling of TfR to the cell surface also appeared to be unaffected, suggesting the PM may be more susceptible to sterol depletion than other intracellular compartments (Subtil et al., 1999). A plausible explanation for these phenomena may lie in the transbilayer asymmetry of PM sterol distribution and directionality of sterol flux as lipid gradients maintained across organelles have been proposed to provide a self-organizing directionality to both the secretory and endosomal pathways (Levental et al., 2020).

Our studies suggest that reduced endocytic activity may be an important contributor to the cellular phenotypes observed within SLOS, potentially providing a unifying explanation for some of the varied and highly tissue-specific abnormalities reported within SLOS. As endocytosis and exocytosis are functionally coupled, impairment of CME may be linked to trafficking abnormalities previously described within secretory granules of the pancreas, pituitary and adrenal glands in *Dhcr7*^{-/-} and *Sc5d*^{-/-} mouse models (Gondre-Lewis et al., 2006). Similarly, impaired endocytic traffic may lead to dysregulation of the endosomal-lysosomal pathway which may be related to both aberrant Sonic Hedgehog signaling (Blassberg and Jacob, 2017; Blassberg et al., 2016) and compromised phagosome maturation (Futter et al., 2012; Ramachandra Rao et al., 2018) described within SLOS models. During embryonic development, CME is necessary for maintenance of pluripotency through regulation of pluripotent and differentiation signals (Narayana et al., 2019). Impaired Wnt/ β -catenin and cadherin-related signaling was recently identified within SLOS patient-derived stem cells which display precocious differentiation (Francis et al., 2016), suggesting a possible role for CME deficits within cell fate decisions and developmental pathologies. Within the nervous system, dynamic communication between neurons is highly dependent upon intact vesicular trafficking and CME

for rapid neurotransmission, receptor desensitization, dendritic spine plasticity, and myelination (Murthy and Stevens, 1998; Saheki and De Camilli, 2012; Watanabe et al., 2014; Winterstein et al., 2008). Interestingly, impairment of CME through *DNM1* mutations causes epileptiform discharges (Dhindsa et al., 2015), a prevalent finding in SLOS individuals (Schreiber et al., 2014). Rapid endocytosis is also of significant relevance to the brush boarder of small intestinal enterocytes whose microvilli exhibit high curvature and are highly enriched in cholesterol (Hansen et al., 2003; Huttner and Zimmerberg, 2001). As such, endocytic deficits could contribute to feeding difficulties and gastrointestinal intolerance commonly observed in SLOS. Previous studies have also observed increased levels of Tfn protein present within the retina of AY9944-treated rats (Tu et al., 2013) and cerebrospinal fluid isolated from SLOS subjects (Cologna et al., 2016), possibly due to reduced TfR-mediated internalization. The varied and complex role of cholesterol in different cellular functions indicates that malformations and functional deficits observed within disorders of cholesterol synthesis could be due to competing and disparate effects of the lipid. As such, the extent to which impairment of CME contributes to disease pathogenesis and clinical phenotypes observed will require additional careful study.

Current treatment strategies for SLOS include empirical dietary cholesterol supplementation, which has been shown to normalize plasma cholesterol with variable outcomes on sterol precursors (Linck et al., 2000). However, cholesterol supplementation does not universally correct plasma cholesterol concentrations nor does plasma cholesterol necessarily reflect tissue sterol content. Furthermore, the benefits of dietary cholesterol supplementation have been modest (Svoboda et al., 2012) and longitudinal studies indicate that baseline cholesterol levels are a better predictor of developmental trajectory than age of supplementation or increase in cholesterol serum levels (Sikora et al., 2004). Depressed CME activity may be linked to suboptimal efficacy of cholesterol supplementation by further reducing cholesterol bioavailability. Our studies indicate that once CME is effectively inhibited by AY9944, recovery

of clathrin trafficking requires upwards of 6-12 hours to resume in the presence of FBS, yet recovers within minutes if cholesterol is delivered directly to the PM via M β CD carrier. Interesting, a secondary defect in LDL metabolism has been described within SLOS fibroblasts, where decreased LDL apolipoprotein degradation products were observed (Wassif et al., 2002). While this mechanism likely involves impaired NPC1 function(Wassif et al., 2002), it's possible that a defect in LDL internalization due to impaired CME efficiency also contributes to this effect. A potential strategy to overcome impaired internalization and utilization of cholesterol *in vivo* may be direct delivery of cholesterol via a mechanism independent of receptor-mediated endocytosis, such as a cyclodextrin (CD) carrier. This seemingly straightforward approach could preferentially target PM properties likely mediating CME impairment as well as bypass impaired lysosomal function, necessary for liberation of cholesterol esters. Furthermore, our sterol substitution studies indicate that cholesterol precursors are more readily mobilized by M β CD, with cholesterol preferentially retained. Thus, cyclodextrin-mediated cholesterol delivery may have the added benefit of aiding clearance of sterol precursors. CDs have been widely studied for various applications within pharmacology (Vecsernyes et al., 2014). Most recently “empty” 2-Hydroxypropyl- β -Cyclodextrin (HP β CD) received regulatory approval for treatment of Niemann-Pick Type C1, currently undergoing Phase II and III clinical trials (Ory et al., 2017). A reversal of this strategy as a cholesterol delivery mechanism in SLOS could theoretically overcome several challenges hindering current cholesterol supplementation therapy.

In summary, we find that maintenance of cholesterol homeostasis is essential for efficient clathrin mediated endocytosis, demonstrate an active role for sterols in mediating membrane remodeling during this process, and provide mechanistic detail into the sterol functional requirements necessary for this process. While the extent to which altered CME dynamics and function contribute to the pathogenesis of disorders of cholesterol metabolism is unclear, further investigation into CME's role within these orphan disease is warranted.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.H.A. and K.R.F.; Methodology, R.H.A., K.R.F., A.D.H., B.L.S., J.G.K.; Investigation, R.H.A., M.M.S., K.A.S., B.L.S., and E.M.B.; Software, H.V. and B.L.S; Writing—Original draft, R.H.A; Writing—Review & Editing, R.H.A, K.R.F., A.D.H, B.L.S, E.M.B, K.A.S., J.W.T; Funding Acquisition, R.H.A. and K.R.F.; Supervision, K.R.F., A.D.H, and J.W.T.

DECLARATION OF INTERESTS

The authors declare no competing or financial interests.

FIGURE TITLES AND LEGENDS

Figure 1. Sterol homeostasis is required for endogenous clathrin trafficking. **A)** Schematic illustrating the final steps of post-squalene cholesterol synthesis. Structural alterations of sterol precursors relative to cholesterol are indicated in red. Small molecules used in this study are noted in blue. **B)** Sterol profiles of HEK293T hCLTA^{EN}-mTq2 cells cultured in cholesterol-replete FBS or 7.5% delipidated (LPDS) serum ± treatment with Atorvastatin, AY9944, or U18666A for 48 h. Acute cholesterol depletion was achieved by 1h MβCD treatment (Mean ± SD). **C)** FBS prevents AY9944-mediated CME trafficking defects. Scale bar = 10 μm. **D)** Tfn uptake under sterol depleted conditions relative to controls cultured in 7.5% LPDS for 48 h (Mean ± SD). ***, P < 0.001; ****, P < 0.0001; one-way ANOVA (F(4,40) = 38.57, p < 0.0001) and Dunnett's test versus LPDS control (N = 9 biological replicates from 3 independent experiments, 2,000 – 4,000 cells per replicate). **E)** Sterol depletion results in accumulation of clathrin at the cell periphery and functional impairment of Tfn internalization by confocal microscopy in HEK293T hCLTA^{EN}-mTq2 cells. Scale bar = 10 μm. **F, G)** Tfn uptake correlates with sterol content by GC/MS analysis (Mean ± SD). N = 3 biological replicates for Tfn uptake and N = 2 biological replicates for sterol analysis performed in parallel.

Figure 2. AY9944 induced clathrin trafficking deficits are rescued by direct cholesterol loading with MβCD. **A)** Overview of recovery conditions. Following AY9944 sterol depletion, HEK293T hCLTA-Tq2^{EN} cells were exposed to cholesterol-loaded MβCD (MβCD-Chol, and chased in LPDS media) **[Top]**, supplemented continuously with lipoprotein-rich 15% FBS in the presence of AY9944 **[Middle]**, or incubated continuously in 7.5% LPDS media in the absence of AY9944 to allow endogenous cholesterol synthesis **[Bottom]**. **B)** Mid-plane live-cell confocal microscopy of HEK293T hCLTA-Tq2^{EN} following addition of transferrin-conjugated to AF-555 (Tfn-555) during recovery as described in (A). Representative images from different fields of view shown. Tfn and clathrin distributions in normal and arrested CME **[Inset]**. Scale bar = 10

μm. C) Corresponding cellular sterol profiles quantified by GC/MS (Mean ± SD). N = 3 biological replicates from independent experiments. Dotted and dashed lines represent total sterol abundance of untreated FBS and LPDS cultured cells, respectively.

Figure 3. Loss of cholesterol homeostasis disrupts clathrin-coated pit dynamics. A)

Representative polTIRFM kymographs comparing SK-MEL-2 (hCLTA-mTq2^{EN}/hDNM2-eGFP^{EN}) cells grown in 10% LPDS [**Left**] to moderate sterol depletion with addition of 2.5 μM AY9944 [**Middle**] or severe sterol depletion with 5 mM MβCD [**Right**]. Positive curvature generation is indicated by the ratio of P/S fluorescence in Dil labeled membrane. Time = 400 s. Scale bar = 1

μm. **B)** Representative kymograph of CLTA-mTq2, membrane bending (P/S), and DNM2-eGFP and corresponding intensity traces during a single CME event. **C)** Unique clathrin events tracked per cell area analyzed across a 400 s time-lapse acquisition (Mean ± SD). *, P < 0.05; ****, P < 0.0001; one-way ANOVA (F(4,25) = 18.86, p < 0.0001) and Dunnett's test versus LPDS control (N = 6 cells from 2-4 independent experiments, 3,000-30,000 tracks analyzed per cell). **D)** Number of persistent tracks (lifetime > 400 s) per cell area analyzed (Mean ± SD).

Adjusted P values; *, P < 0.05; Welch ANOVA (F(4, 11.43) = 7.531, p = 0.0032) and Dunnett's T3 test versus LPDS control (N = 6 cells from 2-4 independent experiments). **E)** Productive CME events per cell area (Mean ± SD). *, P < 0.05; **, P < 0.01; Welch ANOVA (F(4,11.24) = 8.948, p = 0.0017) and Dunnett's T3 test versus LPDS control (N = 6 cells from 2-4 independent experiments). **F)** Average clathrin lifetimes per cell across the indicated treatment groups (Mean ± SD). Lifetime analysis was limited by truncation of clathrin tracks extending beyond the first or last frame (400 s). **, P < 0.01; ***, P < 0.001; one-way ANOVA (F(4,25) =

10.46, p < 0.0001) and Dunnett's test versus LPDS control (N= 6 cells from 2-4 independent experiments). **G)** Average lifetime of observable CME events per cell across the indicated treatment groups (Mean ± SD). Lifetime of CME limited to observed events > 400 s, such that true lifetimes exceed the estimates presented (400 s). *, P < 0.05; one-way ANOVA (F(4,15) =

4.408 , $p < 0.0312$) and Dunnett's test versus LPDS control (N= 6 cells from 2-4 independent experiments). **H**) Relative frequency of clathrin tracks observed associated with curvature generation (Mean $\pm$ SEM, N = 6 cells). Flat clathrin tracks were classified as exhibiting neither curvature nor dynamin recruitment, may represent persistent tracking or abortive clathrin events visiting the TIRM field. Curved clathrin events were classified as exhibiting positive P/S curvature signal, but failed to recruit dynamin. Curved CME events were classified by the ability of clathrin positive events to recruit dynamin.

Figure 4. Aberrant clathrin-coated pit ultrastructure under conditions of sterol depletion.

A) Representative platinum-replica TEM images from LPDS cultured cells, AY9944 treated cells, and M β CD treated cells. **B**) Relative frequency of clathrin structures by curvature classification (Mean $\pm$ SEM). **C**) Area of unroofed cells covered by clathrin structures (Mean $\pm$ SD). **D**) Histogram of spherical clathrin structures showing the distribution of coated pit asymmetry by aspect ratio (major/minor width). **E**) Average area of clathrin-coated structures by curvature classification relative to LPDS controls (Mean $\pm$ SD). ****, $P < 0.0001$; one-way ANOVA ($F(4,15) = 12.91$, $p < 0.0001$) and Dunnett's test versus LPDS control. N = 4 replicas per condition from two independent experiments. Total number of clathrin structures analyzed: FBS = 1164; LPDS = 988; AY9944 = 1700; Atorvastatin = 1003; M β CD = 3818. **F**) Representative platinum-replica TEM images of caveolae ultrastructure and relative frequency of observed structures by curvature classification (Mean $\pm$ SEM). **G**) Total number of caveolae structures on the membrane per area (Mean $\pm$ SEM). N = 3-4 replicas per condition from two independent experiments. Total number of caveolae structures analyzed: FBS = 171; LPDS = 89; AY9944 = 21; Atorvastatin = 31; M β CD = 13.

Figure 5. Sterol mediated phase separation is required to support CME. The ability of structurally diverse sterols to rescue CME inhibition due to sterol depletion by AY9944 treatment was evaluated by direct delivery of sterols to the PM via M β CD carrier. **A**) Summary of physical

properties and phase separation behavior of sterols. Topological Polar Surface Area (TPSA) computed surface sum over all polar atoms. Ordered lipid (L_o) domain (raft) stabilizing (+) or disrupting (-) sterols indicated relative to cholesterol (++) . Refer to **Table S2** for additional details. **B)** Sterol profiles of AY9944 treated HEK293T cells following 1h incubation with sterol loaded M β CD (Mean $\pm$ SD). N = 4 independent biological replicates from two M β CD-sterol preparations. **C)** Tfn uptake relative to controls cultured in 7.5% LPDS for 48 h (Mean $\pm$ SD). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; one-way ANOVA (F(12, 51) = 11.23, p < 0.0001) and Dunnett's test versus LPDS control (N = 5 biological replicates from 3 independent experiments, ~1,500 cells per replicate). Cholesterol, desmosterol, 7DHC, and lathosterol support formation of L_o domains in model membranes as designated. **D)** Representative confocal images taken mid-plane following 30 min incubation with AF-555 conjugated transferrin (Tfn). Scale bar = 20 μ m. (c), Commercially available, pre-loaded M β CD-Chol.

Figure 6. CME dynamics are inhibited within Smith-Lemli-Opitz syndrome patient-derived fibroblasts. **A)** Representative images demonstrating AF-555 conjugated Tfn uptake in control and SLOS patient-derived fibroblasts grown under cholesterol replete and deplete conditions. Scale bar 50 μ m. **B)** Validation of SLOS fibroblast biochemistry by GC/MS analysis. **C)** Tfn uptake relative to unaffected BJ fibroblasts grown in 15% FBS for 10 d (Mean $\pm$ SEM). Adjusted P values, one-way ANOVA (F(3,10) = 2.916 , p = 0.0869) and Dunnett's test versus BJ control (N = 3-4 independent replicates, 25 cells per replicate). **D)** Tfn uptake relative to unaffected BJ fibroblasts grown in 7.5% LPDS for 10 d (Mean $\pm$ SEM). **, P < 0.01; ***, P < 0.001, one-way ANOVA (F(4,13) = 9.464 , p = 0.0008) and Dunnett's test versus BJ control (N = 2-4 independent replicates, 25 cells per replicate).

Figure 7. Model of cholesterol-mediated stress relaxation during clathrin-mediated endocytosis. Proposed mechanism of sterol dynamics during membrane remodeling and associated energy landscape of local curvature generation during CME. As sterols influence the

683 tight packing of lipid bilayers, rapid cholesterol interleaflet flip-flop may relieve compression and
 684 expansion of the PM leaflets to negate energy costs associated with deformation of the
 685 membrane [inset]. This cholesterol-mediated stress relaxation reduces the energetic
 686 requirements necessary for membrane remodeling. Experimental evidence presented herein
 687 supports the model that cholesterol-mediated stress relaxation may facilitate initiation of
 688 curvature generation as well as formation of the highly curved vesicle neck. Tight lipid packing
 689 of sterols, thought to underlie formation of L_o and L_d domains (e.g. lipid rafts), may serve a
 690 driving force for membrane budding as increasing curvature minimizes line tension between
 691 phase boundaries.

MATERIALS AND METHODS

Cell culture. HEK293T (parental and hCLTA^{EN}-Tq2) and human SK-MEL-2 (hCLTA^{EN}-Tq2 hDNM2^{EN}-eGFP, as previously described(Scott et al., 2018)) were maintained in DMEM (Gibco, 4.5 g/L glucose, 110 mg/L pyruvate), supplemented with 10% (v/v) FBS (Hyclone), and 1,000 U/mL penicillin/streptomycin. For inhibition of cholesterol synthesis, cells were rinsed twice in serum-free DMEM and cultured under cholesterol deplete conditions in 7.5% delipidated serum (LPDS) for 48 h with small molecule inhibitors AY9944 (2.5 μ M, Cayman Chemical; *DHCR7* inhibitor), U18666A (20 nM, Cayman Chemical; *DHCR24* inhibitor), Atorvastatin (1 μ M, Cayman Chemical), or Simvastatin (Cayman Chemical), or at concentrations as otherwise specified. Acute sterol depletion was achieved by 1 h incubation with 5 mM M β CD (Alfa Aesar, 1303.31 g/mol) at 37°C. De-identified fibroblasts cultured from skin punch biopsies from Smith-Lemli-Opitz subjects (kind gift from Dr. Forbes Porter, NICHD) were maintained in DMEM (Gibco, 4.5 g/L glucose, 110 mg/L pyruvate), supplemented with 15% (v/v) FBS (Hyclone), and 1,000 U/mL penicillin/streptomycin. SLOS fibroblasts were rinsed twice in serum-free DMEM and cultured for 10 days in 7.5% (v/v) LPDS to induce biochemical profiles.

CRISPR/Cas9 gene editing of clathrin light chain. C' terminal targeting of the *CLTA* gene to enable fluorescence tracking with mTuroquoise2 (mTq2) was performed using a homology-directed repair strategy as previously described (Anderson et al., 2018; Scott et al., 2018). Briefly, a guide RNA (5'-GCAGATGTAGTGTTCACCA-3') targeting the open reading frame in the immediate vicinity of the stop codon was cloned into the Cas9 expression vector pX330-U6-Chimeric_BB-CBh-hSpCas9 (gift from Feng Zhang (Cong et al., 2013), Addgene plasmid #42230). The donor vector consisted of 1 kB homology arms flanking a cassette containing puromycin N-acetyl-transferase expressed in-frame with *CLTA* and Tq2 via the self-cleavable peptide sequence P2A. 1×10^6 HEK293T cells were transfected with 4 μ g donor and 2.5 μ g pX330 Cas9 plasmid (CalPhosTM Mammalian Transfection kit, Clontech). After 72 h, Tq2

positive cells were isolated with a FACSJazz Cell Sorter (BD Biosciences) and characterized as described previously (**Figure S1**) (Anderson et al., 2018).

Preparation of delipidated serum. To facilitate cell culture under defined lipid conditions, fetal bovine serum (FBS) devoid of sterols, triglycerides, and other neutral lipids was prepared based on previously described techniques (Cham and Knowles, 1976). To prevent oxidation due to trace peroxides, 0.1 mg ethylenediamine tetraacetate (EDTA) was added per 50 mL FBS. Serum was mixed 1:2 with organic phase (consisting of 3:2 ratio diisopropyl ether:n-butanol) followed by vigorous stirring for 1 h protected from light. After centrifugation at 4°C for 15 min at 2200 rpm, the aqueous layer was collected and lyophilized. Following resuspension in molecular grade H₂O, ITS-G supplement (Invitrogen) was added, the solution filter-sterilized, and stored at -20°C. GC/MS analysis indicated nearly undetectable cholesterol levels. Delipidated serum (LPDS) was substituted for regular FBS to stimulate expression of de novo cholesterol biosynthesis.

Sterol loading of M β CD. Methyl- β -cyclodextrin (M β CD)-cholesterol complex commercially available (Sigma, C4951). For M β CD-sterol complexation, 50 mg/mL sterol stocks in 1:1 chloroform:methanol were prepared for desmosterol, lathosterol, cholestenone, cholesterol sulfate, 4 β -hydroxycholesterol (Avanti Polar Lipids), 7DHC and cholesterol (Sigma or Avanti Polar Lipids). Stoichiometry of M β CD-cholesterol complexes typically consists of one cholesterol molecule entrapped within two CDs (1:2 molar ratio). We found optimal loading conditions using a 1:7 molar ratio, where desired sterol concentration was dried under nitrogen flow, followed by addition of 5 mM M β CD (Alfa Aesar, 1303.31 g/mol) prepared in serum-free DMEM. Sterol suspensions were sonicated for 5 min and incubated at 37°C with agitation overnight. Sterol crystals were subsequently removed via 0.45 μ m filtration. Sterol loading rescue experiments were performed by incubation for 1 h at 37°C supplemented with 0.5%

BSA, followed by removal and addition of 7.5% LPDS for imaging or subsequent analysis to limit prolonged exposure to M β CD.

GC/MS sterol analysis. Cell pellets flash frozen on dry ice were reconstituted in 1 mL of water and lysed by successive freeze/thaw cycles in a 50°C bead bath. 50 μ L per sample was removed for protein quantification (MicroBCA Protein Assay, Pierce Biotechnology). 1 mL saponification buffer containing 7% KOH in 92% ethanol with 10 μ g/mL coprostan-3-ol (Abcam) was added to 900 μ L cell lysate. Following saponification at 60°C for 1 h, an additional 1 mL of water was added to each sample and the aqueous phase extracted with 3 mL ethyl acetate by vortexing and centrifugation at 2200 rpm. The organic phase was then extracted with 2 mL water, concentrated to dryness by heating at 50°C under nitrogen flow, residue dissolved in 50 μ L pyridine (CHROMASOLV Plus, Sigma), and sterols derivatized in 50 μ L N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS, Thermo Fisher TS-38831) at 60°C for 1 h. Sterol levels were determined by GC/MS analysis as previously described (Kelley, 1995). Samples were analyzed by automatic injection of 1 μ L of the derivatized sterol mixture into an Agilent 7890 GC using a split injection port (4 mm ID x 78.5 mm quartz wool liner, Restek 23309) leading to a 0.18 mm ID x 20 m 1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane column (Restek, 43602). Helium was used as a carrier gas at a linear rate of 46.9 cm/sec. After 0.5 min at 170°C, the oven temperature was raised to 250°C at 18°C/min, then to 280°C at 3°C/min, and finally to 320°C at 20°C/min and held for 7 min. An Agilent 5977B mass spectrometer was operated in the electron impact mode at 70 eV with an ion source temperature of 275°C. Analysis was performed using MassHunter software. Identification of TMS ethers of natural sterols was determined through comparison to commercially available standards for cholesterol, 7DHC, lathosterol, and desmosterol (Avanti Polar Lipids, Inc.), as well as comparison to MS spectra through the National Institute of Standards and Technologies Standard Reference Database when available.

Identification of 8DHC was inferred as an isomer of 7DHC and comparison to SLOS fibroblasts; zymostenol identification was based on spectra from Conradi-Hünemann-Happle syndrome (CDPXD2) fibroblasts. Retention times and mass to charge (m/z) ratios are summarized in **Table S1**. Representative MS fragmentation patterns available upon request. TMS derivatives of sterols exhibiting abundance <3% were excluded from analysis. For peak quantitation, sterol abundance was normalized to both the internal standard (coprostanol) and protein concentration. Data is presented relative to control samples using GraphPad Prism software.

Transferrin uptake assay. HEK293T cells were plated onto 0.1% gelatin coated 12 mm coverslips or 96 well glass bottom plates (Cellvis) and treated for 48 h under respective treatment conditions. For AY9944 rescue experiments, cells were placed in DMEM/BSA (DMEM containing 0.5% w/v BSA; bovine serum albumin) alone or DMEM/BSA containing 5 mM M β CD-sterol complexes for 1 h in a 37°C humidified incubator containing 5% CO₂. HEK293T cells were then incubated with transferrin (Tfn) conjugated to Alexa Fluor™ 555 (25 ug/mL, Invitrogen) for 30 mins at 37°C, followed by rinsing in ice-cold PBS, and fixation in 4% paraformaldehyde for 20 min. For high-content imaging analysis, 24 well glass bottom plates (Cellvis) were imaged using CX7 Rescue High-Content Screening (HCS) Navigator software (PerkinElmer, Waltham, MA) and standard HCS imaging protocol.

Patient fibroblasts were plated onto 0.1% gelatin coated 12 mm coverslips 3 days prior to imaging. Uptake was performed as described above using Tfn conjugated to Alexa Fluor™ 555 (25 ug/mL, Invitrogen) for 15 min at 37°C. Following incubation, cells were rinsed with ice-cold PBS. Cells were fixed in 4% paraformaldehyde for 20 min, rinsed three times in PBS, and mounted onto glass slides with Dako mounting media (Agilent). Cell outlines were traced using the freehand selection tool and the integrated density of 25 cells from 8-12 fields was quantified using ImageJ software (v1.8.0).

Confocal microscopy. Images were captured using a Nikon A1R resonant scanning multispectral confocal microscope (Nikon Instruments, Inc. Melville, NY), equipped with a live cell chamber (37°C with humidified 5% CO₂), Nikon Ti Perfect Focus system, and NIS-Elements analysis software (Nikon). 48 h prior to live-cell imaging, HEK293T cells plated onto 0.1% gelatin-coated FluoroDish™ culture plates (35 mm, World Precision Instruments Inc.) were rinsed twice with DMEM (Gibco) to remove residual lipids and respective treatments were added. Prior to imaging, culture media was refreshed. Time-lapse videos were obtained at 100x magnification (1.45 NA oil objective) at 5-20 s intervals over a 5 min duration.

Polarized TIRF microscopy. SK-MEL-2 hCLTA^{EN}-Tq2 hDNM2^{EN}-eGFP cells were seeded onto fibronectin-coated (Corning) 25 mm coverslips at a density of 8.0 x 10⁴ cells/cm² and imaged within 4-6 hours of plating. To facilitate membrane labeling, a fresh Dil (1,1'-Diocetyl-3,3,3',3'-teramethylindocarbocyanine perchlorate) solution containing 1 µg/mL Dil in 2.5% DMSO/HBSS (Corning) was prepared for each coverslip from a 1 mg/mL Dil stock dissolved in DMSO and stored under nitrogen. After incubating the freshly prepared Dil solution at 37°C for 5 min, 200-300 µL was added dropwise to coverslips containing 1 mL HBSS. Cells were labeled for 20 s with gentle pipetting, rinsed three times in HBSS, and placed into imaging buffer. Imaging buffer for all treatment conditions consisted of Leibovitz's L-15 media supplemented with either 10% FBS or delipidated (LPDS) serum (Hyclone) to monitor basal CME events. Following Dil labeling, coverslips were immediately imaged for no more than 45 min. To ensure consistency in Dil labeling, only cells with mean S polarization intensities between 350-800 were included in the analysis.

As previously detailed, TIRFM images were captured on a custom built Till iMic (Till Photonics, Germany) inverted microscope outfitted with a 60x/1.49 NA oil immersion objective and environmental chamber kept at 37°C (Scott et al., 2018). In brief, excitation for mTurquoise2, eGFP, and polTIRF was achieved by 445 (470/22), 488 (510/10), 561 (595/50) nm lasers

respectively, collected on three EMCCD cameras (iXon3 885, Andor Technology). Back focal plane centering was performed daily to optimize the incidence angle using MatLab analysis to map the angles of TIRF reflectance and determine mirror angle adjustments to center the optical plane. 2-point TIRF illumination facilitated polarization of the 561 nm laser to enable monitoring of membrane curvature. Focused excitation at 90° and 270° positions preferentially excite vertically oriented, Dil-labeled membrane (P polarization), while excitation at positions 0° and 180° selectively excite horizontally labeled membrane (S polarization).

Polarized TIRFM image analysis. Image registration was performed to align Turquoise2 and eGFP channels onto the polTIRF channel. Calibration images of fluorescent beads on a glass coverslip were acquired simultaneously on all three detectors and used to determine the geometric transformation during pre-processing. Bias images, acquired by capturing images with a closed shutter, were subtracted from each frame of the raw data. Using MatLab, detection and tracking of fluorescence over time was performed using a modified version of cmeAnalysis software (Aguet et al., 2013) and custom scripts were used for association and classification of mTq2, eGFP, and P/S events (Scott et al., 2018). Detection criteria included a clathrin peak SNR > 4, dynamin SNR >2, clathrin lifetime >7 seconds, and a buffer of two consecutive frames before and after each clathrin track. Faulty tracks were filtered from the analysis due to clathrin events crossing paths with another track and curvature signal unrelated to the tracked clathrin event itself.

Transmission electron microscopy. SK-MEL-2 cells were prepared as described for polTIRF imaging by seeding onto fibronectin-coated 25 mm coverslips and allowed to adhere for 5 h. Coverslips were transferred into 2 mL stabilization buffer (70 mM KCl, 5 mM MgCl₂, 3 mM EGTA, 30 mM HEPES, pH 7.4) prior to unroofing with a 10 mL syringe and 22 gauge, 1.5 in needle containing 2 mL 4% PFA (Electron Microscopy Sciences) diluted in stabilization buffer. Coverslips were transferred to fresh 2% PFA/stabilization buffer and allowed to fix at RT for 20

min. Coverslips were mounted in 2% glutaraldehyde and sealed with VALAP (1:1:1 vasoline, lanolin, paraffin) for shipping. Sample preparation and TEM imaging of platinum cell membrane replicas was performed as previously described (Sochacki et al., 2017; Sochacki et al., 2014). Representative full EM images available upon request.

Statistical analysis. All statistical analysis was performed using GraphPad Prism 8.0.2 (GraphPad Software, Inc., CA, US). Homogeneity of variances was tested by the Brown-Forsythe test. In cases of equal variances, data was analyzed by one-way ANOVA and post hoc Dunnett t-test for multiple comparisons relative to LPDS control group. In cases of unequal variances, Welch ANOVA and post-hoc Dunnett's T3 test was performed. $P < 0.05$ was accepted as significant. Statistical details for each experiment can be found within figure legends.

Data availability. Datasets generated in this study are available from the corresponding authors upon request.

Code availability. Programming generated in this study is available from the corresponding authors upon request.

SUPPLEMENTAL INFORMATION TITLES AND LENGENDS

Video 1. Confocal video of HEK293T hCLTA-mTq2^{EN} cells demonstrating immobilization

of clathrin trafficking at the PM shown in Figure 1. Endogenous clathrin trafficking in

HEK293T cells cultured in 7.5% LPDS without **[Panel 1]** or with addition of AY9944 **[Panel 2].**

Rescue of clathrin trafficking following sterol depletion with AY9944 by addition of cholesterol-

loaded MβCD (MβCD-Chol) **[Panel 3].** Confocal images taken at midplane. Acquisition:

~8s/frame. Frame rate: 15 frames/s.

Video 2. Confocal video of HEK293T hCLTA-mTq2^{EN} cells demonstrating rescue of

clathrin trafficking deficits by delivery of cholesterol or 7DHC via MβCD carrier as shown

in Supplemental Figure 2. Comparison of endogenous clathrin trafficking in HEK293T cells

cultured in 7.5% LPDS **[Panel 1]** or following sterol depletion with 48 h AY9944 treatment

[Panel 2]. AY9944 induced clathrin trafficking deficits could be rescued by sterol reconstitution

with cholesterol-loaded MβCD (MβCD-Chol) **[Panel 3]** or 7DHC-loaded MβCD (MβCD-7DHC)

[Panel 4]. Confocal images taken at midplane. Acquisition: ~16s/frame. Frame rate: 15

frames/s.

Video 3. polTIRFM time-lapse video of SK-MEL-2 hCLTA-mTq2^{EN} hDNM2-eGFP^{EN} cell

shown in Figure 3. Clathrin, dynamin, and membrane curvature dynamics within 10% FBS

control cell. hCLTA-Tq2, Dynamin2-eGFP, and P/S ratio indicating positive curvature generation

pseudocolored cyan, green, and magenta respectively. Acquisition: 2s/frame. Frame rate: 20

frames/s.

Video 4. TIRFM time-lapse videos of SK-MEL-2 hCLTA-mTq2^{EN} hDNM2-eGFP^{EN} cells

under altered sterol conditions as shown in Figure 3. hCLTA-Tq2 pseudocolored cyan,

Dynamin2-eGFP pseudocolored green. Acquisition: 2s/frame. Frame rate: 20 frames/s.

REFERENCES

- Aguet, F., Antonescu, C.N., Mettlen, M., Schmid, S.L., and Danuser, G. (2013). Advances in analysis of low signal-to-noise images link dynamin and AP2 to the functions of an endocytic checkpoint. *Dev Cell* 26, 279-291.
- Anantharam, A., Onoa, B., Edwards, R.H., Holz, R.W., and Axelrod, D. (2010). Localized topological changes of the plasma membrane upon exocytosis visualized by polarized TIRFM. *J Cell Biol* 188, 415-428.
- Anderson, R.H., Kerkvliet, J.G., Otta, J.J., Ross, A.D., Leiferman, P.C., Hoppe, A.D., and Francis, K.R. (2018). Generation of a CLTA reporter human induced pluripotent stem cell line, CRMi001-A-1, using the CRISPR/Cas9 system to monitor endogenous clathrin trafficking. *Stem Cell Research*.
- Avinoam, O., Schorb, M., Beese, C.J., Briggs, J.A., and Kaksonen, M. (2015). ENDOCYTOSIS. Endocytic sites mature by continuous bending and remodeling of the clathrin coat. *Science* 348, 1369-1372.
- Ayee, M.A., and Levitan, I. (2016). Paradoxical impact of cholesterol on lipid packing and cell stiffness. *Frontiers in bioscience* 21, 1245-1259.
- Bacia, K., Schwille, P., and Kurzchalia, T. (2005). Sterol structure determines the separation of phases and the curvature of the liquid-ordered phase in model membranes. *Proc Natl Acad Sci U S A* 102, 3272-3277.
- Batchelder, E.M., and Yazar, D. (2010). Differential requirements for clathrin-dependent endocytosis at sites of cell-substrate adhesion. *Mol Biol Cell* 21, 3070-3079.
- Blassberg, R., and Jacob, J. (2017). Lipid metabolism fattens up hedgehog signaling. *BMC Biol* 15, 95.
- Blassberg, R., Macrae, J.I., Briscoe, J., and Jacob, J. (2016). Reduced cholesterol levels impair Smoothed activation in Smith-Lemli-Opitz syndrome. *Human molecular genetics* 25, 693-705.
- Boulant, S., Kural, C., Zeeh, J.C., Ubelmann, F., and Kirchhausen, T. (2011). Actin dynamics counteract membrane tension during clathrin-mediated endocytosis. *Nat Cell Biol* 13, 1124-1131.
- Bozelli, J.C., Jr., Jennings, W., Black, S., Hou, Y.H., Lameire, D., Chatha, P., Kimura, T., Berno, B., Khondker, A., Rheinstadter, M.C., *et al.* (2018). Membrane curvature allosterically regulates the phosphatidylinositol cycle, controlling its rate and acyl-chain composition of its lipid intermediates. *J Biol Chem* 293, 17780-17791.
- Bruckner, R.J., Mansy, S.S., Ricardo, A., Mahadevan, L., and Szostak, J.W. (2009). Flip-flop-induced relaxation of bending energy: implications for membrane remodeling. *Biophys J* 97, 3113-3122.
- Bucher, D., Frey, F., Sochacki, K.A., Kummer, S., Bergeest, J.P., Godinez, W.J., Krausslich, H.G., Rohr, K., Taraska, J.W., Schwarz, U.S., *et al.* (2018). Clathrin-adaptor ratio and membrane tension regulate the flat-to-curved transition of the clathrin coat during endocytosis. *Nat Commun* 9, 1109.
- Cenedella, R.J. (2009). Cholesterol synthesis inhibitor U18666A and the role of sterol metabolism and trafficking in numerous pathophysiological processes. *Lipids* 44, 477-487.
- Cham, B.E., and Knowles, B.R. (1976). A solvent system for delipidation of plasma or serum without protein precipitation. *J Lipid Res* 17, 176-181.
- Chen, C., and Tripp, C.P. (2012). A comparison of the behavior of cholesterol, 7-dehydrocholesterol and ergosterol in phospholipid membranes. *Biochim Biophys Acta* 1818, 1673-1681.
- Choubey, A., Kalia, R.K., Malmstadt, N., Nakano, A., and Vashishta, P. (2013). Cholesterol translocation in a phospholipid membrane. *Biophysical journal* 104, 2429-2436.
- Cocucci, E., Aguet, F., Boulant, S., and Kirchhausen, T. (2012). The first five seconds in the life of a clathrin-coated pit. *Cell* 150, 495-507.
- Collins, A., Warrington, A., Taylor, K.A., and Svitkina, T. (2011). Structural organization of the actin cytoskeleton at sites of clathrin-mediated endocytosis. *Curr Biol* 21, 1167-1175.

923 Cologna, S.M., Shieh, C., Toth, C.L., Cougnoux, A., Burkert, K.R., Bianconi, S.E., Wassif, C.A., and Porter,
924 F.D. (2016). Altered cerebrospinal fluid proteins in Smith-Lemli-Opitz syndrome patients. *American*
925 *journal of medical genetics Part A* *170*, 2060-2068.

926 Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., *et*
927 *al.* (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* *339*, 819-823.

928 Conner, S.D., and Schmid, S.L. (2003). Regulated portals of entry into the cell. *Nature* *422*, 37-44.

929 Cuniff, C., Kratz, L.E., Moser, A., Natowicz, M.R., and Kelley, R.I. (1997). Clinical and biochemical
930 spectrum of patients with RSH/Smith-Lemli-Opitz syndrome and abnormal cholesterol metabolism.
931 *American journal of medical genetics* *68*, 263-269.

932 Danino, D., Moon, K.H., and Hinshaw, J.E. (2004). Rapid constriction of lipid bilayers by the
933 mechanochemical enzyme dynamin. *Journal of structural biology* *147*, 259-267.

934 Das, A., Goldstein, J.L., Anderson, D.D., Brown, M.S., and Radhakrishnan, A. (2013). Use of mutant 125-I-
935 Perfringolysin O to probe transport and organization of cholesterol in membranes of animal cells.
936 *Proceedings of the National Academy of Sciences* *110*, 10580-10585.

937 Davis, C.G., Lehrman, M.A., Russell, D.W., Anderson, R.G., Brown, M.S., and Goldstein, J.L. (1986). The
938 J.D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes
939 internalization of LDL receptors. *Cell* *45*, 15-24.

940 de Duve, C. (1971). Tissue Fraction-Past and Present. *Journal of Cell Biology* *50*, 20d-55d.

941 Dhindsa, R.S., Bradrick, S.S., Yao, X., Heinzen, E.L., Petrovski, S., Krueger, B.J., Johnson, M.R., Frankel,
942 W.N., Petrou, S., Boumil, R.M., *et al.* (2015). Epileptic encephalopathy-causing mutations in DNM1
943 impair synaptic vesicle endocytosis. *Neurol Genet* *1*, e4-e4.

944 Diz-Munoz, A., Fletcher, D.A., and Weiner, O.D. (2013). Use the force: membrane tension as an organizer
945 of cell shape and motility. *Trends Cell Biol* *23*, 47-53.

946 Doherty, G.J., and McMahon, H.T. (2009). Mechanisms of Endocytosis. *Annual Review of Biochemistry*
947 *78*, 857-902.

948 Doyon, J.B., Zeitler, B., Cheng, J., Cheng, A.T., Cherone, J.M., Santiago, Y., Lee, A.H., Vo, T.D., Doyon, Y.,
949 Miller, J.C., *et al.* (2011). Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited
950 mammalian cells. *Nat Cell Biol* *13*, 331-337.

951 Fernandez, C., Martin, M., Gomez-Coronado, D., and Lasuncion, M.A. (2005). Effects of distal cholesterol
952 biosynthesis inhibitors on cell proliferation and cell cycle progression. *J Lipid Res* *46*, 920-929.

953 Francis, K.R., Ton, A.N., Xin, Y., O'Halloran, P.E., Wassif, C.A., Malik, N., Williams, I.M., Cluzeau, C.V.,
954 Trivedi, N.S., Pavan, W.J., *et al.* (2016). Modeling Smith-Lemli-Opitz syndrome with induced pluripotent
955 stem cells reveals a causal role for Wnt/beta-catenin defects in neuronal cholesterol synthesis
956 phenotypes. *Nat Med* *22*, 388-396.

957 Frey, F., and Schwarz, U.S. (2020). Competing pathways for the invagination of clathrin-coated
958 membranes. *Soft Matter*.

959 Futter, C., Meschede, I.P., Wavre, S.T., Silva, M.L.D., Tolmachova, T., and Seabra, M.C. (2012).
960 Phagosome Maturation And Interactions With The Endocytic Pathway In Retinal Pigment Epithelial Cells.
961 *Investigative Ophthalmology & Visual Science* *53*, 3701-3701.

962 Gaoua, W., Chevy, F., Roux, C., and Wolf, C. (1999). Oxidized derivatives of 7-dehydrocholesterol induce
963 growth retardation in cultured rat embryos: a model for antenatal growth retardation in the Smith-
964 Lemli-Opitz syndrome. *J Lipid Res* *40*, 456-463.

965 Ge, S., White, J.G., and Haynes, C.L. (2010). Critical role of membrane cholesterol in exocytosis revealed
966 by single platelet study. *ACS chemical biology* *5*, 819-828.

967 Gondre-Lewis, M.C., Petrache, H.I., Wassif, C.A., Harries, D., Parsegian, A., Porter, F.D., and Loh, Y.P.
968 (2006). Abnormal sterols in cholesterol-deficiency diseases cause secretory granule malformation and
969 decreased membrane curvature. *J Cell Sci* *119*, 1876-1885.

970 Gou-Fabregas, M., Macia, A., Anerillas, C., Vaquero, M., Jove, M., Jain, S., Ribera, J., and Encinas, M.
971 (2016). 7-dehydrocholesterol efficiently supports Ret signaling in a mouse model of Smith-Opitz-Lemli
972 syndrome. *Sci Rep* 6, 28534.
973 Grassart, A., Cheng, A.T., Hong, S.H., Zhang, F., Zenzer, N., Feng, Y., Briner, D.M., Davis, G.D., Malkov, D.,
974 and Drubin, D.G. (2014). Actin and dynamin2 dynamics and interplay during clathrin-mediated
975 endocytosis. *J Cell Biol* 205, 721-735.
976 Hamilton, J.A. (2003). Fast flip-flop of cholesterol and fatty acids in membranes: implications for
977 membrane transport proteins. *Curr Opin Lipidol* 14, 263-271.
978 Hansen, G.H., Pedersen, J., Niels-Christiansen, L.L., Immerdal, L., and Danielsen, E.M. (2003). Deep-apical
979 tubules: dynamic lipid-raft microdomains in the brush-border region of enterocytes. *The Biochemical*
980 *journal* 373, 125-132.
981 Hassinger, J.E., Oster, G., Drubin, D.G., and Rangamani, P. (2017). Design principles for robust
982 vesiculation in clathrin-mediated endocytosis. *Proceedings of the National Academy of Sciences*,
983 201617705.
984 Haucke, V., and Kozlov, M.M. (2018). Membrane remodeling in clathrin-mediated endocytosis. *J Cell Sci*
985 131.
986 Henriksen, J., Rowat, A.C., and Ipsen, J.H. (2004). Vesicle fluctuation analysis of the effects of sterols on
987 membrane bending rigidity. *European biophysics journal : EBJ* 33, 732-741.
988 Hilgemann, D.W., Lin, M.J., Fine, M., and Deisl, C. (2020). On the existence of endocytosis driven by
989 membrane phase separations. *Biochimica et biophysica acta Biomembranes* 1862, 183007.
990 Hirama, T., Lu, S.M., Kay, J.G., Maekawa, M., Kozlov, M.M., Grinstein, S., and Fairn, G.D. (2017).
991 Membrane curvature induced by proximity of anionic phospholipids can initiate endocytosis. *Nat*
992 *Commun* 8, 1393.
993 Honda, M., Tint, G.S., Honda, A., Nguyen, L.B., Chen, T.S., and Shefer, S. (1998). 7-Dehydrocholesterol
994 down-regulates cholesterol biosynthesis in cultured Smith-Lemli-Opitz syndrome skin fibroblasts. *J Lipid*
995 *Res* 39, 647-657.
996 Huttner, W.B., and Zimmerberg, J. (2001). Implications of lipid microdomains for membrane curvature,
997 budding and fission. *Current opinion in cell biology* 13, 478-484.
998 Irons, M., Elias, E.R., Salen, G., Tint, G.S., and Batta, A.K. (1993). Defective cholesterol biosynthesis in
999 Smith-Lemli-Opitz syndrome. *Lancet (London, England)* 341, 1414.
1000 Jiang, X.S., Wassif, C.A., Backlund, P.S., Song, L., Holtzclaw, L.A., Li, Z., Yergey, A.L., and Porter, F.D.
1001 (2010). Activation of Rho GTPases in Smith-Lemli-Opitz syndrome: pathophysiological and clinical
1002 implications. *Hum Mol Genet* 19, 1347-1357.
1003 Jülicher, F., and Lipowsky, R. (1993). Domain-induced budding of vesicles. *Physical review letters* 70,
1004 2964-2967.
1005 Jülicher, F., and Lipowsky, R. (1996). Shape transformations of vesicles with intramembrane domains.
1006 *Physical review E, Statistical physics, plasmas, fluids, and related interdisciplinary topics* 53, 2670-2683.
1007 Kaksonen, M., and Roux, A. (2018). Mechanisms of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol*
1008 19, 313-326.
1009 Kaur, S., Fielding, A.B., Gassner, G., Carter, N.J., and Royle, S.J. (2014). An unmet actin requirement
1010 explains the mitotic inhibition of clathrin-mediated endocytosis. *eLife* 3, e00829.
1011 Keller, R.K., Arnold, T.P., and Fliesler, S.J. (2004). Formation of 7-dehydrocholesterol-containing
1012 membrane rafts in vitro and in vivo, with relevance to the Smith-Lemli-Opitz syndrome. *J Lipid Res* 45,
1013 347-355.
1014 Kelley, R.I. (1995). Diagnosis of Smith-Lemli-Opitz syndrome by gas chromatography/mass spectrometry
1015 of 7-dehydrocholesterol in plasma, amniotic fluid and cultured skin fibroblasts. *Clinica chimica acta;*
1016 *international journal of clinical chemistry* 236, 45-58.

1017 Khatibzadeh, N., Gupta, S., Farrell, B., Brownell, W.E., and Anvari, B. (2012). Effects of cholesterol on
1018 nano-mechanical properties of the living cell plasma membrane. *Soft Matter* 8, 8350-8360.

1019 Kim, J., Singh, A., DelPoeta, M., Brown, D.A., and London, E. (2017). The effect of sterol structure upon
1020 clathrin-mediated and clathrin-independent endocytosis. *J Cell Sci*.

1021 Kolf-Clauw, M., Chevy, F., Wolf, C., Siliart, B., Citadelle, D., and Roux, C. (1996). Inhibition of 7-
1022 dehydrocholesterol reductase by the teratogen AY9944: a rat model for Smith-Lemli-Opitz syndrome.
1023 *Teratology* 54, 115-125.

1024 Krishna, A., and Sengupta, D. (2019). Interplay between Membrane Curvature and Cholesterol: Role of
1025 Palmitoylated Caveolin-1. *Biophysical Journal* 116, 69-78.

1026 Kusumi, A., Fujiwara, T.K., Chadda, R., Xie, M., Tsunoyama, T.A., Kalay, Z., Kasai, R.S., and Suzuki, K.G.
1027 (2012). Dynamic organizing principles of the plasma membrane that regulate signal transduction:
1028 commemorating the fortieth anniversary of Singer and Nicolson's fluid-mosaic model. *Annual review of*
1029 *cell and developmental biology* 28, 215-250.

1030 Kwik, J., Boyle, S., Fooksman, D., Margolis, L., Sheetz, M.P., and Edidin, M. (2003). Membrane
1031 cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of
1032 cell actin. *Proc Natl Acad Sci U S A* 100, 13964-13969.

1033 Lange, Y., Dolde, J., and Steck, T.L. (1981). The rate of transmembrane movement of cholesterol in the
1034 human erythrocyte. *J Biol Chem* 256, 5321-5323.

1035 Lange, Y., Swaisgood, M.H., Ramos, B.V., and Steck, T.L. (1989). Plasma membranes contain half the
1036 phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J Biol Chem*
1037 264, 3786-3793.

1038 Lentz, B.R., Siegel, D.P., and Malinin, V. (2002). Filling potholes on the path to fusion pores. *Biophys J* 82,
1039 555-557.

1040 Levental, I., Levental, K.R., and Heberle, F.A. (2020). Lipid Rafts: Controversies Resolved, Mysteries
1041 Remain. *Trends Cell Biol* 30, 341-353.

1042 Linck, L.M., Lin, D.S., Flavell, D., Connor, W.E., and Steiner, R.D. (2000). Cholesterol supplementation
1043 with egg yolk increases plasma cholesterol and decreases plasma 7-dehydrocholesterol in Smith-Lemli-
1044 Opitz syndrome. *American journal of medical genetics* 93, 360-365.

1045 Liu, J., Sun, Y., Drubin, D.G., and Oster, G.F. (2009). The mechanochemistry of endocytosis. *PLoS Biol* 7,
1046 e1000204.

1047 Lu, F., Liang, Q., Abi-Mosleh, L., Das, A., De Brabander, J.K., Goldstein, J.L., and Brown, M.S. (2015).
1048 Identification of NPC1 as the target of U18666A, an inhibitor of lysosomal cholesterol export and Ebola
1049 infection. *Elife* 4.

1050 Luo, J., Jiang, L.Y., Yang, H., and Song, B.L. (2019). Intracellular Cholesterol Transport by Sterol Transfer
1051 Proteins at Membrane Contact Sites. *Trends in biochemical sciences* 44, 273-292.

1052 Maxfield, F.R., and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature* 438,
1053 612-621.

1054 McMahon, H.T., and Gallop, J.L. (2005). Membrane curvature and mechanisms of dynamic cell
1055 membrane remodelling. *Nature* 438, 590-596.

1056 Megha, Bakht, O., and London, E. (2006). Cholesterol precursors stabilize ordinary and ceramide-rich
1057 ordered lipid domains (lipid rafts) to different degrees. Implications for the Bloch hypothesis and sterol
1058 biosynthesis disorders. *The Journal of biological chemistry* 281, 21903-21913.

1059 Miller, S.E., Mathiasen, S., Bright, N.A., Pierre, F., Kelly, B.T., Kladt, N., Schauss, A., Merrifield, C.J.,
1060 Stamou, D., Höning, S., *et al.* (2015). CALM regulates clathrin-coated vesicle size and maturation by
1061 directly sensing and driving membrane curvature. *Developmental cell* 33, 163-175.

1062 Mitsche, M.A., McDonald, J.G., Hobbs, H.H., and Cohen, J.C. (2015). Flux analysis of cholesterol
1063 biosynthesis in vivo reveals multiple tissue and cell-type specific pathways. *Elife* 4, e07999.

Morlot, S., Galli, V., Klein, M., Chiaruttini, N., Manzi, J., Humbert, F., Dinis, L., Lenz, M., Cappello, G., and Roux, A. (2012). Membrane Shape at the Edge of the Dynamin Helix Sets Location and Duration of the Fission Reaction. *Cell* **151**, 619-629.

Murthy, V.N., and Stevens, C.F. (1998). Synaptic vesicles retain their identity through the endocytic cycle. *Nature* **392**, 497-501.

Najafinobar, N., Mellander, L.J., Kurczy, M.E., Dunevall, J., Angerer, T.B., Fletcher, J.S., and Cans, A.-S. (2016). Cholesterol Alters the Dynamics of Release in Protein Independent Cell Models for Exocytosis. *Scientific Reports* **6**, 33702.

Narayana, Y.V., Gadgil, C., Mote, R.D., Rajan, R., and Subramanyam, D. (2019). Clathrin-Mediated Endocytosis Regulates a Balance between Opposing Signals to Maintain the Pluripotent State of Embryonic Stem Cells. *Stem Cell Reports* **12**, 152-164.

Nohturfft, A., Brown, M.S., and Goldstein, J.L. (1998). Sterols regulate processing of carbohydrate chains of wild-type SREBP cleavage-activating protein (SCAP), but not sterol-resistant mutants Y298C or D443N. *Proc Natl Acad Sci U S A* **95**, 12848-12853.

Ory, D.S., Ottinger, E.A., Farhat, N.Y., King, K.A., Jiang, X., Weissfeld, L., Berry-Kravis, E., Davidson, C.D., Bianconi, S., Keener, L.A., *et al.* (2017). Intrathecal 2-hydroxypropyl- β -cyclodextrin decreases neurological disease progression in Niemann-Pick disease, type C1: a non-randomised, open-label, phase 1-2 trial. *Lancet (London, England)* **390**, 1758-1768.

Pandit, S.A., Jakobsson, E., and Scott, H.L. (2004). Simulation of the early stages of nano-domain formation in mixed bilayers of sphingomyelin, cholesterol, and dioleoylphosphatidylcholine. *Biophys J* **87**, 3312-3322.

Raghunathan, K., and Kenworthy, A.K. (2018). Dynamic pattern generation in cell membranes: Current insights into membrane organization. *Biochimica et biophysica acta Biomembranes* **1860**, 2018-2031.

Rahier, A., and Taton, M. (1996). Sterol biosynthesis: strong inhibition of maize delta 5,7-sterol delta 7-reductase by novel 6-aza-B-homosteroids and other analogs of a presumptive carbocationic intermediate of the reduction reaction. *Biochemistry* **35**, 7069-7076.

Ramachandra Rao, S., Pfeffer, B.A., Mas Gomez, N., Skelton, L.A., Keiko, U., Sparrow, J.R., Rowsam, A.M., Mitchell, C.H., and Fliesler, S.J. (2018). Compromised phagosome maturation underlies RPE pathology in cell culture and whole animal models of Smith-Lemli-Opitz Syndrome. *Autophagy* **14**, 1796-1817.

Rodal, S.K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B., and Sandvig, K. (1999). Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell* **10**, 961-974.

Róg, T., Stimson, L.M., Pasenkiewicz-Gierula, M., Vattulainen, I., and Karttunen, M. (2008). Replacing the cholesterol hydroxyl group with the ketone group facilitates sterol flip-flop and promotes membrane fluidity. *The journal of physical chemistry B* **112**, 1946-1952.

Roux, A., Uyhazi, K., Frost, A., and De Camilli, P. (2006). GTP-dependent twisting of dynamin implicates constriction and tension in membrane fission. *Nature* **441**, 528-531.

Saheki, Y., and De Camilli, P. (2012). Synaptic vesicle endocytosis. *Cold Spring Harbor perspectives in biology* **4**, a005645-a005645.

Saleem, M., Morlot, S., Hohendahl, A., Manzi, J., Lenz, M., and Roux, A. (2015). A balance between membrane elasticity and polymerization energy sets the shape of spherical clathrin coats. *Nature Communications* **6**, 6249.

Schreiber, J.M., Lanham, D.C., Trescher, W.H., Sparks, S.E., Wassif, C.A., Caffo, B.S., Porter, F.D., Tierney, E., Gropman, A.L., and Ewen, J.B. (2014). Variations in EEG discharges predict ADHD severity within individual Smith-Lemli-Opitz patients. *Neurology* **83**, 151-159.

Scott, B.L., Sochacki, K.A., Low-Nam, S.T., Bailey, E.M., Luu, Q., Hor, A., Dickey, A.M., Smith, S., Kerkvliet, J.G., Taraska, J.W., *et al.* (2018). Membrane bending occurs at all stages of clathrin-coat assembly and defines endocytic dynamics. *Nat Commun* **9**, 419.

1112 Sengupta, P., Baird, B., and Holowka, D. (2007). Lipid rafts, fluid/fluid phase separation, and their
1113 relevance to plasma membrane structure and function. *Seminars in cell & developmental biology* *18*,
1114 583-590.

1115 Serfis, A.B., Brancato, S., and Fliesler, S.J. (2001). Comparative behavior of sterols in
1116 phosphatidylcholine-sterol monolayer films. *Biochim Biophys Acta* *1511*, 341-348.

1117 Sikora, D.M., Ruggiero, M., Petit-Kekel, K., Merckens, L.S., Connor, W.E., and Steiner, R.D. (2004).
1118 Cholesterol supplementation does not improve developmental progress in Smith-Lemli-Opitz syndrome.
1119 *J Pediatr* *144*, 783-791.

1120 Skruzny, M., Brach, T., Ciuffa, R., Rybina, S., Wachsmuth, M., and Kaksonen, M. (2012). Molecular basis
1121 for coupling the plasma membrane to the actin cytoskeleton during clathrin-mediated endocytosis. *Proc*
1122 *Natl Acad Sci U S A* *109*, E2533-2542.

1123 Smith, D.W., Lemli, L., and Opitz, J.M. (1964). A newly recognized syndrome of multiple congenital
1124 anomalies. *The Journal of Pediatrics* *64*, 210-217.

1125 Sochacki, K.A., Dickey, A.M., Strub, M.P., and Taraska, J.W. (2017). Endocytic proteins are partitioned at
1126 the edge of the clathrin lattice in mammalian cells. *Nat Cell Biol* *19*, 352-361.

1127 Sochacki, K.A., Shtengel, G., van Engelenburg, S.B., Hess, H.F., and Taraska, J.W. (2014). Correlative
1128 super-resolution fluorescence and metal-replica transmission electron microscopy. *Nat Methods* *11*,
1129 305-308.

1130 Song, J., and Waugh, R.E. (1993). Bending rigidity of SOPC membranes containing cholesterol. *Biophys J*
1131 *64*, 1967-1970.

1132 Srinivasan, S., Burckhardt, C.J., Bhawe, M., Chen, Z., Chen, P.H., Wang, X., Danuser, G., and Schmid, S.L.
1133 (2018). A noncanonical role for dynamin-1 in regulating early stages of clathrin-mediated endocytosis in
1134 non-neuronal cells. *PLoS Biol* *16*, e2005377.

1135 Steck, T.L., Ye, J., and Lange, Y. (2002). Probing red cell membrane cholesterol movement with
1136 cyclodextrin. *Biophys J* *83*, 2118-2125.

1137 Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M.A., Keen, J.H., and McGraw, T.E. (1999). Acute
1138 cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci U S A* *96*, 6775-6780.

1139 Sun, L.P., Li, L., Goldstein, J.L., and Brown, M.S. (2005). Insig required for sterol-mediated inhibition of
1140 Scap/SREBP binding to COPII proteins in vitro. *J Biol Chem* *280*, 26483-26490.

1141 Svoboda, M.D., Christie, J.M., Eroglu, Y., Freeman, K.A., and Steiner, R.D. (2012). Treatment of Smith-
1142 Lemli-Opitz syndrome and other sterol disorders. *American journal of medical genetics Part C, Seminars*
1143 *in medical genetics* *160c*, 285-294.

1144 Taylor, M.J., Perrais, D., and Merrifield, C.J. (2011). A high precision survey of the molecular dynamics of
1145 mammalian clathrin-mediated endocytosis. *PLoS Biol* *9*, e1000604.

1146 Tint, G.S., Irons, M., Elias, E.R., Batta, A.K., Frieden, R., Chen, T.S., and Salen, G. (1994). Defective
1147 cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N Engl J Med* *330*, 107-113.

1148 Tint, G.S., Salen, G., Batta, A.K., Shefer, S., Irons, M., Elias, E.R., Abuelo, D.N., Johnson, V.P., Lambert, M.,
1149 Lutz, R., *et al.* (1995). Correlation of severity and outcome with plasma sterol levels in variants of the
1150 Smith-Lemli-Opitz syndrome. *J Pediatr* *127*, 82-87.

1151 Tontonoz, P., and Mangelsdorf, D.J. (2003). Liver X receptor signaling pathways in cardiovascular
1152 disease. *Molecular endocrinology (Baltimore, Md)* *17*, 985-993.

1153 Tu, C., Li, J., Jiang, X., Sheflin, L.G., Pfeiffer, B.A., Behringer, M., Fliesler, S.J., and Qu, J. (2013). Ion-
1154 current-based proteomic profiling of the retina in a rat model of Smith-Lemli-Opitz syndrome. *Molecular*
1155 *& cellular proteomics : MCP* *12*, 3583-3598.

1156 Tulenko, T.N., Boeze-Battaglia, K., Mason, R.P., Tint, G.S., Steiner, R.D., Connor, W.E., and Labelle, E.F.
1157 (2006). A membrane defect in the pathogenesis of the Smith-Lemli-Opitz syndrome. *J Lipid Res* *47*, 134-
1158 143.

1159 van Rheenen, J., Achame, E.M., Janssen, H., Calafat, J., and Jalink, K. (2005). PIP2 signaling in lipid
1160 domains: a critical re-evaluation. *The EMBO journal* **24**, 1664-1673.

1161 Vecsernyes, M., Fenyvesi, F., Bacskay, I., Deli, M.A., Szente, L., and Fenyvesi, E. (2014). Cyclodextrins,
1162 blood-brain barrier, and treatment of neurological diseases. *Arch Med Res* **45**, 711-729.

1163 Vedhachalam, C., Duong, P.T., Nickel, M., Nguyen, D., Dhanasekaran, P., Saito, H., Rothblat, G.H., Lund-
1164 Katz, S., and Phillips, M.C. (2007). Mechanism of ATP-binding cassette transporter A1-mediated cellular
1165 lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles. *J Biol Chem* **282**,
1166 25123-25130.

1167 Wang, N., Kwan, C., Gong, X., de Chaves, E.P., Tse, A., and Tse, F.W. (2010). Influence of cholesterol on
1168 catecholamine release from the fusion pore of large dense core chromaffin granules. *The Journal of*
1169 *neuroscience : the official journal of the Society for Neuroscience* **30**, 3904-3911.

1170 Wang, W., Yang, L., and Huang, H.W. (2007). Evidence of cholesterol accumulated in high curvature
1171 regions: implication to the curvature elastic energy for lipid mixtures. *Biophys J* **92**, 2819-2830.

1172 Wassif, C.A., Krakowiak, P.A., Wright, B.S., Gewandter, J.S., Sterner, A.L., Javitt, N., Yergey, A.L., and
1173 Porter, F.D. (2005). Residual cholesterol synthesis and simvastatin induction of cholesterol synthesis in
1174 Smith-Lemli-Opitz syndrome fibroblasts. *Mol Genet Metab* **85**, 96-107.

1175 Wassif, C.A., Kratz, L., Sparks, S.E., Wheeler, C., Bianconi, S., Gropman, A., Calis, K.A., Kelley, R.I., Tierney,
1176 E., and Porter, F.D. (2017). A placebo-controlled trial of simvastatin therapy in Smith-Lemli-Opitz
1177 syndrome. *Genet Med* **19**, 297-305.

1178 Wassif, C.A., Maslen, C., Kachilele-Linjewile, S., Lin, D., Linck, L.M., Connor, W.E., Steiner, R.D., and
1179 Porter, F.D. (1998). Mutations in the human sterol delta7-reductase gene at 11q12-13 cause Smith-
1180 Lemli-Opitz syndrome. *Am J Hum Genet* **63**, 55-62.

1181 Wassif, C.A., Vied, D., Tsokos, M., Connor, W.E., Steiner, R.D., and Porter, F.D. (2002). Cholesterol
1182 storage defect in RSH/Smith-Lemli-Opitz syndrome fibroblasts. *Mol Genet Metab* **75**, 325-334.

1183 Watanabe, S., Trimbuch, T., Camacho-Pérez, M., Rost, B.R., Brokowski, B., Söhl-Kielczynski, B., Felies, A.,
1184 Davis, M.W., Rosenmund, C., and Jorgensen, E.M. (2014). Clathrin regenerates synaptic vesicles from
1185 endosomes. *Nature* **515**, 228-233.

1186 Waterham, H.R., and Hennekam, R.C. (2012). Mutational spectrum of Smith-Lemli-Opitz syndrome.
1187 *American journal of medical genetics Part C, Seminars in medical genetics* **160c**, 263-284.

1188 Wenz, J.J., and Barrantes, F.J. (2003). Steroid structural requirements for stabilizing or disrupting lipid
1189 domains. *Biochemistry* **42**, 14267-14276.

1190 Winterstein, C., Trotter, J., and Krämer-Albers, E.-M. (2008). Distinct endocytic recycling of myelin
1191 proteins promotes oligodendroglial membrane remodeling. *Journal of Cell Science* **121**, 834-842.

1192 Yue, H.Y., and Xu, J. (2015). Cholesterol regulates multiple forms of vesicle endocytosis at a mammalian
1193 central synapse. *J Neurochem* **134**, 247-260.

1194 Zidovetzki, R., and Levitan, I. (2007). Use of cyclodextrins to manipulate plasma membrane cholesterol
1195 content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* **1768**, 1311-1324.

Figure 1

A

B

C

D

E

F

G

Figure 2

A

B

C

Figure 3

A

B

C

D

E

F

G

Figure 5

A

Cholesterol
20.2 Å² TPSA (Polarity)
++ Liquid ordered (*L_o*) domain stability

B

C

D

Figure 6

Figure 7