Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling

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Despite efforts to understand the interactions between nanoparticles and cells, the cellular processes that determine the efficiency of intracellular drug delivery remain unclear. Here we examine cellular uptake of short interfering RNA (siRNA) delivered in lipid nanoparticles (LNPs) using cellular trafficking probes in combination with automated high-throughput confocal microscopy. We also employed defined perturbations of cellular pathways paired with systems biology approaches to uncover protein-protein and protein–small molecule interactions. We show that multiple cell signaling effectors are required for initial cellular entry of LNPs through macropinocytosis, including proton pumps, mTOR and cathepsins. siRNA delivery is substantially reduced as ≥70% of the internalized siRNA undergoes exocytosis through egress of LNPs from late endosomes/lysosomes. Niemann-Pick type C1 (NPC1) is shown to be an important regulator of the major recycling pathways of LNP-delivered siRNAs. NPC1-deficient cells show enhanced cellular retention of LNPs inside late endosomes and lysosomes, and increased gene silencing of the target gene. Our data suggest that siRNA delivery efficiency might be improved by designing delivery vehicles that can escape the recycling pathways.

siRNAs are a potential new class of medicines that can selectively silence disease-causing genes.¹⁻³ LNPs are the most advanced siRNA delivery system to date, having recently shown therapeutic efficacy in clinical trials.⁴ Although efforts to define the physicochemical properties of nanoparticles that contribute to efficient cellular delivery are ongoing, the cellular factors that regulate nanoparticle-mediated siRNA delivery remain largely unclear.⁵⁻⁷

Nanoparticles utilize different endocytosis pathways for cellular entry.⁸ Intracellular trafficking of nanoparticles is a dynamic process, through which macromolecules are transported to different subcellular destinations, with the majority of material targeted to the lysosomes for degradation.⁹ It has been hypothesized that the buffering capacity of nanoparticles activates the proton pump that raises osmotic pressure inside an endosome, resulting in its swelling and subsequent escape of siRNA from the endosome.¹⁰⁻¹² Alternatively, it has been suggested that cationic lipids in LNPs interact with anionic lipids of the endosome, causing destabilization of the endosomal membrane.¹³ Regardless of the release mechanism, imaging studies have shown that nucleic acids are largely trapped inside the endosomes and lysosomes with only a small fraction being released to the cytoplasm.¹⁰⁻¹² Endosomal transport requires multiple heterotypic or homotypic endocytic fusion events with a constantly changing vesicular milieu regulated by a diverse set of endosomal proteins and different cell signaling cascades.¹³,¹⁴ The effect of this interplay between cell signaling and endocytosis on LNP trafficking needs to be further defined.

In this study, we used automated, high-resolution microscopy to screen a library of small molecules that have a range of effects on cell signaling, endocytosis and autophagy, and identify important key regulators of cellular uptake of cationic LNPs that can encapsulate siRNA (Supplementary Data). These LNPs were formed by the complexation of siRNA with the cationic lipid C12-200. They were further formulated with different excipients using a previously reported microfluidic process that generates slightly positively charged nanoparticles (~70–80 nm in size).¹⁵ They have demonstrated unprecedented efficacy in the delivery of siRNA in vivo.¹⁶,¹⁷ The potency of this delivery system, tested in rodents and nonhuman primates, is in a range similar to that of LNPs at the forefront of clinical trials.¹⁴ In rodents, no liver toxicity was observed at 1 mg/kg, a dose 100-fold above the efficacious range of silencing.¹⁶

When we incubated HeLa-GFP cells with LNPs containing Alexa Flour 647–labeled siRNA against GFP (siAF647-GFP) for 3 h, the particles appeared to localize at the perinuclear region within 1 h after exposure (Supplementary Fig. 1a, b). Subcellular localization of LNP-siRNA in the presence of small-molecule inhibitors under similar conditions was visualized and the amount of internalized siRNA was quantified. The small-molecule library consisted of molecules that affect cell signaling and intracellular trafficking and that either induce or inhibit autophagy (for a list of the inhibitors used and the quantification

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Systems survey for endocytosis of lipid nanoparticles (LNP). LNPs containing siAF647 were incubated with HeLa-GFP cells (50 nM) for 3 h in the presence or absence of small molecules were imaged and quantified using an automated high-throughput confocal microscope. (a) Small molecules that inhibit >80% of internalization (with no more than 10–15% loss of cell viability) and their network interactions in the form of a systems diagram. (b) Localization of siAF647-LNPs in the presence or absence of bafilomycin is represented as an individual image (top; arrows indicate peripheral localization) and a three-dimensional z-stack (bottom). Scale bars, LNP: 70 µm and LNP+baf: 100 µm. (c) Dual HeLa cells were exposed to LNP (siLuc, 10 nM) in the presence or absence of bafilomycin (1 µM). Luciferase to Renilla levels were measured for silencing activity. Experiments were done in triplicate; errors are plotted as s.e.m. (d,e). Time-lapse TIRF microscopy of cells exposed to siAF647-LNPs (50 nM, 3 h) in the presence or absence of bafilomycin combined with multiple particle tracking (MPT). A snapshot of vesicular tracks indicating the movement of LNPs in the presence or absence of bafilomycin (d). The ratio between the MPT parameters for untreated and/or bafilomycin-treated cells was calculated to provide a quantitative measure of differences in LNP trafficking with or without bafilomycin (e). Errors of ratios are plotted through propagation of errors from division.

Figure 1 Systems survey for endocytosis of lipid nanoparticles (LNP). LNPs containing siAF647 were incubated with HeLa-GFP cells (50 nM) for 3 h in the presence or absence of small molecules were imaged and quantified using an automated high-throughput confocal microscope. (a) Small molecules that inhibit >80% of internalization (with no more than 10–15% loss of cell viability) and their network interactions in the form of a systems diagram. (b) Localization of siAF647-LNPs in the presence or absence of bafilomycin is represented as an individual image (top; arrows indicate peripheral localization) and a three-dimensional z-stack (bottom). Scale bars, LNP: 70 µm and LNP+baf: 100 µm. (c) Dual HeLa cells were exposed to LNP (siLuc, 10 nM) in the presence or absence of bafilomycin (1 µM). Luciferase to Renilla levels were measured for silencing activity. Experiments were done in triplicate; errors are plotted as s.e.m. (d,e). Time-lapse TIRF microscopy of cells exposed to siAF647-LNPs (50 nM, 3 h) in the presence or absence of bafilomycin combined with multiple particle tracking (MPT). A snapshot of vesicular tracks indicating the movement of LNPs in the presence or absence of bafilomycin (d). The ratio between the MPT parameters for untreated and/or bafilomycin-treated cells was calculated to provide a quantitative measure of differences in LNP trafficking with or without bafilomycin (e). Errors of ratios are plotted through propagation of errors from division.
after 60 min (Fig. 2b,c and Supplementary Fig. 3b,c). Notably, after 60 min a fraction of the labeled siRNA starts to show co-localization with markers of the tubulovesicular endocytic recycling compartment (Rab11-GFP, antibody against Rab-11, transferrin) whereas a decrease in localization with lysosomal positive vesicles was observed (Fig. 2b,c and Supplementary Fig. 3c). Inhibition of Rab11 (Rab11 siRNA and dominant negative (DN)-Rab-11) to interfere with early endocytic recycling causes a 1.5-fold reduction in LNP internalization (Supplementary Fig. 3d,e). This decreased internalization may be a result of reduced endocytic recycling of trafficking regulators required for LNP entry.

Autophagy, another catabolic process that routes cytosolic proteins to the lysosomes3,24, was apparently not involved in LNP-mediated gene silencing as LNPs showed little co-localization with markers of autophagy (Supplementary Fig. 4a-b). Autophagy-deficient cells (Atg5−/−)24 showed little if any differences in LNP-mediated gene silencing when compared to autophagy competent (Atg5+/+) cells (Supplementary Fig. 4c).

Overall, C12-200–based cationic LNPs utilize macropinocytosis to enter cells and bypass the early endosomes; a small fraction is directed to the endocytic recycling compartment whereas most LNPs traffic toward the late endosome from where they are routed to the lysosomes. Our results are consistent with previous studies that cationic LNPs utilize distinct mechanisms of entry when compared to ionizable LNPs, which internalize through ApoE-dependent entry mechanisms16,25.

In addition to the moderate co-localization of LNPs with endocytic recycling compartment, our imaging studies also indicated a considerable redistribution of LNPs in the presence of a wide variety of compounds from the screen that cause stress on the endoplasmic reticulum (Supplementary Data). Therefore, we wanted to quantify the kinetics of LNP disassembly and the relative contributions of internalization and recycling on siRNA delivery using a fluorescence resonance energy transfer (FRET) probe26. The probe contains two identical siRNAs, each labeled with a different fluorophore that together form a FRET pair. The siRNA-bound fluorophores aggregate with the cationic lipids in the nanoparticle mixture and undergo FRET, which is indicative of intact LNPs. Flow cytometry data showed a time–dependent decrease in FRET-based fluorescence signal within 1 h of internalization, which was indicative of LNP disassembly inside cells (Fig. 3a). However, the fluorescence emanating from a single fluorophore (AF647) remained constant during this time. A decline in AF647 signal was observed only after 1–4 h, which substantially diminished after 24 h. Therefore, LNPs disassembled rapidly within the first hour of entry followed by degradation or recycling of siRNA within 24 h (Fig. 3a). Labeled siRNA was located in vesicular compartments alongside microtubules 1 h after incubation; indicating that most siRNAs remain trapped inside endosomes after nanoparticle disassembly (Fig. 3b).

To show that the reduction of intracellular siRNA-linked fluorescence was due to recycling of the siRNA to the extracellular media, we measured the fluorescence in the supernatant at multiple time points.

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**Figure 2** Cellular trafficking of LNPs. (a) Quantitative image analysis of siAF647-LNP cellular uptake (3 h) in HeLa cells silenced with siRNA against key endocytic regulators (Cdc42, Rac-1, clathrin heavy chain (CHC), caveolin-1 (Cav-1)). siRNA against luciferase serves as a negative control. (b,c) Image-based quantitative analysis of siAF647-LNPs co-localization (3-h pulse; 15-, 30-, 60-min chase) with markers of endocytosis, anti-EEA1 (early endosomes), anti-LAMP1 (late endosome/lysosomes), anti-LAMP2 (late endosomes/lysosomes), Rab11-GFP and anti-Rab11 (both mark endocytic recycling compartment). High-resolution z-stack confocal images representative of 60-min chase are presented in different panels (b). Quantification of LNP-siRNA cellular uptake using high-throughput confocal microscopy after indicated times (a,c). Error bars, mean ± s.e.m.

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**Figure 3** Quantitative analysis for disassembly and recycling of LNPs. (a) HeLa cells were exposed to LNP with the FRET pair (AF647/AF594 siRNA), washed and replensed with media. Changes in FRET (excitation-561 nm, emission-641 nm) intensity were monitored at different time points using flow cytometry to quantify LNP disassembly. The emission from a single fluorophore (excitation-633 nm, emission-641 nm) was used to measure intracellular siRNA at these time points. (b) Cellular uptake of FRET probe-LNP (siAF647/siAF594, 3 h) was imaged in stably transfected GFP-tubulin cells 1 h after incubation. (c) siAF647-LNP was pulsed for 3 h, washed and incubated with fresh media to remove noninternalized particles. Media was removed at multiple time points and analyzed using a fluorescent reader to determine amount recycled. 1% Triton-X was later added (red curve) to the media and fluorescence was remeasured. (d) Concentration of siRNA was obtained from fluorescence values from c that were extrapolated using the standard curve (inset). All experiments were performed in triplicate. Error bars, mean ± s.e.m.
after transfection. We found an increase in the fluorescence intensity that appeared within 6 h after incubation and reached saturation at 24 h in the extracellular milieu (Fig. 3c). Because intact nanoparticles partially quench fluorescence from the siRNA encapsulated inside them, we exposed the supernatant to Triton-X to dissociate any intact LNPs. This suggested that most LNPs had, in fact, disassembled inside the cells, confirming our FRET-based data. Approximately 36% of the initial concentration of siRNA incubated with the cells recycled into intact cationic LNPs enter through the plasma membrane (Exosomes secretion) (5). In NPC1-deficient cells, the late endosome recycling mechanisms are impaired, causing LNP-siRNA to accumulate in enlarged late endosomes leading to persistent escape of siRNA that improves gene silencing. (Intact nanoparticle—siRNA complex—). All experiments were performed in triplicate. Error bars, mean ± s.e.m.

causes late endosomal or lysosomal dysfunction, cholesterol accumulation and is implicated in a lysosomal storage disease that causes liver and neural degeneration in human patients and animal models. As the components of an LNP show some similarities to the endogenous lipids that use NPC1 as a receptor for recycling, we compared LNP retention in mouse embryonic fibroblasts (MEFs) devoid of NPC1 (NPC1+/−) with their wild-type counterparts (NPC1+/+ MEFs) (Fig. 4a). NPC1-deficient cells accumulated cholesterol due to defects in recycling (Supplementary Fig. 6). A markedly increased level (~15-fold) of AF647-siRNA was observed in perinuclear enlarged endosomes in NPC1−/− compared to NPC1+/+ MEFs at 24 h after incubation, presumably due to enhanced cellular retention at a wide range of LNP concentrations (Fig. 4a,b). Furthermore, enhanced cellular retention was observed in multiple cell types deficient in NPC1, including those isolated from human patients (Supplementary Fig. 7a,b). We further tested for differences in the kinetics of LNP

Figure 4 Enhanced cellular retention and efficacy of siAF647-LNPs in NPC1-deficient MEFs. (a) Immunoblot analysis with anti-NPC1 antibody was used to validate wild-type and NPC1-deficient MEFs. (b) Automated confocal microscopy on NPC1+/+ or NPC1−/− MEFs exposed to different concentrations of labeled LNPs imaged and quantified 24 h after incubation. Inset from a representative image (100 nM) shows siRNA accumulation in individual cells. (c) Flow cytometry analysis on LNP-siRNA uptake in (i) NPC1+/+ or NPC1−/− cells or in (ii) NPC1−/− cells transfected with pEGFP or pNPC1-GFP. The mean fluorescent intensity represents LNP uptake. (d) NPC1−/− cells treated with LNP-AF647-siRNA (red) (3-h pulse, 30-min chase) and immunostained with anti-LBPA antibody (green). (e) LNPs containing siRNA against β1 integrin were added to wild-type or NPC1-deficient MEFs as in b. mRNA levels were quantified at 24 h after incubation using branched DNA analysis. The experiment was done in triplicate and the errors are reported as s.e.m. (f) A schematic representation of LNP trafficking in (i) NPC1−/− and (ii) NPC1+/+ cells. Intact cationic LNPs enter through macropinocytosis (1); a small fraction of LNPs transport from macropinosomes to the endocytic recycling compartment (ERC) (2) whereas the majority is directed to late endosomes (3). Late endosome sort LNPs to lysosomes for degradation or utilize multiple recycling pathways to traffic them to the extracellular milieu. These mechanisms include recycling through transport to the ER-Golgi route (4) or direct fusion of late endosomes containing multivesicular bodies, with the plasma membrane (Exosomes secretion) (5). After transfection. We found an increase in the fluorescence intensity that appeared within 6 h after incubation and reached saturation at 24 h in the extracellular milieu (Fig. 3c). Because intact nanoparticles partially quench fluorescence from the siRNA encapsulated inside them, we exposed the supernatant to Triton-X to dissociate any intact LNPs. Exposure to Triton-X resulted in little change in fluorescence. This suggested that most LNPs had, in fact, disassembled inside the cells, confirming our FRET-based data. Approximately 36% of the initial concentration of siRNA incubated with the cells recycled into the supernatant and 2.5 times (70%) the concentration of the stable siRNA was present in the supernatant relative to inside cells after 24 h (Fig. 3d and Supplementary Fig. 5).

Subcellular trafficking of lipids (e.g., cholesterol) from late endosomes and lysosomes toward the extracellular milieu has been reported to utilize the 13 transmembrane glycoprotein NPC1, which is located on the surface of multivesicular late endosomes. Absence of NPC1
uptake in these cells. NPC1−/− cells had accumulated more siRNA after 2 h of incubation as compared to NPC1+/+ cells where at early time points the amount of internalization was not affected. Moreover, a rescue experiment in which we transfected an NPC1-GFP plasmid into NPC1−/− cells reduced the amount of intracellular LNP-siRNA to that of wild-type cells (Fig. 4c, i,ii). We conclude that the greater amount of siRNA in late endosomes is based on accumulation of LNPs due to defects in constitutively active NPC1-mediated recycling. Small molecules that impair cholesterol metabolism28 and increase cholesterol accumulation in cells failed to yield a significant increase in LNP accumulation (Supplementary Fig. 7c). Thus, the increased accumulation of LNPs was not due just to increased endosomal cholesterol but rather to lack of direct interaction with putative intraluminal domains of NPC1 required for egress of LNPs. NPC1−/− cells have been reported to have high numbers of enlarged late endosomes, containing intraluminal vesicles enriched in lysobisphosphatidic acid (LBPA)29. LNPs accumulated in these structures and co-localized with antibody against LBPA in these cells (Fig. 4d).

To study the broader potential relationship between lipid structure and NPC1-dependent LNP retention, we tested cationic siAF647-LNPs formulated with a small set of lipidoids consisting of two different amine cores, each with lipid tails of increasing chain lengths (Supplementary Fig. 11, reaction scheme). Different LNPs containing related cationic lipids also showed increased retention in NPC1-deficient cells. We also found that increased carbon chain lengths of these cationic lipids led to better uptake and retention in both NPC1+/+ and NPC1−/− MEFs (Supplementary Fig. 8a,b). This marked retention was consistent with studies that have shown that lipids with higher chain length can be inserted inside NPC1 carrying late endosome membranes30. Notably, liposomes containing small molecules showed similar increases in cellular retention in knockout cells (Supplementary Fig. 8c).

To determine whether the absence of NPC1 also leads to increased retention with polymeric nanoparticles, we tested polystyrene nanoparticles of different sizes and charges. We found a moderate increase in retention and perinuclear distribution with positively and negatively charged particles at sizes of 100 or 200 nm in NPC1−/− cells whereas negatively charged particles 20 nm in size (known to use a caveolae-mediated pathway31) showed similar retention when compared to wild-type cells (Supplementary Fig. 8d). We found that dextran, a marker for macropinocytosis, shows increased retention in knockout cells as compared to markers of caveola- or clathrin-dependent endocytosis (data not shown). Thus, 20-nm particles that can access 80-nm caveolae3,31 due to their smaller size are able to be retained inside both cell types (NPC1−/− and NPC1+/+) when compared to larger particles that may only use macropinocytosis to gain entry. It is possible that macropinosomes directly fuse with NPC1-positive late endosomes and are recycled to the extracellular milieu, in the absence of which the cargo remains diverted to the endosomal-lysosomal system. It is believed that in the case of certain types of cargo, NPC1-independent recycling allows for partial escape from the endosomal-lysosomal system30. This may not be the case with longer chain lipids that require interaction with NPC1 for cellular exit30. This may be the reason for the major retention of LNPs as compared to polystyrene nanoparticles.

We hypothesized that enhanced cellular retention could translate to improved gene silencing. LNPs containing β-integrin siRNA were added to either NPC1+/+ or NPC1−/− MEFs at different concentrations. A substantial improvement in LNP potency was observed in NPC1−/− cells in comparison to wild-type cells (Fig. 4e). The median inhibitory concentration (IC50) of 1.5 nM in NPC1 deficient cells was roughly one-tenth of wild-type MEFs, and the improved potency was seen across all LNP doses tested (Fig. 4e). To further measure cellular retention and siRNA efficacy, NPC1+/+ and NPC1−/− MEFs (stably transfected with GFP) were treated with LNsps containing GFP siRNA; high-throughput microscopy was used to simultaneously measure cellular retention and GFP fluorescence 72 h after incubation in both cell types. Enhanced cellular retention of siAF647-GFP and a decrease in normalized GFP fluorescence in NPC1−/− compared to that in NPC1+/+ cells were observed, confirming our previous results (Supplementary Fig. 9a–c). Next, we used siRNA against NPC1 to experimentally lower NPC1 protein levels in wild-type cells and a 60% reduction in protein level was achieved (Supplementary Fig. 9d,e). A fourfold increase in LNP efficacy was observed in wild-type cells treated with NPC1 siRNA when compared to the luciferase control (Supplementary Fig. 9f). Although this LNP efficacy was lower than what was achieved in NPC1−/− cells, 40% NPC1 protein still remained in NPC1+/+ cells treated with siNPC1 and this amount may still sufficient to recycle some LNPs, albeit at a less efficient rate. The use of lipids for transfection of NPC1 siRNA may alter intracellular trafficking or recycling and further explain why these effects were partial with siRNA knockdown versus the effect seen in complete NPC1−/− MEFs.

This led us to further investigate which endocytic recycling pathways participate in exocytosis of LNPs, and in particular whether inhibition of these pathways could enhance cellular retention and LNP-mediated gene silencing. Late endosomes have been shown to exchange material with the Golgi and endoplasmic reticulum (ER) from which the cargo can be secreted to the extracellular milieu32,33 and/or multivesicular bodies that can fuse with the plasma membrane to release their intracellular content as exosomes34. Therefore, we examined the effects of siRNA that blocks secretion from the Golgi/ER (Rab8a)32 or exosomes secretion and/or fusion with the plasma membrane (Rab27a and Rab27b)34. Rab8a and Rab27b siRNA treatment led to a substantial (fivefold) increase in retention of LNP in cells. A moderate increase in LNP retention was observed after reduction of Rab27a expression (Supplementary Fig. 10a–f). Notably, only in the case of Rab27b depletion did we detect a substantial improvement in LNP-mediated gene silencing (Supplementary Fig. 10g). Recent reports have shown that Rab27b inhibition leads to redistribution of late endosomes in the perinuclear region34. Concurrently, in our experiments both Rab27b-depleted and NPC1-deficient cells show similarly increased perinuclear retention of LNPs, which contains functional siRNA that contributes to enhanced gene silencing. On the other hand siRNA against Rab11 did not improve retention and gene silencing (Supplementary Fig. 10h,i), these results are consistent with previous reports that NPC1-mediated recycling of endogenous lipids is independent of Rab11 function even though they show some co-localization with Rab11-positive endocytic recycling compartment35.

In summary, to enter cells, cationic LNPs require Cdc42-dependent macropinocytosis, which is shown to be regulated through Vθ-ATPase, once internalized remain largely trapped within endosomes with little escape to the cytosol. LNP-mediated siRNA delivery can be impaired by either lack of LNP disassembly inside endosomes or their subsequent degradation in the lysosomes as well as recycling from the cell. In the absence of NPC1, due to decrease in motility of late endosomes30, LNPs remain in enlarged late endosomes/lysosomes and are not recycled back out of the cell. One hypothesis to explain how this leads to a greater efficiency in siRNA-mediated gene silencing is that the increased residence time of more siRNA inside cells results in a slow, controlled diffusion of the siRNA into the cytoplasm.
(Fig. 4f (i,ii)). It appears that late endosome/multivesicular late endosomes may serve as a transient reservoir where LNP recycle through multiple pathways. Prevention of direct egress of LNPs from the late endosomes leads to enhanced retention, and perinuclear positioning of these vesicular structures may serve as a persistent site for endosomal escape. Future studies that target proteins and lipids involved in biogenesis, trafficking and recycling of the late endosomes may provide further clues into the cellular factors that may improve drug delivery. Finally, the RNA-induced silencing complex (RISC) has been shown to be associated with membranes of late endosomes, and certain RISC complex components can be seen inside intraluminal vesicles of late endosomes/exosomes as well as the cytosolic side of the perinuclear rough endoplasmic reticulum. It is possible that in NPC1-deficient as well as Rab27b-depleted cells, LNPs remain stalled in a manner that affects siRNA delivery to RISC. From a therapeutics perspective, it is tempting to speculate that LNPs may demonstrate enhanced siRNA/drug delivery in lysosomal storage diseases where NPC1 function is impaired.

METHODS

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

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

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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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**ONLINE METHODS**

**Cell culture.** All cell types were grown in DMEM/10% FBS, including (i) HeLa cells—wild type, stable cells that co-express Luciferase and Renilla (Dual HeLa) or GFP—were provided by Alnylam Pharmaceuticals. (ii) NPC1 immortalized MEFs (NPC−/−) and NPC−/− or primary MEFs (NPC−/−, NPC+/−, NPC+/+) were kind gifts from P. Lobel at Rutgers University. (iii) NPC1-deficient human patient fibroblasts and age-matched NPC1 competent control were obtained from Coriell Institute of Medical Research. (iv) Atg5+/+ and Atg5−/− immortalized MEFs (NPC+/+ and NPC−/−) or primary MEFs (NPC−/−, NPC−/+, NPC+/+) were kind gifts from S. Varkar (Rudolph Jaenisch laboratory, MIT). (v) Stably transfected LLC-PK1 epithelial tubulin-GFP cell line were kind gifts from D. Brown of Massachusetts General Hospital, Boston.

**Endocytosis markers and small molecule library.** Plasmid DNA. Cdc42-GFP construct (Origene), Arf6-GFP construct (Origene), Traffic light C3-GFP-mRFP construct and NPC1-GFP (provided by S. Varkar, Rudolph Jaenisch laboratory, MIT), Rab7-GFP, Rab11-GFP and dominant negative DNARab11-GFP (provided by Roberto Zoncu, from David Sabatini’s laboratory at MIT), Rab5-RFP inside baculovirus (Life Technologies) were used for transfections in HeLa cells. Small-molecule library was purchased from Enzo Life Sciences and concanamycin, imipramine, fluphenazine and U18666A were obtained from Sigma Aldrich. **Endocytic tracers.** AF (Alexa Fluor) 594 Transferrin (Life Technologies) and AF 594 Ovulamine (Life Technologies), Lysotracker Red (Life Technologies) were used as markers for select endocytic vesicles in HeLa cells. Antibodies Mouse Anti Lysosomal Associate Membrane Protein-1 (LAMP1 Developmental Studies Hybridoma Bank; 1:200, H4A3), Mouse Anti Lysosomal Associated Membrane Protein-2 (LAMP2, Santa Cruz; 1:200, H4B4), Mouse anti-LBPA (lysophosphatidic acid, Echelon Biosciences; 1:100, 6C4), Mouse anti-EEA1 (BD Transduction; 1:50, 14/EEA-1), Mouse Anti Rab11 (BD Transduction; 1:100, A-11008/A10680). Goat anti-mouse Alexa Fluor 488 IgG antibody was used as a secondary antibody (Life Technologies).

**siRNA.** Purchased from Dharmacon. (a) siRNA NPC1 pool (purchased from Dharmacon) - siNPC1-09-CGAGCACACGUUGCGGAAU, siNPC1-10-CCUUAGAAUAUGAAGGAAU, siNPC1-11-ACACAGGCGCGUGUGAA, siNPC1-12- GGAGAGCUACCGGGAUU (b) siRNA Rab 11 pool (purchased from Dharmacon) -siRab11-7-GCAAAUGAUGGGUUCUCUUU, siRab11-8-CAAGAGCGAUAUGCUCAU, siRNA-9- GUGCGAGCGUGCGACAGCA (most efficacious, data not shown), siRNA-10-GAGAAUAUCGCGAUUU

Provided by Alnylam Pharmaceuticals. (2′-OMe modified nucleotides are in lower case and phosphorothioate linkages are represented by ‘s’):

a. siRNA Alexa Fluor 647 (siAF647GFP). GFP sense: AcAuGAAGc′-AF594-GAUUAUGUCCGGUUAUGUAU-3′

**Antisense:** UCUAAAUUGAUUUCCAUUCUtTdStD

Silencer Select Validated siRNA purchased from Ambion (Life Technologies).

a. Rab8a- Sense:GCAAGAGAAUUAAACUGCAAtt, Antisense:UGCAGAUUAAUCUUGGCG
b. Rab27a-Sense GCCUCUGACGGCUCUUAGGGCat, Antisense:U gccUGAUGCUUAAUGCGCGAGG Cat

c. Rab27b- Sense GACUUAUACUGAGGAAtt Antisense:UCGCGUU CAUGAUAUAGC

Life Technologies GAPDH siRNA was validated and used as a control.

**FRET probe.** siRNA duplexes labeled at the 5′ end of the sense strand with either Alexa Fluor 594 or Alexa Fluor 647 dyes were purchased from Integrated DNA Technologies. Sense 5′-A5FS94-GAUUAUGUCGGGCUUAGUUGAA-3′, 5′-AF647-GAUUAUGUCGGGCUUAGUUGAA-3′ and antisense 5′-UAACAU ACGCGACAUACUCUU-3′.

**Nanoparticles preparation.** Lipid nanoparticles. Cationic LNPs were prepared by rapidly mixing equal volumes of an ethanol solution of lipidoid and aqueous solution of siRNA on a microfluidic device, followed by dilution by PBS buffer15. The lipidoid solution contained C12-200 (ref. 16) or lipidoids consisting of two different amine cores, each with lipid tails of increasing chain lengths15 (new cationic lipids tested have been reported in the reaction scheme shown below), cholesterol (Avanti Polar Lipids), distearoyl phosphatidylcholine (DSPC, Avanti Polar Lipids), and a polyethylene glycol modified lipid (IR3-3-[(o-methoxy-PEG2000-carboxamyl) 1,2-di-O-tetradecyl-sn-glyceride (mPEG2000-DMG); synthesized by Alnylam, Cambridge, MA as previously described25) solubilized in 90% ethanol at mass concentrations of C12-200 (2.0 mg/ml); DSPC (0.28 mg/ml); cholesterol (0.52 mg/ml); mPEG2000-DMG (0.13 mg/ml). The siRNA was dissolved in 10 mM citrate, pH 3.0 buffer at a concentration of 0.4 mg/ml. To prepare LNPs, the siRNA solution, the lipidoid solution, and PBS buffer were injected into a microfluidic mixing device at volumetric flow rates of siRNA (300 liters/min); lipids (300 liters/min); buffer (600 liters/min), using three syringes (Gastight syringes, Hamilton Company), which were controlled by two syringe pumps (PHD 2000, Harvard Apparatus). Ethanol was removed by dialyzing LNPs against PBS buffer using membranes with MWCO of 3.5 kD (PierceProtein Research). In the case of liposomes designed to carry small molecules, hydrogenated soybean phosphatidylcholine (HSPC, Avanti Polar Lipids) was dissolved in ethanol and hydrated in a 5% dextrase solution with 0.2 mg/ml Alexa Fluor 67 (AF647) hydroxide tris(triethylammonium) salt (Molecular Probes) at 52 °C to form multilamellar vesicles (MLV). The MLV were downsized to form small unilamellar vesicles (SUV) by stepwise extrusion through polycarbonate membranes (SterilTech) using a Lipex extruder (Northern Lipids) starting at a pore diameter of 400 nm and ending at 100 nm. Nonencapsulated dye was removed by dialysis. LNPs generated through microfluidics were around 100 nm in size; the physicochemical properties of these nanoparticles have been previously characterized15.

**Poly styrene fluorophores.** Polystyrene nanoparticles of different sizes and charge—negatively charged were carboxylate modified (200 nm, 100 nm and 20 nm), positively charged were amine modified (200 nm)—were purchased from Life Technologies (Carlsbad).

**Confocal microscopy.** High-throughput confocal microscopy. HeLa-GFP cells were seeded at 1.5 × 104 cells per well in black 96-well plates (Corning Bio-one). Cells were pulsed with 50 nM of lipid nanoparticle (C12-200) encapsulated with Alexa 647 dye for multiple time points (0 min, 5 min, 15 min, 30 min and 60 min), fixed and counterstained with Hoechst. In select experiments to study the effect of small molecules on cellular uptake, cells were first pre-incubated for 30 min (or 24 h for cholesterol modulators) with the small molecules at a concentration of 10 μM (concentration for bafilomycin and concanamycin were kept at 1 μM and 500 μM, respectively). The cells were then incubated with siAF647 LNP for 3 h, washed and fixed after 60-min chase. For determination of subcellular localization of LNPs, HeLa cells (3 h pulse, 0–60 min chase) were fixed, permeabilized with 0.05% saponin and incubated with antibodies against LAMP1, LAMP2, Rab11, EEA1 for 2 h in room temperature or 24 h at 4 °C, washed, and followed by addition of secondary antibody for staining. In case of experiments that require transfection of plasmid DNA, cells were transfection (Lipofectamine 2000) 24 h before addition of LNPs. Furthermore, experiments that required treatment with Lyso-tracker (100 nM) or transferrin (5 μg/ml) were conducted with addition of these markers in the last 30 min of the 3 h LNP incubation period, followed by similar processing as discussed above. High-throughput microscopy was also conducted on NPC1+/+ and NPC1−/− that were plated in the same 96-well plates (half plate each), for simultaneous visualization and quantification of LNP retention (as well as GFP silencing) with equal exposure. In select experiments NPC1−/− cells exposed to LNPs (3 h pulse, 30-min chase) were subjected to permeabilization and incubation with anti-LBPA followed by detection using a secondary antibody. In all experiments cells were then counterstained in OPTI-MEM containing Hoechst.
Cells were washed with PBS and imaged (63×) using a Perkin Elmer Spinning Disk Confocal Microscope. A 2-stack was taken using the velocity software and an extended z-focus has been presented for analysis. After identification of cell location and perimeter, intracellular vesicles containing endocytic markers were pseudo-colored green, unless otherwise stated. The same defined pattern of 20 fields from each well was conducted as mentioned above. Cells were fixed and permeabilized (0.05% saponin) followed by addition of primary antibodies and secondary antibodies. For localization studies based on overexpression of endocytic markers or other controls, plasmid DNA was transfected 24 h before addition of LNPs. The cells were washed with PBS and imaged (63×) using a Perkin Elmer Spinning Disk Confocal Microscope. A 2-stack was taken using the velocity software and an extended z-focus has been presented for analysis. The images were done in the presence or absence of bafilomycin in OPTI/MEM. In select experiments LLC-PK1 epithelial tubulin-GFP cells were treated with LNP-FRET probe for 3 h, washed and imaged 1 h after incubation; individual fluorophores were imaged. AF647 signal: pseudo-colored green; AF594 signal: pseudo-colored red; tubulin-GFP signal: pseudo-colored gray.

Fluorescence-activated cell sorting (FACS)/flow cytometry. After aspirating conditioned medium, cells were washed with PBS and detached with 0.25% trypsin-EDTA (25 μg/ml, Life Technologies). FACS running buffer (50 μl), consisting of 98% PBS and 2% FBS, was added to each well. Cells were mixed thoroughly and then transferred to a 96-well round-bottom plate, and the AF647 signal was measured by FACS on a BD LSR II equipped with a fluid-handling robot (Becton Dickinson, San Jose, CA, USA). Cell gating and analysis were performed using FlowJo v8.8 software (TreeStar, Ashland, OR, USA). For transfection experiments involving GFP fusion proteins, GFP signal was collected in addition to the AF647 signal; two-dimensional gating was used to separate increased auto-fluorescence signals from increased GFP signals, and in these experiments transfected cells within the top 25th percentile of GFP expression were considered positive for significant transgene expression. Cell gating and analysis were done using FlowJo v8.8 software (TreeStar, Ashland, OR, USA).

Stable cells generation. Replication-incompetent lentiviral particles containing the GFP ORF were packaged in 293T cells with a VSV-G coat and used to infect MEVs. Virus-containing supernatant was collected 48, 60 and 72 h after transfection. 2 μg/ml of polybrene was added and the supernatant was filtered through a 0.45-μm filter and used for the infection. Culture medium (DMEM supplemented with 10% FBS (Hyclone), penicillin/streptomycin, l-glutamine and nonessential amino acids) was changed 12 h after the last infection.

siRNA transfection. β-integrin siRNA transfection. 50,000 NPC1+/+ or NPC1−/− were plated in 24-well plates. LNPs (C12-200/siβ-integrin) were added to cells grown overnight at different dilutions (the final concentration of siRNA in cells ranged from, 1.5–50 nM in OPTI-MEM). The cells were washed after 3 h and replenished with complete media. The cells were lysed after 24 h and B-DNA assay was performed. C12-200/siLUC containing LNP (50 nM) served as a positive control. In select experiments wild-type cells treated with siNPC1 twice (as discussed below) were further treated with LNP (siβ-integrin) followed by B-DNA assay after 24 h. A similar experiment was performed with Aig5+/+ and Aig5−/− cells. NPC1 siRNA transfection. NPC1−/− MEVs were exposed to siRNA against NPC1 (0.1–10 nM) using Lipofectamine 2000 (Invitrogen). The cells were washed after 24 h and B-DNA assay and 72 h for western blot analysis. In select experiments cells were retransfected (48 h after first transfection) for achievement of complete silencing of NPC1 followed by western blot analysis after 48 h (96 h of reverse transfection). siRNA to silence endocytic regulators. siRNA against Cdc42, Rac1, CHC, Cav-1, Rab11 were validated (data not shown). The most efficacious

Supplementary Movies 1 represents membrane level LNP trafficking whereas Supplementary Movie 2 is representative of LNP trafficking in the presence of bafilomycin.

Multiple particle tracking. Supplementary Movies 1 and 2 were subjected to multiple particle tracking using the Imaris software. An algorithm based on Autoregression motion model was applied to each movie. Spots/vesicles detected via Imaris Tracker were further processed by setting a threshold for spot detection based on visual accuracy of the predicted spots; a sphere was applied to represent each vesicle (8 μm). A gap size of 3 μm and maximum distance of 15 μm were set for prediction of the movement of a given spot to its next subsequent time point. The tracks were then filtered based on the accuracy of vesicular motion; the color-coded tracks (based on track duration) represent an average of duplicate experiments. For stability assays FRET studies were done in the presence or absence of bafilomycin in OPTI/MEM. In select experiments LLC-PK1 epithelial tubulin-GFP cells were treated with LNP-FRET probe for 3 h, washed and imaged 1 h after incubation; individual fluorophores were imaged. AF647 signal: pseudo-colored green; AF594 signal: pseudo-colored red; tubulin-GFP signal: pseudo-colored gray.

Fluorescence-activated cell sorting (FACS)/flow cytometry. After aspirating conditioned medium, cells were washed with PBS and detached with 0.25% trypsin-EDTA (25 μg/ml, Life Technologies). FACS running buffer (50 μl), consisting of 98% PBS and 2% FBS, was added to each well. Cells were mixed thoroughly and then transferred to a 96-well round-bottom plate, and the AF647 signal was measured by FACS on a BD LSR II equipped with a fluid-handling robot (Becton Dickinson, San Jose, CA, USA). Cell gating and analysis were performed using FlowJo v8.8 software (TreeStar, Ashland, OR, USA). For transfection experiments involving GFP fusion proteins, GFP signal was collected in addition to the AF647 signal; two-dimensional gating was used to separate increased auto-fluorescence signals from increased GFP signals, and in these experiments transfected cells within the top 25th percentile of GFP expression were considered positive for significant transgene expression. Cell gating and analysis were done using FlowJo v8.8 software (TreeStar, Ashland, OR, USA).

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siRNA was transfected to HeLa cells using Lipofectamine RNAi Max (50 nM, 6 h). After 48 or 72 h, the cells were retransfected with the lipid/siRNA complex (50 nM, 3 h) and the cells were exposed to siAF647-LNPs (50 nM, 3 h) after 24 h. The cells were fixed, counterstained with Hoechst and subjected to high-throughput confocal microscopy. **siRNA to silence late endocytic recycling regulators.** siRNA against Rab8a (10 nM), Rab27a (10 nM), Rab28b (10 nM), GAPDH (10 nM) and Rab11 (50 nM, twice) were validated (data not shown). siRNA was transfected to Dual HeLa cells using Lipofectamine RNAi Max for 6 h, cells were washed and their media replaced. After 72 h the cells were exposed to siAF647-LNPs (50 nM, 3 h); cells were washed and incubated with fresh media for 3 h then fixed, counterstained with Hoechst and subjected to high-throughput confocal microscopy. In case of efficacy studies the (siRab) pretreated cells (post 72 h) were transfected with siLuc-LNP (0.15, 1.5, 15 and 150 nM). Relative firefly luciferase silencing was assessed 24 h after transfection using a Dual-Glo luciferase assay kit (Promega, Madison, WI). Normalization to Renilla luciferase values served as a control for potential toxicity. **Western blot analysis.** Cell pellets were lysed on ice in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) for 30 min in the presence of complete protease inhibitor cocktail (Roche Diagnostics), boiled for 5–7 min at 100 °C and subjected to western blot analysis, as previously described24. Primary antibodies include anti-NPC1 and anti-actin (kind gifts from Sovan Sarkar at Rudolph Jaenisch laboratory). Blots were probed with anti-mouse or anti-rabbit IgG–HRP secondary antibody (1:4,000 dilutions) and visualized using ECL detection kit (GE Healthcare). **Branched-DNA (B-DNA) assay.** HeLa cells treated with LNPs were lysed in cell lysis buffer (Epicenter) containing Proteinase K. The Quantigene assay 2.0 (Affymetrix, previously Panomics) was used to measure mRNA knockdown (β-integrin or NPC1) according to manufacturer’s instructions. Levels of the target gene were compared to a known housekeeping gene GAPDH and then normalized to controls.

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