

Homeostatic Role of Autophagy in Hepatocytes

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Abstract

Autophagy actively participates in the physiological process of the liver. While the direct effect of autophagy may be limited to the sequestration and degradation of a selective cargo, its overall impact can be broad, affecting many more physiological processes regulated by the particular cargo. This review will discuss two aspects of the importance of autophagy in the liver: metabolic regulation in response to feeding and starvation, and pathological consequences in the absence of autophagy. These two aspects illustrate the homeostatic functions of autophagy in the liver, one in a more direct fashion, regulating the cellular nutrient supply, and the other in a more indirect fashion, controlling the pathological signaling triggered by the abnormal accumulation of cargos. Remarkably, the hepatic pathology in autophagy-deficient livers does not seem different from that presented in other chronic liver diseases. Autophagy deficiency can be a model for the study of the relevant molecular mechanisms.

Keywords

- ▶ autophagy
- ▶ ductular reaction
- ▶ HMGB1
- ▶ metabolism
- ▶ tumorigenesis

Several high-quality review articles have been published in recent years on the topics of autophagy and its role in the liver system.^{1–5} Hence, we will focus only on the recent development in two subjects in this field following a brief visit of the basic autophagy machinery. The two subjects, one on the regulation of autophagy in response to fasting and feeding, and the other on the pathological consequences of autophagy disruption in parenchymal cells, illustrate the physiological roles of autophagy in metabolism and in homeostasis in the liver (▶ Fig. 1).

Basic Review of the Autophagy Machinery

While three types of autophagy—macroautophagy, microautophagy, and chaperon-mediated autophagy (CMA)—have been described,^{1,2} this review only discusses macroautophagy and its role in the liver. To simplify the description, the term “autophagy” will be used throughout the text to refer to “macroautophagy” (▶ Table 1).

The basic autophagy machinery operates to maintain a basal level of autophagy activity in cells, which is required for the constitutive turnover of cytosolic components, that is, the cargos. The activity of this machinery is enhanced or reduced in response to the changing environment and the

cellular needs.⁶ Signaling events lead to the modulation of autophagy machinery at the transcriptional or translational level, which then drives the change in activity.⁷

The three types of autophagy differ mainly in the way the cargos are transported to the lysosome. In macroautophagy, the cargos are delivered to the lysosome by the double-membraned vesicle, known as the autophagosome. At the cellular level, the macroautophagy process begins with the formation of a double-membraned cup-shaped structure, called phagophore, whose edges extend and sequester cytosolic components to sequester them in the growing autophagosomal vesicles.⁸ Formation of the autophagosomes are the morphological signature of autophagy induction. Autophagosomes then fuse with lysosomes to deliver and break down its contents.⁹ The source of the phagophore membrane has not been completely resolved but may include the endoplasmic reticulum (ER), the Golgi apparatus, the mitochondria, the endocytic membrane, or the plasma membrane.¹⁰

At the molecular level, autophagy is driven by a concerted action of a suite of ATG, or “autophagy-related” molecules. The number of these ATG molecules varies in different species, but the core members are evolutionarily conserved.¹¹ The majority of autophagy molecules have been shown to function as complexes to drive the autophagy process. Seven functional

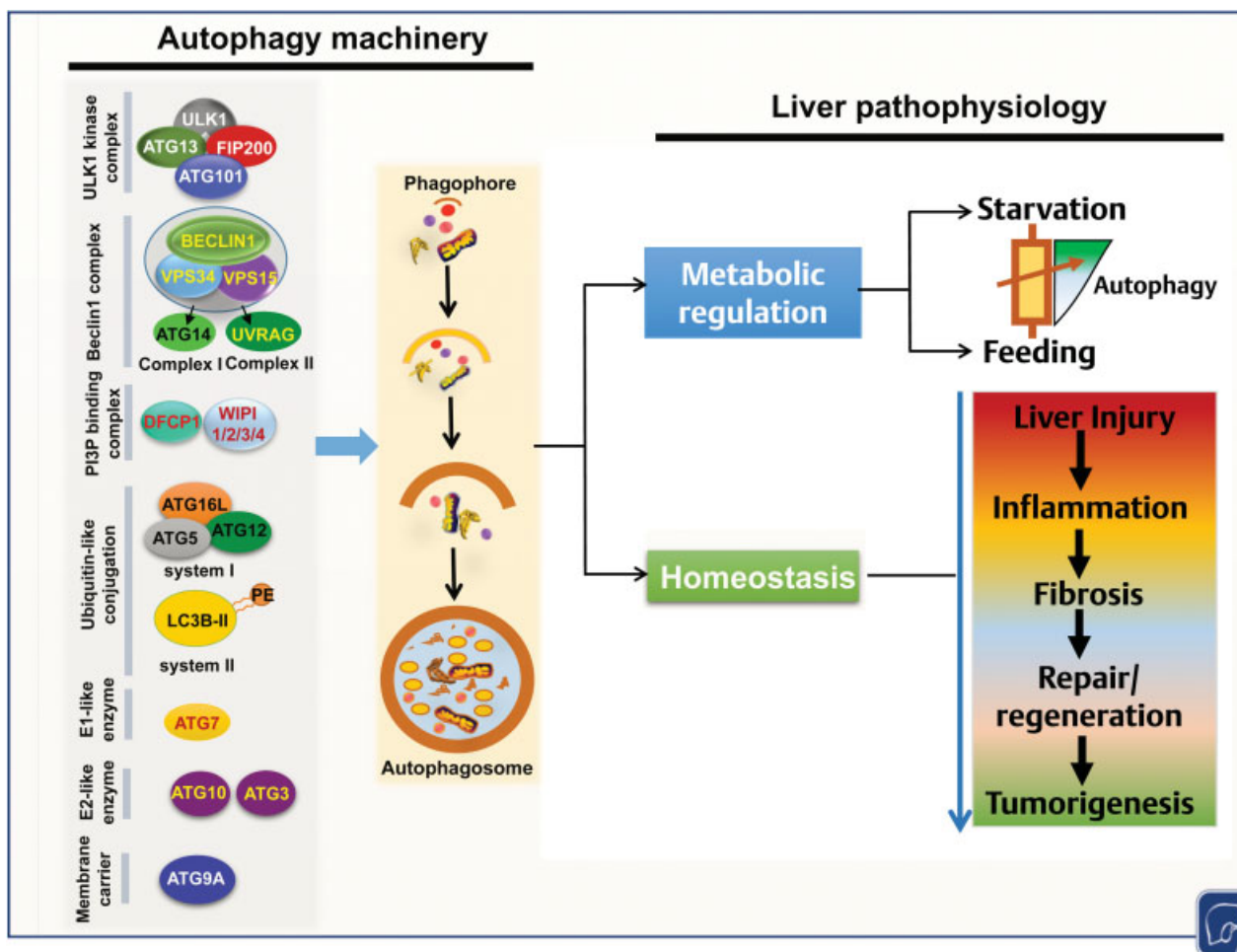


Fig. 1 Autophagy regulates liver functions and maintains the homeostasis of the liver. In macroautophagy, cellular materials are delivered by the autophagosome to the lysosome for degradation. The biogenesis of autophagosome is complicated, with the participation of multiple protein complexes, whose functions are to convert membranes from different sources into double-membraned autophagosomal vesicles, which enwrap the cellular materials to be degraded. The macroautophagic function is important to the liver and the hepatocytes to properly manage the dynamic changes in metabolism, for example, the switch between starvation and feeding. Disruption of macroautophagy leads to the disruption of hepatic homeostasis with the pathological consequences from injury to tumorigenesis.

groups or complexes have been described in the mammalian cells to perform the key steps in autophagosome formation.¹¹

- **ULK1 tetrameric kinase complex:** This is also called initiator complex and is composed of ULK1, FIP200, Atg13L, and Atg101.
- **Beclin1 complex:** The basic components are Beclin1, VPS34 (also known as phosphatidylinositol 3-kinase [PI3K] catalytic subunit 3), and VPS15 (also known as PI3K regulatory subunit 4), which are joined by Atg14 to form complex I, or by UVRAG to form complex II. Beclin1 also interact with several autophagy regulatory molecules, including NRB2F2, Ambra-1, RUBICON, and Bcl-2 with either inhibitory or stimulating effects.
- **Phosphatidylinositol 3-phosphate (PtdIns3P) binding complex:** It includes proteins such as Zinc finger FYVE domain-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting proteins (WIPI) 1/2/3/4 that are recruited to PI3P. These proteins bind to
- **PI3P through its FYVE domains and promote the formation of the omegasome.**
- **Ubiquitin-like conjugation system I:** This includes an ubiquitin-like molecule, Atg5, and its conjugating partner Atg12, which then forms a supramolecular complex with Atg16L1.
- **Ubiquitin-like conjugation system II:** This includes another ubiquitin-like molecule, LC3B, or other Atg8 homologues. They are conjugated to phosphatidylethanolamine (PE).
- **E1-like and E2-like enzymes:** They include the E1-like molecule Atg7, and the E2-like molecule, Atg10 (for Atg5-Atg12 conjugation), or Atg3 (for LC3B-PE conjugation).
- **Atg9A:** This is a multimembrane spanning Atg protein postulated to serve as a membrane carrier for phagophore extension.

The initiation of phagophore begins with the activation of ULK1 kinase complex and the formation of ULK1 punctae at a discrete location on the ER. Activated ULK protein complex

Table 1 Major concepts and learning points

Term	Concept	Learning points
Autophagy	An evolutionarily conserved process for delivering intracellular materials for degradation in the lysosome	1. Three types of autophagy: macroautophagy, microautophagy, chaperon-mediated autophagy, which differ in the way by which cellular materials are delivered to the lysosome.
		2. Virtually all cellular constituents could be delivered in a bulk or selective way.
		3. Its activity is regulated by multiple environmental and internal cues in response to different needs and stress.
		4. In macroautophagy, multiple molecules, designated as ATG molecules, are involved in the core process of autophagosome biogenesis, cargo recognition, and delivery to the lysosome.
DAMP and HMGB1	Damage-associated molecular patterns (DAMPs), also known as danger-associated molecular patterns, danger signals, or alarmin, refer to a heterogeneous group of biological substances that triggers sterile inflammation in response to stress or following cell death.	1. High-mobility group box 1 (HMGB1) is one of the best characterized DAMPs. This protein normally binds to DNA with uncharacterized function. But it translocates to the external space either due to cell breakdown or by an active mechanism that involves inflammasomes. Its effect can be mediated by one of the following receptors that are present on different types of cells: RAGE, TLR2, or TLR4.
		2. HMGB1 has diverse functions, not only in promoting inflammation or fibrosis but also in promoting repair/regeneration, such as angiogenesis and proliferation. It has also been linked to the regulation of tumorigenesis, autophagy, and other processes. Its function can thus be context-dependent.
		3. Other DMAP members can include heat shock proteins, adenosine triphosphate, uric acids, DNA, etc.
Ductular Reaction and hepatic progenitor cells	Ductular reaction is the histology manifestation of the expansion of ductular cells, also known as hepatic progenitor cells, in the hepatic parenchyma. Ductular reaction is frequently present in chronic liver diseases.	1. The significance of ductular reaction is still not well understood. Some consider it as a regenerative/repair process, whereas others consider it to be associated with poor prognosis and severe cirrhosis.
		2. The origin of ductular cells is not well defined. They may be derived from cholangiocytes and/or hepatocytes, as they carry markers from both types of cells. Ductular cells may give rise to regenerating hepatocytes or cholangiocytes, and hence they are also known as hepatic progenitor cells.
		3. The mechanism of their expansion and contraction is not defined well. Certain proteins have been found to have growth factor-like activity.

recruits and activates the Beclin1 complex to the phagophore through phosphorylation. Beclin1-/Atg14-directed activation of the VPS34/VPS15 kinase generates a local pool of PtdIns3P. This alteration in the lipid composition changes the membrane curvature, further expansion of phagophore and local recruitment of the oligomers of Atg5-Atg12-Atg16L. The latter facilitates LC3B conjugation to PE on the autophagosomal membrane. LC3B lipidation causes further expansion and enclosure of the autophagosomal membrane. Formation of autophagosomes may be followed by a maturation process in

the mammalian cells through fusion with other endocytic membranes. The amphisome, product of the maturation process, eventually fuses with the lysosome for cargo degradation.

For the precise and efficient fusion of the double-membraned autophagosomes with the single-membraned lysosome, tethering factors (HOPS complex, RAB7, and adaptors) and fusion machinery SNARE proteins are involved.¹ Lysosomal hydrolases degrade the inner autophagosomal membrane and expose the autophagosomal cargo for further degradation. The degradation products, including amino acids, sugar, and

free fatty acids (FFAs) are transported out of the autolysosome via the lysosomal permeases/efflux transporters. During the termination step of autophagy, lysosome is regenerated by a process called autophagic lysosome reformation (ALR). ALR involves autolysosome tubulation, scission and budding, and generation of the protolysosomes, which is matured to become the lysosomes.^{12,13}

Interestingly, autophagosomes are generally formed in the peripheral region of the cells whereas lysosomes are clustered around perinuclear regions during cellular stress such as starvation.¹⁴ To achieve efficient fusion of autophagosomes and lysosomes, autophagosomes are transported along microtubules (dynein mediated) toward perinuclear region where lysosome are located.¹⁴

Besides bulk cargo degradation, autophagy can selectively transport a given type of cargos for degradation. The *selectivity* of the autophagy process seems to be governed by the organelle that give rise to the membrane. The molecule/s that confers *exclusivity* during the selective process is not clear. Depending on the particular type of cargo involved, autophagy may be named as mitophagy (damaged mitochondria), proteophagy (protein aggregates), pexophagy (peroxisomes), lipophagy (lipid droplets), ferritinophagy (ferritin), ribophagy (ribosomes), or xenophagy (intracellular microorganisms).^{15,16} Selective cargo degradation relies on a plethora of selective autophagy receptors or adaptors such as p62/SQSTM1, NBR1 (neighbor of BRCA1 gene 1), NDP52 (nuclear domain 10 protein 52kDa), and OPTN (optineurin). They generally bind to the cargo (often ubiquitinated) and key components of the autophagy machinery, especially the LC3 protein to mediate the selective engulfment of the cargo by the autophagosome.^{15,17}

Role of Autophagy in Metabolic Homeostasis

In the light of the liver's function in the metabolic control of the body, it is not surprising that autophagy is an integrated part of the mechanism. Hepatic autophagy is important for the maintenance of systemic nutrient and energy balance. The catabolic role of autophagy can be responsible for the breakdown of glycogen, lipids, and proteins in response to the needs of the body.

Degradation of Macromolecules for Nutrient Needs

Hepatic autophagy contributes significantly to the maintenance of the blood glucose. While in adults hepatic glycogen is mainly mobilized through the action of the phosphorylase and debranching enzymes in the cytosol, it is almost exclusively hydrolyzed by lysosomal acid glucosidase in the neonatal stage.¹⁸ Immediately after birth or following the treatment of glucagon, the number and size of autophagic vacuoles containing glycogen are increased.¹⁹ In newborn hepatocytes, autophagic vacuoles are distributed predominantly at the border of hyaloplasmic glycogen stores. They mostly contain engulfed glycogen at various states of disintegration. Interestingly, liver glycogen in the newborn hepatocytes (fetal-type glycogen) differs from that in adult cells, for example, in the degree of branching. Other than glycogen degradation, autophagy also degrades proteins to

generate amino acids for hepatic gluconeogenesis,²⁰ which can also contribute to glucose homeostasis.

Protein degradation maintains the intracellular amino acid pool and the plasma level of amino acids. Other than meeting metabolic needs, autophagic degradation of protein may also contribute to protein quality control by removing misfolded and aggregated proteins, alleviating proteotoxicity caused by an abnormal level of proteins. Degradation of a particular protein may have a particular impact. Degradation of hexokinase 2 (HK2) can directly affect liver glycolysis.²¹ Degradation of p62/SQSTM1, an adaptor/receptor of selective autophagy, is not only a part of the normal autophagy process, but also is key to prevent abnormal activation of Nrf2, which can otherwise cause significant liver pathology.

Breakdown of triglycerides (TGs) by lipophagy may provide the needed energy during nutrient deprivation. In hepatocytes, newly formed TGs are stored as cytoplasmic lipid droplets (LDs). Mobilization of neutral lipids from LDs generates FFAs for mitochondrial oxidation and energy production. TGs in LDs could be enzymatically hydrolyzed to FFA either by cytosolic lipolysis (known as neutral lipolysis, via adipose TG lipase i.e., ATGL) or by lipophagy (known as acidic lipolysis via lysosomal acid lipase [LAL]).²² The functional coordination of the two lipolysis pathways is not well studied. Surprisingly, ATGL is recently shown to be both necessary and sufficient to drive autophagy/lipophagy in the liver in response to starvation.²³ Promotion of ATGL-mediated LD turnover and FFA oxidation was completely abrogated when autophagy was pharmacologically or genetically blunted. Mechanistically, ATGL promotes SIRT1 activation to activate hepatic autophagy.²³ These studies indicate that the two lipolysis pathways in the hepatocyte are well connected and coordinated in a much closer way than we have expected.

Regulation of Autophagy during the Switch of Fast-Fed State

Liver metabolism dynamically alters between anabolism and catabolism depending on the nutrient status. As a catabolic process, hepatic autophagy occurs at low rates under fed condition (constitutive or basal autophagy), but it is strongly induced under fasting or starvation conditions (induced autophagy). Multiple signaling events can occur to regulate the autophagy activity.

Cells integrate the up-stream signals (such as these mediated by the AMPK and/or mTORC1 pathway) and the transcriptional regulation to maintain appropriate control of autophagy. Many transcriptional regulators have been known to regulate autophagy in mammalian cells, including p53, STAT3, NF- κ B, FoxO1/3, SIRT1, TFEB, HIF1 α , GATA, and ATF4.²⁴ Notably, FXR and PPAR α are two nutrient sensing nuclear receptors that modulate autophagy at the transcriptional level in close association with the liver metabolism.^{25,26}

In the fasting condition, glucagon is activated and acts on hepatocytes to raise the level of cAMP. The subsequent activation of the cAMP/PKA signaling pathway leads to (1) inactivation of FXR to allow PPAR α to access to the PPAR α promoters of several lysosomal and autophagy genes; (2)

phosphorylates Ser¹³³ of CREB to promote its nuclear translocation; and (3) inhibition of salt-inducible kinases (SIKs), which phosphorylate Ser¹⁷¹ of CREB-regulated transcription coactivator 2 (CRTC2) to retain it in the cytoplasm. These events lead to the formation of CREB/CRTC2 complex and their nuclear translocation. Once in the nucleus, CREB/CRTC2 (1) upregulates the expression of genes of the gluconeogenesis pathway; (2) upregulates expression of TFEB, which in turn promotes autophagy and lysosomal biogenesis genes; (3) increases PPAR α and its co-activator PGC1 α expression to elevate the expression of genes involved in autophagy, lysosome biogenesis, and β -oxidation; and (4) increase LAL gene to increase lysosomal lipid degradation.

In parallel, lower levels of hepatocellular nutrients, particularly amino acids, cause downregulation of mTORC1 activity, leading to de-phosphorylation of cytosolic pool of TFEB and S6 kinases 2 (S6K2). Both of these events cause increased nuclear translocation of TFEB and cytosolic export of a transcriptional repressor, NCoR1, leading to increased expression of autophagy and lysosome biogenesis genes.

Conversely, in the fed condition, elevations of amino acids, glucose, and fatty acids, together with an abundant level of insulin, promote mTORC1 activation at lysosomal surface, leading to (1) phosphorylation of TFEB to prevent its nuclear translocation, which will induce expression of several autophagy and lysosome-related genes, and (2) phosphorylation of ribosomal protein S6K2. Phosphorylated S6K2 forms complex with NCoR1 and then translocate to the nucleus to repress the expression of catabolic genes, such as β -oxidation pathway genes.^{25,26}

In parallel, fed-state sensing nuclear receptor FXR (1) binds to CREB and disrupts the functional complex between CREB and CRTC2; (2) competes with another nutrient-sensing regulator, PPAR α , for binding on the promoter sites, known as the DR1 element, which presents in many autophagy genes, and inhibits their expression. All these molecular events assure the prevention of autophagy activation. However, basal autophagy level is maintained during this process.

Role of Autophagy in Fasting-Induced Hepatic Steatosis

One of the interesting features during fasting is the development of hepatic steatosis. FFAs generated by lipolysis in adipose tissue enter hepatocytes and are temporarily stored in the form of LDs before they are oxidized by the hepatic mitochondria, or secreted as VLDL. Surprisingly, autophagy-deficient hepatocytes fail to mount fasting-induced steatosis.²⁷⁻²⁹ Thus, autophagy is critical for starvation-induced LD formation in liver cells. It is thought that LC3-II binding to LDs is essential for the LD enlargement in hepatocytes.²⁹ The mechanism of impaired fasting-induced LD biogenesis in autophagy-deficient livers is unknown. It seems that this failure is not related to any adaptive changes in de novo lipogenesis, β -oxidation, or VLDL secretion, but it is related to Nrf2 activation since co-deletion of Nrf2 in autophagy-deficient livers restored the adaptive steatosis.³⁰ This dual role of hepatic autophagy in lipophagy versus LD biogenesis

and its impact in hepatic lipid metabolism is an enigmatic issue that requires further studies.

The Homeostatic Role of Autophagy: Consequences of Autophagy Deficiency

Autophagy dysfunction due to external (improper diet or chemicals) or internal (molecular defects) factors can render hepatocytes vulnerable to a variety of stress, resulting in severe liver pathogenesis. Notably, genetically inherited pathogenic mutations in any of the reported autophagy genes are rarely reported in humans. A study identified a homozygous missense mutation in Atg5 that caused mental retardation, developmental delays, and congenital ataxia.³¹ The same mutation in the yeast and the fly caused reduced autophagy and movement disorder, respectively. But no liver presentation was reported. Constitutive deletion of key autophagy genes in mice causes perinatal lethality,³² which may explain the lack of loss-of-function mutation in human. Tissue-specific deletion of autophagy genes allows the characterization of important function of autophagy in specific organs and the pathology resulted from the lack of such function (**► Table 2**)

Regarding the liver, autophagy genes have been deleted in parenchymal cells, stellate cells, or Kupffer cells, leading to different phenotypes (**► Table 2**). It has to be pointed out that autophagy-deficient mice created by crossing mice with a floxed autophagy gene to the Albumin-Cre transgenic mice will have deletion of the floxed autophagy gene not only in hepatocytes but also in cholangiocytes, since the albumin promoter starts working at E15 in embryonic hepatoblasts, which give rise to both hepatocytes and cholangiocytes. Thus these mice have autophagy deficiency in parenchymal cells (*Atg^{ΔPar}*). By crossing the same floxed mice to transgenic mice expressing a modified tamoxifen-inducible *Alb-Cre* promoter (*Alb-CreERT2*), one can induce the deletion of the floxed autophagy gene in adult mice so that the impact can be limited to the hepatocytes, since the albumin promoter only works in hepatocytes in adult livers (*Atg^{ΔHep-ERT2}*). While several autophagy genes have been deleted in the parenchymal cells (**► Table 2**), *Atg7^{ΔPar}* and *Atg5^{ΔPar}* mice were best characterized,^{28,33-38} which have a most complicated pathologic presentation, including hepatomegaly, injury, inflammation, ductular reaction (DR), fibrosis, and tumorigenesis.^{28,33,37,38} This phenotype has been largely replicated in the *Atg7^{ΔHep-ERT2}* mice,³⁸ suggesting that autophagy deficiency in the hepatocytes is the main driving force for the alterations.

Loss of autophagy in stellate cells reduces fibrotic response in injury as autophagy is required for the maturation of these cells.³⁹ The phenotype in mice bearing Atg5 deletion in the myeloid compartment is not restricted to the liver but the autophagy-deficient macrophages had abnormalities in polarization, which enhanced hepatic inflammatory response and liver injury in fatty liver disease models.⁴⁰

Interestingly, the liver phenotypes caused by autophagy deficiency in parenchymal cells resemble those seen in clinically common chronic liver diseases caused by other etiologies, such as hepatitis virus, alcohol, or high fat diet.

Table 2 Systemic and conditional *Atg* gene knock out mouse, and associated phenotype

Type	Genotype	Tissue	Gene Function	Phenotype	Citation (s)
Conventional	<i>Atg3</i> $-/-$	Whole body/systemic	Phosphatidylethanolamine (PE) conjugation enzyme to Atg8 like E2	Death immediately after birth, suckling defect	83
	<i>Atg5</i> $-/-$	Whole body	E3 like activity for Atg8 conjugation system in corporation with Atg12	Death immediately after birth, suckling defect	32
	<i>Atg7</i> $-/-$	Whole body	Atg8- or Atg12-activating enzyme like E1	Death immediately after birth, suckling defect	28
	<i>Atg9</i> $-/-$	Whole body	The only membrane protein among Atg genes	Death immediately after birth, suckling defect	84
	<i>Atg12</i> $-/-$	Whole body	Ubiquitin-like protein covalently attached to Atg5	Death on postnatal day 1	85
	<i>Atg13</i> $-/-$	Whole body	ULK1 protein complex, Autophagy initiation step	Death in utero, growth retardation of embryos and myocardial growth defect	86
	<i>Atg16L</i> $-/-$	Whole body	Interact with Atg12-Atg5 complex	Death immediately after birth, suckling defect	58
	<i>Bclin 1</i> $-/-$	Whole body	Bcl-2 binding protein and a component of PI3 kinase complexes	Developmental defect and death in utero (E7.5d-E8.5d)	72,73
	<i>FIP200</i> $-/-$	Whole body	Protein kinase	Embryonic death at mid/late gestation associated with heart failure and liver degeneration	87
Conditional	<i>Alb-Cre-Vps34</i> $-/-$	Hepatocyte, Cholangiocyte	Class III phosphoinositide 3-Kinase (PI3K)	Hepatomegaly, steatosis, increased fatality	88
Conditional	<i>Alb-Cre-Atg7</i> $-/-$	Hepatocyte, Cholangiocyte	Atg8- or Atg12-activating enzyme like E1	Hepatomegaly, liver injury, inflammation, fibrosis, hepatoma	34,38
Conditional Inducible	<i>Atg7</i> <i>F/F</i> <i>Mx1-Cre</i>	Hepatocyte	Atg8- or Atg12-activating enzyme like E1	Hepatomegaly, liver injury, inflammation, fibrosis, hepatoma	28
Conditional Inducible	<i>Atg5</i> <i>F/F</i> <i>Mx1-Cre</i>	Hepatocyte	E3-like activity for Atg8 conjugation system in corporation with Atg12	Hepatomegaly, liver injury, inflammation, fibrosis, hepatoma	89
Conditional Inducible	<i>Ert-Atg7</i> <i>F/F</i>	Hepatocyte	Atg8- or Atg12-activating enzyme like E1	Hepatomegaly, liver injury, inflammation, fibrosis, hepatoma	38,90
Conditional	<i>ULK1/2</i> $-/-$	Hepatocyte, Cholangiocyte	Autophagy initiation	Hepatomegaly	91
Conditional	<i>FIP200</i> $-/-$	Hepatocyte, Cholangiocyte	Autophagy initiation	Hepatomegaly, liver injury, inflammation, and hepatic fibrosis	27
Conditional	<i>Atg7</i> <i>F/F</i> <i>GFAP-Cre</i>	Stellate cell	Atg8- or Atg12-activating enzyme like E1	Normal liver architecture and intact stellate cell ultrastructure, no liver injury, attenuated liver fibrosis following liver injury	39
Conditional	<i>Atg5</i> <i>F/F</i> <i>Lzy2-Cre</i>	Macrophage	E3-like activity for Atg8 conjugation system in corporation with Atg12	Polarization of macrophage into proinflammatory M1 type	40

Thus, it is possible that these pathologies share similar or common molecular mechanisms although unique signaling components may be required to trigger these pathological changes in response to specific etiologies. Thus, the study of the pathology in autophagy-deficient liver can provide important mechanistic insights to the similar process in other chronic liver diseases.

p62/SQSTM1 and Nrf2 Key Cellular Players

The specific signaling leading to the common pathological changes in autophagy-deficient livers is not well understood. Although the direct effect of autophagy deficiency is the failure to degrade certain cargos, not all cargos would have pathologic significances if accumulated. Abnormal mitochondria accumulated due to autophagy deficiency are often considered to be pathogenic because they can be the major source of ROS.^{41,42} However, the real culprit may actually be something else, which amplifies the dire consequence of autophagy deficiency.

It has been found that nuclear factor (erythroid-derived 2)-like 2, also known as Nrf2, is persistently activated in autophagy-deficient hepatocytes due to the accumulation of p62/SQSTM1.³⁵ p62/SQSTM1 is a major cargo of autophagy, and its amount is massively elevated in autophagy deficiency.^{28,33,41} p62/SQSTM1 binds to Keap1 (Kelch-like ECH-associated protein 1), weakening the interaction of Keap1 with its target protein, Nrf2, which allows Nrf2 to escape from the ubiquitination by a E3-ligase complex recruited by Keap1 and degradation by the proteasome.³⁵ Undegraded Nrf2 translocates from the cytosol to the nucleus and activates a whole series of transcriptional targets.

Genetic deletion of *Nrf2* in *Atg7^{ΔPar}* or *Atg5^{ΔPar}* mice largely suppressed autophagy deficiency-induced liver pathologies.^{33,35,38} Consistently, genetic deletion of *p62* in autophagy-deficient liver also alleviated most of the liver pathophysiology.^{34,41} It is not entirely clear how persistent activation of Nrf2 could lead to all the major pathological changes. We have evidence that controlling hepatic high-mobility group box 1 (HMGB1) release is one of the mechanisms (see later). It is possible that some of the genes under the transcriptional regulation of Nrf2 are responsible for the pathological outcomes. Paradoxically, Nrf2 is known to activate a variety of antioxidant genes (HO-1, Nqo1, Gstm, etc.) and hepatic detoxification enzymes under normal conditions in response to oxidative stress.⁴³ Indeed, these genes were highly overexpressed in autophagy-deficient livers. It is possible that the Nrf2 toxicity in this context is mediated by other Nrf2's targets.

The almost complete suppression of the liver pathologies by *Nrf2* deletion raises another important issue. *Atg7^{ΔPar}* or *Atg5^{ΔPar}* mice with *Nrf2* deletion appear normal morphologically and in function. However, these hepatocytes do not regain the macroautophagic function enabled by *Atg7* or *Atg5*. It is possible that this deficiency is well compensated by the noncanonical *Atg7*-/*Atg5*-independent pathway,⁴⁴ or by the CMA.⁴⁵ An argument against this possibility is that these compensatory mechanisms, if effective, do not seem to be potent enough to prevent or alleviate Nrf2-mediated pathology in the *Atg7*- or *Atg5*-deficient hepatocytes. An alternative but radical hypothesis is that *Atg7*-/*Atg5*-mediated macroau-

tophagic degradation may not be as important for hepatocyte function as expected. These considerations are important but are yet to be examined further experimentally.

Injury in Mice with Autophagy Deficiency in the Liver

Liver injury, as measured by elevated blood AST, ALT, and ALP levels, is a prominent feature in *Atg7^{ΔPar}* or *Atg5^{ΔPar}* livers. However, several puzzling observations can be made. One is that liver injury is more significant in *Atg7^{ΔPar}* livers than in *Atg5*-deficient livers³⁸ (Bilon Khambu and Xiao-Ming Yin, unpublished observations, 2018). In fact, liver injury is alleviated in older *Atg5^{ΔPar}* mice.³³ The reason for this difference is not clear but may be related to the fact that *Atg7* lies at the upstream of *Atg5* in the conjugation system, although deletion of either gene results in completely defective LC3 conjugation and accumulation of p62/SQSTM1 to the similar level.^{28,33,38}

The other interesting observation is that the extent of protection against liver injury by the deletion of *p62/SQSTM1* in *Atg7^{ΔPar}* mice is not as robust as that of the deletion of *Nrf2*,^{34,35} suggesting that there might be unidentified factor/s other than p62/SQSTM1 involved in the persistent Nrf2 activation. In addition, while *Nrf2* deletion in *Atg7^{ΔPar}* mice rescues most of the injury phenotypes, the level of ALP remains high³⁵ (Bilon Khambu and Xiao-Ming Yin, unpublished observations, 2018), suggesting that there are Nrf2-independent pathological events. Finally, despite the robust liver injury observed, cell death does not seem to be widely spread in the liver and caspase activation seemed to be minor.^{33,38}

Overall, these observations recapitulate the question on the mechanism of liver injury in *Atg7^{ΔPar}* mice, or the question on how persistent activation of Nrf2 can cause liver injury. Toward that, there could be several possible hypotheses. They are not mutually exclusive and in fact could be well overlapped, resulting in the actual scenarios.

Quantitative Proteotoxicity

Misfolded proteins can be toxic to cells, a phenomenon known as proteotoxicity. In the autophagy-deficient cells, both correctly folded and misfolded proteins are dramatically increased due to inhibition of the degradation of normal long-lived protein and abnormal aggregated proteins by autophagy. In addition, persistent Nrf2 activation leads to overproduction of many of its target molecules. Proteomic analysis of autophagy-deficient liver shows increases in total protein mass, and there are no specific changes in the protein composition in these livers.⁴⁶ An excess amount of proteins, even correctly folded, could be toxic to cells, which is thus provisionally termed as quantitative proteotoxicity. This toxic effect may be manifested as hepatocyte hypertrophy, hepatomegaly, and liver injury. Treating mice with a generic protein synthesis inhibitor, cycloheximide, reduced liver injury to some degree (Bilon Khambu and Xiao-Ming Yin, unpublished observations, 2018), which could support the proteotoxicity hypothesis.

Hepatic Lobular Structure Alterations and Cholestatic Injury

These hypertrophied hepatocytes may alter the hepatic lobular structure, sinusoid deformation, hepatocyte detachment from

the matrix, cholestasis, or hemostasis. All of these may lead to liver injury. We have observed deformation of bile canaliculi and elevation of blood bile acid level (Bilon Khambu and Xiaoming Yin, unpublished observations, 2018), which in combination of extensive DR in the autophagy-deficient livers (see later) may indicate cholestatic injury.

Intrinsic Molecular Defects

The deficiency of autophagy in the hepatocytes and/or the persistent activation of Nrf2 causes intrinsic cellular defect as the molecular pathways have been altered. These alterations result in defective physiological process and hence cell injury.

Extrinsic Cause of Hepatocyte Injury

In this hypothesis, cellular injury is not caused by the intrinsic cell-autonomous failure but rather due to secondary events, the recruitment and activation of immune cells, which are responsible for the inflammation and cellular injury in the $Atg^{\Delta Par}$ livers.^{28,33,38}

Role of HMGB1 in Hepatic Pathogenesis Caused by Autophagy Deficiency

Autophagy deficiency leads to inflammation, fibrosis, DR, which represents the expansion of hepatic progenitor cells (HPCs) or ductular cells (DCs), and tumorigenesis. These presentations resemble those observed in many common chronic liver diseases caused by other etiologies, such as virus, alcohol, or high fat. However, the mechanisms are complicated and there might be individual signaling pathways initiated by specific etiology and common pathways that are accountable for the common features.

Two recent studies^{38,47} indicate that one of the damage-associated molecular pattern molecules, HMGB1, can serve as a signaling mechanism to two of these changes, the DR and tumorigenesis, one in the context of autophagy deficiency,³⁸ and the other in the autophagy-competent condition,⁴⁷ again indicating common mechanism can be present in chronic liver injury caused by different etiologies.

Mechanism of HMGB1 Release

HMGB1 is a nuclear protein but its role in the nucleus is not very clear.⁴⁸ Acetylation of HMGB1 can lead to the translocation from the nucleus to the cytosol, which can be a pathological response.⁴⁹ Release of HMGB1 to the extracellular space is often due to cellular breakdown as the result of cell death. However, active secretion of HMGB1 from live macrophages is known to be mediated by the inflammasome.^{50,51}

Inflammasomes are multimeric protein complexes formed to produce inflammatory cytokines, IL-1 β and IL-18. There are several inflammasomes, which are activated in response to different pathogen-associated molecular patterns. Activation of all types of inflammasome leads to the recruitment and activation of Caspase-1. Activated Caspase 1 proteolytically processes IL-1 β and IL-18, as well as a pore-forming protein Gasdermin D (GSDMD), which is responsible for the release of the two cytokines.⁵²⁻⁵⁴ Notably, besides Caspase-1, Caspase-11 can also recognize and cleave GSDMD.^{53,54}

Externalization of HMGB1 by hepatocytes under a variety of pathological conditions have been described.^{38,47,55,56} While in most cases this release seems to be caused by cellular breakdown, it is by active secretion via the inflammasome in autophagy-deficient hepatocytes.³⁸ In earlier studies, the link of autophagy and inflammasome had been observed in macrophages for the secretion of IL-1 β .⁵⁷ Association of inflammasome activities with certain autophagy genes, such as ATG16L, LC3B, and Beclin1, has been observed in other mammalian cells.^{58,59} In the liver, activation of the inflammasome has been reported in not only nonparenchymal cells, but also in parenchymal cells under other cases.^{60,61} However, the molecular mechanisms had not been mapped out in these cases. In the case of autophagy-deficient hepatocytes, we now find that Nrf2 is required for the activation of Caspase-1, and that both Nrf2 and Caspase-1 are required for HMGB1 release independent of cellular injury.³⁸ In fact, Nrf2 activation alone in the absence of autophagy deficiency is sufficient to trigger HMGB1 release in an inflammasome-dependent manner. This study thus provides a molecular example of how inflammasomes in hepatocytes may be activated.

Role of HMGB1 in Ductular Reaction

The function of HMGB1 is quite diverse and is likely context-dependent.^{48,62} It is known as a danger-associated molecular pattern (DAMP) and is in general associated with sterile inflammation, fibrosis, and angiogenesis among other functions. However, when HMGB1 was deleted in the autophagy-deficient livers, neither inflammation nor fibrosis was noticeably affected,³⁸ indicating that these two processes were mainly mediated by other molecular pathways that have yet to be defined. Surprisingly, HMGB1 plays important roles in two other pathological presentations in autophagy-deficient livers, DR, and tumor development.³⁸

Ductular reaction is commonly seen in liver diseases with chronic injury⁶³ and represents the expansion of DCs, also known as oval cells in rat and HPCs in some literatures.^{63,64} Morphologically, DCs are small cells with a high nuclear/cytosol ratio. Their origin is controversial and may represent different populations depending on the nature of injury. Both biliary epithelial cells and hepatocytes have been postulated to give rise to DCs, as DCs seem to be mainly originated from the portal-parenchymal interface. They express several biliary cell markers, such as CK19, Sox9, and EpCAM, and hepatocyte markers, such as CK18 and albumin.^{63,64}

The driving force for the expansion of DCs is still far from clear. In autophagy-deficient livers, HMGB1 seems to be a major driving force. It possesses a mitogenic effect in vitro on DCs. Genetic deletion of HMGB1 or pharmacological inhibition of HMGB1 release results in long-term suppression of DR.³⁸ Notably, in several other mouse models of liver injury, including that caused by the genetic deletion of *Mdr2*, by the DDC diet, the choline-deficient, ethionine-supplemented diet, or by diethylnitrosamine (DEN) treatment, HMGB1 was important for the expansion of DCs.^{38,47} RAGE (receptor for advanced glycation endproducts) is an HMGB1 receptor, which is also required for DR in autophagy-deficient livers,³⁸

as well as for DR in *Mdr2*-deficient livers,⁶⁵ DDC-treated livers,^{38,47} and DEN-treated livers,⁴⁷ whereas other HMGB1 receptors, such as TLR4, TLR9 did not seem to be involved.⁴⁷ These studies indicate that the HMGB1-RAGE signaling axis could be generally important for the expansion of DCs.

Several other factors have been indicated in the DR, including FGF7, TNF-related WEAK inducer of apoptosis (TWEAK) and TWEAK receptor Fn14,^{66,67} connective tissue growth factor, integrin α V β 6, and ADAMTS7.⁶⁴ The relationship of these signaling pathways with that of HMGB1-RAGE would be important to be delineated.

The functional relevance of DR in liver diseases is not entirely understood. It is widely believed that DCs act as “facultative” progenitor cells that could give rise to either cholangiocytes or hepatocytes, depending on which compartment is injured. Thus DR seems to represent a repair/regeneration response. Liver injury is elevated in autophagy-deficient livers when HMGB1 is deleted,³⁸ which could be related to a poor DR and thus a poor repair process.

Role of HMGB1 in Tumorigenesis

Autophagy-deficient liver develops spontaneous tumor that steadily increased in size and number as the mice get older.^{37,38,68,69} In *Atg7*-deficient liver, tumors start to appear beginning from 9 months of age, which increases steadily through 15 to 17 months. As discussed in the following section, there could be several molecular events involved in the tumorigenesis in autophagy-deficient livers. Interestingly, HMGB1 and its receptor RAGE are also important in promoting tumor development.³⁸

No tumors were grossly observable in the liver of HMGB1-codetected or RAGE-codetected autophagy-deficient livers at the age of 9 months. The tumors were developed in these mice at a later time point of 15 to 17 months. However, the size and number of tumor were still not as significant as in the presence of HMGB1.³⁸ We still do not understand how HMGB1-RAGE may affect tumor development in this context, but it does not seem to be related to some of the early tumorigenic events, such as metabolic reprogramming or hedgehog signaling.³⁸

It is intriguing that HMGB1 regulates both DR and tumorigenesis as DCs has been considered by some studies to be a cellular compartment susceptible to malignant transformation.⁷⁰ However, in several models of murine hepatic tumors, direct evidence that DCs gave rise to HCC had not been found.⁷¹ Thus, the connection of the two pathological processes regulated by HMGB1 and how HMGB1 promotes tumor development have yet to be determined.

Role of Autophagy in Hepatic Tumorigenesis

Autophagy is important for the maintenance for hepatocellular homeostasis and its dysfunction can lead to hepatic tumors. In general, autophagy maintains the homeostasis of normal cells and thus suppresses tumorigenesis. However, autophagy is also used in tumor cells as an alternative mechanism for nutrients supply and elimination of damaged organelles. It can thus function as a pro-tumor mechanism. In the liver context, most of the studies based on autophagy deficiency suggest the antitumorigenic role of autophagy.

The first genetic evidence showing disruption of autophagy executing genes may be an important mechanism of hepatic tumorigenesis was based on observation of tumor lesions in haplo-sufficient *Beclin1*-deficient mice.^{72,73} Loss of autophagy genes *Atg7*⁶⁸ or mosaic *Atg5*-deletion³⁷ in rodent liver also lead to hepatic tumors in aged mice. Besides the observations in the mouse models, association of autophagy deficiency with the development of HCC has been reported in human cases.⁷⁴ In human HCC tissues, decreased *Atg5* or *Beclin1* gene expression together with p62 accumulation (low autophagic activity) were observed in comparison with that in adjacent non-tumor tissues.⁷⁵ Selective recruitment and autophagic degradation of oncogenic miR-224 or cell cycle regulator Cyclin D were reported to affect liver tumorigenesis.⁷⁶ This may indicate that autophagy can have a more direct impact on tumorigenesis.

The Role of Mitochondria Damage and ROS

One potential mechanism could be aberrant signaling regulated to hepatocellular mitochondrial dysfunction and ROS production. Studies in *Atg7*- or *Atg5*-deficient models have established that autophagy-deficient hepatocytes have mitochondrial dysfunction, oxidative stress, and DNA damage, which are key contributing factors for tumor development.^{33,37,42} All these factorial changes in autophagy-deficient hepatocytes may culminate in hepatocellular adenoma.^{33,37,42} Liver-specific *Atg5*-deficient mice fed with an oxidant N-acetylcysteine had a reduced level of oxidative stress in the liver and reduced liver tumor incidence.⁴²

The Role of Nrf2

Abnormal accumulation of p62/SQSTM1 could lead to dysregulated activation of NF- κ B pathway,^{77,78} Wnt signaling,⁷⁹ or Nrf2 activation.³⁵ Simultaneous deletion of Nrf2 in mice with hepatic deficiency in *Atg7* or *Atg5* strikingly reduced the size and numbers of tumors.^{33,35,38} Notably, hyperactivation of Nrf2 associated with p62 accumulation has been observed in certain types of human HCC.^{36,68}

In addition to its classical role of Nrf2 in redox homeostasis, Nrf2 has a new role in metabolic reprogramming.^{36,80} In autophagy-deficient livers, phosphorylation of p62 at Ser351 activates Nrf2 to elevate the expression of Nrf2 target genes, which include those encoding enzymes involved in the pentose phosphate pathway and glutamine metabolism.⁸¹ Thus, Nrf2 redirects glucose metabolism to the gluconate pathway, directs glutamate toward glutathione synthesis, and hence supports cell proliferation in addition to enhancing cytoprotection.³⁶ This dual role of Nrf2 between redox and metabolic regulation seems to provide the survival benefits to the tumorigenic hepatocytes. Finally, as mentioned earlier, Nrf2 can also control the release of HMGB1, which promotes tumor development in autophagy-deficient livers (see earlier).

Invasive Hepatocellular Carcinoma vs. Noninvasive Hepatic Adenoma

Hepatic tumors in autophagy-deficient livers seem to be well-capsulated noninvasive hepatoma by morphology.

Interestingly, treatment of a well-defined carcinogen diethyl nitrosamine (DEN) caused invasive HCC in wild type livers, but not in autophagy-deficient livers.⁴² The tumor suppressor genes *p53* seems to play an important role in arresting the development of a more malignant phenotype. The levels of *p53* and several other tumor-suppressor genes, such as *p21*, *p16*, and *Rb1*, are increased in liver adenoma tissues of *Atg5*-deficient livers.⁴² In the absence of mitophagy, *p53* is phosphorylated by *PINK1* at *Sr*ine-392, and is translocated into the nucleus to suppress the expression of *NANOG*,⁸² which could be important for the development of cancer stem cells. It is thought that the activation of CSC is important for the emergence of the malignant invasive HCC, which could be lacking in autophagy-deficient livers due to *p53* activation.

Conclusion and Outlook

The degradative function of autophagy has global impacts on the physiology of hepatocytes. The evidence stems from the development of different liver pathologies under the autophagy-compromised condition. Our current understanding of the role of hepatocyte autophagy on health and disease is expanding. Additional works are needed to fully understand the molecular mechanisms. This will help to better understanding the autophagy's role in liver metabolism and homeostasis. Moreover, dissecting the molecules and pathways directly involved in the development of liver pathologies will also aid in the understanding of similar pathologies in other types of chronic liver diseases.

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