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Review

How cholesterol interacts with proteins and lipids during its intracellular transport☆

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ABSTRACT

Sterols, as cholesterol in mammalian cells and ergosterol in fungi, are indispensable molecules for proper functioning and nanoscale organization of the plasma membrane. Synthesis, uptake and efflux of cholesterol are regulated by a variety of protein–lipid and protein–protein interactions. Similarly, membrane lipids and their physico-chemical properties directly affect cholesterol partitioning and thereby contribute to the highly heterogeneous intracellular cholesterol distribution. Movement of cholesterol in cells is mediated by vesicle trafficking along the endocytic and secretory pathways as well as by non-vesicular sterol exchange between organelles. In this article, we will review recent progress in elucidating sterol–lipid and sterol–protein interactions contributing to proper sterol transport in living cells. We outline recent biophysical models of cholesterol distribution and dynamics in membranes and explain how such models are related to sterol flux between organelles. An overview of various sterol-transfer proteins is given, and the physico-chemical principles of their function in non-vesicular sterol transport are explained. We also discuss selected experimental approaches for characterization of sterol–protein interactions and for monitoring intracellular sterol transport. Finally, we review recent work on the molecular mechanisms underlying lipoprotein-mediated cholesterol import into mammalian cells and describe the process of cellular cholesterol efflux. Overall, we emphasize how specific protein–lipid and protein–protein interactions help overcoming the extremely low water solubility of cholesterol, thereby controlling intracellular cholesterol movement. This article is part of a Special Issue entitled: Lipid–protein interactions.

Contents

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1. Introduction

Cholesterol attracts continuous interest of physicians, cell biologists, biochemists and biophysicists due to its uttermost importance in human pathobiology, its central role in regulating cellular functions, its complex metabolism and its impact on membrane structure and dynamics. The last three decades have witnessed a tremendous increase in our understanding of cholesterol transport between tissues and control of cholesterol homeostasis at an organism level [1–3]. Similarly, a lot of effort has been put into characterizing the membrane organization of cholesterol as well as in determining its effect on membrane proteins. In contrast to that progress, the dynamical aspects, energetics, protein-dependence and overall regulation of intracellular cholesterol transport are only beginning to become resolved. Cholesterol in mammalian cells and ergosterol in fungi play a central role in regulating the permeability barrier and overall structural organization of the plasma membrane (PM) which is why both sterols are highly enriched in that membrane compared to most intracellular organelles. Intermediate cholesterol concentrations are found in early endosomes and the trans-Golgi network (TGN), while mitochondria and the endoplasmic reticulum (ER) harbor only low amounts of cholesterol (i.e. the amount of cholesterol in the ER is below 5% of total relative to the PM with about 60–70% of total). A central question is how the low abundance of cholesterol in the ER is maintained despite the fact that this is the site of cholesterol synthesis and esterification. The ER contains several proteins with essential function in cholesterol metabolism. For example, hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase catalyzes formation of mevalonate in the cytoplasm, which is the rate-limiting step in cholesterol synthesis. Its activity is regulated in a fascinating manner via a protein complex with sterol response element binding protein 2 (SREBP2), its escort proteins Scap and Insig, which all localize to the ER in cells with normal cholesterol levels. Cholesterol homeostasis is regulated in a convergent feedback loop involving these proteins, and sudden changes in ER cholesterol trigger metabolic responses [4–7]. If cholesterol levels in the ER drop, Insig dissociates from Scap/SREBP2 and gets degraded by the proteasome [8]. The Scap/SREBP2 complex is transported in COPII vesicles to the Golgi following normal anterograde membrane traffic, and in the Golgi, two serine proteases cleave the N-terminal domain of SREBP2. This domain travels to the nucleus, where it acts as transcription factor promoting the expression of several genes involved in cholesterol synthesis, uptake and transport [8]. Scap binds cholesterol directly, likely via its sterol sensing domain, while expression of Insig (at least Insig-1) is controlled by the SREBP2 transcription factor. Once normal cholesterol levels are restored and sufficient new Insig is synthesized, the Insig/Scap/SREBP2 complex reforms in the ER, and acquisition of new cholesterol by the cell (via synthesis or uptake of low density lipoprotein (LDL)) ceases. Several excellent reviews have been published in the last few years about these processes [8–11], and we will not discuss the details further here. Instead, we continue with a brief overview of experimental approaches for analysis of cholesterol transport (Sections 2.1 and 2.2). This is especially important due to the fact that our limited knowledge about intracellular cholesterol trafficking is largely a consequence of technical hurdles. We will also briefly discuss several experimental approaches for studying protein–sterol interactions and protein-mediated inter-membrane sterol transport (Section 2.3.). Kinetic and thermodynamic aspects of cholesterol–lipid interaction and inter-membrane cholesterol transfer are explained in Section 3. A survey of specific protein–lipid and protein–protein interactions during intracellular cholesterol movement, uptake and efflux is provided in Section 4.

2. Tools for studying sterol trafficking and sterol–protein interactions

2.1. Quantitative approaches for analysis of intracellular transport processes

Intracellular transport studies in general demand specific and robust labeling of the transported entity with minimal alteration of the investigated transport process by the labeling strategy. This aspect is particularly critical for elucidation of lipid transport processes, in which tagging with a reporter moiety can significantly affect the properties of the studied molecule. The experimenter must also decide whether steady state or dynamic information will be gathered in a particular investigation. For example, the polyene macrolide filipin will report about the steady state distribution of cholesterol or other sterols bearing a free 3-hydroxy group, while no kinetic information can be inferred by that approach [12]. Also, filipin staining would not report about the origin of a particular sterol pool. Similar arguments apply to studies using sterol-binding proteins, as fluorescence tagged perifringolysin O (PFO) [12]. Dynamic information about cellular transport can be inferred in one of three ways; I) one deflects the cellular system from its steady state and follows the system response as a function of time. This approach is used in pulse-chase experiments of isotope- or fluorescence-labeled molecules as well as in fluorescence recovery after photobleaching (FRAP) of suitable fluorescent sterols [13,14]. Pulse-chase studies are also widely used for deciphering lipoprotein-mediated sterol import into cells, and such studies can be easily coupled to metabolic experiments, for example of cholesteryl ester (CE) hydrolysis and reesterification of cholesterol [15] as well as of cholesterol synthesis and transport [16]. II) transport can be studied by creating a permanent sink as a consequence of constantly removing some tagged molecules from the system, as is done in fluorescence loss in photobleaching (FLIP) [17]. In contrast, to the first strategy, the system is permanently disturbed and no new global steady state can be re-established in FLIP studies. III) cells become steady-state labeled with the tagged molecule of interest, for example cholesterol with an attached BODIPY group in the side chain (BChol or TopFluor-cholesterol as trade name) and stochastic fluctuations around the steady state value are monitored as function of time [18,19]. This approach is only applicable with bright and photostable fluorescent reporter groups allowing for either long term tracking of sterol-rich vesicles or for unbiased analysis of fluorescence fluctuations due to molecular transport [18]. According to the fluctuation–dissipation theorem, comparable transport rates can be inferred from the response of the system to a perturbation (i.e., strategies I) and II), above) as well as from long-term analysis of fluctuations around a mean value (i.e., strategy III), above) [20]. In the latter case, transport coefficients, as diffusion constants, are derived from time correlation functions, as in fluorescence correlation spectroscopy (FCS) and its imaging variants [21,22]. Alternatively, statistical ensembles are generated by single particle tracking (SPT) followed by moment analysis of the experimentally determined probability density function (PDF). For example, in SPT the second moment of the step length distribution for recorded trajectories gives the mean square displacement from which diffusion constants or flow speed can be extracted using adequate transport models [23–26]. FRAP and SPT have been shown to provide comparable information in case of membrane diffusion [26], while similar transport coefficients were found for intracellular vesicles using either fluorescence fluctuation analysis or SPT [27].

2.2. Radioactive and fluorescent probes for studying sterol transport and metabolism

Cholesterol synthesis can be investigated using ^{3}H-acetate or other isotope-labeled precursors [16,28]. Cholesterol esterification is often determined by feeding cells ^{3}H-cholesterol or ^{3}H-oleate and analyzing the amount of formed radioactive CEs [29,30]. Isotope-labeled

cholesterol is also used in cellular efflux studies for determining protein dependence and kinetics of sterol clearance from cells [31]. Cyclodextrin is often used in such studies, either to introduce the labeled sterols selectively into the plasma membrane or to allow for sterol extraction from cells in efflux experiments [31–33]. Cyclodextrin is similarly used in studies involving fluorescent cholesterol analogs, and cholesterol-containing cyclodextrin is useful for exchanging a labeled sterol pool or for acute cholesterol loading of cells [13,34,35]. Uptake of sterol-containing lipoproteins can be followed by appropriate fluorescence-tagging of the apoprotein or by scintillation chromatography based on ^{125}I-tagged apoproteins [36,37]. Hydrolysis of isotope-labeled CEs will, combined with assessment of apoprotein degradation using trichloracetic acid precipitation, report about intracellular degradation of the lipoprotein [36,38]. Double-isotope studies using ^{3}H- or ^{13}C-sterols are also possible, either to assess re-esterification of LDL-liberated cholesterol or to determine the extent of esterification of different cholesterol pools [39]. Another tool to generate strong concentration gradients is to use hydrophobic amines, such as U18666A, and steroid hormones, as progesterone, to trap cholesterol in late endosomes and lysosomes (LE/LYSs) [15,40]. Washout of these compounds allows for kinetic and metabolic analysis of the intracellular fate of cholesterol released from these compartments [32,39]. Use of fluorescent analogs of cholesterol combined with suitable imaging technology provides an alternative strategy for gaining insight into sterol transport pathways and dynamics in cells. Fluorescent cholesterol analogs bear either a covalently linked fluorescent dye of suitable color and brightness or are intrinsically fluorescent due to a small conjugated system in the steroid backbone of the sterols. Most cholesterol analogs with extrinsic reporter group fail to mimic cholesterol adequately in model membranes and in cellular studies [12,41]. A somehow reasonable exception is TopFluor-cholesterol which has been successfully used in cells and model organisms, even though it is miss-targeted in cells with high fat content [35,42]. However, BChol or TopFluor-cholesterol fails to order fatty acyl chains in model membranes, an important property of cholesterol to be mimicked by suitable cholesterol analogs [43,44]. Ultraviolet (UV)-sensitive wide field (UV-WF) and multiphoton microscopy of intrinsically fluorescent sterols, such as cholestatrienol (CTL) and dehydroergosterol (DHE) as close analogs of cholesterol and ergosterol has provided new insight into cellular sterol trafficking [13,17,34,45,46]. Their poor fluorescence properties justify the quest for development of further sterol probes. Importantly, metabolic studies on intracellular esterification are also possible with fluorescent sterols [34,35]. A different tagging strategy is to synthesize alkyne-labeled cholesterol or oxysterols, which after cellular uptake become chemically linked to a fluorescent dye, as BODIPY [47,48]. A disadvantage here is the need for fixation of cells making live-cell imaging studies impossible. For further details, the reader is referred to recent technical reviews [12,49–51].

2.3. Biophysical characterization of sterol–protein interactions

Non-vesicular transport of cholesterol and ergosterol in mammalian and yeast cells, respectively depends on cytosolic lipid transfer proteins (LTPs). Non-specific LTPs, as cytosolic sterol carrier protein 2 (SCP2) and fatty acid binding proteins (FABPs) transfer sterols and a variety of other lipids between model and cell membranes and bind their ligands with micromolar affinity [52,53]. Specific LTPs bind and transfer only one or two types of lipids, sometimes in exchange against each other and often show affinities in the low nanomolar range [54,55]. Examples of the latter category are members of the family of oxysterol binding proteins (OSBPs), steroidogenic acute regulatory (StAR) proteins and elicitins — plant defensins to which the Niemann Pick C2 protein (NPC2) shows high structural similarity [56–59]. All LTPs were shown to bind their respective ligand and to transfer it between liposomes. In case of sterols, typical binding assays consist of making an often aqueous solution of either radiolabeled or fluorescent sterol and adding the protein in the absence or presence of excess unlabeled sterol [54]. In such competition assays, apparent dissociation constants (K_D) for cholesterol or other sterols, as oxysterols, to LTPs have been determined. Sterol binding to LTPs can be also measured by NMR spectroscopy or by H/D exchange followed by mass spectrometry detection [60,61]. When using fluorescent analogs, intrinsically fluorescent sterols as DHE or CTL are clearly preferable compared to tagged cholesterol, as NBD-, Dansyl or TopFluor-cholesterol, since binding equilibria are strongly affected by the presence of the fluorophore in the latter analogs [62]. For example, side-chain tagged NBD-cholesterol binds to Niemann Pick C1 protein (NPC1) but not to NPC2 [62]. This is likely a direct consequence of the opposite orientation of the sterol in the respective binding pocket; while in NPC1, sterols bind with their 3′-hydroxy group buried inside the binding pocket [63], in NPC2, the sterol side chain points into the binding region (see Section 4.3., below) [64]. Accordingly, the fluorophore in NBD-cholesterol will interfere with binding to NPC2 but not to NPC1. On the other hand, DHE and/or CTL have been used to demonstrate sterol binding of NPC2 [65,66], FABPs [52], SCP2 [67], NPC1 [62], yeast homologs of OSBPs [68,69], StAR proteins [14] and elicitins [70]. Photo-crosslinking of a suitable cholesterol analog to the protein of interest can be also used as evidence for sterol-binding capacity [49,62,71]. This strategy can be combined with click-chemistry via a side-chain alkyne group to generate a bifunctional cholesterol probe [72]. Combined with stable-isotope labeling by amino acids in cell culture (SILAC) mass spectrometry, such a bifunctional cholesterol probe has been employed to identify over 250 sterol-interacting proteins [72]. Beside many known sterol-binding proteins, several new candidates were identified, which were formerly known only from their function in sugar and glycerolipid metabolism. To determine specific sterol binding to a reconstituted membrane protein, immunoaffinity tagging to sepharose beads has been used followed by adding radioactive cholesterol in a buffer solution, centrifugation of the bead-protein/ligand complex and extraction of bound sterol with organic solvent [73]. Estimation of comparable K_D values is often impeded by the low monomeric solubility of cholesterol and its analogs, as DHE, which is far below 100 nM [74,75]. Accordingly, cholesterol or fluorescent sterols will form crystals or micelles, which, however, are not taken explicitly into account in most published assays reporting K_D values. Also, different solubility of cholesterol and its oxidized metabolites can bias the measured affinity values to LTPs [54,66]. Such problems can be circumvented by inserting the sterol ligand into a supported bilayer followed by detection of protein binding using surface plasmon resonance [68]. Alternatively, the critical concentration of sterol aggregate formation is explicitly taken into account in the estimation of binding equilibria [76].

Binding stoichiometries of sterols to LTPs have been estimated by fluorescence approaches, calorimetry or circular dichroism [52,53,76]. Again, cholesterol analogs with extrinsic fluorophore, as NBD-cholesterol gave deviating binding stoichiometry (2:1) compared to native cholesterol (1:1) to the StAR protein, the mitochondrial cholesterol importer [76–78]. Chemical modification of the sterol structure can also have a large impact on inter-membrane sterol transfer catalyzed by LTPs. For example, the BODIPY-group in TopFluor-cholesterol was found to block interbilayer transport of that analog by StARD4, a cytoplasmic sterol transport protein in mammalian cells (Fred Maxfield, Cornell Medical College; personal communication). In contrast, StARD4 transferred DHE with similar efficiency as cholesterol between model membranes and between the PM and the endocytic recycling compartment (ERC) in living CHO cells [14]. Inter-bilayer exchange of DHE and CTL has become a standard method for determining transfer activity of a variety of LTPs. Two readouts of these sterols can be used for that purpose: (1) relief of Förster resonance energy transfer from DHE or CTL to Dansyl-tagged phosphatidyl ethanolamine (Dansyl-PE). This assay has been originally developed to assess DHE's flip-flop in model membranes by stopped flow-based monitoring of cyclodxtrin-mediated sterol extraction [79]. Two kinetic phases were found in

such experiments, and the slow one could be associated with DHE transbilayer migration. Later, this assay was used to monitor DHE and CTL transfer between membranes by a variety of LTPs including NPC2 [80], StARD4 [14], the yeast OSBP homologue Osh 4 [69], OSBP [81] and OSBP related protein 9 (ORP9) [82]. (2) DHE shows a concentration-dependent decline in fluorescence polarization or anisotropy which has been used to predict sterol transbilayer dimers at low sterol concentration [75,83]. This property was used first by Schroeder and colleagues to quantify sterol transport by SCP2 and FABPs between model and cellular membranes [84,85]. Similarly, Slotte and colleagues has used anisotropy as readout for equilibrium partitioning of CTL between membranes [86]. Both assays do not depend on separation of donor and acceptor vesicles, a problem which otherwise has been tackled, when using radioactive or unlabeled cholesterol [87,88].

3. Modes and models of intracellular cholesterol transport

3.1. Is the heterogeneous distribution of cholesterol in cells based on equilibrium thermodynamics?

More than 30 years ago, Silbert and colleagues found that isotope-labeled cholesterol resides preferentially in purified PM fractions compared to membrane fractions made of ER or mitochondria in LM cells [89]. The same tendency (but not the same numbers) was found in lipid extracts made from these membrane fractions. Given the highly different phospho- and sphingolipid composition of PM versus ER and mitochondria, the authors speculated that the properties of the host lipid species in a particular organelle determine the different membrane affinities of cholesterol (and related sterols) and consequently the heterogeneous intracellular cholesterol distribution [89]. This is plausible, given that cholesterol clearly shows preferred interaction with membranes consisting of saturated sphingomyelin (SM) and phosphatidylcholine (PC), as found in the PM compared to membranes made of unsaturated PC and other lipids, as found in the ER [90,91]. Indeed, the half-time of passive cholesterol transfer between liposomes was found to decrease in the order dipalmitoyl-PC (DPPC; 4.7 h), dimyristoyl-PC (DMPC; 3.9 h), dioleoyl-PC (DOPC; 2.6 h), and soy-bean-PC (1.8 h) [92]. Similarly, Mitropoulos and co-workers found a strong correlation between net cholesterol transfer between liposomes and ER membranes purified from rat liver and the activity of ACAT in these microsomes [91]. Thus, the higher the measured partitioning of cholesterol into microsomes was, the larger the activity of ACAT in these membranes. The activation energy for cholesterol transfer from liposomes containing bovine brain SM was about 45% higher than that from liposomes made of egg yolk PC [91]. The transfer kinetics was first order with a half-time of ~28 h versus ~3 h at comparable liposome/microsome ratios for SM- and PC-containing membranes, respectively. Given, that bovine brain SM contains much more saturated fatty acyl species than egg yolk PC these observations could be a consequence of the more saturated acyl chain species in membranes made of SM than those made of PC. Alternatively, preferred interaction between cholesterol and SM compared to PC even for similar acyl chain order could explain the observations by Mitropoulos and colleagues [91]. Partition experiments have indeed shown that cholesterol has the highest affinity for liposomes made of SM followed by those made from phosphatidylserine (PS), PC and finally phosphatidylethanolamine (PE) [90]. Similar results were found using CTL by Slotte and colleagues using a fluorescence assay based on CTL [93]. These authors showed additionally that increased membrane order as assessed by diphenylhexatriene fluorescence in SM containing membranes is not the primary cause of higher sterol partitioning into SM compared to PC bilayers [93]. Thus, energetically favorable interfacial properties of SM compared to PC might stabilize cholesterol in sphingolipid containing bilayers. Structural differences in the ceramide compared to the glycerol backbone must account for such properties, as the phosphorylcholine head group of PC and SM is the same. For example, hydrogen bonding between the 3′-hydroxy group of cholesterol and the NH-group of the ceramide part of SM could stabilize such interactions. Others, however, could not find evidence for a specific interaction of cholesterol with SM [94]. Independent of the molecular details, the observed partition differences of cholesterol for varying head group and acyl chain composition are fully in accordance with equilibrium thermodynamics. Let's consider a two-membrane system; membrane *A* and membrane *B* with differing phospholipid composition (for example *A* contains saturated PC and *B* contains unsaturated PC) and cholesterol as solute within such membranes. Cholesterol's mole fraction in both membranes reads

$$x^A_{Chol} = \frac{n^A_{Chol}}{n^A_{Phl} + n^A_{Chol}} \quad \text{and} \quad x^B_{Chol} = \frac{n^B_{Chol}}{n^B_{Phl} + n^B_{Chol}}. \tag{1a, b}$$

Here, n^i_{Chol} and n^i_{Phl} with $i = A, B$ correspond to the number of cholesterol and phospholipid molecules in the respective membrane *A* and *B*. Let's assume that cholesterol transfer between both membranes takes place via the aqueous solution, we can write the scheme

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} water \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} B. \tag{2}$$

Here, k_1 and k_{-1} are the desorption and insertion rate constants for cholesterol into membrane *A*, respectively. The aqueous sterol fraction is indicated by '*water*'. For membrane *B*, k_2 is the insertion rate constant and k_{-2} is the corresponding desorption rate constant. Typical experimentally determined values for DHE insertion into and desorption from POPC membranes are $k_2 = 5.1{\cdot}10^7\ M^{-1}{\cdot}s^{-1}$ and $k_{-2} = 1{\cdot}10^{-3}\ s^{-1}$ [95]. The equilibrium constants for the individual steps in Eq. (2) are given by $q_1 = k_1/k_{-1}$ and $q_2 = k_2/k_{-2}$, respectively. Whether aqueous or collisional transfer dominates for cholesterol is still debated due to cholesterol's low water solubility [87,96]. For example for DHE, the partition coefficient between water and POPC liposomes has been estimated after normalizing to phospholipid concentration to $q = 1.3{\cdot}10^6$ corresponding to 13 million DHE molecules in the membrane for one molecule in water [95]. At increasing concentration of acceptor membranes, sterol transfer seems to be dictated by the frequency of vesicle collisions [96]. After sufficient time, the cholesterol distribution between membranes *A* and *B* will be independent of the aqueous sterol pool, anyway, according to:

$$K = q_1 \cdot q_2 = \frac{water}{A} \cdot \frac{B}{water} = \frac{B}{A} = \frac{k_1 \cdot k_2}{k_{-1} \cdot k_{-2}}. \tag{3}$$

Thus, cholesterol solubility in the aqueous phase might play a role for the kinetics of sterol transfer between membranes but not for the equilibrium distribution. Accordingly, we can ignore an eventual intermediate cholesterol pool between the membranes in what follows. At thermodynamic equilibrium cholesterol's chemical potential, μ_{Chol}, defined as change in Gibbs free energy due to an infinitesimal change in cholesterol abundance according to the Gibb–Duhem equation, is identical in both membranes, i.e.

$$\mu^A_{Chol} = \frac{\partial G}{\partial n^A_{Chol}} = \mu^{A,0}_{Chol} + k_b \cdot T \cdot \ln x^A_{Chol} \equiv \mu^B_{Chol} = \frac{\partial G}{\partial n^B_{Chol}} = \mu^{B,0}_{Chol} + k_b \cdot T \cdot \ln x^B_{Chol}. \tag{4}$$

Here, k_b is Boltzmann's constant and *T* is temperature. The partition coefficient of cholesterol between both membranes, assuming ideal mixing, will therefore become

$$K = \frac{x^A_{Chol}}{x^B_{Chol}} = \exp\left(\frac{-\left(\mu^{A,0}_{Chol} - \mu^{B,0}_{Chol}\right)}{k_b \cdot T}\right). \tag{5}$$

Thus, how cholesterol partitions, depends on its chemical potential in the respective membrane under standard conditions. This, in turn depends on the property of the host lipid matrix in the particular membranes. Lipid membranes, however, are not dilute isotropic solvents for cholesterol, but instead crowded, anisotropic media with many internal degrees of freedom. If we want to take such effects into account, we can introduce the activity coefficient, γ, leading to a modification of the expression for the chemical potential:

$$\mu_{Chol} = \mu^0_{Chol} + k_b \cdot T \cdot \ln x_{Chol} \cdot \gamma_{Chol}. \quad (6)$$

From that, we define an apparent equilibrium constant, K', as:

$$K' = \frac{\gamma^B_{Chol}}{\gamma^A_{Chol}} \cdot K. \quad (7)$$

The activity coefficient for cholesterol determines the free volume accessible for cholesterol in each membrane [97]. Thus, if for example, $\gamma^B_{Chol} = 5 \cdot \gamma^A_{Chol}$ because the available volume for cholesterol in membrane *A* made of saturated PC is several-fold that in membrane *B* made of unsaturated PC, membrane *A* could accommodate five times the amount of cholesterol compared to membrane *B* at equilibrium.

For many membrane solutes, the activity coefficient can be directly related to the work required to create a cavity for the molecule in the bilayer, as can be derived from scaled particle theory [97,98]. However, cholesterol reduces the available free volume in the bilayer, and consequently, lowers drastically the membrane permeability for many solutes [99–101]. So, why would a membrane consisting of saturated PC molecules accommodate more cholesterol than a membrane made of unsaturated PC? The answer lies in cholesterol's condensing effect on phospholipid bilayers, a well-known phenomenon tracing back to experimental mapping of binary phase diagrams of PC and cholesterol and their theoretical interpretation [102,103]. Due to its planar, non-polar structure, cholesterol reduces the conformational space available for fatty acyl chains, thereby reducing their area requirement under the PC head groups [103–105]. In membranes made of unsaturated PC, however, higher flexibility of the kinked fatty acyl chains prevents equally tight packing with cholesterol. Consequently, the space requirement of the non-polar bilayer region (i.e. sum of fatty acyl chains and cholesterol) in a membrane is higher, if the host phospholipid is unsaturated PC (e.g. DOPC in membrane *B*) than if it is saturated PC (e.g. DPPC in membrane *A*). Since both membranes have the same head group, more cholesterol gets exposed to the interfacial area in membrane *B* compared to membrane *A*, thereby making unfavorable contacts with water. Exposure of cholesterol to water lowers the entropy of the water molecules in vicinity of the sterol, which gives a positive contribution to the Gibbs free energy (i.e. the free enthalpy). This is therefore highly unfavorable, and there is a tendency to shield sterols under phospholipid head groups to lower the total free enthalpy. The efficiency of this shielding, though, depends also on the ability of cholesterol to pack with the adjacent phospholipid acyl chains, which in turn are ordered by the presence of cholesterol. The same reasoning applies to other host lipid species, as for example, the amount of sphingolipids will positively correlate with the partition preference of cholesterol in a given membrane [89,90,106]. Instead of free-volume theory, as for other membrane solutes, the extent of sterol tilt seems to be an adequate measure for cholesterol's effects on membrane properties [107,108]. The lower the tilt, i.e. the more cholesterol is aligned with the bilayer normal, the larger is the extent of membrane condensation and the stronger is the interaction with the fatty acyl chains of the host phospho- or sphingolipids. A low tilt means also high mechanical stability and thickening of the membrane, thereby likely affecting protein interaction with the bilayer [107–110]. Equally important, a low sterol tilt in the membrane is associated with lowered propensity for sterol flip-flop, which has been suggested to involve rotation of the sterol perpendicular to its long axis [111,112]. The sterol tilt does not only depend on the host lipid composition but also on the sterol type and mole fraction in the bilayer. In full accordance with that are recent experiments and theoretical studies showing i) faster flip-flop and higher tilt of ketosterols than cholesterol [113], ii) decreasing cholesterol tilt for increasing cholesterol mole fraction in the membrane [108,114], iii) higher partitioning of cholesterol in cholesterol-rich PC than in cholesterol-poor PC membranes [111,115,116] and iv) decreasing flip-flop rates of cholesterol for increasing membrane cholesterol content [111].

Taken together, the specific properties of the host membrane including phospho- and sphingolipid composition and sterol content determine cholesterol's chemical activity in the respective membrane allowing for differing sterol mole fractions in two bilayers at thermodynamic equilibrium (Eq. (7)). Deviation from thermodynamic equilibrium upon a small perturbation will cause a non-vesicular sterol flux between both membranes. This flux is proportional to the difference in chemical potentials between both membranes (i.e. to $\Delta\mu_{Chol} = \mu^A_{Chol} - \mu^B_{Chol}$). Thus, the chemical potential difference of cholesterol between the membranes acts as thermodynamic driving force for sterol flux and this flux vanishes at equilibrium when $\Delta\mu_{Chol}$ approaches zero. This connection between differences in chemical potential and flux is called a linear flux–force relationship in linear irreversible thermodynamics and can be derived from a Taylor expansion of the flux with respect to the force around a reference state. Of course, lipid membranes are anisotropic solvents, causing additional terms in the Gibbs free energy change associated with sterol transfer [98,114]. In contrast, classical partition experiments upon which the above formalism is based use an oily phase, made of isotropic solvents as alkanes with no preferred orientation of the hydrocarbon chains. In cells, proteins could additionally affect the cholesterol solubility of membranes, as could be shown for transmembrane peptides affecting the partition coefficient of fluorescent sterols as CTL or TopFluor-cholesterol between liposomes [117].

3.2. How do membrane properties of cholesterol relate to its intracellular transport?

Several lines of evidence indicate that intracellular sterol flux becomes non-linearly dependent on the membrane sterol mole fraction. For example, above certain threshold concentrations of total cell cholesterol a non-linear raise in cholesterol content in the ER accompanied by a halt in SREBP2 activation and acute proteolytic inactivation of HMG-CoA reductase has been observed [4,6,7]. An increase of cellular cholesterol beyond some threshold causes also abrupt stimulation of cholesterol esterification by ACAT [5,7], sudden acceleration of cholestenone formation by cholesterol oxidase and enhanced extractability of cholesterol with cyclodextrin [7,118], accelerated DHE influx in ATP-depleted macrophages [34] and increased accessibility of cholesterol to PFO binding [51,57]. These observations cannot be explained by a linear flux–force relationship, since they point to a non-linear increase of μ_{Chol} at critical cholesterol mole fractions. Three physico-chemical models have been invoked to explain such sudden changes in μ_{Chol} at critical cholesterol mole fractions. In all models, as in our considerations above, two membranes can have largely differing cholesterol amounts and still be in thermodynamic equilibrium. In the superlattice model, cholesterol is supposed to prefer regular packing geometries in the phospholipid matrix due to a mismatch in the cross-sectional area of the different lipid species [119]. This causes long-range repulsive forces between cholesterol molecules and sudden spikes in μ_{Chol} at critical mole fractions of the constituents, where preferred packing geometries are disturbed [120]. In the umbrella model, cholesterol is believed to avoid contacts with water due to its small polar OH-group resulting in preferred association with certain membrane phospholipids, which can shield the sterol under their head groups [121–123]. Due to increasing order of the fatty acyl chains and consequently condensing of the membrane, the ability to 'hide' cholesterol under the phospholipid head group diminishes as a non-linear function of cholesterol

concentration. The umbrella model considers explicitly multi-body interactions in the energy functional and predicts several jumps in μ_{Chol} at particular packing geometries of phospholipids and cholesterol at high sterol mole fractions in the bilayer [121,122]. Each jump in μ_{Chol} corresponds to a transition from one type of a regular cholesterol distribution to another. In the third model, the condensed complex model, stoichiometric interactions are assumed between cholesterol and phospholipids with saturated fatty acyl chains, called reactive phospholipid (e.g. DPPC), while no complex formation is assumed for unsaturated phospholipids (called 'unreactive'; e.g. DOPC) [124]. The expression for the Gibbs free energy of the system contains an ideal term and a mixing term for all four species (i.e. cholesterol, reactive and unreactive phospholipid and the complex formed between reactive lipid and cholesterol). From a thermodynamic analysis of this system and its temperature dependence deuterium NMR quadrupole splittings and order parameters were predicted, and phase diagrams were constructured [124,125]. The non-linear behavior in that model stems from formation of higher order complexes implying some form of cooperativity between cholesterol and certain phospholipid species [126,127]. The condensed complex model predicts a jump in μ_{Chol} at a stoichiometric composition for a particular cholesterol fraction (i.e., 33 mol% to 45 mol% cholesterol, dependent on the exact model parameters).

Recently, many observations made on living cells are interpreted based on the condensed complex model by conjecturing two pools of cholesterol to reside in each intracellular membrane, a free, that is non-complexed and a lipid-complexed cholesterol pool [4,127–129]. The equilibrium constant between both intra-membranous pools is set by the differing cholesterol affinity of the host lipids in that model [127,128]. The non-complexed pool, increasing substantially above the threshold concentration, is sometimes called 'active cholesterol' [129]. Although intriguing and certainly an attractive view point for understanding cellular homeostatic responses [9], several questions remain, as for example the physical identity of the active and condensed cholesterol pool in a biological membrane are not clear. Even though the authors of the condensed complex model emphasize, that observation of sudden increases in μ_{Chol} in a membrane do not require phase separation [124,125], suggestive figures in recent reviews on sterol transport indicate two lateral sterol pools (i.e., non-complexed and complexed) in cellular membranes [128,130,131]. However, lateral microscopic sterol-enriched domains have never been observed in membranes of living cells. In fact, recent results show a homogeneous lateral distribution of cholesterol and its fluorescent analogs in the PM of living cells [19,132–134]. This was supported by high resolution secondary ion mass spectrometry reporting that cholesterol is homogeneously distributed and not enriched in SM domains in the PM of fibroblasts, even though the cells were fixed using glutaraldehyde in these experiments [134]. Stimulated emission-depletion based FCS allowing for testing of diffusion laws by spot-size variation found free diffusion but no trapping of TopFluor-cholesterol and other dye-tagged cholesterol probes down to less than 80 nm in the PM of living cells [19,135]. Thus, if cholesterol forms indeed lateral inhomogeneities in cellular membranes, such domains must be highly dynamic and very small with diameters much less than 80 nm [19,136]. Interestingly, Monte Carlo simulations and recent neutron scattering experiments indicate cholesterol-PC domains in binary mixtures at high sterol mole fractions with an upper diameter of 22 nm and an average lifetime of 100 ns [52,70]. Currently, no experimental technique is available to determine whether cholesterol forms similar domains in membranes of living cells. Could the two PM leaflets resemble the active versus condensed or restrained cholesterol pools? While this cannot be ruled out, the known rapid sterol flip-flop in lipid membranes as well as the numbers found in recent studies with only about 30% sterol in the SM-rich outer leaflet and the majority of sterol in the likely less packed inner leaflet of the PM make this explanation also questionable [79,106,137]. The prediction of jumps in μ_{Chol} in the condensed complex and in the umbrella model, as applied to bilayers, occur for particularly high cholesterol mole fraction (i.e., about 40–50 mol% depending on the exact parameters used for lipid interaction potentials) [123,124]. At these concentrations, a given membrane might simply have reached its capacity to solubilize cholesterol, in which case the 'active' cholesterol pool would resemble excess cholesterol beyond the membrane solubility limit [122]. The cholesterol solubility limit depends of course on the phospholipid head group and the acyl chain length and saturation, such that all arguments of the above analysis apply. Above the solubility point, cholesterol would precipitate from the bilayer in form of crystals [122], unless other acceptor membranes with remaining capacity to solubilize cholesterol or soluble LTPs are available in the system. Thus, the active cholesterol could simply resemble partly water-exposed cholesterol, which cannot be shielded under phospho- and sphingolipid head groups and needs to be exported to other membranes for lowering the total system Gibbs free energy. Experiments on acutely cholesterol-loaded cells support that view, since the cholesterol content of intracellular membranes increases rapidly up to five-fold under these conditions [138]. Also, non-vesicular DHE influx to lipid droplets and other membranes as well as efflux to cyclodextrin is enhanced in cholesterol-loaded murine macrophages [34]. Interestingly, these cells do not sequester excess sterol in domains in the PM [133], but rather stimulate their PC synthesis to generate more membrane for accommodation of excess sterol [139]. After prolonged cholesterol loading in such macrophage foam cells cholesterol crystals have been observed, indicating that the sterol solubility limit of all membranes has been passed [140,141]. Neutron scattering experiments support the view, that cholesterol can partially protrude from the bilayer, especially at high mole fractions [142]. Recent molecular dynamics simulations indicate that the distance of cholesterol's hydroxyl group from the bilayer center diminishes non-linearly for high sterol mole fractions [143]. This partially protruding cholesterol could resemble the active cholesterol pool without need for separate domains. Increased transverse excursion of cholesterol at high mole fractions could resemble the onset of cholesterol precipitation unless being picked up by LTPs to trigger non-vesicular sterol exchange to a given acceptor membrane. Several other thermodynamic models have been put forward to explain binary and ternary phase diagrams of cholesterol and phospholipids; for example [102,103,144–146]. Not all of them invoke formation of condensed complexes, indicating that existence of complexes is not a requirement for the observed mixing characteristics of cholesterol and phospholipids. Such models do not attempt to relate the proposed biophysical mechanisms of cholesterol-phosholipid interactions to cholesterol transfer between membranes. This could be an interesting topic for future research.

Interesting but also somehow contradictive evidence for the hypothesis of 'active' cholesterol comes from recent studies using the cholesterol dependence of PFO binding to model and cellular membranes as readout for changes in μ_{Chol} in the bilayer. In model and cell membranes, there is a threshold concentration of cholesterol above which binding of PFO and its derivatives becomes detectable [147,148]. This threshold does not seem to relate to cooperative oligomer formation of the proteins, since it also occurs for truncated PFO variants not forming oligomers [149]. For the PM of intact cells, the threshold for PFO binding is around 35% cholesterol, while for ER membranes this threshold is only 5 mol% cholesterol [4,148]. Intriguingly, the latter value coincides exactly with the threshold for SREBP2 processing and nuclear targeting as a switch for regulating cholesterol homeostasis [4,150]. Based on these observations, it has been proposed that the threshold for PFO binding resembles the jump in μ_{Chol}, as predicted in the condensed complex and umbrella model [143,147,148]. In strong support of this argument is the sequence of threshold cholesterol concentrations for PFO binding to PC model membranes of varying acyl chain saturation ranging from about 35 mol% for DOPC over 45 mol% for POPC to approx. 49 mol% for DPPC [143,147,148]. Also, the mean depth of cholesterol's hydroxyl group in MD simulations coincided convincingly with the PFO binding affinity measured in the same study [143]. Neither

membrane fluidity as assessed by diphenylhexatriene fluorescence anisotropy nor detergent solubility correlated with the threshold of PFO binding [147]. Thus, one can argue that PFO detects the 'active' cholesterol pool in the membrane, when the bilayer approaches its cholesterol solubility limit [143]. The extremely low threshold value for PFO binding to ER membranes, however, could not be reconciled in any model membrane experiment and cannot be explained by the condensed complex model, either [4]. Since the same threshold was observed for liposomes made from ER lipids, a specific role of ER proteins can be excluded [4]. Liposomes made of ER lipids should have bulk biophysical properties (i.e. fluidity, bending rigidity and microviscosity) resembling those of liposomes made from unsaturated PC species, as DOPC [89,91,151]. Thus, the very different threshold values for cholesterol accessibility of liposomes made of DOPC compared to those made of ER-derived lipids as measured by PFO binding remains enigmatic [4]. Since some cholesterol-dependent cytolysins require cholesterol in both membrane leaflets [152], and cholesterol might form transbilayer dimers at very low concentrations [75,153,154], a specific transverse cholesterol organization in the ER membrane might be responsible for the low threshold in PFO binding. Alternatively, PFO binding might require a specific lipid co-factor found in the complex mixture of ER lipids but not in model membranes. Quantitative lipid mass spectrometry of purified ER membranes might help to clarify this issue.

In the PM of living cells additional leaflet-specific cholesterol–lipid interactions might be at play in regulating sterol transport and triggering physiological responses to control cholesterol metabolism. For example, the majority of SM and all glycosphingolipids will be restricted to the outer PM leaflet. Treatment of fibroblasts with bacterial sphingomyelinase (SMase) causes the formation of ceramide in the PM paralleled by rapid ATP-independent cholesterol transport to and esterification in the ER [155,156]. The same treatment increases cholesterol efflux to cyclodextrin, while degradation of PC with a specific phospholipase had a much lower effect on this process [157]. SMase treatment also triggered energy-independent formation of endocytic vesicles which, however, do not appear to be enriched in PM-derived sterol [34,158]. These observations can be explained by a preferred interaction of cholesterol with sphingolipids, as discussed in Section 3.1., above and further reviewed elsewhere [159]. Consumption of SM as substrate in the SMase-catalyzed reaction would reduce the number of cholesterol-SM pairs, thereby weakening cholesterol interactions in the PM. Alternatively, the non-polar reaction product, ceramide, competes with cholesterol for shielding by phospholipid head groups and pushes cholesterol out of the bilayer above some critical concentration. This explanation is fully in line with the umbrella model, described above and further substantiated by several experimental studies. For example, ceramide replaces cholesterol from ordered domains in model membranes and triggers cholesterol precipitation into the crystal phase [160,161]. The maximum solubility of cholesterol in the latter study decreased concomitantly with the increase in ceramide concentration, completely in line with the umbrella model of cholesterol–lipid interactions. Ceramide also directly competes with cholesterol for association with SM in model membranes [86]. A recent study combined the PFO binding assay with SMase treatment of cells and other biochemical assays on human fibroblasts incubated with LDL [162]. PFO binding was increased after incubating cells with LDL for several hours, and this effect was enhanced when cells where treated with SMase prior to PFO binding. Similarly, SMase caused increased cholesterol esterification, in agreement with earlier results [155,156,162]. The authors argued that three pools of cholesterol exist in the PM; 1) a PFO-detectable pool comprising about 15 mol% of PM lipids, which is expanded upon LDL uptake. The other two pools are accordingly not accessible to PFO and were suggested to resemble 2) a SM-sequestered pool and 3) a so-called essential pool, not responding to any treatment but being important for cell viability. As discussed above for the 'active' vs. lipid-complexed cholesterol pool, the physical nature of these biochemically defined cholesterol pools remains to be determined, especially in light of accumulating evidence for a homogeneous lateral cholesterol distribution in the PM [162–164].

Experiments with collisional quenchers and intrinsically fluorescent cholesterol analogs suggest that the majority of cholesterol resides in the inner leaflet of the PM. This is a striking but also puzzling observation given the evidence for preferred interaction of cholesterol with SM and other sphingolipids in the outer leaflet (see above). However, evidence for preferred enrichment of cholesterol in the inner leaflet has been provided also in other much earlier studies [165–167]. Recently, a model has been put forward which suggests that cholesterol is drawn to the inner leaflet by the high abundance of PE in this leaflet [168]. PE has a small headgroup and by itself a high spontaneous curvature forming inverted hexagonal phases at physiologic temperature due to dominating entropy of its fatty acyl chains. This causes a strong bending energy being quadratic in the PE concentration, which would be counteracted by cholesterol [168]. At first glance, this suggestion is in contradiction to partition experiments, in which cholesterol has a lower affinity for liposomes made of PE compared to those made of PC and SM (see Section 3.1., above) [90]. However, the important point in the model by Giang and Schick (2014) is the asymmetric PE distribution in the membrane causing an energy penalty due to negative bending and PE's spontaneous curvature [168]. Since cholesterol is shielded under the PE head group in accordance with the umbrella model, the spontaneous curvature caused by PE's molecular shape gets compensated when cholesterol is flipped to the inner leaflet [121,168]. The model predicts about 48-58% of PM cholesterol in the inner leaflet, depending on the parametrization of the spontaneous curvature. These modeling results are in line with earlier theoretical studies on phospholipid translocation in the erythrocyte membrane by an ATP-dependent translocase, which also predicted about 50% of cholesterol in the inner leaflet [169]. Active translocation of PE and PS or adding exogeneous SM to the membranes outer leaflet would cause a transient increase of cholesterol in the inner leaflet to compensate for any area imbalance. Thus, fast cholesterol flip-flop in the PM could be an efficient mechanism for keeping the difference in lipid numbers between both leaflets small, especially under non-stationary conditions due to membrane fusion or bending [169]. The latter is indeed supported by experiments [170].

Taken together, passive processes and specific cholesterol–lipid interactions play a dominant role in cholesterol movement and distribution in the cell, especially when cellular cholesterol exceeds some threshold concentration. Understanding the detailed nature of such cholesterol–lipid interactions in individual cellular membranes and the specificity of LTPs carrying cholesterol along seem to be the key aspects in determining the control of non-vesicular sterol transport in cells. How other biophysical properties of cellular membranes, as protein content [117], free fatty acids [171] or bilayer curvature [153,154,170] affect inter-organelle cholesterol transfer remains open. Similarly, understanding the impact of constant vesicle trafficking and ATP-consuming lipid remodeling on non-vesicular sterol fluxes awaits future research.

4. Protein-mediated uptake, intracellular targeting and efflux of cholesterol

In the following, we will give an overview of several cholesterol transport pathways and their protein dependence in living cells. We will focus on mammalian cells, since several excellent reviews have recently discussed sterol trafficking in yeast and other organisms [56,130,172].

4.1. How lipid transfer proteins mediate non-vesicular sterol transport — two examples

Several cases of non-vesicular cholesterol transport in cells have gained a lot of interest and insight in the last decades. One example is

cholesterol import into mitochondria in steroidogenic cells, which involves StAR on the mitochondria and cholesterol-donating StARD3/MLN64 and NPC2 on late endosomes (LEs) [173–175]. StAR and MLN-64 are both involved in cholesterol transport, and they were found to contain the same domain named steroidogenic acute regulatory-related lipid transfer (START) domain. The START domain of MLN64 and StAR was shown to bind cholesterol at an equimolar ratio [77]. The C-terminal domain of MLN-64 contains 37% amino acids identical to the StAR protein and nearly 60% of amino acids, which show similarities to StAR [176]. Little is known about the mechanistic details of this transport route, and current knowledge has been reviewed elsewhere [173,174]. Another case is transport of cholesterol from the ER, where cholesterol is synthesized, to the cell surface and the third case is cholesterol import from the PM to the ERC and ER. We will consider these latter two processes in the following section and use them for delineating basic principles of LTPs function in sterol transport. De novo synthesized cholesterol traffics to the PM in about 10 min, largely bypassing the Golgi apparatus [16,28,177,178]. This transport required ATP and stopped below 15 °C, while maintenance of the established cholesterol gradient between PM and ER was independent of metabolic energy [16,28]. Thus, vesicular and non-vesicular transport modes seem to be at play for export of cholesterol from the ER. Sterol exchange between ER and Golgi depends on the activity of OSBP, whose two structural motifs create membrane contact sites between both organelles. The N-terminal plekstrin homology (PH) domain binds to phosphatidyl inositol-4-phosphate (PI-4-P) in the TGN, while the FFAT (two phenylalanine in an acid tract) motif binds to VAMP associated protein (VAP) in the ER [56,81]. The OSBP-related domain (ORD) of the protein transfers sterols between both membranes, as suggested by in-vitro experiments, in which about 30 DHE molecules were transferred per

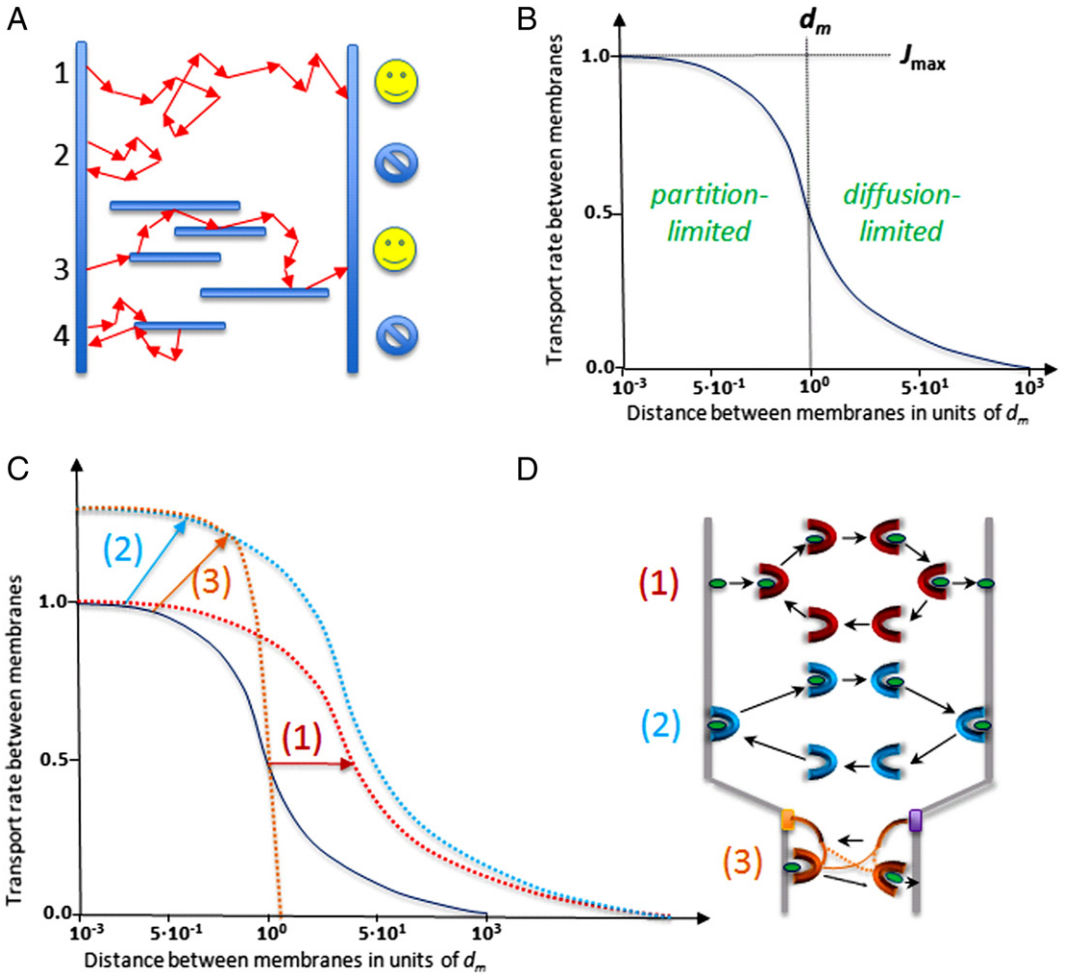

Fig. 1. Possible mechanisms underlying non-vesicular sterol transport by transfer proteins. A, possible outcomes of desorption of a cholesterol molecule from a donor membrane: 1) The molecule performs a random walk from the donor membrane (left) to the acceptor membrane (right), where it gets inserted giving a successful excursion, indicated by a smiley. This process is likely very inefficient and requires transfer proteins to take place at significant rates. 2) The cholesterol molecule desorbs and diffuses up to approx. its mean excursion distance, d_m, before it gets reabsorbed by the donor membrane. The definition of d_m is given in Eq. (8) of the main text. 3) Membrane structures located in between the donor and acceptor membrane might bind the sterol transiently allowing for its lateral diffusion in that membrane and desorption closer to the acceptor membrane. Together, this allows for extending d_m so much as to enable the sterol molecule to travel to the acceptor membrane, where it gets inserted (smiley). 4) An intermediate membrane, as there are plenty in the cytoplasm, might extend d_m somehow by allowing for transient absorption of the sterol, but this is not sufficient to prevent its reabsorption by the donor membrane. B, Sterol transport rate as function of distance between the membranes is given in units of d_m. If $d < d_m$ the sterol molecule can move to the acceptor membrane approaching the maximal flux, J_{max}, for very short distances. In this regime, sterol flux is limited by partitioning of sterol between the membranes. For inter-membrane distances larger than d_m the diffusion constant of cholesterol in the medium limits its flux. C, D, soluble transfer proteins (i.e., carriers which do not interact with membranes; case (1) — upper part in panel D) will increase d_m several fold without changing J_{max} (red dotted line in C). In case (2), membrane-binding transfer proteins will increase both, d_m and J_{max} (cyan dotted line in C and middle part of panel D). In case (3), transfer proteins tether two membranes together, thereby increasing J_{max} and pulling the membranes to a distance $d \sim d_m$, such that diffusion will never limit the net sterol flux between membranes (orange dotted line in C and lower part of panel D).
Adapted from [187].

minute per OSBP tethered between donor and acceptor liposomes [81]. Addition of 25-hydroxycholesterol to cells recruits OSBP to the Golgi interface, while the PH-FFAT region induces tethering and formation of several membrane appositions of about 20 nm distance between ER and PM [81]. Such close apposition will enhance the likelihood of sterol transfer instead of reabsorption by the donor membrane (see Fig. 1 and below) [179]. The ORD binds and transfers not only sterols but also PI-4-P, and a model has been put forward, in which ATP-consuming hydrolysis of PI-4-P in the ER by Sac1 drives directional lipid exchange between both organelles [81]. Further information about the function of OSBP and other family members in lipid transfer can be found in recent review articles [56,130,180]. Establishment of membrane contact sites by a putative sterol transporter has been recently also observed between LEs and the ER via interaction of MLN64 with VAP [181]. This result suggests that MLN64 can catalyze non-vesicular sterol transfer between these organelles by a similar mechanism, as proposed for OSBP (for further details on cholesterol transport from LEs, see Section 4.3. and Fig. 3, below).

Compelling evidence for non-vesicular sterol transport from the PM came from imaging studies of DHE in TRVb1 cells, a Chinese hamster ovarian (CHO) cell line expressing the human transferrin receptor [13]. DHE was inserted into the PM from a cyclodextrin complex, and sterol transport to the ERC was studied. Interestingly, energy-poisoning reduced the steady state level of DHE in the ERC only by 30%, and transport of DHE to the ERC continued even after cell fixation [13]. A simple flux–force relationship can explain these observations, as long as the influx rate is proportional to the size of the perturbation. Here, a strong gradient of fluorescent sterol is established at the moment, the tracer DHE is added to the cells (i.e., pulse-chase conditions; Section 3.1., above). Note, that in this experiment DHE is exchanged against PM cholesterol, such that overall sterol equilibrium should not be perturbed. Specific properties of the endosomal membranes might create a sink for sterol transport resulting in DHE entrapment in the ERC [13]. This could be a consequence of the specific lipid composition of this organelle, as discussed in Section 3.2. Non-vesicular cholesterol transport between PM and ERC/ER was found to be accelerated by overexpressing some LTPs, as SCP2, liver-specific FABP and ORP2, an OSBP homologue, as well as StARD4 [14,85,182–184]. The abundance of StARD4 had a pronounced effect on DHE targeting to the ERC and ER, on cholesterol esterification and on proteolytic procession of SREBP2 [14]. FRAP experiments have shown that DHE transport to the ERC was rapid and largely ATP-independent with a half-time of $t_{1/2} \sim 2.5$ min in TRVb1 cells [13]. Reduced expression of StARD4 but not its over-expression slowed also the FRAP kinetics of DHE in the ERC of HepG2 cells and resulted in increased free cholesterol in the PM [185]. Importantly, injection of cyclodextrin mimicked the effects of StARD4 including acceleration of DHE transfer between PM and ERC [14]. In vitro, sterol transfer by StARD4 and by SCP2 was enhanced in the presence of negatively charged lipids, which likely interact electrostatically with surface residues on the proteins [14,186]. StARD4 was also more efficient than cyclodextrin in transferring DHE between liposomes, when comparing the same carrier concentrations [14].

Given the extremely low water solubility of cholesterol, pulling one cholesterol molecule out of a bilayer into water requires overcoming an energy barrier of 80–90 kJ/mol corresponding to hydrolysis of about 1.5 ATP molecules [111]. So, what is the molecular mechanism underlying enhanced interbilayer sterol transport by these LTPs? Rapid binding of sterol to LTPs after cholesterol desorption from the bilayer would prevent reinsertion of the sterol into the donor membrane. The average distance, a cholesterol molecule diffuses into water before rebinding to the donor membrane can be quantified as mean diffusional excursion, d_m, as proposed in a steady state transport model developed by Weisiger and Zucker (2002) to describe cytosolic fatty acid transport by FABPs [179]. Applied to non-vesicular sterol transport, the presence of a LTP with concentration [*LTP*] and dissociation constant for the ligand, K_D, will increase d_m according to

$$d_m = \frac{2 \cdot \left(D_f + [LTP] \cdot D_b \cdot K_D^{-1}\right)}{P_{mw}}. \tag{8}$$

Here, D_f and D_b are the diffusion constants of the free and protein-bound sterol, respectively, and P_{mw} is the permeability of the membrane–water interface defining the rate of rebinding [179,187]. From Eq. (8), one can see that the largest values for d_m results from highly abundant LTPs with high diffusion constants and strong binding affinity. The diffusive flux between two membranes at steady state, J, was calculated in that model as function of membrane separation distance, d, to [179,187]:

$$J = \frac{J_{\max}}{1 + d/d_m}. \tag{9}$$

Here, $J_{\max}$ is half the rate of sterol dissociation (desorption) from the donor membrane, and thereby directly related to the chemical potential difference between the membranes, as discussed in Section 3.2. For non-zero cholesterol concentration in the donor and acceptor membrane with concentration difference Δc, one gets $J_{\max} = \Delta c \cdot P_{mw}/2$ [187]. Thus, the maximally achievable flux follows a simple flux–force relationship, as discussed in Section 3.1., in which the concentration difference resembles the thermodynamic force, and P_{mw} is the generalized transport coefficient (e.g. 1. Fick's law on diffusion) [20]. Fig. 1A illustrates the possible fates for a sterol molecule after desorption from a donor membrane, while Fig. 1B shows, how the inter-bilayer flux depends on the inter-membrane distance, d. Adding a soluble LTP will strongly increase d_m, thereby preventing reabsorption of the sterol molecule (Fig. 1C). If $d_m \gg d$, one sees from inspection of Eq. (9), that the steady state flux J approaches $J_{\max}$, as indeed found for membrane-inactive LTPs (e.g., fatty acid transport by L-FABP, Fig. 1C) [187]. If the membrane separation distance, d, is much smaller than d_m, J approaches $J_{\max}$, even without soluble LTPs, since the flux in this case is not diffusion-limited (Fig. 1B) [187]. For cholesterol, which has a much lower desorption rate from membranes (i.e. hours, see Section 3.1.) than fatty acids (i.e. milliseconds to seconds) [188], soluble LTPs might be not very efficient. Instead, for most known sterol carrier proteins and even for cyclodextrin, transient interaction with the donor membrane is instrumental for catalyzing inter-bilayer cholesterol transfer [14,186,189,190]. In their pioneering modeling study, Weisiger and Zucker (2002) also determined the impact of membrane interactions of the LTPs on steady state flux of fatty acids [179,187]. They showed that transient membrane associations of the LTPs also affects $J_{\max}$, as they lower the Gibbs free energy barrier for sterol desorption and thereby increase the permeability at the membrane–water interface, P_{mw} (Fig. 1D). Interaction of LTPs with certain cellular membranes, either due to electrostatic interactions with acidic lipids as shown for StARD4 or due to dual binding motifs to proteins residing in different organelles as known from OSBP fall into this category [14,81]. Membrane interaction of a LTP will therefore not only raise specificity but also increase the inter-bilayer sterol flux even if the membrane–membrane distance, d, is smaller than the mean diffusional excursion, dm. Interestingly, membrane-interacting LTPs as StARD4 show up to thousandfold higher per-molecule transfer activity of compared to cyclodextrin [14]. Similarly, absolute rates of intralysosomal sterol transfer by NPC2 are higher than for cyclodextrin [191]. As in case with StARD4, NPC2 was found experimentally to interact at least transiently with membranes [190]. Molecular simulations including free energy calculations suggest also absorption of cyclodextrin to membranes [189], but whether this interaction is weaker compared to StARD4 or NPC2 awaits further comparative studies. Dual binding motifs to the donor and acceptor membrane, as found in OSBP or as recently suggested for tethering LEs to the ER via MLN64, might be a particularly efficient way of raising

sterol flux, since beside of increasing J_{max}, such proteins reduce the interbilayer distance by creating close membrane contact zones [56]. Via such membrane contacts, a situation might be generated in which $d_m \sim d$, such that monomeric sterol diffusion is not limiting and very efficient sterol flux between organelles is ensured (Fig. 1C and D, case (3)).

4.2. Cholesterol endocytosis and its functional importance

In addition to non-vesicular transport described above in Section 3.2., cholesterol trafficking between PM and ERC takes place by vesicle traffic as well. For example, recycling of DHE from the ERC back to the cell surface required the vesicle export machinery involving the Rme-1 protein and occurred with a half-time of about 24 min in TRVb1 cells [13]. Transport of DHE from the PM to the ERC studied in pulse-chase experiments in the same study reached steady state values after about 15 min, which was similarly found in macrophages and human hepatoma HepG2 cells [17,34]. Similarly, transport of DHE to the ERC took place with a half time of about 8 min in rat hepatoma cells [45]. We compared recently membrane partitioning and intracellular transport of DHE with that of TopFluor-cholesterol and showed that both sterols are targeted to the ERC of Baby hamster kidney (BHK) cells with identical kinetics [42]. Uptake of both sterols from the cell surface was strongly reduced in BHK cells overexpressing a dominant-negative clathrin heavy chain and after ATP depletion, suggesting that clathrin-dependent endocytosis makes a significant contribution to overall sterol uptake in these cells. Similarly, Ge et al., found that uptake of cholesterol from the plasma membrane of rat hepatoma cells depended on clathrin and required expression of human Niemann–Pick C1 like 1 (NPC1L1) which directly interacted with clathrin heavy chain in this study [192]. NPC1L1 is a target of ezetimibe, a cholesterol absorption inhibitor in the intestine and is thought to play a central role in intestinal cholesterol absorption [193,194]. The sequence and structure similarity of NPC1L1 to NPC1 including sterol binding in the N-terminal domain would suggest a similar function of NPC1L1 in cholesterol export from early endosomes and/or LE/LYSs [195]. This, however, was not reported so far. NPC1L1 contains a sterol sensing domain, as NPC1 or HMG-CoA reductase and binds cholesterol at its N-terminal domain [196]. NPC1L1is found in the apical, canalicular membrane of hepatocytes and polarized hepatoma cells, where it is highly mobile, as shown by FRAP [45,197]. This protein was found to be important for cholesterol reabsorption from the bile compartment, probably to a subapical recycling compartment, thereby regulating cholesterol homeostasis also in the liver [198]. We observed recycling of NPC1L1 between cell surface and ERC and slightly enhanced vesicular targeting of DHE to this endocytic compartment in non-polarized rat hepatoma cells [45]. However, the majority of DHE uptake in this and other cell types takes place also in the absence of NPC1L1. Similarly, recycling of DHE or cholesterol from the ERC to the cell surface does not require NPC1L1, but rather depends on other proteins orchestrating endocytic recycling, as the ED domain protein RME-1 [13]. Several publications by the Song-group have provided evidence for a microtubule-based NPC1L1-mediated endocytic net cholesterol transport route from the PM to the ERC requiring flotilin and the clathrin adaptor Numb and for recycling of the cholesterol-NPC1L1 complex back to the cell surface in dependence of Myosin Vb, Rab11-FIP2 and the small GTPase Cdc42 [192,199–201]. While this transport route is likely important for maintenance of the steady state distribution of NPC1L1, the used cholesterol transport assay is not beyond reproach; filipin has been used to detect cholesterol replenishment from a cyclodextrin–cholesterol complex, but first after acute cholesterol depletion of the cells for 60 min with cyclodextrin [192]. While this treatment allowed for establishing a filipin-based pulse-chase transport assay, it seems to be far from mimicking the demand for cholesterol absorption in enterocytes. Thus, eventual artefacts known to be caused by prolonged cholesterol depletion using empty cyclodextrin cannot be ruled out [202]. Indeed, intestinal cholesterol absorption is a complex process, and the involvement of NPC1L1 is not without question [203]. Given the high sterol mole fraction in the PM of mammalian cells, formation of endocytic vesicles will probably always cause some internalization of cholesterol, likely in several endocytic pathways (i.e., clathrin-dependent and -independent pathways). Thus, specific sterol recruitment to a particular membrane protein, as NPC1L1, in the PM, prior to internalization is likely not necessary for sterol endocytosis. Indeed, we observed endocytosis of DHE and TopFluor-cholesterol by time-lapse microscopy in cells not expressing NPC1L1 [18,46]. Due to lack of conclusive experimental evidence, a convincing mechanistic model on involvement of NPC1L1 in sterol uptake and transport between PM and ERC is therefore currently not available. Interestingly, both fluids from which NPC1L1 has been proposed to mediate cholesterol absorption into cells, the digestive fluid in the intestine and the bile fluid in the liver, contain cholesterol derived detergents, i.e. bile salts. It would be interesting to study, how bile salts affect NPC1L1-mediated cholesterol absorption into cells, thereby eventually shedding light onto the function of this protein in these specialized tissues.

After internalization, the dynamics of vesicles carrying TopFluor-cholesterol has been studied in CHO cells using two-photon excited SPT [18]. Such vesicles were independently shown to contain DHE and TopFluor-cholesterol avoiding any artefacts caused by eventual miss-targeting of TopFluo-cholesterol [12,42]. We found that the majority of sterol-rich vesicles moved slowly by anomalous diffusion with $D \sim 2°10^{-3}\ \mu m^2/s^{\alpha}$, and $\alpha \sim 0.6$ [18]. Both, actin and microtubule disruption affected the diffusion of such vesicles in the cytosol. A sub-pool of vesicles, however, showed faster, directed motion, and endosome fission and fusion was found in time-lapse sequences of TopFluor-cholesterol [18]. Slow confined diffusion was also found for DHE-rich vesicles by SPT on a wide field set up in macrophages and by temporal image correlation spectroscopy on a multiphoton microscope in HepG2 cells [46,204]. We showed also recently that SPT and TICS provide comparable results on diffusion and directed transport of vesicles [27]. Together, recent data suggest that under normal physiological conditions sterol trafficking between PM and the sterol-rich ERC takes place on a time scale of 15 to 20 min. Vesicular and non-vesicular modes seem to contribute each about half to that sterol transport, but exact numbers depend on cell type and used transport assay.

4.3. Cholesterol import via low density lipoprotein and intracellular processing of its sterol load

This section is dedicated to cholesterol import into cells via lipoproteins. Since lipoproteins are very diverse and their physiology is a huge field of research, we will focus on the paradigm of LDL-mediated cholesterol uptake. This pathway and its discovery represents one important example, in which a genetic disease, here familial hypercholesterolemia, led to important mechanistic insights into a fundamental biochemical process [36,205]. There are, of course, many earlier examples in which genetic mutations were mechanistically linked to human diseases, as sickle cell disease or phenylketonuria, but the history of the discovery of the LDL pathway follows that tradition [206]. LDL is about 22–27 nm in diameter, and each particle contains abundant CEs and triacylglycerides (TAGs) in its core. The surface coat of the LDL particle contains phospholipids (25% by weight), a single 550 kDa apoB-100 protein (25%) and non-esterified cholesterol. TAGs, cholesterol and CEs represent the remaining 50% by weight of each LDL particle [207]. LDL binds to the LDL receptor (LDL-R) at the cell surface at neutral pH, upon which the ligand-receptor complex gets recruited to clathrin-coated pits via interaction with adaptor protein 2 (AP2). After endocytosis and uncoating, the newly formed vesicle fuses with sorting endosomes (SEs), a sub-population of the early endocytic pathway. SEs lose their fusion competence for incoming vesicles and mature into LEs, a process accompanied by a switch in endosome-associated rab proteins, from rab5 to rab7 and by acquisition of intraluminal vesicles (ILVs) (Fig. 2) [208,209]. The lysosomal pathway can be divided

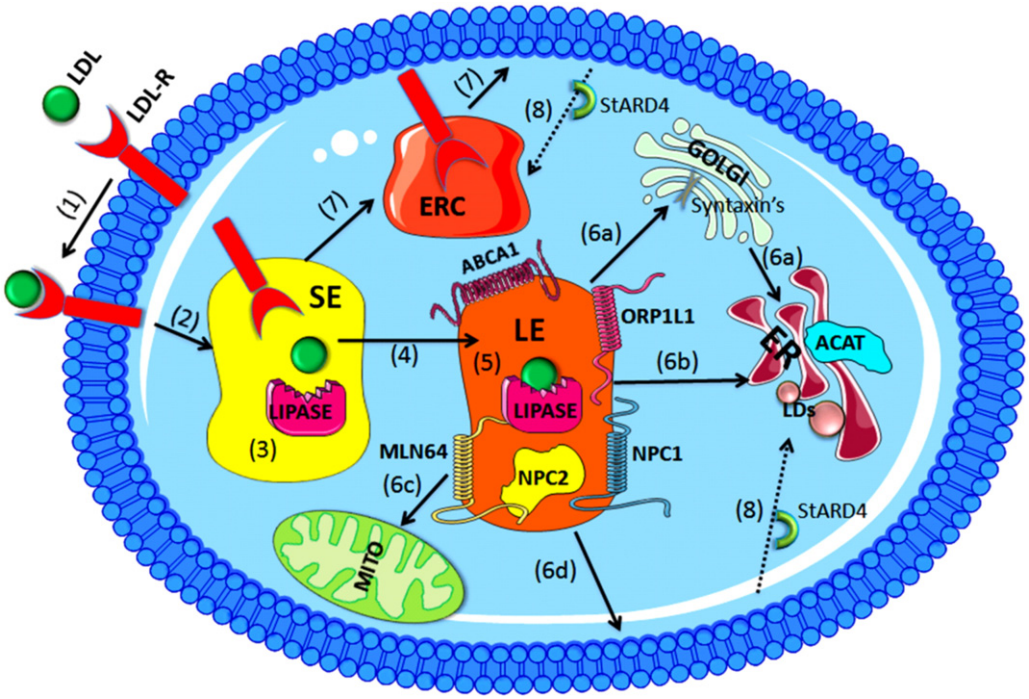

Fig. 2. Schematic diagram for endosomal cholesterol transport. (1) LDL particles bind to the LDL receptor (LDL-R) at the cell surface and become internalized by clathrin-dependent endocytosis followed by (2) fusion with sorting endosomes (SEs). (3) in the SEs, LDL dissociates from its receptor and some hydrolysis by acid lipase starts. (4) SEs mature into late endosomes (LEs). (5) the majority of LDL degradation including hydrolysis of cholesteryl esters to cholesterol and fatty acid occurs in LEs. LDL-liberated cholesterol can exit the LEs via several pathways; (6a) to the Golgi/TGN in transport vesicles which fuse with the acceptor compartment via TGN-specific SNAREs (e.g. syntaxin 16); (6b) to the endoplasmic reticulum (ER) in a pathway involving NPC2, NPC1 and ORP1L1; (6c) to mitochondria in steroidogenic cells in a pathway involving NPC2 and MLN64 but not NPC1 (a similar pathway might also operate between LEs and ER, not shown); (6d) to the plasma membrane (PM) via a poorly defined pathway. (7) some sterol follows also the normal endocytic recycling route from SEs via the endocytic recycling compartment (ERC) to the PM. (8) from the PM, some cholesterol can be internalized by a non-vesicular mechanism involving StARD4. In this process, cholesterol gets targeted to the ERC and ER, respectively (dashed line). Excess cholesterol arriving in the ER becomes esterified by acyl-CoA acyl transferase (ACAT) and stored in lipid droplets (LDs). See text for further explanations.

into LEs, a subpopulation of which is sometimes called multivesicular bodies, and lysosomes (LYSs) — acidic organelles with large content of hydrolytic enzymes [210]. Formation of ILVs inside LEs depends on specific lipids such as bis(monoacylglycero)phosphate (BMP), ceramide and cholesterol. This process is also strictly dependent on the endosomal sorting complex required for transport (ESCRT), as recently reviewed in detail [210]. The ILVs fulfill diverse functions in endosome maturation, protein degradation and lipid metabolism [210,211]. LE/LYSs can also secrete material from cells, often in form of small vesicles called exosomes. These secreted vesicles likely form from ILVs and have diverse functions in cell-to-cell communication and lipid metabolism [212]. Traffic between LEs and LYSs is extensive and several theories exist about the relation of both organelles [210]. Degradation of ingested cargo seems to take place in LEs or hybrid organelles between LEs and LYSs, while LYSs fulfill a role as enzyme reservoir [210,213]. However, as assignment of protein markers and molecular function is not unequivocal in the literature, we will use in the following the term LE/LYSs to address transport through the degradative organelles, unless otherwise specified. The LDL-R contains six building domains: the binding domain, an epidermal growth factor domain (EGF), a tyrosine–tryptophan–threonine–aspartic acid (YWTD) rich-domain, an O-linked sugar domain, the transmembrane domain and the cytosolic NPxY motif [214]. There are around 1400 LDL-R mutations known, which lead to fatal diseases such as hypercholesterolemia, tendinous xanthoma and premature coronary heart disease [3]. The ligand binding domain in the extracellular portion of the LDL-R contains 7 cysteine-rich regions, which were shown to be negatively charged. Therefore, they can bind to positively charged residues on the apoB-100 of LDL and VLDL. Lipoprotein binding to the LDL-R can be blocked by chemical modification of R1–R7 in LDL-R [3]. The extracellular domain of the LDL-R undergoes a conformational change upon switching the pH from 7.4 to 5.0, which is a precondition of ligand dissociation within SEs in cells [215]. Fass et al. (1997) showed binding of calcium ions into the binding site on the R5 at pH = 5.0, and the crystal structure of this complex could be resolved [216]. Recent results show that, beside an acidic pH, low endosomal calcium is essential for release of LDL from its receptor, likely as a consequence of structural changes in R1–R7 upon calcium release [217].

The ligand-freed LDL-R returns to the cell surface, either directly from SEs or from the ERC for another round of LDL uptake [208,218]. Each LDL-R can import about 120 LDL particles and thereby about 200,000 CE molecules. Within a population of LEs, acid lipase hydrolyzes LDL-associated CEs to cholesterol and fatty acids. Lack or reduced expression of the acid lipase causes Wolman disease and CE storage disease, respectively [219]. These diseases are characterized by accumulation of CEs in LE/LYSs. Export of the LDL-derived cholesterol from LEs is only partly understood, but important insight came from another genetic disease. NPC disease is a rare neurodegenerative disorder caused by a mutation in one of two proteins. NPC1 protein contains several transmembrane helices and locates mostly to LE/LYSs but is also found in the TGN [220]. NPC2 protein is small and soluble in the lysosomal lumen, but it is also present in several body fluids including milk, bile and epididymal fluid [59,220]. Loss of function in either NPC1 or NPC2 protein has been shown to cause severe lysosomal accumulation of cholesterol, glycosphingolipids, sphingosine, and SM [220,221]. The resulting cellular phenotype is characterized by large amounts of lipid-laden crescent shaped LE/LYSs, sometimes called lysosomal storage organelles [220]. Fibroblasts lacking functional NPC1 protein hydrolyze LDL derived CEs normally but have a strongly reduced ability to elicit normal regulatory responses, as stimulation of esterification and suppression of synthesis of cholesterol [59,220]. It is unclear whether cholesterol liberated from ingested LDL is first targeted to the PM, the

TGN or to the ER, where most cholesterol metabolizing enzymes reside [222]. NPC1 and NPC2 bind cholesterol, various oxysterols as well as intrinsically fluorescent sterols like DHE with nanomolar to micromolar affinity [62,65,66,223]. Infante et al. (2008) purified and characterized the N-terminal domain (NTD) of NPC1, later called NPC(NTD) [223]. This loop contained 240 amino acids, and it was isolated as a highly water soluble protein with tendency to form a homodimer, as suggested by gel filtration [54,223]. One cholesterol molecule is able to bind to NPC(NTD), but 25-hydroxycholesterol, was found to be favored in the binding site in comparison to cholesterol, eventually due to formation of additional hydrogen interactions of the oxysterol in the binding site [223]. In fact, Infante et al. (2008) found that the majority of the oxysterol binding protein found in the liver is NPC1 [54]. The crystal structure of bovine NPC2 was first determined by Friedland et al in 2003 [65]. Crystals suitable for the X-Ray experiment were grown from NPC2 purified from bovine milk using a hanging drop method at neutral pH stabilized by Tris buffer. The structure contains 7 β strands, which are arranged into two β sheets. The hydrophobic interior responsible for sterol binding in NPC2 is loosely packed with an available space for one sterol molecule (i.e. with a volume of 84 $Å^3$ [64,65]). The mechanisms underlying NPC1/ NPC2 function in export of LDL-derived cholesterol from LE/LYS are not known. Mutations in both genes cause inhibition of LDL-stimulated cholesterol re-esterification in the ER and failed suppression of SREBP- and LXR-dependent gene expression [224]. These defects take place despite increased total cellular free cholesterol and are consistently more severe for mutations in NPC2. Treatment of NPC disease fibroblasts with the oxysterol, 25-hydroxycholesterol can partially overcome the cholesterol loading in these cells [224]. It has been proposed that NPC1 and NPC2 protein work in tandem in mediating lysosomal cholesterol export [59]. Pioneering studies by Goldstein & Brown and co-workers published from 2008 on have shown that the N-terminal soluble luminal domain of NPC1, named NPC1(NTD) can bind cholesterol and oxysterols with high affinity [54,223]. Ligand binding and transfer assays showed that NPC2 can efficiently transfer cholesterol to and from liposomes, while transfer between vesicles and NPC1(NTD) was very slow. This transfer was, however, more than a hundredfold accelerated in the presence of NPC2 [225]. Subsequent alanine mutagenesis studies combined with X-ray crystallography revealed distinct subdomains in NPC1(NTD) for cholesterol binding and transfer [63]. Key amino acids on the surface of NPC1(NTD) and NPC2 were identified for interaction of both proteins and for cholesterol transfer [226]. The suggested hydrophobic hand-off of cholesterol between both proteins resembles substrate channeling in metabolic reactions and has been investigated further using free energy calculations based on molecular dynamics simulations [225,227]. The latter study used an implicit solvent description based on the generalized Born approximation and a simulation technique called nudge elastic band calculations to suggest likely (i.e. energetically favourable) conformational transitions during cholesterol transfer between NPC1(NTD) and NPC2 [227]. Importantly, the authors found that the protein complex is most stable if cholesterol associates with NPC2 and least stable if the apoproteins NPC1(NTD) and NPC2 were simulated [227]. However, based on existing experimental and computational data, the directionality of cholesterol transport between both proteins could not be unequivocally established, yet [225,227]. The cholesterol binding pockets of both proteins are bent with respect to each other during cholesterol transfer in the simulation study by Wiest and colleagues, indicating that either the sterol ligand or one of the NPC proteins needs to change its conformation during sterol transfer (Fig. 3, inset A) [227]. Another computational study on the NPC1(NTD)–NPC2 complex was published recently by Elghobashi-Meinhardt (2014), in which the possibility of conformational changes in the side chain of cholesterol during sliding from the NPC2-pocket to the NPC1(NTD) binding site was explored [228]. Using a quantum mechanical (QM) description of the sterol ligand and a classical molecular mechanics (MM) force field for the proteins, the energy barrier for rotation of the C17–C20–C22–C23 dihedral angle was determined during sliding of cholesterol from NPC2 to NPC1(NTD) (Fig. 3, inset B) [228]. This was motivated by the earlier simulation study showing that this dihedral angle is 71.6° for cholesterol in NPC2 but −157.3° in the NPC1(NPD) binding pocket, while in the respective crystal structures, this torsion angle is nearly identical with −164° [63,64,227,228]. Using QM/MM simulations, the likely 'reaction pathway' for cholesterol transfer and the energy barrier along that path was calculated giving a barrier of ~22 kcal/mol in total. Structural changes in several torsion angles in the cholesterol side chain were suggested to allow for its isomerization during transfer [228]. In both simulation studies, point mutations found in the alanine mutagenesis screen were found to reduce either the efficiency of transfer or the stability of the complexes [63,226–228]. Additional interactions between NPC2 and other intraluminal loops of NPC1 have been recently determined by surface plasmon resonance [229].

Despite this progress and mechanistic insight, the hydrophobic handoff model for cholesterol transfer between NPC2 and NPC1 is based solely on in vitro experiments but not (yet) grounded in thorough cellular studies. For example, a direct interaction between NPC1 and NPC2 protein has never been demonstrated in living cells. NPC1 seems to have a rather complex distribution between several organelles including not only LEs but also early endosomes, the Golgi and even partly the PM [230–232]. In contrast, NPC2 is restricted to LE/LYSs, and a recent quantitative imaging study showed that internalized bovine NPC2 can remove cholesterol efficiently from a subset of the sterol storage compartments visualized using filipin [25]. Also, export pathways for internalized cargo from LE/LYSs have been described, which depend on NPC2 but not on the NPC1 protein [233–235]. For example, the StAR-domain containing MLN64 was found to export cholesterol from LE/LYSs independent of NPC1 and was found to reside in a distinct population of LE/LYSs [233,236]. How such results can be incorporated into the structural model of NPC2–NPC1-mediated cholesterol transfer described above and in Fig. 3 is a challenge for future research. Maybe, several exit pathways exist from LE/LYSs for cholesterol; e.g. a NPC1-mediated route as well as a pathway dependent on MLN64-and/or the ATP-binding cassette transporter A1 (ABCA1, discussed below in Section 4.3.; Fig. 2). NPC2 could feed all these pathways by efficiently shuttling cholesterol from ILVs to such transporters in the limiting endosomal membrane which then transfer them to various acceptor organelles. On the cytoplasmic side, OSBP-related protein (ORP5) and OSBP-related protein like protein 1 (ORPL1) were additionally implicated in shuttling of LDL-derived cholesterol between LE/LYSs and the ER [237,238]. Recent multi-color imaging studies suggest functional subcompartmentalization of LEs in 'early LEs' containing LDL, an ABC transporter (ABCA3) and MLN64 and 'late LEs' harboring most NPC1 and ORPL1 [236]. ORP1L1 seems to be essential to target LEs to the perinuclear region in proximity of the ER, which would be a precondition for cholesterol transfer [237]. Again a virus trafficking study provided direct evidence for concerted action of NPC1 and ORPL1 in targeting cholesterol from LEs to the ER; adenovirus RIDα can rescue the cholesterol storage phenotype in cells lacking NPC1 [239]. This pathway led to increased formation of CEs derived from hydrolysis of LDL and in formation of ER-derived lipid droplets [240]. Importantly, this transport depended strictly on functional NPC2 supporting the view that NPC2, NPC1 and ORPL1 act in tandem [240]. Direct observation of endocytic processing of LDL-derived CEs was made possible using a TopFluor-cholesteryl ester reconstituted into LDL [241]. Egress of hydrolyzed TopFluor-cholesterol from LE/LYSs was found to depend not only on acid lipase and NPC1 activity but also on the small GTPase rab8. This supports earlier results by the same group showing that rab8 overexpression can partially restore the NPC1 lipid storage phenotype [242]. The motility of LEs containing the fluorescent CE analog depended on actin, similar as previously found again by Ikonen's group for MLN64 [243]. Members of the ESCRT family, as Hrs/Vps27, and rab7, a key GTPase for LEs formation and positioning, are also involved in processing of LDL and release of its cholesterol after CE hydrolysis [244,245].

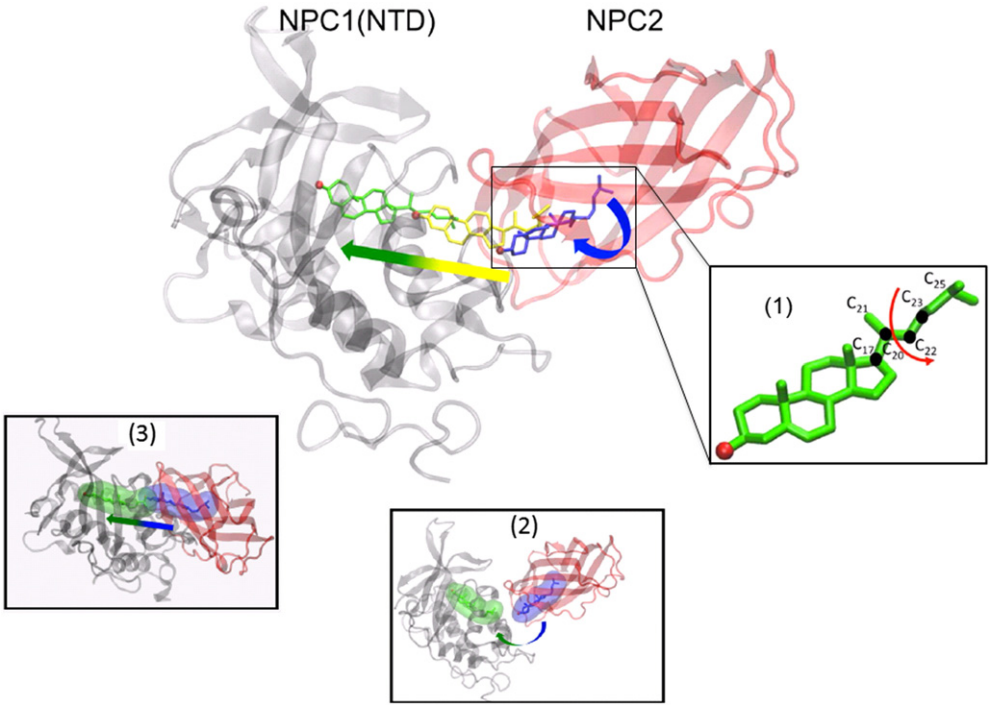

Fig. 3. Complex between the N-terminal loop of NPC1 (NPC1(NTD)) and NPC2 in endosomes and its suggested conformational changes allowing for cholesterol transfer. Based on the crystal structure of NPC1(NTD) (PDB ID: 3GKI) and NPC2 (PDB ID: 2HKA), molecular simulations have been carried out to uncover the energetic and conformational changes associated with cholesterol transfer from NPC2 to NPC1 [228]. A putative complex forms between NPC2 and NPC1, located in the late-endosomal lumen and limiting membrane, respectively [226]. In NPC2, the sterol (blue stick representation) tail is buried in the binding pocket, and the 3β-hydroxyl group (red ball) extends toward the NPC1(NTD) binding site. In NPC1(NTD), the orientation of the ligand (green stick representation) is reversed, with the ligand 3β-hydroxyl group (red ball) pointing toward the interior of the protein, while the isooctyl sterol tail points toward the sterol opening. The transfer of the cholesterol ligand from one binding pocket to the other requires a conformational change in the ligand–protein complex. One possible mechanism includes the conformational rearrangement of the cholesterol ligand inside the NPC2 binding pocket (blue arrow), for example, isomerization of the cholesterol side chain dihedral torsion angle C17–C20–C22–C23, as shown in inset (1). Via an intermediate structure (yellow), the sterol ligand could slide to the binding pocket in NPC1(NTD) (yellow to green arrow). Inset (2) shows, how the NPC2 binding pocket (in blue) and the NPC1(NTD) binding pocket (in green) are bent with respect to each other in this scenario during cholesterol transfer [227]. Inset (3) shows an alternative scenario in which the protein moieties are rotated with respect to each other such that the binding pockets are aligned, allowing for transfer of cholesterol with negligible change in its conformation.
Reproduced and adapted from [228] with permission.

In addition, evidence has been provided for an export route of LDL-derived cholesterol from LEs over the Golgi to the ER [246]. Further information about the protein machinery regulating trafficking through the endosomal pathway and thereby affecting also cellular cholesterol homeostasis can be found elsewhere [210,222,247,248].

Some evidence has been provided that the primary offending metabolite in NPC1 disease is SM, while other studies point to gangliosides or accumulation of sphingosine (the product of ceramide degradation by acid ceramidase) [221]. Sphingolipids, as SM, were found to block calcium import into LE/LYSs, thereby impairing endosome/lysosome fusion and lysosomal exocytosis, a mechanism suggested for NPC1 disease [221,249]. Agonists of the transient receptor potential (TRP) channel in LE/LYSs have been shown to restore normal calcium levels in LE/LYSs (~150 μM) and to rescue the cholesterol and sphingolipid storage phenotype in NPC1 disease cells [249]. Similar effects were described for the polyphenol curcumin found in yellow curry and the blocker of the ER calcium pump thapsigargin [250]. It was speculated that lysosomal exocytosis was stimulated by such treatments, resulting in enhanced secretion of cholesterol-rich exosomes from the NPC1 disease cells [251]. How calcium homeostasis is linked to function of the NPC2 protein and eventual lysosomal exocytosis remains to be determined. In in vitro assays, NPC2 protein was found to enhance inter-membrane cholesterol transfer in a ceramide- and BMP-dependent manner [211]. Production of ceramide from SM by an acid SMase (aSMase) seems to be essential for cholesterol efflux from LE/LYSs [211,252]. In fact, treatment of NPC1 disease cells with aSMase could also ameliorate the cholesterol storage phenotype [252]. Similar experiments have not been performed for NPC2 disease cells, but in vitro experiments indicate, that NPC2 and aSMase act in concert in transferring cholesterol between liposomes [211,253]. However, though much argues for cholesterol, the offending metabolite remains to be unequivocally identified.

4.4. ATP-binding cassette transporters and their role in cholesterol efflux from cells

Plasma concentrations of high density lipoprotein (HDL) are negatively correlated with the risk for developing cardiovascular disease. This is, because HDL-mediated reverse cholesterol transport to the liver is an efficient way to remove excess cholesterol from the circulation. Formation of HDL starts with secretion of lipid-poor apoprotein A1 (apoA1) by the intestine and liver, and apoA1 gets subsequently lipidated by interaction with peripheral tissue cells. ApoA1 receives cellular cholesterol and phospholipids in a process dependent on ABCA1. Within the nascent HDL particles, cholesterol is esterified by lecithin-cholesteryl acyl transferase (LCAT) associated with the growing lipoprotein. Using a fatty acyl chain of apoprotein-associated PC, LCAT esterifies cholesterol in the nascent HDL particles thereby creating a sink for further cholesterol efflux from cells. Patients with mutated ABCA1, as observed in Tangier disease, have an increased risk of developing cardiovascular diseases due to a strongly impaired cellular lipid efflux to apoprotein A1 (apoA1), and consequently dramatically reduced plasma HDL levels [254,255]. Cholesterol efflux to mature HDL is primarily mediated by the ABC half transporter ABCG1 and takes place either on the cell surface or during endocytic recycling of HDL [256]. Targeted disruption of the gene coding for ABCG1 in mouse results in massive accumulation of CEs, TAGs and phospholipids in hepatocytes and macrophages [257]. Recent evidence indicates ABCG1's involvement in regulating endosomal cholesterol levels, thereby indirectly affecting

the transbilayer sterol distribution in the PM upon fusion of endocytic vesicles with the cell surface [258]. Expression of ABCA1 and ABCG1 is under control of the Liver X receptor (LXR) and can be stimulated by LXR ligands, like 25-hydroxycholesterol [259]. It is currently not known, which cellular cholesterol pool is dominantly used for ABCA1-mediated sterol efflux. The prevailing hypothesis is that ABCA1 acts in concert with apoA1 on the cell surface to remove PM cholesterol being constantly replenished by intracellular sources [254,260]. Other studies indicate that ABCA1, which follows a complex intracellular trafficking scheme [255,261,262], mediates lipidation of apoA1 during its passage through the cell, likely by a retroendocytic pathway [260]. Interestingly, ABCA1-mediated cholesterol efflux is not only impaired in fibroblasts from patients with Tangier disease but also in fibroblasts from patients suffering from NPC disease and CE storage disorder [219,263,264]. Reduced conversion of LDL-derived cholesterol into oxysterols is likely causing the diminished expression of ABCA1 in these diseases, since oxysterols act as LXR agonists and induce expression of ABCA1 [224,259]. Importantly, activation of ABCA1 using LXR agonists is sufficient to trigger cholesterol efflux to apoA1 in cells lacking functional NPC1 protein [263], while ABCA1-mediated cholesterol efflux is strictly dependent on the NPC2 protein [234]. These results demonstrate the importance of the lysosomal cholesterol pool for ABCA1-dependent cholesterol efflux and indicate again different mechanistic roles for NPC1 and NPC2 in cholesterol mobilization from lysosomes. Interestingly, recent work by Molday and colleagues on reconstituted ABCA1 shows that this transporter, though being so central for cellular cholesterol efflux, does not bind cholesterol; in fact, its ATPase activity in phospholipid liposomes actually declines in the presence of cholesterol [73]. Reconstituted ABCA1 actively translocated fluorescent analogs of PC, SM and PS from the cytoplasmic to the exoplasmic leaflet of membranes [265]. In line with this observation, active translocation of PS analogs to the outer leaflet of the PM by ABCA1 has been shown in intact cells [266]. This causes altered inner surface membrane potential and reduced rate of endocytosis [266–268]. Side-specific quenchers of DHE and CTL have been used to demonstrate ABCA1/ABCG1-dependent sterol redistribution from the inner to the outer leaflet of the PM in living cells [269]. Similar experiments on CHO cells which lack ABCA1 revealed that most sterol resides in the inner leaflet [137], while sterol flip-flop across membranes is very rapid [79,270,271]. Together with the absence of cholesterol binding to ABCA1 and the inhibitory effect on its ATPase activity [73], a mechanistic understanding of the enhanced ABCA1-dependent sterol translocation to the exoplasmic leaflet of the PM is lacking. One can hypothesize that active flopping of PS and other phospholipids 'pulls' some cholesterol to the outer leaf of the PM. In line with this idea are recent observations by Smith and colleagues, showing that depletion of SM by treating cells with SMase or by inhibiting sphingolipid synthesis cause PM remodeling with enhanced PS exposure on the outer leaflet and higher cholesterol efflux from cells by ABCA1-dependent and -independent mechanisms [272]. Importantly, LXR-dependent expression of ABCA1 depends on another ABC transporter, ABCA12, which was first described to mediate formation of lipid lamellae in the skin. Mutations in ABCA12 cause harlequin ichthyosis, a devastating skin disease with abnormal lipid granules in keratinocytes resulting in impairment of the skin barrier function [273]. ABCA12 deficiency caused foam cell formation and decreased reverse cholesterol transport in mice due to a posttranslational impact on ABCA1 [274]. Thus, as discussed for the NPC proteins, mechanisms underlying ABCA1 mediated cholesterol transport are complex and regulated on several levels. Further discussions of ABC transporters and scavenger receptors involved in intestinal cholesterol absorption and biliary cholesterol secretion can be found elsewhere [275,276].

Conflict of interest

The authors, Dr. Daniel Wüstner and Dr. Katarzyna Solanko, declare to have no conflict of interest.

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