

REVIEW

GCKIII kinases in lipotoxicity: Roles in NAFLD and beyond

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is defined by excessive accumulation of lipid droplets within hepatocytes. The STE20-type kinases comprising the germinal center kinase III (GCKIII) subfamily – MST3, MST4, and STK25 – decorate intrahepatocellular lipid droplets and have recently emerged as critical regulators of the initiation and progression of NAFLD. While significant advancement has been made toward deciphering the role of GCKIII kinases in hepatic fat accumulation (i.e., steatosis) as well as the aggravation of NAFLD into its severe form nonalcoholic steatohepatitis (NASH), much remains to be resolved. This review provides a brief overview of the recent studies in patient cohorts, cultured human cells, and mouse models, which have characterized the function of MST3, MST4, and STK25 in the regulation of hepatic lipid accretion, meta-inflammation, and associated cell damage in the context of NAFLD/NASH. We also highlight the conflicting data and emphasize future research directions that are needed to advance our understanding of GCKIII kinases as potential targets in the therapy of NAFLD and its comorbidities. *Conclusions:* Several lines of evidence suggest that GCKIII proteins govern the susceptibility to hepatic lipotoxicity and that pharmacological inhibition of these kinases could mitigate NAFLD development and aggravation. Comprehensive characterization of the molecular mode-of-action of MST3, MST4, and STK25 in hepatocytes as well as extrahepatic tissues is important, especially in relation to their impact on carcinogenesis, to fully understand the efficacy as well as safety of GCKIII antagonism.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), defined as fatty infiltration in >5% of hepatocytes (steatosis) in the absence of excessive alcohol consumption, is emerging as a leading cause of chronic liver disease worldwide.^[1,2] In a subgroup of patients with NAFLD, the disease aggravates to nonalcoholic steatohepatitis (NASH), which in addition to liver lipid accumulation is characterized by local inflammation and hepatocellular

damage in the form of ballooning and apoptosis, with different degrees of hepatic fibrosis.^[3] NAFLD contributes to the pathogenesis of type 2 diabetes mellitus and cardiovascular disease, and patients with NASH are also at risk of progressing into cirrhosis, liver failure, and hepatocellular carcinoma (HCC).^[1,4–6] Despite extensive research being invested in this area of high unmet medical need, the molecular pathogenesis of NAFLD and NASH remains elusive, and no approved drugs are available for their treatment.^[7]

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Among genetic variants that confer susceptibility to NAFLD/NASH, the best characterized are a single-nucleotide polymorphism (rs738409) in the *PNPLA3* (patatin-like phospholipase domain containing 3) gene and a splice variant (rs72613567:TA) in the *HSD17B13* gene,^[8,9] both of which encode proteins anchored to the intrahepatocellular lipid droplets. Importantly, recent evidence has revealed that the STE20-type kinases comprising the GCKIII subfamily (MST3, MST4, and STK25) also regulate the initiation and aggravation of NAFLD via association with hepatocellular lipid droplets. Together, these data highlight the critical function of liver lipid droplet proteome in NAFLD etiology.

In the following sections, we discuss the newly described roles of GCKIII kinases in the molecular pathogenesis of NAFLD and NASH and explore the underlying mechanisms.

DOMAIN STRUCTURE OF THE HUMAN GCKIII SUBFAMILY OF STE20 KINASES

The human genome features a large branch of STE20 kinases, named after the founding member yeast Sterile20 kinase involved in the mating pathway.^[10] Mammalian STE20-type kinases (~30 described to date) are distinguished by a high degree of homology within the catalytic domain, and they fall into two subfamilies: the p21-activated kinases (PAKs) with a C-terminal

kinase domain and an N-terminal p21 GTPase-binding domain and the germinal center kinases (GCKs), which have an N-terminal kinase domain and lack GTPase-binding domains.^[11] Functionally, STE20 kinases have been reported to regulate a broad range of biological processes including cell differentiation, proliferation, apoptosis, motility, polarity, metastasis, angiogenesis, and stress responses.^[12–20]

MST3 (also known as STK24), MST4 (also known as STK26 or MASK), and STK25 (also known as YSK1 or SOK1) comprise the GCKIII subgroup of STE20 proteins. The members of the GCKIII family are ubiquitously expressed and have been implicated in the regulation of Golgi integrity, cytoskeletal organization, cell polarity, proliferation and migration, apoptosis, as well as neuronal and immune functions.^[21–27] In humans, *MST3* is located on chromosome 13q32.2, whereas *MST4* and *STK25* are located on Xq26.2 and 2q37.3, respectively.

The kinases in the GCKIII subgroup are the shortest within the STE20 family, with the human proteins ranging from 416 to 431 amino acids in length. At the N-terminal end of GCKIIIs, a short variable sequence is followed by a highly conserved kinase domain of 251 residues (about 90% similarity in amino acid sequence).^[19,20,25,28,29] This catalytic domain encompasses the adenosine triphosphate-binding (ATP) site (GXGX[F]GX₆K) and a protein substrate binding site, which also contains the STE20 signature peptide (GTPFWMAPE) (Figure 1).^[12,30] Molecular modeling and crystallographic studies have revealed that the GCKIII catalytic domains

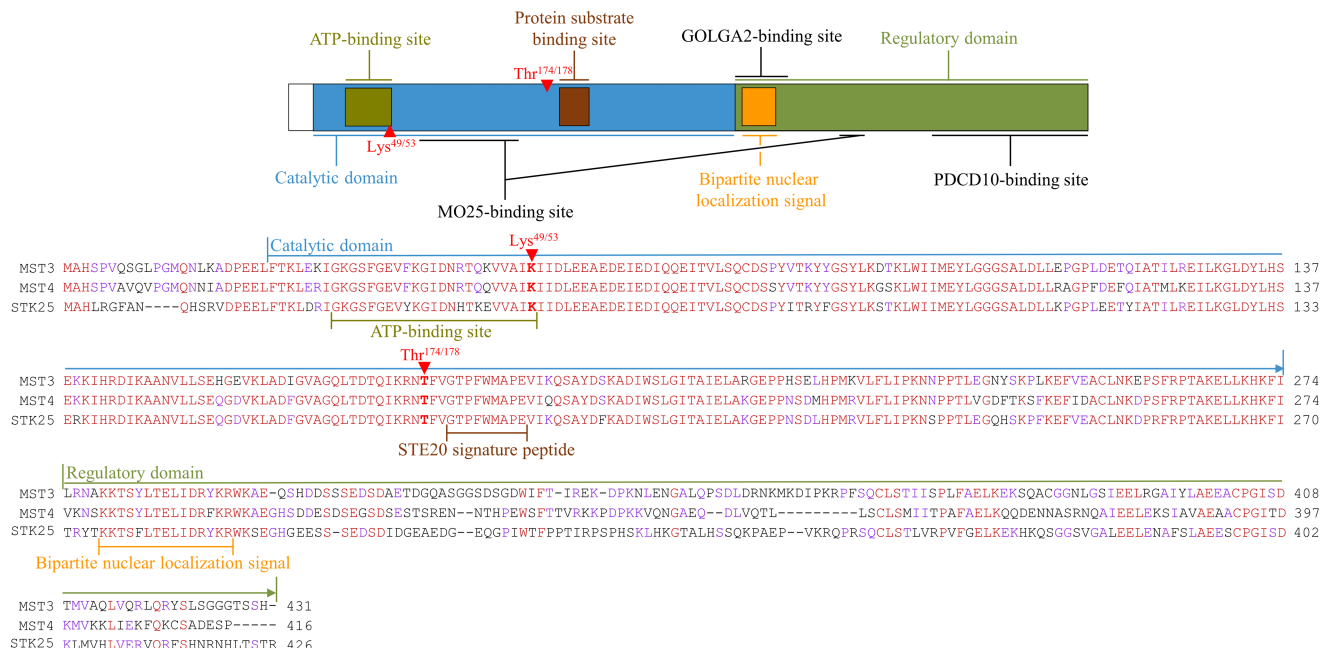


FIGURE 1 Domain structure and alignment of human GCKIII kinases. Sequences of *MST3* (GenPept accession number NP_001027467), *MST4* (GenPept accession number NP_057626), and *STK25* (GenPept accession number NP_001258906) were aligned using Clustal Omega (1.2.2; Conway Institute UCD, Dublin, Ireland). Fully, partially, or not aligned sequences are shown in red, violet, or black, respectively. ATP, adenosine triphosphate.

display a typical structure of protein kinase domains, with a smaller N-lobe and a larger C-lobe, and the substrate-binding cleft placed between them.^[25,31–36] Notably, the lysine residue (Lys⁴⁹ in STK25 and Lys⁵³ in MST3/MST4) and the threonine residue (Thr¹⁷⁴ in STK25 and Thr¹⁷⁸ in MST3/MST4) within the kinase domain have been identified as instrumental for the catalytic activity of GCKIII kinases.^[29,33,37–39] Rather than requiring activation by upstream kinase(s), GCKIIIs appear to be activated by constitutive autophosphorylation of Thr^{174/178}, suggesting that the primary regulation of MST3, MST4, and STK25 activity lies at the level of inhibition of the Thr^{174/178} dephosphorylation by protein phosphatase(s).^[25,33,40–43]

The regulatory domain of MST3, MST4, and STK25 lies at the C-terminal to the catalytic domain and carries a putative bipartite nuclear localization signal at its N-terminal end, which is relatively well conserved (Figure 1).^[44,45] This sequence is followed by a variable region of 119–134 residues (about 20% similarity in amino acid sequence), which is predicted to be highly disordered and is suggested to interact with various signaling molecules and regulatory proteins.^[25,30] As an example, the regulatory domain of all three GCKIIIs has been shown to heterodimerize with PDCD10 (programmed cell death 10, also known as CCM3).^[46–58] The regulatory region of GCKIIIs also interacts with GOLGA2 (Golgin subfamily A member 2, also known as GM130) in the control of Golgi assembly.^[25] Furthermore, through both regulatory and catalytic domains, GCKIIIs associate with MO25 protein in the LKB1/STRAD/MO25 complex.^[34,35,59,60] The regulatory regions appear to be involved in homodimerization of GCKIII kinases, although the exact residues engaged in these interactions are not clearly determined.^[29,61] Notably, several caspase cleavage sites are predicted *in silico* within the regulatory domain of MST3, MST4, and STK25, some of which have been experimentally verified (Figure 1).^[25,37,38,44,62–66]

GCKIII KINASES DECORATE INTRAHEPATOCELLULAR LIPID DROPLETS

Intracellular lipid droplets are composed of a neutral lipid core of primarily triacylglycerol (TAG) and cholesterol esters surrounded by a phospholipid monolayer that harbors specific proteins.^[67–69] By using immunofluorescence microscopy, we found that MST3, MST4, and STK25 are exclusively localized around lipid droplets in human hepatocytes (exemplified by STK25 in Figure 2).^[70–72] Consistently, GCKIII kinases are associated with hepatic lipid droplets in mice, as demonstrated by global proteomic analysis of the lipid droplet fraction isolated from steatotic livers of obese mice as well as using immunofluorescence microscopy of mouse liver sections (Figure 2).^[71–74]

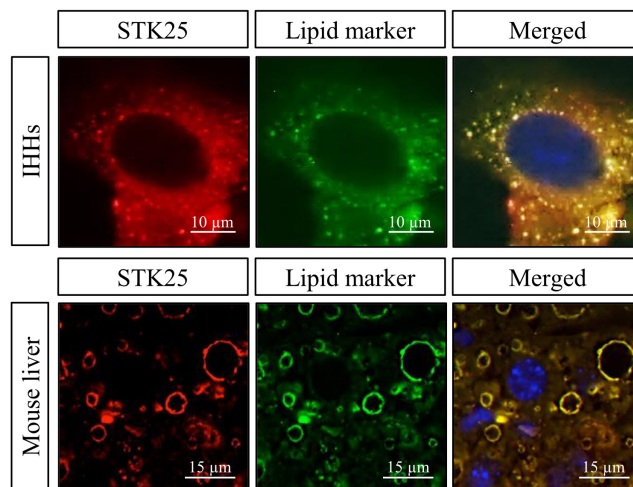


FIGURE 2 STK25 associates with intrahepatocellular lipid droplets. Representative immunofluorescence images of oleate-loaded immortalized human hepatocytes (IHHs) and liver sections from high fat-fed mice, where the merged images show colocalization of STK25 and lipid droplets. (Figure adapted from Refs. 70 and 74).

In contrast, MST4 and STK25 have been shown to localize to the Golgi apparatus in the human cervical carcinoma cell line (HeLa) and human embryonic kidney cell line (HEK293), where they function in a signaling cascade required for cell migration and polarization.^[29,49,50] MST3 has been reported to localize predominantly to the cytosol in HeLa and HEK293 cells; however, following caspase cleavage, the protein translocates to the nucleus inducing apoptosis.^[37,44] Similarly, a cleaved form of STK25 can translocate from the Golgi to the nucleus in HEK293 cells after chemical anoxia, promoting cell death.^[38] Together, these observations suggest that GCKIII kinases display a different subcellular localization pattern in the liver compared with extrahepatic tissues. Furthermore, it is important to investigate whether the localization of GCKIII kinases may change in hepatocytes in response to challenges such as fasting, when very few lipid droplets are present.

HEPATIC EXPRESSION OF GCKIII KINASES CORRELATES WITH THE SEVERITY OF HUMAN NAFLD

We have reported a significant positive correlation between *MST3*, *MST4*, and *STK25* transcript abundance in human liver biopsies and all three individual lesions of the NAFLD Activity Score (NAS) used for clinical diagnosis (i.e., histological scores of hepatic steatosis, lobular inflammation, and hepatocellular ballooning) as well as total NAS ($n = 62$; Figure 3A–D).^[71,72,75] Moreover, messenger RNA (mRNA) levels of *MST3*, *MST4*, and *STK25* were about 2–3-fold higher in subjects with NAS ≥ 5 (defines definite NASH; $n = 24$) compared to participants

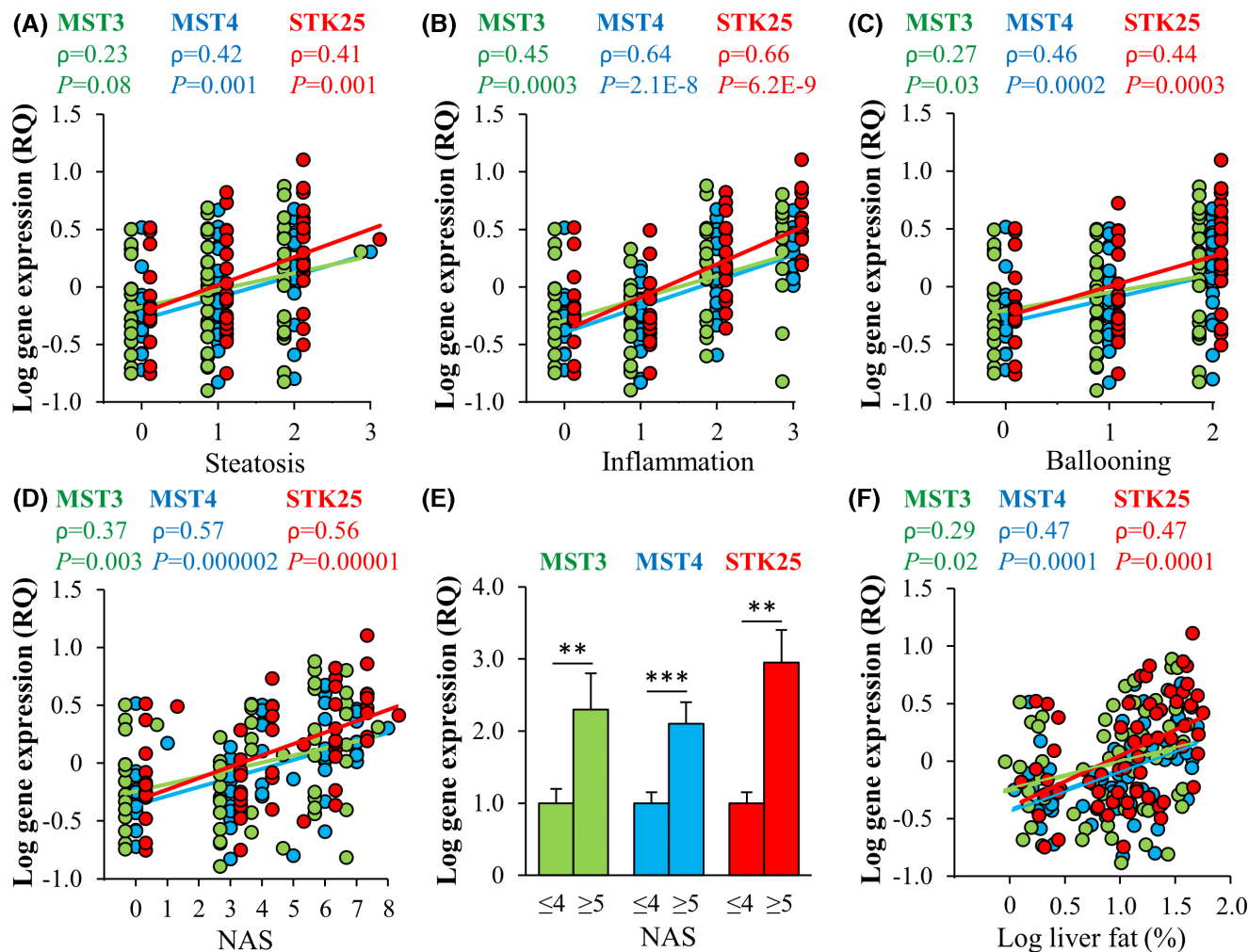


FIGURE 3 Hepatic expression of GCKIII kinases is positively correlated with nonalcoholic fatty liver disease (NAFLD) severity. Correlation between messenger RNA (mRNA) levels in human liver biopsies and the individual histological lesions of NAFLD Activity Score (NAS; A–C), the total NAS (D), and subjects with low versus high NAS (E). (F) Correlation between mRNA levels and hepatic fat content measured by magnetic resonance spectroscopy. RQ, relative quantification. (Figure combines results from several publications (Refs. 70–72 and 75).

with NAS ≤ 4 (defines simple steatosis or borderline NASH; $n = 38$) (Figure 3E).^[71,72,75] We also found a positive correlation between the hepatic expression of GCKIII kinases and the liver fat content assessed by magnetic resonance spectroscopy (Figure 3F).^[70–72,75]

GCKIII KINASES CELL-AUTONOMOUSLY CONTROL LIPID STORAGE AND METABOLIC STRESS IN HUMAN HEPATOCYTES

Our recent studies reveal that small interfering RNA silencing of MST3, MST4, or STK25 in human hepatocytes markedly reduces intracellular lipid accumulation, which is mediated by increased β -oxidation and very low-density lipoprotein (VLDL)-TAG secretion (i.e., output), combined with decreased TAG synthesis (i.e., input) (Figure 4).^[70–72] Importantly,

MST3-, MST4-, or STK25-deficient hepatocytes are also substantially protected against oxidative and endoplasmic reticulum (ER) stress (Figure 4),^[71–73,75] which are considered the key triggers of disease progression from simple liver steatosis to NASH.^[76–78] Reciprocally, a substantial increase in lipid deposition as well as oxidative/ER stress was detected in human hepatocytes overexpressing GCKIII kinases.^[70–72,75] With these data describing the functional implications of modified MST3, MST4, and STK25 abundance on lipid partitioning and metabolic stress being highly consistent, the underlying mechanisms of GCKIII signaling in the regulation of intrahepatocellular lipid homeostasis still remain elusive. Further unbiased investigations combining global phosphoproteomics, interactomics, and substrate mapping would be valuable to identify the interaction partners and direct targets of GCKIII kinases to decipher their molecular mode-of-action in hepatic lipid metabolism. It would

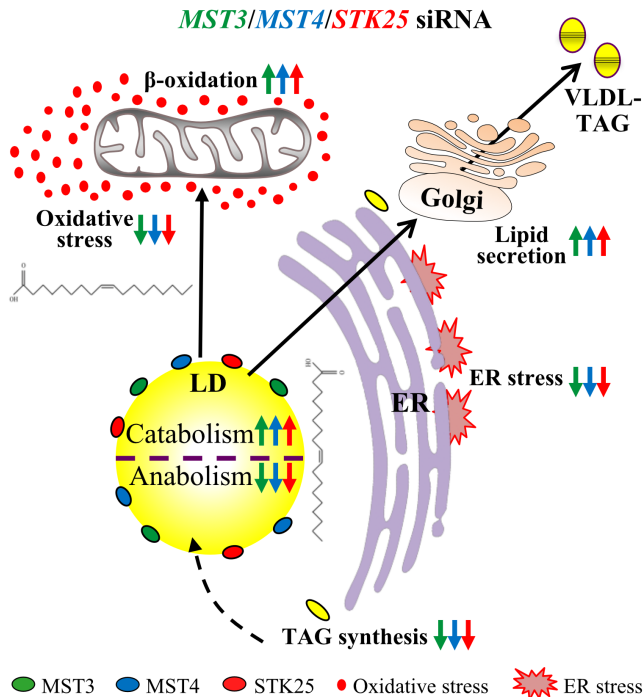


FIGURE 4 Proposed mechanisms through which GSKIII kinases control liver lipotoxicity. Silencing of MST3, MST4, or STK25 in human hepatocytes lowers fat storage by inhibiting lipid droplet anabolism through reduced triacylglycerol (TAG) synthesis, and by stimulating lipid droplet catabolism through enhanced β -oxidation and very low-density lipoprotein (VLDL)-TAG secretion, resulting in protection against oxidative and endoplasmic reticulum (ER) stress. LD, lipid droplet. (Figure combines results from several publications (Refs. 70–73 and 75).

also be interesting to use advanced imaging (e.g., super-resolution microscopy or correlative light-electron microscopy) to examine whether GSKIII kinases affect the interactions of liver lipid droplets with other cellular organelles including ER, mitochondria, peroxisomes, and lysosomes.

The possible role of MST3, MST4, and STK25 in glucose metabolism has not been studied in detail in hepatocytes. However, *in vivo* data support the impact of GSKIII kinases on the control of glucose and insulin homeostasis (see subsequent section). Of note, STK25 was shown to negatively regulate the aerobic glycolysis in colorectal cancer cells via mammalian target of rapamycin (mTOR) signaling.^[79]

SILENCING OF STK25 OR MST3 HINDERS THE DEVELOPMENT OF NASH IN MICE

The main pathophysiological characteristics of human NALFD can be replicated in rodent models by different dietary challenges. We found that, when challenged with a NASH-inducing diet, the livers from *Stk25* knockout mice and mice treated with *Stk25*-targeting

antisense oligonucleotides (ASOs) display substantial suppression of integral NASH features compared with wild-type livers: Along with less steatosis, we observed attenuation of oxidative stress, inflammation, and fibrosis, combined with reduced hepatocellular damage (Figure 5).^[80–82] This improvement of the liver phenotype correlates with a decrease in the hepatic levels of acetyl-CoA carboxylase (ACC) protein, a key regulator of both lipid oxidation and synthesis.^[80–82] Reciprocally, we show that diet-induced NASH is markedly aggravated in *Stk25*-overexpressing transgenic versus wild-type mice.^[74,75] Of note, high-fat-fed *Stk25*^{-/-} mice also accumulate less ectopic fat in extrahepatic tissues such as skeletal muscle, kidney, and vascular wall, and have healthier adipose tissue,^[82–85] which is accompanied by improved whole-body glucose tolerance and insulin sensitivity^[82] compared with wild-type littermates.

Similarly to the silencing of STK25, we found that mice treated with *Mst3*-targeting ASOs are significantly protected against diet-induced NASH, which is also paralleled by a lower abundance of liver ACC.^[86] In light of the recent evidence of an association and causal link between NASH and insulin resistance,^[5,87] our results are in line with a study by Iglesias et al. reporting that *Mst3* genetrapped mice (which have a significant but not complete reduction of *Mst3* expression) fed with a high-fat diet display improved hepatic insulin sensitivity compared with wild-type controls.^[88] In the livers from high fat-fed mice, the loss of MST3 was also shown to increase the signaling of the insulin receptor pathway at the level of AKT phosphorylation, accompanied by high activity of mTOR complex 2.^[88] In contrast, Qin et al. found that elimination of MST3 in mice promoted high-fat diet-induced systemic glucose intolerance and insulin resistance, and attenuated phosphorylation of AKT in adipose tissue and liver.^[89] It is possible that the differences in the genetic background of mouse strains studied, and composition of the high-fat diets used, have contributed to this variability in results.

To date, there are no studies in nonclinical *in vivo* models that implicate MST4 in the development of NAFLD/NASH. However, there is some indirect evidence suggesting that MST4 may regulate liver fibrosis,^[90] and MST4 has also been described as a critical mediator limiting inflammatory responses in extrahepatic tissues.^[23,91]

ROLE OF GSKIII KINASES IN HCC

Recently, NASH has been reported in several studies as a rapidly increasing underlying etiology of HCC,^[92–94] which is one of the most fatal and fastest-growing cancers.^[95] Interestingly, we found that the genetic ablation of STK25 protects mice against NASH-driven HCC via

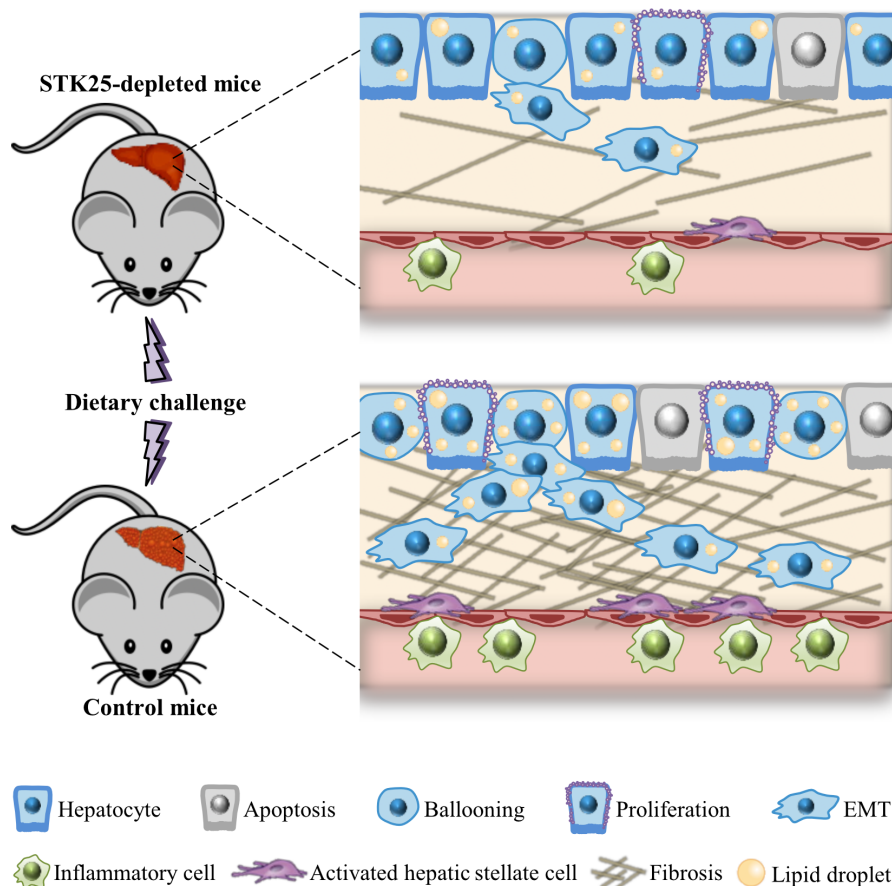


FIGURE 5 A schematic model: STK25 antagonism protects mice against diet-induced nonalcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC) by suppressing lipotoxicity-induced inflammatory and profibrotic responses, reducing hepatocellular ballooning and apoptosis, decreasing compensatory proliferation and epithelial–mesenchymal transition (EMT). (Figure combines results from several publications (Refs. 75,80–82 and 96).

reduced hepatocellular apoptosis and lowered compensatory proliferation, by a mechanism that involves the suppression of hepatic lipotoxicity and inactivation of liver signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 signaling—the key pathways implicated in human HCC (Figure 5).^[96] In line with these observations, short hairpin RNA knockdown of STK25 has been demonstrated to inhibit tumor growth in human HCC xenografts in nude mice.^[97] The silencing of STK25 also suppresses proliferation, migration, and invasion in human hepatocarcinoma cells *in vitro*, which is accompanied by a lower expression of the markers of epithelial–mesenchymal transition (EMT) and augmented autophagic flux.^[96,97] Furthermore, high STK25 expression was found to be correlated with adverse clinicopathological characteristics and poor survival in patients with HCC.^[97]

A report by Lim et al. has described STK25 as an activator of the Hippo pathway in the liver by phosphorylation of LATS.^[98] The Hippo signaling is known to maintain tissue homeostasis and organ size by negatively regulating the oncogenic transcriptional co-activators yes-associated protein 1 (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ).^[99]

Consistently, STK25 knockdown was reported to result in a moderate increase in liver weight in aged mice; however, hepatic carcinomas were not detected.^[98] In contrary, a recent study by Bae et al. has identified STK25 as a suppressor of the Hippo pathway in human embryonic kidney cells by inhibition of STE20-type kinase MST2.^[100] In all, STK25 may display both positive and negative impact on Hippo signaling depending on the downstream targets involved.

Evidence supporting the involvement of MST4 in HCC is contradictory. A high level of liver MST4 expression, associated with large tumor size, microvascular invasion, and presence of intrahepatic metastasis, has been reported as an adverse prognostic factor for overall survival and time to recurrence after hepatectomy in patients with HCC.^[101] In the same study, the knockdown of MST4 in human HCC cell lines (MHCC-97H and SMMC7721 cells) was shown to inhibit proliferation, colony formation, and invasion, whereas up-regulation of MST4 appeared to aggravate these processes by promoting EMT via the activation of ERK signaling.^[101] Conversely, a low MST4 expression has also been reported to correlate with HCC progression and poor prognosis, and MST4 inactivation was

found to induce EMT in human HCC cell lines (Bel-7402, Bel-7404, and Huh7 cells), promoting their migratory and invasive potential *in vitro*, and facilitating intrahepatic metastasis *in vivo*, with the opposite phenotype observed when MST4 is overexpressed.^[102,103]

Similarly to STK25, MST4 is suggested to act in the Hippo signaling pathway: MST4 is described as a bona fide kinase of YAP, phosphorylating YAP at Thr⁸³ and causing an impaired nuclear importing of YAP.^[104,105]

MST3 has also been implicated in the Hippo pathway: Under certain circumstances, MST3 directly phosphorylates the nuclear Dbf2-related kinase, which in turn phosphorylates and inhibits YAP.^[106,107] The possible role of MST3 in HCC initiation and progression is yet to be explored; however, MST3 has been reported to act both as a positive or negative regulator of tumorigenesis in extrahepatic tissues.^[40,108–110]

CONCLUSIONS

Recent translational studies in patient cohorts, cultured human cells, and mouse models provide several lines of experimental evidence suggesting that GCKIII kinases MST3, MST4, and STK25 are important components of the hepatocellular lipotoxic milieu. Antagonizing hepatic GCKIII signaling appears to enable targeting of the initiating nexus for lipid-triggered liver injury in NAFLD, warranting further investigations in basic biology as well as clinical implications of these kinases.

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CONFLICT OF INTEREST

Nothing to report.

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