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The formation of giant plasma membrane vesicles enable new insights into the regulation of cholesterol efflux

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Abstract

Aberrant cellular cholesterol accumulation contributes to the pathophysiology of many diseases including neurodegenerative disorders such as Niemann-Pick Type C (NPC) and Alzheimer's Disease^{1–4}. Many aspects of cholesterol efflux from cells remain elusive. Here we describe the utility of cholesterol-rich giant plasma membrane vesicles (GPMVs) as a means to monitor cholesterol that is translocated to the plasma membrane for secretion. We demonstrate that small molecules known to enhance lipid efflux, including those in clinical trials for lipid storage disorders, enhance this GPMV formation. Conversely, pharmacological inhibition of cholesterol efflux blocks GPMV formation. We show that microtubule stabilization via paclitaxel treatment and increased tubulin acetylation via HDAC6 inhibition promotes the formation of GPMVs with concomitant reduction in cellular cholesterol in a cell model of NPC disease. The pan-deacetylase inhibitor panobinostat, which has been shown to reduce the severity of cholesterol storage in NPC, elicited a similar response. Further, the disruption of actin polymerization inhibits the formation of GPMVs, whereas the small GTP-binding protein Arl4c promotes actin remodeling at sites overlapping with GPMV formation. Thus, monitoring the formation of GPMVs provides a new avenue to better understand diseases whose pathology may be sensitive to alterations in cellular cholesterol.

Graphical Abstract

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Keywords

Cholesterol efflux; giant plasma membrane vesicles; cytoskeleton; Niemann-Pick Type C

Introduction

Normal cholesterol homeostasis at the cellular and systemic levels is imperative for health. Consequently, the aberrant trafficking or storage of cholesterol is implicated in many disease processes^{5,6}. Unesterified cholesterol, in particular, can exert toxicity when accumulated, and in healthy cells this is minimized by its efflux or esterification for storage in lipid droplets⁷⁻⁹. The plasma membrane contains much of the cellular cholesterol^{6,10} and provides vital feedback signals that modulate cholesterol synthesis and homeostasis^{11,12}. The trafficking of cholesterol to and from the plasma membrane, between intracellular compartments, as well as its removal from the cell, have been shown to be dependent on one or more cellular cytoskeletal components¹³⁻²⁰. While a significant amount of research has focused on understanding how these processes are regulated, many facets remain to be elucidated.

Lipids are not homogeneously distributed in the plasma membrane, but instead are selectively trafficked and sorted for distribution into isolated domains such as lipid rafts²¹, and for efflux from the cell²². Multiple cellular cholesterol efflux mechanisms have been identified, including aqueous diffusion, facilitated diffusion by scavenger receptor class B (SR-BI), and movement by the cholesterol transporters ABCA1 and ABCG1²². Intracellular cholesterol trafficking is facilitated by vesicular and non-vesicular processes^{23,24}, with microtubules playing a role in the movement of cholesterol between intracellular compartments and the cell surface^{19,25}. A role for the actin cytoskeleton in cholesterol efflux has been suggested by the observation that binding of the cholesterol acceptor apolipoprotein A-I (ApoA1) stimulates actin remodeling at the cell surface²⁶.

Lipids such as cholesterol have been shown to be present on extracellular vesicles (EVs)^{27–32}. EVs have emerged as critical mediators of intercellular communication in normal development and physiology, as well as during systemic pathophysiological events accompanying various disease states^{33–36}. They comprise a large group of heterogeneous particles, including exosomes and microvesicles, and are released from virtually all cell types. Cholesterol has been proposed to regulate membrane fluidity and the stability of vesicles in the extracellular environment^{31,37}, and to play a role in the formation of those that are induced by promoting membrane phase separation, such as giant plasma membrane vesicles (GPMVs)³⁸.

Here we demonstrate that GPMVs, known to be enriched in cellular lipids³⁹, provide a novel means for studying the population of cholesterol that has been trafficked to the cell surface for efflux. We validated this approach by demonstrating that small molecules known to enhance cholesterol efflux enhance GPMV formation, and conversely, inhibiting the movement of cholesterol to the plasma membrane inhibits GPMV formation. This was accomplished using multiple, well-established approaches for altering cellular cholesterol levels, including U18666A treatment to aggregate cholesterol intracellularly, as well as cyclodextrin treatment, exposure to the cholesterol acceptor ApoA1, and the intrinsic stimulation of cholesterol efflux by liver X receptor agonist treatment to promote efflux. We demonstrated a correlation between efflux induction and increased GPMV formation, and found that the formation of GPMVs subsequently decreased as cellular cholesterol levels were lowered. We utilized this method to then investigate the contributions of the cytoskeleton in cholesterol efflux, and found that microtubule stabilization via paclitaxel treatment and increased tubulin acetylation via HDAC6 inhibition promotes the formation of GPMVs, with a subsequent reduction in cellular cholesterol in a model of the cholesterol storage disorder Niemann-Pick Type C Disease. Treatment with the pan-deacetylase inhibitor panobinostat, which has been shown to ameliorate the cholesterol storage in NPC, elicited a similar response, which could be abrogated upon microtubule depolymerization with nocodazole. Further, we demonstrated the importance of actin dynamics in these processes, as disruption of actin polymerization inhibited the formation of GPMVs, whereas the small GTP-binding protein Arl4c promoted actin remodeling at sites overlapping with GPMV formation.

Results

Cholesterol is present on extracellular vesicles

To better understand the role of cholesterol in EV biogenesis, EVs released from the melanoma cell line LOX were stained with filipin III to label free cholesterol (FC). For this assay, the cells were plated on a thick layer of fluorescent gelatin, as this system has been characterized to promote microvesicle shedding⁴⁰, and allows the visualization of shed extracellular vesicles which are trapped within the matrix. While we observed FC in the traditionally-characterized MV population, we noted an additional population of vesicles, which contained FC but not β_1 integrin or filamentous actin, which are commonly used markers for MVs^{40–42} (Fig. 1a, b). These vesicles were typically larger than microvesicles, ranging from 5–10 μm in diameter, and were occasionally greater than 10 μm . As described

below, these large FC-rich vesicles were subsequently identified to be a form of giant plasma membrane vesicles (GPMVs), previously described^{36–39}. GPMV formation results from induced blebbing of the plasma membrane⁴³, commonly elicited in live cells with the treatment of a buffer containing a low concentration of paraformaldehyde^{38,43}. This type of blebbing has also been characterized in aldehyde fixed cells, thought to be due to the retained mobility of lipids upon protein fixation⁴⁴.

While GPMV composition may vary based upon the method of their induction, they have been well-characterized to be enriched in many lipids, including free cholesterol³⁹. GPMV formation can be induced by multiple methods such as laser irradiation and salt solution treatment, however the combination of paraformaldehyde and dithiothreitol (DTT) is the most commonly used^{39,45,46} and results in the formation of abundant, large vesicles at the cell periphery, which can be visualized forming on live cells (Fig. 2a). To allow for the visualization of proteins interacting with these cholesterol-rich structures, cells were fixed and stained with filipin III, which allows the bright labeling of FC, followed by labeling for proteins of interest, and mounting. We found that GPMV stimulation prompts the elevation of plasma membrane cholesterol above the cell surface into the vesicles that we had previously observed in LOX cells as described above (Fig. 2b). This was confirmed by loading live cells with the fluorescent cholesterol analog TopFluor-cholesterol (Fig. 3a), which show many immature vesicles at the cell surface following PFA fixation. Note that the turgidity of the vesicles is reduced, resulting in a stretched and wrinkled appearance as compared to those in live cells (Fig. 3b). This appears to be due to the permeabilization of the vesicles, resulting in the loss of bulk cytoplasmic contents, as evidenced by the loss of cytoplasmic GFP (Fig. 3b). This can be advantageous, as it allows for the analysis of proteins which exhibit a specific interaction with the lipid vesicle, rather than bulk cytoplasmic inclusion. We confirmed the preservation of an additional lipid raft marker, GM1, in GPMVs by labeling with cholera toxin (Fig. 3c).

GPMV formation correlates with cholesterol efflux

Cholesterol is trafficked through the cell and presented to the cell surface for its removal via transporters and acceptors^{8,22}. To gain insight into the relevance of GPMVs in monitoring cholesterol which has redistributed to the cell surface for efflux, we used a variety of well-established methods to modulate cellular cholesterol levels and efflux, and evaluated GPMV formation. We found that supplementing the culture medium with exogenous cholesterol in the form of water-soluble cholesterol augments GPMV formation, in addition to the intracellular cholesterol pool (Fig. 4a). Blocking efflux and the movement of cholesterol to the cell surface by prompting its intracellular aggregation with the small molecule inhibitor U18666A^{16,47} severely abrogates GPMV formation (Fig. 4a). Cells were also treated with the cholesterol acceptor ApoA1, which is well-characterized to promote cholesterol efflux^{19,48}. Cells were evaluated after 24 and 48 hours of treatment, to monitor efflux progression. After 24 hours of ApoA1 exposure, an increase in GPMV formation was noted at the cell surface, and a significant decrease in intracellular cholesterol was noted at the 48 hour time point (Fig. 4b, c), indicating that the availability of this acceptor prompts the enrichment of cholesterol at the cell surface, and therefore, GPMV induction. At the later time point, this population decreases as cholesterol is removed from the cell (Fig. 4c).

GPMV formation requires the microtubule and actin cytoskeleton

In order to better understand the cytoskeletal proteins that regulate cholesterol trafficking and efflux, GPMVs were evaluated for the presence of proteins that potentially mediate these processes. Intermediate filaments were absent from GPMVs as demonstrated by a lack of appreciable vimentin or keratin 17 labeling (Fig. 5a). However, microtubules were frequently detected in GPMVs, shown both by immunostaining for α -tubulin and also by the expression of GFP-tagged α -tubulin (Fig. 5a). Substantial staining for EB1, a microtubule (MT) plus-end binding protein, is also evident (Fig. 5a). These data suggest that a population of microtubules, likely anchored in the plasma membrane, associate with GPMVs⁴⁹. Various populations of microtubules connected with the plasma membrane have been characterized⁴⁹. Of note, plasma membrane tubulin may regulate Na⁺/K⁺-ATPase (NKA) and other membrane ATPases, and these ATPases may function as anchorage sites for microtubules⁵⁰. The association of microtubules with NKA is thought to induce hydrophobic behavior in tubulin⁴⁹. The knockdown of NKA has been shown to induce a reduction in cholesterol found at the plasma membrane and a redistribution to intracellular compartments⁵¹. Likewise, a reduction in plasma membrane cholesterol via treatment with U18666A results in decreased NKA expression⁵². We also noted the presence of the Golgi marker GM130 at some GPMVs (Fig. 5b). Interestingly in this regard, in neuronal cells, structures termed Golgi outposts have been characterized to nucleate acentrosomal microtubule formation⁵³. It is unclear if they are mediating microtubule dynamics in a similar way in this context, to modulate the formation of these cholesterol domains.

Since actin has also been shown to play roles in cholesterol regulation, we have evaluated its contribution to the movement of cholesterol to the cell surface. The binding of the cholesterol acceptor ApoA1 has been noted to induce actin polymerization²⁶, and depleting cholesterol levels has been noted to alter membrane stiffness through a proposed alteration in cortical F-actin dynamics⁵⁴. As noted above, we found that GPMVs were devoid of F-actin, distinctly arising above the cortical actin cytoskeleton (Fig. 6a). The treatment of cells with a low dose of latrunculin A to subtly disrupt actin dynamics severely impaired GPMV formation. Cells formed either very small or no GPMVs (Fig. 6b). Intriguingly, sites of cholesterol enrichment and GPMV formation overlapped with actin-rich pseudopodia induced by Arl4c expression (Fig. 7a), and Arl4c expression increased GPMV formation (Fig. 7b). Arl4c, a small GTP-binding protein also known as Arl7, has been characterized to regulate actin rearrangement⁵⁵ and to promote ApoAI-mediated cholesterol efflux via ABCA1, with its mRNA expression upregulated by LXR activation (the only ARF or ARL member regulated as such)⁵⁶. Latrunculin A treatment inhibited these actin rearrangements, and also inhibited the redistribution of FC into those actin domains (Fig. 7c). Taken together, these findings demonstrate the importance of the actin cytoskeleton in cholesterol dynamics at the plasma membrane for efflux.

GPMV formation in a cellular model for Niemann-Pick Type C

Next, we evaluated GPMV formation under conditions of abnormal cellular cholesterol storage. NPC1 is frequently mutated in Niemann-Pick Type C disease, resulting in the aberrant accumulation of lipids and the dysfunction of multiple organs^{3,57}. NPC1 is a multipass transmembrane protein that localizes primarily to the late endosome and lysosome

and modulates cholesterol trafficking in cells⁵⁸. Notably, NPC1 mutant cells were still competent in forming GPMVs (Fig. 8a). The cyclodextrin compounds methyl- β -cyclodextrin (M β CD) and (2-hydroxypropyl)- β -cyclodextrin (HP β CD) are some of the most intensively studied therapeutics for ameliorating the cholesterol accumulation seen in NPC, and a large body of work supports their effectiveness in cellular cholesterol removal^{16,59–62}. HP β CD is currently under evaluation in NPC clinical trials⁶³. When normal skin fibroblasts (GM05659) and NPC1 patient-derived fibroblast cell lines (GM03123, GM17923, GM18436) were treated with M β CD and HP β CD for 24 hours, GPMV formation increased (Fig. 8a, b), reflecting the movement of cholesterol to the cell surface for efflux. The number of GPMVs formed in response to cyclodextrin treatment correlated with the cholesterol burden of the mutant versus wild type cells (Fig. 8a, b). The upregulation in vesicle formation corresponds to increased efflux of a fluorescent cholesterol analog into the culture medium (Fig. 9a), and treatment with U18666A blocks cholesterol efflux as expected (Fig. 9a). It is interesting that we note U18666A, at a commonly used treatment concentration^{2,52,64}, to result in a more striking intracellular accumulation of cholesterol than is seen in NPC1 patient skin fibroblasts, with less cholesterol at the cell surface. Although used for decades to study lipid behavior and model NPC1 pathogenesis, the target of U18666A has only recently been elucidated as the NPC1 protein itself⁶⁵. The more pronounced lipid storage phenotype elicited by U18666A treatment is likely due to U18666A exerting a stronger inhibition of NPC1 function than that of mutated NPC1 protein, as the mutant protein has been noted to retain a level of functionality in mediating cholesterol transport^{66,67}. M β CD was more effective than HP β CD at moving cholesterol to the plasma membrane, even at lower doses (Fig. 8a, b). A significant reduction in intracellular cholesterol was subsequently apparent at 72 hours (Fig. 9b), when GPMV formation had decreased. It should be noted that the speed of cholesterol removal exhibits a degree of variability from one experiment to the next, due to variables such as the level of cholesterol present in the culture medium, and differences in behavior between cell lines. In this model of increased cholesterol storage we also evaluated liver X receptor modulation as an intrinsic means to prompt the movement of cholesterol to the plasma membrane. Liver X receptors (LXRs) are ligand-activated transcription factors which regulate cholesterol homeostasis and efflux, and function as sterol sensors^{68–71}, and serve as a mechanism to reduce cholesterol storage⁷⁰. Treatment with the LXR agonist GW3965 also leads to an upregulation in GPMV formation prior to a reduction in cellular cholesterol pools in NPC mutant fibroblasts (Fig. 9c, d).

Deacetylase inhibitors also promote the efflux of cholesterol from cells with cholesterol storage disease, and panobinostat, also known as LBH-589 (trade name Farydak), a drug recently approved by the FDA for the treatment of multiple myeloma, has been identified as one of the most effective⁶⁶. Panobinostat is a pan-deacetylase inhibitor characterized to promote acetylation of diverse targets including α -tubulin, Hsp90, and histones^{72–74}. Two cell lines were used to evaluate panobinostat in the formation of GPMVs: HeLa, with normal cholesterol trafficking, and the GM03123 NPC1 mutant skin fibroblasts described above. In both cell lines, panobinostat significantly increased the formation of GPMVs prior to a reduction in free cholesterol within the cells (Fig. 10a, b, c), while also frequently eliciting morphological changes including cell spreading or elongation. Co-treatment with

nocodazole significantly impaired the ability of panobinostat to reduce cholesterol levels (Fig. 10c), highlighting the importance for microtubules in this process. Treatment with nocodazole alone prompted increased intracellular cholesterol accumulation (Fig. 10c), in accordance with published literature which indicates a role for MT in delivering cholesterol to the plasma membrane in the context of NPC mutation¹⁶. We observed a strong increase in α -tubulin lysine 40 acetylation in the lysate of panobinostat treated cells as described⁷³ (Fig. 10d), however K40 acetylated microtubules were not detected at GPMVs (Fig. 10e). Treatment of cells with paclitaxel, which stabilizes microtubules^{75,76}, increases GPMV formation at the cell surface in this NPC1 fibroblast model (Fig. 11a), and causes a subsequent decrease in intracellular cholesterol (Fig. 11b), indicating that increased MT stability could promote lipid movement from cells. Treatment with tubacin, a histone deacetylase 6 (HDAC6)-selective inhibitor, generated similar results (Fig. 11c, d). HDAC6 is a cytosolic deacetylase with non-histone targets, the most prominent being α -tubulin, and has been investigated as a target in a variety of diseases^{77–81}. Tubacin-treated NPC1 mutant cells display an increase in GPMV formation after 24 hours of treatment (Fig. 11c), with a significant decrease in intracellular cholesterol at 72 hours (Fig. 11d), again pointing to the importance for tubulin acetylation in cholesterol efflux. Tubacin treated cells show a decrease in NKA- α expression as cholesterol is removed at a 24 hour time point (Fig. 11e), corroborating previous reports that a reduction in plasma membrane cholesterol results in diminished NKA expression⁵². Treatment with U18666A, which inhibits the availability of cholesterol for the formation of GPMVs at the cell surface, while promoting cholesterol accumulation intracellularly, results in a much larger decrease in NKA expression, again supporting the contention that plasma membrane cholesterol, and not total cellular cholesterol, regulates NKA expression.

Discussion and Conclusions

The data presented here support the contention that the simple induction of GPMV formation affords a useful means to study the population of cholesterol redistributed to the cell surface for efflux, and also demonstrates the importance of the actin and microtubule cytoskeletons for the movement of this cholesterol population. GPMVs are enriched in free cholesterol and as such represent a useful tool for studying the mechanisms of cholesterol efflux. This was verified by demonstrating that their formation could be augmented by loading cells with supplemental cholesterol, and blocked by the aggregation of cholesterol intracellularly using the small molecule inhibitor U18666A. We also demonstrated that their formation could be upregulated by the treatment with compounds that promote cholesterol efflux via multiple different mechanisms including cyclodextrins, deacetylase inhibitors, LXR agonism, and apolipoprotein A-I. This was also demonstrated in Niemann-Pick Type C mutant skin fibroblasts using drugs currently under evaluation for their therapeutic potential, providing a visible readout for cholesterol efflux progression. Vesicle formation was noted to be highest at early time points following efflux stimulation as cellular cholesterol levels were beginning to be lowered, and reduced at later time points when cellular cholesterol levels had been significantly reduced.

We have utilized GPMV formation to investigate the contributions of the cytoskeleton to cholesterol pools at the plasma membrane for removal from cells. Both the microfilament

and microtubule cytoskeletons were found to regulate lipid efflux. The disruption of actin polymerization inhibited the formation of GPMVs, as did microtubule depolymerization, while also inhibiting cholesterol efflux. The Arf-like small GTPase Arl4c, known to modulate cholesterol efflux⁵⁶, resulted in significant actin reorganization, promoting the formation of cholesterol-rich vesicles at peripheral ruffles. Arl4c-mediated redistribution of cholesterol at the plasma membrane requires normal actin dynamics, as treatment with latrunculin A to disrupt actin polymerization abrogated these effects of Arl4c. Likewise, the promotion of tubulin acetylation and stabilization promoted vesicle formation and resulted in an upregulation of cholesterol efflux. We observed an increase in GPMV formation and reduction of cellular cholesterol levels following treatment with the microtubule-stabilizing chemotherapeutic paclitaxel, which is interesting in light of studies which showed paclitaxel treatment reducing the severity of atherosclerotic lesions^{82,83}. Dyslipidemia has been reported as a side-effect of paclitaxel cancer treatment⁸⁴. The stimulation of GPMV formation ahead of cellular cholesterol reduction was also shown with the pan-deacetylase inhibitor panobinostat, which has previously been demonstrated to reduce cholesterol burden in NPC1 mutant cells⁶⁶. While the mechanism of action for panobinostat in ameliorating cholesterol storage is unclear, it is thought to be due to enhanced transcription and post-translational stability of the mutant NPC1 protein^{66,85,86}. Our data indicate that its effects on microtubules also contribute, supported by the observation that the HDAC6-selective inhibitor tubacin yielded similar results, and microtubule depolymerization inhibited the ability of panobinostat to induce cholesterol efflux. This supports work which has demonstrated that HDAC6 inhibition to promote tubulin acetylation in a model of cystic fibrosis also ameliorates abnormal cholesterol storage⁸⁷. Of note, HDAC6 is highly expressed in Purkinje cells⁸⁸, the neurons most critically affected by NPC mutation³. We also noted NKA- α expression to be reduced in tubacin treated cells, reflecting a reduction in plasma membrane cholesterol as has been previously published to modulate NKA expression⁵². Interestingly, NKA- α 1 expression has been shown to be reduced in NPC^{-/-} mice⁵², most prominently in the brain and liver, two of the organs most critically affected by NPC pathogenesis.

It is understood that cholesterol-rich lipid rafts play an important role in the coupling of the plasma membrane to the underlying cytoskeleton⁸⁹, and cytoskeletal components are important for regulating cellular cholesterol homeostasis^{14,16,18}. Further elucidation of these mechanisms may yield useful information for the development and application of therapeutics for diseases characterized by abnormal cholesterol storage or whose pathology may be sensitive to cholesterol alterations.

Methods

Cell culture, transfection, and cholesterol modulation

All cells were maintained and treated in complete media containing serum. Cells were plated 16 hours prior to drug treatments. Working drug concentrations and suppliers were as follows: M β CD 1 mM (Sigma), HP β CD 3 mM (Sigma), cholesterol-M β CD (water-soluble cholesterol) 1 mM (MP Biomedicals), U18666A 5 μ g/mL (Calbiochem), panobinostat 40 nM (LC Labs), paclitaxel 100 nM (LC Labs), tubacin 10 μ M (ApexBio), GW3965 1 μ M

(Cayman Chemical), ApoA1 10 µg/mL (Sigma), latrunculin A 100 nM (Enzo), TopFluor cholesterol 500 nM (Avanti Polar Lipids). For efflux assays, cells were loaded with TopFluor cholesterol for 16 hours, washed once with fresh medium, and then treated with drug in phenol-free complete media for 24 hours prior to imaging or analyzing fluorescence of the cell conditioned media at 450 nm on a fluorescence plate reader.

Immunofluorescence reagents, staining, and microscopy

All transfections were carried out using the lipid-free transfection reagent PolyExpress (Excellgen), following the manufacturer's suggested protocol. For cytoplasmic GFP expression, cells were transfected with pEGFP-C1. For Arl4c overexpression, cells were transfected using pDEST47-Arl4c-GFP, a gift from Richard Kahn (Addgene plasmid # 67402). AlexaFluor555 conjugated cholera toxin B was purchased from Invitrogen. Phalloidin conjugates were purchased from Molecular Probes. The antibodies used were as follows: rat anti- β_1 integrin clone AIIB2 (Iowa DSHB), mouse anti-EB1 (BD Pharmingen), mouse anti- α -tubulin (Sigma), mouse anti-vimentin (Sigma), rabbit anti-keratin 17 (Cell Signaling), mouse anti-GM130 (BD Pharmingen).

For immunostaining, cells were washed once briefly with warm PBS, fixed with warm 2% paraformaldehyde in PBS for 30 minutes at 37°C, washed two times with PBS for 5 minutes each, washed one time with wash buffer containing 0.2% gelatin dissolved in 1x PBS, and subsequently stained with filipin (Sigma) as described previously⁹⁰. After filipin staining, cells were washed and stained with antibodies diluted in incubation medium containing 20% goat serum and 0.2% gelatin in 1x PBS. It should be noted that due to their unique structure, great care must be taken when processing samples for fluorescence staining of cholesterol-rich vesicles, as the vesicles are labile to loss during fixation and staining due to improper handling or reagent selection. Also of note, visualization is lost following alcohol fixation or permeabilization, harsh detergents, or vigorous handling. Imaging was performed using a Zeiss Observer.Z1 fluorescence microscope, with a 350 nm excitation wavelength for filipin.

GPMV induction

The formation of GPMVs was induced by following published methods⁴⁵. Briefly, live cells were washed twice with "GPMV buffer" (10 mM HEPES, 150 mM NaCl, 2 mM CaCl₂, pH 7.4), then incubated at 37°C for 30–60 minutes (as noted in figure legends) in GPMV buffer containing 25 mM PFA and 2 mM DTT to induce vesiculation.

Quantification and statistical analysis

Corrected total cell fluorescence for filipin staining was calculated utilizing ImageJ to obtain the integrated density, area of each cell, and the mean fluorescence of the background. Microscope camera exposure settings were identical for each condition within a cell line for fluorescence measurements. Quantification of vesicles was accomplished by counting filipin⁺/actin⁻ vesicles forming on the cell surface, or released and within one cell's diameter of an isolated cell. All quantification data were compiled from 3 independent experiments, and all error bars indicate the standard error of the mean. GraphPad Prism was used to calculate statistical significance, using the following tests: Welch's t-test (Fig. 10b, 11d, 7b), Mann-Whitney U test (Fig. 4b, 9b, 9c, 11b, 11c), one-way ANOVA with Holm-Sidak's multiple

comparisons test (Fig. 8b), Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test (Fig. 9a, 10c).

Abbreviations

EV	extracellular vesicle
FC	free cholesterol
GPMV	giant plasma membrane vesicle
HDAC	histone deacetylase
HPβCD	(2-hydroxypropyl)- β -cyclodextrin
LXR	liver X receptor
MβCD	methyl- β -cyclodextrin
NKA	Na ⁺ /K ⁺ -ATPase
NPC	Niemann-Pick Type C
PFA	paraformaldehyde

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Highlights

- Cholesterol-rich giant plasma membrane vesicles (GPMVs) serve as a means to monitor cholesterol that is translocated to the plasma membrane for secretion.
- Small molecules and cellular regulators known to enhance lipid efflux, enhance this GPMV formation. Conversely, pharmacological inhibition of cholesterol efflux blocks GPMV formation.
- Microtubule stabilization and increased tubulin acetylation promotes the formation of GPMVs with concomitant reduction in cellular cholesterol in a cell model of NPC disease.
- Actin dynamics modulate plasma membrane cholesterol organization. Arl4c promotes actin remodeling at sites overlapping with GPMV formation.

Figure 1- GPMVs are distinct from microvesicles.

A: Large filipin-labeled cholesterol-rich vesicles that lack β_1 integrin (arrows), are observed in LOX cells on FITC-labeled gelatin. These vesicles are distinct from β_1 -integrin positive microvesicles (arrowheads). The lower right panel shows a magnified image of the area indicated by the white box. **B:** This population of large, cholesterol-rich vesicles (arrows) are also absent of filamentous actin.

Figure 2- Characterization of GPMVs in live and fixed cells.

A: HeLa cells expressing GFP were treated with PFA/DTT for 30 minutes to form GPMVs (arrows). **B:** Phase contrast images of live HeLa cells, both untreated (left) and treated with PFA/DTT for 60 minutes (center). The right panel shows cells after fixation and filipin labeling. Filipin labeled cells are shown at a higher magnification to enable easier GPMV visualization. GPMVs indicated by arrows.

Figure 3- Analysis of GPMV characteristics in fixed cells.

A: Live and fixed HeLa cells that were loaded with TopFluor-cholesterol (green) display GPMVs (arrows) after fixation. **B:** GPMV-stimulated HeLa cells expressing cytoplasmic GFP, following fixation and staining. GPMVs, devoid of cytoplasmic GFP, indicated by arrows. **C:** LOX stained with filipin and CtxB, to label cholesterol and GM1, respectively. GM1 labels GPMVs (arrows).

Figure 4- GPMV formation as a visible readout of cholesterol efflux.

A: Treatment of cells with U18666A to aggregate cholesterol intracellularly inhibits GPMV formation. Treatment with water-soluble cholesterol (cholesterol-M β CD) to augment the cholesterol pool increases GPMV formation. Cells labeled with filipin. GPMVs indicated by arrows. **B:** Intracellular filipin was quantitated (see methods) and the corrected total cell fluorescence for cells treated with ApoA1 at 48 hours is shown. Error bars indicate the standard error of the mean. **C.** HeLa treated with ApoA1 for 24 and 48 hours show increased GPMV formation (arrows) most prominently at 24 hours, and decreased cellular cholesterol levels at 48 hours. Cells labeled with filipin.

Figure 5- GPMVs contain α -tubulin, EB1 and cis-Golgi components but are devoid of intermediate filaments.

A: Cells stained for intermediate filament and microtubule components, as indicated. Arrows indicate GPMVs. Scale bars 20 μ m. **B:** Cells stained for GM130 show cis-Golgi localized at GPMVs. Arrow indicates GPMV.

B: Cells stained for GM130 show cis-Golgi localized at GPMVs. Arrow indicates GPMV.

Figure 6- GPMV formation is regulated by the actin cytoskeleton

A: HeLa cells labeled with filipin and phalloidin show GPMVs (arrows) arising above the cortical actin cytoskeleton. **B:** HeLa cells treated with latrunculin A and stained with filipin show a decrease in GPMV formation. GPMVs indicated by arrows.

Figure 7- Arl4c-regulated actin dynamics alter cholesterol distribution at the cell surface.

A: HeLa transiently transfected with GFP (control) or Arl4c-GFP and stained with filipin and phalloidin. Arl4c localizes to sites of GPMV formation (arrows). In the merged image, filipin is shown in red and GFP in green, for co-localization analysis. **B:** Quantification of GPMV formation described in A. Error bars indicate the standard error of the mean. **C:** HeLa transiently transfected with Arl4c-GFP and treated with latrunculin A, and stained with filipin and phalloidin. Latrunculin A inhibits GPMV formation induced by Arl4c. In the merged image, filipin is shown in red and GFP in green, for co-localization analysis.

Figure 8- Cyclodextrin-induced cholesterol efflux promotes GPMV formation in a cellular model of NPC1.

A: WT (GM05659) and NPC1 mutant skin fibroblasts (GM03123, GM17923, GM18436) were treated with cyclodextrins for 24 hours as indicated and stained with filipin. Arrows indicate GPMVs. **B:** Quantification of GPMVs formed by cells in A show enhanced formation of GPMVs in MβCD and HPβCD-treated NPC1 mutant cells.

Figure 9- Increased GPMV formation precedes efflux of cholesterol in an NPC1 model.

A: GM03123 were loaded with TopFluor-cholesterol and treated as indicated. Measurement of fluorescence in media shows an increase in cholesterol efflux elicited by M β CD treatment and inhibition by U18666A. **B:** Quantification of intracellular cholesterol in GM03123 fibroblasts treated with M β CD for 72 hours. Corrected total cell fluorescence of filipin shows a decrease in cellular cholesterol levels. **C:** Quantification of intracellular cholesterol in GM03123 treated with LXR agonist GW3965 for 72 hours. Corrected total cell fluorescence of filipin shows a decrease in cellular cholesterol levels. **D:** Cells treated with GW3965 for 24 hours show an increase in GPMV formation. Cells stained with filipin. Arrows indicate GPMVs. All error bars indicate the standard error of the mean.

Figure 10- Regulation of GPMV formation by treatment with deacetylase inhibitors.

A: NPC1 mutant skin fibroblasts (GM03123) and HeLa cells treated with panobinostat for 24 hours show increased GPMV formation (arrows). **B:** Quantification of GPMVs at the surface of GM03123 cells treated with panobinostat for 24 hours. **C:** Quantification of intracellular cholesterol in cells treated with panobinostat, nocodazole, or a combination of both drugs, for 72 hours. Corrected total cell fluorescence of filipin shows a decrease in cellular cholesterol levels following panobinostat treatment, which is blocked with nocodazole co-treatment. Nocodazole alone increases cellular cholesterol levels. **D:** Western

blot of NPC1 mutant skin fibroblasts treated with vehicle or panobinostat for 24 hours and probed for proteins as indicated, shows an increase in K40 acetylated α -tubulin following panobinostat treatment. **E:** Cells labeled with filipin, phalloidin, and for K40 acetylated α -tubulin. GPMVs (arrows) do not contain K40 Ac- α tubulin.

Figure 11- Regulation of GPMV formation and intracellular cholesterol levels via microtubule stabilization and HDAC6 inhibition.

A: GM03123 treated with paclitaxel and stained with filipin show an increase in GPMV formation (arrows). Panels on left show matched exposure settings, panels on right show brightened images of boxed area. Areas selected for magnification in boxes were selected to visualize a comparable number of cells. **B:** Corrected total cell fluorescence of filipin of GM03123 treated with paclitaxel for 72 hours shows a decrease in cellular cholesterol levels. **C:** Quantification of GPMVs formed by GM03123 treated with tubacin for 24 hours shows an increase in GPMV formation. **D:** Corrected total cell fluorescence of filipin of GM03123 treated with tubacin for 72 hours shows a decrease in cellular cholesterol levels. **E:** Western blot of lysates of cells treated with tubacin, U18666A, or a combination of both, for 24 hours

and probed for the proteins indicated shows a decrease in NK α expression. All error bars indicate the standard error of the mean.

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