Insights into the Molecular Mechanisms of Cholesterol Binding to the NPC1 and NPC2 Proteins

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Abstract In recent years, a growing number of studies have implicated the coordinated action of NPC1 and NPC2 in intralysosomal transport and efflux of cholesterol. Our current understanding of this process developed with just over two decades of research. Since the cloning of the genes encoding the NPC1 and NPC2 proteins, studies of the biochemical defects observed when either gene is mutated along with computational and structural studies have unraveled key steps in the underlying mechanism. Here, we summarize the major contributions to our understanding of the proposed cholesterol transport controlled by NPC1 and NPC2, and briefly discuss recent findings of cholesterol binding and transport proteins beyond NPC1 and NPC2. We conclude with key questions and major challenges for future research on cholesterol transport by the NPC1 and NPC2 proteins.

Keywords Sterol-sensing domain · Transport · Binding · Structure · Lysosome · Niemann-Pick Disease Type C

Abbreviations

CLR	Cholesterol
CTD	C-terminal domain
MLD	Middle luminal domain

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NPC Niemann-Pick Type C
NTD N-terminal domain
SSD Sterol sensing domain

1 Overview

Cholesterol (Fig. 1a) plays a critical role in multiple cellular functions. These include regulating the physical properties of the plasma membrane to ensure cell viability, growth, proliferation, and serving as a signaling and precursor molecule in biochemical pathways [1–7]. Regulation of cellular cholesterol levels is tightly controlled via multiple pathways that include *de-novo* biosynthesis, uptake, recycling and release [7–11]. In particular, delivery of extracellular cholesterol to cells is achieved by receptor-mediated uptake of low density lipoproteins (LDLs) that carry both free and esterified cholesterol [12]. Subsequent to entering the vascular tissue, LDL particles reach the endosomal-lysosomal system via endocytosis [13]. In this system, cholesterol esters are converted back to free cholesterol. Proteins are then harnessed to export the hydrophobic cholesterol molecule through the hydrophilic environment of endosomes and lysosomes. In particular, coordination of the NPC1 and NPC2 proteins (Fig. 2) facilitates cholesterol trafficking through the lysosome. Genetic mutations of either gene result in the accumulation of unesterified cholesterol in the endo-lysosomal system [14]. While clinical manifestations were reported in the early 1900's [15, 16], it was not until the 1980s that an understanding of the relationship between the genetic defect and the clinical phenotype began to emerge [17–28]. This chapter introduces the disease component related to the proteins involved in lysosomal cholesterol trafficking as well as covers the cloning and structural discoveries that represent our current understanding of cholesterol movement via NPC1 and NPC2 interplay.

2 Discovery of NPC Disease

The discovery of Niemann-Pick Type C (NPC) disease occurred in the early 1900s by Albert Niemann reporting combined hepatosplenomegaly and central nervous system defects in a young child [15] (also reviewed in [29]). Clinical and pathological evaluation continued in the 1920s when the distinct disease was established by Ludwig Pick [16]. In 1961, Crocker proposed to classify Niemann-Pick Disease into Types A, B, C and D based upon differential clinical and biochemical phenotypes [30]. Further studies have linked Types A and B to mutations in the *SMPD1* gene and sphingomyelinase deficiency, and are now regarded as a distinct disorder [31]. Collectively, today NPC represents the previously termed C and D Types and includes progressive cerebellar neurodegeneration, which manifests in ataxia, seizures, enlarged liver and spleen and other clinical features (as reviewed in [32]). To

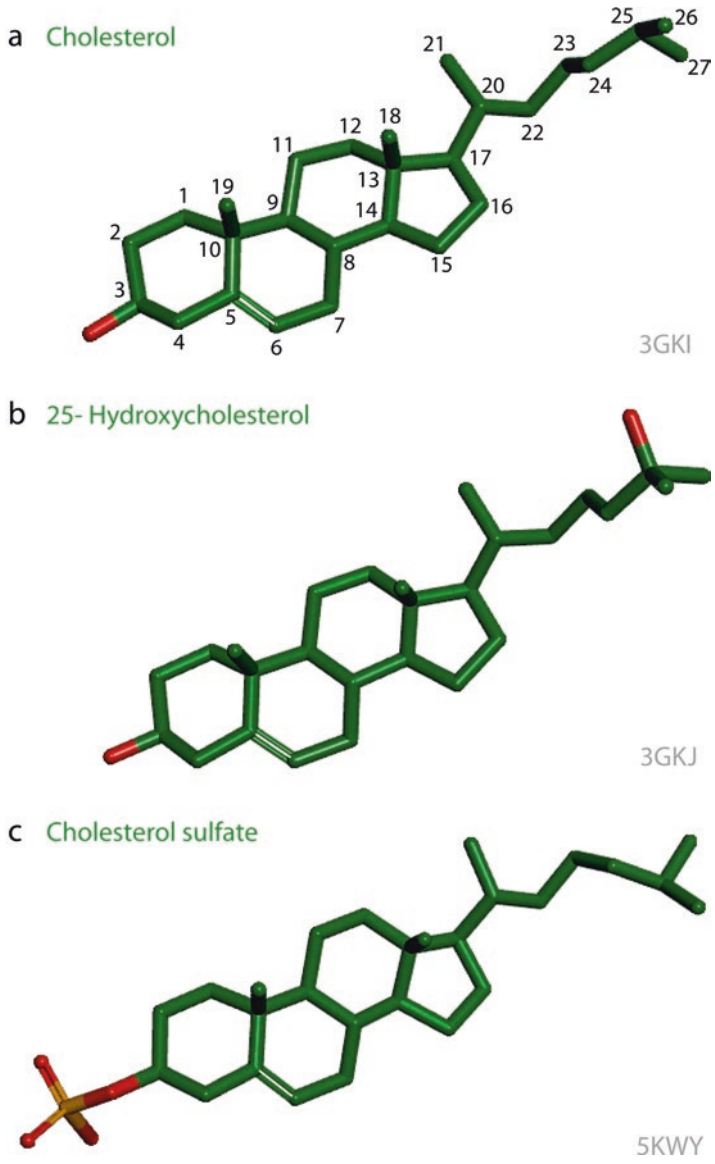

Fig. 1 Structure of cholesterol, 25-hydroxycholesterol and cholesterol sulfate. Stick representation of (**a**) cholesterol based on the cholesterol molecule bound to the N-terminal domain of NPC1 (PDB ID: 3GKI) showing the numbering of the carbon atoms in the molecule, (**b**) 25-hydroxycholesterol based on the 25-hydroxycholesterol molecule bound to the N-terminal domain of NPC1 (PDB ID: 3GKJ), and (**c**) cholesterol sulfate based on the cholesterol sulfate molecule bound to NPC2 (PDB ID: 5KWY)

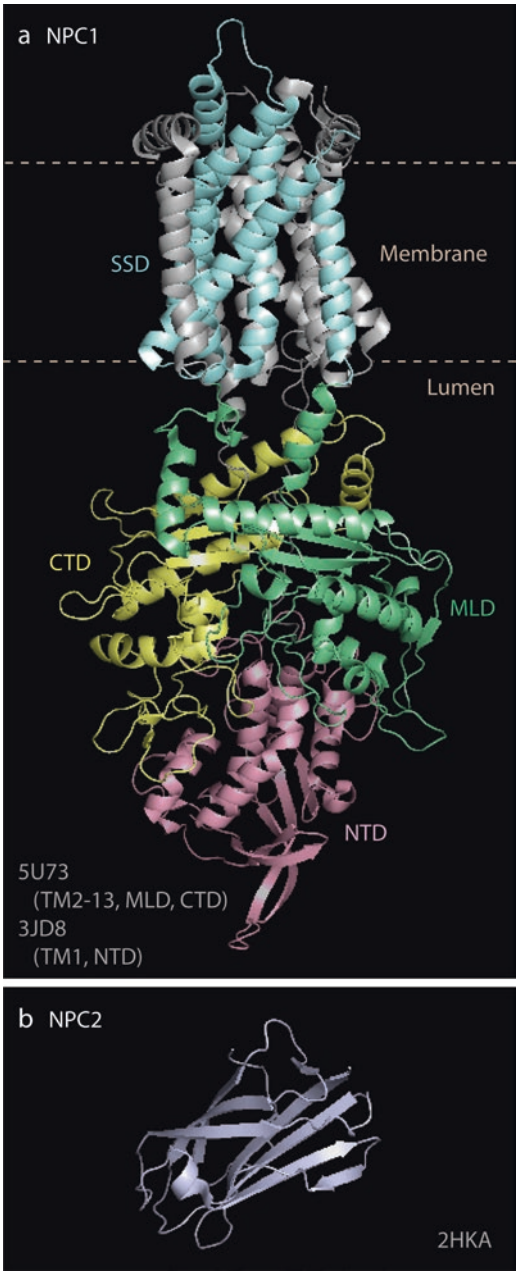

Fig. 2 Structure of NPC1 and NPC2. (**a**) Cartoon representation of NPC1 as constructed by aligning the 2017 Li et al. [81] crystal structure (PDB ID 5U73) and the 2016 Gong et al. [73] cryo EM structure (PDB ID 3JD8). The structure depicted includes TM2-13, the middle luminal domain, and the C-terminal domain based on PDB ID 5U73. TM1 and the N-terminal domain are based on PDB ID 3JD8. Critical domains for cholesterol binding are shown including the sterol sensing domain (SSD) comprised of TM3-7 (in light cyan), the N-terminal domain (NTD) (in pink), the middle luminal domain (MLD) (in green), and the C-terminal domain (CTD) (in yellow). (**b**) Cartoon representation of NPC2 based on the 2007 Xu et al. structure (PDB ID 2HKA)

indicate the genetic cause of the disease, the field has more recently taken forth the notations of NPC1 and NPC2 to represent the genetic causation of NPC disease.

Pioneering work by Roscoe Brady and Peter Pentchev [33, 34] provided biochemical insights into the function of both NPC1 and NPC2. In the spontaneous occurring NPC mouse model, cholesterol storage, lysosomal enzyme activity defects and similarities with the human described disease were observed [18, 35]. Moreover, esterification of cholesterol was defective in this mouse model similarly to the case in NPC patients [17]. Other biochemical features of patient cultured fibroblasts and in the NPC mouse model included alterations of LDL-mediated processes [19, 36, 37], demyelination [38, 39], cerebellar degeneration [40–43], oxidative stress [44, 45], altered calcium homeostasis [46–48] and others [49–52]. Additionally, further studies have demonstrated that many cell types are defective in NPC [53, 54]. Notably, NPC1 and NPC2 mutations lead to similar NPC disease phenotypes [55] suggesting that the two proteins may function either together or sequentially in a common pathway affecting cholesterol transport.

3 NPC2 Gene and Protein Structure

The NPC2 protein, also known as HE1, was reported in 2000 by Peter Lobel's group to be the other gene affected in NPC [28]. NPC2 is a small, ubiquitous, lysosomal protein that is often found in epididymis fluid and was cloned in the late 1990's [56, 57]. In the latter study, the NPC2 protein was shown to bind cholesterol in a porcine model with a 1:1 stoichiometry and micromolar affinity [57]. The crystal structure of the protein at 1.7 Å was reported in 2003 revealing an immunoglobulin-like β-sandwich fold consisting of seven β-strands arranged in two β-sheets forming a loosely packed hydrophobic core (Fig. 2b) [58]. This observation led to the suggestion that the hydrophobic core constitutes an incipient internal cholesterol binding pocket [58]. However, as potential hydrophobic pockets in NPC2 were too small to accommodate cholesterol, it was subsequently proposed that a shift in the two β sheets of NPC2 would be necessary upon cholesterol binding [59]. Specific clues to the location of the putative binding pocket of cholesterol in NPC2 were obtained from mutagenesis studies showing that the F66A, V96F, and Y100A mutations in NPC2 lead to a decrease in cholesterol binding to the protein *in vitro*. When added to NPC2 deficient cells, these mutants were unable to clear elevated cholesterol levels, further supporting the notion that the ability of NPC2 to bind cholesterol is necessary for normal protein function. However, with the discovery that the K32A, D72A, and K75A NPC2 mutants all exhibited normal cholesterol binding, but were unable to correct the cholesterol accumulation phenotype of the cells, it was proposed that cholesterol binding may not be the only requirement for normal NPC2 function [59].

An indication that NPC2 may have a cholesterol transport function emanated from experiments demonstrating that the absolute cholesterol transfer rates from NPC2 to the membrane were orders of magnitude faster than its off-rates from

NPC2 to an aqueous buffer [59]. These experiments also led to the hypothesis that subsequent to binding cholesterol from internal lysosomal membranes, NPC2 interacts with NPC1, thereby facilitating post-lysosomal export of cholesterol [59]. The idea that NPC2 alone was not sufficient for cholesterol egress from lysosomes was further supported by genetic considerations [33, 60]. Additional experiments demonstrated that the rate of transfer of cholesterol from NPC2 to membrane vesicles increased with the frequency of NPC2-membrane electrostatic interactions, particularly in an acidic environment such as that in lysosomes, supporting the proposed role of NPC2 in lysosomal cholesterol transport [61]. Consequently, NPC2 could significantly accelerate the rates of cholesterol transport from and between membranes, as well as the extent of cholesterol transfer. It was shown that transfer of cholesterol occurred rapidly via direct NPC2-membrane interactions via a collisional mechanism, and suggested that NPC2 bound to the membrane surface without penetration into the bilayer hydrophobic core [61]. More recently, in a study carried out in 2015 by the Storch laboratory, it was demonstrated that multiple different mutations in several surface regions of NPC2 exhibited deficient cholesterol transport properties, and were unable to promote egress of accumulated intracellular cholesterol from NPC2 knock out fibroblasts [62]. The point mutations caused changes in the surface charge distribution of NPC2 with minimal conformational changes. Furthermore, complementary molecular modeling showed that NPC2 was highly plastic, with several positively charged regions across the surface that could interact with negatively charged membrane phospholipids. This led the authors to suggest that the plasticity of NPC2 may allow for multiple mechanisms for sterol transfer, and that NPC2 could bind to more than one membrane simultaneously. Consequently, NPC2 may act to traffic cholesterol rapidly at zones of close apposition between membranes such as those that exist in the interior of endo/lysosomes [62].

Structural insights into the molecular basis for sterol binding by NPC2 were obtained in 2007 when NPC2 was co-crystallized with cholesterol sulfate (Fig. 1c) at a resolution of 1.81 Å [63] (Table 1). The sulfate moiety was the only portion of the ligand exposed to solvent, peeking out of the hydrophobic sterol binding pocket.

Table 1 Structures of NPC1 and/or NPC2 in complex with cholesterol or cholesterol derivatives

PDB ID	Protein	Ligand	Release date	Resolution (Å)
2HKA	NPC2	C3S (cholesterol sulfate)	6/26/2007	1.81
3GKI	NPC1 N-terminal domain (NTD)	CLR (cholesterol)	7/14/2009	1.8
3GKJ	NPC1 N-terminal domain (NTD)	HC3 (25-hydroxycholesterol)	7/14/2009	1.6
3JD8	NPC1	CLR (cholesterol)	6/1/2016	4.43
5KWY	NPC1 middle lumenal domain (MLD) bound to NPC2	C3S (cholesterol sulfate)	8/24/2016	2.4

In contrast, the cholesterol iso-octyl tail was shielded from the hydrophilic environment by the interior residues of the NPC2 protein. Comparison between the holo NPC2 structure with a bound cholesterol sulfate and the apo NPC2 structure corroborated that NPC2 had a sterol incipient binding pocket, which was formed from several adjacent small cavities that expanded to accommodate the closely sequestered steroid nucleus of cholesterol sulfate. The structures showed that during this process, the β-strands of NPC2 separated slightly while undergoing substantial side chain reorientation [63].

4 NPC1 Gene Cloning, Structure and Cholesterol Binding

While the pathology of NPC disease as a sterol transport defect was described already in the early 1900s [15, 16], the genetic cause remained unknown until the cloning of the *NPC1* gene in both humans and mice in the late 1990s [27, 64]. Since these reports, and with the implementation of gene sequencing in clinical research, it has become evident that the majority of NPC patients have NPC1 mutations [65]. However, while the structural and functional basis for the consequences of genetic mutations in the NPC2 protein began to unravel in the late 1990's, the structure and role of the NPC1 protein in cholesterol binding and transport remained unknown for several more years.

Initial clues regarding the role of NPC1 started to emerge in 1999 in a mutagenesis study by Watari et al. that suggested that the transmembrane region encompassing helices 3-7, which is thought to form a sterol sensing domain (SSD) (Fig. 2), was required for normal cholesterol egress from the endosome/lysosome system [66]. This concept was further supported by a 2004 study by Ohgami et al., in which a photoactivatable cholesterol analog was implicated in binding to NPC1 with low affinity [67]. The study demonstrated that the SSD was required for NPC1 to bind the cholesterol analog. It was thus suggested that NPC1 may be involved in cholesterol transport at the late endosomal membrane and/or that cholesterol may regulate the activity of NPC1. However, whether and how NPC1 function was linked to NPC2 function remained unclear. While it was proposed in 2001 that NPC1 activity may depend on NPC2 [68], it was found that the interaction between the photoactivatable cholesterol analog and NPC1 did not require NPC2 [67].

In a subsequent study carried out by the Brown and Goldstein labs in 2008, the N-terminal domain (NTD) of NPC1 (Fig. 2a) was implicated as a sterol binding site with a sub-micromolar affinity [69]. Comparison of the differential ability of a variety of oxysterols to bind to NPC1 suggested that in contrast to the orientation of cholesterol when it binds to NPC2, upon binding to NPC1, the hydroxyl group of the cholesterol molecule faces the interior of the NTD of NPC1 whereas the iso-octyl tail is exposed [69]. In 2009, the cholesterol-bound structure of the NTD of NPC1 was solved using X-ray crystallography at a resolution of 1.8 Å confirming the predicted orientation of cholesterol in the NTD binding site [70] (Table 1). As a comparison, the structures of the apo and the 25-hydroxycholesterol (Fig. 1b) bound NTD of NPC1

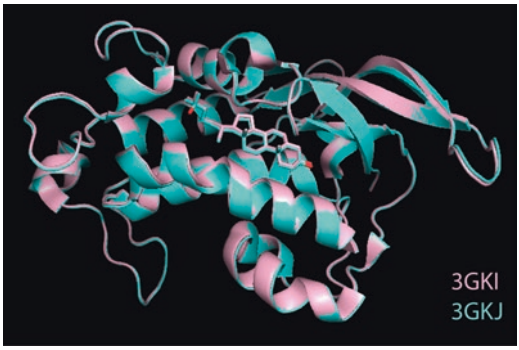

Fig. 3 Cholesterol and 25-hydroxycholesterol share the same binding site in the N-terminal domain of NPC1. Alignment of the structures of the cholesterol- and 25-hydroxycholesterol-bound N-terminal domain of NPC1 in the 2009 Kwon et al. [70] structures (PDB IDs 3GKI and 3GKJ, respectively). The protein is depicted in ribbon representation, and the sterols in stick representation

were also determined. Cholesterol and 25-hydroxycholesterol bind in a similar manner to the NTD of NPC1, interacting with the same protein residues (Fig. 3). The 25-hydroxyl group on the 25-hydroxycholesterol molecule formed a water-mediated interaction with the main chain of L175. The sterol binding pocket was lined primarily with hydrophobic residues including W27, L83, F108, P202, F203, and I205. Two polar residues, N41 and Q79 formed hydrogen bonding with the cholesterol hydroxyl group. E30 formed a water-mediated interaction with the hydroxyl group of the cholesterol molecule, thereby stabilizing the interaction between the NTD of NPC1 and cholesterol, and imposing stereospecificity. When added to NPC1-deficient cells, alanine mutants of these residues failed to restore function. At each end of the sterol-binding pocket was an opening toward the surrounding solvent. One opening was located near the cholesterol hydroxyl group, and was large enough for a single water molecule to enter or exit. In contrast, the second opening was located at the end of the cholesterol iso-octyl side chain, and was not large enough to permit passage of the tetracyclic ring without a conformational change indicating that it would need to expand to facilitate cholesterol entry [70].

The growing structural and functional insights into the binding of cholesterol to the NTD of the NPC1 protein, along with the realization that the orientation of cholesterol binding to this NPC1 domain was opposite to the way that cholesterol binds to NPC2, supported the notion that there is cooperation among NPC1 and NPC2 in cholesterol transport. Accordingly, reversal of the orientation of cholesterol during its transfer from NPC2 to NPC1 would allow its iso-octyl hydrophobic tail to lead the way into the outer lysosomal membrane [70].

In a parallel line of research, in vitro work raised the possibility that the NTD may not be the only cholesterol binding site in NPC1, and that NPC1 could possess a second binding site, possibly at the SSD [71]. NPC1 with alanine point mutations of L175/L176, D180/D182, N185, T187/N188, E191/Y192, and G199/Q200 in a helical subdomain of the SSD consisting of helices 7, 8 and the intervening loop could not restore cholesterol exit from lysosomes in NPC1-deficient cells. In line

with these results, a later study that explored the binding of oxysterol derivatives to NPC1 demonstrated that they bound directly and selectively to a low-affinity or transient non-NTD sterol binding site [72]. In 2016, two structures that included the SSD domains were utilized to further explore the possibility of a second sterol binding site in the SSD [73, 74]. The first was a cryo-EM structure of the full length NPC1 that was obtained at 4.4 Å resolution [73] (Table 1). The structure showed that the five SSD-forming membrane helices (3–7) were exposed to the lipid bilayer suggesting that the SSD was available for potential interactions with membrane-embedded sterols. The second structure was a crystallographic structure obtained at 3.6 Å resolution, and included 12 of the 13 transmembrane domains of NPC1 [74]. In this structure, the NPC1 SSD formed a cavity that was accessible from both the luminal bilayer leaflet and the endosomal lumen. Complementary computational modeling suggested that this cavity was large enough to accommodate one cholesterol molecule, further supporting the notion that the SSD may harbor a second cholesterol binding site [74]. Combining the accumulating evidence that NPC2, the NTD of NPC1, and the SSD of NPC1 all possessed a sterol binding site paved the way to a comprehensive model of the molecular mechanism of cholesterol transport by NPC1 and NPC2 in the lysosomes involving cholesterol derived LDL uptake in the lysosome, followed by binding to NPC2 which would then 'handoff' cholesterol to NPC1 for recycling out of the lysosome.

5 Development of a Cholesterol Transport Mechanism Model from NPC2 to NPC1

In 2008, the Brown and Goldstein laboratories demonstrated that cholesterol can transfer between the NTD of NPC1 and NPC2 in a bidirectional fashion facilitated by NPC2 [75]. While the transfer of cholesterol to and from NPC2 was rapid, the transfer of cholesterol to and from the NTD of NPC1 was very slow. The latter, however, was significantly accelerated in the presence of NPC2, supporting the notion that the two proteins act together to facilitate cholesterol egress from lysosomes [75].

Then, subsequent to determining the structure of the cholesterol-bound NTD of NPC1 in 2009 [70], the groups of Brown and Goldstein further evaluated in 2010 the cholesterol 'handoff' mechanism between NPC2 and the NTD of NPC1, and showed that in the presence of mutations of surface residues such as V81 of NPC2 and L175/L176 of the NTD of NPC1, cholesterol binding could occur but 'handoff' did not [76]. This raised the possibility that these 'transfer mutants' that clustered in surface patches of NPC2 and the NTD of NPC1 interacted with each other to facilitate the opening of the binding pocket in the NTD of NPC1, thereby allowing cholesterol to transfer between the two proteins. Further support of the notion that NPC2 and NPC1 interacted with each other came in 2011 from the Pfeffer laboratory who showed that NPC2 directly interacted with the NPC1 middle luminal domain (MLD) (Fig. 2a) in an acidic environment of pH 5.5 with a low micromolar affinity and a cholesterol dependent binding strength that increased when NPC2

was carrying a cholesterol molecule [77]. In alignment with these observations, the disease causing mutations R404Q and R518Q in the NPC1 MLD interfered with the ability of NPC1 to bind to NPC2. These results stimulated the idea that the NPC1 MLD may bring NPC2 into close proximity with the NPC1 NTD to facilitate the transfer of cholesterol between NPC2 and the NTD of NPC1. Once transferred, the loss of cholesterol from NPC2 would trigger its release from the MLD following a reduction in the binding strength between the two proteins. The NPC1 NTD-bound cholesterol would then be transferred to the lysosomal membrane bilayer [77].

In 2013, the Wiest group carried out computational studies of the NPC2-NTD(NPC1) system to gain further mechanistic insights into cholesterol binding and transfer between the two proteins (Fig. 4) [78] based on working models from the Brown and Goldstein laboratories [75]. The results of the simulations suggested that when bound to NPC2, the cholesterol hydroxyl group formed multiple interactions with NPC1 residues, thereby stabilizing the interaction between the NTD of NPC1 and NPC2. The simulations also suggested that a large reorganization occurred in the binding pocket of the NPC1 NTD upon cholesterol binding. These results inspired the 'sliding model' that enhanced the mechanistic description of the 'handoff model'. According to this model, cholesterol binding to NPC2 would lead to an increase in the association constant for the formation of the complex between NPC2 and the NTD of NPC1. Once the NPC1-NPC2 complex was formed, cholesterol would transfer from NPC2 to the NTD of NPC1. This transfer would proceed through the displacement of multiple NTD helices, followed by the actual transfer of cholesterol through the opened pathway. The transfer of the cholesterol molecule from the NPC2 protein would then lead to a decrease in the association constant of

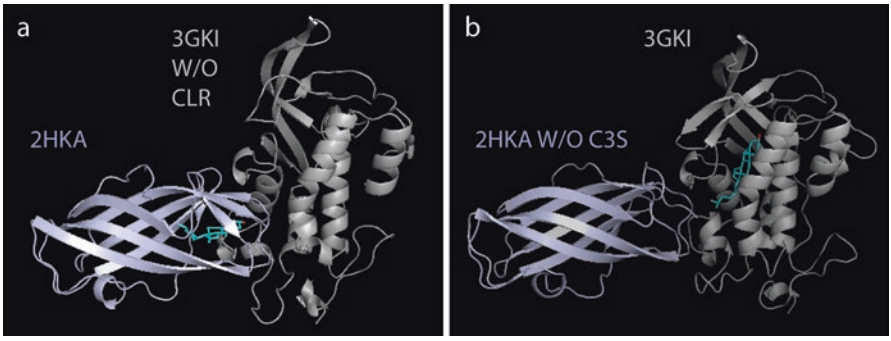

Fig. 4 Models of the putative interactions between the N-terminal domain of NPC1 and NPC2, and the cholesterol binding sites in each protein. (**a**) Ribbon representation of the final structure of the 2013 Wiest et al. [78] simulation of the docked structures of NPC2 (PDB ID 2HKA) and the N-terminal domain of NPC1 (PDB ID 3GKI) with a stick representation of cholesterol bound to NPC2 based on a 2008 working model by Infante et al [75]. Based on bi4005478_si_005.pdb from the Supplementary information of [78]. (**b**) Ribbon representation of the final structure of the 2013 Wiest et al. [78] simulation of the docked structures of NPC2 (PDB ID 2HKA) and the N-terminal domain of NPC1 (PDB ID 3GKI) with a stick representation of cholesterol bound to the N-terminal domain of NPC1 based on a 2008 working model by Infante et al [74]. Based on bi4005478_si_003.pdb from the Supplementary information of [78]

the protein-protein complex, facilitating the dissociation of the complex, and the transport of the cholesterol molecule by NPC1 through the membrane [78].

A follow-up simulation study carried out by Elghobashi-Meinhardt in 2014 explored potential isomerization of the C17–C20–C22–C23 dihedral angle in the tail of the cholesterol molecule (Fig. 1a) during its transfer from NPC2 to the NTD of NPC1 [79]. The cholesterol molecule sampled different geometries inside the binding pockets of the NPC1–NPC2 complex during the simulations [78, 79]. In the final structures of the simulations carried out by the Wiest group [78], the C17–C20–C22–C23 dihedral angle was 71.6° for cholesterol in NPC2 but −157.3° in the NTD(NPC1) binding pocket. Notably, in the respective crystal structures of NPC2 and NTD(NPC1) in complex with cholesterol or a cholesterol derivative, the value of the C17–C20–C22–C23 dihedral angle was nearly identical ranging from −162.2° to −164.5° (NPC2 in complex with cholesterol sulfate (PDB ID 2HKA): −164.5°; NPC1 NTD in complex with cholesterol or 25-hydroxycholesterol (PDB IDs 3GKI, 3GKJ): −163.9° and −162.2°, respectively). Further simulations suggested that cholesterol may isomerize in the NPC2 pocket either before or after docking to the NTD of NPC1 to ensure an efficient transfer. By calculating the energy barrier for rotation of the C17–C20–C22–C23 dihedral angle during the sliding of cholesterol from NPC2 to NTD(NPC1), the likely 'reaction pathway' for cholesterol transfer was predicted. The energy barrier along that path was ~22 kcal/mol in total [79]. The primary contribution to the energy barrier was attributed to the distorted geometry of the tail of the cholesterol molecule within the constrained binding pocket in the NTD of NPC1. This energy barrier was in agreement with semi quantitative experimental kinetic rates corresponding to half-lives of up to ~100 sec at 37 °C [79]. Further studies are required to corroborate experimentally the possibility that cholesterol undergoes isomerization of its tail to facilitate its transfer between NPC2 and NPC1.

In addition to providing insights into the structure of the SSD of NPC1, the 2016 cryo-EM structure of the full length NPC1 determined by Gong et al. and discussed above provided insights into the mechanism of cholesterol transfer from NPC2 to NPC1 delineating the structural relationship between the NTD and the MLD of NPC1 [73]. In this structure, a number of polar and charged residues in the NTD (e.g. Q88, Q92, R96) and MLD (e.g. R518) appeared to form an interface between these two luminal domains. Notably, the single point disease mutations R518W or R518Q in the MLD led to reduced interaction between an isolated MLD construct and NPC2. Similarly, the NPC1 mutants L175A/L176A and Q88A/Q92A/R96A showed decreased binding to NPC2 at pH 6.0. While interaction between NPC2 and an isolated NPC1 NTD was not detected, deletion of the NTD resulted in reduced binding between NPC1 and NPC2. The proximity between L175/L176 and the interface residues suggested that the NTD and the MLD of NPC1 may together constitute a docking site for orienting NPC2, thereby facilitating the transfer of cholesterol to the pocket of NTD [73].

This role of the involvement of the MLD of NPC1 was further supported by a crystal structure of the complex of the MLD of NPC1 and NPC2 with a bound cholesterol sulfate molecule that was determined by the Pfeffer laboratory at 2.4 Å resolu-

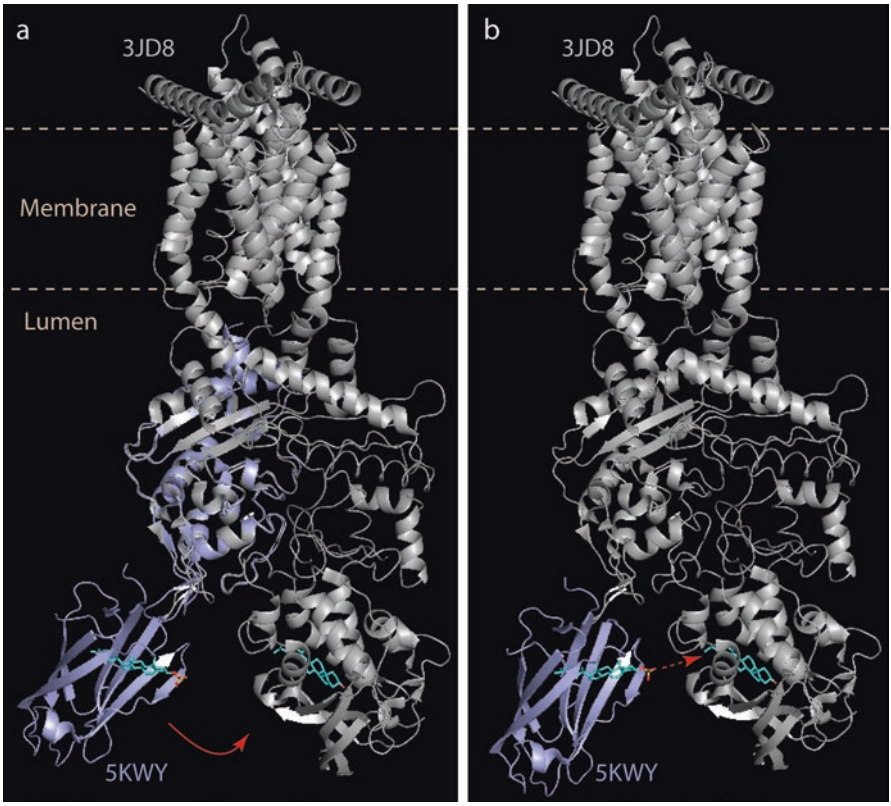

Fig. 5 Model of the putative cholesterol transfer from NPC2 to the N-terminal domain of NPC1. Ribbon representations of the NPC1-NPC2 complex obtained by aligning the structure of the middle luminal domain of NPC1 from the 2016 Li et al. [80] MLD(NPC1)-NPC2 complex (PDB ID 5KWY) with the 2016 Gong et al. [73] cryo-EM structure (PDB ID 3JD8) of NPC1. The MLD(NPC1)-NPC2 complex is in violet, and the NPC1 cryo-EM structure is in gray. The sterol molecules are shown in stick representation (cyan). The alignment in (**a**) is based on the middle luminal domain, and the alignment in (**b**) is based on the two loops of the middle luminal domain that interact with NPC2. By aligning the structures based on these loops, NPC2 approaches closer to the NPC1 N-terminal domain to form a sterol transfer tunnel leading from NPC2 to NPC1. For clarity, the middle luminal domain from the MLD(NPC1)-NPC2 complex was removed in (**b**)

tion in the same year [80] (Table 1). The complexed structure revealed that the MLD of NPC1 binds the top of the NPC2 sterol-binding pocket. Aligning the MLD(NPC1)-NPC2 complex onto the full-length NPC1 cryo-EM structure by aligning the MLDs in the two structures suggested a spatial proximity between NPC2 and the NTD of NPC1 (Fig. 5). The distance between NPC2 and the NTD of NPC1 was further decreased and formed a cholesterol-transfer tunnel when the alignment was done based on the two protruding loops of the MLD of NPC1 that comprised its principal binding interface with NPC2 (Fig. 5) [80]. This interface involved interactions between both polar and hydrophobic residues. For example, interactions were formed between Q421 in the MLD of NPC1 and Q146 of NPC2, and between Y423 of the MLD of NPC1 and M79 of NPC2. Hydrophobic interactions involving F503 and

F504 of the MLD of NPC1 and NPC2 were also observed. Alanine mutations of the equivalent two residues in a murine model significantly reduced the binding affinity between NPC1 MLD and NPC2. Further reduction in binding affinity was observed following mutation of polar MLD residues (e.g. the equivalent residue to Q421) in addition to the above aromatic ones. Notably, a significant rearrangement of the side-chains of NPC2 residues (e.g. K25, M79, K123, and Q146) that contributed substantially to the interface between the MLD of NPC1 and NPC2 was observed in the cholesterol sulfate bound NPC2 structure compared to the apo structure of NPC2. The orientations of the sterol molecules in the binding pockets in NPC2 and in the NTD of NPC1 were compatible with molecular transfer supporting the 'hydrophobic handoff' transfer model between NPC2 and NPC1, and led to the proposal that NPC2 binding to NPC1 may trigger a conformational change(s) that would reorient the NTD of NPC1 into a more planar configuration in relation to NPC2 to accomplish actual cholesterol transfer, as previously modeled [76]. It is important to note, however, that the orientation of the NTD of NPC1 relative to NPC2 in this model [80] somewhat differed from prior computational models (Fig. 4) [78], indicating that further studies are required to establish the precise manner by which NPC2 interacts with the NTD of NPC1 to facilitate cholesterol transfer between the two proteins. With that in mind, the picture emerging from this study was that subsequent to binding cholesterol, NPC2 underwent a subtle conformational change that enhanced its binding to the MLD of NPC1, which in turn led to interactions of NPC2 with the NTD of NPC1. These interactions then slightly impacted the structure and orientation of the MLD protruding loops to orient the NPC2 pocket directly adjacent to the cholesterol binding pocket in the NTD of NPC1 forming a cholesterol transfer tunnel. After the transfer of the cholesterol molecule, the prominent NPC2 residues located at the interface with the MLD of NPC1 would revert to their apo NPC2 conformation, thereby triggering the release of NPC2.

Further insights into the emerging mechanism of lysosomal cholesterol transport was provided by the crystal structure of NPC1 released by the Blobel laboratory, also in 2016 (see above) [74]. This structure included 12 of the 13 transmembrane domains of the protein and extended the proposed model for NPC1 function in cholesterol sensing and transport to include the SSD. According to this extended model, cholesterol would bind first to NPC2, which would then dock to the NTD of NPC1, permitting cholesterol transfer between their binding pockets. The link between the NTD and the remainder of NPC1 would permit the NTD to reorient in such a way that would allow cholesterol transfer to the transmembrane domain. Luminal entry would then be used to transfer cholesterol from its binding site in the NTD of NPC1 that could then exit via the membrane pocket's lateral opening. This lateral opening in the membrane could also provide access for free cholesterol from the lipid bilayer (Fig. 6). It was further proposed that the access properties of the putative binding pocket in the SSD of NPC1 may enable monitoring of the concentration of cholesterol in the lysosome lipid bilayer [74]. While this and prior studies support a mechanism of 'pocket brigade' in which cholesterol would transfer from one pocket to another (NPC2 to the NTD of NPC1 to the SSD of NPC1), the subsequent step involving the transfer of cholesterol to the cytoplasmic leaflet of the endosomal membrane remains completely elusive.

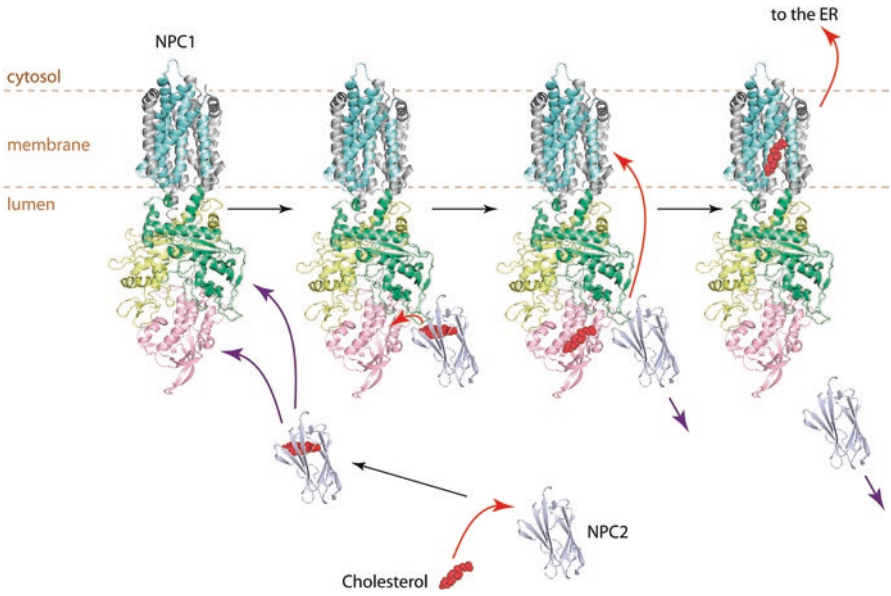

Fig. 6 Putative steps in the mechanism of NPC1/NPC2 mediated cholesterol egress from lysosomes. A combined picture of models proposed to describe the binding of cholesterol to NPC2 followed by its transfer to NPC1, and subsequently its egress from the lysosome via a yet to be determined mechanism. Conformational changes that occur during the process are not depicted. Also, based on current information, it is unclear whether NPC2 remains bound to NPC1 when cholesterol is transferred to the NPC1 sterol sensing domain, and subsequently, when cholesterol is exported from the lysosome. The model depicts one of the possible scenarios that need to be tested. Black arrows show the sequence of events, red arrows refer to the movement of cholesterol, and purple arrows, to the movement of NPC2. NPC1 and NPC2 are depicted in ribbon representation using the same color scheme as the one used in Fig. 2. Cholesterol is depicted in ball representation in red

In 2017, a crystal structure of NPC1 determined by the Blobel laboratory at 3.3 Å resolution added another player to the picture, the C-terminal luminal domain (CTD) of NPC1 (Fig. 2a) [81]. NPC1 possesses three distinct luminal domains. However, in the absence of a high resolution structure that included the CTD, significant effort has been previously put primarily into determining the roles of the NTD and MLD in the cholesterol transfer from NPC2 to NPC1. Yet, while this crystal structure offered increased resolution of the CTD of NPC1, it lacked the NTD and the TM1 helix of NPC1. Thus, to obtain a more complete structure of NPC1, the crystal structure [81] was aligned to the full-length 2016 Cryo-EM structure [73] that included the missing domains (Fig. 2a). Using the aligned the structures of NPC1, the interactions of the CTD with the NTD were delineated revealing an interaction surface larger than the interaction surface between the NTD and the MLD. These predominantly involved hydrophobic interactions (G910, G911, M912, and G913 of the CTD and V234, T235, and A236 of the NTD) between two distinct loops. In addition, a secondary interface between the CTD and NTD was found to involve primarily polar residues (Q60, E233 of the NTD interacting with L982, Q988 of the CTD, respectively). Furthermore, disruption of the interface between the NTD and CTD of NPC1 via mutagenesis dis-

rupted the transfer of cholesterol from the late endosome to the ER. It was thus suggested that the CTD-NTD interaction may play a critical role in orienting the NTD of NPC1 to facilitate the transfer of cholesterol from NPC2, as well as in modulating the interaction between the NTD and the SSD that would facilitate cholesterol export from the late endosomes [81].

6 Recently Reported Cholesterol Transporters

In addition to NPC1 and NPC2, a number of proteins have been shown or implicated in cholesterol transport [82]. Recent reviews have covered topics ranging from lipoprotein involvement, ABC lipid transporters [83, 84], steroidogenic acute regulatory domain (StARD) and StAR-related lipid transport (START)-domain proteins among others [85–87]. Below are examples of recent findings of proteins involved in cellular cholesterol transport.

1. Sandhu and colleagues reported earlier this year on the involvement of endoplasmic reticulum (ER) proteins termed 'Aster' proteins in cholesterol transport [88] The Aster-A, B and C proteins are structurally similar to StARD and START-domain containing proteins that facilitate cholesterol transport from the plasma membrane to the ER in a non-vesicular mediated process that requires a 'bridge-like' structure to mediate the cholesterol transfer.
2. The protein Patched1 homolog 1 (PTCH1) receptor serves to bind Hedgehog (Hh) proteins and downstream signal transduction including activation of Smoothened (SMO). The regulation of SMO has for some time been known to require cholesterol [89–95]. Most recently, a cryo-EM study provided evidence that PTCH1 has an NPC1-like structural topology, acts as a cholesterol transporter, and alters inner leaflet cholesterol in cells [96]. The latter is reversed by Hedgehog stimulation, suggesting that PTCH1 regulates Smoothened by controlling cholesterol availability [96]. Further structural evidence of PTCH1 and NPC1 similarities was also reported by Qi et al. [97].

While many proteins have been predicted or shown to bind cholesterol, direct evidence of cholesterol transport remains challenging and many transport mechanisms are based on indirect evidence. Nonetheless, combined structural, in vitro and genetic investigations have provided our current understanding of cholesterol transport in the cell.

7 Concluding Remarks and Outlook

Since the observation in 1996 that mutations in NPC1 and NPC2 result in similar NPC disease phenotypes, and may thereby function in a common pathway affecting cholesterol transport [26], the molecular mechanism underlying this process has been gradually unraveling via biochemical, structural and computational efforts (summarized in Fig. 7). Subsequent to the identification of sterol binding sites in the

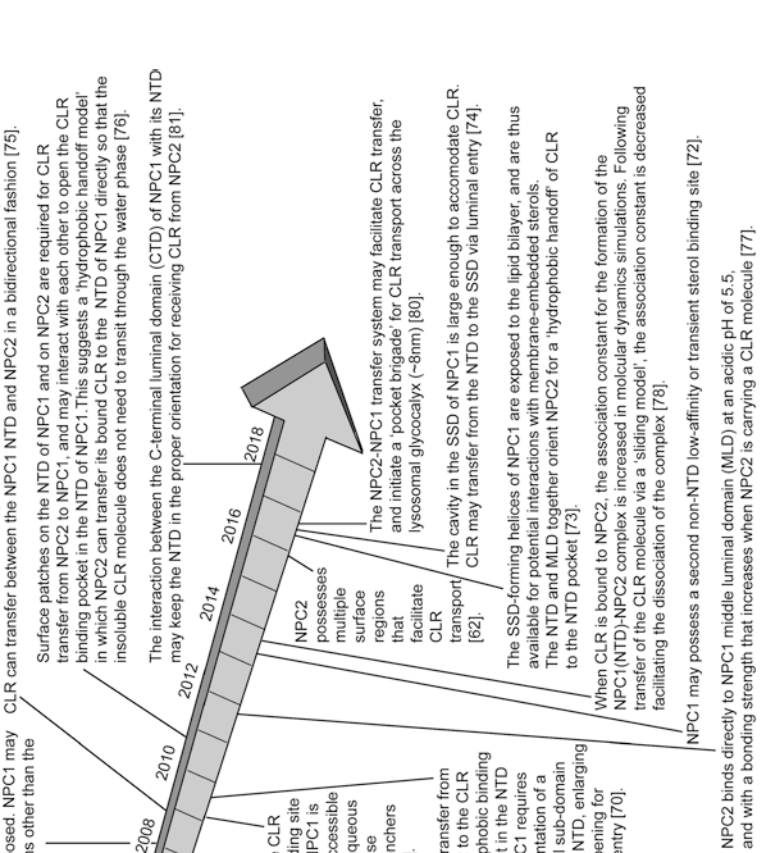

Fig. 7 Developments in the discovery of the molecular mechanism of cholesterol transfer from NPC2 and NPC1. Highlights of major discoveries involving the binding of cholesterol to NPC2 and NPC1, and the proposed mechanisms underlying the transfer of cholesterol from NPC2 and NPC1 during the egress process of cholesterol from lysosomes. In this figure, cholesterol is represented by the three letter abbreviation CLR

two individual proteins, clues have begun to emerge regarding the potential mechanism of cholesterol transfer from NPC2 to NPC1, and the involvement of the different NPC1 domains in this process (summarized in Fig. 6). Notably, the proposed interaction between NPC1 and NPC2 that forms to facilitate cholesterol egress from lysosomes may underlie the common disease phenotype traced to mutations in the two proteins.

However, while it is well accepted that NPC2 and NPC1 play important roles in cholesterol trafficking, several parts of the puzzle are still missing. For example, does cholesterol undergo a conformational change when it is transferred from NPC2 to NPC1? How is NPC2 oriented with respect to the NPC1 luminal domains before and after transferring a cholesterol molecule to NPC1? If cholesterol is transferred from the NTD of NPC1 to the SSD, how does this occur? Is NPC2 released before the cholesterol molecule transfers to the SSD or before it is exported? Most importantly, how cholesterol is exported from NPC1 remains to be shown.

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