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The ubiquitous role of ubiquitination in lipid metabolism

Melanie Loix^{1,2}, Noam Zelcer³, Jeroen F.J. Bogie^{1,2†}, Jerome J.A. Hendriks^{1,2†}

¹ Biomedical Research Institute, School of Life Sciences, Hasselt University, Diepenbeek, Belgium.

² University MS Center Hasselt, Pelt, Belgium.

³ Department of Medical Biochemistry, Amsterdam UMC, Amsterdam Cardiovascular Sciences and Gastroenterology and Metabolism, University of Amsterdam, Amsterdam, the Netherlands.

[†] Authors contributed equally (last author)

*Correspondence: jerome.hendriks@uhasselt.be

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Abstract

Lipids are essential molecules that play key roles in cell physiology by serving as structural components, for storage of energy, and in signal transduction. Hence, efficient regulation and maintenance of lipid homeostasis is crucial for normal cellular and tissue function. In the past decade, increasing research showed the importance of ubiquitination in regulating the stability of key players in different aspects of lipid metabolism. This review describes recent insights into the regulation of lipid metabolism by ubiquitin signaling, discusses how ubiquitination can be targeted in diseases characterized by lipid dysregulation, and identifies areas which require further research.

Ubiquitination and lipid metabolism

Controlled degradation of proteins by the ubiquitin-proteasome system (UPS) (**Box 1**) is implicated in the breakdown of 80% of intracellular proteins. Accordingly, abnormal UPS function perturbs cellular homeostasis and underpins disease pathology in cancer, metabolic diseases, and neurodegenerative disorders such as Parkinson's (PD) and Alzheimer's disease (AD) [1, 2]. Emerging evidence indicates that ubiquitination is a hub and driver of cellular lipid metabolism. Lipids are important in preserving membrane structure, regulating energy metabolism, and controlling signal transduction. Hence, balanced lipid homeostasis is crucial for cellular well-being [3, 4]. In this review, we discuss current knowledge of implications for ubiquitination in regulating lipid metabolism, highlight links between abnormalities in lipid homeostasis and ubiquitination, and identify areas that require more research. **As an enormous body of research has been performed on UPS-mediated regulation of lipid metabolism, this review aims to provide a broad and comprehensive overview of existing evidence on this topic.**

UPS-mediated control of cholesterol metabolism

Cholesterol is vital in cellular physiology and metabolism by acting as a major structural component of (intra)cellular membranes and as a precursor for the production of bioactive steroids and derivatives. Imbalances in cholesterol metabolism are linked to cancer and cardiovascular and neurodegenerative diseases [5, 6]. Alongside transcriptional regulation, cholesterol homeostasis is maintained via **post-translational mechanisms**. Concerning the latter, ubiquitination is a common post-translational modification controlling cholesterol metabolism (**Figure 1**). The UPS mainly induces negative regulation of cholesterol metabolism, with high cholesterol load promoting ubiquitination of proteins involved in cholesterol biosynthesis and uptake, while reducing that of proteins involved in cholesterol efflux. Vice versa, a decrease in cholesterol levels promotes cholesterol uptake and synthesis by, amongst others, reducing the ubiquitination of proteins involved in these processes, while enhancing the ubiquitination of efflux transporters [7].

Transcriptional regulation of cholesterol metabolism

Essential in preserving cholesterol homeostasis is the transcriptional control of genes involved in the biosynthesis and metabolism of cholesterol. This regulation occurs primarily by the transcription factors Sterol Regulatory Element-Binding Protein (SREBP) and Liver X Receptor

(LXR). While SREBP predominantly promotes the expression of genes involved in cholesterol and fatty acid (FA) synthesis and uptake, LXRs promote the removal of excess cholesterol.

Sterol regulatory element-binding proteins (SREBPs) and its regulatory proteins

SREBPs are a family of membrane-bound transcription factors, with SREBP1a and -1c originating from distinct transcriptional start sites of *SREBF1*, and SREBP2 from *SREBF2*. While SREBP1 preferentially activates genes involved in FA synthesis (discussed later in this review), SREBP2 closely regulates genes involved in cholesterol synthesis and uptake. Under conditions of high intracellular cholesterol, trafficking factor SREBP cleavage-activating protein (SCAP) binds to the ER-resident insulin-induced gene (INSIG) protein, which maintains SCAP on the ER. When cholesterol levels are low, SCAP undergoes a conformational change which induces its detachment from INSIG and allows its association with SREBP. By doing so, SCAP transports SREBP from the endoplasmic reticulum (ER) to the Golgi complex, where it is cleaved by proteases. Cleaved SREBP enters the nucleus and binds sterol-regulatory elements (SRE) in the promotor region of sterol-increasing genes such as HMG-CoA reductase (*HMGCR*), squalene epoxidase (*SQLE*), and low-density lipoprotein receptor (*LDLR*) [8].

SREBP2 was shown to be a substrate for UPS-mediated regulation, and Ring Finger Protein (RNF) 139 was identified as the responsible E3 ligase in HepG2 cells. RNF139 has a sterol-sensing domain and binds the SREBP-SCAP complex upon high cholesterol load, thereby hindering its translocation towards the Golgi [9]. Regulatory proteins INSIG and SCAP are substrates for UPS-mediated degradation as well [10-13]. RNF145 is an ER membrane E3 ligase that triggers the ubiquitination of SCAP, thereby inhibiting SREBP shuttling to the Golgi apparatus upon high cholesterol content. RNF145 expression was induced in both human and mouse primary cells and cell lines upon LXR activation, and in mice after high-cholesterol diet feeding, thereby providing a molecular link between hepatic cholesterol levels and the reciprocal mechanisms of SREBP and LXR signaling [14]. More recently, ER-anchored E3 ligase RNF5 was demonstrated to mediate SCAP ubiquitination in diverse cell lines as well [11]. Finally, INSIG is a target for ubiquitination by gp78 in sterol-depleted cells [12, 13]. INSIG degradation releases the SCAP-SREBP complex to travel to the Golgi for subsequent activation and attenuates degradation of HMGCR, the key rate-limiting enzyme of the cholesterol synthesis pathway.

Liver X Receptors (LXRs)

LXRs belong to the family of cholesterol-sensing **nuclear receptors**. Upon ligand binding, LXRs undergo a conformational change resulting in recruitment of co-activator complexes to DNA-responsive elements (LXRE). This induces the expression of cholesterol-lowering genes, including ATP-binding cassette transporter A1 and G1 (*ABCA1* and *ABCG1*) and apolipoprotein E (*APOE*). LXRs also directly regulate cholesterol homeostasis via the UPS by inducing the transcription of E3 ligases Inducible Degradator of the LDLR (IDOL) and RNF145 [14, 15]. With respect to the former, LXR activation promotes the expression of IDOL, thereby triggering the ubiquitination of LDLR on its intracellular cytoplasmic domain that subsequently directs the receptor towards lysosomal degradation [16]. In parallel, several earlier studies suggested that the UPS controls LXR degradation. With respect to the latter, LXR α ubiquitination reduces ABCG1-mediated cholesterol efflux in interferon- γ (IFN γ)-stimulated macrophages, thereby promoting foam cell formation and atherogenesis [17]. High-density lipoprotein (HDL) tetrapeptide repeat domain protein 39B (TTC39B) was shown to promote LXR ubiquitination and degradation, attenuating the expression of cholesterol-lowering genes and promoting fatty liver formation in mice [18].

De novo cholesterol synthesis

De novo cholesterol biosynthesis is a complex process involving many intermediates and 22 different enzymes. Accumulation of different intermediates and cholesterol derivatives increases the recruitment of INSIG to the sterol-sensing domain of HMGCR to promote its ubiquitination. HMGCR is a multispanning membrane protein and the extraction of ubiquitinated HMGCR is dependent on its dislocation from UbiA Prenyltransferase Domain Containing 1 (UBIAD1) and subsequent translocation to the cytosol by the 'ATPases associated with diverse cellular activities' (AAA ATPase) valosin-containing protein (VCP)/p97 and recruitment factor UBX Domain-Containing Protein 8 (UBXD8). Released HMGCR is subsequently degraded by the 26S proteasome [19-21]. **Several E3 ligases have been proposed to participate in HMGCR ubiquitination, possibly reflecting the high degree of regulation of this central metabolic enzyme. Meanwhile, HMGCR is one of the most intensely studied cholesterol metabolism substrates of the UPS as it receives major clinical interest by being the target of statins, and because it is the key rate-limiting enzyme in the cholesterol synthesis pathway. This might contribute to the fact that divergent E3 ligases are found to regulate**

HMGCR. With respect to the latter, initial studies defined HMGCR degradation protein 1 (HRD1) as being responsible for basal degradation of HMGCR. Stimulated degradation of HMGCR by lanosterol and other sterol derivatives was proposed to be mediated by gp78 and RNF139. Later, the LXR-responsive RNF145 was also proposed to contribute to HMGCR ubiquitination **in diverse cell lines**, likely in concert with gp78 and HRD1 [22]. As such, it is possible that the identification of the E3 partaking in HMGCR ubiquitination is context and signal dependent. In parallel, deubiquitination by ubiquitin-specific protease (USP)20 is essential in stabilizing HMGCR levels in the fed state. Specifically, feeding mice a high-sucrose, low-fat diet stimulated mTORC1 to phosphorylate USP20 at S132 and S134. By doing so, mTORC1 enhanced the USP20-HMGCR interaction and prevented HMGCR degradation. Deletion of USP20 attenuated hyperlipidemia and weight gain, and was suggested as an attractive therapeutic target to reduce cholesterol levels [23].

A second cholesterol biosynthesis enzyme highly regulated by the UPS is SQLE, which catalyzes the first oxygenation of squalene to mono-oxidosqualene downstream of HMGCR. The regulatory domain of SQLE is strongly responsive to sterols, and high cellular cholesterol causes SQLE to undergo a conformational change, allowing its ubiquitination. Membrane-associated ring-CH-type finger 6 (MARCHF6) was identified as the responsible E3 **in HEK293 cells**, which is stabilized by high cholesterol levels itself by inhibiting MARCHF6 autoubiquitination [24, 25]. **Importantly, silencing of MARCHF6 was demonstrated to stabilize basal HMGCR levels as well. Yet, the contribution of MARCHF6 to HMGCR regulation is not fully clear as sterols were still able to promote HMGCR degradation in a mutant 25-hydroxycholesterol-resistant hamster cell line (SRD-1 cells) [24].** In support of MARCHF6 controlling cholesterol synthesis, follow-up studies demonstrated that it drives the ubiquitination of cholesterol synthesis enzymes lanosterol 14 α -demethylase (LDM), dehydrocholesterol reductases (DHCR) 24, and **sterol-C4-methyl oxidase-like (SC4MOL) in diverse cell lines** [26, 27]. Several other cholesterol biosynthesis enzymes, such as DHCR7 and 14, were recently described to be regulated via the UPS, albeit the underlying mechanisms are still poorly understood [28-30].

Cholesterol uptake

As cholesterol biosynthesis is a complex and energy-intensive process, receptor-mediated **endocytosis** often meets cellular cholesterol requirements. Ample evidence demonstrated

that the UPS is crucial in regulating key receptors involved in cellular cholesterol uptake, including LDLR, very low-density lipoprotein receptor (VLDLR), and ApoE receptor 2 (ApoER2). As described above, LDLR is transcriptionally regulated by SREBP2 when cellular sterol content is low. Vice versa, LXRs reduce LDLR protein abundance when sterol levels rise by inducing IDOL-mediated ubiquitination and degradation [15, 31, 32]. IDOL also promotes the ubiquitination and subsequent degradation of related receptors VLDLR and APOER2 [33]. As these receptors are implicated in neuronal development, IDOL is situated at the crossroad of lipid metabolism and central nervous system (CNS) physiology. Accordingly, loss of IDOL in the CNS of mice protects from diet-induced obesity, mimicking the phenotype of a whole-body IDOL knock-out [34, 35]. Therefore, the brain IDOL-VLDLR axis regulates systemic energy homeostasis. As an extra layer of regulation, LDLR was demonstrated to be degraded by proprotein convertase subtilisin/kexin type 9 (PCSK9), a serine protease in which gain-of-function mutations have been linked to familial hypercholesterolemia. In HEK293T cells, PCSK9 co-internalized with LDLR through endocytosis and induced its ubiquitination. This resulted in lysosomal LDLR degradation instead of recycling to the cell membrane [36]. In aggregate, these studies indicate that the UPS curbs cellular cholesterol levels by driving the degradation of various members of the LDLR family [37].

Cholesterol esterification

Cholesterol esterification is essential for cellular storage and transfer of cholesterol, and protects cells from the lipotoxic impact of excess free cholesterol. Cholesterol esterification enzyme acyl-CoA:cholesterol acyltransferase 2 (ACAT2) was shown to be regulated by a less common cysteine-linked form of ubiquitination [38]. Here, treatment of multiple mammalian cell types with sterols or FAs resulted in a rapid increase in ACAT2 protein level through ROS-mediated oxidation of the ubiquitin-conjugation site of ACAT2, which allowed efficient storage of sterols in the cell. The authors identified gp78 and its cofactor INSIG as ACAT2 interactors responsible for its ubiquitination. In addition, ACAT1 is a target for proteasomal degradation, as USP19 was found to enhance cholesterol esterification through deubiquitinating and stabilizing ACAT1 in hepatocellular carcinoma cells [39]. Together, these studies indicate that the UPS controls cholesterol esterification by regulating ACAT1 and 2 levels by divergent E3 ligases.

Cholesterol efflux

As most cell types are unable to metabolize cholesterol, active cholesterol export is essential to reduce intracellular sterol levels and maintain homeostasis. Several ABC transporters, including ABCA1, ABCG1, ABCG5, and ABCG8 have been demonstrated to mediate cholesterol efflux. In mammalian cells, cholesterol efflux is predominantly mediated by ABCA1 and ABCG1, which export free cholesterol towards the extracellular ApoA-I acceptor protein or to HDL, respectively, thereby promoting 'reverse cholesterol transport' [40]. Proteasomal inhibition increases the abundance of both transporters and cholesterol efflux *in vitro* and *in vivo* in macrophages. Yet, the underlying mechanisms underpinning the latter are poorly understood [41, 42]. First, HUWE1 (HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1) and NEDD4-1 (neural precursor cell expressed developmentally down-regulated protein 4-1)-mediated ubiquitination was demonstrated to control ABCG1 in a human monocyte-derived macrophage cell line [43]. In a follow-up study, ABCA1 was identified as a target for HECTD1 (HECT domain E3 ubiquitin protein ligase 1) ubiquitination in non-cholesterol-loaded macrophages as well, without affecting ABCA1 in cholesterol-loaded cells [44]. Later, cullin-3 was proposed to mediate ABCA1 ubiquitination in response to the activation of thrombin-protease-activated receptor (PAR)1 signaling in macrophages and smooth muscle cells, thereby promoting atherosclerotic lesion formation in ApoE^{-/-} mice [45, 46]. Finally, ubiquitin-protein E3 ligase A (UBE3A)-mediated ubiquitination of ABCA1 plays a crucial role in driving myelin-induced foam cell formation in models for CNS repair (unpublished). Lipid accumulation induced a more inflammatory macrophage phenotype, which suppresses myelin repair in an *ex vivo* organotypic brain slice model [47-49]. In aggregate, the UPS plays an important role in regulating cellular cholesterol efflux capacity, although underlying mechanisms are still poorly understood. Identification of responsible E3 ligases and metabolic cues can lead to the discovery of new therapeutic targets for diseases involving cholesterol metabolism.

UPS-mediated control of fatty acid (FA) metabolism

FAs are pivotal in the human body as they are obligatory constituents of membranes, are the most energy-dense substrate for ATP generation, serve as ligands for nuclear receptors, and affect protein function by post-translational acylation of target proteins. FA homeostasis is governed by their uptake, *de novo* synthesis, and oxidation (β -oxidation). As discussed in the

following sections, increasing evidence supports that UPS-mediated protein degradation acts as a crucial regulator for each of these processes (**Figure 2**).

Transcriptional regulation of FA metabolism

Peroxisome proliferator-activated receptors (PPARs) are crucial for maintaining energy homeostasis by regulating glucose, FA, and insulin metabolism at a transcriptional level.

Several studies have shown that the UPS is essential in fine-tuning PPAR γ activity by promoting ubiquitination of its ligand-binding domain upon ligand binding, thereby serving as a negative feedback loop that limits transcriptional activity of PPAR γ [50]. Follow-up research demonstrated that the inflammatory mediator IFN γ enhances proteasomal degradation of PPAR γ **in adipocytes**, resulting in the development of insulin resistance [51]. E3 ligases which ubiquitinate PPAR γ include Siah2 [52] and NEDD4-1 [53]. In contrast, USP22 was found to be involved in PPAR γ stabilization, thereby increasing the expression of **rate-limiting enzyme** acetyl-CoA carboxylase (ACC) and ATP Citrate Lyase (ACLY), and promoting *de novo* FA synthesis in hepatocellular carcinoma cells [54]. Of note, PPAR γ holds E3 ligase activity as well, as its activation induces Lys48-linked ubiquitination of the Rel Homology Domain region of nuclear factor κ B (NF κ B)/P65, which halts pro-inflammatory signaling in cancer tissue [55].

Next to PPARs, SREBP1 plays a key role in regulating FA synthesis. Few studies indicate that the UPS controls SREBP degradation [9, 56, 57]. First, phosphorylation of transcriptional active SREBP1 was demonstrated to serve as a degron for ubiquitination by the Skp1–Cul1–FBXW7 E3 ligase complex (SCF^{FBXW7}), resulting in its proteasomal **degradation in a HepG2 cell line** [56]. **Vice versa, acetylation of SREBP1 improves its stability by competing with ubiquitin for SREBP lysine residues** [58]. Alongside SCF^{FBXW7}, RNF20 mediates proteasomal degradation of SREBP1 upon nutrient deprivation, thereby inhibiting lipogenesis in primary hepatocytes when precursor metabolites are limited [57].

FA synthesis and remodelling

Early studies already demonstrated an essential role for the UPS in controlling **FA synthesis** by Tribbles Pseudokinase 3 (TRB3)-COP1-mediated ubiquitination of ACC and FA synthase (FASN) **in adipose tissue** [59]. Furthermore, USP2 and USP38 were lately reported to deubiquitinate and stabilize ACC and FASN [60]. ACC and FASN deubiquitination enhanced triglyceride production in gastric cancer cells, thereby promoting gastric **cancer** cell growth, migration, and tumorigenesis. Recently, ubiquitination of ACLY, the enzyme responsible for the

conversion of citrate towards acetyl-CoA, was demonstrated [61]. ACLY ubiquitination and degradation enhanced FA oxidation at the expense of *de novo* FA biosynthesis in regulatory T cell (Tregs). The authors further showed that this was closely associated with tumor growth factor- β signaling, a pathway involved in Treg differentiation, which alleviated autoimmunity in a mouse colitis model.

Alongside FA synthesis, the UPS controls FA desaturation as well. In a **murine adipocyte cell line**, stearoyl-CoA desaturase 1 (SCD1), a key enzyme that catalyzes the formation of monounsaturated FAs, was demonstrated to be controlled by the UPS, although E3 ligases involved remain to be uncovered [62]. **FA desaturation plays a vital role in maintaining cellular membrane fluidity, and increased lipid saturation causes membrane rigidification and lipotoxicity. An interesting connection between membrane fluidity, ER-associated degradation (ERAD) and the UPS has been uncovered. In yeast, ERAD was demonstrated to closely regulate membrane fluidity in a ubiquitin-dependent manner [63]. Specifically, increased lipid saturation enhanced ubiquitination of the transcription factor Mga2 by E3 ligase RSP5. Consequently, Mga2 undergoes proteasomal processing, resulting in the production of transcriptionally active Mga2. Activated Mga2 promotes the expression of FA cis- Δ 9-desaturase OLE1, thereby increasing membrane lipid desaturation and restoring membrane fluidity. Another critical player involved in counteracting saturated FA (SFA)-induced membrane rigidification is lipid hydrolase adiponectin receptor 2 (ADIPOR2), which was identified as a substrate of lipid-sensitive RNF145 [64]. In unsaturated membranes, RNF145 remains stable and ubiquitinates ADIPOR2, inducing its degradation. However, when membranes become enriched with SFAs, RNF145 is auto-ubiquitinated, leading to its degradation. The latter stabilizes ADIPOR2 and allows restoring of membrane homeostasis.**

FA uptake

Alongside biogenesis, cellular FA content is regulated via endocytic processes. The FA translocase cluster of differentiation 36 (CD36) is a well-studied receptor involved in the uptake of FAs and is proposed to be sensitive to UPS-dependent regulation [65-68]. Smith *et al.* were the first to report ubiquitin-mediated regulation of CD36 and found that changes in CD36 abundance upon exposure to FAs and insulin are, at least partially, due to changes in its ubiquitination status. While insulin exposure reduced CD36 ubiquitination and increased its membrane abundance in Chinese hamster ovary and HEK293 cells, FA treatment enhanced

CD36 ubiquitination and reduced CD36 levels [65]. In contrast, ubiquitination was demonstrated to play a role in CD36 stabilization as well. Specifically, Parkin-deficient mice show lower hepatocellular C36 levels and are resistant to weight gain, and reconstitution of Parkin increased CD36 and FA uptake in liver cells [66]. Interestingly, Parkin is defective in patients with early-onset PD. Hence, impaired Parkin-mediated CD36 stabilization may provide a molecular rationale for the observed lipid abnormalities in these patients. Later, deubiquitinating enzymes ubiquitin C-terminal hydrolase 1 (UCHL1) and USP14 were shown to decrease oxidized LDL-induced foam cell formation by reducing CD36 ubiquitination, and were suggested to be promising targets for atherosclerosis treatment [67, 68].

FA oxidation

To date, few studies demonstrated a role for the UPS in controlling FA oxidation (FAO), also called β -oxidation. By using **mass spectrometry**, hydroxysteroid 17-beta dehydrogenase 4 (HSD17B4) and carnitine-palmitoyl-transferase 2 (CPT2), enzymes necessary for FAO and transport into mitochondria, were defined as substrates for the E3 ligase HRD1 in human liver HepG2 cells. Loss of HRD1 induced a metabolic profile that favored FAO, inhibited lipogenesis, and protected mice from diet-induced obesity [69]. Later, ubiquitination of CPT2 by HRD1 was confirmed in **human triple-negative breast cancer** (TNBC) cells. As a result, HRD1 expression is decreased in TNBC cells, which enhances FAO and tumorigenesis [70]. HRD1 acts as a homeostatic ERAD-associated E3 and therefore has a promiscuous substrate specificity.

Triglyceride metabolism

Excessive levels of free FAs (FFAs) are toxic as their amphipathic structure can perturb membranes. To prevent lipotoxicity, FFAs can be stored in triglycerides, which are formed from the combination of glycerol and three FA molecules, and are generally stored in **lipid droplets (LDs)**. **When stored energy has to be mobilized**, LD-associated triglycerides can be hydrolyzed by a series of cytosolic lipases in a process called **lipolysis**, **releasing glycerol and FFAs** [71]. Several recent studies demonstrated UPS-dependent regulation of adipose triglyceride lipase (**ATGL**) [72, 73]. First, ubiquitination of ATGL was shown in hepatocytes in a COP1-dependent manner, thereby reducing triglyceride hydrolysis and increasing hepatocellular lipid content. In support of this notion, COP1 inhibition ameliorated hepatic steatosis in high-fat diet-fed mice [72]. Second, RNF213 was demonstrated to ubiquitinate

299 ATGL on LDs, thereby attenuating triglyceride hydrolysis and increasing LD content [73].
300 Whether the UPS affects other lipases or triglyceride synthesis remains to be explored.

301 **Ubiquitination of lipids**

302 Although ubiquitination was formerly considered a protein-linked modification, in the past
303 decade, various other types of substrates such as lipids were found to be targeted by
304 ubiquitination.

305 **Ubiquitination of phospholipids**

306 Phospholipids consist of a hydrophilic glycerophosphate head linked to two hydrophobic FA
307 tails. A recent study by Sakamaki *et al.* showed that ubiquitin can tag phospholipid species
308 [74]. Specifically, endosomal and lysosomal phosphatidylethanolamine (PE) was shown to be
309 ubiquitinated in yeast and human cells by the ubiquitin enzymes Uba1 (E1), Ubc4/5 (E2), and
310 Tul1 (E3). The C-terminal glycine of ubiquitin was found to form a linkage bond with the amino
311 group of PE, resulting in the recruitment of endosomal sorting complex required for transport
312 (ESCRT) components, and was postulated to regulate membrane dynamics and the formation
313 of cellular vesicles. Vice versa, phospholipids control protein ubiquitination. For instance,
314 cardiolipin promotes the auto-ubiquitination of MARCH5, a mitochondrial membrane-
315 resident E3 ligase important in regulating mitophagy [75]. In aggregate, these studies show an
316 intricate relationship between the UPS and phospholipid dynamics.

317 **Ubiquitination of bacterial lipids**

318 In bacteria-infected host cells, ubiquitin is crucial for facilitating targeted xenophagic
319 destruction. Next to tagging bacterial membrane proteins, recent research shows that
320 bacterial lipids are earmarked by ubiquitin as well. Otten and colleagues demonstrated that
321 lipid A, a component of lipopolysaccharide in the outer bacterial membrane, is a target of
322 ubiquitination. They further identified the giant ~600 kDa E3 ligase RNF213 as the responsible
323 enzyme [76]. Hence, lipid regulation by the UPS represents an important defense response
324 against invading pathogenic microorganisms, and is crucial in preserving human health.

325 **Ubiquitin-dependent regulation of lipid droplet (LD) dynamics**

326 LDs can be metabolized to release FFAs and free cholesterol. This dynamic process occurs via
327 cytosolic lipolysis or lipophagy, a selective form of **autophagy** of LDs [77]. To date, mechanisms
328 underpinning LD degradation remain poorly understood, but increasing evidence indicates

that ubiquitination is involved (**Figure 3**). With respect to the latter, ubiquitin can associate with light-chain 3 (LC3, autophagosome marker) and sequestosome 2 (SQSTM2) on LDs during lipophagy in foamy macrophages. The authors identified several LD-associated proteins (LDAPs) related to the ubiquitination machinery, such as ancient ubiquitous protein 1 (AUP1) and UBE2G2, to be required for lipophagy and consequent lipid efflux [78]. Similarly, other studies demonstrated that LDAP stability is closely monitored by the UPS machinery, with LD breakdown and biogenesis in lipid-deprived and -sufficient conditions being associated with increased proteasomal degradation or stability of LDAPs, respectively. Examples of LDAPs that the UPS degrades include perilipins (PLIN) [79], ATGL [80], and LD fusion protein FSP27 (fat-specific protein 27) [81]. In addition, several proteins which function in the ubiquitination pathway or contain ubiquitin-binding motifs have been identified in the LD proteome and can function to regulate UPS-mediated LDAP degradation directly [80, 82, 83]. For instance, it was demonstrated **in mouse embryonic fibroblasts** that UBXD8 can recruit VCP/p97 to LDs, thereby inhibiting ATGL activity and increasing LD size [80]. Also, LD membrane-resident Spartin and AUP1 were identified to serve as adaptors for E3 ligases **in several cell lines**, thereby providing a direct molecular connection between LDs and the ubiquitination machinery [82, 83]. All in all, significant evidence argues for an important role of the UPS in the regulation of the LD proteome and dynamics.

Dysregulated ubiquitination in lipid disorders

Altered lipid metabolism is one of the most significant metabolic changes in cancer cells, and dysregulation of ubiquitination of lipid metabolism proteins was demonstrated to affect tumor development. In line with this, most currently available therapies which target the UPS are developed for malignancies and may mediate their effects, at least partly, through intervening with tumor cell lipid metabolism [84]. Atherosclerosis, a metabolic disorder characterized by disrupted lipid homeostasis and plaque formation, has been linked to altered ubiquitin-mediated regulation of lipid metabolism pathways as well. Here, E3 ligases are shown to be important contributors to the development of this chronic inflammatory vascular disease, and treatment strategies focused at targeting E3 ligases have gained increasing attention in the recent years [85]. Furthermore, UPS dysfunction is an important pathological hallmark of several neurodegenerative disorders such as AD and PD. Specifically, a derangement of the UPS is correlated with toxic amyloid β ($A\beta$) aggregation and dysregulation

of lipid levels in AD [2]. In addition, IDOL is the primary post-translational regulator of LDLR in the brain and was shown to control ApoE-containing HDL particles and A β accumulation, thereby increasing plaque burden and neuroinflammation [86]. Finally, mutations in the E3 ligase gene *PARKIN* are associated with PD development and multiple groups demonstrated that loss of PARKIN leads to lipidomic alterations in the mitochondria of mice in an SREBP-dependent manner [87].

Therapeutic targeting of the ubiquitin-proteasome system

As ubiquitination involves a three-step catalytic pathway with a broad array of proteins involved, multiple strategies could be applied to target the UPS. Bortezomib (trade name Velcade) and its derivatives were the first approved UPS-targeting drugs and showed potent effects for the treatment of multiple malignancies [88]. Later, additional UPS-directed drugs were developed, and it was demonstrated that combining different UPS inhibitors was more effective, while avoiding the development of drug resistance. Yet, given the complex UPS network, it is more interesting to have a selective approach of UPS targeting. One promising avenue involves intervening with substrate-E3 interactions, which are increasingly recognized as attractive targets with substantial potential. However, this approach has been considered one of the most challenging therapeutic strategies due to several factors. The interaction between substrates and E3 ligases occurs over a large and flat interface area, making it difficult to find small molecular ligands for reference. Additionally, the high molecular weight of protein-protein interactions complicates the development of therapeutics. Alternatively, specificity could be provided by targeting E2 or E3 ligases since these determine, respectively, the nature of the poly-ubiquitin chain and substrate specificity. Although proteasomal inhibitors are widely used in the clinic for cancer therapy already, small molecules that target specific E2 or E3 ligases are still under development. The identification of these using high-throughput screens is challenging due to the complexity of the ubiquitination reactions and the weak interaction between ligases and their substrates [89, 90]. As a result, inhibitors remain poorly defined, and further characterization of their structure and mechanisms should benefit the development of future modulators.

Concluding remarks

In the past decades, significant progress has been made in understanding the molecular action of ubiquitination, and increasing evidence shows a crucial role for ubiquitination events in regulating divergent aspects of lipid metabolism. As a result, dysregulation of ubiquitination actions can lead to aberrant activation or repression of lipid metabolism pathways and have been linked to the development and progression of a number of disorders. **Importantly, ubiquitination events are highly context-dependent and can vary significantly between cell types, tissues, and organs. Studies investigating ubiquitination typically focus on specific cell types or tissues, which can limit the generalizability of the findings to other contexts. In addition, despite the existence of a broad range of E3 ligases, certain ligases are more often identified as enzymes responsible for ubiquitinating lipid metabolism substrates. Given this complexity and cell-specific nature of ubiquitination, it is crucial to consider these factors when interpreting experimental data.** Mass spectrometry-based approaches and bioinformatics have emerged as powerful tools to define ubiquitinated substrates and ubiquitin ligases involved in a high-throughput and more global manner. Further identification of underlying enzymes, targets, and their context will improve our understanding of the role of ubiquitination in lipid metabolism, and provide more insights into implications for disease development and treatment (**see Outstanding Questions**).

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410 **Declaration of Interests**

411 The authors declare no competing interests.

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628 **Glossary**

629 **26S Proteasome:** The major protease in eukaryotic cells responsible for degradation of
630 ubiquitinated proteins in both cytoplasm and nucleus.

631 **ATGL:** Adipose triglyceride lipase, an enzyme critical for the release of FAs from TGs on the
632 surface of LDs.

633 **Autophagy:** A homeostatic cellular recycling process that mediates the lysosomal delivery and
634 clearance of various cellular components and damaged organelles in double-membraned
635 vesicles termed autophagosomes.

636 **Beta-oxidation:** Beta-oxidation, also called fatty acid oxidation, is the mitochondrial aerobic
637 process of breaking down a fatty acid into acetyl-CoA units.

638 **Cholesterol esterification:** The linkage of cholesterol with a fatty acid in a reaction catalyzed
639 by ER-resident acyl-CoA:cholesterol acyltransferases (ACAT), also called sterol O-
640 acyltransferases (SOAT).

641 **Endocytosis:** The cellular process by which molecules are brought into the cell.

642 **ERAD:** Endoplasmic-reticulum-associated protein degradation – Degradation of ubiquitinated
643 proteins at the ER.

644 **Fatty acid synthesis:** Initiates with the carboxylation of acetyl-CoA to malonyl-CoA, a
645 conversion catalyzed by the rate-limiting enzyme acetyl-CoA carboxylase (ACC). Next, fatty
646 acid synthase (FASN) converts malonyl-CoA to palmitate, which is a substrate for elongation
647 or desaturation by other enzymes, generating fatty acids with different lengths and saturation
648 degrees.

649 **Lipid droplet (LD):** Ubiquitously expressed organelles in which neutral lipids such as
650 triglycerides and cholesterol esters are sequestered. Emerging evidence indicates that the
651 function of lipid droplets goes beyond mere storage of neutral lipids, as they control cell
652 signaling, gene expression, histone sequestration, and drug activity as well.

653 **Lipolysis:** The hydrolysis of LD-associated triglycerides by the consecutive action of cytosolic
654 adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol
655 lipase (MGL), resulting in the release of free fatty acids and glycerol.

656 **Mass spectrometry:** An analytic technique which determines the mass-to-charge ratio of
657 molecules and allows the identification of proteins.

658 **Nuclear receptors:** A group of proteins which sense certain molecules such as steroids and
659 vitamins and respond by regulating the expression of certain genes.

660 **PPAR:** Peroxisome proliferator-activated receptor; A group of NRs which controls FA and
661 glucose metabolism, and adipogenesis. The PPAR family consists out of PPAR α , β , and γ , which
662 are activated by free FAs, after which they undergo a conformational change that facilitates
663 their heterodimerization with retinoid X receptors (RXRs). The PPAR-RXR complex assembles
664 at PPAR response elements (PPREs) and promotes transactivation of target genes.

665 **Post-translational mechanisms:** Any process which happens with a protein after its
666 translation.

667 **Reverse cholesterol transport:** Movement of cholesterol from the periphery to the liver for
668 elimination out of the body.

669 **Ubiquitin:** A small, highly conserved protein that can be reversibly attached to proteins. It
670 regulates substrate degradation, cellular location, activity, or interaction with other proteins.

671

Elements

Box 1. Protein degradation by the ubiquitin-proteasome system (UPS). The UPS plays a fundamental role in maintaining protein homeostasis and is crucial for many central cellular processes such as apoptosis, cell cycle progression, DNA repair, development, immune responses, and membrane transport. It involves a three-step catalytic cascade in which proteins are marked for degradation by covalent conjugation of **ubiquitin**, a small evolutionarily conserved 76-amino-acid protein. Initially, ubiquitin is activated by an E1 ubiquitin-activating enzyme, generating a high-energy thioester intermediate. Next, ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme that escorts ubiquitin towards an E3 ubiquitin-protein ligase. E3 ligase enzymes mediate the final transfer of ubiquitin towards a protein substrate and can be classified in three different families: RING (really interesting new gene), HECT (homologues to the E6AP carboxyl terminus), and RBR (RING-between-RING) E3 ligases, which differ in their mode of ubiquitin acceptance. Within each group of UPS enzymes, different variants have been identified: 26 for E1, over 105 for E2, and more than 1000 for E3 enzymes, with E3 ligases conferring substrate specificity. E3 ligases recognize a substrate protein by their degron, which is a short amino acid sequence on which ubiquitin is conjugated. Proteins can either be mono- or poly-ubiquitinated, the latter requiring two or more ubiquitination cycles.

Most commonly, ubiquitinated proteins are recognized by the **26S proteasome** for degradation, after which the ubiquitin-chain is disassembled by deubiquitinating enzymes (DUBs), and ubiquitin molecules can be recycled for new ubiquitination cascades [90]. Yet, ubiquitination controls lysosomal degradation, protein trafficking and localization, and cell signaling pathways as well. **The fate of a ubiquitinated protein is determined by the ubiquitin-linkage type and the site of ubiquitin attachment. Ubiquitin can be linked through amino- or hydroxy-ubiquitination, with amino-ubiquitination being the most common linkage form regulating diverse aspects, such as protein degradation, activity, and location. Hydroxy-ubiquitination is less common and has been identified in certain cellular processes, including DNA repair and endocytosis. Ubiquitin can be conjugated to proteins via amide- or ester-bonds, with amide-bonds generally being more stable and involved in proteasomal degradation, and the less common ester-linked conjugates being associated with reversible modifications. Finally, the most well-studied and common sites of ubiquitin attachment are**

703 linkages to lysin residues, N-terminal amino acids, or cysteine residues. This diversity of
704 enzymes, ubiquitin-linkages, and location all contribute to the complexity and versatility of
705 the UPS [91].

Box 2. Degradative pathways for lipid metabolism. Various cellular degradation systems regulate lipid metabolism. Next to the UPS, autophagy and ERAD are important in maintaining lipid homeostasis. Autophagy is involved in the breakdown and recycling of cellular components, including proteins, organelles, and lipids. A type of selective autophagy, called lipophagy, plays a key role in LD turnover. During this process, parts of LDs are sequestered in autophagosomes which fuse with lysosomes. This allows the breakdown of LDs, releasing FFAs and glycerol [92]. ERAD is a cellular quality control mechanism that targets misfolded or unassembled proteins in the ER for proteasomal degradation. More than a dozen E3 ligases have been demonstrated to be involved in the ubiquitination of ERAD substrates, including Hrd1 and gp78. ERAD impacts lipid metabolism by regulating the turnover of ER-resident misfolded proteins involved in lipid synthesis and transport. This prevents their accumulation and potential disruption of cellular lipid homeostasis [93]. In summary, the UPS, autophagy, and ERAD are all important in lipid metabolism through different mechanisms. While the UPS is involved in regulating the degradation of specific lipid-related proteins, autophagy is responsible for LD turnover and recycling, and ERAD ensures the quality control of ER-resident proteins involved in lipid metabolism. Together, these systems maintain cellular lipid homeostasis and prevent the aberrant accumulation of lipids and of damaged or misfolded proteins that can interfere with lipid-related processes.

Table 1. List of ubiquitin ligases which regulate lipid metabolism.

E3	Aliases	Substrates	Function of substrate	References
RING E3 ligases				
COP1	RNF200, CFAP78, FAP78, RFWD2	ACC	Rate-limiting enzyme of FA synthesis	[59]
		FASN	Involved in FA synthesis	[59]
		ATGL	Hydrolysis of triglycerides	[72]
Cullin 3	CUL3	ABCA1	Cholesterol efflux transporter	[45, 46]
FBXW7	SEL-10, FBW7, CDC40, AGO	SREBP1	Regulates the expression of genes involved in cholesterol and FA biosynthesis and uptake	[58]
gp78	AMFR, RNF45, SPG89	INSIG	SREBP-associated molecule	[12, 13]
		HMGCR	Key rate-limiting enzyme of cholesterol biosynthesis pathway	[22]
		ACAT2	Catalyses cholesterol esterification	[38]
HRD1	Synoviolin 1, DER3	HMGCR	See above	[22]
		HSD17B4	Enzyme involved in FAO	[69]
		CPT2	Transport of FA into mitochondria	[70]
IDOL	MYLIP	LDLR	Mediates LDL-derived cholesterol endocytosis	[16]
		VLDLR	Involved in cholesterol endocytosis	[16]
		ApoER2	Involved in cholesterol endocytosis in the brain	[27]
MARCHF6	MARCH6, RNF176, TEB4	SQLE	Enzyme in cholesterol biosynthesis pathway	[24, 25]
		LDM	Enzyme involved in cholesterol biosynthesis	[26]
		DHCR24	Enzyme involved in cholesterol biosynthesis	[26]
		SC4MOL	Enzyme involved in cholesterol biosynthesis	[30]
		PLIN2	LDAP	
RNF5	RMA1, RING5, NG2	SCAP	See above	[11]
RNF20	BRE1A, HBRE1	SREBP1	See above	[57]
RNF139	HRCA1, TRC8	SREBP2	Regulates the expression of genes involved in cholesterol and FA biosynthesis and uptake	[9]
RNF145	/	SCAP	SREBP trafficking factor	[14]
		HMGCR	See above	[94]
		ADIPOR2	Lipid hydrolase which maintains membrane fluidity	[64]
RNF213	KIAA1618, ALO17	ATGL	See above	[73]
		lipid A	Component of lipopolysaccharide (LPS) in the outer bacterial membrane	[76]
Rsp5	/	Mga2	Transcription factor which regulates membrane fluidity	[63]
Siah2	/	PPAR γ	See above	[52]
Tul1	/	phospholipids	Hydrophilic glycerophosphate head linked to two hydrophobic FA tails	[74]
RING-between-RING (RBR) E3 ligases				
Parkin	AR-JP, PARK2, PDJ	CD36	Fatty acid translocase	[66]
HECT E3 ligases				
HUWE1	UREB1, Ib772, KIAA0312	ABCG1	Cholesterol efflux transporter	[43]
NEDD4-1	RPF1	ABCG1	Cholesterol efflux transporter	[43]
		PPAR γ	Nuclear receptor involved in regulating FA metabolism	[53]
HECTD1	KIAA1131	ABCA1	Cholesterol efflux transporter	[44]
UBE3A	E6AP	ABCA1	Cholesterol efflux transporter	<i>unpublished</i>

FIGURE LEGENDS

Figure 1. Role of ubiquitination in cholesterol metabolism. Ubiquitination is a common post-translational modification controlling cholesterol metabolism at multiple levels. Key cholesterol metabolism proteins which are described to be targets for ubiquitination are indicated by the orange ubiquitin (Ub)-circles. Orange frames list identified E3 ligases involved. At the level of transcription, ubiquitination of sterol regulatory element-binding protein 1 (SREBP) and SREBP cleavage-activating protein (SCAP) hinders its translocation to the nucleus. In contrast, ubiquitination of insulin-induced gene 1 (INSIG) allows the SREBP-SCAP complex to travel from the Golgi to the nucleus. Besides inducing the expression of E3 ligase inducible degrader of low-density lipoprotein receptor (IDOL), liver X receptor (LXR) is degraded by the UPS itself. At the level of cholesterol synthesis, both 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) and squalene monooxygenase (SQLE) are substrates for ubiquitination. Ubiquitination induces the degradation of cholesterol uptake receptor low-density lipoprotein receptor (LDLR) and cholesterol efflux transporters ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1). Finally, cholesterol esterification enzyme acyl-CoA:cholesterol acyltransferase (ACAT) was shown to be regulated by E3 ligase gp78. *UBE3A: ubiquitin protein ligase E3A; HECTD1: HECT Domain E3 Ubiquitin Protein Ligase 1; HUWE1: HECT, UBA And WWE Domain Containing E3 Ubiquitin Protein Ligase 1; RNF: Ring finger proteins; MARCH6: membrane-associated ring finger (C3HC4) 6; TRC8: translocation in renal carcinoma, chromosome 8 gene; SCF^{FBW7}: Skp1–Cul1–FBW7 E3 ubiquitin ligase complex. Enzymes in bold denote rate-limiting activity.*

Figure 2. Role of ubiquitination in fatty acid metabolism. Fatty acid (FA) homeostasis is governed by their uptake, de novo synthesis, and oxidation (β -oxidation), and UPS-mediated protein degradation acts as a crucial regulator for each of these processes. FAs activate SREBP1, which regulates FA synthesis and is target for ubiquitination by the Skp1–Cul1–FBW7 E3 ligase complex (SCF^{FBW7}) and Ring Finger Protein (RNF) 20. FAs also activate peroxisome proliferator-activated receptors (PPARs) whose activity is fine-tuned by the UPS. PPARs promote the expression of cluster of differentiation 36 (CD36), a receptor involved in the uptake of FAs which is regulated by the UPS in a PARKIN-dependent manner. Internalized free FAs (FFAs) can be converted to monoglyceride (MGs), diglycerides (DGs), and triglycerides (TGs), which are stored in lipid droplets (LDs). Upon energy demand, TGs are hydrolyzed by

lipolysis which requires the action of adipose triglyceride lipase (ATGL) and is subject to ubiquitination. On the other hand, FFAs can be used to generate unsaturated FAs [monounsaturated (MUFAs) and polyunsaturated FAs (PUFAs)], which requires the action of carnitine palmitoyltransferase 2 (CPT2), which is necessary for FA transport into mitochondria, acyl-protein thioesterases (ACLY), acetyl-CoA carboxylase (ACC), fatty acid synthetase (FASN) and stearoyl-CoA-desaturase-1 (SCD1), which are all targets for ubiquitination. Orange frames list the identified E3 ligases involved. *RNF*: ring finger proteins; *TRB3*: tribbles 3. Enzymes in **bold** denote rate-limiting activity.

Figure 3. Ubiquitination in lipid droplet metabolism. Overview of lipid droplet (LD) and lipophagy proteins which are described to be regulated by ubiquitination. Ubiquitin (Ub) can associate with light-chain 3 (LC3) and sequestosome 2 (SQSTM2) on LDs during lipophagy. Several LD-associated proteins (LDAPs) are related to the ubiquitination machinery, such as ancient ubiquitous protein 1 (AUP1) and ubiquitin conjugating enzyme E2 (UBE2G2). Furthermore, LDAPs adipose triglyceride lipase (ATGL), fat-specific protein 27 (FSP27) and members of the perilipin (PLIN) family are degraded by the ubiquitin-proteasome system (UPS). *EC*: esterified cholesterol; *TG*: triglycerides; *FFA*: free fatty acid; *FC*: free cholesterol.