



# Molecular functions and clinical impact of thyroid hormone-triggered autophagy in liver-related diseases

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## Abstract

The liver is controlled by several metabolic hormones, including thyroid hormone, and characteristically displays high lysosomal activity as well as metabolic stress-triggered autophagy, which is stringently regulated by the levels of hormones and metabolites. Hepatic autophagy provides energy through catabolism of glucose, amino acids and free fatty acids for starved cells, facilitating the generation of new macromolecules and maintenance of the quantity and quality of cellular organelles, such as mitochondria. Dysregulation of autophagy and defective mitochondrial homeostasis contribute to hepatocyte injury and liver-related diseases, such as non-alcoholic fatty liver disease (NAFLD) and liver cancer.

Thyroid hormones (TH) mediate several critical physiological processes including organ development, cell differentiation, metabolism and cell growth and maintenance. Accumulating evidence has revealed dysregulation of cellular TH activity as the underlying cause of several liver-related diseases, including alcoholic or non-alcoholic fatty liver disease and liver cancer. Data from epidemiologic, animal and clinical studies collectively support preventive functions of THs in liver-related diseases, highlighting the therapeutic potential of TH analogs. Elucidation of the molecular mechanisms and downstream targets of TH should thus facilitate the development of therapeutic strategies for a number of major public health issues.

Here, we have reviewed recent studies focusing on the involvement of THs in hepatic homeostasis through induction of autophagy and their implications in liver-related diseases. Additionally, the potential underlying molecular pathways and therapeutic applications of THs in NAFLD and HCC are discussed.

**Keywords:** Thyroid hormone, Thyroid hormone receptor, Autophagy, non-alcoholic fatty liver disease, hepatocellular carcinoma

## Background

Thyroid hormones (TH) serve as potent regulators of cellular development, growth and metabolism in mammals [1] and control several metabolic activities related to anabolism or catabolism of macromolecules, including carbohydrates, proteins, lipids and damaged organelles within cells to maintain homeostasis under different physiological conditions [2]. In addition to their critical regulatory roles in cellular homeostasis, imbalance of TH levels in the

body is associated with multiple chronic diseases, including diabetes mellitus [3, 4], cardiovascular disease [5, 6] and liver-related disorders [7]. Liver is one of the most important target organs whereby THs regulate components involved in cellular metabolism, such as fatty acids, supporting the possibility that disruption of TH action in liver contributes to development of non-alcoholic fatty liver disease (NAFLD). Indeed, recent studies have reported associations between alterations in cellular TH signaling and several liver-related diseases, including NAFLD and hepatocellular carcinoma (HCC). Earlier epidemiological findings suggest that long-term

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hypothyroidism is positively associated with high risk of NAFLD and HCC incidence, independent of other risk factors [8, 9]. Moreover, treatment with  $T_3$  or its analogs has been shown to prevent a spectrum of liver-related diseases ranging from hepatic steatosis to HCC in rodents subjected to high-fat diet (HFD) or carcinogens [10–17]. These collective findings support the potential utility of TH analogs as therapeutic drugs to prevent liver disease progression. Analysis of the downstream signals of TH in liver may further shed light on the underlying TH pathways that induce therapeutic effects against liver-related diseases.

Autophagy is a self-digestion process primarily involving recycling of cellular fuel stores in lysosomes to generate amino acids, glucose and fatty acids [18]. Catabolism of lipids through autophagy is termed lipophagy [19]. In addition to metabolic functions, autophagy presents a cellular surveillance mechanism to suppress accumulation of toxic protein aggregates and impairment of organelles, thus facilitating maintenance of organelle integrity and cellular homeostasis [20]. The specific regulatory functions of autophagy in hepatic homeostasis have been increasingly explored in recent years. Dysregulation of the autophagic process is reported to cause an imbalance in energy metabolism in the liver and consequently affect hepatic physiology and trigger disease [21–24]. Several research groups, including ours, have shown that hepatic lipid turnover is stimulated by THs through lipophagy, preventing hepatosteatosis, both *in vitro* and *in vivo* [25, 26]. Additionally, TH stimulates the metabolic rate accompanied by increased mitochondrial turnover through mitophagy, leading to elimination of mitochondrial dysfunction induced by hepatic carcinogens or hepatitis B virus HBx protein [16, 17, 27]. The finding that THs and Thyroid hormone receptors (THR) prevent hepatic damage, hepatosteatosis and hepatocarcinogenesis via autophagy stimulation supports their therapeutic potential in clinical applications. In the current report, we have reviewed studies published by our research group and other investigators on the involvement of TH-induced autophagy in liver-related diseases, particularly NAFLD and HCC. Elucidation of the network of molecular mechanisms underlying the effects of TH/THR on hepatic metabolism may aid in the design of effective therapeutic strategies for a range of liver-related diseases.

## Molecular actions of thyroid hormones and receptors

### Genomic actions of TH

$T_3$  (triiodothyronine) and  $T_4$  (L-thyroxine) are the two major thyroid hormones affecting almost every organ system. Under physiological conditions,  $T_4$  is the main hormone secreted into the bloodstream by the thyroid gland. However, the thyroid hormone receptor (THR)

binding affinity of  $T_4$  is considerably lower (10-fold less) than that for  $T_3$ . The conversion of  $T_4$  to  $T_3$  is regulated via iodothyronine deiodinases (DIO1, DIO2, and DIO3) in extrathyroidal tissue. Type I and type II iodothyronine deiodinases (DIO1, DIO2) deiodinate circulating  $T_4$  to produce biologically active  $T_3$ . Conversely, type III deiodinase (DIO3) suppresses intracellular thyroid activity by converting  $T_4$  and  $T_3$  to the comparatively inactive forms, reverse  $T_3$  ( $rT_3$ ) and  $T_2$ . Recently,  $T_2$  was shown to possess thyromimetic activity and mimic some of the effects of  $T_3$  on liver metabolism [28, 29], implying that  $T_2$  or  $rT_3$  may not just be inert metabolites as originally suggested. Expression levels and activities of DIO1, DIO2 and DIO3 vary among different tissues, causing a tissue-specific increase or decrease in circulating TH levels or availability of active hormones for THR binding [7, 30]. To exert genomic effects, cytoplasmic  $T_3$  enters the nucleus, most likely through passive diffusion, and binds THRs associated with thyroid hormone response elements (TRE) within the promoter regions of downstream genes of TH/THR [31–33]. Typical TREs within promoter regions of downstream genes contain two half-site sequences (A/G)GGT(C/A/G)A in a palindromic, direct repeat or inverted repeat arrangement that are recognized by THR [1].

THRs are  $T_3$ -inducible transcription factors belonging to the nuclear receptor superfamily that are encoded by two tissue-specific genes, *THRA* ( $TR\alpha$ ) and *THRB* ( $TR\beta$ ). The *THRA* gene encodes one active  $T_3$ -binding receptor,  $TR\alpha1$ , and two dominant-negative spliced variants,  $TR\Delta\alpha1$  and  $TR\Delta\alpha2$  [34]. that lack  $T_3$  binding ability [35].  $TR\alpha1$  is the predominant subtype highly expressed in brain, cardiac and skeletal muscle [36]. *THRB* encodes two functional  $T_3$ -binding  $TR\beta$  isoforms ( $TR\beta1$  and  $TR\beta2$ ) and another dominant-negative isoform,  $TR\beta4$  [34].  $TR\beta1$  is predominately expressed in brain, liver and kidney whereas  $TR\beta2$  is limited to the hypothalamus, retina and pituitary. THRs exert transcriptional effects via formation of homodimers or heterodimers with other nuclear receptors, such as retinoid X receptor (RXR), Vitamin D receptors (VDR) and other retinoic acid receptor subtypes. RXR generally functions as a partner of several nuclear receptors to regulate target genes [47]. THRs form heterodimers with RXR on TREs within the promoter regions of target genes. In addition, recent ChIP-Seq studies have shown that THRs bind to specific response element motifs with non-conserved sequences and in non-promoter regions [37–39], implying that interactions with other transcription factors are required to regulate chromatin remodeling and gene expression.

In the absence of TH, THRs still bind to TREs but are associated with co-repressors displaying histone deacetylase (HDAC) activity, leading to modifications in

chromatin structure and repression of transcription. For instance, nuclear receptor corepressor 1 (NCoR1) and silencing mediator for retinoid or thyroid-hormone receptors (SMRT), well-characterized co-repressors with histone deacetylase activity, serve as platforms for repressor complex-mediated chromatin remodeling [40]. Binding of  $T_3$  induces conformational changes of THR and recruitment of transcriptional coactivators with histone acetyl transferase (HAT) activity to increase histone acetylation at specific promoter regions, facilitating generation of a permissive chromatin state and further recruitment of general transcriptional machinery (Fig. 1). For instance, steroid hormone receptor coactivator (SRC), PCAF (p300/CBP-associated factor) and p160 family members facilitate ligand-bound THRs to activate  $T_3$  target genes through histone acetyltransferase activity [41]. Moreover, transcriptional activities of THRs are stimulated by TR-associated protein (TRAP) family independently of HAT activity [42]. Alterations in THR-associated co-regulator complexes may induce differential responses for appropriate target gene expression (Table 1).

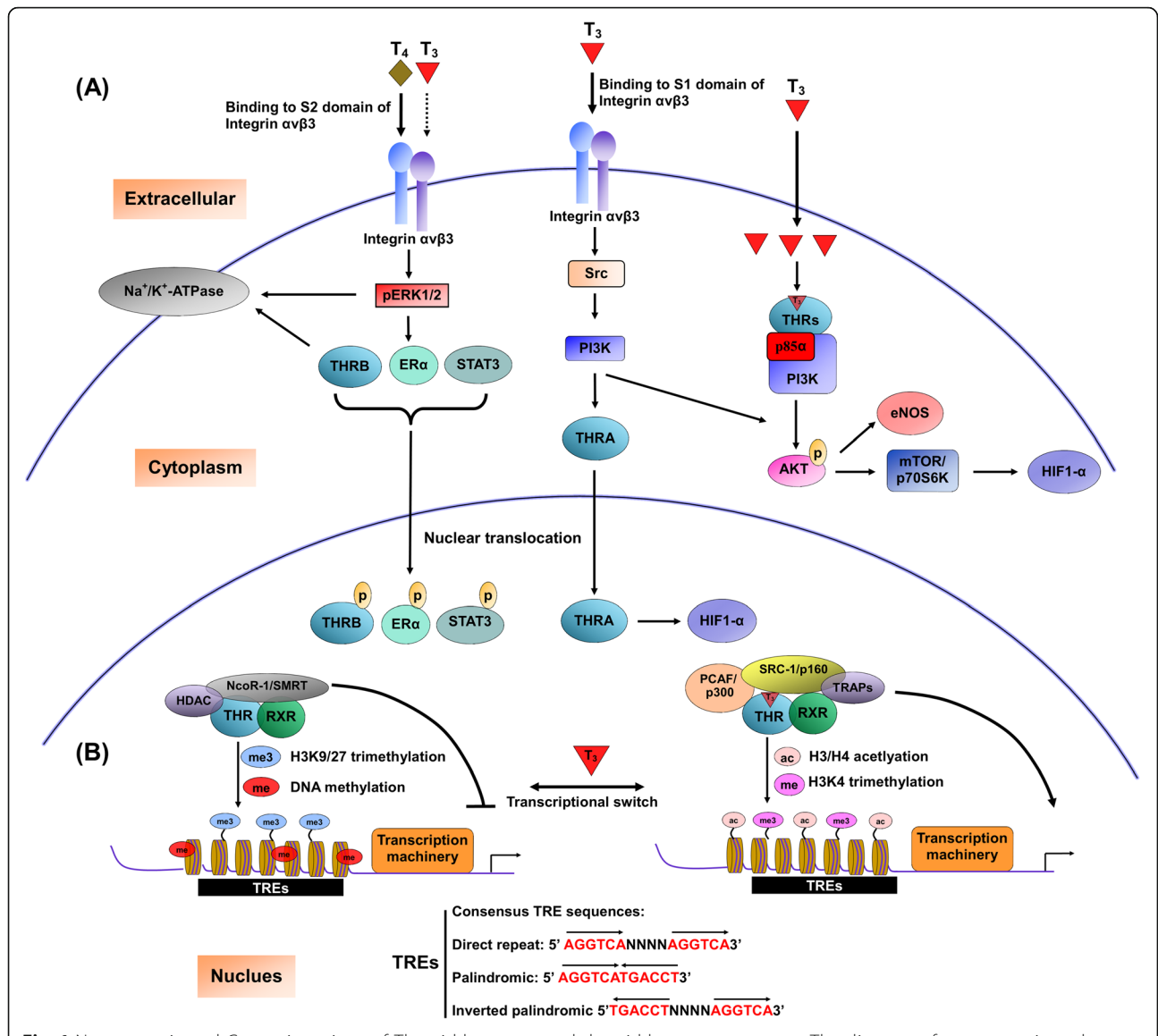
In addition to positively regulating downstream targets, TH binding to THR can lead to negative regulatory effects. Notably, these negatively regulated target genes are upregulated in the absence but downregulated in the presence of THs [30, 43] although the precise details remain to be established. In a typical case, high levels of THs exert a negative feedback effect on thyrotropin-releasing hormone (TRH) and thyroid stimulating hormone (TSH), and low levels of THs stimulate secretion of TSH from the anterior pituitary. This critical negative feedback loop regulates the hypothalamic-pituitary-thyroid axis [44–46]. TREs of genes negatively regulated by TH are frequently located near proximal promoter regions. However, binding of THRs to these putative regions is generally weak, suggesting that interactions between THRs and other co-factors may contribute significantly to negative regulatory effects of THs. Alterations in chromatin remodeling through histone modification via recruitment of HDACs and HATs may be involved in negative transcriptional regulation by THs. Recently, a novel THR co-repressor, LCOR, was identified as an inhibitor of TR $\beta$ -dependent lipogenic gene activity. LCOR serves as a competitor for binding of coactivators SRC-1/3 to TR $\beta$  leading to reduced recruitment of SRCs to TREs within the promoter regions of downstream target genes of TR, potentially representing a novel mechanism by which LCOR regulates gene transcription [47].

THRs are additionally reported to interact with transcription factors to negatively regulate expression of several genes, including Sp1 [48], p53 [49], Oct-1 [50], GHF-1 [51, 52] and CTCF [53, 54], although the underlying mechanisms remain largely unknown at present.

### **Nongenomic actions of TH**

In addition to transmission of signals through interactions with nuclear THRs, activities of THs in the plasma membrane or cytoplasm are termed nongenomic effects (Fig. 1, Table 1). THs bind to integrin  $\alpha\beta3$  membrane receptor protein independently of nuclear THRs [55–57], which has been further characterized as a membrane-bound THR. Integrin  $\alpha\beta3$  was originally shown to contain the Arg-Gly-Asp (RGD) recognition region that interacts with extracellular matrix ligands [55]. Unexpectedly, TH could bind integrin  $\alpha\beta3$  near its RGD recognition site [58]. Integrin  $\alpha\beta3$  contains two TH-binding domains with no homology to nuclear THRs. The S1 domain mainly recognizes  $T_3$ , consequently activating the phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B (PKB) pathway through Src kinase [59]. Both  $T_4$  and  $T_3$  bind to the S2 domain and activate the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ ERK1/2) pathway. Moreover, S1 and S2 domains mediate the specific effects of TH. For instance, S1 directs Src and PI3K-mediated TR $\alpha$  translocation from the cytoplasm to the nucleus and promotes expression of target genes, such as hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) while S2 activates MAPK1 and MAPK2, leading to nuclear trafficking of TR $\beta$ 1 from the cytoplasm and tumor cell proliferation [58, 60].

THs also cause serial phosphorylation and nuclear localization of other critical genes responsible for several cellular functions (Table 1). For instance, TH-activated ERK1/2 has been shown to promote estrogen receptor- $\alpha$  (ER $\alpha$ ), signal transducer and activator of transcription-3 (STAT3), and several THR-associated proteins [61–67]. TR $\alpha$ 1 interacts with the p85 $\alpha$  subunit of PI3K in a  $T_3$ -dependent manner, leading to activation of Akt and endothelial nitric oxide synthase (eNOS) [7, 68]. In addition, liganded TR $\beta$ 1 associates with p85 $\alpha$  in the cytoplasm to activate Akt via phosphorylation. Activated Akt subsequently triggers the nuclear mammalian target of rapamycin (mTOR)-p70S6K cascade and sequential induction of several HIF-1 $\alpha$  target genes, including glucose transporter 1 (GLUT1), platelet-type phosphofructokinase (PFKP) and monocarboxylate transporter 4 (MCT 4) [69–71]. TR $\beta$ 1 is additionally reported to modulate Na<sup>+</sup>/K<sup>+</sup>-ATPase activity by PI3K or ERK1/2. For instance, PI3K signaling slows potassium voltage-gated channel, subfamily H, member 2 (KCNH2) channel deactivation in the plasma membrane of pituitary cells [72–74]. Moreover, THRB-PV, a THRB mutant that shows loss of  $T_3$  binding ability but interacts more significantly with the PI3K regulatory subunit, p85, triggers a greater increase in PI3K kinase activity and activation of the PI3K-AKT- mTOR-p70S6K pathway in cytoplasmic and nuclear compartments, with predisposition to tumor



**Fig. 1** Nongenomic and Genomic actions of Thyroid hormone and thyroid hormone receptor. The diagram of nongenomic and transcriptional actions of thyroid hormone (TH) and thyroid hormone receptor (THR). **a** Nongenomic effects of THs are initiated from Integrin αvβ3 localized on the plasma membrane or occurs at cytoplasm. T<sub>3</sub> interacts with S1 domain of Integrin αvβ3 to activate the PI3K signal pathway via Src kinase, leading to trafficking of THRA from the cytoplasm to nucleus and increases HIF-1α expression. THs, mainly T<sub>4</sub>, also interact with S2 domain of Integrin αvβ3 to activate ERK 1/2 signal, causing phosphorylation and nuclear localization of THRβ, estrogen receptor α (ERα) and STAT3. Activated ERK1/2 and cytosolic THRβ increase the activity of the sodium pump (Na, K-ATPase). T<sub>3</sub>-liganded THRs in the cytoplasm interact with the PI3K regulatory subunit, p85α, to activate Akt, subsequently triggering mTOR/p70S6K and eNOS signals. **b** In the nucleus, THRs form heterodimers with the retinoid X receptor (RXR) at thyroid hormone response elements (TREs), within the regulatory regions of downstream genes. In the absence of T<sub>3</sub>, the co-repressor complex involving histone deacetylase (HDACs), NCoR1 and SMRT deacetylate histones in the regulatory regions. Consequently, trimethylation of histone H3 at lysine 9 and 27 along with DNA methylation causes a more closed conformation in chromatin and blocks the transcriptional machinery access to the DNA, causing suppression of downstream targets transcription. Binding of T<sub>3</sub> induces conformational changes of THRs and recruitment of transcriptional coactivators (such as PCAF/P300 and SRC-1/p160) with histone acetyltransferase (HAT) activity to increase histone acetylation at specific promoter regions, facilitating generation of a permissive chromatin state and further recruitment of general transcriptional machinery. Typical TREs within promoter regions of downstream genes contain two half-site sequences (A/G)GGT(C/A/G)A in a palindromic, direct repeat or inverted repeat arrangement that are recognized by THR

**Table 1** Summary of the genes/signals regulated by genomic or nongenomic action of TH/THR signal axis

Molecular function	Gene/signal name	Reference
Nongenomic regulation by TH/THR		
Membrane receptor of TH	Integrin $\alpha\text{v}\beta 3$	[55, 56]
	Src kinase	[59]
Signal transductor	PI3K/Akt	[7, 59, 68]
	p-ERK1/2	[58, 61–66, 60, 67]
	mTOR/p70S6K	[69]
	eNOS	[7, 68]
	Estrogen receptor	[62, 67]
Transcriptional factor	STAT3	[64]
	HIF1- $\alpha$	[58, 60, 70, 69, 71]
	$\beta$ -catenin	[77]
Metabolic regulator	GLUT1	[71]
	PFKP	[71]
	MCT 4	[71]
Na-K-ATPase	KCNH2	[72]
Apoptosis regulator	FOXO1	[136, 137, 176]
	BCL2L11	[176]
Genomic regulation by TH/THR		
Transcriptional coregulator of THR	SP1	[48]
	p53	[49]
	Oct-1	[50]
	GHF-1	[51, 52]
	CTCF	[53, 54]
	LCOR	[47]
Autophagy regulator	DAPK2	[16]
	Betatrophin	[26]
Cell cycle regulator	UHRF1	[171]
	STMN1	[172]
	Mir-214	[173]
	BC200	[174]
Apoptosis regulator	TRAIL	[175]
Metastatic regulator	BSSP4	[178]
	LCN2	[180]
	mir-130b	[177]
	mir-21	[179]

development in several cancer types, including thyroid and mammary tumors [75, 76]. The THRB-PV mutant additionally associates with  $\beta$ -catenin to regulate cell proliferation in thyroid tumors of THRB<sup>PV</sup>/PV mice [77]. This interaction favors the unliganded state of TR $\beta$ , and T<sub>3</sub>-independent interactions between  $\beta$ -catenin and TR $\beta$  promote activation of  $\beta$ -catenin-related downstream targets.

## The TH/THR axis in regulation of hepatic autophagy

### The autophagic process

Autophagy was originally characterized as a catabolic process targeting cellular constituents, including unfolded proteins, damaged organelles and intracellular pathogens, to lysosomes for degradation [19, 78]. Autophagy is categorized into three main types: macroautophagy, chaperone-mediated autophagy and microautophagy [79]. Macroautophagy, hereafter known as autophagy, is generally considered the major route for directing cytoplasmic components into lysosomes for degradation. The autophagic process involves membrane biogenesis and formation of a double-membrane phagophore (termed autophagosome), which sequesters partial cytoplasmic components or entire organelles and subsequently fuses with lysosomes for degradation. Amino acids and other metabolic compounds generated by this process are consequently released for energy production or recycling. Chaperone-mediated autophagy involves sequestration of proteins or polypeptides harboring the KFERQ-like motif by chaperone proteins. This process promotes translocation of target proteins into lysosomes for degradation through interactions with lysosome-associated membrane protein type 2A (LAMP2A). Microautophagy is implicated in invagination of cellular constituents within endosomes or lysosomes but small fractions of cytoplasmic constituents in the close vicinity of lysosomes are sequestered.

Under basal conditions, autophagy is implicated in the degradation of long-lived proteins while another catabolic system, the ubiquitin-proteasome process, is responsible for the turnover of short-lived proteins [80, 81]. However, under specific conditions, such as nutrient deprivation, the autophagy pathway leads to selective degradation of cytosolic materials (termed selective autophagy). Selective autophagy directs degraded products into highly spatiotemporally controlled metabolic pathways. When specific autophagic cargo, such as misfolded proteins or damaged cellular organelles, appear within the cytoplasm, they are tagged with molecular markers, such as ubiquitin [82, 83], resulting in assembly of autophagic adapter proteins, such as SQSTM1, that bind to both molecular marker-harboring cargo and LC3-II. A number of core autophagy proteins, such as the ULK-FIP200 complex, also recognize these tagged targets [84, 85], initiating autophagosome formation. Selective autophagy is predominantly regulated by cargo labeling as well as recruitment of adaptor proteins to cargo.

Significant links between the regulation of selective autophagy and liver complications associated with NAFLD and HCC have been reported, supporting the manipulation of this process as a potential therapeutic strategy for liver-related diseases.

### **Autophagy in liver-related diseases**

In addition to the fundamental function of starvation-induced autophagy, basal and selective autophagy contribute to maintaining the quality and quantity of cellular organelles and cytosolic proteins efficiently in the liver. Consequently, dysregulation or malfunction of the autophagic process is associated with the pathogenesis of multiple disorders and liver-related diseases, such as age-related hepatic disorders, NAFLD and HCC [86].

**Aging in liver** Aging is positively associated with severity and poor prognosis of several liver-related diseases, including alcoholic liver disease, NAFLD and HCC [87]. Furthermore, the age-dependent frequent decrease in autophagic activity underlies the pathogenesis of hepatic diseases. The initial finding of age-dependent decrease in hepatic autophagy was based on a marked increase in oxidative damage-triggered protein carbonyl derivatives in liver of 27-month-old rats, compared to 2-month-old rats [88]. Further studies indicated that the efficiency of autophagic degradation and capacity of autophagic proteolysis of exogenous amino acids of primary hepatocytes from older rats is dramatically decreased relative to that in young rats [89, 90]. Moreover, decreased expression of LC3-II and number of autophagosomes in mice were age-dependent [91]. These results suggest that the age-dependent decrease in efficiency of autophagy leads to substantially diminished clearance of inactive organelles, including mitochondria, generating increased oxidative stress and consequent accumulation of oxidized protein aggregates.

**NAFLD** Fatty liver is attributed to continuous intake of excess dietary fat without consumption of excessive alcohol [92]. Nonalcoholic fatty liver disease (NAFLD) incorporates a spectrum of liver-related diseases ranging from steatosis to steatohepatitis, fibrosis and cirrhosis. Non-alcoholic steatohepatitis (NASH) presents as a hepatic disease histologically similar to alcoholic hepatitis but occurs without consumption of excessive alcohol, representing a stage within NAFLD [93, 94]. Recent metabolic studies on animals and humans demonstrated that NAFLD represents one feature of metabolic syndrome closely associated with several metabolic diseases, such as diabetes and insulin resistance. Moreover, diabetes or insulin resistance conditions accelerate the entire pathological spectrum of NAFLD [94]. Chronic hepatic steatosis can trigger inflammatory responses [95]. In some cases, NAFLD progresses to NASH, which frequently advances into fibrosis and cirrhosis, and 4–27% NASH cases develop HCC [96].

In response to accelerated lipid availability or nutrient starvation, hepatic autophagy degrades lipid droplets to produce free fatty acids (FFA) for ATP generation. This

autophagy-induced degradation of hepatic lipid droplets is termed lipophagy [97]. Since lipophagy involves the selective degradation of hepatic lipid droplets, autophagy in liver could serve as a preventive mechanism against NAFLD. In contrast, several studies indicate that lipotoxic effects, including oxidative stress or insulin resistance, elicited by excess triglycerides and free fatty acids in NAFLD, inhibit activation of autophagy [19, 98, 99]. Hepatic autophagy regulates lipid metabolism through elimination of triglyceride accumulation in liver and prevents the development of steatosis [97]. Enhancement of autophagic activity using pharmaceutical agents, such as rapamycin or carbamazepine, has been shown to retard liver steatosis [99–102]. Moreover, pharmacological inhibition of autophagy via 3-methyladenine or knock-down of the essential autophagy gene, *atg5*, in hepatocytes challenged with a lipid load induced a dramatic increase in the cellular triglyceride level. Excessive triglyceride and cholesterol ester accumulation in hepatic lipid droplets was observed owing to decreased lipolysis and fatty acid  $\beta$ -oxidation in cells with low autophagy activity.

Compared to hepatocytes, autophagy in stellate cells exerts opposite effects on NAFLD progression. In NAFLD, quiescent hepatic stellate cells are activated and transdifferentiate into myofibroblasts, which express a large number of inflammatory cytokines and collagen, thereby promoting hepatic fibrosis [103]. In stellate cells from livers of autophagy-deficient mice,  $\text{CCl}_4$ -induced hepatic fibrosis was dramatically inhibited [104].

Abnormal structural and functional alterations of hepatic mitochondria in NAFLD are frequently observed [105]. Mitochondria are the powerhouse of cells and decreased mitochondrial function concomitant with alterations in structural and molecular pathways may elicit a metabolic imbalance, contributing to NAFLD progression. Mitochondrial biogenesis and mitophagy, a highly selective form of autophagy that functions in removal of damaged mitochondria, are the major pathways that regulate mitochondrial mass [86, 105]. The balance of mitochondrial biogenesis and mitophagy is a precisely regulated process that influences cellular homeostasis. Activation of hepatic mitophagy is reported to eliminate the lipid content and oxidative stress, and dysregulation of mitophagy implicated in the progression of NAFLD [19, 86, 97].

Under oxidative stress conditions, SQSTM1 is phosphorylated and subsequently binds to KEAP1 with high affinity. KEAP1 is an adaptor of the ubiquitin ligase complex for nuclear factor-erythroid 2-related factor-2 (NRF2). Downstream target proteins of NRF2, such as NAD(P)H, dehydrogenase quinone 1 (NQO1) and glutathione *S*-transferase (GST), ameliorate ROS production by damaged mitochondria [106, 107]. Selective

autophagic degradation of the SQSTM1-KEAP1 complex inhibits KEAP1-driven ubiquitylation and degradation of NRF2. In NAFLD, the turnover of hepatic cytoplasm fractions is substantially impaired due to dysfunctional autophagy/mitophagy, leading to accumulation of damaged mitochondria and elevated oxidative stress, which activates the SQSTM1-KEAP1-NRF2 pathway to protect hepatocytes against oxidative stress. However, under conditions where ROS levels exceed the antioxidant capacity of NRF2-related signals, various harmful effects, including lipid peroxidation, protein oxidation, and DNA damage, trigger liver injury [106, 107].

**HCC** As autophagy plays important roles in maintenance of the quality of organelles and supply of energy to cancer cells, autophagy-related pathways are considered important for cancer cell survival [108]. Previous studies indicate that loss of autophagy inhibits KRAS-triggered tumorigenesis of non small-cell lung cancer [109, 110]. Indeed, several clinical trials using a combination of existing anticancer drugs and autophagy inhibitors, such as chloroquine and hydroxychloroquine, are currently underway for several cancer types [111, 112]. However, the specific functions of autophagy in different tumors are complex and context-dependent. Pancreas-specific activated KRAS in mice leads to the development of pancreatic ductal adenocarcinoma (PDAC), which is suppressed by inhibition of autophagy [113]. In contrast, in mice lacking *Tp53*, loss of autophagy facilitates tumor progression [113].

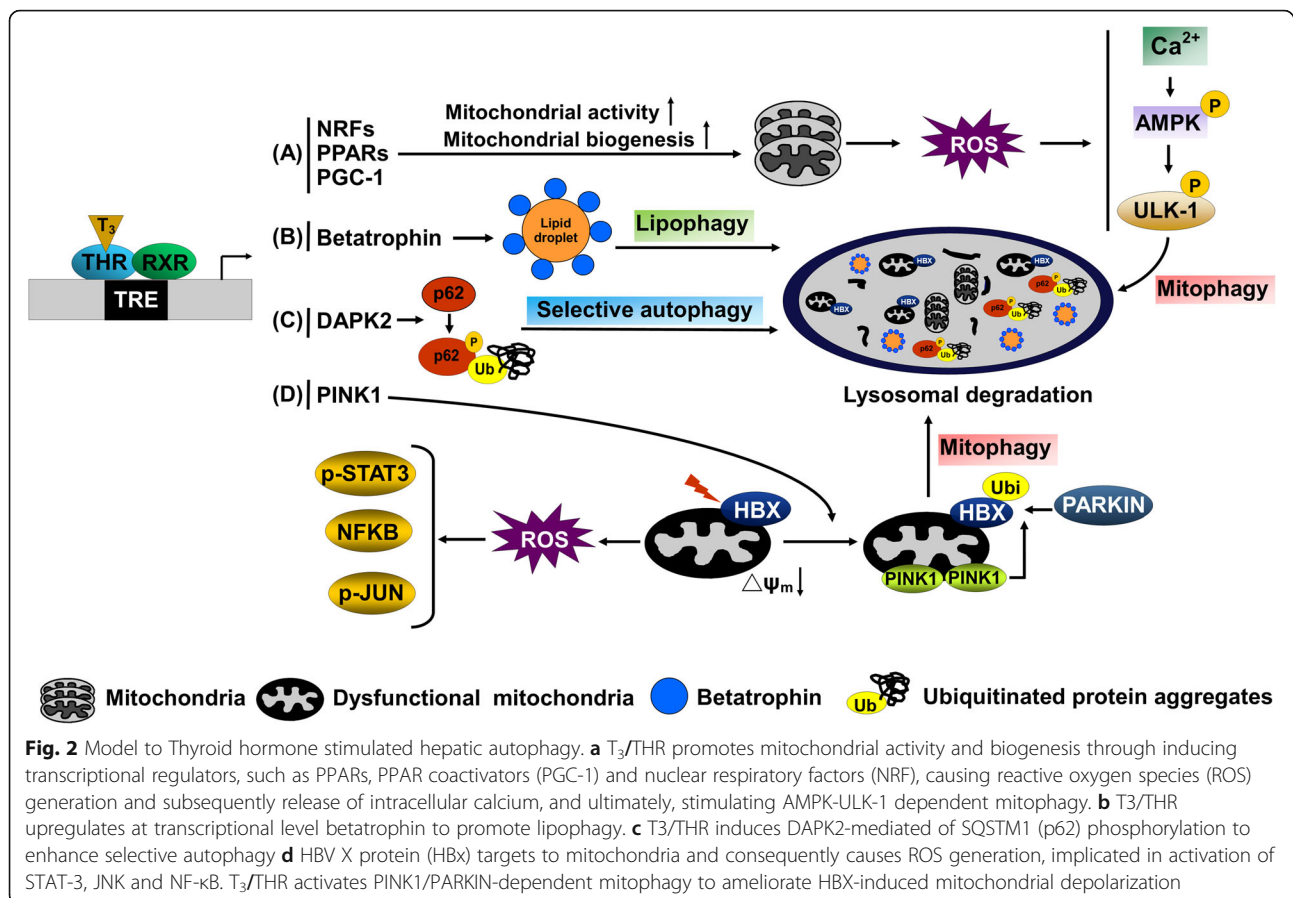
In the liver, autophagy appears to function as a tumor suppressor. For instance, mosaic depletion of *Atg5*, liver-specific *Atg7*, or *Beclin-1* in mice causes accumulation of degenerated protein aggregates, lipid droplets and damaged cellular organelles, including mitochondria and peroxisomes, as well as persistent activation of NRF2 owing to sequestration of KEAP1 by SQSTM1-positive cytoplasmic aggregates, leading to spontaneous hepatic carcinogenesis [114–116]. Simultaneous loss of *nrf-2* or *sqstm1* in mice with *Atg5* or *Atg7*-deficient liver suppresses tumor development [117]. Additionally, the cargo receptor degraded by autophagy, SQSTM1, accumulates in the hepatic tumor region [118], implying that the SQSTM1-KEAP1-NRF2 axis contributes to tumor growth. Further studies have revealed that heterozygous deletion of a major regulator of autophagy, *Beclin1*, increases the frequency of development of spontaneous tumors and HBV-induced hepatic premalignant lesions in mice [116, 119]. Additionally, ATG5 and BECLIN-1 levels are downregulated in hepatic tumor, compared to adjacent non-tumor regions [120]. HCC patients with low BECLIN-1 accompanied by high Bcl-xL (a crucial anti-apoptotic protein) expression display poorer disease-free and overall survival rates [120], indicating that

normal autophagic flux is important for HCC prevention in this apoptosis compromised background.

In view of the protective function of autophagy against hepatocarcinogenesis, researchers have focused on the mechanisms underlying autophagy-dependent tumor cell death identified in several cancer types [121–123]. The PI3K/Akt/mTOR axis is a known crucial signaling pathway for cell growth, survival and metabolism in tumor cells [124]. The mTOR pathway is activated in HCCs and manipulation of mTOR inhibitors shown to effectively exert anti-tumor effects in HCC [125, 126]. Rapamycin and its derivatives are mTOR inhibitors reported to serve as autophagy inducers with anti-tumor activity in a phase II study on 25 advanced HCC patients [127]. Liver transplantation is an important therapeutic option for the selected patients with unresectable HCC. In another study, rapamycin-directed immunosuppression was associated with improved survival after liver transplantation in HCC patients, but showed a trend toward lower survival in non-HCC patients, further showing the clinical evidence of its anti-cancer impact [128]. However, the utility of rapamycin and its derivatives in HCC therapy is controversial due to insufficient and conflicting clinical results. For example, everolimus (RAD001) exerted an anti-tumor effect in xenografts of human HCC models [129] whereas a recent clinical phase III trial disclosed no benefits on advanced HCC prognosis [130]. Co-targeting of mTOR via everolimus along with a PI3K/mTOR dual inhibitor, BEZ235, displayed greater efficacy through activating autophagy, specifically mitophagy, in tumors and led to decreased tumor sizes in a mouse model of HCC [131]. Interestingly, recent findings suggest that combination of mTOR inhibitors with SBI-0206965, a highly selective ULK1 inhibitor acting as a specific blocker of autophagy, has a promising effect on HCC [132]. Further investigations are required to validate the clinical utility of rapamycin. Sorafenib, a multi-kinase inhibitor used as first-line systemic therapy for advanced HCC, promotes autophagy-dependent cell death through Mcl-1 signaling [127]. A combination of sorafenib and autophagy inhibitors was also shown to induce an enhanced therapeutic effect. Sorafenib-induced autophagy-dependent cell death is reported to cause drug resistance in HCC [133]. Further research is therefore warranted to determine the utility of autophagy inducers in improving the current limits of HCC therapy and treatment outcomes. The involvement of autophagy as a function of tumor type, pathological stage and genetic context remains to be established.

#### **TH/THR regulation of hepatic autophagy**

The effects of TH on hepatic lysosomal activity and proteolysis were first described in 1978 [134], although the



underlying mechanisms were yet to be elucidated. Recently, T<sub>3</sub> was shown to enhance hepatic lysosomal activity accompanied by formation of autophagosomes in hepatic cells or livers of mice [25, 26]. The effects of T<sub>3</sub> on autophagy were THR-dependent and binding of NCoR-HDAC3, the corepressor of THR, abolished T<sub>3</sub>-induced hepatic autophagy [25].

The T<sub>3</sub>/THR axis is known to promote fatty acid β-oxidation in liver via activation of autophagy (Fig. 2A). Additionally, THs upregulate several critical genes involved in the autophagic process, including ULK1, PINK1, Beclin-1, DAPK2, betatrophin and LC3 (Fig. 2B-D) [16, 17, 25–27, 135]. These autophagy-related genes could be regulated directly by T<sub>3</sub>/THR at the transcriptional level or indirectly through FOXO1 activation by dephosphorylation and deacetylation via TH-activated SIRT1 [136, 137]. SIRT1 is a NAD<sup>+</sup>-dependent deacetylase activated by increases in cellular NAD<sup>+</sup> levels that serves as an energy sensor of cells to control transcriptional activity by T<sub>3</sub> and FOXO1. Furthermore, SIRT1 mediates T<sub>3</sub>-induced autophagy through stimulation of expression as well as deacetylation of autophagy-related genes [136]. The master transcription factor, transcription factor EB (TFEB), regulates autophagy and lysosome-related genes may additionally be modulated by TH [44].

Other than transcriptional regulation, TH/THR complexes also regulate autophagy through post-transcriptional mechanisms. For instance, TH/THR is reported to activate the autophagy process through AMPK signaling. T<sub>3</sub> induces mitochondrial activity and biogenesis through inducing transcriptional regulators, such as PPARs, PPARγ coactivator-1 (PGC-1) and nuclear respiratory factors [138], which causes the generation of reactive oxygen species (ROS) and subsequently release of intracellular calcium, and ultimately, CAMKK2 activation. Activated CAMKK2 phosphorylates AMPK, in turn, inhibiting mTOR signaling and stimulating autophagy via ULK1 phosphorylation [27]. SQSTM1 is a key adapter protein of autophagy, and accumulating evidence has demonstrated that phosphorylation of this protein facilitates clearance of ubiquitinated protein aggregates through the autophagic process [139, 140]. We previously showed that T<sub>3</sub>/THR interactions induce transcription of DAPK2, which, in turn, phosphorylates SQSTM1 to promote clearance of protein aggregates through autophagy. Our results collectively indicate that the TH/THR signaling axis coordinates both transcriptional and post-translational regulation of hepatic autophagy [16].



**TH regulation of selective autophagy** Lipophagy is implicated in the digestion of neutral lipid droplets to release free fatty acids for mitochondrial lipid oxidation [97] and considered a major lipolytic pathway in hepatic cells [141]. Recently, we and other groups showed that  $T_3$  induces lipophagy in both human and mouse hepatic cells that is essential for  $T_3$ -mediated lipid catabolism [25, 26]. Although the specific receptors for recognition of lipid droplets by autophagosomes have not been identified, our results indicate that  $T_3$  upregulates hepatic betatrophin transcription, which localizes to lipid droplets and possibly targets lipids for autophagic degradation [26].

TH is considered an inducer of mitochondrial activity and oxidative stress in the liver [142]. However, TH also induces mitophagy to prevent accumulation of damaged mitochondria and prevents hepatic injury by excessive ROS production [27]. TH is reported to stimulate the AMPK-ULK1 axis to initiate mitophagy. This process is dependent on translocation of ULK1 to mitochondria and subsequent recruitment of autophagic proteins, such as SQSTM1 and LC3-II, to damaged mitochondria for eventual degradation in autolysosomes. PINK1/PARKIN-mediated mitophagy is another pathway that contributes to protecting mitochondria against cellular ROS [143, 144]. PINK1 accumulates at the outer mitochondrial membrane upon membrane depolarization and subsequently recruits PARKIN to ubiquitinate damaged mitochondria, facilitating autophagic degradation. Experiments by our group showed that  $T_3$  activates PINK1/PARKIN-dependent mitophagy to ameliorate HBX-induced mitochondrial depolarization [17]. Selective removal of damaged mitochondria by TH is crucial for preventing oxidative damage in the liver.

Recently, we demonstrated that transcriptional regulation of DAPK2 by TH promotes phosphorylation of SQSTM1 to facilitate clearance of diethylnitrosamine (DEN)-induced protein aggregates through autophagy, which may protect hepatocytes from DEN-induced hepatocarcinogenesis [16].

#### **Potential application of TH and analogs in NAFLD and HCC**

Thyroid hormones affect energy metabolism, glucose homeostasis and lipid utilization. Hypothyroidism is positively associated with high risk of NAFLD and HCC incidence, independent of other risk factors [8, 9]. THs may thus be useful in a therapeutic capacity in hyperlipidemia and NAFLD. However, excessive production or administration of exogenous THs triggers several side-effects, such as muscle wasting, increased heart rate with possible atrial arrhythmia, and heart failure [145]. Over the past few decades, biologists have focused on the possibility that TH derivatives have the beneficial actions of the thyroid hormone but without associated deleterious effects [145–149]. Recently, several analogs of TH

specific for THRB have been generated, which have therapeutic activity in liver-related diseases with limited side-effects in organs, such as heart or bone, with abundant THRA expression.

GC-1 was the first synthetic THRB agonist that could be used as a scaffold compound for developing other TH derivatives, which are easily modified and synthesized more efficiently than native TH [168]. GC-1 binds all major isoforms of THRB with similar affinity to  $T_3$ . The binding affinity of GC-1 to THRB is 10-fold higher than that to THRA. GC-1 accumulates predominantly in the liver but its uptake is low in other organs, including skeletal muscle and heart. Due to the specific binding of GC-1 to THRB in liver, it may exhibit gene-specific actions relative to the native form of the thyroid hormone [145].

In the CMD diet-triggered NAFLD rat model, administration of either  $T_3$  or GC-1 could prevent steatohepatitis. Notably, GC-1 treatment not only caused a more significant reduction in hepatic TG levels but also did not elicit significant side-effects, such as increased heart rate and muscle wasting [150, 151]. These findings support the potential therapeutic application of THs on NAFLD prevention. Furthermore,  $T_3$  and GC-1 exert therapeutic effects on HCC [16, 29, 152, 153]. Upon treatment of rats with DEN combined with a choline-deficient (CD) diet for weeks, development of preneoplastic lesions was observed. Administration of  $T_3$  or GC-1 dramatically reduced the preneoplastic lesions caused by DEN.

Recently, a liver-selective prodrug, MB07811, was developed. Following hepatic enzymatic cleavage, the active form, MB07344, is generated that has been characterized as a liver-selective THRB agonist [154]. In HFD-exposed or diabetic fatty animals, two weeks of MB07811 treatment significantly reduced both hepatic and plasma triglyceride levels with no other side-effects of TH [154]. Clearance of hepatic lipid droplets by MB07344 may be attributable to acceleration of mitochondrial activity and fatty acid catabolism [12].

KB2115 has been identified as another THRB-selective agonist preferentially taken up in the liver. In both animal and clinical studies, administration of KB2115 significantly lowered serum total and LDL cholesterol and prevented the development of hepatic steatosis [155–157]. Furthermore, treatment with KB2115 as well as GC-1 in rats induced hepatomitogenic activity with no evidence of hepatic toxicity [158], supporting its potential for regenerative therapy, including liver transplantation and other surgical modalities.

Despite encouraging results from human clinical studies showing that GC-1, MB07811 and KB2115 exert therapeutic effects via lowering the levels of serum LDL

cholesterol and triglycerides, these compounds have not reached human clinical trials or been developed into therapeutic agents. Phase II trials on GC-1 and MB07344 are yet to be performed. Clinical studies on KB2115 were discontinued due to cartilage damage and hepatic toxicity observed following long-term dosing in dogs [29, 159].

More recently, two liver-directed THRB selective agonists, MGL-3196 and VK2809, have been developed [160, 161]. Results from phase II trials showed preventive effects on NAFLD accompanied by a decrease in serum levels of LDL cholesterol and triglycerides as well as hepatic lipids with none of the side-effects of the thyroid hormone axis. Thus, therapeutic application of THs in liver-related diseases in the clinic is feasible.

Recent studies by our group revealed a mechanistic link between TH and HCC prevention [16, 17]. DEN-treated liver cells have been shown to cause ROS accumulation accompanied by increased DNA damage and hepatic injury [162]. Increased oxidative stress may occur due to the accumulation of SQSTM1-associated protein aggregates and damaged organelles. SQSTM1 is the major component of inclusion bodies in hepatocytes (termed Mallory bodies), which have been identified in the livers of patients diagnosed with alcoholic hepatitis and NAFLD [163, 164].  $T_3$ -treated mice exhibit higher DAPK2 expression, and consequently,  $T_3$ -driven autophagy alleviates DEN triggered hepatic injury and hepatocellular carcinogenesis [16].

Chronic infection of hepatitis B virus in liver is one of the major risk factors for HCC development, and the HBV X protein (HBx) exerts powerful disruptive effects on mitochondrial dysfunction and ROS production, leading to progression of HCC [165]. Our group further showed that mitophagy triggered by the TH-PINK1-Parin axis is a putative pathway implicated in protection of HBx-induced hepatocellular carcinogenesis. Additionally, TH-triggered autophagy was shown to reduce hepatic lipid droplets and mitochondrial fatty acid oxidation [25, 27]. These results collectively support the involvement of TH-triggered autophagy in regulating mitochondrial metabolism in the development of NAFLD and HCC and provide insights into the physiological significance of THs in prevention of liver-related diseases [17]. However, the role of autophagy in the preventive and therapeutic potential of TH analogs (GC-1, MB07344, KB2115, MGL-3196 and VK2809) have not yet been determined.

## Conclusions

Over the past decade, molecular mechanisms and physiological effects of THs in liver have gradually been elucidated. Disruption of TH signals is known to cause multiple organ dysfunction that is closely associated with several diseases [5, 6]. Liver is one of the major target

tissues of TH, and people with low thyroid function are closely associated with multiple liver-related diseases. The cross-sectional and systemic view studies indicated that subclinical hypothyroidism, and even in the upper normal limit of TSH levels were significantly associated with the risk of NAFLD and advanced fibrosis [166–169]. Interestingly, high level of TSH itself may be an important risk factor points to the pathogenesis of NAFLD, independent of thyroid hormones [168, 169], and the supplementation of levothyroxine shows clear benefits on NAFLD in subclinical- and mild subclinical- hypothyroidism patients with dyslipidemia [170]. Moreover, both in *vitro* and in *vivo* experiments demonstrated THs and THs analogs exhibit the potential therapeutic and preventive application in NAFLD and HCC [7, 16, 17, 142, 145–149], highlighting the urgent need to understand the complex mechanisms underlying the effects of thyroid hormone. The TH/THR axis is a strong inducer of hepatic autophagy, which promotes lipid droplet degradation as well as mitochondrial biogenesis and turnover. This process has been implicated in the removal of damaged mitochondria and ROS that cause hepatic injury [7, 16, 17, 26, 27, 28]. In the present review, we have discussed the growing complexity of TH-regulated autophagy, highlighted advantages associated with the TH/autophagy axis-based therapeutic strategy for liver-related diseases, and discussed recent findings that may be exploited for improving the therapeutic outcomes of NAFLD and HCC [11, 12, 148, 150, 151, 154].

However, several challenges in TH-based therapies for hepatic diseases, HCC in particular, remain to be overcome, since the actions of individual TH derivatives within the same tissue may be different. Alterations in TH deiodinases, transporters, co-activators or co-repressors may influence the cellular level and molecular actions of THs, in turn, causing metabolic changes [44]. Moreover, the processes of cancer progression are complex. Individual THR isoforms exert different effects in a cancer type- and stage-specific manner. The TH/THR signals and interacting partners may facilitate the switch from tumor suppression in the premalignant stages to promotion in the later stages of HCC [7]. For instance, administration of TH not only reduces the size of preneoplastic lesions in the livers of rats suffering with HCC, but suppresses the aberrant cellular growth via control the expression of cell cycle regulators, such as CDK2, Cyclin E, UHRF1, STMN1 mir-214 and *BC200* lncRNA [7, 171–174]. Our recent studies further support the preventive effect of TH on hepatocarcinogenesis via activating autophagy [16, 17], whereas TH/THR is reported to promote metastasis and chemoresistance through control the expressions of BSSP4, TRAIL, BCL2L11, LCN2, mir-21, and mir-130b [7, 173, 175–180]. This characteristic of THs

supports the double-edged sword effect of autophagy in cancer progression. Autophagy mitigates stress-caused damage by removing damaged cellular organelles and protein aggregates and impaired autophagy causes accumulation of excess oxidative stress and DNA damage, leading to initiating hepatocarcinogenesis. Mosaic depletion of *Atg5*, liver-specific *Atg7* or *Beclin-1* in mice causes accumulation of degenerated protein aggregates, lipid droplets and damaged cellular organelles, leading to spontaneous hepatic carcinogenesis [114, 116]. By contrast, after the initiation of tumorigenesis, autophagy can also facilitate tumor cell survival under metabolic stress, becoming dormant and regenerating with anti-stress capacity that promotes tumor progression [181]. For instance, malfunction of autophagy inhibits KRAS-triggered tumorigenesis of non small-cell lung cancer and DEN-induced HCC. [109, 110, 182]. Moreover, inhibition of autophagy increases the sensitivity of chemotherapy and triggers cellular apoptosis and necrosis of HCC by activating several tumor suppressor genes, including p53, PTEN, CDKN1, CDKN2 and Rb1 [182–184]. Therefore, further animal and clinical studies are warranted to establish the specific functions of THs-induced autophagy in the different processes that lead to HCC development.

#### Abbreviations

CD: Choline-deficient; DEN: Diethylnitrosamine; eNOS: Nitric oxide synthase; ERK: Extracellular signal-regulated kinase; ER $\alpha$ : Estrogen receptor- $\alpha$ ; GLUT1: Glucose transporter 1; GST: Glutathione S-transferase; DIO: Iodothyronine deiodinase; HAT: Histone acetyl transferase; HCC: Hepatocellular carcinoma; HDAC: Histone deacetylase; HFD: High-fat diet; HIF-1 $\alpha$ : Hypoxia-inducible factor-1 $\alpha$ ; KCNH2: Potassium voltage-gated channel, subfamily H, member 2; LAMP2A: Lysosome-associated membrane protein type 2A; MAPK: Mitogen-activated protein kinase; MCT 4: Monocarboxylate transporter 4; mTOR: Mammalian target of rapamycin; NAFLD: Non-alcoholic fatty liver disease; NCoR1: Nuclear receptor corepressor 1; NQO1: NAD(P)H, dehydrogenase quinone 1; NRF2: Nuclear factor-erythroid 2-related factor-2; PCAF: p300/CBP-associated factor; PDAC: Pancreatic ductal adenocarcinoma; PFKF: Platelet-type phosphofructokinase; PGC-1: PPAR $\gamma$  coactivator-1; ROS: Reactive oxygen species; RGD: Arg-Gly-Asp; RXR: Retinoid X receptor; SMRT: Silencing mediator for retinoid or thyroid-hormone receptors; SRC: Steroid hormone receptor coactivator; STAT3: Signal transducer and activator of transcription-3; TFE3: Transcription factor EB; TH: Thyroid hormone; THR: Thyroid hormone receptor; TRAP: TR-associated protein; TRE: Thyroid hormone response elements; TRH: Thyrotropin-releasing hormone; TSH: Thyroid stimulating hormone; VDR: Vitamin D receptors

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#### Authors' contributions

HCC and CYT composed the idea and wrote the manuscript. CYT and MMT were involved in preparing the Figure content. CTY and KHL supervised the process. All authors read and approved the final manuscript.

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