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Niemann-Pick C2 protein regulates sterol transport between plasma membrane and late endosomes in human fibroblasts

Running title: Sterol transport in NPC2 deficient fibroblasts

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Highlights

- Plasma-membrane derived sterol is transported to late endosomes/lysosomes but becomes trapped in these organelles in NPC2 deficient cells.
- A small sterol pool remains mobile as shown by fluorescence recovery after photobleaching (FRAP).
- Quantitative lipid mass spectrometry reveals NPC2-dependent but little esterification of plasma-membrane derived sterol.
Internalized NPC2 rescues sterol storage and increases the mobile fraction of DHE in FRAP experiments.
Our data supports that NPC2 mobilizes sterol from vesicles inside late endosomes for non-vesicular export to other compartments.

Abstract

Niemann-Pick disease type C2 is a lipid storage disorder in which mutations in the NPC2 protein cause accumulation of lipoprotein-derived cholesterol in late endosomes and lysosomes (LE/LYSs). Whether cholesterol delivered by other means to NPC2 deficient cells also accumulates in LE/LYSs is currently unknown. We show that the close cholesterol analog dehydroergosterol (DHE), when delivered to the plasma membrane (PM) accumulates in LE/LYSs of human fibroblasts lacking functional NPC2. We measured two different time scales of sterol diffusion; while DHE rich LE/LYSs moved by slow anomalous diffusion in disease cells (D~4.6·10^{-4} μm^2/sec; α~0.76), a small pool of sterol could exchange rapidly with D~3 μm^2/sec between LE/LYSs, as shown by fluorescence recovery after photobleaching (FRAP). By quantitative lipid mass spectrometry we found that esterification of 13C-labeled cholesterol but not of DHE is reduced 10-fold in disease fibroblasts compared to control cells. Internalized NPC2 rescued the sterol storage phenotype and strongly expanded the dynamic sterol pool seen in FRAP experiments. Together, our study shows that cholesterol esterification and trafficking of sterols between the PM and LE/LYSs depends on a functional NPC2 protein. NPC2 likely acts inside LE/LYSs from where it increases non-vesicular sterol exchange with other organelles.

Abbreviations
ACAT, acyl-Coenzyme A acyl transferase; Alexa647-Tf, Alexa647-tagged transferrin; \(^{13}\)C\(^3\)-cholesterol, cholesterol with heavy carbon (C\(^{13}\)) at position 3; CHO; Chinese Hamster Ovarian; ER, endoplasmic reticulum; D, diffusion coefficient; DHE, dehydroergosterol; DHE/CD, dehydroergosterol-cyclodextrin complex; DHE/BSA, complex of dehydroergosterol with bovine serum albumin; DIT, dynamic intensity threshold; FRAP, fluorescence recovery after photobleaching; LE/LYSs; late endosomes/lysosomes; LPDS, lipoprotein depleted serum; MSD, mean square displacement; NPC1, Niemann Pick type C1 protein; NPC2; NPC2, Niemann Pick type C2 protein; PC, phosphatidylcholine, NPC2+/+ cells; human fibroblasts from healthy subjects; NPC2-/- cells; human fibroblasts from patients lacking functional NPC2; OG-dextran; Oregon-green-tagged dextran; PC, phosphatidylcholine; PDF, probability density function; PM, plasma membrane; REs, recycling endosomes; , Rh-dextran, tetramethyl-rhodamine-tagged dextran; ROI, region of interest; SM, sphingomyelin; TGN, trans-Golgi network

Key words: cholesterol; vesicle tracking; fluorescence recovery after photobleaching; diffusion, Niemann-Pick disease type C2; lysosome; non-vesicular; lipid mass spectrometry; kinetics

Introduction

Niemann-Pick type C (NPC) disease is a lysosomal storage disorder characterized by accumulation of cholesterol and sphingolipids in various organs of affected individuals. NPC patients experience progressive neurodegeneration and hepatosplenomegaly as a consequence of mutations in either of two genes (Mukherjee and Maxfield, 2004; Storch, 2009). One gene encodes NPC1, a large 1278-amino acid polytopic membrane protein localized mainly to the limiting membrane of late endosomes (LEs). The other gene encodes a small protein of 132 amino acids residing mostly in lysosomes (LYSs). Current treatments of NPC disease are largely symptomatic and the life expectancy of affected patients is variable (Rosenbaum and Maxfield, 2011). NPC1 and NPC2 bind cholesterol as well as the intrinsically fluorescent cholesterol analog dehydroergosterol
(DHE) (Friedland, 2003; Liu et al., 2009; Poongavanam et al., 2016). The exact mechanisms underlying NPC1/NPC2 function in export of cholesterol derived from lysosomal hydrolysis of low density lipoprotein (LDL) are not known. NPC2 has been shown to accelerate sterol exchange between liposomes, especially under conditions mimicking the intra-endosomal milieu (Babalola et al., 2007; Cheruku et al., 2006; Gallala et al., 2011; McCauliff et al., 2015; Xu et al., 2008). How this in-vitro function of NPC2 relates to the observed cellular disease phenotype is not known.

Intracellular trafficking of cholesterol is mediated by vesicular and non-vesicular modes of transport, but their relative contribution to the establishment and maintenance of the characteristic distribution of sterols in cells remains poorly understood (Mesmin and Maxfield, 2009; Wüstner, 2009). It was found that in addition to the plasma membrane (PM), the recycling endosomes (REs), sometimes also called endocytic recycling compartment, the trans-Golgi network (TGN) and the internal vesicles of LEs called intraluminal vesicles (ILVs) contain most of the cellular cholesterol (Hao et al., 2002; Ikonen, 2008; Möbius et al., 2003). In contrast, the LYSs were found to contain only low amounts of cholesterol in healthy human cells (Lange et al., 2002; Lange et al., 1998; Möbius et al., 2003). The classical model is that intracellular transport of LDL-derived cholesterol is defective in NPC1 disease fibroblasts (Liscum et al., 1989; Pentchev et al., 1985). Fibroblasts lacking functional NPC1 hydrolyze LDL-derived cholesteryl esters normally but have a strongly reduced ability to elicit regulatory responses. This includes a lack of stimulation of cholesterol esterification and diminished suppression of cholesterol de novo biosynthesis by ingested LDL (Kruth et al., 1986; Pentchev et al., 1985). Despite the low steady state concentration of cholesterol in LYSs in human fibroblasts, Lange and co-workers showed using radioactive cholesterol and subcellular fractionation, that most PM-derived sterol circulates through LYSs with a rate of about 5%/h (Lange et al., 1998). These authors argued that also in NPC1 deficient fibroblasts the then strongly expanded lysosomal cholesterol pool is dynamic, just with strongly
reduced exit rates (Lange et al., 2000). They found that PM-derived cholesterol accumulated in LYSs of NPC1 deficient fibroblasts even in the absence of LDL in the medium (Lange et al., 2000). Pipalia et al. (2006) reported that DHE gets trapped in aberrant lysosomal compartments called lysosomal storage organelles in Chinese Hamster Ovarian (CHO) cells lacking functional NPC1 protein over a time course of six hours (Pipalia et al., 2006). This was accompanied by misrouting of fluorescent lipid probes and transferrin to the lysosomal storage organelles, suggesting a general trafficking defect in cells with perturbed NPC1 function. Chang and co-workers reported that even endogenously synthesized cholesterol accumulated in CHO cells with mutated NPC1 protein (Cruz and Chang, 2000) and in various cell types isolated from a NPC1 mouse disease model (i.e. homozygous BALB/c NPC1 mice) (Reid et al., 2003). Together, these results suggest that NPC1 protein is necessary to mediate efflux not only of LDL-derived cholesterol but also to prevent lysosomal accumulation of endogenous cholesterol. Whether the same holds also for NPC2 with its intraluminal location and likely different function in lysosomal cholesterol egress is not known.

We have performed a detailed quantitative imaging and mass spectrometry study to monitor the intracellular destinations and metabolic fate of PM-derived sterol in healthy fibroblasts and in NPC2 deficient fibroblasts. We show that the fluorescent cholesterol analog DHE is transported between the PM, REs and LE/LYSs, but becomes slowly trapped in the latter compartment in disease fibroblasts. Using a new image analysis protocol, we determine that the amount of DHE internalized from the PM and accumulating in LE/LYSs is increased ~2.5-fold in disease compared to control fibroblasts. By quantitative lipid mass spectrometry of $^{13}$C$_3$-cholesterol we demonstrate that esterification of PM-derived sterol is defective in NPC2 disease cells. Internalized NPC2 rescues the sterol storage in LE/LYSs. Using fluorescence recovery after photobleaching ((FRAP) we demonstrate that NPC2 increases non-vesicular sterol exchange between LE/LYSs and other organelles. We conclude that NPC2 acts from inside LE/LYSs, where
it mediates non-vesicular export of cholesterol, likely by supplying it from ILVs to proteins in the limiting membrane of LE/LYSs.

**Materials and Methods**

**Reagents**

Fetal calf serum (FCS) and DMEM were from GIBCO BRL (Life Technologies, Paisley, Scotland). All other chemicals including human lipoprotein depleted serum (LPDS) were from SIGMA Chemical (St. Louis, MO). Rhodamine-labeled dextran (Rh-dextran; 70kD), Mitotracker Red, succinimidy esters of Alexa488, Alexa546 and Alexa647 as well as C6-nitrobenzoxadiazole (NBD)-Ceramide (C6-NBD-Cer) and Alexa488-tagged bovine serum albumin (Alexa488-BSA) were purchased from Invitrogen/Molecular Probes (Inc. USA). Buffer medium contained 150 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM glucose and 20 mM HEPES (pH 7.4) as described (Hao et al., 2002). Release medium was buffer medium supplemented with 25.5 mM citric acid, 24.5 mM sodium citrate, 100 mM deferoxamine mesylate, was adjusted to pH 5.2, and contained 280 mM sucrose instead of glucose (see above). Transferrin (Tf) was iron loaded as previously described (Yamashiro, 1984). Succinimidyl ester of either Alexa647 dye (emission in infrared) or of Alexa546 dye (emission in red) were then conjugated to the iron-loaded Tf following the manufacturer’s instructions. Synthetic lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Chemicals and solvents for lipidomics were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Rathburn Chemicals (Walkerburn, Scotland). DHE was either purchased from SIGMA Aldrich Chemical (St. Louis, MO) or synthesized using a modified protocol of Schroeder and colleagues (McIntosh et al., 2008). Purification of NPC2 from bovine milk was carried out as described previously (Larsen et al., 1997; Lund et al., 2014).
**Cell culture**

Human skin fibroblasts from control subjects (Coriell Institute #GM08680; male healthy donor) and from NPC2 patients (Coriell Institute #GM18455) were used. The NPC2 deficient cells were from a male patient who is a clinically affected heterozygote at the NPC2 gene locus: allele 1 carries a substitution (G>T) at nucleotide 58 (c.58G>A) in exon 1, resulting in a nonsense mutation at codon 20, while allele 2 carries a substitution (G>T) at nucleotide 140 (c.140G>T) in exon 2, resulting in a missense mutation at codon 47. The fibroblasts showed no detectable activity in a cholesterol esterification assay and abnormal accumulation of recycling glycoproteins (Mbua et al., 2013; Park et al., 2003). Cells were grown at 37°C in an atmosphere of 5% CO₂ until 90% confluence in complete DMEM culture medium supplemented with 1% glutamine, 1% penicillin and 10% FBS for control cells or 20% FBS for disease cells. The cells were placed on microscopy dishes and allowed to settle for another 48-72 h before the experiment in the same culture medium.

**Labeling of cells with DHE, 13C₃-cholesterol and organelle markers**

A stock solution of DHE was made in ethanol and stored under nitrogen at -80°C. For staining cells, a DHE labeling solution containing a complex of DHE with methyl-β-cyclodextrin (MCD) to get DHE/MCD was generated as described (Hao et al., 2002; Wüstner et al., 2002). Alternatively, 20 µg/mL DHE was loaded onto bovine serum albumin (BSA) in a solution of 40 mg/ml BSA under sterile conditions.

**Uptake and sorting of endocytic markers.** For uptake experiments, control and NPC2 mutant fibroblasts were labelled with 5 µg/ml Alexa546-Tf in cell culture medium at 37°C for the indicated time points. After labelling, the cells were washed with buffer-medium, chilled with ice-cold buffer-medium and incubated in release medium for 10 min on ice to remove surface-bound Tf by a mild acid-wash (Ghosh et al., 1994; Wüstner et al., 2002). For analysis of endocytic sorting, cells were co-incubated with 5 µg/ml Alexa647-Tf and 0.5 mg/ml Rh-dextran in complete growth medium for
the incubated time points. Cells were washed with buffer medium and imaged as described below. In separate experiments, Rh-dextran labeled cells were subsequently labeled for 2 h with LysoTracker® DeepRed following the manufacturer’s instructions.

**Pulse-chase experiments with DHE.** Cells were pulse-labeled for 3 min with DHE/MCD, washed with buffer medium, and either imaged directly, or chased for up to 5 h at 37°C in buffer medium (Wüstner, 2007; Wüstner and Færgeman, 2008). In some of these experiments, cells were preincubated with 0.5 mg/ml Rh-dextran overnight in sterile growth medium to visualize LE/LYSs, followed by a wash in buffer medium and by pulse-labeling with DHE/MCD and long-term chase, as described above. During that chase, cells were eventually labeled with 1 mg/ml Oregon-green-dextran (OG-dextran). Alternatively, they were labeled with 5 µg/ml Alexa647-Tf during the last 20 min of that chase to selectively stain REs. In other experiments, no selective labeling of the endocytic pathway was performed, but cells were after pulse-labeling with DHE/MCD and chase for up to 5 h incubated with a sterol MCD/cholesterol exchange solution for 3 to 10 min to replace DHE in the PM by cholesterol, as described (Wüstner et al., 2005).

**Continuous labeling experiments with DHE or $^{13}$C$_3$-cholesterol.** Cells were incubated with the DHE/BSA solution in LPDS containing growth medium for 24-72 hrs under sterile conditions. In some experiments designed to determine sterol transport to the REs and to LE/LYSs during continuous uptake, cells were preincubated with sterile DMEM containing and 0.5 mg/ml Rh-dextran overnight. Subsequently, cells were washed with sterile DMEM and incubated with DHE/BSA in DMEM with LPDS for the indicated times. Cells were washed with buffer medium followed by a chase in buffer medium containing 5 µg/ml Alexa647-Tf to label the REs. For lipid mass spectrometry cells were cultured in 75cm$^2$ flasks until confluent at 37°C in an atmosphere of 5% carbon dioxide in DMEM containing 100 units/mL penicillin and 100 mg/mL streptomycin sulfate, and 10% FBS. Cells were washed with 10 ml 10% PBS, trypsinized for 5 min, and 10 ml
fresh growth media was added to culture flasks. Two ml growth media and 250 µl of cell culture were added to each 6-well plate. A continuous uptake experiment was performed as described above after 4 hrs chase in DMEM with LPDS using a final concentration of 50 µM DHE in a DHE/BSA complex or 13C3-cholesterol in a 13C3-cholesterol/BSA complex, respectively. Cells were incubated for 24 hrs with the sterol/albumin complex in DMEM containing LPDS, washed extensively and harvested by scraping in ice-cold 155 mM ammonium acetate, followed by centrifugation at 4000 g at 4°C for 5 min and washing three times with ice-cold 155 mM ammonium acetate. The supernatant was removed and cells were stored at -80°C until further processing.

Fluorescence microscopy

Wide field epifluorescence microscopy was carried out on a Leica DMIRBE microscope with a 63 x 1.4 NA oil immersion objective (Leica Lasertechnik GmbH) with a Lambda SC smart shutter (Sutter Instrument Company) as illumination control. Images were acquired with an Andor Ixon blue EMCCD camera operated at -75°C and driven either by the Solis software supplied with the camera or by µManager, a Java-based open source software (Edelstein et al., 2014). DHE was imaged in the UV using a specially designed filter cube obtained from Chroma Technology Corp. with 335-nm (20-nm bandpass) excitation filter, 365-nm dichromatic mirror and 405-nm (40-nm bandpass) emission filter. Alexa546-NPC2 and Rh-dextran were imaged using a standard rhodamine filter set [535-nm, (50-nm bandpass) excitation filter, 565-nm longpass dichromatic filter and 610-nm (75-nm bandpass) emission filter], while Alexa488-NPC2, Alexa488-BSA, OG-dextran and C6-NBD-Cer were imaged using a standard fluorescein filter set [470-nm, (20-nm bandpass) excitation filter, 510-nm longpass dichromatic filter and 537-nm (23-nm bandpass) emission filter]. Alexa647-Tf and LysoTracker® DeepRed were detected with an infrared filter cube [620-nm, (20-nm bandpass) excitation filter, 660-nm dichromatic mirror, and 700 nm (75-nm
bandpass) emission filter. For detecting co-localization of DHE with organelle markers, a correction for chromatic aberration was performed as described (Wüstner and Færgeman, 2008).

Fluorescence recovery after photobleaching (FRAP) and time-lapse experiments. Cells were preincubated with 0.5 mg/ml Rh-dextran overnight to visualize LE/LYSs, as described above. Cells were either pulse-labeled with DHE/CD for 3 min or continuously labeled with DHE/BSA for 48 h as described above. Pulse-labeled cells were chased for the indicated time in buffer medium. Cells were placed on the stage of the wide field microscope and images were taken in the UV channel (the DHE prebleach image) and in the red channel (for Rh-dextran) by taking chromatic aberration into account, as described (Wüstner and Færgeman, 2008; Wüstner et al., 2002). The bleached region was determined by closing the field aperture in the microscope optical train thereby narrowing the field of view to a circular region of radius 15 µm. Another reference image was taken in the red channel to determine position and size of the region of interest (ROI) for the bleach before the same ROI was bleached in the DHE channel for five sec by continuous illumination from the mercury arc lamp (Wüstner et al., 2002). Subsequently the field aperture was opened, a 50% neutral density filter was inserted and images were acquired in the DHE channel every 20 sec. A short acquisition time (typically 5-10 msec) was chosen to minimize photobleaching during recording of fluorescence recovery sequences. Based on the red channel image of Rh-dextran, the focus was set to the LE/LYSs while taking the chromatic shift between the UV- and red channel into account. For FRAP in the PM, the ROI was moved to the cell periphery to avoid bleaching underlying organelles. For time-lapse imaging, cells were labeled either by using the pulse-chase or the continuous labeling protocol (see above), washed with buffer medium and placed on the stage of the wide field microscope. Subsequently, cells were imaged with a short acquisition time and in the presence of a 50% neutral density filter to reduce photobleaching of DHE. The frame rate was set to
0.167 Hz and the EMCCD settings were set to electron multiplication in the Andor software control to maximize the signal-to-noise ratio.

**Mass spectrometric lipid analysis**

Samples corresponding to 20 µg protein were spiked with 10 µl lipid internal standard mixture containing 500 pmol cholesterol+3H7, 100 pmol CE 19:0, 70 pmol PC 36:6 and 70 pmol SM 35:1:2 in methanol. Sample amount was adjusted to 200 µl with 155 mM ammonium acetate and extracted with 990 µl chloroform/methanol (10:1, v/v) for 2 h at 4°C (Sampaio et al., 2011). The lower organic phase was collected, evaporated and kept at -20ºC until analyzed. Lipid extracts were dissolved in 80 µl chloroform/methanol (1:2, v/v) and subjected to mass spectrometric analysis using a QSTAR Pulsar-i instrument (MDS Sciex, Concord, Canada) equipped with a TriVersa NanoMate nanoflow ion source (Advion Biosciences), as previously described (Ejsing et al., 2009).

Phosphatidylcholine (PC) and sphingomyelin (SM) species were monitored using positive ion mode precursor ion scanning for m/z 184.0733 (Ekroos et al., 2002). Sterol esters were monitored using parallel reaction monitoring (Ejsing et al., 2015). PC, SM and sterol esters were identified using LipidView (Sciex, Concord, Canada) (Ejsing et al., 2006) and quantified using Microsoft Excel.

Analysis of cholesterol, cholesterol+13C3, cholesterol+3H7 and DHE was performed by chemical sulfation of lipid extracts (Carvalho et al., 2012; Sandhoff et al., 1999) followed by high resolution Fourier transform mass spectrometry in negative ion mode using an LTQ Orbitrap XL (Thermo Fischer Scientific) equipped with a TriVersa NanoMate (Advion Biosciences), as previously described (Carvalho et al., 2012; Casanovas et al., 2015). The sterols were identified using ALEX software (Husen et al., 2013) and quantified using Microsoft Excel.

**Image analysis**
Quantification of endocytic capacity of control and disease fibroblasts. For quantification of probe uptake by digital image analysis, images were first background-corrected as described (Ghosh, 1995; Mukherjee et al., 1999). Macros were programmed for the open-source image analysis software ImageJ (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij), which measure cell-associated probe fluorescence above a threshold value defined by the autofluorescence of the non-labeled cells in the respective channel in an automated manner. Mean intensity per field was exported as ASCII file and data was plotted as mean ± SE of 2 experiments with at least 8 images per time point and condition and 2-5 cells per image. The Pearson co-localization coefficient was calculated between background-corrected images of disease cells double-labeled with Rh-dextran and Alexa657-Tf.

Assessment of DHE fluorescence compared to autofluorescence. Primary cells like human fibroblasts can have significant autofluorescence over the whole spectral range (Andersson et al., 1998; Monici, 2005). It is therefore important that the relatively weak DHE fluorescence can be unequivocally distinguished from cellular autofluorescence under all experimental conditions. We have recently developed a pixel-wise bleach rate analysis implemented as a plugin ‘PixBleach’ to ImageJ (Wüstner et al., 2010). When applying this analysis to fibroblasts, we can clearly discriminate DHE fluorescence from cellular autofluorescence, (not shown here, but see (Wüstner et al., 2011; Wüstner et al., 2010)). Autofluorescence appeared as dim and constant background in the UV channel for bleach image stacks, both in control and in disease fibroblasts, as described (Modzel et al., 2017; Wüstner et al., 2011). DHE fluorescence was about sixfold and at least tenfold the autofluorescence in the UV channel in the pulse-chase and continuous uptake experiments, respectively. The impact of uneven illumination in the image field was assessed by calculating the time-integrated emission (TiEm) of DHE from selected bleach stacks, as described (Wüstner et al., 2014). The TiEm of a probe is independent of quantum yield and illumination shading and can be
used to correct for cellular autofluorescence (Hirschfeld, 1976; Wüstner et al., 2014). We found the effect of these parameters on quantification of DHE fluorescence from many cell images negligible (not shown). We therefore continued the quantification of sterol fluorescence with background-corrected images only (see below).

Automated image-based measurement of organelle sterol content in pulse-chase experiments. To be able to perform a reliable quantification of DHE targeting to intracellular organelles within the purpose of this study, we developed a new plugin to ImageJ. This plugin generates first a binary mask from up to three channels of organelle markers (for example, C6-NBD-Cer in green, Rh-dextran in red and Alexa647-Tf in infrared) using a dynamic intensity threshold (DIT). We found that cell-to-cell variation of marker uptake can compromise simple intensity thresholding, as the threshold would need to be adjusted for each condition, thereby introducing some ambiguity into data analysis. Using a DIT, the organelle-associated intensity of Rh-dextran or Alexa647-Tf becomes weighted by the respective mean value of total cellular fluorescence of the organelle markers. Any variation in probe uptake, e.g. of Rh-dextran, from cell to cell and between various co-localization experiment can be accounted for by this method, since the DIT allows for weighting the marker intensity in the selected region by the total cellular intensity of the respective probe. The user can determine this threshold interactively for each image session with the degree of overlap between the organelle markers being calculated and shown in some separate images (see Fig. S2 in Supplemental data). By adjusting the DIT, the cell area covered by a given organelle marker can be defined and converted to a binary mask. We chose the DIT for both reference channels such that marker overlap between Alexa647-Tf in REs and Rh-Dextran in LE/LYSs was minimal (see Fig. S2). Separately, a whole cell mask is generated from the DHE images using a low intensity threshold, which segments the cells from the background. The binary masks obtained from the marker channels are subsequently multiplied separately with the background corrected DHE images.
thereby creating a measure of DHE content in each of the stained organelles. The plugin quantifies
the fluorescent sterol inside PM, LE/LYSs or REs compared to the fluorescent sterol in the total
cell. The program also takes into account apparent sterol intensity in the organelles originating from
the overlaying PM as performed manually previously (Hao et al., 2002). The plugin works with
stacks, and one can thanks to its intuitive graphical user interface (GUI; Fig. S2A), change the
respective thresholds with a slider per mouse click. The plugin allows one to generate binary
organelle masks based on up to three organelle markers, and it accounts for partial marker overlap
and differences in organelle labeling. The plugin is available from the authors upon request.

Quantification of fluorescence recovery of DHE. A macro was written within ImageJ, which
automatically performs a background subtraction, measures the integrated intensity in the bleached
area and in the whole cell, as outlined manually, and calculates the ratio of DHE fluorescence in the
bleached region divided by the total cellular DHE intensity. This procedure efficiently accounted
for any photobleaching during image acquisition, as we showed in experiments (Wüstner et al.,
2002) and simulations (Solanko et al., 2013). Subsequently, the resulting fractional recovery was
normalized to the initial postbleach intensity (i.e., the first data point in the recovery curve). To
determine the lateral diffusion coefficient, the recovery curves were fitted to

\[
f(t) = \frac{f_\infty \cdot t / t_{1/2}}{1 + t / t_{1/2}}
\]

where \(f_\infty\) is the final fluorescence intensity and \(t_{1/2}\) is the half time of recovery. From this the lateral
diffusion constant is given by:

\[
D = \frac{r^2}{4 \cdot t_{1/2}}
\]
For visualization of the recovery, as shown in Fig. 4 in the Results section, another macro was developed which measures the pixel-wise intensity and normalizes each image to the average intensity in the pre-bleach image. This macro generates a new stack in which the recovering DHE fluorescence in the bleached ROI was not obscured by photobleaching during image acquisition. It was only used for visualization purposes but not for quantification.

**Automated tracking of vesicles containing DHE.** Vesicle tracking was performed in SpatTrack, our recently developed automated tracking program (Lund et al., 2014; Lund and Wüstner, 2013). Prior tracking, the image sequences were denoised in SpatTrack using a boxcar filter as described (Lund et al., 2014; Lund and Wüstner, 2013). After recording particle positions over time, the trajectories can be further analyzed in SpatTrack, for example by calculating the mean squared displacement (MSD) as

$$MSD(\Delta t = nh) = \frac{1}{N-n} \sum_{i=1}^{N-n} \{[x(ih) - x(ih + nh)]^2 + [y(ih) - y(ih + nh)]^2\}, \quad (3)$$

where \(N\) is the number of frames, \(h\) is the time between subsequent frames, and \(\Delta t\) is the time lag corresponding to \(n\) frames. The MSD was fitted to either a model of normal diffusion according to

$$MSD(t) = 4Dt, \quad (4)$$

, to a model of anomalous subdiffusion

$$MSD(t) = 4D_\alpha t^\alpha. \quad (5)$$

or to an extended model for anomalous diffusion including a component for directed diffusion (Lund et al., 2012a):

$$MSD(t) = 4D_\alpha t^\alpha + v^2t^2. \quad (6)$$

Here, \(D\) is the diffusion constant, \(v\) is the velocity and \(t\) is the time lag. The anomalous exponent, \(\alpha\), defines for \(0 < \alpha < 1\) particle confinement called anomalous subdiffusion.
Results

Pathways and kinetics of DHE transport from the plasma membrane in human fibroblasts

First, we studied the uptake and intracellular targeting of the cholesterol analog DHE from the PM to intracellular organelles in healthy and NPC2 disease fibroblasts. For that purpose, both fibroblast types were pulse-labeled with DHE/MCD which resulted in exclusive location of the fluorescent sterol to the PM (data not shown, but see (Hao et al., 2002; Wüstner et al., 2002; Wüstner et al., 2005) for other cell types). After washing to remove the labeling solution, the cells were chased for various times, and the intracellular localization of DHE was compared to that of Alexa647-Tf accumulating in the REs during a 20-min labeling at the end of the chase period. By an overnight pre-incubation with Rh-dextran, the LE/LYSs were independently loaded with this fluid phase marker (Mukherjee, 1997). We verified in independent experiments that REs labeled with Alexa647-Tf and LE/LYSs labeled with Rh-dextran can be clearly separated under the conditions used here, both, in control (NPC2+/+) and disease (NPC2/-) fibroblasts (Fig. S1). In both cell types, some DHE co-localized already after 5-15 min of uptake with Alexa647-Tf in the REs and with Rh-dextran in LE/LYSs (Fig. S1A, B). This pattern did not change significantly in NPC2+/+ cells, while in NPC2/- cells, co-localization with Rh-dextran became more pronounced over time. After 4 h chase, the majority of intracellular DHE overlapped with Rh-dextran in LE/LYSs in fibroblasts lacking functional NPC2. Little DHE co-localized with Alexa647-Tf at that time (lower panels in Fig. 1B). NPC2/- cells showed also reduced growth compared to NPC2+/+ cells, consistent with an overall trafficking defect (not shown). For quantification of organelle sterol content, we developed a specific image analysis protocol implemented as a plugin to ImageJ (Fig. S2). This plugin allows for quantifying sterol in REs, here stained with Alexa647-Tf and in spatially separated LE/LYSs labeled with Rh-dextran (compare Fig. 1B at 15 and 30 min as an example with
Fig. S2B). Using this new tool, our analysis revealed that about 20% of PM-derived DHE became internalized in control cells, while the internalized sterol fraction comprised about 40% of total DHE in the disease cells (Fig. 1C-D, black circles and triangles). In the LE/LYSs (identified based on fluorescence of Rh-dextran), the initial rapid increase in fractional DHE fluorescence between 15-30 min was followed by a second slower phase of sterol accumulation continuing up to the end of the experiment in NPC2 deficient fibroblasts (i.e., 6 h chase; Figure 1D, red symbols). This steady increase in lysosomal sterol content was not found in NPC2+/+ cells (Figure 1C, red symbols). The sterol fraction in the PM, REs and LE/LYSs of NPC2+/+ cells was ~76%, 7% and 12% at the end of the chase period, respectively. In NPC2/- cells, we found ~60% DHE in the PM, 4% in the REs and ~29% in the LE/LYSs. The actual sterol content in the REs might be somehow higher, as our image analysis is based on 2D images, and the area covered by REs is about 35-50% of that covered by LE/LYSs in the 2D projections of the fibroblasts. We conclude that the fraction of DHE in the Tf-positive REs was almost constant during the experiment and comparable in both cell types (compare green symbols in Fig. 1C and D).

Thus, the lysosomal sterol pool is almost 2.5-fold expanded in the disease cells after only 5 h chase from the PM, while the sterol fraction in the REs remains largely unaffected by the NPC2 mutation.

In parallel experiments, we used the lysosomal marker LysoTracker® DeepRed to identify LYSs as a subpopulation of LE/LYSs labeled with Rh-dextran (not shown). We found that LysoTracker® DeepRed is retained in the same vesicles as Rh-dextran, supporting the notion that they are acidic organelles (not shown). Additionally, we performed measurements of the pH in vesicles containing double-labeled rhodamine-fluorescein dextran on a confocal microscope as described previously (Dunn et al., 1994). These experiments confirmed the acidic nature of the sterol-rich vesicles with pH≤5.0, clearly identifying them as LE/LYSs (not shown). To identify subpopulations in the heterogeneous population of dextran-containing vesicles, we pre-labeled cells overnight with Rh-
dextran, pulse-labeled them with DHE/MCD and incubated them further in the presence of the green-emitting OG-dextran (Fig. S4). The rationale behind this experiment is that gradual passage of the green cargo OG-dextran will allow us to distinguish kinetically different endosome populations; continuous uptake of OG-dextran for 5 h will primarily label early endosomes and ‘early’ LEs, while overnight incubation with Rh-dextran followed by a 5 h chase should result in preferred enrichment of the red-emitting dextran in ‘late’ LEs and in LYSs. Indeed, LEs have been recently divided into ‘early’ and ‘late’ populations based on various membrane markers (van der Kant et al., 2013). Any co-localization of both dextran types in our experiment should identify the respective organelles as LEs, since it is there, where both markers overlap. By this protocol, we found that after a 5 h chase, DHE co-localizes in vesicles containing OG- and Rh-dextran (‘early’ LEs; arrowheads in Fig. S3A and B) and becomes enriched in vesicles containing primarily Rh-dextran (‘late’ LEs; grey-red overlap Fig. S4). Together, these results show that sterol-enriched LE/LYSs are intact acidic organelles which can be further distinguished based on the kinetics of cargo flow from the PM. LE/LYSs are the major sites of accumulation of PM-derived sterol in NPC2 disease fibroblasts.

Sterol enrichment in the LE/LYSs in the NPC2 disease cells could be a consequence of lysosomal trapping, i.e. defective sterol export from LE/LYSs (Scenario I in Fig. 2). Less likely but possible, normal sterol recycling but enhanced sterol transport to the degradative compartments could account for DHE enrichment in LE/LYSs (Scenario II in Fig. 2) (Lange et al., 2000). To distinguish between these possibilities, we pulse-labeled disease fibroblasts with DHE, chased them for 2 h, exchanged the PM associated DHE for cholesterol using a Chol/CD exchange solution (Wüstner et al., 2005) and chased the cells without exchange solution for another 2 h (Fig. 2A). Recycling of DHE from the LE/LYSs to the PM would result in increasing PM labeling by the fluorescent sterol during the second chase. This, however, was not observed; clearly establishing
that DHE becomes trapped in the LE/LYSs of the disease cells. Enhanced sterol uptake as cause of the extensive sterol accumulation in the lysosomal compartments can be thereby excluded.

**Sterol enriched endosomes are enwrapped by the endoplasmic reticulum**

To assess the extent of sterol trafficking to the ER and Golgi apparatus, cells were loaded with DHE in a continuous uptake experiment from DHE/BSA complexes for 48 h. By that protocol, we increase the total cell-associated DHE by a factor of 1.7 compared to the pulse-chase experiment. This allows for detecting organelles with low sterol content. Subsequently, they were labeled with Alexa647-Tf for 1h and co-stained with C6-NBD-Cer, a marker which brightly stains the trans-Golgi network (TGN) and faintly labels the ER (Fig. 3A and B) (Chen et al., 1997; Pagano et al., 1991). The contribution of DHE fluorescence in the TGN varied between cells but was generally very low (Fig. 3A). The cellular area covered by the TGN overlapped by more than 30% with REs in the 2D-projection of the cell images (not shown). For these reasons, we were not able to discern REs unequivocally from the TGN in our analysis protocol. When focusing the microscope close to the bottom of triple-labeled cells (i.e., when focusing on the region close to cell-substrate attachment) we could discern sterol containing endosomes that also have Alexa647-Tf (Fig. 3C and D). The DHE staining pattern was punctuate/vesicular in NPC2+/+ and NPC2-/- cells and differed from the morphology of the ER tubules labeled with C6-NBD-Cer. Small, DHE containing vesicles were often found in close proximity to ER tubules (see arrows in inset to Fig. 3B). Some of the endosomes containing fluorescent sterol and Tf aligned with or became even enwrapped by ER tubules stained with C6-NBD-Cer (insets in Fig. 3C-F). Also, such ER tubules emanated to the cell periphery in close contact to Alexa647-Tf positive vesicles. We surmise that the ER forms contact sites to both, sterol rich early endosomes and REs and to the sterol rich PM in control and NPC2 deficient fibroblasts.

**Fluorescence recovery after photobleaching of DHE after pulse-labeling cells**
The observed trapping of DHE in LE/LYSs of NPC2-/- cells prompted the question as to whether this process is irreversible or whether a mobile sterol pool remains. To answer this question, we performed a FRAP experiment of DHE in the LE/LYSs of NPC2-/- fibroblasts (Fig. 4). Cells were pre-stained with Rh-dextran overnight to visualize the LE/LYSs followed by a pulse-labeling with DHE/MCD for 3 min. Cells were subsequently chased for the indicated times, a FRAP experiment was performed with recovery of DHE fluorescence in the bleached ROI (i.e., the LE/LYSs) being monitored as a function of time. Significant recovery stems from DHE diffusing in the overlaying PM, such that we corrected the fraction in the LE/LYSs for fluorescence recovery in the overlaying PM (see Fig. 4 and Materials and Methods). The recovery kinetics could be accurately fitted with a hyperbolic function from which we can extract a mean half-time of fluorescence recovery (see Eq. 1 in Materials and Methods). The half-time values were 157.2 sec, 163.6 sec and 204.9 sec for the cells chased for, respectively 30 min, 120 min and 240 min. In order to determine a diffusion constant for sterol exchange between cytoplasm and LE/LYSs, we used a 2D description of the cells, which is adequate as the fibroblasts are very thin (Braga et al., 2004). Using a radius of the bleach region in our wide field setup of 15 µm, one can derive an effective diffusion constant of DHE as given in Eq. 2 in Materials and Methods, yielding $D = 3.3 \, \mu m^2/sec$, $D = 3.1 \, \mu m^2/sec$ and $D = 2.5 \, \mu m^2/sec$ after 30 min, 120 min and 240 min chase, respectively. Thus, the diffusion constant of DHE between cytoplasm and LE/LYSs dropped only slightly for increasing chase times ranging from 30 min to 240 min. Note that this is only an efficient diffusion constant, as likely rapid binding and release of sterol to and from LE/LYSs participate in the recovery process. Measurement of fluorescence recovery of DHE in the PM was performed in the cell periphery lacking the majority of vesicles, and here we observed diffusion of sterol into the bleached region in this experiment (Fig. 4C). In such experiments, the recovery fraction approached one and the quantification gave $D = 2.5 \, \mu m^2/sec$. This is fast compared to lateral diffusion of other membrane lipid probes, but it is in
accordance with diffusion of BODIPY-tagged cholesterol analogues, as measured by confocal FRAP, STED-FCS or single molecule tracking in the PM of living cells (Hiramoto-Yamaki et al., 2014; Solanko et al., 2013). We also fitted the normalized recovery curves to a model of free diffusion with an immobile fraction (Yguerabide et al., 1982). This, however, did not result in an improved fit (not shown).

Note, that we used the PM recovery kinetics to correct the FRAP curves for DHE in LE/LYSs for the recovering DHE intensity in the overlaying PM (see Materials and Methods). Still, we find that the mobile sterol fraction exchanging inside the cells between cytoplasm and LE/LYSs recovers as fast as or even slightly faster than DHE in the PM. Given that the measured effective $D$ is likely comprised of diffusion and rapid binding of sterol to and release from LE/LYSs, free diffusion of sterol in the cytoplasm is likely even faster (Wüstner et al., 2015). We found that the extent of recovery in the LE/LYSs was very low and decreased as a function of the chase duration from 10.2% to 7.0% after 30 min and 240 min chase, respectively. This again demonstrates increasing immobilization, i.e., trapping of the sterol in the LE/LYSs. Since during the chase the sterol pool in LE/LYSs expands due to continuous transport from the PM from about 14.6% at 30 min chase to 28.7% at 240 min chase (see above and Fig. 1D), the mobile fraction in the FRAP experiment corresponds to 1.5% and 2% of total cellular DHE. In other words, the mobile pool of PM-derived DHE in LE/LYSs remains very small and almost constant during continuous lysosomal sterol trapping. We propose that this small dynamic sterol pool resembles non-vesicular sterol exchange between cytoplasm and sterol rich vesicles.

**Time-lapse analysis of sterol rich vesicles in NPC2 disease fibroblasts**

To compare the recovery kinetics of DHE in LE/LYSs with the overall mobility of these organelles in NPC2-/- cells we performed time-lapse experiments, in which we followed the movement of
individual DHE containing vesicles. Cells were loaded with the sterol by a continuous labeling protocol with DHE/BSA in the medium for 24 or 48 h. In time-lapse series, we found mobile sterol vesicles mostly in the periphery of the strong sterol accumulation in LE/LYSs (Fig. 5B and zoomed areas in Fig. 5A, A’ and C). Using our recently developed tracking program SpatTrack (Lund et al., 2014), we were able to track DHE enriched LE/LYSs in the cell periphery for up to 50 frames (Fig. 5A-C). From the vesicle trajectories, the MSD was calculated for all trajectories in each cell according to Eq. 3 in Materials and Methods and plotted as a function of tracking time (Fig. 5D). The MSD is getting less reliable for long times due to the diminishing number of data points entering the calculation (see Eq. 3 and inset of Fig. 5D). In addition, the unavoidable bleaching of DHE vesicles obscured particle detection accuracy for longer times than 50 frames. Only the initial MSD up to 5 min was therefore used and fitted either to a model of Brownian diffusion, anomalous subdiffusion or anomalous subdiffusion with drift, as described previously (Lund et al., 2014; Lund et al., 2012b) (see Eqs. 5-7 in Materials and Methods). In most cases (i.e. for most cells with about 20-36 tracked vesicles over at least 30 frames), the model of anomalous subdiffusion with flow gave the best regression, as judged from the parameter confidences and the Bayesian information criterion (BIC) (Lund et al., 2014). The BIC weights the goodness-of-fit given by low residuals against the model complexity to avoid overfitting (Lund et al., 2014; Türkcan et al., 2012). The residuals for all three models were low but the lowest for the model of anomalous diffusion plus flow (see inset of Fig. 5D).

**Uptake and esterification of DHE and $^{13}$C$_3$-cholesterol**

To determine the metabolic fate of internalized sterols, cells were loaded with DHE or the stable cholesterol isotope $^{13}$C$_3$-cholesterol in a continuous uptake experiment from sterol complexes with BSA for 24 h, and sterols were subsequently detected by lipid mass spectrometry (see Materials and
Methods for details). By this approach, we can compare NPC2+/+ and NPC2-/ cells with respect to their lipid composition, sterol uptake and esterification. In parallel, we can assess how similar DHE is compared to cholesterol, as $^{13}$C$_3$-cholesterol is chemically identical to cholesterol. We found that the level of total sterols (i.e., unlabeled, endogenous cholesterol and cholesteryl esters + respective cholesterol tracer; DHE or $^{13}$C$_3$-cholesterol) is ~2-fold higher in NPC2 deficient cells as compared to control cells (Fig. 6A). Separate determination of the different sterol species shows that NPC2-/cells contain more endogenous cholesterol and cholesteryl esters than NPC2+/+ cells (Fig. 6B).

Thus, NPC2 deficient fibroblasts are not only characterized by a strong redistribution of cholesterol to LE/LYSs but also by increased total cellular cholesterol content. Similarly, assessing the total levels of DHE-based lipids or $^{13}$C$_3$-cholesterol-based lipids showed that their uptake was about sixfold higher in NPC2 deficient cells as compared to control cells (Fig. 6B).

Moreover, when comparing the difference in uptake of DHE vs $^{13}$C$_3$-cholesterol in both control cells and NPC2 deficient cells we found that the amount of internalized DHE was about 25% of the internalized $^{13}$C$_3$-cholesterol (Figure 6B). This finding suggests that in fibroblasts cholesterol is preferentially incorporated into cells as compared to DHE. The fatty acid composition of cholesteryl esters was comparable in NPC2+/+ and NPC2-/ cells, irrespective of whether the cells had been labeled with DHE or $^{13}$C$_3$-cholesterol (Fig. 6C, D). Thus, ACAT activity is not affected by the NPC2 mutation and not compromised by the sterol labeling protocol. Only about 1.5% of internalized DHE but almost 6% of internalized $^{13}$C$_3$-cholesterol became esterified during the continuous labeling of fibroblasts from healthy donors. In NPC2-/ fibroblasts, the extent of DHE esterification was similar as compared to control cells. In contrast, conversion of $^{13}$C$_3$-cholesterol to $^{13}$C$_3$-cholesterol esters was strongly reduced in disease compared to control cells (i.e. ~0.4% of internalized $^{13}$C$_3$-cholesterol as ester which is 10-fold lower than what was found in control fibroblasts; see above). We conclude that PM-derived sterols are to some extent targeted to the ER,
where they become esterified by ACAT, both in control and NPC2 deficient fibroblasts. As synthesis of $^{13}$C$_3$-cholesterol esters is reduced in NPC2 disease cells, it is likely that the lysosomal trapping of sterol prevents $^{13}$C$_3$-cholesterol from being effectively esterified in the ER. This cannot be detected for DHE as less DHE is internalized and less is esterified by ACAT, such that the amount of available DHE cannot activate ACAT allosterically, as much as cholesterol (Liu et al., 2005). We found in separate experiments, that some albumin is internalized under our uptake conditions, suggesting that this labeling protocol delivers DHE into the PM and into LE/LYSs (Fig. S5). Incubating cells with DHE/BSA or $^{13}$C$_3$-cholesterol/BSA over prolonged time only minimally affected the composition and relative amount of PC and SM species compared to unlabeled cells, as shown by quantitative lipidomics (Fig. S6). Thus, the labeling protocols used in our study should not perturb metabolism and turnover of other lipids, as here shown for the abundant membrane lipids PC and SM.

**Internalized NPC2 protein rescues sterol storage and increases non-vesicular sterol exchange**

In independent imaging experiments carried out with the same cell batch as used for lipid mass spectrometry, we observed that after 24 and 48 h of continuous sterol uptake from a DHE/BSA complex, the fraction of fluorescent sterol in the LE/LYSs surmounts to about 50% of total cell-associated DHE in NPC2-/- cells (Fig. 7A and S6). Incubating such cells subsequently for 48 h with 100 nM of bovine NPC2 in medium containing LPDS removed a large portion of this excess sterol, almost to levels found in fibroblasts from healthy subjects. At the end of the 48 h incubation period, the fraction of intracellular DHE was ~0.47 in disease cells not treated with NPC2, while it was ~0.31 in disease cells treated with NPC2 (compare open and coarse white bars in Fig. S6). In addition to a strongly reduced DHE signal in LE/LYSs the fractional fluorescence of DHE in the PM was increased in NPC2 treated disease cells (Fig. 7A; most right panel). Thus, internalized
NPC2 can efficiently rescue the sterol storage phenotype and might reallocate sterol from LE/LYSs directly to the PM. Under exactly the same conditions, the intracellular DHE fraction was ~0.22 in control fibroblasts incubated in LPDS without NPC2 (black bar in Fig. S6). To get further insight into this efflux process, we performed a FRAP experiment in cells treated with NPC2 prior to labeling with DHE. NPC2 deficient fibroblasts were incubated with 100 nM NPC2 for 48 h with Rh-dextran being present during the last 12 h to visualize the degradative pathway. Cells were subsequently pulse-labeled with DHE for 3 min as described above, washed and chased for 5 h in buffer medium. Quantification of the FRAP experiment revealed that significantly more DHE fluorescence recovered in cells pre-incubated with NPC2 than in cells without NPC2 (Fig. 7B and compare blue and pink symbols in Fig. 7C). The mobile fraction of DHE increased from about 0.07 in the absence to ~0.19 in the presence of NPC2. In contrast, the recovery half time was slightly slower in cells pre-incubated with NPC2 ($t_{1/2} = 245.1$ sec, blue line in Fig. 7C) than in cells incubated in the absence of NPC2 ($t_{1/2} = 162.4$ sec, pink line in Fig. 7C). In both cases, the recovery kinetics is too fast to be accounted for by vesicle transport, since vesicles containing DHE (Figure 5) or fluorescent NPC2 (Lund et al., 2014) are of comparably low mobility in the disease cells. Thus, the FRAP experiment suggests that internalized NPC2 enhances fast, non-vesicular sterol exchange in disease fibroblasts.

**Discussion**

Niemann-Pick type C2 disease is a devastating lysosomal storage disorder in which lack of functional NPC2 causes accumulation of cholesterol in LE/LYSs. Consequently, the feedback regulation in which liberated cholesterol blocks synthesis of the LDL-receptor and thereby downregulates further LDL uptake is attenuated, resulting in further LDL uptake and cholesterol accumulation. In this study, we demonstrate that transport of endogenous cholesterol between the
PM, REs and LE/LYSs contributes to this aberrant process, as also PM-derived sterol builds up in lysosomal storage compartments in NPC2 deficient cells. Some DHE is rapidly transported from the PM to LE/LYSs in healthy human fibroblasts (Fig. 1), supporting previous findings with isotope-labeled cholesterol in intact cells and in isolated lysosome fractions (Castellano et al., 2017; Lange et al., 1997; Lange et al., 1998). We show that this endo-lysosomal sterol pool is about 2.5-fold expanded in cells lacking functional NPC2 (Fig. 1 and S6). Accumulation of PM-derived stable isotope-labeled cholesterol and fluorescent cholesterol analogs in LE/LYSs has been previously described in fibroblasts lacking functional NPC1 protein (Hölttä-Vuori et al., 2008; Lange et al., 1997; Lange et al., 1998; Pipalia et al., 2006; Sezgin et al., 2016), although the mechanism underlying this process is not undisputed (Lange et al., 2000). Our results demonstrate that lack of NPC2 leads to build up of PM-derived sterol specifically in LE/LYSs labeled with Rh-dextran but not in REs co-stained with Alexa647-Tf (Fig. 1). While our image analysis protocol captures and quantifies the majority of all intracellular DHE (Fig. S7), the fraction of DHE in REs might be somehow underestimated due to the 2D projections of the three-dimensional cells, in which LE/LYSs cover a much larger area than the REs. Still, the key observation is that for a given amount of sterol in the PM the fraction in LE/LYSs is specifically increased in NPC2-/- cells. In addition to this sterol trafficking defect we observed miss-sorting of fluorescent Tf and reduced recycling of dextran, pointing to a more complex function of NPC2 in endocytic trafficking (Goldman and Krise, 2010). In support of our findings are the observations of abnormal recycling of fluid phase markers such as dextran as well as of various glycoproteins in NPC1 and NPC2 disease cells (Goldman and Krise, 2010; Mbua et al., 2013; Neufeld et al., 1999). Similarly, miss-targeting of fluorescent Tf and of lipid probes, normally following the recycling route to LE/LYSs has been described in cells lacking functional NPC1 (Pipalia et al., 2006). Thus, our findings expand the view that absence of both, functional NPC1 and NPC2, lead to similar general
membrane trafficking defects. This aberrant membrane trafficking affects also the growth rate, as we found that NPC2 disease fibroblasts grow slower and go earlier in senescence compared to wild type fibroblasts (not shown). Moreover, we observed in separate experiments that calcium homeostasis between ER and LE/LYSs is disturbed in NPC2-/- cells (not shown), a feature shared with fibroblasts deficient in NPC1 (Lloyd-Evans et al., 2008). The similarity of endocytic trafficking defects in fibroblasts lacking functional NPC1 and NPC2 supports the notion of both proteins functioning in the same pathway. We also show that in fibroblasts from healthy subjects conversion of cholesterol to cholesteryl esters is low. Less than 6% of $^{13}$C$_3$-cholesterol delivered to healthy fibroblasts as complex with BSA became esterified (Fig. 6). This result is in line with earlier observations in various cell types; suggesting that direct transfer of cholesterol from the PM to the ER domain harboring ACAT is low under normal physiological conditions (Hölttä-Vuori et al., 2008; Infante and Radhakrishnan, 2017; Lange et al., 1997; Wüstner et al., 2005). In fibroblasts lacking functional NPC2, the late-endosomal sterol pool originating from the PM is almost 2.5fold expanded (Fig. 1). An almost 2.5fold expansion of endo-lysosomal sterol pool size is also found in a continuous sterol uptake experiment in which DHE was delivered to cells from albumin complexes overnight (Fig. S6). Using the same labeling protocol, total cellular $^{13}$C$_3$-cholesterol (Fig. 5) was almost 2fold higher in cells lacking NPC2 than in control cells. In contrast, esterification of internalized $^{13}$C$_3$-cholesterol is about 10-fold reduced compared to control cells (see Fig. 6B). Significantly less DHE becomes esterified by ACAT compared to $^{13}$C$_3$-cholesterol. One explanation for that observation is that DHE is less efficiently recognized as substrate for ACAT (Liu et al., 2005). Another possibility is that the two-fold lower uptake of DHE compared to $^{13}$C$_3$-cholesterol (Fig. 6A) did not suffice to activate ACAT, which is known to require a threshold concentration of sterol in the ER (Sokolov and Radhakrishnan, 2010; Xu and Tabas, 1991). Differences in esterification of cholesterol and DHE were not of concern in previous studies
(Mesmin et al., 2011; Wüstner et al., 2005). However, they are important here, as a small proportion of DHE escapes the trapping in LE/LYSs. Based on the FRAP experiments, we propose that non-vesicular sterol transport feeds cholesterol to ACAT in the ER (Fig. 8). The same amount of ‘mobile’ $^{13}$C$_3$-cholesterol (if it would be detectable by FRAP) will not suffice to activate ACAT and thereby to replenish the esterified cholesterol in the ER, which results in the lowered sterol cholesterol esterification in NPC2-/− cells.

What are the molecular mechanisms underlying the enhancement of non-vesicular sterol exchange from LE/LYSs by internalized NPC2? In in-vitro experiments, bi-directional exchange of cholesterol and DHE between liposomes is several-fold enhanced by NPC2 in a concentration-dependent manner (Babalola et al., 2007; Cheruku et al., 2006; Xu et al., 2008). The effect of NPC2 on sterol desorption from the donor membrane and transfer to the acceptor membrane is highest at acidic pH and for liposomes containing ceramide and LBPA, thus for vesicles mimicking the ILVs inside LE/LYSs (Abdul-Hammed et al., 2010; Cheruku et al., 2006; Gallala et al., 2011). Thus, we propose, that NPC2 catalyzes sterol exchange between ILVs and protein acceptors in the limiting membrane of LE/LYSs (Fig. 8). In the absence of NPC2, PM- and LDL-derived sterol might get trapped in ILVs inside LE/LYSs. Addition of functional NPC2 to NPC2-/− cells rescues the sterol storage and enhances the non-vesicular sterol export from LE/LYSs, as we found in FRAP experiments (Fig. 7B). As NPC2 is located inside the vesicles (Lund et al., manuscript in preparation), it must have caused the enhanced sterol exchange from the endo-lysosomal lumen, likely by supplying sterol from ILVs for transfer to NPC1 or other acceptors in the limiting membrane of LE/LYSs (Figure 8) (Boadu et al., 2012; Deffieu and Pfeffer, 2011; Infante et al., 2008; Wang et al., 2010). That we find a second slow phase of transport of PM-derived DHE to and sterol trapping in LE/LYSs in cells lacking functional NPC2 (Fig. 1D and Fig. 2) is totally in line with the proposed function of NPC2 in shuttling sterol between ILVs and the
limiting membrane of LE/LYSs. Indeed, additional multi-compartment kinetic modeling can account for the bi-phasic transport kinetics of DHE to LE/LYSs in the pulse-chase experiments in NPC2−/− cells (see Fig. 1D) by assuming slowed sterol exchange between ILVs and the membrane of LE/LYSs (not shown). One likely acceptor for sterol transported by NPC2 from ILVs to the endo-lysosomal membrane is NPC1 (Fig. 8), but also other membrane proteins have been described which might fulfill this function, such as MLN64/STARD3, ABCA1 or LAMP2 (Boadu et al., 2012; Charman et al., 2010; Li and Pfeffer, 2016). The observed fast cytoplasmic diffusion necessary to replenish sterol in LE/LYSs as we found for DHE in FRAP experiments could be due to cytosolic sterol transfer proteins accepting the exported sterol from NPC1, such as STARD4 (Mesmin et al., 2011). Alternatively, endosome-ER contacts mediated by NPC1 and ORP5 or via other proteins, such as the STARD3-VAP complex cause sterol transfer to the ER (Du et al., 2011; Wilhelm et al., 2017). This is then followed by rapid cytosolic sterol diffusion due to sterol movement along ER tubules which emanate through the whole cell (Fig. 2 and 7). In fact, sterols could be also replenished from the ER to LE/LYSs, as recently shown to take place via membrane contacts mediated by Annexin 1 and suggested for the STARD3-VAP complex (Eden et al., 2016; Wilhelm et al., 2017). Bi-directional transport of a small sterol portion between PM and ER is likely to contribute to increased PM fluorescence of DHE during NPC2 mediated efflux (Fig. 7A) and could in parallel deliver PM-derived sterols to ACAT in the ER (Fig. 6). Such a process has been suggested to contribute to cholesterol homeostasis (Infante and Radhakrishnan, 2017), and it could take place via membrane contact sites between both compartments (Saheki and De Camilli, 2017).

Direct quantitative imaging combined with mass spectrometric analysis of the close cholesterol analogue DHE provides a useful toolbox for studying this process. Further studies are warranted, which specifically investigate other sterol uptake pathways, such as the import of LDL-derived cholesteryl esters in our cell system.
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**Figure captions**

**Figure 1. Quantification of DHE transport to REs and LE/LYSs**
Cells were pre-labeled with 0.5 mg/ml Rh-dextran overnight, washed and labeled with DHE/MCD for 3 min followed by a chase with 5 µg/ml Alexa647-Tf as described in Materials and Methods. Panel A, C and B, D show the data for NPC2+/+ and NPC2-/- cells, respectively. DHE (most left column) co-localizes as early as after 5 min chase with Alexa647-Tf in peripheral vesicles in both cell types. DHE is found in LE/LYSs after ~15 min in control and disease fibroblasts. Already after 30 min chase, DHE is clearly accumulating in the Rh-dextran stained LE/LYSs in disease but not in control cells. This trend continues and is well apparent after 6 h chase. The far right panels show a color overlay with DHE in grey, Rh-dextran in green and Alexa647-Tf in red, respectively. The pulse-chase experiments were quantified for NPC2+/+ cells (C, circles) and NPC2-/- cells (D, triangles) as described in Materials and Methods and Fig. S2. The DHE fraction normalized to total cellular DHE is plotted as function of time for the PM (black symbols), LE/LYSs (red symbols) and REs (green symbols) for control cells (circles) and disease fibroblasts (triangles). Bar, 10 µm. Data in panel C and D represent the mean ± SEM of 3-5 biological replicates. Each biological replicate consisted of 5-8 images having 2-10 cells per channel for each time point.

**Figure 2. Lysosomal trapping of HE in disease cells**
Fibroblasts lacking functional NPC2 were pulse-labeled with DHE/CD for 3 min, washed and chased for 2 hrs in medium 1 (left panel in A; ‘2hrs chase’). Subsequently, cells were incubated for 10 min in medium 1 containing a cholesterol/CD exchange solution to remove selectively DHE from the PM (middle panel in A; ‘2hrs chase + Chol/CD’). Finally, cells were washed and chased for another 2hrs in medium 1 (right panel in A; ‘2hrs chase + Chol/CD + 2hrs chase’). The DHE intensity in the lysosomal area remained constantly high (red to yellow color in a FIRE LUT), with little DHE appears in the PM. Bar, 10 µm.

B, Sterol accumulation in LE/LYSs could be due to defective transport from that compartment back to the PM in disease cells (scenario I) or due to normal recycling from LE/LYSs but enhanced sterol uptake from the PM (scenario II). The data shown in panel A supports scenario I.

**Figure 3. Endosomes containing DHE and fluorescent Tf are enwrapped by the ER**
NPC2 deficient fibroblasts were incubated with DHE/BSA for 48 hrs, washed, labeled with 5 µg/ml Alexa647-Tf for 1hr, washed and labelled with C6-NBD-Cer for 30 min at 37°C. Images were post-processed by deconvolution, as described in Materials and Methods. C6-NBD-Cer labels faintly the ER tubular network and strongly the TGN. A, B, DHE (green) and C6-NBD-Cer (red), intensity adjusted to see the TGN (A) or the ER (B) with overlaying DHE image with insets 1 and 2 highlighting the peripheral ER and perinuclear TGN region, respectively. C-F, Co-localization of DHE in grey (C) with Alexa647-Tf in red (D) and C6-NBD-Cer in green (E) gives a color overlay, as shown in panel F. The rectangular box is zoomed in the lower right corner, and vesicles having
DHE and Alexa647-Tf being enwrapped by or aligned with the ER stained with C6-NBD-Cer are indicated by arrows and small boxes. Bar, 20 µm

**Figure 4. Fluorescence recovery after photobleaching of DHE in disease fibroblasts**
NPC2 deficient fibroblasts were pre-labeled with 0.5 mg/ml Rh-dextran overnight, washed, pulse-labeled with DHE/MCD for 3 min and chased for various times. FRAP was performed on a UV-sensitive wide field microscope in which the bleach area is defined by the closed field aperture in the image of Rh-dextran (A). DHE fluorescence was bleached by illuminating that region for 5 sec, the aperture was opened and images were taken with reduced intensity and a short acquisition time. B shows normalized FRAP curves after 30 min (green symbols), 120 min (red symbols) and 240 min chase (pink symbols). The symbols with error bars show mean ± SD of at least six recovery curves for each condition. Recovery in the ROI was fitted to Eq. 1 after 30 min (green line), 120 min (red line) and 240 min chase (pink line), respectively. FRAP of DHE in the PM was determined in regions of the cell being located apart from DHE-rich organelles (C). In these experiments, DHE recovered first at the edge of the bleach ROI with subsequent spreading of intensity, revealing the diffusive nature of sterol transport in the PM. Bar, 20 µm

**Figure 5. Tracking and diffusion of vesicles containing DHE in NPC2-/- cells**
NPC2 deficient fibroblasts were incubated with DHE/BSA for 48 hrs, washed and imaged with a frame rate of 0.17 Hz. Panel B shows the cell with regions enlarged in panel A and C indicated as boxes. In A and C, the first frame is colored in red and subsequent frames sequentially in green. The box in the lower left corner in A is magnified in panel A’, where one can see directed movement of a DHE containing vesicle (red arrows). Vesicles were tracked using SpatTrack, and the mean square displacement (MSD) was calculated as a function of lag time in panel D (blue symbols with error bars indicate mean ± SE as determined from 36 tracked vesicles in this particular cell). The initial MSD up to 300 sec (see upper inset for full MSD up to 480 sec) was fitted to various diffusion models with the best fit found for the model describing sub-diffusion plus flow (red line). Residuals of the non-linear regression to all diffusion models is shown in the lower inset (grey bars, normal diffusion; black bars, subdiffusion; red bars, subdiffusion plus flow). Bar in panel B is 5 µm

**Figure 6. Lipid mass spectrometry of sterol uptake and esterification**
NPC2+/+ and NPC2-/- fibroblasts growing in 6-well plates were labeled with DHE or \(^{13}\)C\(_3\)-cholesterol from a BSA complex according to the continuous uptake protocol for 24 h in LPDS. Cells were washed and harvested by scraping into ice-cold buffer and subjected to quantitative lipid mass spectrometry (MS) after lipid extraction into chloroform/methanol. A QqTOF mass spectrometer was used in positive ion mode for analysis of \(^{13}\)C\(_3\)-cholesterol esters (13C3 CholE), DHE esters (DHEE). Free sterols were converted to sterol sulfates using a sulfur trioxide pyridine
complex prior to MS analysis in negative ion mode on an LTQ Orbitrap XL hybrid FTMS mass spectrometer. Quantification of total cell-associated sterols (A), of sterol uptake and esterification (B) or of fatty acyl chain composition of total cellular cholesteryl esters (C, D) in NPC2+/+ (C) and NPC2−/− cells (D) was carried out as described in Materials and Methods. Data is presented in units of pmol/µg protein (A, B) or expressed as mole percentage (Mol%) of total cholesteryl ester (C, D). The inset in B shows the concentration of DHE ester (DHEE) and 13C3-cholesterol (13C3-CholE) in a concentration range of 0-20 pmol/µg protein) for NPC2+/+ cells (dark grey bars) and NPC2−/− cells (light grey bars), respectively

Figure 7. Internalized NPC2 enhances fluorescence recovery after photobleaching of DHE
A, NPC2 deficient fibroblasts were incubated with DHE/BSA for 48 h, washed and incubated in culture medium containing LPDS in the presence of 100 nM NPC2 for 48 h. Intensity images of DHE color-coded with a FIRE LUT showing high intensities in yellow/white and low intensities in blue. Most left panel (after labeling with DHE/BSA) and middle panel (after additional incubation with 100 nM NPC2) are identically scaled. The most right panel shows the same cell as in the middle, just intensity scaled for visualization of sterol distribution. B, NPC2 deficient fibroblasts were pre-incubated with 100 nM NPC2 in LPDS containing medium for 48 h with 0.5 mg/ml Rhodexan being present in the last 12 h to visualize the LE/LYSs (red in B). Cells were washed and labeled with DHE/MCD for 3 min, washed and chased for 4 h in buffer medium. FRAP was performed as described in legend to Figure 4. B shows normalized FRAP curves after 240 min in the presence (blue symbols) or absence of NPC2 (pink symbols, compare Fig. 4).

Figure 8. Model of NPC2’s function in cholesterol transport between LEs and the PM
There is constant inflow of sterol from the PM (I), likely via REs (not shown). There is also sterol export from LEs back to the PM and other destinations by vesicular transport (II, II’). Cholesterol derived from the PM or from ingested lipoproteins, such as LDL is typically transported to ILVs inside LEs, which provide the important membrane interface for lipid metabolism. NPC2 inside LEs picks up sterol from ILVs (1) and delivers it to acceptor proteins in the limiting membrane of LE/LYSs, as here shown for NPC1 (2). On the cytoplasmic site of the LE/LYSs, other proteins contact NPC1 (e.g. ORP5 or StARD3) to mediate non-vesicular sterol exchange to the ER (3) and other membranes (not shown). Once a threshold of cholesterol concentration is exceeded, ACAT (not shown) is activated in a subdomain of the ER to promote cholesterol esterification (4). The generated cholesteryl esters (CEs) are stored in lipid droplets. Between the ER and the PM, non-esterified sterol might be exchanged via membrane contact sites (III). See text for further explanations
Fig. 3