

## REVIEW

# Mitochondria, mitophagy, and the role of deubiquitinases as novel therapeutic targets in liver pathology

Elham Aryapour | Thomas Kietzmann 

Faculty of Biochemistry and Molecular Medicine, and Biocenter Oulu, University of Oulu, Oulu, Finland

**Correspondence**

Thomas Kietzmann, Faculty of Biochemistry and Molecular Medicine, and Biocenter Oulu, University of Oulu, Aapistie 7C, Oulu 90220, Finland.  
Email: [Thomas.Kietzmann@oulu.fi](mailto:Thomas.Kietzmann@oulu.fi)

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**Abstract**

Liver diseases such as nonalcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), fibrosis, and hepatocellular carcinoma (HCC) have increased over the past few decades due to the absence or ineffective therapeutics. Recently, it has been shown that inappropriate regulation of hepatic mitophagy is linked to the pathogenesis of the above-mentioned liver diseases. As mitophagy maintains cellular homeostasis by removing damaged and nonfunctional mitochondria from the cell, the proper function of the molecules involved are of utmost importance. Thereby, mitochondrial E3 ubiquitin ligases as well as several deubiquitinases (DUBs) appear to play a unique role for the degradation of mitochondrial proteins and for proper execution of the mitophagy process by either adding or removing ubiquitin chains from target proteins. Therefore, these enzymes could be considered as valuable liver disease biomarkers and also as novel targets for therapy. In this review, we focus on the role of different DUBs on mitophagy and their contribution to NAFLD, NASH, alcohol-related liver disease, and especially HCC.

**KEYWORDS**

cancer, DUBs, HIF, liver, mitophagy, NAFLD, NASH, ubiquitylation

## 1 | MULTIPLE FUNCTIONS OF THE LIVER

The liver is an organ with a wide range of functions that are achieved by the cooperation of the different cell types such as hepatocytes, liver resident macrophages (Kupffer cells), hepatic stellate cell (HSCs), liver endothelial cells, and liver-specific natural killer (NK) cells (Pit cells). It is a critical hub for many crucial physiological functions, including macronutrient

metabolism, endocrine control of growth signaling pathways, regulation of blood volume, lipid and cholesterol metabolism, and the metabolic breakdown of xenobiotics. This organ stores fat-soluble vitamins, iron, and copper. It is also involved in enzymatic heme breakdown into bilirubin and its conjugates.<sup>1,2</sup> In addition, the liver assists to remove pathogens and exogenous antigens from the systemic circulation. Thereby, Pit cells and Kupffer cells are involved in the body's immune system.<sup>2</sup>

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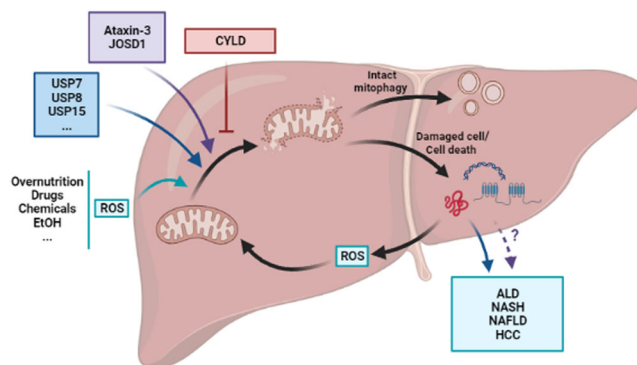
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## 2 | LIVER DISEASE, MITOCHONDRIA, AND MITOPHAGY

Worldwide, millions of people suffer from liver diseases. As a result of improvements in disease prevention, diagnosis, and treatments, viral hepatitis is declining in most developed countries. Many countries have also seen a significant reduction in the number of new cases of hepatitis B following the implementation of expanded immunization programs against the virus. However, as living standards improve, the prevalence of metabolic liver diseases such as nonalcoholic fatty liver disease (NAFLD), or as recently suggested metabolic associated fatty liver disease (MAFLD), and alcohol-related liver disease (ALD) is set to increase, ultimately leading to more end-stage liver diseases like liver failure, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) which is among the malignant tumors with maximum mortality.<sup>3–5</sup>

Recent evidence indicates that hepatic mitochondrial dysfunction, often associated with hypercaloric diet and reduced physical activity, plays an important role in the pathophysiology of NAFLD and ALD. Although mitochondria initially adapt their dynamics and functions to maintain metabolic homeostasis, for example, during NAFLD, it appears that a certain threshold exists where chronic mitochondrial dysfunction contributes to a destructive phase with loss of metabolic homeostasis and promotion of reactive oxygen species (ROS) production, lipid peroxidation, secretion of cytokines, and cell death. The latter is commonly associated with the onset of either ALD or nonalcoholic steatohepatitis (NASH) and progresses with fibrosis and HCC.

Dynamic mitochondrial activities are crucial for liver mitochondrial homeostasis under damage circumstances in both, the adaptation phase as well as in the destructive phase. Thus, to maintain mitochondrial homeostasis, nonfunctional or damaged mitochondria need to be removed from the cell (Figure 1). This occurs in a process known as mitophagy. Mitophagy contributes to the maintenance of hepatic function and to protect the liver from tissue damage by removing mitochondria for lysosomal degradation. Hence, in the adaptive phase, mitophagy is rather associated with metabolic adaptation and homeostasis as with liver diseases, whereas it is associated with liver diseases in the destructive phase. Vice versa, inappropriate regulation of mitophagy is implicated to result itself in cellular damage involving ROS and the appearance of liver-associated diseases, such as steatosis, fibrosis, and cancer (Figure 1).<sup>6,7</sup>



**FIGURE 1** Deubiquitinating enzymes in mitophagy. Several factors such as overnutrition, ethanol consumption, drug abuse, or chemical intoxication cause cellular oxidative stress and mitochondrial damage. To cope with mitochondrial damage and to maintain homeostasis mitophagy is induced. Improper mitophagy or exceeding its capacity contributes to several disease such as ALD, NASH, NAFLD, fibrosis, and HCC. Several DUBs such as USP7, USP8, USP14, USP15, USP30, USP33, USP35, UCHL1, Ataxin-3, and JOSD1 promote mitophagy while so far only one DUB, CYLD, was found to inhibit mitophagy in liver. ALD, alcohol-related liver disease; CYLD, cylindromatosis; HCC, hepatocellular carcinoma; JOSD, Josephin Domain Containing; MAFLD, metabolic associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; UCHL, ubiquitin carboxy-terminal hydrolase; USP, ubiquitin-specific proteases.

## 3 | MITOPHAGY AND THE INVOLVEMENT OF UBIQUITIN LIGASES AND DEUBIQUITINATING ENZYMES

Mitochondria are involved in autophagy regulation as membrane sources and signaling platforms. Damaged mitochondria are removed via mitophagy as part of general autophagy. Thereby, protein ubiquitination via mitochondrial E3 ligases plays an integrative role for degradation of mitochondrial proteins and those proteins localized on the outer mitochondrial membrane (OMM).<sup>8</sup>

Primarily, the phosphatase and tensing homolog (PTEN)-induced kinase (PINK1) and the ubiquitin E3 ligase parkin (parkin/PRKN) are involved in degradation of damaged mitochondria through mitophagy. In healthy mitochondria, PINK1 is normally transported from OMM to the inner mitochondrial membrane (IMM) and cleaved by the presenilin-associated rhomboid-like protein and mitochondrial processing peptidase. Subsequently, cleaved PINK is retro transported to the cytosol where the ubiquitin proteasome system quickly ubiquitinates and degrades the cleaved PINK1. When mitochondria are depolarized, PINK1 cannot be cleaved and instead accumulates at the OMM. This leads to an

increased level of PINK1 at the OMM and phosphorylation of ubiquitin chains that are attached at the OMM. Subsequently, this recruits the E3 ligase parkin to the OMM and leads to its phosphorylation as well as activation. The activated parkin ubiquitinylates the mitochondrial membrane proteins and triggers the recruitment of autophagy receptors such as optineurin, calcium-binding and coiled-coil domain 2 (CALCOCO2), also known as NDP52, and Tax1 binding protein 1 (TAX1BP1).

As a result, the autophagosome is assembled where mitochondrial constituents are finally degraded. In addition to PINK1 and parkin, mitophagy can also be triggered by other OMM receptors such as BCL2 interacting protein 3 (BNIP3), FUN14 domain-containing protein 1 (FUNDC1), neuronal interacting factor X 1 (NIX1), cardiolipin, ceramide, autophagy and beclin 1 regulator 1 (AMBRA1), BCL2 Like 13 (BCL2L13), FKBP prolyl isomerase 8 (FKBP8), NLR family member X1 (NLRX1), and prohibitin 2 (PHB2). The genetic depletion of *PARKIN* increases acute and chronic injury and, acute alcohol binge induced liver injury and steatosis.<sup>7,9,24</sup>

Of note, several other E3 ligases such as mitochondrial E3 ubiquitin protein ligase 1 (MUL1), glycoprotein 78 (Gp78), E3 ubiquitin-protein ligase SMURF1, and E3 ubiquitin-protein ligase HUWE1, have been observed to play a role in mitophagy in addition to parkin. MUL1 was found to act simultaneously with parkin to ubiquitinate the OMM proteins mitofusin-1 (MFN1) and mitofusin-2 (MFN2) in depolarized mitochondria of *Drosophila* and mammalian cells. In a study, single mutant *pink1*, *parkin*, or *mul1* flies were compared to double-mutant models (*pink1/mul1* and *parkin/mul1*), with the double-mutant flies representing a higher mortality, muscle degeneration, damaged mitochondria, and decreased levels of adenosine triphosphate (ATP). However, overexpression or lack of MUL1 in *PARKIN* expressing cells did not affect parkin translocation to depolarized mitochondria suggesting that MUL1 can act *PARKIN*-independent.

GP78 is already known to play a role in endoplasmic reticulum linked degradation. However, studies indicate that overexpression of GP78 also induces the ubiquitination of MFN1/MFN2 leading to their degradation and enhanced mitochondrial fragmentation and mitophagy. Notably, knockdown of GP78 induces mitofusins' levels and decreases depolarization-induced mitophagy independent of parkin.

SMURF1 is an E3 ubiquitin ligase that is also involved in mitophagy. The C2 domain of this ligase is essential for engulfment of injured mitochondria by autophagosomes. It was reported that *Smurf1* lacking mice have a buildup of damaged mitochondria in several organs including heart, brain, and liver.<sup>9,10</sup>

In addition, the E3 ubiquitin ligase HUWE1 has been identified to participate in mitophagy by acting as a co-factor of the autophagy and beclin 1 regulator 1 (AMBRA1) mitophagy receptor. Thereby, HUWE1 removes MFN2 from the OMM and permits that AMBRA1 can be posttranslationally controlled via phosphorylation on serine 1014. This phosphorylation is carried out by the IKK $\alpha$  kinase and promotes interaction of AMBRA1 with LC3/GABARAP (mATG8) proteins and induces mitophagy activity. Overall, these results suggest that AMBRA1 regulates mitophagy via a pathway, in which HUWE1 and IKK $\alpha$  are crucial factors.<sup>11</sup>

## 4 | MITOPHAGY IN ALD AND NAFLD

ALD is the main cause of acute and chronic liver disease. Ethanol consumption results in an extensive ethanol-induced hepatocellular ROS production and mitochondrial depolarization which are reported to cause mitophagy (Figure 1).<sup>12</sup>

Mitochondrial dysfunction is also believed to be a hallmark of NAFLD by considering the crucial role of the mitochondria in fatty acid metabolism and energy generation. Changes in these physiological processes, for example, due to overnutrition are critical for the development of NAFLD. It is understood that mitochondrial dysfunction rises in NAFLD due to alterations in electron transport chain complexes and the formation of a proper membrane potential ( $\Delta\psi_m$ ) as well as reduced ATP production.

In addition, swollen hepatocellular mitochondria followed by loss of cristae were reported in NASH patients. Subsequently, these abnormal mitochondria were identified with decreased activity of respiratory chain enzyme complexes. Both, lower levels of  $\beta$ -oxidation and induced lipogenesis cause lipid accumulation in hepatocytes, and the side production of ROS causing hepatocyte injury promote hepatic inflammation and fibrosis via Kupffer cell and HSCs activation.<sup>9,13</sup>

By removing depolarized mitochondria and lipid molecules, mitophagy can efficiently disrupt ALD, NAFLD, and NASH development, thereby protecting hepatocytes and decreasing liver injury.<sup>14</sup>

## 5 | LIVER CANCER AND MITOCHONDRIAL HOMEOSTASIS AND DYNAMICS

The inflammatory hepatic diseases discussed above are known risk factors for development of liver cancer. As the chronic inflammation affects not only hepatocytes

but also other cell types of the liver including NK and natural killer T cells,  $\gamma\delta$  T cells, Kupffer- and biliary epithelial cells as well as extrahepatic macrophages, the cancerogenic process is highly complex and not limited to a single cell type.

Tumor cells that had undergone malignant transformation display a series of features such as apoptosis resistance, derestricted cell metabolism, and endless ROS production, cell hyperproliferation, the ability to invade as well as loss of mitochondrial integrity and uninhibited mitophagy. There are data showing that mitochondrial dynamics in cancer cells as well as in immune cells play a role in tumor growth, tumor aggression, and defense. In addition to the major signaling relays that play a role in HCC development, such as tumor necrosis factor- $\alpha$ , nuclear factor kappa B (NF- $\kappa$ B), interleukin-6, and c-Jun N-terminal kinase pathways, especially loss of mitochondrial integrity and deregulated mitophagy appear to contribute to the transition of inflammation to cancer.<sup>15</sup> In particular, the primary trigger for this transition is cell death associated with mitochondrial permeabilization, and release of ROS, the proteins BAX and BAK, caspase-activating proteins, as well as mitochondrial DNA, and RNA. The latter two trigger activation of different pattern recognition receptors, the initiation of a type I interferon response and together with ROS activation of NF- $\kappa$ B. In addition, NF- $\kappa$ B appears to be crucial for activating hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ),<sup>16</sup> expression of which increases the transition from NASH to HCC<sup>17</sup>; a similar response on HIF-1 $\alpha$  can also be exerted by other inflammatory mediators such as tumor necrosis factor- $\alpha$ , interleukin-6, and oncostatin M.<sup>18,19</sup> Consequently, HIF-1 regulated genes that are involved in proliferation and promotion of tumor growth are strongly expressed.

At the same time, dysregulated expression of mitochondrial dynamic proteins such as dynamin-related protein 1 (DRP1), MFN1, and MFN2 is reported in several cancers. Additionally, downregulation of MFN2, for example, in breast tumors is associated with poorer outcomes. Further, studies have indicated mitochondrial fusion increases tumor cell resistance to apoptosis, while mitochondrial fission promotes tumor cell invasion and proliferation.<sup>20</sup>

With respect to HCC, several studies indicate changes in mitochondrial fusion proteins. Some data have described a shorter mitochondrial length in HCC tissues compared to control tissues. These findings point to unbalanced mitochondrial fusion and fission in HCC cells. Downregulated levels of MFN1 protein and messenger RNA (mRNA) have been shown in HCC tissue compared to control tissue. Moreover, lack of MFN1 protein in human HCC associates with vascular

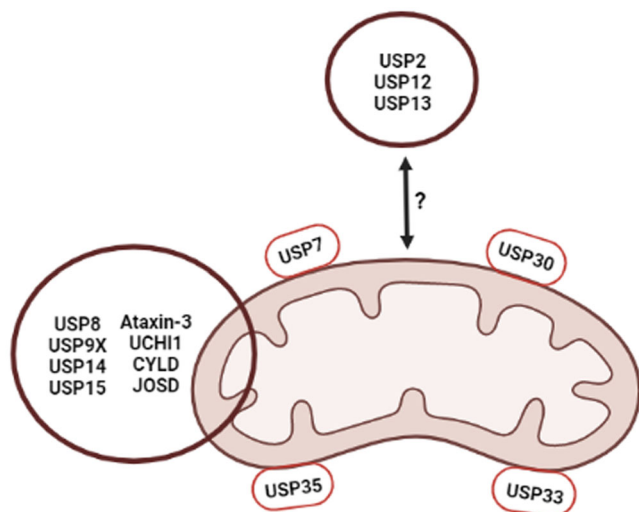
invasion and poor survival. Further, expression of MFN1 is reduced in distant metastases of HCC compared to primary HCC suggesting MFN1 to be associated with metastasis in HCC.<sup>21</sup> Other studies have revealed enhanced mitochondrial fission as a protumorigenic event and linked it with smaller mitochondrial size and increased expression of DRP1 protein and mRNA in HCC. These findings show that enhanced mitochondrial fission has an important role in regulating HCC cell survival. Further, these findings indicate that mitochondria and the process leading to their fission/fusion or mitophagy are crucially contributing to liver disease and HCC.

## 6 | DUBS, MITOPHAGY, AND LIVER CANCER

To prevent mitochondrial ubiquitylation and mitophagy, a factor should be able to inhibit mitochondrial ubiquitylation or improve the deubiquitylation process. The enzyme group that reverses the action of ubiquitin ligases are identified as deubiquitinating enzymes (DUBs). According to studies, DUBs can be localized directly to mitochondria, or to the cytosol and regulate mitophagy via different approaches, that is, by regulating the stability of parkin, by antagonizing the activity of parkin, and by regulating the level of proteasome activity and autophagy (Figure 2).

About 100 human DUBs are known that can be classified into seven subgroups, based on their sequence and structure. Ubiquitin-specific proteases (USP), ubiquitin carboxyl-terminal hydrolases (UCH), ovarian tumor domain proteins (OTU), Ataxin-3-like proteins (Josephin), MIU-containing new DUB family (MINDY), and zinc-finger ubiquitin protease (1 ZUP1/ZUFSP) are six cysteine protease families with ubiquitin peptidase activity and ubiquitin-deconjugating isopeptidases. The last family, known as Jab/MPN domain-associated metalloisopeptidases (JAMM), are zinc-dependent metalloenzymes.<sup>22</sup>

Removing ubiquitin chains from proteins is the main role of DUBs resulting in protein stabilization and protection from proteasomal degradation. Further, DUBs are responsible for maturation of ubiquitin precursors, and recycling of ubiquitin.<sup>23</sup> Numerous studies verified that specific DUBs are involved in multiple types of autophagy pathways such as removing the ubiquitin- and PARKIN-mediated signals thus postponing or disrupting mitophagy.<sup>24</sup> Several DUBs have been reported to be involved in mitophagy<sup>25</sup> and several DUBs with crucial roles in mitophagy and HCC will be described in the following part.



**FIGURE 2** DUBs and mitochondrial localization. Among DUBs that are involved in mitophagy USP7, USP30, USP33, and USP35 are localized to the outer mitochondrial membrane. USP8, USP9X, USP14, USP15, Ataxin-3, UCHL1, CYLD and JOSD are nonmitochondrial DUBs involved in mitophagy. The DUBs USP2, USP12, and USP13 have links to mitophagy that are not completely understood. CYLD, cylindromatosis; JOSD, Josphin Domain Containing; UCHL, ubiquitin carboxy-terminal hydrolase; USP, ubiquitin-specific proteases.

## 7 | MITOCHONDRIA-ATTACHED DUBS DIRECTLY INVOLVED IN MITOPHAGY

### 7.1 | Ubiquitin specific protease 7 (USP7)

USP7, also known as HAUSP, is an evolutionarily conserved protein that is mainly found in the nucleus but also localizes to the mitochondria. A recent study showed that USP7 mediates excessive hepatic lipid accumulation and liver steatosis, the contributing factors of NAFLD, NASH, and HCC via deubiquitylation and transcriptional activation of Zinc finger protein 638 (ZNF638).<sup>26,27</sup>

In addition, varied expression of USP7 has been reported in different human tumor types including HCC where the expression of USP7 is higher than in matched peritumoral tissues. Altered expression of USP7 seems to promote HCC growth at two levels. First, USP7 overexpression stabilizes thyroid hormone receptor-interacting protein 12 (TRIP12), also known as the E3 ubiquitin ligase ULF, that constitutively ubiquitylates and degrades the tumor suppressor p14 (ARF) by deubiquitylation. Second, USP7 affects p53 signaling and mitochondrial apoptosis and mitophagy in response to stress.

Thereby, nuclear USP7 was found to stabilize p53 by deubiquitylation as well as to contribute to the stabilization of the E3 ubiquitin ligases MDM2 and MDM4. Stress, such as severe DNA damage, induces p53 translocation from nucleus to mitochondria with subsequent outer membrane permeabilization. Thereby, homeodomain-interacting protein kinase 2 (HIPK2) mediated p53 phosphorylation reduces its MDM2 mediated polyubiquitylation and degradation and facilitates its monoubiquitylation. The monoubiquitylation of p53 greatly promotes its mitochondrial translocation. Upon arrival at the mitochondria, p53 undergoes fast deubiquitylation by mitochondrial USP7 and then interacts with MDM4, BCL2/BCLXL and other proteins on the OMM to induce membrane rupture and depolarization, and mitophagy or apoptosis.<sup>26–28</sup>

### 7.2 | USP30

USP30, a mitochondrial deubiquitinating enzyme, is embedded in the OMM. Its catalytic domain is facing the cytoplasm, thereby, it can directly interact with parkin. USP30 upregulation reverses ubiquitin chain-linkages on OMM proteins that are added by parkin. Thereby, USP30 especially removes K6- and K11-linked ubiquitin chains from mitochondria. However, USP30 overexpression in cell lines is more promiscuous and eliminates both noncanonical (K6, K11) and canonical (K48, K63) chains. Notably, lack of USP30 resulted in an enhancement of K6-linked chains in cells treated with mitochondrial uncouplers. This suggests, the main targets of USP30 are K6-linked ubiquitin chains. Studying the initial phases of parkin translocation showed that overexpression of USP30 decreases parkin recruitment to altered mitochondria. It is in line with the role of USP30 in eliminating ubiquitin from mitochondrial proteins. In addition, lack of USP30 induced ubiquitination of some of mitochondrial parkin substrates. Notably, USP30 knockdown improves mitophagic flux in cultured neurons or in Hela cells. In mutant parkin cell lines, USP30 knockdown enhanced autophagy receptor sequestosome 1 (SQSTM1) levels, and its recruitment to damaged mitochondria rescued mitophagy deficiency.<sup>29,30</sup>

Further, USP30 interacts with glycerone phosphate O-acyltransferase (GNPAT) and DRP1 whereby the latter is deubiquitylated and stabilized. As a consequence of that reaction, mitochondrial morphology and hepatocarcinogenesis are altered. In addition, inhibition of GNPAT and DRP1 significantly reduced hepatocarcinogenesis.<sup>31</sup> These findings suggest that USP30 and the mediated balance between DRP1 and GNPAT have an important

role in maintenance of mitochondrial homeostasis disturbance of which adds to the progress of HCC.

### 7.3 | Ubiquitin-specific protease 33 (USP33)

The deubiquitinase USP33 is another mitochondrial deubiquitinase localized at the OMM. USP33 deubiquitinates the E3 ubiquitin-protein ligase parkin (PRKN). Vice versa, USP33 deficiency increased K63-linked PRKN ubiquitination. This ubiquitination was significantly enhanced by mitochondrial depolarization. In addition, lack of USP33 in U2OS cells caused PRKN stabilization and translocated it to depolarized mitochondria resulting in mitophagy development.<sup>32</sup>

Studies have shown that abnormal expression of USP33 is involved in development of different types of cancer such as breast, colorectal, gastric cancer, and HCC. In HCC tissues, USP33 expression level is high and correlates with a poor outcome in HCC patients that is in line with the results of The Cancer Genome Atlas (TCGA) database. Additionally, results from *in vivo* and *in vitro* studies showed that USP33 knock-down inhibits invasion, migration, and metastasis in HCC cells. USP33 regulates c-Met expression by increasing the protein stability of the transcription factor SP1 that binds to the c-Met promoter and upregulates c-Met expression. These findings show that USP33 acts as a tumor promoter in HCC.<sup>33</sup>

### 7.4 | USP35

USP35 is another mitochondrial localized USP that is dissociated upon mitochondrial depolarization during mitophagy induction and quickly translocates to the cytosol. It is not well-understood how the USP35 complex (short-USP35 and long-USP35) contributes to mitochondrial quality control, but some evidence suggests that USP35 acts as a housekeeping factor in healthy mitochondria since it maintains the level of mitochondrial morphology proteins such as MFN2. As a result of mitochondrial depolarization, the USP35 complex detaches from altered mitochondria, allowing PARK2 to perform its function.<sup>34</sup>

Clinical studies revealed that USP35 expression is upregulated in HCC when compared to corresponding normal tissues.<sup>35</sup> The upregulation in USP35 seems to be not limited to HCC as other studies showed also upregulation of USP35 expression in lung, ovarian, colon adenocarcinoma, and head and neck cancer. USP35 is also involved in breast cancer progression by stabilizing

and increasing transcriptional activity of estrogen receptors.<sup>35-37</sup>

## 8 | NONMITOCHONDRIAL DUBS DIRECTLY INVOLVED IN MITOPHAGY

### 8.1 | Ubiquitin-specific protease 8 (USP8)

The USP8 is a cytosolic prototypic multidomain deubiquitinating enzyme having pleiotropic roles. USP8 has a significant role in parkin-mediated mitophagy. To recruit parkin to depolarized mitochondria and to eliminate them through mitophagy, USP8 specifically removes K6-linked ubiquitin chains from parkin. Thereby, deubiquitination of K6-linked ubiquitin by USP8 plays a crucial role in mitochondrial quality control.<sup>38</sup>

When studying the expression of USP8 in HCC and its roles in signaling, it was found that this protein is upregulated in HCC compared to normal liver tissue. Inhibition by an USP8 inhibitor and by genetic approaches delayed growth and caused apoptosis in both chemotherapy sensitive and resistant HCC cells. Notably, inhibition of USP8 significantly enhanced efficacy of chemotherapeutic drugs (doxorubicin or sorafenib) in mouse models and HCC cells. Furthermore, USP8 inhibition reduced levels of multiple receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR), and tyrosine-protein kinase Met (c-MET) up to 90%. As shown by decreasing phosphorylated AKT kinase-transforming protein (p-Akt), phosphorylated signal transducer and activator of transcription 3 (p-STAT3), and phosphorylated RAF proto-oncogene serine/threonine-protein kinase (p-RAF) levels, USP8 inhibition also disrupted downstream signaling regulated by RTKs in HCC cells.<sup>39</sup>

Another study shows that USP8 participates in the cellular signaling pathways mediated by TNF receptor-associated factor 6 (TRAF6). TRAF6, TGF-Beta activated kinase 1 (MAP3K7) binding protein 2 (TAB2), mitogen-activated protein kinase kinase kinase 7 (TAK1), p62, and Beclin 1 (BECN1) are factors with an important role in NF- $\kappa$ B activation and autophagy induction. USP8 induces the deubiquitylation of these factors. Patients suffering from HCC with low USP8 mRNA expression had significantly shorter survival time, while there was no significant difference in patients suffering from bladder, breast, colon, kidney, brain, lung, ovary, pancreas, rectum, esophageal, head and neck squamous, stomach carcinoma, glioblastoma, acute myeloid, skin cutaneous melanoma, and leukemia. TCGA and

transcriptome analyses on *USP8* knockout cells showed a noteworthy association between *USP8* and *TRAF6*, *TAB2*, *TAK1*, *p62*, and *BECN1* mRNA levels. In addition, *NF- $\kappa$ B*-dependent, autophagy-related cancer development, and metastasis-associated genes such as forkhead box protein P3 (*FOXP3*) and peroxisome proliferator-activated receptor gamma were increased by lipopolysaccharide stimulation in SK-HEP-1 human hepatic adenocarcinoma cells.

Additionally, mice transplanted with *USP8* knockout cells showed an enhanced migration and invasion of cancer cells upon stimulation of Toll-like receptor 4 (*TLR4*), as well as an increase in tumorigenicity and metastasis. *TLR4* has central role in HCC genesis by promoting the malignant transformation of epithelial cells and these data show that *USP8* negatively regulates *NF- $\kappa$ B* activation and mitophagy induction.<sup>40</sup> Together, *USP8* seems to play a role in mitophagy and to link HCC development with immune function.

## 8.2 | Ubiquitin specific peptidase 9 X-linked (*USP9X*)

*USP9X* is a USP member with a N-terminal ubiquitin-like domain that is highly conserved from *Drosophila* to mammals demonstrating an important role in various cellular functions.<sup>41,42</sup>

It is reported that *USP9X* affects mitochondrial quality and cancer progression. Lack of *USP9X* induces mitochondrial fission in prostate cancer cells. In cell lines with lower levels of *USP9X*, mitochondria carry shorter tubules and the level of p-DRP1, a GTPase that controls mitochondrial fission/fusion balance, was induced.<sup>43</sup>

Interestingly, *USP9X* is involved in the regulation and stabilization of *SMAD1*, a member of the *SMAD* protein family involved in *TGF $\beta$*  signal transduction and hence fibrosis. Consequently, enhanced *USP9X* levels would promote fibrosis by preventing *SMAD1* degradation. This scheme is receiving positive feedback from the action of runt-related transcription factor 1 (*RUNX1*) that was reported to be increased in a mouse model with liver fibrosis. *RUNX1* is able to bind to the *USP9X* promoter and to induce its expression. As a result, the enhanced *USP9X* levels would contribute again to *SMAD1* stabilization. Vice versa, experiments in mouse models with liver fibrosis have shown that lack of *usp9x* reduces migration and viability of HSCs.<sup>44</sup> Together, *USP9X* appears to be a crucial DUB that can regulate mitophagy and HSC activation in a feedback loop involving *SMAD1* and *RUNX1*. The level of *USP9X* expression is also significantly upregulated in HCC tissues and correlates with tumor size and microvascular

invasion. By contrast, *USP9X* depletion causes significant proliferation inhibition in HCC cells. Furthermore, *USP9X* knockdown in the human HCC cell line MHCC97H caused a significant decrease in beta-catenin expression<sup>41</sup> suggesting a role for *USP9X* in the regulation of beta-catenin that is known to be involved in HCC cell proliferation.

## 8.3 | Ubiquitin-specific protease 14 (*USP14*)

*USP14*, a critical enzyme for proteome homeostasis, is one of the major proteasome-associated deubiquitinating enzymes.<sup>45</sup>

*USP14* inhibition, both genetically and pharmacologically, promotes mitophagy in the absence of *PINK1* and *PARKIN*, two well-known mediators of mitophagy. By mitochondrial fragmentation and mitochondrial membrane rupture, *USP14* induces mitophagy through exposure of the *LC3* receptor prohibitin. Inhibition of *USP14* via genetic or pharmacological approaches resulted in mitochondrial dysfunction correction in Parkinson's disease, that is correlated with diminished mitochondrial quality control.<sup>46</sup>

Activation of *USP14* promotes tumor progression in HCC by playing an oncogenic role. Studies on *USP14* expression in tumor tissues revealed that *USP14* is highly expressed in tumor tissues, compared to adjacent noncancerous and normal tissues. *USP14* upregulation was correlated with some pathological symptoms, such as progressing tumor stage. According to a Kaplan–Meier curve study, patients with HCC having *USP14* upregulation showed a worse prognosis after surgery compared to patients with lower *USP14* expression levels. Further, cell proliferation and cell cycle were altered, and cell apoptosis was induced in *USP14* human hepatocarcinoma knockdown cells. In addition, the *Wnt*/beta-catenin pathway was activated in HCC patients overexpressing *USP14*. Thus, these studies suggest that *USP14* is involved in the progression of HCC and could be a therapeutic target in HCC.<sup>47</sup>

## 8.4 | *USP15*

*USP15*, another member of the USP family, counteracts parkin-mediated mitophagy. In particular, the mitophagy in fibroblasts with parkin RBR E3 ubiquitin protein ligase (*PARK2*) mutations and decreased parkin levels is rescued by *USP15* knockdown. While *USP15* acts against parkin-mediated mitochondrial ubiquitination, this protease has no effect on the ubiquitination level of parkin

itself and is also not involved in parkin translocation to mitochondria.<sup>48</sup>

The expression level of USP15 is not constant during progression of HCC. USP15 is overexpressed in HCC and metastatic tissue compared with normal tissue. Further studies in patients with HCC revealed the positive correlation of USP15 expression with reappearance of HCC. Patients having higher USP15 expression also showed a shorter disease-free survival period than those with low USP15 expression. In addition, apoptosis induction and proliferation inhibition can be caused by downregulation of USP15 expression.<sup>49</sup>

## 9 | ATAXIN-3 (ATXN3)

ATXN3 is a significant deubiquitylation enzyme which was first recognized as a transcript from patients with Machado-Joseph disease, also identified as spinocerebellar ataxia type 3 (SCA3).<sup>50,51</sup>

To regulate PARKIN, ATXN3 employs a different mechanism than being a DUBs. Interestingly, ATXN3 forms a complex with PARKIN and the E2 conjugating enzyme. The formation of this complex impedes proper charging of the E2 with ubiquitin and promotes ubiquitin transfer from PARKIN away to ATXN3. As a result, ATXN3 counteracts PARKIN self-ubiquitination and clearance of mitochondria that have lost their membrane potential. Moreover, ATXN3 is able to regulate beclin-1 dependent starvation autophagy. Thereby, the interaction between ATXN3 and beclin-1 enabled deubiquitylation of beclin-1 and protected it from proteasomal degradation to enhance autophagy. This is in line with a rise in parkin self-ubiquitination and ubiquitination of the large GTPases MFN1 and MFN2 during autophagy.<sup>52</sup>

Furthermore, ATXN3 upregulation inhibited the expression of PTEN and triggered the AKT/mTOR pathway. By contrast, ATXN3 inhibition repressed the expression of p-AKT and p-mTOR.<sup>50</sup> Considering that the PI3K-PTEN-AKT-mTOR pathway plays a crucial role in cell growth and is an important driver for different types of human cancer. ATXN3 might have a role in HCC as well, although to date no study has explained the role of ATXN3 in detail.<sup>53</sup>

However, a potential function for HCC may be derived from testicular cancer where ATXN3 overexpression enhanced cell proliferation, and ATXN3 knockdown inhibited cell proliferation. Further studies show that liver cirrhosis patients with different ATXN3 rs8021276 genotypes had different expression levels of ATXN3 protein, suggesting that AT3 polymorphisms could be a risk biomarker for liver cirrhosis and eventually HCC.<sup>51</sup>

## 9.1 | Cylindromatosis (CYLD)

CYLD is a deubiquitylation enzyme with a role in the regulation of different cellular pathways like inflammation, fibrosis, and cancer. It mainly functions through deubiquitylation of specific substrates (such as BCL3, TRAF2, TRAF6, and NEMO) in diverse signaling pathways like NF- $\kappa$ B, Notch, and JNK. CYLD is involved in hepatic homeostasis and restoration following liver damage. Lack of CYLD in animal models causes acute and chronic liver injury, followed by progression to HCC. This is facilitated by a change in the balance between profibrogenic, pro-inflammatory, and pro-oncogenic processes.<sup>54</sup>

Although a recent study reported that CYLD could be localized to mitochondria with a threshold of 20%, there are no data about involvement of CYLD in mitophagy. However, it is reported that CYLD mediated deubiquitylation of receptor associating serine/threonine-protein kinase 1 (RIPK1) is a critical step in necrosome formation. Apart, RIPK1 is involved in many cellular pathways related to both cell survival and death including apoptosis and necroptosis. The formed necrosome shreds the cell membrane and also causes reduction of ATP and loss of mitochondrial membrane potential<sup>54</sup> implying that CYLD could affect mitophagy.

A recent study revealed that *CYLD* knockout in mice induces the resistance of hepatocytes to TNF- $\alpha$  and CD95 caused apoptosis. This indicates an important role in antiapoptotic NF- $\kappa$ B action subsequent to *CYLD* deletion. Therefore, inhibition of CYLD suggests a potential treatment method for acute and chronic liver injury activated by death receptor-induced apoptosis of hepatocytes.<sup>4,55,56</sup>

Further studies show a mechanism via that CYLD acts as a tumor suppressor protecting cells from hepatocellular damage and fibrosis. *CYLD* knockout mice were susceptible to hepatocellular injury, inflammation, fibrosis, and having lower hepatocyte growth factor (HGF) levels, than wild-type animals. The downregulation of HGF occurred in HSCs via binding of histone deacetylase 7 (HDAC7) to the promoter of the HGF gene; while in wild-type cells, CYLD removed HDAC7 from the HGF promoter to enhance expression of HGF. Of note, this interaction happened independent of the deubiquitinase activity of CYLD.<sup>4</sup>

Notably, mice lacking only the deubiquitinase function of CYLD, expressing the mutant CYLDC/S protein, had signs of enhanced aging in many organs such as skin, thymus, pancreas, liver, and lung. Moreover, they spontaneously developed tumors of diverse origin.<sup>57</sup>



## 9.2 | Ubiquitin carboxy-terminal hydrolase L1 (UCHL1)

UCHL1 is a member of a DUBs family that generate ubiquitin monomers by hydrolyzing small C-terminal adducts of ubiquitin.<sup>58</sup>

The cytosolic form of UCHL1 affects mitochondria by regulating MFN2. Knockdown of UCHL1 revealed a significant reduction of MFN2 and an induction of mitochondrial respiratory capacity in neuroblastoma SH-SY5Y cells. In addition, UCHL1 affects proteasomal activity because it also associates with pathological  $\alpha$ -synuclein accumulation and protein aggregation<sup>59</sup>

A significant induction of UCHL1 is associated with HSCs activation and their role in fibrogenesis. Furthermore, genetic and pharmacological inhibition of UCHL1 in mice resulted in prevention of HSC proliferation and showed a therapeutic effect to prevent progression of fibrosis.<sup>60</sup>

Other studies showed that UCHL1 protein expression is increased in ALD in correlation with increased  $\alpha$ SMA levels in human liver. In addition, UCHL1 has not only an important role in fibrosis but also in regulating epithelial to mesenchymal transition (EMT) and expression of pluripotency markers, chemoresistance, and sphere-forming ability in cancer stem-like cells (CSC). The effect of UCHL1 overexpression on CSCs was suppressed by inhibiting the PI3K/AKT pathway which suggests a role of UCHL1 in liver fibrosis and cancer.<sup>58,61</sup>

## 9.3 | Josephin Domain Containing 1 (JOSD1)

JOSD1 appears to be another DUB that affects mitochondrial homeostasis. It was shown that JOSD1 inhibits mitochondrial apoptotic signaling to drive acquired chemoresistance in ovarian cancer cell lines by stabilizing induced myeloid leukemia cell differentiation protein MCL1. By using mass spectrometry, MCL1 was found a JOSD1 substrate. By stabilizing MCL1, JOSD1 inhibits the mitochondrial apoptosis pathway and exerts anti-apoptotic effects. In addition, the antiapoptotic function of JOSD1 was confirmed in vivo where ovarian cancer cells overexpressing, JOSD1 or MCL1 short hairpin RNA (shRNA)s were xenografted into nude mice. Additionally, knockdown of MCL1 reversed the chemoresistance effect produced by JOSD1 overexpression.<sup>62</sup>

Moreover, JOSD1 was aberrantly expressed in head and neck squamous cell carcinoma (HNSCC) specimens, particularly in the chemo-resistant ones. The overexpression of JOSD1 showed poor clinical outcome of HNSCC patients. In contrast, JOSD1 depletion reduced cell

proliferation and colony formation, and promoted cisplatin-induced apoptosis of HNSCC cells in vitro. Additionally, *JOSD1* suppression inhibited the tumor growth and improved chemosensitivity against cisplatin in a xenograft mouse model.<sup>63</sup>

Together, these aspects defined above may also be relevant for HCC or hepatocellular functions in general, but direct proof is currently missing.

In addition to the above DUBs, there are more deubiquitinating enzymes that promote HCC with a potential role in mitophagy (Table 1). They will be described in the following part.

## 10 | NONMITOCHONDRIAL DUBS PROMOTING HCC WITH INDIRECT LINKS TO MITOPHAGY

### 10.1 | Ubiquitin-specific peptidase 2 (USP2)

USP2 seems to be another regulator of mitochondrial homeostasis. In myoblasts, *USP2* knockout decreased the accumulation of intracellular ATP and oxygen consumption. Furthermore, *USP2* knockout cells carried fragmented mitochondria, indicating that mitochondrial respiration was not active. The lack of USP2 did not affect the enzymatic activities of respiratory chain complexes I, III, IV, and V. However, mitochondrial membrane permeability was increased whereas the membrane potential of *USP2* knockout cells was obviously reduced. *USP2* knockout cells accumulated ROS in the mitochondria. Likewise, ML364, a USP2 selective inhibitor enhanced the levels of mitochondrial ROS and regulated the membrane potential and morphology of the mitochondria. These data support a role of USP2 for mitochondrial homeostasis.<sup>64</sup>

Furthermore, knockdown of USP2 inhibited actinomycin D/TNF- $\alpha$ -induced hepatocyte apoptosis, that was associated with increased levels of the antiapoptotic protein c-FlipL/S and a concurrent decrease in cellular levels of the ubiquitin ligase ITCH. Accordingly, elevated c-FlipL/S protein levels correlate with a switch from JNK and ERK to p38 signaling after galactosamine/TNF challenge suggesting that USP2 downregulation is cytoprotective in hepatocytes.<sup>65</sup>

Moreover, USP2 was found to be an inducible regulator of hepatic gluconeogenesis. In the liver, adenoviral mediated expression of USP2 promoted the production of hepatic glucose and aggravates glucose intolerance in diet-induced obese mice.<sup>66</sup>

In addition to liver, USP2 is upregulated in triple-negative breast cancer CSC populations. Genetic and

**TABLE 1** Role of different DUBs in liver disease and their contribution to mitophagy

DUB	Liver diseases			HCC		Mitochondrial contribution		
	NAFLD	NASH	ALD	Promoter	Suppressor	Mitophagy	Localized to mitochondria	Mitochondrial quality
USP7	✓					✓	✓	
USP8				✓		✓		
USP9X				✓				✓
USP14				✓		✓		
USP15				✓		✓		
USP30				✓		✓	✓	
USP33				✓		✓	✓	
USP35				✓		✓	✓	
Ataxin-3				?		✓		
CYLD					✓	✓		
UCHL1			✓	✓		✓		
USP2				✓		✓		
USP12				✓				
USP13				✓		✓		
JOSD1				?		✓		

pharmacological inhibition of USP2 significantly inhibited the self-renewal, growth and chemoresistance of CSCs. Thereby, USP2 preserves the CSC population by activating self-renewing factor BMI1 and EMT through TWIST upregulation.<sup>67</sup>

Thus, these data suggest that USP2 contributes to mitochondrial homeostasis and hepatocyte function but its targets at the mitochondrial level remain to be detected.

## 10.2 | Ubiquitin specific protease 12 (USP12)

USP12 is specifically upregulated in tumor tissues of HCC patients compared to the corresponding adjacent control tissues. However, the association between USP12 and the growth of HCCs is not well-understood. In vitro experiments in HCC cell lines showed that USP12 knockdown inhibited cell proliferation through G2/M arrest. A similar effect has also been observed in prostate cancer cells where USP12 deficiency leads to decreased proliferation, as well as increased apoptosis and G1 arrest. In addition, USP12 knockdown increased apoptosis in HCC cell lines. In line, the growth of Huh7 HCC cells expressing USP12 shRNA was significantly inhibited when these cells were injected into nude mice and it

was revealed that USP12 regulates the proliferation of human HCC cells primarily via the p38/MAPK pathway.<sup>68</sup>

## 10.3 | Ubiquitin-specific peptidase 13 (USP13)

USP13 primarily deubiquitinates and therefore upregulates ATP citrate lyase and oxoglutarate dehydrogenase, two crucial enzymes that control glutaminolysis and fatty acid synthesis and also mitochondrial respiration. USP13 has been reported to be involved in the genesis of some tumors including ovarian cancers and HCC. USP13 is upregulated in HCC tumor tissues and cell lines. Furthermore, HCC patients with high USP13 expression have lower overall survival or relapse-free survival compared with patients having low USP13 expression. In HCC cell lines, knockdown of USP13 by shRNAs significantly reduced cell growth, and decreased levels of c-Myc. Moreover, overexpression of c-Myc attenuated USP13 knockdown on HCC cell growth. In addition, implanting USP13 knockdown cells to nude mice showed that knockdown of USP13 significantly inhibited HCC tumor growth.<sup>68</sup>

While inhibiting USP13 significantly suppresses HCC and ovarian tumor progression is unknown whether

these effects are mediated by ATP citrate lyase and oxoglutarate dehydrogenase. Further, it remains open whether USP13 knockdown sensitizes tumor cells to the treatment of PI3K/AKT inhibitors in HCC which was shown for ovarian cancer.<sup>69</sup>

## 11 | CONCLUSION

The liver is critical for numerous physiological processes that require dynamic mitochondrial activities. By removing damaged mitochondria for lysosomal degradation, mitophagy contributes to the maintenance and protection of hepatic function. Conversely, inappropriate regulation of mitophagy is linked to the development of liver diseases such as NAFLD, ALD, and liver cancer. Because of fast progression and lack of targeted drugs, the survival rate of liver cancer is significantly low. Protein ubiquitylation via mitochondrial E3 ligases and deubiquitylation by DUBs that reverse the action of ubiquitin ligases play an important role for mitophagy and maintenance of cellular homeostasis.

As several DUBs appear to be overexpressed in HCC, they may represent valuable biomarkers and serve as novel targets for cancer therapy. Following a dramatic development in DUB screening technologies and biochemical analyses, various DUB inhibitors have been developed. At current, it is unknown whether DUBs inhibitors that could regulate mitophagy will find their way into the therapy of liver disease, especially into HCC therapy. This opens a new field of therapeutical options with DUBs inhibitors in general. As there are several DUBs involved in the regulation of mitophagy in liver, they may therefore be also crucial in the pathogenesis of liver disease, and the use of selective DUBs inhibitors targeting mitophagy would be very attractive. However, before those inhibitors become available for therapy much more knowledge from preclinical models is required. This applies specially to models of ALD, NAFLD, and NASH as well as fibrosis where the knowledge of participating DUBs is rather limited in contrast to HCC. Altogether, DUBs and their role in the maintenance of mitochondrial hemostasis just opens up to a new field that might be relevant to understand a number of diseases that are not only limited to liver.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

## ORCID

Thomas Kietzmann  <http://orcid.org/0000-0003-0242-8636>

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