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## Role of Autophagy in Alcohol and Drug-induced Liver Injury

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### Abstract

Alcohol-related liver disease (ALD) and drug-induced liver injury (DILI) are common causes of severe liver disease, and successful treatments are lacking. Autophagy plays a protective role in both ALD and DILI by selectively removing damaged mitochondria (mitophagy), lipid droplets (lipophagy), protein aggregates and adducts in hepatocytes. Autophagy also protects against ALD by degrading interferon regulatory factor 1 (IRF1) and damaged mitochondria in hepatic macrophages. Specifically, we will discuss selective autophagy for removal of damaged mitochondria and lipid droplets in hepatocytes and autophagy-mediated degradation of IRF1 in hepatic macrophages as protective mechanisms against alcohol-induced liver injury and steatosis. In addition, selective autophagy for removal of damaged mitochondria and protein adducts for protection against DILI is discussed in this review. Development of new therapeutics for ALD and DILI is greatly needed, and selective autophagy pathways may provide promising targets. Drug and alcohol effects on autophagy regulation as well as protective mechanisms of autophagy against DILI and ALD are highlighted in this review.

### Graphical Abstract

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#### Author Contributions

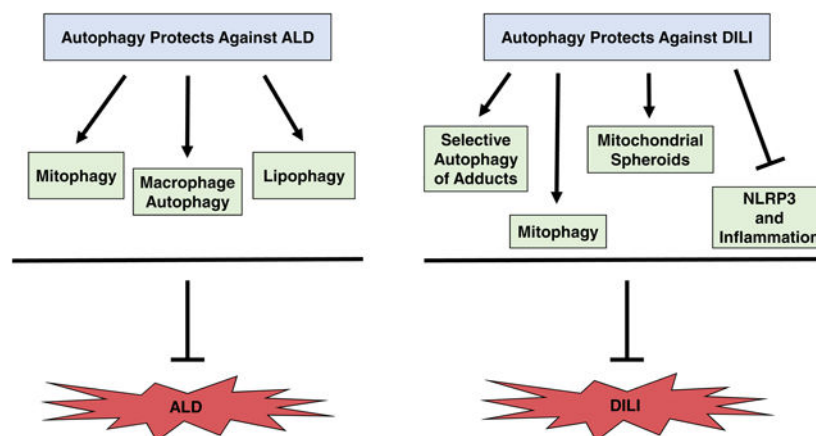
Wen-Xing Ding helped develop ideas for the manuscript and proof-read the manuscript and figures.

Jessica A. Williams helped develop ideas for the manuscript, wrote the manuscript, and designed figures for the manuscript.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



## Keywords

Alcohol; Autophagy; Lipophagy; Liver; Mitophagy; DILI

## 1. Introduction

Autophagy has been shown to play a protective role against drug-induced liver injury (DILI) and alcohol-related liver disease (ALD). Drug and alcohol effects on autophagy regulation as well as protective mechanisms of autophagy against DILI and ALD are highlighted in this review.

### 1.1. Drug-induced liver injury (DILI)

DILI is the most common cause of acute liver failure in the United States with cases continuing to rise as more drugs are developed (Lee, 2013; Reuben et al., 2010; Ye et al., 2018). Many drugs cause DILI including chemotherapeutics, antipyretics, non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressive drugs, antibiotics, and antivirals among others, and over 300 drugs have been linked to liver injury (Bjornsson and Hoofnagle, 2016; Licata, 2016; Ye et al., 2018). Even though occurrence of DILI is rare, it is the most common reason a drug is withdrawn from the market or from clinical trials (Licata, 2016). Parent drugs and their metabolites can cause hepatocyte death and subsequent liver injury by inducing mitochondrial dysfunction, oxidative stress, or inflammation. DILI can be dose-dependent, such as in acetaminophen toxicity, or idiosyncratic / dose-independent (Lee, 2013; Licata, 2016). Unfortunately, the mechanisms for initiation of liver injury by drugs are largely unknown, and management of DILI in patients is challenging due to a lack of biomarkers and tests available for diagnosis. In addition, better treatment options are needed for DILI, which will likely arise from a better understanding of mechanisms involved in initiation of liver injury by various drugs (Licata, 2016; Ye et al., 2018).

### 1.2. Alcohol-related liver disease (ALD)

ALD is a major cause of liver injury, steatosis, and end-stage liver disease. Alcohol abuse causes liver injury by various mechanisms including oxidative and non-oxidative ethanol metabolism, production of oxidative stress, mitochondrial and lysosomal damage and

dysfunction, endoplasmic reticulum stress, inflammation and cytokine release, and initiation of cell death (Gao and Bataller, 2011; Williams et al., 2014). ALD pathogenesis includes fatty liver, alcoholic hepatitis, fibrosis, cirrhosis, and eventual hepatocellular carcinoma. Alcohol metabolism and ALD pathogenesis have been thoroughly reviewed previously (Crabb et al., 2019; Gao and Bataller, 2011; Rehm et al., 2013; Seitz et al., 2018; Shen et al., 2019; Teschke, 2019; Williams et al., 2014) and will not be discussed in detail in this review. Similar to DILI, ALD also lacks good treatment options. Main treatment options for ALD patients include abstinence from alcohol use and liver transplantation. Many transplant programs have removed a previous requirement for ALD patients to be sober for 6 months before receiving liver transplantation due to positive outcomes in patients receiving early liver transplantation. However, only patients with severe disease are eligible for liver transplantation, and 17 to 22% of these patients experience alcohol relapse after transplantation (Diehl, 2002; Godfrey et al., 2019). Therefore, novel therapeutic strategies are necessary to decrease ALD and mortality.

### 1.3. Autophagy

Autophagy has recently been shown to have roles in both DILI and ALD, and targeting this pathway may provide future therapeutic options for both DILI and ALD patients. Autophagy is an intracellular pathway responsible for maintaining cellular homeostasis by directing cytosolic macromolecules and damaged or excess organelles to lysosomes for degradation. The autophagy pathway is generally considered to be a protective pathway, and it has been shown to play a critical role in maintaining cellular homeostasis in physiological conditions as well as in disease. Regulation of the autophagy pathway has been extensively reviewed and will only be briefly discussed here (Mizushima et al., 2008; Parzych and Klionsky, 2014; Ueno and Komatsu, 2017; Yu et al., 2018).

Initiation of the autophagy pathway involves phagophore formation at the phagophore assembly site (PAS), which recruits other autophagy-related gene (Atg) proteins necessary for autophagosome formation. Phagophore formation is regulated by the Unc-51 like kinase 1 (ULK1) complex, which is composed of ULK1, FAK family-interacting protein of 200 kDa (FIP200), and Atg13 (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Mizushima, 2010). ULK1 and autophagosome formation is positively regulated by AMP-activated protein kinase (AMPK) and negatively regulated by the mechanistic target of rapamycin complex 1 (mTORC1). During low energy conditions, AMPK phosphorylates ULK1 at several sites to activate it, which induces autophagy. When nutrients are plentiful, mTOR phosphorylates ULK1 at S757, inhibiting autophagy (Egan et al., 2011; Kim and Guan, 2011; Kim et al., 2011). Activated ULK1 initiates autophagy via Beclin1 phosphorylation and subsequent activation of the class III phosphoinositide 3-kinase (PtdIns3K) complex to induce autophagosome formation (Russell et al., 2013). After PtdIns3K activation, ubiquitin-like conjugation system complexes Atg12-Atg5-Atg16L1 and microtubule-associated protein light chain 3 (LC3)-Atg7-Atg3 induce phagophore elongation and expansion to form the autophagosome by regulating phosphatidylethanolamine conjugation with LC3, which forms LC3-II. LC3-II is stable on the autophagosome membrane and is commonly used as a marker for autophagy activation (Ichimura et al., 2000; Kabeya et al., 2000; Ohsumi, 2001; Yoshii and Mizushima, 2017).

The elongated phagophore then closes around its cargo to form a complete autophagosome. Mechanisms for autophagosome closure are not completely understood but may require the endosomal sorting complex required for transport III (ESCRT-III) subunit CHMP2A, the AAA ATPase VPS4, and VPS37A (Takahashi et al., 2018; Takahashi et al., 2019). Completed autophagosomes fuse with lysosomes to form autolysosomes for cargo degradation. Autophagosome-lysosome fusion requires the GTPase Ras-related protein 7 (Rab7), the SNARE protein syntaxin 17 (STX17), and the lysosomal membrane protein LAMP-2 (Huynh et al., 2007; Itakura et al., 2012; Jager et al., 2004). After fusion, autophagosome inner membrane LC3-II is degraded in the lysosome along with autophagosome cargo (Tanida et al., 2008).

Autophagy has been shown to be both a non-selective and selective process. Non-selective autophagy occurs during nutrient deprivation to degrade cellular contents for energy production. Selective autophagy mediates degradation of specific organelles that are damaged or in excess, such as in mitophagy and lipophagy for selective degradation of mitochondria and lipid droplets (LDs), respectively (Gatica et al., 2018). Several selective autophagy pathways have roles in ALD and / or DILI. Mitophagy and lipophagy in hepatocytes and selective autophagy for removal of IRF1 and mitochondria in hepatic macrophages are protective mechanisms in ALD, and mitophagy and selective autophagy for removal of protein adducts are protective mechanisms in DILI.

#### 1.4. Mitophagy

Mitophagy is a form of selective autophagy specific for degradation of damaged mitochondria. Mitophagy has been shown to be protective against both DILI and alcohol-induced liver injury and steatosis. Selective removal of damaged mitochondria helps maintain a healthy population of mitochondria, which is important for maintaining cellular homeostasis. Removal of damaged mitochondria via mitophagy prevents cell death by decreasing oxidative stress. In addition, maintenance of a healthy mitochondrial population preserves mitochondrial bioenergetics and respiratory chain function. Finally, healthy mitochondria allow for  $\beta$ -oxidation and removal of LDs to prevent steatosis (Ding and Yin, 2012; Williams and Ding, 2015, 2018).

Regulation of the mitophagy pathway has been recently reviewed by our group and others and will not be discussed at length here (Ding and Yin, 2012; Ni et al., 2015; Palikaras et al., 2018; Rodger et al., 2018; Williams and Ding, 2015, 2018). The best characterized mitophagy pathway is the Parkin and phosphatase and tensin homolog (PTEN)-induced kinase (PINK1) pathway. Parkin is an E3 ligase that remains in the cytosol under normal conditions, but it is recruited to damaged or depolarized mitochondria for mitophagy induction (Narendra et al., 2008). PINK1 is required for Parkin recruitment to damaged mitochondria and for activation of its E3 ligase activity (Matsuda et al., 2010). In healthy mitochondria, PINK1 is cleaved by the inner mitochondrial membrane protease PARL, and the truncated form is released into the cytosol for degradation by the proteasome (Jin et al., 2010; Meissner et al., 2011; Yamano and Youle, 2013). When mitochondria are damaged or depolarized, PINK1 becomes stabilized on the mitochondrial membrane and initiates self-phosphorylation to recruit Parkin to the mitochondrial membrane (Okatsu et al., 2012).

PINK1 then phosphorylates both Parkin and ubiquitin at the mitochondrial membrane to activate Parkin's E3 ligase activity and tether it to the mitochondrial membrane. Activated Parkin adds ubiquitin chains onto mitochondrial outer membrane proteins, which are then phosphorylated by PINK1 to additionally recruit and activate Parkin using a feed-forward mechanism (Ordureau et al., 2014; Shiba-Fukushima et al., 2014). Various mitophagy receptor proteins, such as p62/SQSTM1, NDP52, Huntingtin, and optineurin (OPTN) may then be recruited to ubiquitinated mitochondria to help further recruit autophagosomes (Rui et al., 2015; Williams and Ding, 2018). These receptor proteins all have a ubiquitin-associated (UBA) domain for binding to ubiquitinated proteins and an LC3-interacting region (LIR) domain for binding to LC3 on autophagosomes to help initiate autophagosome recruitment and cargo recognition (Birgisdottir et al., 2013).

The Parkin-PINK1 mitophagy pathway is generally considered to be a linear pathway with PINK1 acting upstream of Parkin (Park et al., 2006; Yang et al., 2006). However, PINK1 was recently shown to activate mitophagy alone by ubiquitinating mitochondria and recruiting selective autophagy receptors NDP52 and OPTN (Lazarou et al., 2015). There are also several other Parkin-independent pathways for induction of mitophagy including induction by E3 ligases March5 or Mulf1 or mediation of mitophagy by Fun 14 domain containing 1 (Fundc1), Nix, or cardiolipin (Rogov et al., 2014; Williams and Ding, 2018).

### 1.5. Lipophagy

Lipophagy is a form of selective autophagy specific for degradation of intracellular LDs (Singh et al., 2009) that occurs during nutrient deprivation to provide energy or during cellular stress to remove excess LDs (Kounakis et al., 2019; Schulze et al., 2017b). Lipophagy may require cytosolic triglyceride lipase (ATGL) to initiate lipolysis of large LDs into small LDs to subsequently facilitate their removal by autophagosomes (Sathyanarayan et al., 2017; Schott et al., 2019). In addition, lysosomes can directly enwrap LDs for degradation by micro-lipophagy. After autophagosome or lysosome sequestration, LDs are broken down by lysosomal lipases into fatty acids (Li et al., 2017; Singh et al., 2009)

Exact mechanisms for induction of the lipophagy pathway are still unknown, but several processes important for lipophagy have been discovered. Similar to mitophagy, lipophagy likely involves selective autophagy receptors such as p62, OPTN, Huntingtin, or NDP52 for autophagosome recognition of LDs. The exact receptor necessary has not been identified, but Huntingtin and p62 may act together to initiate autophagosome recruitment to LDs (Kounakis et al., 2019; Rui et al., 2015; Schulze et al., 2017b).

The GTPases Rab7 and Rab10 may have important roles in lipophagy regulation. Rab7 is a regulator of late endocytic and autophagosome trafficking and is associated with the LD membrane (Gutierrez et al., 2004; Jager et al., 2004; Vitelli et al., 1997). Rab7 was shown to possibly recruit autophagosomes and lysosomes to LDs (Schroeder et al., 2015). Rab7 on the LD surface has been shown to co-localize with autophagosomes and lysosomes (Lizaso et al., 2013), and starvation promoted Rab7 activation on the LD surface, which recruited lysosomes to LDs. Rab7 deletion also increased numbers of LDs in hepatoma cells (Schroeder et al., 2015). Schroeder *et al.* described a "kiss and run" mechanism for Rab7-mediated lipophagy where lysosomes are recruited to LDs, docked for a short period of

time, and then depart, suggesting that lysosomes may clear away portions of a large LD at a time (Carmona-Gutierrez et al., 2015; Schroeder et al., 2015).

In addition to Rab7, Rab10 is also on the LD surface and may be involved in lipophagy in hepatocytes. Similar to Rab7, deletion of Rab10 increased numbers of LDs in hepatocytes, and Rab10 on LDs was shown to co-localize with autophagosomes and lysosomes. In addition, starved Huh-7 cells treated with the mTOR inhibitor Torin1 to activate autophagy had increased numbers of Rab10-labeled LDs, further suggesting a role for Rab10 in lipophagy regulation (Li et al., 2016).

Transcription Factor EB (TFEB) also likely plays a role in mediating lipophagy because mice with acute TFEB knock-down had greater steatosis after alcohol treatment compared to control mice (Chao et al., 2018c). TFEB is the master regulator for transcription of genes necessary for lysosome biogenesis and autophagy (Settembre et al., 2011). In addition, Dynamin2 (Dyn2), which is responsible for recycling lysosomes from autolysosomes to maintain autophagy (Schulze et al., 2013) may play a role in lipophagy because inhibition of Dyn2 by alcohol decreased lipophagy in rats, resulting in increased liver steatosis (Li and Ding, 2017). The roles of TFEB and Dyn2 in lipophagy are further discussed in the alcohol section of this review. Overall, the mechanisms regulating lipophagy are becoming more understood, but there is still a lot unknown about regulation of this pathway.

## 2. Role of Autophagy in Alcohol-induced liver injury and steatosis

Alcohol has been shown to both activate and inhibit autophagy, depending on the alcohol model used. Autophagy was activated in primary mouse hepatocytes and in mice after acute alcohol binge. Alcohol increased LC3-II levels and autophagosome co-localization with lysosomes in mice and primary hepatocytes. LC3-II levels were further increased by alcohol after pre-treatment with chloroquine (CQ) to block lysosome degradation, indicating autophagy flux and activation (Ding et al., 2010; Lin et al., 2013; Ni et al., 2013a; Thomes et al., 2015). Alcohol-induced activation of autophagy in the alcohol binge model was found to be dependent on ethanol metabolism, production of reactive oxygen species (ROS), and inhibition of mTOR (Ding et al., 2010; Lu and Cederbaum, 2015; Scherz-Shouval et al., 2007).

Chronic alcohol feeding was shown to both activate and inhibit autophagy. Two independent studies reported that chronic alcohol feeding activates autophagy. Lin *et al.* showed increased autophagic flux in mouse livers after chronic alcohol feeding (Lin et al., 2013), and Eid *et al.* showed increased autophagosome numbers in rat livers after chronic alcohol feeding (Eid et al., 2013). Two independent groups also showed alcohol-induced inhibition of autophagy. Menk *et al.* showed that chronic alcohol feeding in rats had no effect on LC3-II levels and that p62 levels increased after alcohol feeding, suggesting inhibition of autophagy (Menk et al., 2018). Thomes *et al.* demonstrated that alcohol feeding in mice increased autophagosome numbers, similar to mice treated with alcohol binge. However, alcohol-fed mice had increased p62 protein expression along with decreased lysosome numbers and decreased levels of autophagosome-lysosome fusion, suggesting autophagy inhibition (Thomes et al., 2015). Autophagy inhibition in this case was due to a lack of



available lysosomes for autophagosomes to fuse with to degrade their cargo; this form of autophagy inhibition was recently defined as “insufficient autophagy” (Chao et al., 2018a). Mice treated with the chronic alcohol feeding plus acute binge (Gao-binge) alcohol model also had insufficient autophagy, similar to chronic alcohol-fed mice (Chao et al., 2018c). Even though chronic alcohol feeding has been shown to both activate and inhibit autophagy, chronic alcohol feeding likely inhibits autophagy because it has been reported to cause hepatic protein accumulation and hepatomegaly (Baraona et al., 1975). Alcohol-induced hepatomegaly is likely associated with autophagy inhibition or decreased lysosome function. In support of this, chronic alcohol feeding decreased cathepsin activity in lysosomes (Kharbanda et al., 1995) and reduced lysosomal biogenesis mediated by TFEB (Chao et al., 2018c; Thomes et al., 2015).

Interestingly, TFEB is differentially regulated by alcohol binge and chronic alcohol feeding, which may provide one answer to why these alcohol models have contrasting effects on autophagy activation. Alcohol binge was associated with increased liver autophagosome numbers, lysosome numbers, and TFEB nuclear content and subsequent autophagy activation. Chronic alcohol feeding was also associated with increased autophagosome numbers, but lysosome numbers and TFEB nuclear content were decreased in chronic alcohol fed mice compared to control fed mice, leading to insufficient autophagy and subsequent autophagy inhibition (Thomes et al., 2019; Thomes et al., 2015). Gao-binge alcohol treated mice also had decreased TFEB nuclear content in addition to decreased TFEB expression levels and transcriptional activity (Chao et al., 2018c).

Regulation of alcohol-induced changes in TFEB localization are likely through post-translational modifications of ERK 1/2 and / or mTORC1, which both regulate TFEB (Chao et al., 2018c; Li and Ding, 2016; Thomes et al., 2015). Phosphorylated (inactive) TFEB remains in the cytosol while unphosphorylated (active) TFEB is able to travel to the nucleus to induce gene transcription (Settembre et al., 2012). A recent idea for alcohol-mediated TFEB regulation suggests involvement of the proteasome. Alcohol is known to suppress proteasome activity (Donohue et al., 2007), and the phosphorylated form of TFEB is ubiquitinated by STIP1 homology U-Box containing protein (STUB1) for subsequent degradation by the proteasome (Sha et al., 2017). It is possible that chronic alcohol feeding indirectly inhibits autophagy via suppression of proteasome activity, which may cause an accumulation of phosphorylated TFEB in the cytosol that would prevent entry of unphosphorylated TFEB into the nucleus (Yang et al., 2019).

Even though TFEB regulation varies with alcohol models used in mice, TFEB plays an important protective role in ALD. Mice with acute TFEB knock-down had significantly greater liver injury and steatosis after Gao-binge alcohol treatment compared to control mice, and mice with overexpressed TFEB were protected against Gao-binge alcohol induced liver injury and steatosis (Chao et al., 2018c). Intriguingly, TFEB nuclear content was restored in alcohol-fed rats after withdrawal from alcohol for 7 days, and liver injury and steatosis were reduced (Thomes et al., 2019; Yang et al., 2019). More importantly, nuclear TFEB accumulation was decreased in hepatocytes from human alcoholic hepatitis patients, confirming that TFEB plays an important role in human ALD pathogenesis (Chao et al., 2018c).

It is not clear why various alcohol models regulate autophagy differently. It should be noted that autophagy flux analysis was not always implemented when evaluating alcohol-mediated regulation of autophagy. Due to the dynamic nature of autophagy, measurement of autophagic flux is important for determining autophagy activation or suppression (Klionsky et al., 2012). Future alcohol studies should include autophagy flux assays to determine autophagy activation. In addition, autophagosome-lysosome fusion analysis should be performed to confirm that alcohol-induced autophagy flux produces sufficient autophagy.

Even though autophagy may be regulated differently by chronic and acute alcohol exposure, activation of autophagy is protective against alcohol-induced liver injury and steatosis. Pharmacological activation of autophagy by rapamycin alleviated alcohol-induced steatosis and liver injury in both alcohol binge and chronic alcohol feeding models (Ding et al., 2010; Lin et al., 2013; Lu and Cederbaum, 2015) while pharmacological inhibition of autophagy by 3-methyladeline (3-MA) and CQ exacerbated alcohol-induced hepatocellular apoptosis, steatosis, and liver injury in mice using both the alcohol binge and chronic alcohol feeding models (Ding et al., 2010; Lin et al., 2013; Lu and Cederbaum, 2015). In addition, augmenter of liver regeneration (ALR) was shown to protect against alcohol-induced liver injury by activating autophagy (Liu et al., 2019). ALR is a hepatic growth factor that plays an important role in liver regeneration (Gupta and Venugopal, 2018). Mice overexpressing ALR had decreased liver injury, and ALR knockdown mice had increased liver injury compared to WT mice after alcohol treatment. ALR-mediated protection against alcohol-induced liver injury correlated with autophagy activation (Liu et al., 2019). Therefore, development of therapeutics for autophagy activation may prove useful for treatment of ALD. In addition, development of therapeutics targeted at TFEB regulation may be beneficial for ALD patients.

Interestingly, alcohol-induced autophagy activation was shown to be selective for removing damaged mitochondria (mitophagy) and LDs (lipophagy) in the liver. Alcohol binge in mice did not cause long-lived protein degradation, which is characteristic of non-selective autophagy, but instead enhanced selective degradation of fragmented mitochondria and LDs (Ding et al., 2010; Lin et al., 2013). Alcohol binge also increased degradation of the selective autophagy receptor p62 (Ding et al., 2010; Ding et al., 2011). In addition, chronic alcohol feeding increased autophagic sequestration of mitochondria and LDs in rats (Eid et al., 2013). Therefore, the selective mitophagy and lipophagy pathways are important for protection against ALD pathogenesis and will be further discussed below.

## 2.1. Mitophagy in ALD

Mitophagy has been shown to be protective against alcohol-induced liver injury and steatosis by removing damaged mitochondria (Ding et al., 2010; Ding et al., 2011). Mechanisms involved in activation of mitophagy after alcohol treatment are not fully understood, but they may involve the PINK1- Parkin signaling pathway. Acute alcohol binge in rats caused Parkin and PINK1 co-localization to mitochondria. Alcohol-treated rats also had increased LC3 puncta formation compared to control rats, and Parkin co-localized with LC3 and mitochondrial markers after alcohol treatment (Eid et al., 2016). In addition, Parkin knockout (KO) mice had increased liver injury and mitochondrial damage and dysfunction



compared to Wild-type (WT) mice after treatment with alcohol using both the acute-binge and Gao-binge models. Parkin also translocated to mitochondria after Gao-binge alcohol treatment in mice, suggesting that Parkin-induced mitophagy is activated by Gao-binge alcohol treatment (Williams et al., 2015a). Furthermore, numbers of autophagosomes containing mitochondria were decreased in Parkin KO mice compared to WT mice after alcohol treatment in both acute-binge and Gao-binge alcohol models, suggesting a defect in mitophagy induction in Parkin KO mice. Defective mitophagy in Parkin KO mice likely contributed to their increased liver injury after alcohol treatment (Williams et al., 2015a). Further investigation into alcohol-induced regulation of the mitophagy pathway is needed.

## 2.2. Lipophagy in ALD

Lipophagy occurs as a protective mechanism to reduce alcohol-mediated steatosis in the liver (Ding et al., 2010; Ding et al., 2011; Eid et al., 2013; Lin et al., 2013). Mechanisms for lipophagy regulation by alcohol are still not completely understood, but they likely involve Rab7 and Dyn2 GTPases. Alcohol was recently shown to inhibit Rab7 activity, which resulted in impaired recruitment of autophagosomes to LDs (Li and Ding, 2017; Schulze et al., 2017a). Hepatocytes from rats fed an alcohol diet for 6 weeks were resistant to starvation-induced lipophagy, likely through alcohol-mediated inhibition of Rab7 activity. Cultured primary hepatocytes from rats treated with alcohol had 80% decreased Rab7 activity, and pharmacological inhibition of Rab7 produced similar resistance to starvation-induced lipophagy as alcohol treatment (Schulze et al., 2017a). The mechanism by which alcohol inhibits Rab7 activity requires further investigation.

Alcohol was also recently found to inhibit activation of Dyn2 (Li and Ding, 2017; Rasineni et al., 2017), which is responsible for recycling lysosomes from autolysosomes to maintain autophagy (Schulze et al., 2013). Inhibition of Dyn2 activity led to decreased lipophagy and subsequent liver steatosis in alcohol-fed rats. Rats were fed alcohol for 6 to 8 weeks, and their isolated hepatocytes were subjected to starvation to activate lipophagy. Triglyceride loss in hepatocytes from alcohol-fed animals was slower during fasting compared to control-diet hepatocytes. Lipophagy was activated in control and alcohol-fed rat livers during fasting because LC3 and p62 levels were increased in liver and LD fractions, and lysosomes colocalized with autophagosomes and LDs. However, lysosome numbers were decreased by 40% in alcohol-fed rats resulting in an accumulation of autophagosomes and insufficient autophagy. The reduction in lysosome availability was at least partly due to an alcohol-mediated inhibition of Dyn2 activity (Rasineni et al., 2017). The mechanism for how alcohol regulates Dyn2 activity is unknown.

TFEB also likely has a role in lipophagy regulation by alcohol because TFEB-induced lysosome biogenesis was decreased by alcohol, and mice with TFEB overexpression were protected against alcohol-induced fatty liver (Chao et al., 2018c; Thomes et al., 2015). TFEB-mediated lysosomal biogenesis can either promote macro-lipophagy (involving the formation of autophagosomes and fusion of autophagosomes with lysosomes) or micro-lipophagy (direct engulfment of LDs by lysosomes) for the removal of LDs to protect against alcohol-induced steatosis. It would be interesting to determine the role of TFEB in

these two types of lipophagy using alcohol models in the future. In addition, the effect of alcohol on Rab7 and Dyn2 *in vivo* should be investigated.

### 2.3. Hepatic Macrophage Autophagy in ALD

Hepatic macrophages are the resident macrophages in the liver, and hepatic macrophage autophagy has recently been shown to have an important role in alleviation of alcohol-induced liver inflammation, injury, and steatosis. Chronic alcohol feeding was shown to inhibit liver macrophage autophagy because mice fed alcohol for 6 weeks had decreased autophagic flux in liver macrophages compared to macrophages from mice fed control diet (Liang et al., 2019). However, macrophage autophagy is important for preventing alcohol-induced liver injury and steatosis because myeloid-specific Atg7 KO mice co-treated with chronic alcohol feeding and LPS had increased liver injury, steatosis, and cell death compared to WT mice (Liang et al., 2019). Mechanistically, macrophage autophagy prevented accumulation of damaged mitochondria and ROS production, resulting in decreased activation of the NLRP3 inflammasome and inhibition of liver injury and steatosis (Liang et al., 2019). Additionally, macrophage autophagy promoted p62-mediated degradation of IRF1, which inhibited transcription of CCL5 and CXCL10, two chemokines known for promoting liver inflammation (Liang et al., 2019). Interestingly, myeloid-specific Atg5 KO mice had similar levels of liver injury, inflammation, and steatosis as WT mice after Gao-binge alcohol treatment (Denaes et al., 2016). It is unknown why myeloid-specific Atg5 KO and Atg7 KO mice behave differently after alcohol treatment, but it may also be due to variations in alcohol models used for these mice.

The Cannabinoid receptor 2 (CB2) has recently been shown to have a protective role in ALD by activating autophagy of hepatic macrophages. Cannabinoid receptors are G-protein coupled receptors that have been shown to play a protective role against liver injury. They are mostly expressed in immune cells (Mallat et al., 2013). CB2 in hepatic macrophages was shown to alleviate alcohol-induced inflammation and steatosis by preventing macrophage activation via induction of macrophage autophagy in a heme-oxygenase-1 (HO-1) dependent pathway (Denaes et al., 2016). Myeloid-specific CB2 KO mice treated with Gao-binge alcohol had increased steatosis, pro-inflammatory gene expression, and neutrophil recruitment compared to WT mice. In addition, Gao-binge treated mice given a CB2 agonist had decreased pro-inflammatory gene expression and steatosis compared to Gao-binge treated mice not given a CB2 agonist. Protection against alcohol-induced steatosis and inflammation provided by the CB2 agonist was lost in Atg5 myeloid-specific KO mice, emphasizing the need for macrophage autophagy in CB2-mediated protection from alcohol (Denaes et al., 2016). Further investigation into the effects of CB2 receptor agonists on alcohol-induced liver injury and steatosis may be beneficial for future therapeutic development for ALD. In addition, it would be interesting to further investigate whether the combination of CB2 agonists with other known autophagy inducers such as mTOR inhibitors in hepatic macrophage autophagy prevents alcohol-induced liver injury, inflammation, and steatosis.

### 3. Autophagy in DILI

#### 3.1 Acetaminophen (APAP)

APAP overdose is responsible for most cases of acute liver failure in the United States (Craig et al., 2010; Larson et al., 2005). APAP metabolism has been extensively reviewed and will not be discussed in detail here (McGill and Jaeschke, 2013; Ramachandran and Jaeschke, 2018). Briefly, APAP is metabolized by cytochrome p450s, mainly by Cyp2e1, to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). After therapeutic doses of APAP, NAPQI is bound and detoxified by glutathione (GSH). However, APAP overdose leads to depletion of GSH, which allows for NAPQI binding to proteins and subsequent mitochondrial dysfunction, hepatocyte necrosis, liver injury and possible liver failure (McGill and Jaeschke, 2013; Ramachandran and Jaeschke, 2018). The only current therapeutic option for APAP overdose is N-acetylcysteine (NAC), which is a prodrug for GSH synthesis. The downfall to NAC treatment is that it must be given early after overdose to replenish GSH supply before NAPQI has the opportunity to bind to proteins. If NAC is not administered early enough, the only other therapeutic option for APAP-induced acute liver failure is liver transplantation (Corcoran et al., 1985; Craig et al., 2010). Since APAP is one of the most widely-used antipyretic drugs in the United States (Herndon and Dankenbring, 2014), a better understanding of mechanisms leading to APAP-induced liver injury and development of therapeutic options for preventing APAP-induced acute liver failure are needed.

The role of autophagy in APAP-induced liver injury was recently reviewed in detail by our group (Chao et al., 2018b) and will be briefly summarized here. Autophagy has been shown to be both activated and inactivated by APAP. Autophagy is activated by APAP in mice, as demonstrated by increased autophagic flux in GFP-LC3 mice treated with APAP (Ni et al., 2012). The mechanism for APAP-induced activation of autophagy in hepatocytes is still uncertain but may be via inhibition of mTORC1 at early time points (Ni et al., 2012). Adiponectin was also shown to protect against APAP-induced liver injury by activating autophagy via AMPK and ULK1 (Lin et al., 2014). Interestingly, APAP was also recently shown to inhibit autophagy. Kang *et al.* showed impaired autophagic flux after APAP treatment in mice (Kang et al., 2019). APAP also inhibited autophagy in a model of non-alcoholic fatty liver disease (NAFLD), which led to aggravated disease in mice (Shi et al., 2019). Reasons for discrepancies in APAP-induced activation or inhibition of autophagy are unknown but may involve different mouse models used and / or differences in time points of autophagic flux assessment.

Regardless if autophagy is activated or inhibited by APAP, activation of autophagy protects against APAP-induced liver injury. Inhibition of autophagy by CQ or 3-MA both increased APAP-mediated necrosis in mouse hepatocytes while co-treatment and post-treatment with the autophagy activator rapamycin decreased APAP-mediated hepatocyte necrosis. Furthermore, mice co-treated with APAP and CQ had increased liver injury compared to APAP treated mice, and co-treatment with APAP and rapamycin inhibited APAP-induced liver injury (Ni et al., 2012). In addition, treatment with various drugs in addition to rapamycin have been shown to protect against APAP-induced hepatotoxicity via autophagy

activation. Similar to alcohol-fed mice, ALR treatment protected mice from APAP-induced liver injury by activating autophagy (Hu et al., 2019). Treatment with the resveratrol derivative pterostilbene (Kang et al., 2019) and with IL-22 (Mo et al., 2018) both protected against APAP-induced liver injury by activating autophagy. Therefore, autophagy is overall protective against APAP-mediated liver injury, likely via selective removal of APAP adducts and damaged mitochondria, as discussed below.

Formation of APAP-adducts, particularly mitochondrial protein adducts, during APAP overdose is thought to contribute to liver injury during APAP overdose (McGill et al., 2012; Qiu et al., 1998; Ramachandran and Jaeschke, 2018; Xie et al., 2015), and autophagy was suggested to remove and degrade APAP adducts as a protective mechanism (Ni et al., 2016). APAP-adducts co-localized with autophagosomes and lysosomes in APAP-treated primary mouse hepatocytes, and autophagosomes and autolysosomes isolated from APAP-treated mouse livers contained APAP-adducts. Torin-1 induced activation of autophagy decreased blood levels of APAP-adducts in mice treated with APAP, which correlated with decreased liver injury and hepatocellular necrosis. In addition, autophagy inhibition by leupeptin and CQ both increased APAP-adduct levels, liver injury, and hepatocellular necrosis in APAP-treated mice (Ni et al., 2016). The specific mechanisms for selective removal of adducts by autophagy require further investigation, but p62 likely plays a role in targeting APAP adducts for recognition by the autophagosome. p62 co-localized with APAP-adducts in primary mouse hepatocytes treated with APAP. In addition, p62 depleted hepatocytes had reduced clearance of APAP-adducts and increased necrosis compared to p62-expressing hepatocytes after APAP treatment (Ni et al., 2016).

Autophagy has also been shown to be protective against APAP-induced liver injury via selective removal of damaged mitochondria by mitophagy. APAP-induced mitochondrial dysfunction and oxidative stress play a key role in mediating APAP-induced liver injury (Jaeschke et al., 2012). Therefore, timely removal of damaged mitochondria by mitophagy is an important protective mechanism against APAP-induced liver injury. APAP-induced autophagy in primary mouse hepatocytes caused sequestration of mitochondria in autophagosomes and degradation of mitochondrial proteins, suggesting activation of mitophagy by APAP overdose (Ni et al., 2012; Ni et al., 2013b; Wang et al., 2019; Williams et al., 2015b).

The mechanism for APAP-induced activation of mitophagy is not completely understood but may involve Parkin and PINK1. Parkin translocated to mitochondria after APAP treatment, which correlated with increased ubiquitination of mitochondrial proteins (Wang et al., 2019; Williams et al., 2015b). Parkin translocation to mitochondria after APAP treatment was dependent on PINK1 (Wang et al., 2019). Mice with acute knockdown of Parkin had increased liver injury after APAP treatment compared to control mice (Williams et al., 2015b). Parkin and PINK1 double KO mice also had increased liver injury and mortality after APAP treatment compared to WT mice (Wang et al., 2019), suggesting an importance for the Parkin / PINK1 mitophagy pathway in alleviating APAP-induced liver injury. However, Parkin KO mice were surprisingly protected against APAP-mediated liver injury (Wang et al., 2019; Williams et al., 2015b), and PINK1 KO mice had decreased liver injury after APAP treatment compared to WT mice (Wang et al., 2019). Mitophagy was

significantly decreased in Parkin and PINK1 double KO mice after APAP treatment, but Parkin KO and PINK1 KO mice still had active mitophagy (Wang et al., 2019; Williams et al., 2015b). These data suggest that PINK1 and Parkin may compensate for each other to activate mitophagy. In addition, other mediators of mitophagy may be activated in the absence of Parkin and / or PINK1 in response to APAP overdose.

Parkin KO mice also had other mechanisms unrelated to mitophagy that protected them from APAP-induced liver injury, such as increased hepatocyte proliferation and decreased JNK activation after APAP treatment compared to WT mice (Williams et al., 2015b). JNK activation is well known to exacerbate APAP-induced liver injury (Gunawan et al., 2006; Hanawa et al., 2008), and increased hepatocyte proliferation likely helped Parkin KO mice recover faster from APAP-induced hepatocyte necrosis than WT mice. Interestingly, JNK activation was increased in PINK1 KO mice compared to WT mice after APAP treatment (Wang et al., 2019). Proliferation wasn't investigated in PINK1 KO mice after APAP treatment, but PINK1 also has a role in mediating proliferation (O'Flanagan and O'Neill, 2014).

Mitochondrial spheroids may also have a protective role against APAP-induced liver injury (Ding et al., 2012). Mitochondrial spheroids are mitochondria with cup-like morphology with their lumen connected to the cytosol by a small opening. Mitochondrial spheroids can enwrap cellular contents, such as lipid droplets or other mitochondria. Mitochondrial spheroids co-localize with lysosomes, but their ability to degrade their contents requires further investigation (Ni et al., 2013b). Together, it is clear that timely removal of damaged mitochondria either via PINK1-Parkin dependent or independent mitophagy plays a critical role in protection against APAP-induced liver injury. Future studies are needed to determine whether removal of damaged mitochondria by mitophagy would also be beneficial for hepatocyte proliferation and liver regeneration.

### 3.2. Efavirenz

Efavirenz (EFV) is a commonly used non-nucleoside reverse transcriptase inhibitor for AIDS treatment. EFV is generally considered to be a safe drug, but it has been linked to mitochondrial depolarization and damage, oxidative stress production, hepatocyte cell death, and liver injury (Apostolova et al., 2011b; Blas-Garcia et al., 2014; Bumpus, 2011; Chen et al., 2014). EFV induces mitophagy in human hepatocytes, and EFV-induced mitophagy is assumed to be a protective mechanism because pharmacological inhibition of autophagy increased EFV-induced hepatocellular apoptosis, which was likely due to a build-up of damaged mitochondria (Apostolova et al., 2011a, b).

The exact mechanism for EFV-induced liver injury is not completely understood, but it may involve cell death caused by autophagic stress. Autophagic stress occurs when the autophagic degradation capacity for damaged mitochondria by mitophagy is exceeded, resulting in a build-up of damaged and dysfunctional mitochondria and subsequent cell death. EFV was shown to induce mitophagy when human hepatocytes were given a clinically-relevant dose of 25  $\mu$ M. However, when human hepatocytes were given a 50  $\mu$ M dose of EFV, autophagic flux was blocked and cell death increased, likely due to levels of

damaged mitochondria exceeding the threshold for successful degradation by mitophagy (Apostolova et al., 2011a, b).

Even though clinically relevant doses of EFV are considered safe and induce mitophagy, AIDS patients are frequently treated with multiple drugs for co-infections, and other drugs used for treatment may also lead to mitochondrial damage and hepatocyte death. Therefore, use of EFV in combination with other hepatocyte-harming drugs may produce results similar to the 50  $\mu\text{M}$  dose of EFV, leading to autophagic stress, cell death and subsequent liver injury (Apostolova et al., 2011a).

In addition to mitophagy induction by EFV, the autophagy adaptor protein p62 was shown to play a protective role against EFV-induced oxidative stress and inflammation independent of the autophagy pathway. Alegre *et al.* recently reported that EFV treatment in Hep3B cells upregulated transcription of the autophagy adaptor protein p62, and increased p62 levels were independent of autophagy regulation. Authors concluded that increased p62 transcription was induced by EFV-mediated mitochondrial damage. In addition, they showed that EFV treatment in Hep3B cells activated the NLRP3 inflammasome, which was exacerbated by p62 silencing. Interestingly, p62 silencing increased EFV-induced ROS production but had no effect on cell death. However, pharmacological inhibition of autophagy alone and in combination with p62 silencing in Hep3B cells increased EFV-induced cell death, confirming a protective role for autophagy against cell death during EFV treatment (Alegre et al., 2018). It would be valuable to study the effects of EFV treatment on mitophagy and p62 regulation *in vivo* in the future.

### 3.3. Diclofenac

Diclofenac is a commonly used NSAID for treatment of pain and swelling in rheumatic diseases (Derry et al., 2012). The cause of diclofenac-induced liver injury is not completely understood, but it likely involves drug-induced mitochondrial damage and dysfunction (Gomez-Lechon et al., 2003; Helfgott et al., 1990; Masubuchi et al., 2002; Ramachandran et al., 2018).

Autophagy, particularly mitophagy, was recently shown to have a potential role in protection against diclofenac-induced hepatotoxicity. Kang *et al.* showed that diclofenac treatment of hepatocytes induced mitophagy, and that co-incubation of hepatocytes with AMPK activator 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) for autophagy induction inhibited mitochondrial depolarization and hepatocyte death. However, determination of autophagy and mitophagy activation was dependent on LC3-II and Parkin levels alone without autophagy flux analysis. Therefore, it is unclear if protection by AICAR was via autophagy induction or by another mechanism. Treatment of hepatocytes with rapamycin did not inhibit diclofenac-induced mitochondrial depolarization or cell death, suggesting that mTOR inhibition-induced autophagy may not be a protective mechanism against diclofenac-induced cell death (Kang et al., 2019). However, autophagy stress may also play a role in diclofenac-mediated liver injury as in EFV discussed above. AICAR was likely protective via another autophagy-independent mechanism, such as by promoting mitochondrial fusion (Kang et al., 2016). Due to diclofenac causing mitochondrial damage and dysfunction, the



role of mitophagy in protection against diclofenac-induced hepatotoxicity should still be further investigated.

### 3.4. Cisplatin

Cisplatin is a widely used chemotherapeutic. Although rare, cisplatin can cause hepatocellular death and subsequent hepatotoxicity. Hepatotoxicity of cisplatin is dose-dependent and may lead to decreasing dosage used or halting treatment in cancer patients. The mechanism for cisplatin-mediated liver injury is unknown (Qi et al., 2019). Cisplatin may mediate liver injury by inhibiting autophagy. Qu *et al.* showed that cisplatin inhibited autophagy because rats treated with cisplatin had decreased LC3-II protein expression and increased p62 protein expression in their livers. Cisplatin treatment in rats also led to upregulation of the NLRP3 inflammasome, release of pro-inflammatory cytokines and subsequent liver injury. NLRP3 activation was suggested to be due to suppression of autophagy (Qu et al., 2018).

In contrast to Qu *et al.*, Chen's group showed that cisplatin induces autophagy in HepG2 cells (Li et al., 2019; Liu et al., 2018). Cisplatin increased protein and puncta levels of LC3-II and decreased protein levels of p62. Cisplatin also increased protein levels of phosphorylated AMPK and decreased phosphorylated levels of mTOR in HepG2 cells, suggesting autophagy activation via mTOR inhibition (Li et al., 2019; Liu et al., 2018). Interestingly, cisplatin slightly decreased viability in human hepatocytes, but it did not affect autophagy (Li et al., 2019).

It is not clear why these studies show differences in autophagy regulation by cisplatin. It may be due to differences in models used. For example, Qu *et al.* used an *in vivo* rat model while Chen's group used HepG2 cells and hepatocytes. It could also be due to different cells used. Chen's group used both a cancer cell line and normal hepatocytes, which may behave differently in regards to autophagy regulation by cisplatin. It is important to note that autophagy flux analysis with a lysosome inhibitor was not performed for any of the above studies, leaving interpretation of cisplatin-induced autophagy regulation open for debate (Li et al., 2019; Liu et al., 2018; Qu et al., 2018).

## 4. Concluding Remarks

Current research progress has significantly increased our understanding of molecular mechanisms involved in autophagy-mediated protection against DILI and ALD, but many questions still remain. For example, different models of ALD and DILI produce contrasting effects on autophagy activation status, which may be clarified by assessing autophagy flux and performing autophagosome-lysosome fusion analysis to ensure autophagy sufficiency in future studies. In addition, assessment of autophagy flux at multiple time points rather than one snap-shot time point may provide a better understanding of autophagy regulation by alcohol and DILI-causing drugs. Moreover, future DILI *in vivo* studies would help elucidate mechanisms involved in induction of liver injury by various drugs. Nevertheless, current evidence generally supports autophagy as a protective mechanism against DILI and ALD, particularly via upregulation of selective autophagy pathways. The role of autophagy in ALD and DILI has been summarized in Figure 1. Further investigation into mechanisms

involved in autophagy regulation by DILI-causing drugs and alcohol may provide better therapeutic options for ALD and DILI patients.

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## Abbreviations

<b>3-MA</b>	3-methyladeline
<b>AICAR</b>	5-Aminoimidazole-4-carboxamide ribonucleotide
<b>ALD</b>	Alcohol-related liver disease
<b>ALR</b>	augmenter of liver regeneration
<b>AMPK</b>	AMP-activated protein kinase
<b>APAP</b>	acetaminophen
<b>Atg</b>	autophagy-related gene
<b>ATGL</b>	cytosolic triglyceride lipase
<b>CB2</b>	cannabinoid receptor 2
<b>CQ</b>	chloroquine
<b>DILI</b>	drug-induced liver injury
<b>Dyn2</b>	Dynamain 2
<b>EFV</b>	efavirenz
<b>ESCRT-III</b>	endosomal sorting complex required for transport III
<b>Fip200</b>	FAK family-interacting protein of 200 kDA
<b>Fundc1</b>	Fun14 domain containing 1
<b>GSH</b>	glutathione
<b>HO-1</b>	heme-oxygenase 1
<b>IRF1</b>	interferon regulatory factor 1
<b>KO</b>	knock out
<b>LC3</b>	microtubule-associated protein light chain 3
<b>LD</b>	Lipid droplet
<b>LIR</b>	LC3-interacting region

<b>MTORC1</b>	mechanistic target of rapamycin complex 1
<b>NAC</b>	N-acetylcysteine
<b>NAFLD</b>	non-alcoholic fatty liver disease
<b>NAPQI</b>	N-acetyl-p-benzoquinone imine
<b>NSAID</b>	non-steroidal anti-inflammatory drug
<b>OPTN</b>	optineurin
<b>PAS</b>	phagophore assembly site
<b>PINK1</b>	phosphatase and tensin homolog (PTEN)-induced kinase
<b>PtdIns3k</b>	class III phosphoinositide 3-kinase
<b>Rab</b>	ras-related protein
<b>ROS</b>	reactive oxygen species
<b>STUB1</b>	STIP1 homology U-Box containing protein
<b>STX17</b>	syntaxin 17
<b>TFEB</b>	transcription factor EB
<b>UBA</b>	ubiquitin-associated
<b>ULK1</b>	Unc-51 like kinase 1
<b>WT</b>	wild-type

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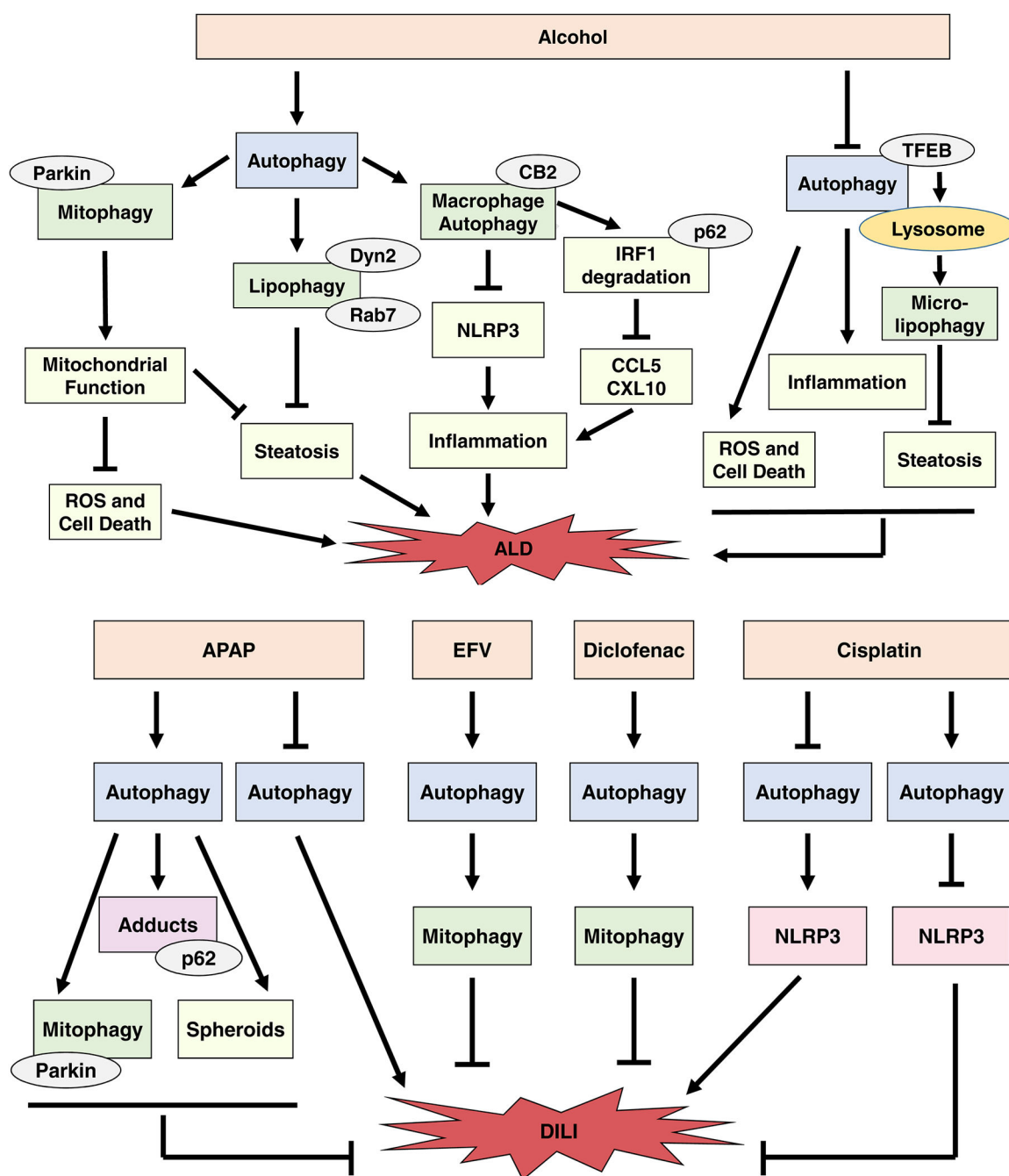
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**Highlights**

- Selective autophagy may provide therapeutic targets for ALD and DILI.
- Mitophagy, lipophagy, and macrophage autophagy protect against ALD.
- Mitophagy and selective autophagy for adducts protect against DILI.
- Autophagy activity requires assessment of autophagic flux and sufficiency.





**Figure 1. Autophagy protects against ALD and DILI.**

A) Autophagy protects against ALD by selectively removing damaged mitochondria, excess LDs, and hepatic macrophages. Alcohol was shown to both induce and inhibit autophagy depending on the alcohol model used. Mitophagy induction during excessive alcohol use degrades damaged mitochondria, which prevents ROS production and subsequent cell death and also inhibits steatosis by maintaining a healthy population of mitochondria capable of  $\beta$ -oxidation. Alcohol-induced mitophagy induction is not completely understood but may rely on the PINK1-Parkin mitophagy pathway. Parkin-independent mechanisms for alcohol-induced mitophagy activation may also exist. Alcohol-induced lipophagy prevents liver

steatosis by selectively degrading lipid droplets. Mechanisms involved in alcohol-induced lipophagy are becoming clearer and may involve TFEB, Dyn2, and / or Rab7. In addition, lysosomes may directly enwrap LDs via micro-lipophagy. Macrophage autophagy prevents accumulation of damaged mitochondria and ROS, which decreases NLRP3 inflammasome activation and subsequent liver injury. Macrophage autophagy also promotes p62-mediated degradation of IRF1, which inhibits transcription of chemokines CCL5 and CXL10, two important initiators of inflammation. Mechanisms for hepatic macrophage autophagy are not completely understood, but CB2 may have a role in its induction. Overall, selective autophagy pathways are important for protection against ALD. **B)** Autophagy protects against DILI initiated by APAP, EFV, Diclofenac, and Cisplatin. APAP was shown to both induce and inhibit autophagy. Discrepancies regarding the effect of APAP on autophagy activation may be due to differences in models used or in time points of autophagy flux assessment. Autophagy protects against APAP-induced liver injury by degrading damaged mitochondria by mitophagy and selectively removing and degrading APAP-adducts. APAP-induced mitophagy likely involves the PINK1-Parkin pathway, but Parkin-independent mechanisms for mitophagy induction may also exist. The mechanism for selective autophagy of APAP adducts is not completely understood, but the adaptor protein p62 may play a role in recognition of APAP adducts by autophagosomes. Mitochondrial spheroids may also protect against APAP-induced liver injury, which requires further investigation. EFV and diclofenac induce mitophagy, which protects against DILI by removing damaged mitochondria. The mechanisms for mitophagy induction by EFV and diclofenac have not been investigated. Cisplatin was shown to both induce and inhibit autophagy. Discrepancies in the effect of cisplatin on autophagy activation status may be due to differences in models or cells used. Inhibition of autophagy by cisplatin causes NLRP3 activation and subsequent liver injury. Autophagy activation during cisplatin use may protect against liver injury by inhibiting activation of NLRP3. Overall, autophagy protects against DILI by selective removal of damaged mitochondria and protein adducts and by preventing inflammation by reducing NLRP3 activation.