Scratching the surface: Regulation of cell surface receptors in cholesterol metabolism

Nelson, J.K.

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Chapter 1
General Introduction & Scope of the Thesis
1. **The Basics of Cholesterol Metabolism**

Cholesterol is an essential lipid constituent of cellular membranes. Its biophysical properties allow it to be inserted into, or extracted from, membranes. Cholesterol influences membrane fluidity and integrity by altering the packing order of other lipids in the bilayer, particularly di-saturated phospholipids and sphingolipids, which otherwise would be in a solid gel phase. Membrane lipids are orderly distributed between, and within, different organelles to facilitate their specific function. For example, the plasma membrane is rich in cholesterol (30–40 mol%), sphingolipids (10–15 mol%) and modified saturated phospholipids (~50 mol%). In contrast, the endoplasmic reticulum (ER) membrane primarily contains modified unsaturated phospholipids and a low concentration of cholesterol (5 mol%) making ER membranes flexible and enabling for incorporation of newly synthesized proteins. Beyond maintaining membrane integrity, cholesterol performs other functional roles as well. Within the plasma membrane, cholesterol can concentrate locally together with sphingolipids and membrane proteins to form micro-domains, termed ‘lipid rafts’, that serve as platforms for protein sorting and signal transduction. Next to the established role in membranes, cholesterol also serves as the precursor for other important steroid molecules, including oxysterols, bile salts, steroid hormones (i.e. estradiol and testosterone) and vitamin D. Given the central roles of cholesterol in cellular and whole-body physiology, complex mechanisms have evolved to tightly regulate the abundance and distribution of this essential lipid.

1.1 **De novo biosynthesis**

To maintain the cellular demand, cholesterol can be obtained either from exogenous (i.e. dietary) sources or through *de novo* synthesis, the latter being the major source in humans. In the body, the liver is the primary site for cholesterol synthesis, which occurs through a series of enzymatic reactions that take place predominantly in the ER membrane of hepatocytes. In 1964, biochemists Konrad Bloch and Feodor Lynen were awarded the Nobel Prize for characterizing the *de novo* cholesterol biosynthetic pathway as a complex process involving 30 enzymatic reactions. Within the ER, this pathway starts with the conversion of acetyl coenzyme A (Acetyl-CoA) into 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). This intermediate is reduced to mevalonate by the enzyme HMG-CoA reductase (HMGCR), an irreversible ‘rate-limiting’ step in the synthesis of cholesterol and other isoprenoids. This control point is highly regulated in the body and HMGCR is the molecular target of the statin class of drugs used to treat hypercholesterolemia. In the second stage of cholesterol synthesis, several ATP–dependent reactions convert mevalonate to 3-isopentenyl pyrophosphate. This intermediate substrate is decarboxylated and condensed through several reactions to eventually form squalene via squalene synthase in the ER. In the last stage, squalene is converted into squalene-2,3-epoxide in the first oxygenation step of cholesterol synthesis, which is catalyzed by squalene monooxygenase (SQLE). This enzyme is a commonly overlooked second ‘rate-limiting’ enzyme in cholesterol synthesis, and in fact the enzyme committing the pathway to producing cholesterol. In the subsequent reactions, squalene epoxide is converted into...
their density that include: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). Apolipoprotein B (ApoB) is an essential structural component of VLDL, IDL, LDL and chylomicrons, whereas apolipoprotein E (ApoE) is a constituent of HDL particles. Metabolism of these lipoproteins is a complex process of absorbance, assembly, secretion, and catabolism which mainly occurs in the intestine and the liver. Dietary fats and cholesterol are absorbed in intestinal enterocytes, packaged and released to the mesenteric lymph as large, triglyceride-rich lipoproteins known as chylomicrons (CMs). CMs function to transport the digested lipids to the peripheral tissues, namely the heart, skeletal muscle, and adipose tissues. On the surface of capillaries in peripheral tissues, both CMs and liver-derived VLDL undergo triacylglycerol hydrolysis through the activation of extracellular lipoprotein lipase. As a consequence, both chylomicrons and VLDL are reduced to protein-rich remnants (referred to as chylomicron remnants and IDL, respectively) and eventually taken up by the liver through specific receptors on the cell surface. IDL particles can also undergo further hydrolysis in the periphery to form LDL. Of the five lipoprotein classes, LDL is the richest in cholesterol, and the main form in which cholesterol is transported to tissues. Alternatively, HDL plays a crucial role in returning excess cholesterol from peripheral tissues to the liver for excretion. In this, the liver can be viewed as the central organ orchestrating cholesterol homeostasis owing to its ability to synthesize (above), uptake, and excrete cholesterol into bile (Figure 1).

### 1.3 Cardiovascular diseases and LDL-cholesterol

In humans, LDL is a major carrier of cholesterol to peripheral cells accounting for over 60% of the total cholesterol in plasma. Extensive epidemiological studies have shown that elevated levels of circulating LDL-cholesterol is a well-established risk factor for cardiovascular diseases (CVDs). To date, CVDs are the leading global cause of death claiming more lives than all forms of cancer combined. The underlying source of various forms of CVD, including myocardial infarction and stroke, is atherosclerosis. Derived from the Greek words atherē (gruel) and skleros (hard), atherosclerosis was initially described over a 100 years ago as waxy plaques formed on the inside of blood vessels. In the early 19th century, cholesterol was identified as the main component of these waxy plaques, and it was recognized that a diet high in cholesterol leads to severe atherosclerosis in laboratory animals. The link between plasma cholesterol and CVDs was further emphasized in the genetic studies of families in which high plasma cholesterol levels were transmitted as an autosomal dominant trait. This genetic disorder was later termed familial hypercholesterolemia (FH), which is characterized by elevated plasma LDL-cholesterol levels and increased risk of CVDs early in life. As such, the transport and uptake of LDL to hepatic and non-hepatic tissues is extensively regulated and is an important determinant of cholesterol levels in the circulation.
2. Discovery of the LDL-Receptor and SREBPs

In an effort to elucidate the molecular mechanism underlying FH, Michael Brown and Joseph Goldstein studied fibroblasts from FH patients. They showed that normal cells obtained cholesterol through endogenous biosynthesis, and that this process could be attenuated once LDL was supplied to the cells. This feedback inhibition mechanism was absent in fibroblasts from FH patients. Collectively, this suggested the presence of a receptor-mediated event for uptake of LDL. In 1974, the existence of the cell surface LDL receptor (LDLR) was demonstrated, and in 1985, the LDLR gene was cloned. In that same year, Brown and Goldstein were awarded the Nobel Prize for their initial discovery and elucidation of the LDLR pathway. Moreover, their initial studies demonstrated that FH could be caused by genetic defects in the LDLR gene, and that these defects disrupt cholesterol homeostasis resulting in elevated levels of circulating LDL and early onset of CVDs.

2.1 The LDLR pathway

Years later, crystal structures and homology modelling of the LDLR identified several domains in the LDLR that mediate LDL binding and intracellular transport. The N-terminus of the LDLR contains the ligand-binding region, composed of seven LDLR type-A repeats, which binds to ApoB present in the LDL lipoprotein. Following this region is a domain that shows strong homology with the membrane-bound precursor of the epidermal growth factors (EGF). It consists of two EGF-like repeats, followed by a β-propeller region and another EGF-like repeat. Located extracellularly to the single membrane-spanning region is an O-linked sugar domain, which is thought to act as a hydrophilic buffer zone that keeps bound lipoprotein particles away from the lipid bilayer. On the plasma membrane, the LDLR clusters in clathrin-coated pits that are capable of pinching off to form clathrin-coated vesicles inside the cell. The intracellular C-terminus of LDLR contains an NPxY sequence motif (Asn-Pro-Val-Tyr), which mediates its interaction and internalization in the clathrin-coated vesicles. Several members of the LDLR superfamily contain this clathrin-endocytosis NPxY motif (where x can be any amino acid) in their cytoplasmic tails. When ligand-bound LDLR is internalized into clathrin-coated endosomes, the decrease in pH partially charges a cluster of Histidine residues in the β-propeller region in the EGF domain of the LDLR. This pH-sensitive switch allows for the formation of salt bridges between the EGF-like domain and the LDLR type-A repeat regions, which causes displacement of the bound lipoprotein. The displaced LDL particle is trafficked to the lysosome, whereas the LDLR is recycled back to the plasma membrane. The LDLR is estimated to make this round trip every 10 minutes, for a total of several hundred trips in its 20-hour lifespan, taking in ~1600 molecules of cholesterol present in each LDL particle. This rapid recycling of the LDLR provides an efficient mechanism for delivery of cholesterol to cells. Therefore, it is not surprising that mutations in the LDLR that affect LDL binding, release, endocytosis, folding or transport of the receptor are reported to result in severe disorders such as FH.
2.2 Discovery of the sterol-responsive transcriptional pathway

Brown and Goldstein’s discoveries of the LDLR pathway lead to another important finding: in parallel to decreased cholesterol synthesis (HMGCR activity), the expression of the LDLR was also repressed in cells loaded with LDL cholesterol. This implied a sterol-responsive transcriptional regulator controlling both cholesterol synthesis and uptake in the cell, later identified as the sterol-responsive element binding proteins (SREBP s). The SREBP s are a family of basic-helix-loop-helix leucine zipper transcription factors, which are synthesized as inactive precursors bound in the ER membrane. Within the ER, SREBP form a tripartite complex with SREBP cleavage activating protein (SCAP) and insulin-induced gene (INSIG) proteins. Formation of this complex is largely governed by the cholesterol content of the ER membrane, which is sensed by a sterol-sensing-domain (SSD) present in SCAP. When the ER cholesterol content decreases below a threshold of 5% of total lipids, SCAP undergoes a conformation change that releases INSIG. Upon this release, the SCAP-SREBP complex is transported to the Golgi apparatus via COPII-coated vesicles and subsequently processed by two membrane-bound proteases: Site-1protease (S1P) and Site-2 protease (S2P). The cleaved N’terminus region of SREBP translocates to the nucleus, where it binds to sterol-response elements (SRE) in promoter regions of target genes.

2.4 SREBP isotype-specific roles

SREBP s are encoded by two genes which generate three different isoforms: SREBP-1a, SREBP-1c and SREBP-2. SREBP-2 is encoded by a gene on human chromosome 22q13. Both SREBP-1a and -1c are derived from a single gene on human chromosome 17p11.2 through distinct transcriptional start sites. In the liver, SREBP-1c and SREBP-2 are the...
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Acetyl-CoA
HMG-CoA
Mevalonate
Squalene
Squalene-2,3-expoxide
Lanosterol
Cholesterol

Extracellular space

Statins
HMGCR
SQLE
SREBP-2
nucleus
ER
lysosome
LDLR
PCSK9
monoclonal antibody

LDLR
Lysosome
predominate isoforms, and show specificity towards their target genes. SREBP-1c regulates expression of genes primarily related to fatty acid and triglyceride metabolism, whereas the cholesterol biosynthesis pathway (i.e. HMGCR, SQLE) and lipoprotein uptake (LDLR) is largely controlled by SREBP-2 (Figure 3). SREBP-1a can regulate expression of all SREBP-responsive genes, including those that regulate the synthesis of cholesterol, fatty acids and triglycerides. Mechanistically, the statin class of drugs used to treat hypercholesterolemia acts directly through SREBP-2. By inhibiting the catalytic activity of HMGCR, statins reduce the de novo synthesis pathway of cholesterol (Figure 3). Subsequently, this further enhances SREBP-2 processing and transcriptional activity of its target: the LDLR. This results in increased cell surface expression of the LDLR and improved LDL clearance from the circulation. In hepatocytes, SREBP-2 also promotes the expression of the secreted protein proprotein convertase subtilisin/kexin type 9 (PCSK9): a protease involved in the post-transcriptional regulation of the LDLR. PCSK9 is auto-catalytically cleaved in these cells, and subsequently secreted, where it can bind the extracellular EGF domain of the LDLR. The PCSK9-bound LDLR complex is then internalized via clathrin-mediated endocytosis and directed to lysosomes for degradation, thus preventing recycling of the LDLR back to the plasma membrane. This negative regulator of the LDLR was first identified in families with autosomal dominant hypercholesterolemia, resulting from gain-of-function mutations in the PCSK9. In addition, several nonsense mutations in PCSK9 (loss-of-function) have been described in individuals with low plasma LDL cholesterol, thus PCSK9 influences variations in circulating LDL levels in humans. In recent years, monoclonal antibodies that target soluble PCSK9 - thereby increasing hepatic LDL levels and LDL clearance - have shown strong LDL-lowering potency (Figure 3) and are being currently introduced into clinical practice.

3. The LXR pathway

Cholesterol homeostasis is achieved through a balance of tightly regulated transcriptional and post-transcriptional mechanisms. Under low sterol conditions the SREBP pathway is activated to induce de novo synthesis and lipoprotein uptake (i.e. LDL), as described above. Activation of this pathway will subsequently lead to the accumulation of intermediate cholesterol metabolites and formation of oxysterols. These molecules act as endogenous activators of LXRs (LXRα and LXRβ) and potently induce its transcriptional activity to reduce the cholesterol burden. The transcriptional activity of LXRs is dependent on the formation of proprotein convertase subtilisin/kexin type 9 (PCSK9), which is a secreted protein that binds to the extracellular domain of the LDLR and targets it for degradation in the lysosome through clathrin-dependent endocytosis.

Figure 3 | The SREBP-2 pathway. Upon low intracellular cholesterol levels, SREBP translocates to the nucleus where it induces the genes required for the de novo cholesterol synthesis pathway, including the rate-limiting enzymes HMGCR and SQLE. SREBP-2 activation also promotes expression of the LDLR, a membrane bound receptor that binds extracellular LDL lipoproteins. LDLR-mediated uptake of LDL requires clathrin-dependent endocytosis and is followed by recycling of the receptor back to the plasma membrane. The internalized LDL particle is trafficked to the lysosome where the cholesterol content is released. By inhibiting de novo cholesterol synthesis, statins further enhance SREBP-2 activity and expression of LDLR to promote LDL uptake. SREBP-2 also regulates the expression of PCSK9, which is a secreted protein that binds to the extracellular domain of the LDLR and targets it for degradation in the lysosome through clathrin-dependent endocytosis.
a heterodimer with retinoid X receptors (RXRs), which binds to the LXR response elements (LXRE) found within the promoter region of target genes \(^{66-69}\). In the basal state, the LXR-RXR complex is bound to the promoter region of its target genes in complex with co-repressors, such as nuclear co-repressor 1 (NCOR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), which inhibit gene activation \(^{70}\). Upon ligand binding, the LXR-RXR heterodimer undergoes a conformational change, leading to the release of the co-repressor and recruitment of nuclear receptor co-activator complexes \(^{71-73}\) to activate gene expression. The most potent oxysterol ligands of LXR are 20(S)-hydroxycholesterol and 22(R)-hydroxycholesterol (intermediates in steroid hormone synthesis), and 24(S)-hydroxycholesterol (the major oxysterol in human plasma and in the brain) \(^{62,65,74-76}\). LXR are also activated through intermediates of the \textit{de novo} biosynthesis pathway, primarily desmosterol and 24(S), 25-epoxycholesterol (abundant in the liver) \(^{64,77}\). In addition to binding LXR, these endogenous ligands attenuate cholesterol synthesis by retaining SREBP in the ER through its interaction with SCAP and INSIG, thus inhibiting SREBP transcriptional activity \(^{78-80}\). Moreover, several synthetic LXR agonists have been described \(^{81,82}\) which bind LXR without inhibiting SREBP. Both LXR\(\alpha\) and LXR\(\beta\) share \(\sim 78\%\) sequence similarities in both the DNA and ligand-binding domains \(^{75}\), but differ in their tissue distribution. LXR\(\alpha\) is primarily found in the liver, spleen, small intestine, kidney, macrophages, and adipose tissues, whereas LXR\(\beta\) is ubiquitously expressed \(^{83-85}\). Functionally, LXR induce the expression of genes whose main function is to reduce the cellular cholesterol burden, described in detail below.

### 3.1 LXR in reverse cholesterol transport and lipogenesis

Several direct LXR target genes regulate the reverse cholesterol transport (RCT) pathway, a process in which excess cholesterol from peripheral tissues (\textit{e.g.} macrophages) is transported to the liver in HDL for biliary excretion (Figure 4). This pathway is primarily mediated through the LXR transcriptional targets ApoE, and the cholesterol efflux ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) \(^{86-91}\). Both ABCA1 and ABCG1 are essential for the first step in the RCT pathway, as they transfer intracellular cholesterol to apolipoprotein acceptors ApoA1 and HDL, respectively. HDL particles from the peripheral tissue are taken up by the liver through the scavenger receptor class B member 1 (SRB1), and the excess cholesterol in the liver (either from lipoproteins or \textit{de novo} synthesis) is converted to bile acid through activity of the LXR target gene (only a target in mouse but not in humans) cytochrome P450-7A1 (Cyp7A1), the rate-limiting enzyme in bile acid synthesis \(^{92}\). Alternatively, cholesterol in the liver can be transported into bile for fecal excretion via the LXR-regulated ABCG5 and ABCG8 cholesterol transporters \(^{88,93,94}\). The importance of LXR in RCT is highlighted in numerous \textit{in vitro} and \textit{in vivo} studies. Mice lacking both Lxra and Lxrb display increased LDL and reduced HDL cholesterol levels, and severe accumulation of foam cells (lipid-laden macrophages present in atherosclerotic lesions) in multiple peripheral tissues \(^{95}\). Moreover, deletion of both LXR on the ApoE-null background results in extreme cholesterol accumulation and foam cell formation, with atherosclerosis and death occurring by 10 weeks of age \(^{96,97}\). Conversely, activating LXR signaling through administration of the synthetic LXR
agonist GW3965 in ApoE-null and Ldlr-null mice inhibited the development of aortic lesions. Specifically, these anti-atherogenic effects of an LXR agonist were lost in Ldlr-null mice transplanted with either Lxraβ(-/-) or Abca1(-/-) bone marrow. In vitro, murine macrophages lacking LXRs accumulate cholesteryl esters and rapidly become foam cells. In line with these findings, Ldlr-null mice transplanted with bone marrow from mice deficient in both ABCA1 and ABCG1 display accelerated atherosclerosis. Taken together, LXR activation of the RCT pathway is central for regulating systemic cholesterol transport. Stimulating this pathway through LXR-based therapeutics would be beneficial in preventing atherosclerosis and CVDs. However, there is one main obstacle in using LXR agonists, in that LXRs induce lipogenesis in the liver by enhancing expression of the lipogenic transcription factor SREBP-1c (Figure 4). Activated SREBP-1c induces the expression of key lipogenesis enzymes, including fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD-1). Accordingly, administration of synthetic LXR agonists to mice promotes triglyceride synthesis in the liver, stimulates VLDL secretion and raises plasma triglyceride levels. Therefore, developing novel LXR-selective agonists that induce the RCT pathway (in peripheral tissues) and circumvent SREBP-1c activation (in the liver) would offer an ideal anti-atherosclerotics therapy.

**Figure 4 | The LXR pathway.** LXRs are activated under conditions of high intracellular cholesterol levels, and induce expression of the cholesterol transporters ABCA1 and ABCG1 to promote cholesterol efflux in the form of HDL. HDL is taken up by the liver though cell surface receptors, such as SRB1, and extracted cholesterol is excreted directly through the ABCG5 and ABCG8 transporters or converted into bile acid for biliary excretion. LXR in the liver also induces the transcription factor SREBP-1C which promotes lipogenesis. Figure adapted from 85.
4. The Basics of Ubiquitylation

Protein ubiquitylation is a post-translational modification (PTM) that regulates nearly all aspects of eukaryotic biology, such as 26S proteasomal and lysosomal degradation, subcellular localization, transcription, DNA repair, and transmembrane signaling. The occurrence and importance of this PTM emerged in the 1980s, when pioneering studies associated the ATP-dependent ubiquitylation of substrates to their degradation by the proteasome. Since then, over 1000 proteins have been identified to regulate ubiquitylation in human cells. This process occurs through a three-step enzymatic cascade, involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. In an ATP-dependent reaction, the C-terminal carboxyl group of ubiquitin is activated through a high-energy thioester linkage to the active Cysteine residue of an E1. The ubiquitin is transferred from the E1 to one of ~40 E2s (in mammals) through a trans-thioesterification reaction. Following this step, ubiquitin is shifted from the E2 to a target protein in an E3-dependent manner. There are two major families of E3 ubiquitin ligases (~600 members): the really interesting new gene (RING) domain and the homologous to the E6AP carboxyl terminus (HECT) domain E3s. The RING E3 ligases mediate the direct transfer of ubiquitin from an E2 to a substrate, whereas the HECT E3s first accept the activated ubiquitin from the E2 on an internal Cysteine residue, forming a HECT-ubiquitin intermediate, prior to target conjugation.

4.1 The diversity of the ubiquitin-code

The conjugation of ubiquitin to target proteins typically takes place on an ε-NH₂ group of an internal Lysine residue, even though Cysteine ubiquitylation have been reported as well. These monoubiquitylation events are highly prevalent, and have been shown to be essential in mediating localization and endocytosis, DNA repair, transcription, and proteasomal degradation. Moreover, several Lysines can be modified in this manner to generate a multi-mono ubiquitylation signal on a substrate, which further diversifies the signal. This modification (monoubiquitylation) can be extended by ligation of additional activated ubiquitin moieties to any of the seven Lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) that are present on the initial conjugated ubiquitin molecule, thereby producing poly-ubiquitin chains of various topologies. In addition, Met1-linked ubiquitin chains (i.e. linear ubiquitin chains) can also be generated when ubiquitin is attached to the N-terminus of a second ubiquitin. Proteomic studies have shown that all chain topographies co-exist in cells, consisting of either homotypic (one linkage type) or heterotypic (several linkage types) chain types. The predominate linkage type in cells is Lys48-linked chains, which have a key role in 26S proteasomal degradation, whereas a chain linked through Lys63 can lead to lysosomal sorting or inducing signaling pathways without involving the proteasome. Additionally, ubiquitin itself can be modified by either ubiquitin-like molecules (such as SUMO or NEDD8), acetylation, phosphorylation, or even a combination of all three types, thus creating a multitude of distinct signals with diverse cellular outcomes.
Figure 5 | Protein ubiquitylation. In an ATP-dependent reaction, ubiquitin is activated through an E1 and transferred to active-site Cysteine of an E2 through a trans-thioesterification reaction. Following this step, ubiquitin is transferred from the E2 to a Lysine residue in a target protein in an E3-dependent manner. The single conjugation of an ubiquitin molecule is known as monoubiquitylation. Several Lysines on a target protein can be modified in this manner to generate a multi-monoubiquitylation signal on a substrate. This modification can be extended by ligation of additional activated ubiquitin moieties to any of the seven Lysine residues that are present on the previously conjugated ubiquitin molecule, thereby producing poly-ubiquitin chains of various topologies. Figure adapted from 106.
4.2  **Breaking the chain, the role of de-ubiquitylases**

Substrate ubiquitylation promoted by E3-ubiquitin ligase can be effectively reversed through the activity of de-ubiquitylases (DUBs). These enzymes can hydrolyze peptide, amide, ester or thiolester bonds at the C-terminus of ubiquitin \(^{124}\), and as such DUBs have the potential to regulate any ubiquitin-mediated process in the cell. Their activities are generally classified into three major categories: (1) generation of free ubiquitin through linear cleavage of ubiquitin (2) removal of mono- or poly-ubiquitylated chains from ubiquitylated proteins, and (3) remodeling ubiquitin chain topographies. The human genome encodes approximately 100 DUBs, \(~80\) of which are predicted to be active \(^{116}\), that can be classified into five families. The largest of the five families is the ubiquitin-specific protease (USP) family (~55 members). The USPs, as well as the ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), and members of the Josephin or Machado-Joseph disease (MJD) are cysteine proteases. The fifth DUB family functions as zinc-dependent metalloproteases, referred to as JAB1/MPN domain-associated metalloisopeptidases (JAMMs). The substrate specificity of DUBs is determined by sub-cellular localization, specific binding interactions, and preference for ubiquitin chain topography.

4.3  **DUBs in cholesterol metabolism**

At the time of writing, the role of DUBs in lipid metabolism, especially in cholesterol metabolism, remains largely unexplored. One particular aspect of DUB activity which will be interesting to investigate is their ability to associate with, and modulate E3 ligase activity. This can be either through direct interaction (functioning as a DUB-E3 ligase pair) or indirectly (as in the proteasome), to counteract the tendency of the E3 ligase to auto-ubiquitylate itself in the absence of substrates \(^{113,125,126}\). Under homeostatic conditions, DUBs could also rapidly edit or remove ubiquitylated proteins to alter a particular signal or cellular outcome. Two well-described DUBs, known as USP8 (also known as UBPY) and AMSH (associated molecule with the SH3 domain of STAM), have been shown to modify ubiquitylated-cell surface receptors and components of the endosomal sorting complexes required for transport (ESCRT) pathway \(^{116,127-129}\). Biochemical and cellular studies have shown that AMSH can process Lys63-linked chains, but not Lys48-linked poly-ubiquitin chains \(^{130,131}\). Alternatively, USP8 can hydrolyze both Lys48- and Lys63-linked ubiquitin chains \(^{127,132}\). These DUBs associate with internalized ubiquitylated-receptors in the endosomal sub-compartments and influence receptor recycling and/or lysosomal degradation \(^{130,133}\). Whether these, and other DUBs play a role in cell surface receptors involved in cholesterol metabolism is explored in this thesis.

5.  **The Discovery of an LXR-induced E3 ligase**

In addition to promoting cholesterol efflux and lipogenesis, it was also observed that LXRs decrease LDL binding and uptake into a variety of cell types \(^{134}\). Activation of LXRs in these cells markedly reduced LDLR abundance without changing expression of the corresponding LDLR mRNA. This post-transcriptional event was rapid and absent in fibroblasts
and macrophages lacking LXRs. Using transcriptional profiling, the E3 ubiquitin ligase myosin regulatory light chain interacting protein (MYLIP) was identified as an LXR target gene and regulator of LDLR degradation. Following its discovery, MYLIP was renamed to reflect its “adopted” function, and is commonly referred to as the inducible degrader of the LDLR (IDOL). Subsequent studies have confirmed that IDOL is a direct transcriptional target of both human and mouse LXR in vitro and in vivo. Gain-of-function experiments in cells and mice have clearly demonstrated that IDOL reduces LDLR abundance and LDL uptake, whereas IDOL loss-of-function studies display the opposite outcome. Akin to PCSK9, IDOL promotes the lysosomal degradation of the LDLR. However, IDOL-mediated degradation is regulated by LXRs, is ubiquitylation-dependent, and mediated through the intracellular domain of the LDLR.

5.1 IDOL-mediated ubiquitylation of the LDLR

Ubiquitylation has recently emerged as a significant PTM in cholesterol metabolism, particularly through the LXR-IDOL-LDLR axis. Recent functional studies have identified and characterized key structural components required for IDOL-mediated ubiquitylation of its substrates. The IDOL gene encodes a 445 amino acid open reading frame with two distinct functional protein domains: a N-terminal FERM and a C-terminal RING. The FERM domain mediates the interaction between membranes and membrane proteins, and the RING domain of IDOL is similar to that found in other RING-containing E3-ubiquitin ligase and mediates its catalytic activity. Modeling and structure-guided mutagenesis studies have demonstrated that the FERM domain of IDOL is essential for interacting with the intracellular tail of the LDLR. Unlike other FERM domain containing proteins, IDOL’s FERM domain contains an insertion that is predicated to function as a phosphotyrosine-binding domain (PTB). This PTB domain is also present in the LDLR-family adaptor proteins DAB1 and ARH, and promotes their interaction with the LDLR, which is essential for clathrin-mediated internalization of the receptor. Similarly, residues in IDOL’s PTB domain are necessary for LDLR binding. However, unlike ARH and DAB1, the NPxY endocytosis motif in the intracellular tail of the LDLR is not required for binding and subsequent degradation by IDOL. IDOL effectively promotes poly-K63 ubiquitin chain formation on the LDLR that target the receptor for lysosomal degradation. Another important determinant of IDOL-mediated degradation of...
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the LDLR is the membrane environment. IDOL interacts with the LDLR at the plasma membrane, and this membrane environment is sufficient to promote degradation of a chimeric receptor containing only the intracellular tail of the LDLR. In contrast, when this same intracellular tail is presented to IDOL the context of soluble cytosolic protein IDOL is unable to stimulate degradation of the chimeric construct. Akin to other E3-ubiquitin ligases, IDOL promotes its own degradation. However, unlike the lysosomal degradation of the LDLR, IDOL auto-ubiquitylation leads to its rapid proteasomal degradation (Figure 7). Structural studies of IDOL's RING have revealed that this domain forms a homodimer, similar to other RING-containing E3 ligases and cellular pull-down experiments have also confirmed that full-length IDOL dimerizes. This event may be necessary for IDOL-mediated degradation, as several studies have shown that dimerization mutants impair IDOL's ubiquitylation activity towards its substrates.

5.2 IDOL targets the VLDL-Receptor and ApoE-Receptor type 2

The structural elements required for IDOL degradation are conserved amongst members of the LDLR family of lipoprotein receptors, and in addition to the LDLR, IDOL targets two closely-related receptors, the VLDL receptor (VLDLR) and Apolipoprotein E receptor-2 (ApoER2). Mechanistically, this occurs similar to targeting of the LDLR and involves ubiquitylation of a highly conserved Lysine in the intracellular tail of these LDLR-related receptors. Accordingly, activation of the LXR pathway, or over-expression of IDOL, has been shown to reduce the VLDLR and ApoER2 in various cell types. Likewise, activation of LXR in vivo reduced VLDLR levels in murine tissues. Unlike the LDLR which is highly expressed...
in liver, the \textit{VLDLR} is predominately expressed in heart, skeletal muscle, adipose tissue, and brain, whereas the \textit{ApoER2} is largely found in brain, placenta and testis. A growing body of evidence has emphasized the contribution of the LDLR, VLDLR and ApoER2 in the central nervous system \textsuperscript{148}. Both the VLDLR and ApoER2 play an essential role in the Reelin signaling pathway of the developing brain \textsuperscript{149-152}. In this signaling pathway, both receptors bind the extracellular matrix protein Reelin and promote downstream signaling through Dab1. In mice, mutations of Dab1, VLDLR and ApoER2 produce a “reeler” phenotype, where neurons are produced normally but fail to migrate and orientate correctly \textsuperscript{149,153}. Similarly, the brains of \textit{Lxr}\textsuperscript{a,b}\textsuperscript{-/-} mice display abnormal anatomical structures and neuronal function \textsuperscript{154}. Whether impaired Reelin signaling contributes to this phenotype is not known. In support of IDOL involvement herein, in a cellular model of this signaling pathway, activation of LXR leads to reduced VLDLR- and ApoER2-mediated binding of Reelin and phosphorylation of DAB1 \textsuperscript{146}. In line with studies, the IDOL-LDLR pathway was recently demonstrated to play a pivotal role in the development of an Alzheimer’s disease (AD)-like phenotype in mice. The hallmark feature of AD is the accumulation of \textit{\beta}-amyloid, and interestingly, \textit{IDol}\textsuperscript{-/-} mice were protected from this. Mechanistically, this occurred through enhanced LDLR surface abundance and clearance of \textit{\beta}-amyloid and ApoE by microglia cells in the central nervous system \textsuperscript{148}. Collectively, IDOL-mediated ubiquitylation of lipoproteins serves an important function in lipid homeostasis in the brain.

5.3 \textbf{IDOL- vs PCSK9-mediated degradation of lipoprotein receptors}

IDOL and PCSK9 share several similarities, such as their ability to promote degradation of the LDLR, VLDLR, and ApoER2. They both preferentially target the plasma membrane pool of LDLR for degradation, even though their modes of action are different. Whereas PCSK9 is a soluble protein that binds to the extracellular domain of the LDLR, IDOL recognizes the short intracellular domain of the receptor. Mechanistically, how PCSK9 routes the LDLR towards lysosomal degradation remains unclear. It was initially hypothesized that IDOL may function down-stream of PCSK9 binding to the LDLR. However, several studies have indicated that PCSK9 can still degrade the LDLR in \textit{IDol}\textsuperscript{-/-} cells \textsuperscript{145}, or an LDLR lacking intracellular ubiquitylation sites or even the entire intracellular domain \textsuperscript{155,156}. It is possible that these two pathways are differentially used in a tissue-specific manner. For example, the expression of annexin A2, an endogenous inhibitor of PCSK9, in peripheral tissues could limit PCSK9 to targeting hepatic LDLR \textsuperscript{157}. In contrast, IDOL is highly active in peripheral tissues \textsuperscript{134,158}. In addition, these two pathways may show different kinetics. In response to high sterols, LXR-induced IDOL can rapidly target and degrade lipoprotein receptors, where SREBP-regulated PCSK9 needs to be catalytically processed and secreted into the circulation before it can bind the LDLR. Future kinetic experiments \textit{in vivo} will be informative to clarify how these two degradation pathways co-regulate LDLR abundance.
Chapter 1

Over the last century, many scientific milestones have deepened our understanding of cholesterol homoeostasis. Deducing the existence of cholesterol in human aortic plaque led to the revelation of elevated plasma LDL and its role in atherosclerosis and CVD. Then, the LDLR and the SREBP pathway were discovered leading to functional characterization of the genetic mutations that cause FH. Collectively, these breakthroughs have laid the groundwork for future discoveries and defined current lipid-lowering therapies aimed at increasing hepatic LDLR on the cell surface, owing to its ability to clear LDL-cholesterol from the circulation. Thus far, three pathways are known to regulate LDLR plasma membrane expression: SREBP-2, PCSK9 and IDOL. Current statin therapies are effective at lowering plasma LDL by indirectly activating the SREBP-2 pathway to drive LDLR expression and promote LDL clearance from the plasma. In the last decade, post-translational mechanisms in protein degradation, mainly PCSK9 and IDOL, have emerged as key modulators of LDLR half-life and function. In humans, the PCSK9 pathway has been thoroughly investigated and strategies for its inhibition are being currently introduced into clinical practice \(^{58-61,159}\). Yet, the physiological relevance of IDOL-mediated LDLR degradation is less understood and largely investigated in this thesis.

Chapter 2 describes quantitative and qualitative methods to assess LDLR protein abundance and function. These methods were used in the following chapters to elucidate the molecular mechanisms that govern the LDLR pathway, the main research focus of this thesis.

Chapter 3 expands our understanding of the endosomal trafficking route the LDLR undertakes following IDOL-mediated ubiquitylation. In this chapter we identify an internalization mechanism for the LDLR that is distinct from its “classical”, clathrin-dependent one. Ensuing IDOL-mediated ubiquitylation at the cell surface, ubiquitylated-LDLR is internalized through a clathrin-, caveolae- and dynamin-independent process, which requires Epsins and the ESCRT endosomal sorting system for lysosomal trafficking.

Chapter 4 identifies NDRG1 as a novel regulator of endosomal trafficking of the LDLR. In the absence of this protein, uptake of LDL-cholesterol is reduced due to increased IDOL-mediated ubiquitylation of the LDLR at the plasma membrane. However, this ubiquitylation event does not result in lysosomal degradation, but rather increased accumulation of the ubiquitylated-receptor in modified early endosomes/MVBs.

Chapter 5 details the first loss-of-function IDOL variant in individuals with low circulating levels of LDL-cholesterol. Biochemical and molecular analysis revealed that this nonsense variant impairs IDOL’s E3 catalytic towards degrading the LDLR. These findings provide the first evidence that IDOL contributes to variation in circulating levels of LDL-cholesterol in humans, and supports the notion that inhibiting IDOL activity may provide a novel therapeutic approach for treating dyslipidaemia.

Scope of the Thesis

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Chapter 6 identifies the DUB USP2 as negative regulator of IDOL-mediated ubiquitylation. Genetic, molecular and biochemical evidence suggest that USP2 is a post-translational regulator of IDOL abundance and activity. Mechanistically, IDOL, USP2 and the LDLR form a tripartite complex at the plasma membrane, leading to less IDOL-mediated ubiquitylation and degradation of the LDLR.

Chapter 7 reports that short-term pharmacological pan-inhibition of cellular DUB activity leads to a sterol-independent mechanism to regulate IDOL expression and IDOL-mediated degradation of lipoprotein receptors. This novel transcriptional induction occurs independently of LXR activity, and requires a 70-bp region in the proximal promoter of IDOL which is distinct from its identified LXR response element.

Chapter 8 identifies (P)RR as a novel regulator of LDL metabolism by controlling the abundance of SORT1 and the LDLR. Silencing of either (P)RR or SORT1 selectively reduces LDLR protein levels and LDL-cholesterol uptake. Mechanistically, this decrease in LDLR does not occur at the plasma membrane, akin to IDOL- or PCSK9-mediated degradation, instead the (P)RR-SORT1 interaction seems to be essential for proper trafficking of the nascent LDLR protein towards the plasma membrane.

Chapter 9 describes an intricate interplay between the E3 ubiquitin ligase IDOL and MARCH6, which together uncouple cholesterol synthesis from lipoprotein uptake in hepatocytes. Mechanically, silencing MARCH6 increases the expression of SREBP-2 target genes (i.e. HMGCR, LDLR) and flux across the mevalonate pathway. Unexpectedly, this was also associated with an increase in LXR signaling and induced expression of the LXR-responsive E3 ligase IDOL. As a result, despite enhanced expression of the LDLR, overall LDLR protein and LDL uptake were reduced in a MARCH6-dependent manner.

Chapter 10 identifies EEPD1 as a novel LXR target gene in human and mouse macrophage. EEPD1 does not seem to regulate the level of LDLR itself, but rather the cell surface expression of the cholesterol transporter ABCA1 that is a critical determinant of circulating levels of HDL cholesterol.

Chapter 11 summarizes the findings presented in this thesis and suggests new directions for future research in this field.
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