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A sterol sensing domain-containing family of proteins

Rodina proteinů obsahujících sterol sensing doménu

Bachelor´s thesis

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Poděkování:

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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1. Abstract

Sterol sensing domain (SSD) is a putative transmembrane region consisting of 5 helices and in few cases it was shown that it interacts with cholesterol. Proteins containing this domain play a role in many cellular pathways connected to cholesterol. It was first described in a study of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase). It is an enzyme playing role in cholesterol biosynthesis and its transcription is regulated by sterol regulatory element binding protein cleavage activating protein (SCAP). HMG-CoA reductase and SCAP were the first proteins where SSD was recognized. After that, other proteins were described; one more protein functioning in cholesterol biosynthesis was described as containing SSD – 7-dehydrocholesterol reductase. Also two proteins playing role in cholesterol endocytosis – Niemann-Pick type C 1 liked 1 – and in its efflux from lysosomes – Niemann-Pick type C 1 – were recognized. Furthermore, proteins playing role in Hedgehog signalling Patched, Patched-related and Dispatched were found as SSD containing structures. In the end, tumour-suppressor gene TRC8 was also recognized as member of SSD family. In this text, structure and function of these proteins is summarized.

Key words: sterol sensing domain, Hedgehog signalling pathway, sterol binding protein, cholesterol synthesis, cholesterol trafficking

Abstrakt

Sterol sensing doména (SSD) je transmembránový úsek proteinu skládající se z 5 helixů. Proteiny, které tuto doménu obsahují, jsou součástí mnoha buněčných drah souvisejících s metabolismem cholesterolu. V některých případech bylo ukázáno, že SSD interaguje s molekulou cholesterolu. Poprvé byla popsána během studia 3-hydroxy-3-metylglutaryl-CoA reduktázy (HMG-CoA reduktáza), enzymu účastnícího se biosyntézy cholesterolu. Jeho transkripce je regulována proteinem Sterol regulatory element binding protein Cleavage Activating Protein (SCAP). 3HMG-CoA reduktáza a SCAP byly prvními proteiny, v jejichž struktuře byla SSD rozeznána. Později byl popsán jiný protein s touto doménou, který je také součástí biosyntézy cholesterolu – 7-dehydrocholesterol reduktáza. Další proteiny s identifikovanou SSD, hrají roli v endocytóze cholesterolu a jeho transportu z lysozomů – Niemann-Pick typ C 1 liked 1 a Niemann-Pick typ C 1. Mimoto, jako struktury obsahující SSD byly také popsány proteiny působící v Hedgehogové signalizaci – Patched, Patched-related a Dispatched. Konečně, protein působící jako tumor-supresor TRC8 byl do této rodiny proteinů zařazen také. V této práci je popsána struktura a funkce jednotlivých členů proteinové rodiny obsahující sterol sensing doménu.

Klíčová slova: sterol sensing doména, signální dráha Hedgehog, proteiny vázající sterol, syntéza cholesterolu, transport cholesterolu

Abbreviations

SSD	Sterol sensing domain
CoA	Coenzyme A
NPC1	Niemann-Pick type C 1
NPC2	Niemann-Pick type C 2
TMD	Transmembrane domain
GFP	Green fluorecsent protein
YFP	Yellow fluorescent protein
NTD	N-terminal domain
NPC1L1	Niemann-Pick type C 1 liked 1
FISH	Fluorescence *in situ* hybridization
SRE	Sterol regulatory element
SREBP	SRE-binding protein
SCAP	SREBP-cleavage activating protein
INSIG	Insulin induced gene
25-HC	25-hydroxycholesterol
[^{3}H]-AC	[^{3}H]-7,7-azocholestanol
HMG-CoA reductase	3-hydroxy-3-methylglutaryl-CoA reductase
SLOS	Smith-Leimli-Opitz syndrome
7-DHCR	7-dehydrocholesterol reductase
HH	Hedgehoh
PTC	Patched

SMO	Smoothened
WG	Wingless
WNT	Wingless/Int 1
SHH	Sonic Hedgehog
DHH	Desert Hedgehog
IHH	Indian Hedgehog
IHOG	Interference of Hedgehog
BOI	Brother of IHOG
CDO	CAM-related/downregulated by oncogenes
BOC	Brother of CDO
CI	Cubitus interruptus
GLI protein	Glioblastoma protein
HSC	Hedgehog signalling complex
PTR	Patched-related
HH-N	N-terminal part of HH
TRC8	Translocation in renal carcinoma, chromosome 8

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2. Introduction

Cholesterol plays an important role in eukaryotic cells – it is one of the membrane building blocks and a precursor of many isoprenoids, hormones, lipoproteins, bile acids etc. Cholesterol can be synthesized through series of enzymatic reactions from acetyl-CoA and can also be absorbed by receptor mediated endocytosis.

During studies of cholesterol metabolism, a particular domain has been rrevealed in many proteins (the most important players are summarized in Fig. 1). This domain was later designated as sterol sensing domain (SSD). It was first described when sequences of two key cholesterol biosynthesis regulators – SCAP and HMG-CoA reductase – were compared. It was shown that this domain, which consists of 5 putative transmembrane helices containing approximately 180 amino acids, is crucial for proper function of these proteins. Later, many new proteins were recognized as members of the SSD protein family; however, the role of SSD in their function still remains unclear.

This family includes NPC1 and NPC1L1 proteins playing role in cholesterol uptake, SCAP, HMG-CoA reductase and 7-DHCR proteins acting in cholesterol synthesis/regulation. Patched, Patched-related and Dispatched, which are proteins functioning in HH signalling. Moreover, tumour-suppressor protein TRC8 was recognized as member of SSD protein family.

Main objective of this work is to summarize new information about these proteins and describe their structure and function in eukaryotic cells and organisms and also their main interactions with other molecules. Some of the disorders caused by mutations in genes of these proteins are also briefly described in following text. I was interested in this issue, because I have already got in touch with Disp3 and I would like to continue in studying this protein during my master studies.

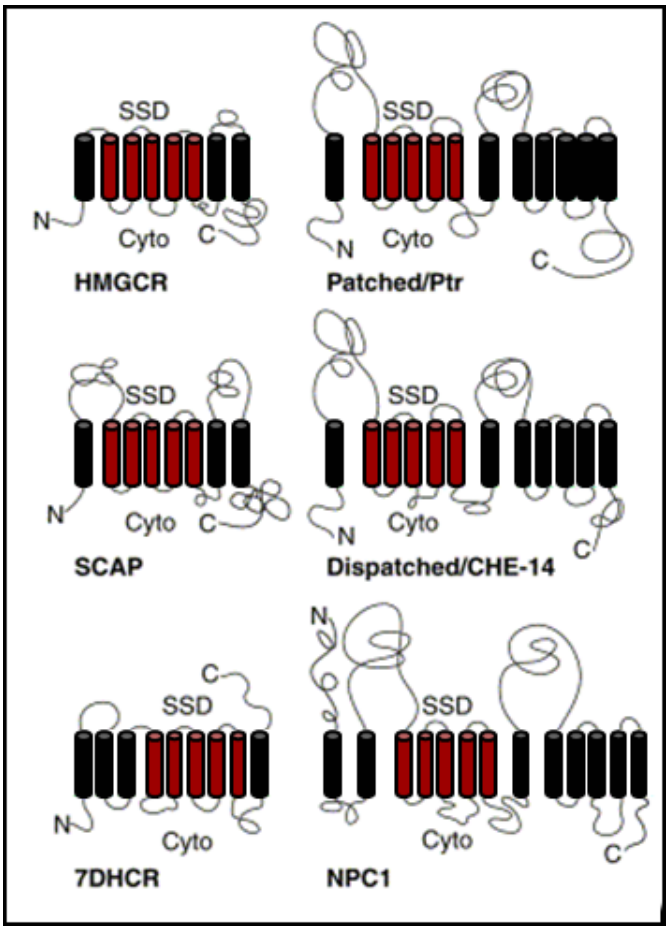

Figure 1. Schema of structures of main SSD family members (reviewed in Kuwabara et al. 2002). In the picture, TMD are labelled by black colour, while for SSD it is used red colour. Orientation of proteins is marked, Cyto means orientation to cytosol.

3. NPC1 protein

3.1 Niemann – Pick type C disease

Niemann-Pick type C disease is a genetic disorder caused by loss-of-function mutations in Npc1 (95% cases) and in Npc2 (remaining 5% cases) genes. Niemann-Pick type C disease is autosomal recessive neurovisceral fatal disorder, which has many phenotypes including hepatic, neural and psychiatric problems. Patients often suffer from hepatosplenomegaly and typically also from cerebellar ataxia, dysarthria or progressive dementia and they usually die before the age of 20 (reviewed in Vanier et al. 2003). At the cellular level, it causes lipid storage disorder by affecting efflux of cholesterol from lysosomes, so that unesterified cholesterol accumulates in late endosome/lysosome system. In the case of Npc mutations, specialized cellular compartment called lysosomal storage organelles (LSO) are formed, in

which steroids and various sphingolipids are stored together with bis(monoacylglycerol)phosphate. Cholesterol is the main component of these organelles in peripheral tissue; in neural tissue, on the other hand, gangliosidoses are prominent (reviewed in Rosenbaum et al. 2011).

3.2 NPC1 protein structure

Mutation in Npc1 gene was first identified as the cause of Niemann–Pick type C disease by Carstea et al. (1997), while the less abundant cause of this disease mutation in Npc2 was described later. NPC1 is a protein which contains 1278 aminoacids and its molecular weight is 142 kDa. It forms 13 transmembrane domains (TMD) and three luminal loops – luminal loop 1, 2 and 3 as predicted from amino acid sequence. First 13 N–terminal amino acids target protein to the endoplasmic reticulum. At the C–terminus, there is a di-leucine motif containing LLNF amino acids which was described as targeting sequence to lysosomes in another lysosomal transmembrane protein – LIMPII (Tabuchi et al. 2002).

By comparison of protein sequence of NPC1 and its orthologs in *Caenorhabditis elegans, Saccharomyces cerevisiae* and in mouse, a highly conserved area at the N–terminus has been discovered. It is in the first luminal loop between amino acids 55 to 165. Authors suggest this domain could be crucial for protein function in efflux of cholesterol. Moreover, leucine zipper motif was discovered within the protein structure, which is known to play a role in multimerization of some transcription factors (Zhang et al. 2013). This motif lies between amino acids 73 to 94 and the authors came up with an idea, that this might be the domain responsible for eventual cooperation with NPC2.

Comparison of NPC1 with other proteins playing role in cholesterol homeostasis such as SCAP or HMG-CoA reductase has been made. These proteins contain SSD domain, which is important for the interaction with cholesterol. By sequencing of Npc1, putative SSD region consisting of 5 transmembrane helices was recognized between amino acids 615 to 797 (Carstea et al. 1997). Whether NPC1 binds cholesterol and if yes, which domain is responsible for cholesterol binding, was the main subject of following research.

3.3 Sterol binding analysis

It was shown that Niemann–Pick type C phenotype is caused by a mutation in Npc1, specifically a frameshift mutation at codon 1205, which leads to premature termination of the protein. Another mutation causing this phenotype is a deletion resulting in termination at codon 632; however there are more mutations leading to abolished efflux of cholesterol from late endosomes/lysosomes (Carstea et al. 1997).

To solve whether NPC1 binds cholesterol Ohgami et al. (2004) performed an experiment with [^{3}H] -7,7-azocholestanol ([^{3}H]-AC), analogue of cholesterol with photoactivity and GFP– (or YFP-) tagged NPC1. After immunoprecipitation it was shown, that [^{3}H]-AC coimmunoprecipitates with a band of the same molecular weight as GFP-tagged NPC1. Moreover, [^{3}H]-AC did not coimmunoprecipitate with GFP alone or VAMP7 (control lysosomal protein with one TMD). Next step was to measure the difference in binding of [^{3}H]-AC to WT and mutated forms of NPC1. It was observed that mutations within SSD (P692S and Y635C) diminished binding. Finally, they concluded SSD plays a role in cholesterol binding to NPC1. Whether NPC2 acts in this process as well was the next subject of their research (Ohgami et al. 2004). NPC2 is lysosomal soluble protein, which was shown to bind cholesterol *in vitro* (Ko et al. 2003). Ohgami discovered NPC2 binds cholesterol also *in vivo*, although, he confirmed that NPC2 is not necessary for cholesterol binding to NPC1.

However, other experiments showed slightly different results. Infante et al. purified NPC1 and measured binding affinity of cholesterol and some other derivatives, e. g. 25–hydroxycholesterol and other oxysterols, cholesterol precursors and steroid hormones. Surprisingly, they discovered that NPC1 binds 25–hydroxycholesterol with a higher affinity than it binds cholesterol; moreover, this interaction is also very specific. Cholesterol binding was affected by the concentration of used detergent, especially by the critical micellar concentration. Exceeding this threshold prevented cholesterol from binding. They also looked for competition in binding between these two substances, and observed 25-hydroxycholesterol affects cholesterol binding, but cholesterol does not affect 25-hydroxycholesterol binding (Infante et al. 2008).

In a related study they were trying to identify cholesterol/oxysterol binding site. They prepared recombinant NPC1 proteins containing C-terminal lysosomal targeting sequence

and a tag. There were 4 forms differing at the N-terminus consisting of: 1) just first luminal loop with the transmembrane helix, 2) first two luminal loops plus the transmembrane helix at the C-terminus, 3) first two luminal loops and the SSD, 4) all three luminal loops with the C-terminal transmembrane helix. Then, the binding to cholesterol and oxysterols was measured. Luminal loop 1 was revealed as the region responsible for cholesterol/oxysterol binding; both these molecules bind as a single molecule per the NPC1 dimer. After that, they wondered which amino acid residues are essential in this process. 14 evolutionary conserved amino acids were chosen for their evolutionary conservation in vertebrates, their hydrogen–bond potential, ring–stacking interaction potential or their known presence in Niemann–Pick patients and they were substituted by alanine. Two of them, Q79A and Q117A seem to play an important role in NPC1 action while especially Q79A showed no 25-hydoxycholesterol binding and diminished cholesterol binding (Infante et al. 2008).

Finally, first luminal loop designated as the N–terminal domain (NTD) was crystalized and its structure was determined (Fig. 2). NPC1 NTD contains 8 α–helices and 3 β–sheets. A sterol binding pocket was identified, which has two openings. One of them is smaller and it has been called the W–opening, because its size is optimal for a water molecule. The larger one was designated as the S–opening (S stands for sterol). Cholesterol/oxysterols binds in this pocket in a specific orientation. Tetracyclic ring is localized inside the pocket towards the W-opening (which is not permeable for sterols). Isooctyl chain is facing outside through the S-opening, which tightly encircles this molecule, and thus the sterol is not able dissociate without conformational change.

Amino acid residues crucial for sterol binding have been identified – hydrophobic Trp27, Leu83, Phe108, Pro202, Phe203 and Ile205, and hydrophilic Asn41 and Gln79 making hydrogen bonds with 3β–hydroxyl group. There is an important role of Leu175, which stabilizes 25–hydroxyl group of 25–hydroxycholesterol. The binding pocket tightly limits sterol molecule at the side of tetracyclic ring and expands behind C20 of isooctyl chain, so modifications on C24, C25 and C27 permit binding (Kwon et al. 2009). These results were consistent with previous data of Infante et al. However, in the analysis of ultracentrifugation sedimentation velocity it was shown that NPC1 NTD is soluble as monomer, which is not in agreement with the binding analysis.

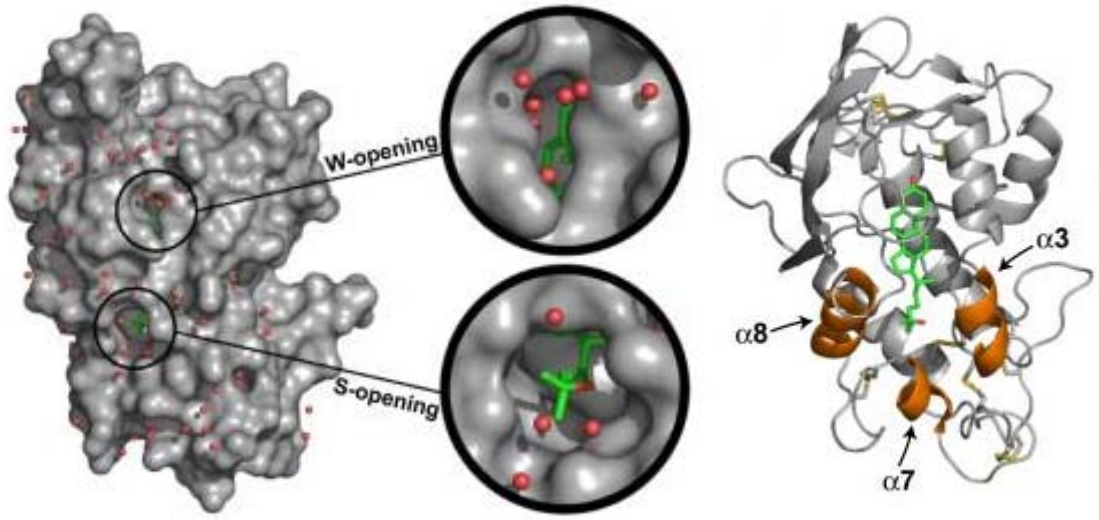

Figure 2. Structure of N-terminal domain of NPC1 with bound 25-hydroxysterol. (Kwon et al. 2009) In the picture, structure of NTD of NPC and orientation of oxysterol in binding pocket is shown.

Two different results have been obtained in the identification of the cholesterol binding domain of the NPC1. These differences might be caused by different experimental setups, e.g. first experiment has been done *in vivo*, whereas the second one *in vitro*. Chromatography used in the second experiment (which showed binding to NTD) could cause different conformation of the protein than the cross-linking method used in the first experiment (binding to SSD). This could have an effect on sterol binding.

There is also a possibility that sterols binds to both domains of NPC1. It was shown that a mutation in SSD could affect sterol binding to SCAP (Nohturfft et al. 1996) and that SSD in these two proteins – SCAP and NPC1 – reveal high similarity and identity (Carstea et al. 1997). This might indicate that, originally, SSD bound sterols as Ohgami et al. (2004) suggested and the NTD evolved later.

3.4 NPC1 and NPC2 cooperation

Niemann–Pick type C disease is described as a cholesterol storage disorder, when cholesterol is not able to exit lysosomes. It is known that this disease is caused by mutations in two genes, Npc1 and Npc2. That suggests that these proteins cooperate in efflux of cholesterol from these organelles. NPC1 cholesterol binding capability has been described in

previous subheads. It was shown, that also NPC2 binds cholesterol *in vitro* (Okamura et al. 1999, Ko et al. 2003) and also *in vivo* (Ohgami et al. 2004). However, Ohgami et al. observed, that NPC2 is not required for sterol binding to NPC1. Infante at al. were looking for the transfer of sterols between liposomes and NPC1 *in vitro*. It was shown that NPC2 plays a role in the transfer of unesterified cholesterol from liposomes to NPC1 and from NPC1 to liposomes. In the presence of NPC2, this movement is much faster than in the absence of this protein (Infante et al. 2008). In a related study, amino acids responsible for the interaction of these proteins were shown; they are located on NTD of NPC1, in the neighbourhood of sterol binding pocket (Kwon et al. 2009).

Crystalization experiment showed that cholesterol or its derivatives bind to NPC2. While isooctyl chain is buried into the binding pocket, β – hydroxyl group is orientated outside to the solvent (Ko et al. 2003). Model of cholesterol pathway in lysosomes (Fig. 3) was suggested. Cholesterol is absorbed into the cell by endocytosis of LDL particles. In late endosomes/lysosomes, esterified cholesterol is unesterified by acid lipases and then it binds to NPC2. NPC2 transfers cholesterol to NPC1 thanks to the orientation of its binding pocket (β-hydroxyl group is buried into protein). NPC1 is then able to insert cholesterol to the lipid bilayer (Kwon et al. 2009, reviewed in Rosenbaum et al. 2011). Cholesterol is then transported to the endoplasmic reticulum.

4. NPC1L1 protein

4.1 NPC1L1 structure

NPC1L1 protein was first described as a new member of NPC1 family, and that is where its name originated – Niemann-Pick C1 Like1. FISH analysis revealed that Npc1l1 is localized on the chromosome 7 (7p13). At first, sequence of Npc1l1 was compared to the sequence of Npc1 and a high percentage of similarity has been revealed - 52 % of nucleotides of Npc1l1 were identical to Npc1. Npc1l1 gene encodes 20 exons of different size ranging from 56 bp to 1526 bp. Moreover, two alternatively spliced variants were identified – one of them has all 20 exons, in the second one exon 15 is missing. Transcript containing all exons produces protein with 1359 amino acids.

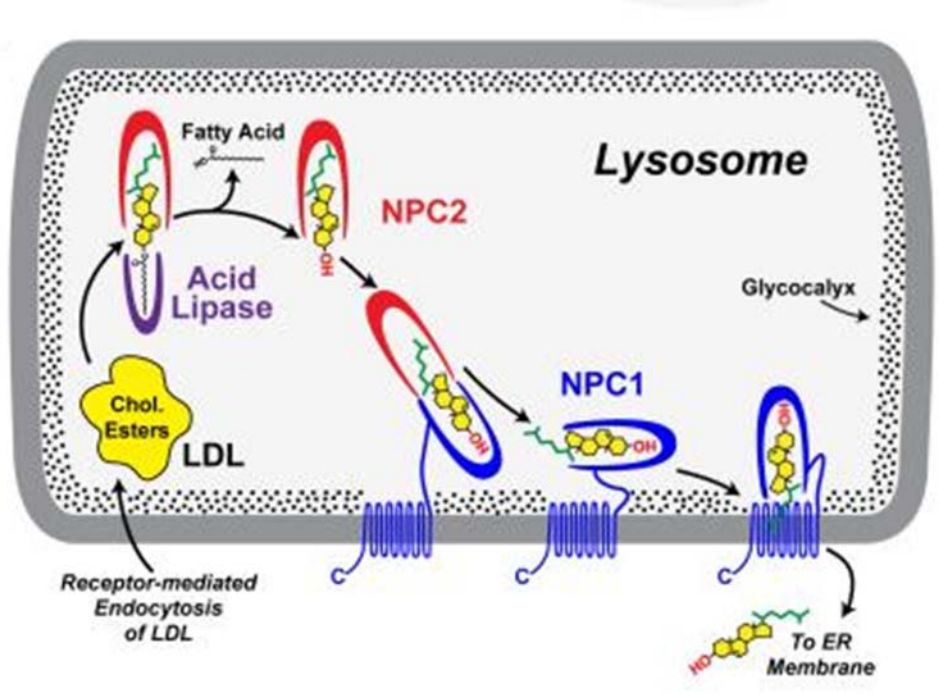

Figure 3. Proposed transfer of cholesterol in lysosome is described in the schema. (Kwon et al. 2009) Cholesterol is transfered from acid lipase to NPC2 and to NPC1, which intermediates its efflux.

Amino acid sequence of NPC1L1 showed 42% identity and 51% similarity to NPC1 and N-terminal domain of NPC1L1 has been designated as „NPC1 domain“. The whole protein has 13 transmembrane domains including SSD, but sequences targeting protein to specific organelles are different. While NPC1 contains lysosomal targeting sequence, NPC1L1 is targeted to the membrane of *trans*-Golgi network via YQRL motif (Davies et al. 2000). N-terminal domain was later recognized as the one responsible for specific cholesterol binding (Zhang et al. 2011).

4.2 NPC1L1 localization

First question to solve was in which tissues is Npc1l1 expressed. Tissue samples from rat, mouse and also from human were used to quantitative RT-PCR. To measure expression in human microarray analysis was performed. These methods have shown that Npc1l1 is expressed mainly in the small intestine in all three species. Human also expresses Npc1l1 in

liver with similar intensity as in small intestine. Expression in other tissues is lower and differs among species.

Rat small intestine was segmented into 10 segments, it was observed that expression increased from first to the third segment and then it slowly decreased to half-fold of the initial expression. NPC1L1 protein was specifically observed in enterocytes (Altmann et al. 2004). NPC1L1 null mouse shown no signs of degeneration – mutation was not lethal and mice were fertile, but they exhibited substantial reduction in absorbed cholesterol (Altmann et al. 2004, Davies et al. 2005).

In cell, the NPC1L1 protein is localized in the membrane. First, it was shown that NPC1L1 resides in the plasma membrane of enterocytes (Altmann et al. 2004); however, in a later study it was not observed. NPC1L1 did not colocalize with different markers for plasma membrane in this experiment, so they suggested protein resides in the internal membrane system (Davies et al. 2005). Differences in these results could be caused by different experimental conditions. Later, it has been shown that protein localization depends on concentration of cholesterol, due to its role in cholesterol absorption (Ge et al. 2008).

4.3 NPC1L1 role in cholesterol absorption

In a related study they looked for NPC1L1 localization depending on the amount of cholesterol in the medium. First, they slowly depleted cholesterol from the medium throughout one hour and then they started to replenish it again. NPC1L1 localized on the cell surface when cholesterol concentration was low, but when the cholesterol concentration increased, NPC1L1 translocated into cytoplasm. It has also been shown that NPC1L1 colocalizes with cholesterol and binds rather this sterol than different phytosterols. They also looked for a cellular mechanism needed for the cholesterol uptake. By using different cytoskeleton affecting drugs they looked whether microfilaments or microtubules are required and it has been shown that cytochalasin D – drug which affects microfilaments – blocks cholesterol uptake. They also showed that clathrin/AP2 complex is required for NPC1L1 endocytosis, but this mechanism is blocked in the presence of ezetimibe – a hypercholesterelemia medication. It also blocks cholesterol binding to NPC1L1.

Mechanism of how NPC1L1 affects uptake of cholesterol has been suggested (Fig. 4). Cholesterol binds to NPC1L1 and this protein is then endocytosed by clathrin/AP2 complex. Endosomes are then transferred by microfilametal cytoskeleton and cholesterol is then transported by other cellular mechanisms. This mechanism is disturbed by drug ezetimibe, which is used to cure hypercholesterelemia. When ezetimibe is present, NPC1L1 cannot be endocytosed (Ge et al. 2008).

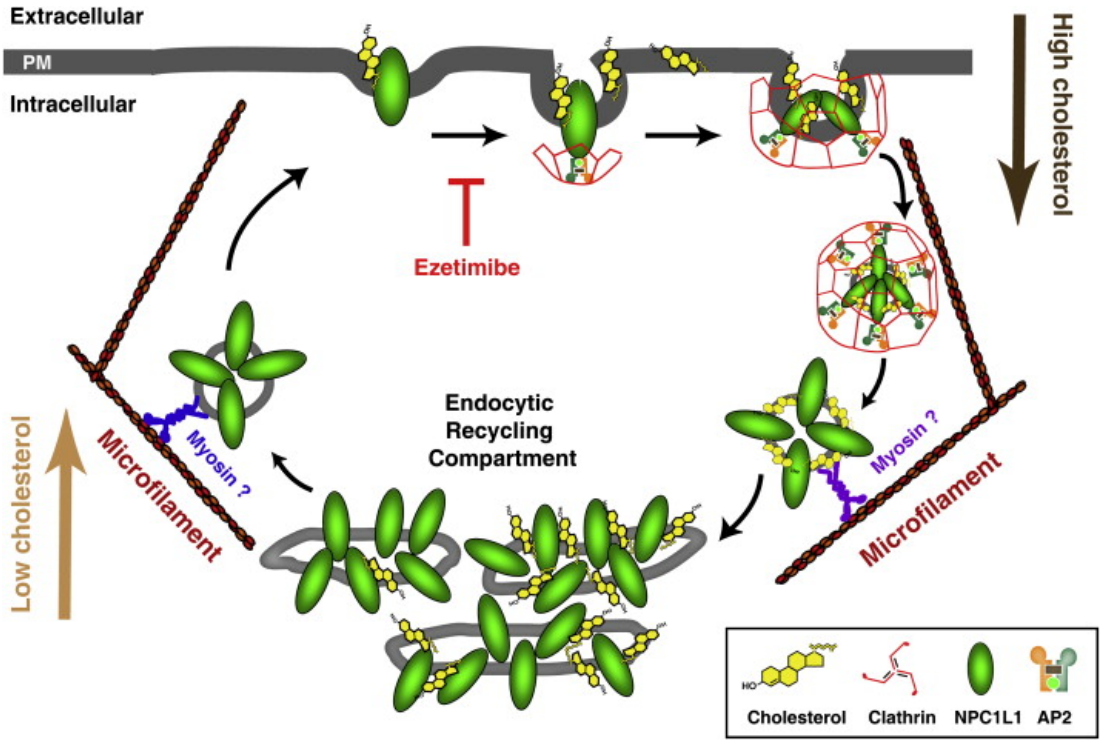

Figure 4. Mechanism of cholesterol uptake by NPC1L1 is marked in the schema. (Ge et al. 2008). NPC1L1 is endocytosed after cholesterol binding, then it is transported via cytoskeleton, is recycled and translocated back to plasma membrane. Legend of main components is marked in the picture.

5. SCAP

5.1 SCAP function

Process of *de novo* synthesis of cholesterol in cell and its regulation has been studied intensively since 30´s in 20th century, when it was shown that mouse is able to synthesize cholesterol without feeding. This was later followed by discovery of HMG-CoA reductase.

This enzyme is a key regulator of cholesterol synthesis. It has been observed that activity of this protein decreased when cholesterol was present in the medium. After cholesterol depletion, the activity of HMG-CoA reductase increased (reviewed in Brown et al. 1986). During the search for regulatory elements, promoter of this gene has been studied and a sterol regulatory element (SRE) has been recognized, which is a 10 bp long sequence (Smith et al. 1990). After that, transcription factors were found and were designated as SREBP – sterol regulatory element binding proteins. These factors affect the expression of some enzymes of the biosynthetic pathway of cholesterol - for instance HMG-CoA reductase - and also the expression of LDL plasma membrane receptors. SREBP is a protein located in the endoplasmic reticulum containing two transmembrane helices. After two cleavages its N-terminal domain moves to nucleus where it binds to SRE.

Mechanism how cholesterol regulates SREBP cleavage has been suggested (Fig. 5). In cholesterol absence, SCAP (SREBP cleavage activating protein) is in a conformation which allows complex of Sec/Sar GTPases to bind its specific MELADL sequence. This complex of GTPases mediates transport of COPII vesicles containing SCAP and SREBP to Golgi, where SREBP is cleaved. When cholesterol is present, it binds to SCAP and changes its conformation. MELADL is not able to bind Sec/Sar complex because of its localization closer to membrane. Specific proteins – INSIG1 and INSIG2 (earlier described as Insulin induced gene) also functioning as prevention against Sec/Sar binding were recognized (Yang et al. 2002). Specific situation occurs when oxysterols are present. Oxysterol molecule binds to INSIG and protects it from releasing SCAP/SREBP proteins. That prevents Sec/Sar from binding to MELADL and intermediate SCAP and SREBP transport to Golgi.

INSIG1 also plays an important role in regulation of HMG-CoA reductase protein. When cholesterol is present, INSIG1 binds to HMG-CoA reductase and recruits it to degradation – binding of INSIG facilitates ubiquitinylation (reviewed in Brown et al. 2009).

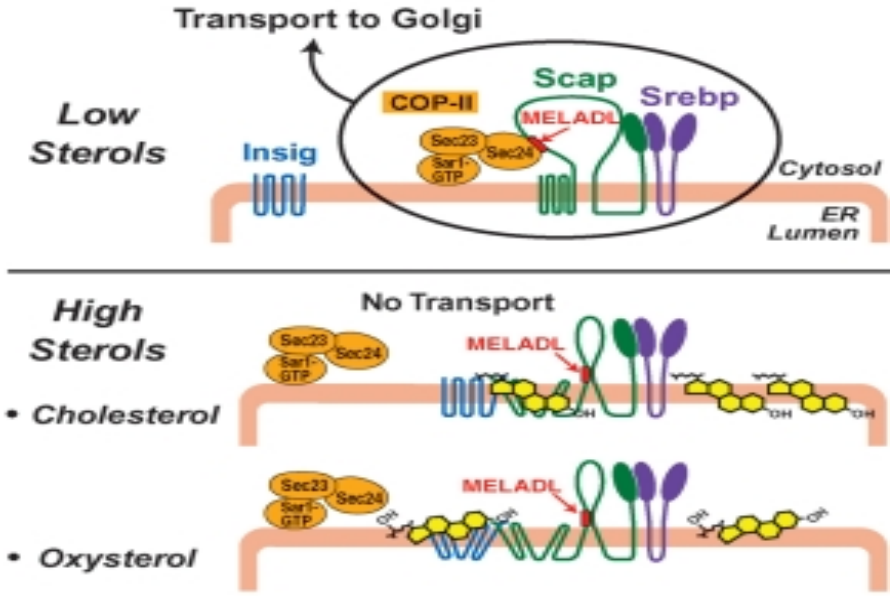

Figure 5. Mechanism of regulation of transporting SCAP/SREBP complex from ER to Golgi (reviewed in Brown et al. 2009). In the picture it is marked conformation of SCAP/SREBP complex under different conditions. Green molecule is SCAP, violet molecule is SREBP, blue one is INSIG. Complex of GTPases Sec and Sar is labelled by orange colour and yellow ones are cholesterol and 25-hydroxycholesterol.

5.2 SCAP structure

Protein SCAP was discovered in studies of cholesterol feedback in cells by Hua et al. (1996). An experiment was performed, when cell culture was transfected by two plasmids. One of them encoded luciferase under promoter with SRE, thus luciferase expression depended on nuclear SREBP. Second plasmid contained different cDNAs from 25-RA cell line cDNA library. It is a cell line with steadily active cholesterol biosynthesis. Cells transfected by these two plasmids were incubated with 25-hydroxycholesterol. When luciferase signal was detected, it suggested plasmid encoded protein abolished 25-HC suppressing SREBP cleavage. One of the hits was protein coding cDNA, which was designated SCAP.

When cDNA sequence was known, protein structure was predicted. SCAP consists of 1276 amino acids and its sequence was compared to its human ortholog (Nagase et al. 1996) and to a related protein from *Caenorhabditis elegans* (Wilson et al. 1994). High identity has been recognized; hamster and human SCAP bear 92% identity. *C. elegans* protein shows only 26%

of identity with the hamster protein. According to a hydropathy plot the N-terminal domain of SCAP containing 800 amino acids revealed high hydrophobicity. 10 transmembrane helices were predicted for this domain. An experiment with tagged protein confirmed that it is localized in membrane fractions. Transmembrane region of SCAP shows high degree of identity and similarity with transmembrane region of HMG-CoA reductase. This region was later designated as the sterol sensing domain – SSD (Nohturfft et al. 1996).The C-terminal domain is a region with high hydrophilicity (Hua et al. 1996). It is orientated into cytosol when the protein is in ER membrane and into lumen of Golgi when protein is in its membrane (reviewed in Brown et al. 2009).

In 25-RA cells, point mutation has been recognized within one of the highly identical regions. In the wildtype protein there is aspartic acid at the position 443 of the protein, whereas in 25-RA cells it is asparagine. An experiment has been made to identify whether this transition has an effect on SCAP function. SCAP variants with mutation D443N inappropriately translocate to the Golgi even when the cholesterol level is high. It suggests that this amino acid residue might play an important role in sterol-binding regulation (Hua et al. 1996). These findings were confirmed in a related study (Nohturfft et al. 1996).

6. HMG-CoA reductase

6.1 HMG-CoA reductase overview

Regulation of cholesterol level in cells is crucial for the function of the whole organism. When this is abolished, cholesterol then accumulates in cells and affects on many cellular processes, e.g. proliferation. Cholesterol belongs to the class of isoprenoids, which can be endocytosed into cell by LDL-receptors or can be synthesized via so-called mevalonate pathway (reviewed in Goldstein et al. 1990).

3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) is an enzyme playing an important role in cholesterol biosynthesis in cells as well as other crucial enzymes, for example HMG-CoA synthase. Reductase has been recognized as a regulator of this pathway. Different levels of HMG-CoA reductase mRNA were observed when cells were incubated with or without cholesterol in the medium. When sterols were present, mRNA decreased

(Chin et al. 1982). It has been shown that HMG-CoA reductase is encoded by a 1.2 kb long cDNA (Chin et al. 1982). Next it was shown that this protein is encoded by a 25 kb gene which contains 20 exons and 19 introns (Reynolds et al. 1984).

6.2 HMG-CoA reductase structure

Later, amino acid sequence and secondary structure of the protein has been predicted. Hydropathy plot was used to identify an area of high hydrophobicity, which is localized at the N-terminus. This domain consists of 339 amino acids and 7 transmembrane helices were predicted there. Between each of them, there were β-sheets structures and loops distinguished. It was also shown, that each of the transmembrane helices is encoded by a different exon. This domain localizes the protein into the membrane which was also confirmed by immunoblotting of ultricentrifugation extracts (Liscum et al. 1985). There is a high degree of similarity to the transmembrane domain of protein SCAP – 25% identity and 55% similarity (Hua et al. 1996). This domain was later designated as a sterol sensing domain – SSD (Nohturfft et al. 1996). However, in related studies, one more transmembrane helix was discovered, so HMG-CoA reductase contains 8 putative transmembrane domains (Roitelman et al. 1992).

C-terminal domain consists of amino acids 340 to 887. In a hydropathy plot these residues reveal high hydrophilicity. Also, cleavage sites of Ca^{2+} dependent protease has been recognized there. Cleavage in the C-terminal domain gives rise to two fragments – 53 kDa and 44 kDa and it has been shown that the 53 kDa fragment retains its catalytic activity. This suggests that the catalytic site of the reductase is in the C-terminal domain. Also a hydrophobic region has been recognized between amino acids 520 and 545. Authors suggested that this cannot be a transmembrane helix, because it lies in 53 kDa water-soluble region of the protein (Liscum et al. 1985).

HMG-CoA reductase is a crucial regulator of cholesterol biosynthetic pathway and it has been studied as target of therapeutical treatment of hypercholesterolemia. Molecules called statins have been discovered, which bind to active site of HMG-CoA reductase and prevent it catalytic function (Istvan et al. 2001).

7. 7-DHCR

7.1 SLOS

Smith-Leimli-Opitz syndrome (SLOS) was first described in 1964 and is defined by many physical malformations including cerebral, ocular, skeletal and genital defects. It also includes intellectual disability and developmental malformations. Patients with SLOS usually reveal prenatal and postnatal growth malformations (reviewed in Nowaczyk et al. 2012). Later it was shown this disorder is caused by mutation in gene of 7-dehydrocholesterol reductase (7-dhcr) (Moebius et al. 1998, Waterham et al. 1998). Lower amount of cholesterol has been observed in the plasma of patients with SLOS as well as 2000–fold higher amount of cholesterol precursor – 7-dehydrocholesterol (Tint et al. 1994). Because cholesterol acts in modification of HH as well as in many other important cellular mechanisms, its defficiency causes these malformations (reviewed in Nowaczyk et al. 2012).

7.2 7-DHCR function and structure

7-DHCR functions as the last step in cholesterol biosynthetic pathway. Cholesterol is made from its precursor, 7-dehydrocholesterol. Cholesterol contains one double bond in its tetracyclic ring while 7-dehydrocholesterol contains two double bonds. One of them – Δ^7 - is reduced by 7-DHCR and this reduction is NADPH dependent. This is one of the options how cell is synthesizing cholesterol. Whether 7-DHCR is used depends on the tissue (reviewed in Waterham et al. 2000). The mRNA expression in different tissues in rat has been studied and the highest expression was shown in liver. 7-DHCR expression was detected also in kidney and brain, but not in other rat tissues (Bae et al. 1999).

Rat cDNA of 7-dhcr was cloned and it consisted of 2356 nucleotides; its identity to mouse enzyme was 96 %, to human enzyme 87 %. Amino acid sequence was predicted for all three orthologs of 7-DHCR; rat and mouse enzyme consists of 471 residues (Fitzky et al. 1998, Bae et al. 1999) and human consists of 475 residues (Moebius et al. 1998). Protein forms 9 transmembrane helices, five of which were recognized as SSD, specifically TMD 4 to 8. This domain seems to be important in protein functioning, because some patients suffering from SLOS bear mutations in SSD. Furthermore, there were also some glycosylation sites and potential phosphorylation sites recognized (Bae et al. 1999).

8. Patched and Hedgehog signalling pathway

8.1 Hedgehog signalling overview

Patched (PTC) – in *Drosophila melanogaster* and its vertebrate ortholog were discovered and studied as important players of the Hedghog (HH) signalling pathway. HH is one of the crucial long-range morphogenes playing roles in development of embryos. But it is also studied its acting in maintenance of stem cells and its role as tumour-suppressor in vertebrates. HH signalling is usually changing behaviour of cells via transcription – HH pathway contains transcriptional factors. These can play an activating or inactivating role depending on the rate and HH signal duration. Pathway contains negative-regulation feedback. However, HH signalling can also affect cells without activating these factors, but some GTPases instead. In embryonic development, HH acts in cooperation with other morphogenes, especially with Wingless (WG, *D. melanogaster*) and WNT (vertebrate ortholog).

To study this pathway, two main models were introduced – fruit fly *D. melanogaster* and mouse as the vertebrate model. Fly embryonic development includes segmentation, where HH signalling plays crucial role. Each segment and their anterior/posterior organization are formed due to concentration gradients of WG and HH. These morphogenes also affect development of wing discs.

In vertebrates,homologs of HH have been discovered, Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH). In fly, there is just HH, but it can be alternatively spliced depending on the tissue. The main function of the SHH is the development of the central nervous system, where its role is to determine different cell types. Neurons and glia cells differentiate in specific concentrations of this morphogene. Vertebrate limbs are also affected by SHH which induce transcription of many other morphogenes and also itself. In the skin development, this network of different factors gives rise to epithelial/mesenchymal interface. Similar process is known in development of bladder (reviewed in Kuwabara et al. 2002 and, Briscoe et al. 2013).

8.2 Hedgehog signalling pathway

HH is a protein molecule with two lipid modifications. At the C-terminus cholesterol binding takes place and at N-terminus palmitic acid binds. Due to these modifications, HH is able to make soluble multimers and, consequently, can be transported between cells in tissue (Fig. 7).

In *D. melanogaster* HH starts its cascade in the cell by binding to PTC. There are also coreceptors as Interference Hedgehog (IHOG) and Brother of Ihog (BOI) which participate. After it, signal is transduced to transmembrane protein Smoothened (SMO). Next step of cascade is HH signalling complex (HSC), which contains many kinases such as Phosphokinase A, Costal2 or Fusion and where the transcription factor Cubitus interruptus (CI) is bound. In the absence of HH, PTC inactivates SMO, which is localized in vesicles inside the cell. HSC is bound to microtubules and CI factors are specifically phosphorylated. The effect of this phosphorylation is that CI is cleaved and repressor domain (CIR) is not degraded in proteasomes. Thus CIR translocates to the nucleus, where it binds to DNA and represses transcription of target genes. When HH binds to PTC, SMO inactivation is terminated and this protein is translocated to the plasma membrane. There its C-terminal intracellular domain is phosphorylated, which leads to binding of HSC to SMO. Phosphorylation of CI is then abolished and these factors are released to translocate to the nucleus. There, they act as activators of transcription of target genes. These genes also include Patched 1 and thus this represents mechanism of negative feedback.

In vertebrates, signalling cascade is a bit different and comprises of vertebrate ortholog – PTC and SMO – and factors specific for the vertebrate cascade – CDO and BOC. SMO is again repressed by PTC, but the HSC complex is missing. Instead, vertebrate kinases bind to the base of cilia. When SHH binds to PTC, SMO translocates to the plasma membrane of cilia and is able to affect complex of kinases with bound transcription factors – in vertebrates those are GLI2 and GLI3 – which move along the microtubules into cilia. Factors are then released to translocate to nucleus, where they can initiate transcription. Without SHH signalling, GLI2 and 3 do not move along the microtubules; they are phosphorylated and cleaved and repressor form – GLI2R and GLI3R – binds to DNA in the nucleus. Vertebrates also have the transcription factor GLI1, which is not cleaved and plays a role as activator only. Possibility of

non-canonical signalling, which does not include transcription factors, has been proposed (reviewed in Beachy et al. 2010 and, Briscoe et al. 2013).

8.3 Patched structure

Patched was first identified by mutation analysis as a protein playing role in *Drosophila* embryonic development (Nusslein-Volhard et al. 1980). Later, cDNA has been cloned from fruit fly cells. It has been shown, that patched transcript is encoded by 4152 bp coding sequence. In comparison to the genomic DNA, it was shown, that patched gene contains 45 kb and consists of 6 exons. Patched protein has been predicted with 1286 amino acids and molecular weight of 143 kDa. After making hydropathy plot of the Patched protein, 12 transmembrane domains were predicted (Hooper et al. 1989). Furthermore, authors recognized one region of the protein related with growth hormone somatotropin (Abdel-Meguid et al. 1987).

It was also studied when and where is patched expressed during *Drosophila* embryonic development. Patched RNA is detectable since 2 hours of development after fertilization of fly embryo, amount is significant when embryo becomes a bit older (4-8 hours old) and then level of patched expression decreases. *In situ* hybridization was used to determine distribution of RNA during development of the fruit fly. It was discovered that Patched appears after 13^{th} nuclear cleavage and is located in in the cortical region of *Drosophila* embryo during its blastodermal cellularization. Patched was not found in dorsal anterior patch and posterior pole cells. In the following development, it is expressed abundantly, but since stage 8, expression is located in stripes. It helps to determine segmental organization of the fly embryo (Hooper et al. 1989).

Protein Patched reveals high evolutionary similarity to other proteins with SSD, especially with NPC1, Dispatched and PTC-related (PTR) protein families (Fig. 6). Many orthologs of PTC have been found in vertebrate, *D. melanogaster* and nematodes – Patched 1, 2 and 3 were found in *Caenorhabditis elegans*; however, their specific role in the organism are still not clear. Patched 1 plays an important role in *C. elegans* development, where it is crucial factor affecting germline cytokinesis; however, in nematodes orthologs of HH and SMO were not found. It seems like Patched 2 is duplicated form of Patched 1 (Burglin et al. 2006). Patched 3 has been recognized as a protein functioning in osmoregulation (Soloviev et al. 2011).

Moreover, plenty of Patched-related proteins have been discovered in nematodes. Those also contain 12 transmembrane domains with SSD and play role with PTC1 in embryonic development (Burglin et al. 2006). Nevertheless many research groups are more interested in Patched 1.

8.4 Hedgehog binding to Patched

In following studies, the Patched role in HH signalling has been analysed. In a study of Hidalgo et al. (1990) it was suggested, that PTC acts as receptor for HH at plasma membrane of cells, starting cascade of processes leading to initiation of transcription – including the Patched gene itself (Hidalgo et al. 1990). To confirm this hypothesis, experiment of binding SHH to vertebrate PTC and SMO was made. At first, it was shown that expression of these two genes is overlapping in mice. Then, they produced recombinant vertebrate PTC and SMO with tags and immunoprecipitation analysis was made. It was shown, that PTC coimmunoprecipitates with labeled SHH and this binding was then confirmed by a cross-linking analysis. SHH binds to PTC with a high affinity. Immunoprecipitation of SMO and SHH was not observed; however, SMO bound to complex of PTC and SHH (Stone et al. 1996). Nevertheless, direct binding of SMO to PTC was not later observed.

Later, new gene family playing role in HH signalling with similar phenotype to Patched has been identified. Proteins encoded by these genes were designated as Interference of Hedgehog (IHOG) and Brother of Ihog (BOI) in *D. melanogaster*. In vertebrates, there are orthologs called CDO and BOC. They are transmembrane proteins containing fibronectin type III domains (FnIII), which consists of subdomains Fn1, Fn2 and Fn3 and these subdomains are well conserved. Binding was observed between these proteins and HH and Fn1 domain was determined to mediate it (Yao et al. 2006).

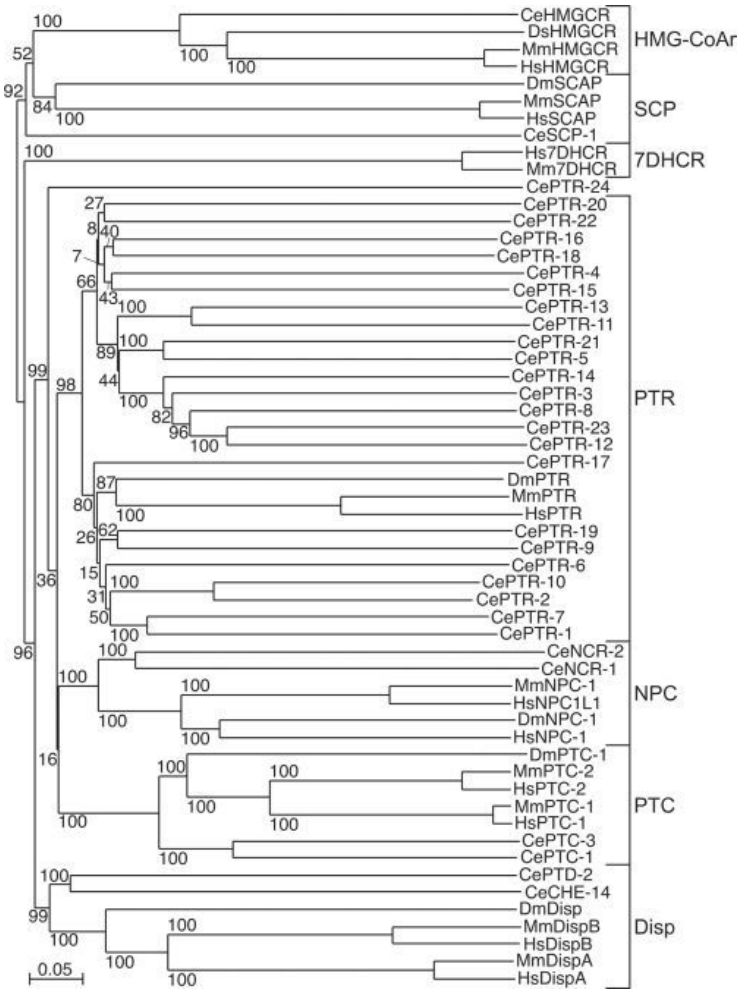

Figure 6. An evolutionary tree generated from an alignement of members of the SSD proteins family (Burglin et al. 2006).

In following study, interaction of protein of IHOG family and Patched was analysed. Because target gene of transcriptional factors initiated by HH signalling cascade is also PTC, its expression can be used as marker of HH signalling. It was shown, that this expression decreased when Ihog and Boi were mutated. When only one of these genes is mutated, substitution of function by the second one is observed. It was also shown, that the expression of Ihog and Boi overlaps with the expression of Ptc with similar phenotype and it is required for HH signalling in tissues. After that, immunoprecipitation was used to solve question whether IHOG/BOI physically interacts with PTC. Coprecipitation was observed, but it was abolished when Fn2 deletion protein was used. It was also shown localization of Patched depends the expression of Ihog, when it is not expressed, protein is localized intracellularly. In conclusion, Patched in HH signalling seems to function as receptor for HH protein, playing its role in complex with IHOG and BOI (Zheng et al. 2010).

8.5 Patched role in SMO inhibition

Patched functions in the cell as the inhibitor of HH signal by repressing SMO, but the mechanism is not entirely clear. At first, Taipale et al. (2002) studied whether PTC and SMO interact directly. An experiment measuring concentration of SMO and PTC was performed, which lead to repression or activation of SMO. It was shown, that PTC functions in 50-fold less concentration than SMO. Based on this nonstoichiometric ratio, indirect regulation by some small molecule has been suggested. Furthermore, high similarity of PTC to RND (resistance-nodulation-cell division) protein family has been shown. Authors suggest, that PTC might transport some small molecule affecting SMO (Taipale et al. 2002).

In related study, lipophorin was suggested as molecule interacting with PTC. Because HH has two lipid modifications; two models of its long-range transport have been predicted – multimerization of HH molecules and association with LDL particles. Based on this, Callejo et al. (2008) looked for interactions of PTC and lipoproteins. Colocalization of Patched, HH and Apolipoprotein (APOL) was observed in early endocytosed vesicles and coprecipitation of PTC and APOL was shown too. After that, PTC bearing mutation in its SSD was not able to repress SMO, although it bound HH and constitutively activated signal cascade. In this study, it was also shown, that PTC regulates cellular lipid homeostasis, but independently to HH. These findings might suggest that lipoproteins play a role in Patched inactivation of SMO, nevertheless, more experiments need to be performed (Callejo et al. 2008).

9. Dispatched

9.1 Hedgehog synthesis overview

Hedgehog is a morphogene which is secreted during embryonic development. HH protein is synthesized in cell as precursor (Fig. 7) and after that, it undergoes autocleavage process in the endoplasmic reticulum. C-terminal part is then degraded in proteasome and N-terminal part (HH-N) needs to be modified. Cholesterol covalently binds to carboxyterminal amino acid of HH-N. N-terminal tail is modified by palmitic acid. After that, modified HH needs to be translocated across the plasma membrane and protein Dispatched was predicted as the main actor in this translocation. It was suggested, cholesterol and palmitic acid modifications

are necessary for its binding and also for its multimerization (reviewed in Briscoe et al. 2013).

9.2 Dispatched function and structure

Dispatched (DISP) was first identified by Burke et al. (1999) and gene dispatched was mapped to third chromosome. It was shown, that the expression of dispatched is necessary for embryonic development, specifically for HH signalling. Study was made on *D. melanogaster* model and authors were looking for expression on anterior/posterior segmental borderline. It was shown DISP is required in cells secreting HH signal but mutation does not affect HH synthesis. Thus, DISP is required for the export of HH molecule outside the cell. Later, DISP functioning in another model organisms has been observed (Ma et al. 2002, Nakano et al. 2004). In human, there was described Dispatched 1 homolog designated as Dispatched 2 (Katoh et al. 2005).

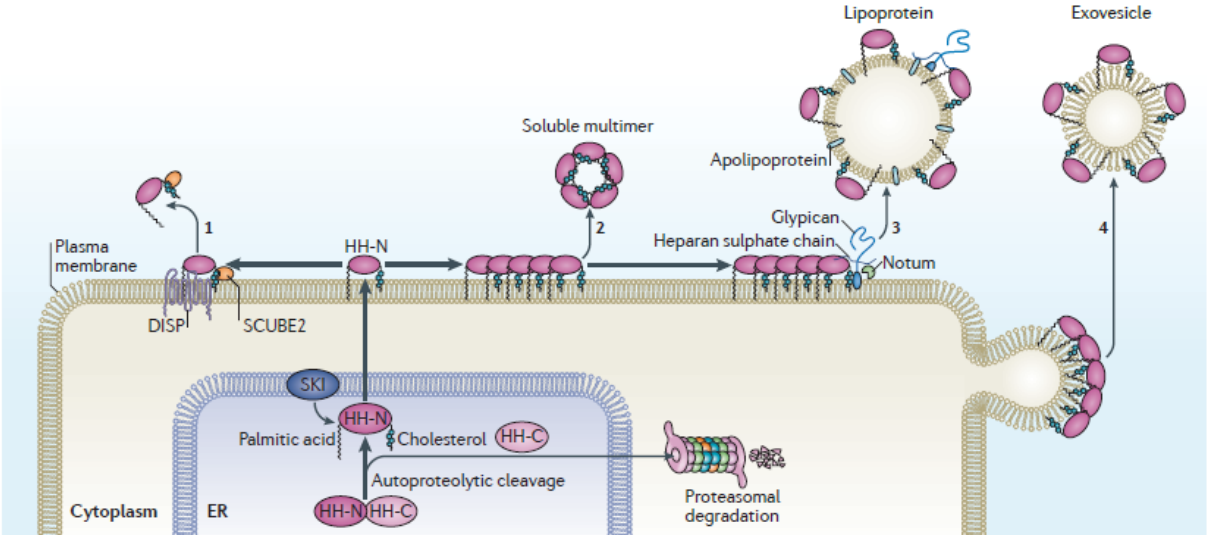

Figure 7. Hedgehog modification and transport outside the cell (reviewed in Briscoe et al. 2013). HH-N - N-terminal part of HH, which is then modified and exported. HH-C - C-terminal part intended to degradation.

After cloning cDNA, protein structure was predicted. It has 1218 amino acids and forms 12 transmembrane helices, which contain the SSD. It reveals high similarity to PTC and NPC1, so they asked whether SSD acts in protein role. An experiment has been made with HH containing cholesterol moiety and HH which does not. It was shown that DISP translocates across membrane cholesterol-modified HH, but the translocation without sterol moiety was not observed. It was shown, that other SSD proteins as SCAP and HMG-CoA reductase interact with cholesterol (as mentioned above). Author suggested SSD of DISP mediates binding of the HH cholesterol moiety. Due to its similarity to PTC within SSD, next suggestion was HH binds to Patched also via SSD – cholesterol interaction (Burke et al. 1999).

In a related study, direct binding of SHH to human Dispatched has been proved using coimmunoprecipitation experiment. To minimalize the effect of detergents, cross-linking experiment has been made with two different photoreactive cholesterol derivatives – first one with labeled at the isooctyl chain of cholesterol and second one at the tetracyclic ring. It was shown DISP interacts with SHH by its cholesterol moiety and rather with its isooctyl chain. Improved binding was observed when disp was mutated - SHH then does not release from cells to the medium. Furthermore, previously designated soluble proteins SCUBE2 (Kawakami et al. 2005) were recognized as proteins binding cholesterol moiety of SHH and interaction was also observed with DISP.

Due to these results, authors suggests model (Fig. 8) of SHH releasing the cell and DISP role in this process. After synthesis and its modification in the endoplasmic reticulum, SHH attaches plasma membrane bilayer. This attachment is mediated by cholesterol and palmitic acid modifications. DISP then binds to SHH molecule, releasing it from the membrane. SCUBE2 protein then interacts with DISP-SHH complex and releases it (Tukachinsky et al. 2012).

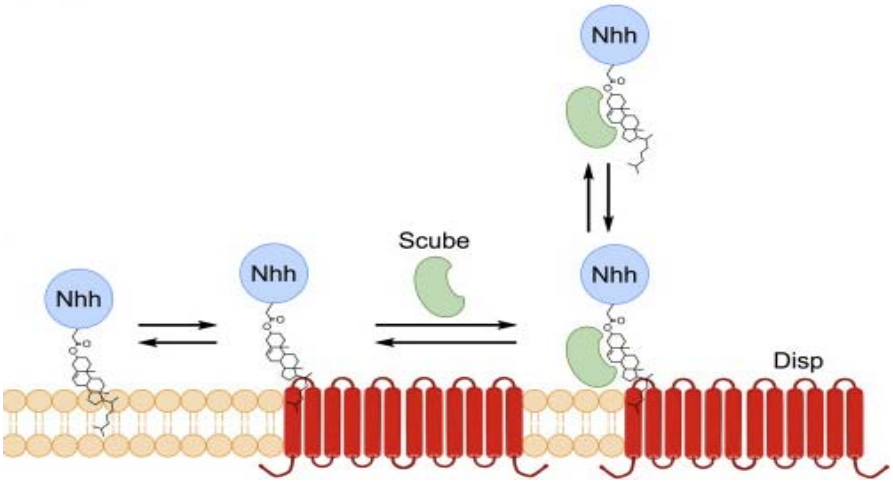

Figure 8. Mechanism of Scube and Disp interaction in N-terminal domain Hh releasing is marked in schema (Tukachinsky et al. 2012). Nhh stands for modified N-terminal part of Hedgehog, green molecule is SCUBE2 and Dispatched is labelled by red colour.

9.3 Dispatched 3

Later, protein related to Dispatched family was discovered and designated as Dispatched 3 (DISP3 also known as PTCHD2 and KIAA1337). It contains 1392 amino acids which form 13 transmembrane helices, 5 of them form SSD. It was shown localization of DISP3 in membrane of endoplasmic reticulum. Expression of DISP3 was observed in embryonic stem cells, testis, brain and retina. In the brain, expression of DISP3 was localized into cerebellum Purkinje cells, hippocampus and brain cortex. In retina DISP3 appears in ganglion and bipolar cells. Also brain tumour revealed expression of Disp3 as well as neuro- and retinoblastoma and lung carcinoid (Katoh et al. 2005).

It was also shown expression of Disp3 might be affected by thyroid hormone. Nevertheless effect of this hormone is dependent on tissue. While Disp3 was down-regulated in testis cell line, in retinoblastoma cell line Disp3 was up-regulated. These data were obtained by *in vitro* experiment. *In vivo* analysis showed DISP3 expression in chicken retina is down-regulated by thyroid hormone.

Moreover, it was studied DISP3 role in cholesterol metabolism in cell. It was shown DISP3 might regulate cholesterol uptake and localization in the cell. It has been observed significant increase in cholesterol amount in the cell ectopically expressing Disp3. Localization of cholesterol in the cell was also affected by expression of Disp3; cholesterol

appears in vesicular structures in contrast to usual perinuclear membrane localization. This effect decreased, when cells expressed Disp3 lacking its SSD. This suggests that affecting cholesterol distribution and amount in the cell by DISP3 is partly mediated by its SSD, but there might be another domain responsible for this phenotype. However, direct mechanism of these interactions is still unclear (Zikova et al. 2009).

10. TRC8

10.1 TRC8 structure

Trc8 gene (stands for translocation in renal carcinoma, chromosome 8) has been discovered due to studies of renal cell carcinoma by Gemmill et al. (1998). It encodes protein consisting of 664 amino acids and with molecular weight 76 kDa. It forms 10 transmembrane helices which include SSD between amino acids 22 – 179. One of the region is highly similar to Patched and other SSD – containing proteins, specifically HMG-CoA reductase or SCAP. There is also second region with high similarity to Patched between 344 and 443 amino acids. Furthermore, region with ring-finger has been recognized there. This motif is suggested to function in protein-protein and protein-lipid interactions (Freemont 1993). Protein localizes in the cell in the ER as its integral protein. Expression of Trc8 in adult human tissues was observed in heart, brain, placenta, also in liver and skeletal muscle and pancreas. And it was also shown TRC8 is highly conserved in mammals (Gemmill et al. 1998).

10.2 TRC8 function

Trc8 has been shown as one of tumour-suppressor genes. It plays role in cholesterol regulation in cell by the influence pre-SREBP and HMG-CoA reductase. In the presence of sterols, HMG-CoA reductase is polyubiquitilated and later degraded in proteasome and this is intermediated by INSIG and among others by TRC8. Also pre-SREBP is targeted to degradation by TRC8; however, TRC8 itself is dependent on the level of sterols, when sterols are present, its stability decreases (reviewed in Drabkin et al. 2012). TRC8 also targets heme oxygenase-1 to degradation. The direct interaction between these proteins has been proved. Moreover, when Trc8 was overexpressed, half-life of heme oxygenase-1 decreased. When

heme oxygenase-1 is known as player in oxidative stress (reviewed in Abraham et al. 2008), authors suggested one of roles of TRC8 as tumour-suppressor (Lin et al. 2013).

11. Conclusion

In this text, new knowledge about proteins containing SSD is summarized. It was described their structures and functions in eukaryotic cells. Also their role in whole organism was slightly mentioned as well as disorders caused by mutations in their genes.

Although interaction of cholesterol molecule with HMG-CoA reductase and SCAP seems to be solved, interactions of other SSD containing proteins are still needed to clarify; e.g. for protein NPC1 two domain interacting with cholesterol are suggested (Ohgami et al. 2004, Kwon et al. 2009). Also research to clear up binding of other proteins with this sterol molecule yet has to be done.

I would like to use these collected information in my next study; I am going to work during my master degree in Laboratory of Cell Differentiation at IMG of ASCR, where I would like to join project focused on DISP3. It is still unclear whether DISP3 directly interacts with cholesterol and if does, which domain is responsible for this binding; this project will by probably focused on this problem. It is area where especially knowledge about NPC1 binding assays might be very helpful.

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