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CAMK2γ Antagonizes mTORC1 Activation during Hepatocarcinogenesis

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Abstract

Hepatocellular carcinoma (HCC) is one of the most deadly cancers that still lacks effective treatments. Dysregulation of kinase signaling has frequently been reported to contribute to HCC. In this study, we used bioinformatic approaches to identify kinases that regulate gene expression changes in human HCCs and two murine HCC models. We identified a role for calcium/calmodulin-dependent protein kinases II gamma isoform (CAMK2**γ**) in hepatocarcinogenesis. CAMK2$\boldsymbol{\gamma}^{-/-}$ mice displayed severely enhanced chemical-induced hepatocarcinogenesis compared with wild-type controls. Mechanistically, CAMK2**γ** deletion potentiates hepatic activation of mechanistic target of rapamycin complex 1 (mTORC1), which results in hyperproliferation of

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Conflict of interest

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hepatocytes. Inhibition of mTORC1 by rapamycin effectively attenuats the compensatory proliferation of hepatocytes in CAMK2$\gamma^{-/-}$ livers. We further demonstrated that CAMK2γ suppressed growth factor- or insulin-induced mTORC1 activation by inhibiting IRS1/AKT signaling. Taken together, our results reveal a novel mechanism by which CAMK2γ antagonizes mTORC1 activation during hepatocarcinogenesis.

Keywords

Hepatocellular Carcinoma; Kinase; CAMK2γ; mTORC1; Rapamycin; AKT

Introduction

Liver cancers, including the hepatocellular carcinoma (HCC), are the second most common cause of cancer deaths worldwide (World Cancer Report 2014, WHO). Patients with liver cancer have poor prognosis due to recurrence and the lack of effective treatments.[1, 2] The development of liver cancer is associated with various risk factors.[3] Chronic infection by virus, certain types of parasites in the liver, abuse of alcohol, or steatohepatitis can all cause long-term hepatocyte necrosis and inflammation, followed by formation of post-necrotic cirrhosis of the liver.[3] Insults from xenobiotics or chemicals such as Aflatoxin and Arsenic not only cause hepatocyte death, but also directly induce oncogenic mutations at tumor suppressor genes such as p53.[1, 4] A common pathological consequence of these risk factors is continuous hepatocyte death accompanied by compensatory hepatocyte proliferation, which promotes hepatocyte transformation and malignant cell expansion.[5] However, the underlying molecular mechanisms controlling hepatocarcinogenesis are still largely unclear.

Kinase regulation plays important roles during hepatocarcinogenesis. Adult hepatocytes are normally in a quiescent state. Upon carcinogen stimuli, pro-inflammatory cytokines, for instance IL-6 and TNFα, are synthesized and released from the Kupffer cells to activate kinase pathways such as JAK/STAT and IKK/IκBα in hepatocytes, which in turn prime hepatocytes out of quiescence and drive them into cell cycling.[5] Growth factors and hormones subsequently stimulate the proliferation of the transformed hepatocytes by interacting with the receptor-tyrosine kinase (RTK) family. Several kinase inhibitors are under active development to treat liver cancer. For example, Sorafenib, which targets both the Raf/MEK/ERK pathway and receptor tyrosine kinases VEGFR/PDGFR in tumor cells, can prolong the survival of liver cancer patients by about 3 months through inhibiting cancer cell proliferation and angiogenesis.[6] Population studies show that metformin decreases cancer risk by activating AMPK to suppress mTORC1, which induces apoptosis of malignant liver cells.[7, 8]

Current approaches to systemically predict kinase activities include Mass Spectrometry (MS)-based phosphor-proteomic analysis and substrate peptide based phosphor-kinase arrays.[9–13] Both approaches have their limitations when applying to *in vivo* studies. MS-based phosphor-proteomic analysis is limited by abundance of a specific phosphorylation peptide, efficiency of phosphor-specific peptide enrichment, as well as sensitivity of MS instruments.[9–11] Peptide based kinase arrays, on the other hand, can only detect a limited

number of kinases with known substrate motifs, and the results are highly influenced by specificity of the substrate peptide towards different kinases.[11–13] Moreover, activity of kinases is regulated by transiently induced cellular chemical and mechanical signals. Differential subcellular localization or kinase/adaptor/scaffold protein interaction could spatially regulate kinase activity or accessibility to specific substrates, which cannot be reflected by the peptide-based kinase arrays.

Taking advantage of the rapidly expanding transcriptome and kinase function studies, new bioinformatics algorithms have recently been developed to predict activation of potential upstream transcription factors and associated kinases.[14–16] These bioinformatics tools use gene expression profiles as input to identify differentially expressed genes and can further examine transcription factor or kinase correlated to gene expression signatures. They can then provide candidate gene lists for further analyses. Among these tools, Expression2Kinases (X2K) and Ingenuity Pathway Analysis (IPA) have successfully been used to predict the change of kinase activity in various human diseases and animal models.[17–20]

mTORC1 is a protein complex that senses nutrient/energy/redox status and regulates protein synthesis.[21] mTORC1 activities are essential for liver cell proliferation as the S6 knockout mice almost fail to display liver regeneration after 70% partial hepatectomy.[22] Noteworthy, up to 50% of liver cancer patients show up-regulation of mTORC1 activities.[7] However, regulation of mTORC1 during hepatocarcinogenesis remains elusive.

Calcium/calmodulin-dependent protein kinases II (CAMK2s) have been extensively studied in central neural systems due to its key roles in learning and memory.[23] The γ and δ isoforms of CAMK2s are key mediators of different upstream signaling pathways, including oxidative and ER stresses, non-canonical Wnt signaling, and GPCRs.[24–28] In this study, we used novel bioinformatic approaches to analyze gene expression profiling from human HCCs and two HCC animal models. Among the identified list of kinases, CAMK2γ stood out to play a critical role during hepatocarcinogenesis by antagonizing mTORC1 activation.

Results

Identification of new kinases in hepatocarcinogenesis

To identify novel kinases involved in liver hepatocarcinogenesis, we analyzed genome-wide mRNA profiling datasets of both animal HCC models and human HCC specimens by computational approaches, including the ChIP-X Enrichment Analysis (ChEA) and Kinase Enrichment Analysis (KEA).[29, 30] Two well-characterized HCC rodent models, the chemical carcinogen diethylnitrosamine (DEN)-treated mice and the spontaneously tumorigenic $FXR^{-/-}$ mice,[31–34] were selected for these analyses. Both models recapitulate the human HCC pathological progression.[31, 35] In addition, a dataset of human HCC specimens was also included,[36] in which the gene expression of HCC and surrounding nontumorous liver tissue was compared. Differentially expressed genes were called for each dataset using limma [37] (see Materials and Methods for details) and these genes were further analyzed for potential upstream transcription factors (TFs) with ChIP Enrichment Analysis (ChEA).[29] ChEA utilizes a database of TF binding sites from chromatin immunoprecipitation studies

(ChIP-seq and ChIP-chip) to determine the over-representation of TF targets for the given set of genes compared to the whole genome. The top-ranked TFs reported by ChEA from each dataset are shown in Fig. 1A (Table. S1). Seven TFs (HNF4A, PPARG, MITF, CREM, FLI1, KDM5B and RUNX1) were identified in the top 10 in at least two of the datasets, indicating that they are involved in gene regulation in the development of HCC. These seven TFs were further used to predict the upstream kinases that contribute to liver tumorigenesis with kinase enrichment analysis (KEA).[30] KEA utilizes an underlying kinase-substrate interaction database to identify the kinases that phosphorylate given proteins/TFs.[38–40] KEA showed that p38α (MAPK14), GSK3α/β, CAMK2γ/δ, ERK2 (MAPK1), AMPKα2 (PRKAA2), Ribosomal s6 kinase (RPS6KA1, RSK1), Casein kinase 1α (CSNK1α1), and TGFBR2 were the top-ranked kinases that most likely to be involved in HCC. Ingenuity Pathway Analysis was then used to characterize connections among these kinases. ERK2 and p38α appear to constitute the center of the liver cancer kinase network and affect each other. Interestingly, CAMK2γ is known to activate both kinases (Fig. S1A).

To account for the fact that kinases may be involved in hepatocarcinogenesis without directly phosphorylating TFs, we further analyzed the three datasets using Expression2Kinases (X2K) to include the kinases whose substrates are not TFs (Fig. 1B).[15] X2K analysis unitizes known protein-protein interactions to construct a network that connects identified TFs and predict upstream regulators.[17–20] For each dataset, the top 10 TFs from ChEA (Fig. 1A) were further used to generate regulation networks based on the known protein-protein interactions of these TFs (Fig. S1B–D). All the proteins identified in these networks were then used to identify the upstream kinases using KEA in each dataset. Because the X2K analyses gave rise to many kinase candidates, we only selected the top 50 kinases enriched from each dataset (Fig. 1B). The kinases that emerged in all 3 datasets as common upstream kinases are listed in Fig. 1B. CAMK2γ was significantly enriched (p-value < 0.05, Fisher's exact test) in all the three datasets. Taken together with the Ingenuity Pathway Analysis (IPA) results (Fig. S1A), these results suggest that CAMK2γ is located upstream of the kinase cascades and plays an important role in hepatocarcinogenesis. Consistent with the bioinformatics study, the phosphorylation of liver CAMK2γ, which indicates its activation, was induced by a genotoxic stress via DEN treatment (Fig. S1E).

Deletion of CAMK2γ promoted DEN-induced hepatocarcinogenesis in mice

Our bioinformatics analyses suggest that CAMK2γ may be involved in hepatocarcinogenesis.[41] Previous studies from our lab also revealed an oncogenic role of CAMK2γ in human chronic myeloid leukemia.[42] We therefore hypothesized that CAMK2γ might promote HCC development. To address our hypothesis, we generated two chemical-induced HCC animal models to determine the roles of CAMK2γ in hepatocarcinogenesis. For the first model, 2-weeks-old male CAMK2γ$^{-/-}$ mice and the wild-type littermates were injected with one single dose of 25 mg/kg DEN. This regimen is sufficient to induce hepatocarcinogenesis in wild-type mice after 8–10 months (Fig. 2A). To our surprise, while wild-type mice only showed small tumors or neoplastic foci in liver, deletion of CAMK2γ resulted in a significant increase in hepatocarcinogenesis (Fig. 2B). Deletion of CAMK2γ did not change liver size, but significantly increased the tumor number and volume. To confirm these observations, we induced HCC using another protocol that DEN was injected

and followed by a tumor promotor (TCPOBOP) as described previously.[43] This model better represents the liver carcinogenesis caused or enhanced by xenobiotic insults. Four weeks old male mice were treated with one single dose of DEN and thereafter TCPOBOP injection every two weeks for 8 times. More significant increase of liver carcinogenesis in CAMK2$\gamma^{-/-}$ mice was observed in this model (Fig. 2C). The CAMK2$\gamma^{-/-}$ mice exhibited more than 3-fold increase of both liver tumor sizes and tumor volumes compared with their wild-type littermates (Fig. 2D).

Histological analyses revealed that the hepatic tissues from CAMK2$\gamma^{-/-}$ mice displayed enhanced liver necrosis, apoptosis, inflammatory cell infiltration (Fig. S2A). An increase in compensatory hepatocyte proliferation was observed in the CAMK2$\gamma^{-/-}$ liver (Fig. S2B). Consistently, quantitative real-time PCR analyses showed an enhanced expression of inflammatory cytokines such as TNFα and IL-6 in the CAMK2$\gamma^{-/-}$ livers (Fig. S3). Loss of p53 and activation of STAT3 are hallmarks of liver carcinogenesis and represents advanced HCC progression. Indeed, hepatic STAT3 phosphorylation at Tyr705 was increased in both tumor and non-tumor adjacent tissues, and the p53 expression was diminished in CAMK2$\gamma^{-/-}$ livers (Fig. S4A). As expected, the expression levels of p53 target genes p21 and Bax were decreased (Fig. S4B). In contrast, the expression levels of STAT3 target genes Socs3, Bcl-2, and Mcl-1 were increased by CAMK2γ deletion (Fig S4C).

CAMK2γ deletion enhanced carcinogen-induced hepatocyte deaths

DEN-induced acute liver injury is a commonly used model to study liver cancer initiation because massive liver cell deaths trigger inflammation, the main driver of liver cell proliferation and transformation.[31, 35, 44–46] Since deletion of CAMK2γ enhances cell deaths and inflammation in liver tumor and surrounding tissues, we speculate whether deletion of CAMK2γ also promotes liver cancer initiation through elevating the levels of cell deaths and inflammation upon acute liver injury. Therefore, CAMK2$\gamma^{-/-}$ mice and wild-type littermates were administrated with 100 mg/kg DEN by i.p. injection. As expected, CAMK2$\gamma^{-/-}$ mice had enhanced body weight loss (Fig. 3A) and significantly higher serum alanine aminotransferase (ALT) levels (Fig 3B), indicating a higher level of liver injury and substantial hepatocyte deaths in CAMK2$\gamma^{-/-}$ mice. The histological analyses of hematoxylin & eosin (H&E)-stained also demonstrate significantly enhanced necrosis caused by CAMK2γ deletion (Fig. 3C). DEN led to significant necrosis in the lobules that is distant from vein area in addition to enhanced necrosis around portal vein in the CAMK2$\gamma^{-/-}$ livers. In contrast, the same dose of DEN did not induce necrosis around portal vein in the wild-type mice (Fig. 3D). Furthermore, compared with wild type livers, TUNEL staining showed that CAMK2γ−/− livers exhibited significantly increased hepatocyte apoptosis induced by DEN, especially around portal veins (Fig. 3E,F). It is also worth mentioning that CAMK2$\gamma^{-/-}$ mice exhibited more spontaneous apoptosis without DEN treatment (Fig. 3F). Severe cell deaths are known to trigger inflammatory responses, which is a key cause of tumor initiation in the liver.[5] In fact, CAMK2γ deletion strongly elevated inflammatory cell infiltration caused by DEN-induced liver injury (Fig. 3G).

CAMK2γ deletion potentiated DEN-induced mTORC1 activation and enhanced compensatory hepatocyte proliferation

A key event of liver tumor initiation is the continuous compensatory proliferation of the premalignant hepatocytes. Compensatory proliferation plays a critical role in DEN-induced hepatocarcinogenesis, and dysregulated compensatory proliferation results in enhanced malignant transformation of liver cells.[31, 35, 44–46] Indeed, CAMK2$\gamma^{-/-}$ livers displayed greatly enhanced hepatocyte proliferation as shown by PCNA immunostaining (Fig. 4A). In contrast, wild-type livers only displayed limited PCNA stains around portal vein areas (Fig. 4B).

NF-κB, STAT3, and MAPK (p38, ERK, JNK) signaling pathways are known to drive the compensatory proliferation of liver cells during initiation stages. On the other hand, CAMK2γ has been known to affect NF-κB activation and phosphorylation of STAT3 and MAPKs.[25, 47, 48] Therefore, we profiled the activation of these pathways in the liver treated with DEN. The results showed that STAT3 and MAPK had marginally increased activation based on the phosphorylation status of STAT3, p38, ERK1/2 and c-Jun. However, no degradation of IκB proteins is observed, indicating that NF-κB may be not involved in the increased compensatory proliferation in CAMK2$\gamma^{-/-}$ livers (Fig. 4C).

Unexpectedly, mTORC1 signaling was robustly activated in CAMK2$\gamma^{-/-}$ mice (Fig. 4D). The autophosphorylation of mTOR at S2481 was much higher in the CAMK2$\gamma^{-/-}$ livers at the 48 h after DEN treatment, indicating that the activities of mTOR were enhanced in the CAMK2$\gamma^{-/-}$ mice.[49] mTORC1 promotes tissue growth by coordinating protein synthesis and inhibiting autophagy. mTORC1 stimulates protein translation by directly phosphorylating S6K and 4E-BP-1.[21] Indeed, DEN treatment dramatically increased phosphorylation of S6K at T389 as early as 4 h after DEN injection in the wild-type mice and subsequently diminished at the 48 h (Fig. 4D). In contrast, the initial induction in the CAMK2$\gamma^{-/-}$ livers was slightly delayed but maintained until the 48 h. This is correlated with the greater enhanced hepatocyte proliferation in the CAMK2$\gamma^{-/-}$ livers. Though DEN did not dramatically increase the phosphorylation of 4E-BP-1, the overall phosphorylation of 4E-BP-1 at T37/46 and S65 was significantly higher in the CAMK2$\gamma^{-/-}$ livers. In addition, the phosphorylation of eIF4E at S209 was also slightly increased in the CAMK2$\gamma^{-/-}$ livers. There was also a slightly reduced autophagosome formation as shown by the LC3 conversion in the CAMK2$\gamma^{-/-}$ livers. Our results suggest that CAMK2γ may antagonize the exaggerated activation of mTORC1 in the livers after DEN treatment.

Because mTORC1 potentially promotes liver tumor initiation in the CAMK2$\gamma^{-/-}$ mice through promoting hepatocyte proliferation, we therefore investigated whether rapamycin could abolish the observed effects. Immunoblotting showed that rapamycin effectively decreased the hyperactivation of mTORC1 in the CAMK2$\gamma^{-/-}$ livers, as shown by the decreased phosphorylation of S6K and 4E-BP-1 (Fig. 5A). Interestingly, rapamycin also significantly increased the autophosphorylation of CAMK2γ as well (Fig. 5A). As expected, rapamycin treatment greatly reduced the hepatocyte proliferation in the CAMK2$\gamma^{-/-}$ livers (Fig 5B, C). However, rapamycin did not rescue the enhanced inflammatory cell infiltration, centrilobular necrosis, and hepatocyte apoptosis (Fig 5D), indicating that mTORC1

hyperactivation caused by CAMK2γ deletion may contribute to only the enhanced proliferation but not the cell deaths or the inflammation in the liver.

CAMK2γ deletion enhanced AKT/mTORC1 signaling

RTK/AKT/mTORC1 pathway plays a broad role in cancer, including liver cancer.[7] Therefore, we next examined whether the deletion of CAMK2γ affected AKT activation. DEN induced significant AKT phosphorylation in the wild-type livers (Fig 6A). Surprisingly, deletion of CAMK2γ not only enhanced DEN-induced AKT phosphorylation but also increased the basal AKT phosphorylation in the livers (Fig 6A, Fig. S5A). Consistently, the substrates of AKT, including mTOR (S2448), FoxO1 (S26), and GSK3β (S9), all showed increased phosphorylation in the DEN-treated CAMK2γ$^{-/-}$ livers. On the other hand, ramamycin, which greatly lowers mTORC1 activities in the liver, did not affect AKT phosphorylation significantly. This is consistent with the notion that mTORC1 is at the downstream of AKT (Fig 6B). Hepatocyte proliferation is triggered by hepatomitogens including cytokines, growth factors, or hormones (etc., EGF and insulin), which are known to activate AKT/mTORC1 pathway in the liver. Indeed, we observed that the insulin or EGF-induced activation of AKT and mTORC1 is greatly enhanced by CAMK2γ deletion in primary hepatocytes (Fig 6C).

To further assess a direct suppressive effect of CAMK2γ on AKT, we generated a tetracycline-inducible cell system to evaluate the effects of CAMK2γ overexpression on insulin-induced AKT activation. As expected, CAMK2γ overexpression repressed the insulin-induced AKT phosphorylation in both HepG2 (Fig 7A) and Huh7 cells (Fig 7B). A chemical inhibitor of CAMK2γ, KN93, but not its non-functional structural analog KN92, also enhanced insulin-induced AKT phosphorylation (Fig 7C). However, this enhancement was largely abolished by chemical compound that are reported to inhibit PI3K activity, which indicated that CAMK2γ might act at the upstream of PI3K.[50] Insulin, EGF, and other hormone/growth factors activate PI3K/AKT pathway by binding to the insulin receptor or EGFR, and then activate the signaling transducer IRS1. We found that DEN could induce strong IRS1 phosphorylation at S612 (Fig 7D). IRS1-S612 is a putative phosphorylation site of PKC and ERK,[51, 52] and phosphorylation of this site leads to desensitization of insulin signals to the cells.[53] However, in the CAMK2γ$^{-/-}$ livers, this robust induction was completely abolished. In contrast, the PTEN protein levels were unchanged. Consistently, CAMK2γ deletion also abolished the insulin-induced IRS1-S612 phosphorylation in the primary hepatocytes (Fig 7E). It is reported previously that CAMK2γ affects S612 phosphorylation in an ERK-dependent manner.[54] However, treating liver cells with ERK inhibitors did not alter the phosphorylation of IRS1 and AKT (Fig. S5B), which suggested that CAMK2γ might affect IRS-S612 activation independent of ERK.

Discussion

Kinases are known to play key roles during hepatocarcinogenesis. In this study, we used bioinformatics approaches to analyze the transcriptomics of HCC patients as well as two animal HCC models. We identified several kinases that are potentially involved in hepatocarcinogenesis. Some of these kinases, for instance, MAPK1 and MAPK14, are well

studied and known to be crucial for HCC development,[55, 56] which confirmed the efficacy and reliability of our computational analyses. Meanwhile, several kinases such as CAMK2γ and CSNK1α1 were less studied and were not clearly linked to hepatocacinogenesis. Specifically, we found that CAMK2γ was activated by HCC associated genotoxic stress. Considering its oncogenic role in hematopoietic malignancies,[42] we first hypothesized that CAMK2γ might play an oncogenic role in HCC development. Unexpectedly, deletion of CAMK2γ in mice accelerated HCC development, indicating that CAMK2γ may play a protective role against hepatocarcinogensis.

CAMK2γ was previously implicated as a glucagon effector in the liver and was involved in regulating glucose homeostasis.[57] Our previous study showed that, in liver cancer cells, CAMK2γ can promote survival in *in vitro* cultured liver cancer cells.[41] This survival-promoting function of CAMK2γ is consistent with the results that we obtained from animal studies here (Fig 3; Fig. S2A, B). Indeed, CAMK2γ prevents hepatocyte death from tissue damage, thereby suppressing the compensatory cell proliferation and expansion of the transformed hepatocytes.[5, 58] Similar animal studies on NF-κB and JNK have also shown that survival signaling for hepatocytes is required to prevent chemical-induced hepatocarcinogenesis.[45, 59] In this scenario, the dual roles of CAMK2γ in liver cancer are similar with those of NF-κB, JNK, and SHP2 in hepatocytes.[58] That is, they prevent malignant transformation by suppressing hepatocyte death and compensatory liver growth in the tumor initiation and early stages. However, in cancer cells, the same functional role may promote the survival and expansion of the transformed cells and tumor progression at the later stages of cancer progression. This phenomenon seems to be common for HCC and has many implications in human HCC pathogenesis and therapy.[58]

Mechanistically, we have identified a novel role of CAMK2γ in antagonizing mTORC1 signaling. It is well documented that activation of AKT/mTORC1 plays essential role during both the initiation and later stage development of HCC.[7, 60] It is observed that AKT/mTORC1pathway is gradually activated from pre-malignant liver tissue towards HCC tissues in both patient and animal models.[61, 62] Abnormal activation of mTORC1 pathway itself is also sufficient to drive the spontaneous development of HCC in transgenic mouse models.[63, 64] Sirolimus and everolimus, two derivatives of rapamycin are believed to suppress liver fibrosis which is an important risk factor of HCC.[65] Administration of Metformin, which can suppress mTORC1 activity through both AMPK dependent and AMPK independent mechanism, show an overall 62% reduction in the risk of liver cancer for patient with type 2 diabetes.[66] It is interesting that members of the RSK family of serine/threonine kinases (RPS6Kα1, RPS6Kα2, RPS6Kα3, RPS6Kα4), well-known regulators of mTORC1,[67] are also identified as key kinases that are involved in hepatocarcinogenesis by our bioinformatics analyses, which further implied the importance of mTORC1 pathway in HCC. However, mTORC1 itself was not identified in our analyses probably due to the fact that mTORC1 is mainly a post-transcriptional regulator. mTORC1 is positively regulated by hormones (e.g. insulin) and growth factors (e.g. IGF1, EGF),[68] but suppressed by stress signaling (genotoxic, oxidative, ER stress, etc.). However, the molecular mechanisms by which mTORC1 is suppressed by stress signaling remain unclear. Because CAMK2s are well-established stress-responsive kinases,[24, 25] our studies thus establish a molecular link between the stress signaling and AKT/mTORC1 regulation. The receptor tyrosine kinases,

which are activated by insulin and growth factors, function through AKT and ERK to phosphorylate TSC2[67, 69] and ERK/RSK1 to phosphorylate Raptor to activate mTORC1.[70] In the CAMK2γ$^{-/-}$ mice, the MAPKs (p38/ERK/JNK) were not significantly changed. In contrast, AKT activation became exaggerated after CAMK2γ deletion. Furthermore, our in vitro experiments clearly demonstrate that CAMK2γ simultaneously inhibits the activation of AKT and mTORC1 by insulin or EGF. A previous report shows that CAMK2γ suppresses AKT by phosphorylating p38 in liver, thereby activating a direct AKT inhibitor TRB3.[71] However, we have not observed any difference of p38 phosphorylation between wild-type and CAMK2γ$^{-/-}$ livers after DEN treatment. Instead, we found that CAMK2γ suppressed AKT, possibly by enhancing IRS1-S612 phosphorylation, which negatively regulated the RTK/IRS/PI3K module (Fig. 7C–E).[54] Because IRS1-S612 is a PKC phosphorylation site,[72] it would be interesting to know whether CAMK2γ affects PKC activities. Alternatively, IRS1-S612 could be a direct CAMK2γ phosphorylation site because CAMK2γ and PKC share many substrate sites such as GluR1-S831 and STAT1-S727.[73–75] In summary, we provide clear evidence to show that activation of CAMK2γ suppresses AKT/mTORC1 activation during HCC initiation in both mouse models and human cell lines. Though we also observed a potential negative correlation between CAMK2 γ and AKT/mTORC1 expression in human pre-malignant HCC samples (data not shown), further studies to confirm this mechanism in human HCC are required to extend its clinical relevance.

Last but not least, through one of our bioinformatics approaches, KEA, we also identified that CAMK2γ is exclusively linked to one of the top 7 TFs, CREM (Fig. S7A). It is reported that CREM can be directly phosphorylated by CAMK2γ at a site that is important for its transactivation.[76] Previous publications have shown that CREM is highly expressed in human HCC cell lines while inhibition of CREM can suppress HCC cell proliferation (Fig S7B, C).[77] This effect of CREM is consistent with the role of CAMK2γ at the later stages of cancer progression.[41] However, knocking down CREM in human HCC cell lines has no significant effect on insulin-induced Akt activation (Fig. S7D). More importantly, the Akt inhibition induced by CAMK2γ overexpression cannot be rescued by knocking down CREM (Fig. S7E). In this case, the effect of CAMK2γ of Akt/mTOR activation is independent from CREM; the CAMK2γ/CREM signaling may play independent roles in hepatocarcinogenesis. In addition, we also cannot exclude the possibility that CAMK2γ may also affect HCC initiation and development through other downstream targets. Further study is required in the future to investigate how CAMK2γ/CREM axle and other CAMK2γ-related signal pathways are involved in early and late stage liver cancer development.

In summary, this study identifies a novel mechanism by which CAMK2γ antagonizes AKT/mTORC1 signaling in liver during hepatocarcinogenesis. Our findings not only provided a better mechanistic insight into our understanding of compensatory hepatocyte proliferation during hepatocarcinogenesis, but also have important implications for developing new HCC therapies.

Materials and methods

Microarray and computational Kinase Enrichment Analysis

Our previously published microarray data [31] from FXR$^{-/-}$ mice versus wild-type mice was used for this study. Two additional microarray datasets were downloaded from Gene Expression Omnibus (GEO): GEO accession numbers GSE32244[34] and GSE12941.[36] For the GSE32244 dataset, the gene expression in liver from wild-type mice treated with the carcinogen DEN was compared to liver from control mice. For the GSE12941 dataset, gene expression from HCC patient liver tissue was compared to surrounding non-tumorous liver tissue. Microarray data was analyzed with GEO2R. Differential gene expression was assessed by limma.[37] *P*-values were adjusted using Benjamini & Hochberg False Discovery Rate method. Genes that were significantly up- or down-regulated (adjusted p-value < 0.01) were selected. Additional details of KEA and ChEA analysis are provided in the supporting documents.

Animals

Camk2γ knockout mice were kindly provided by Dr. Johannes Backs at University of Heidelberg, and were backcrossed to C56BL6/J background. The mice were maintained in a pathogen-free animal facility under standard 12:12-h light/dark cycle, and were fed with standard rodent chow and water ad libitum. To induce hepatocellular carcinogenesis, 25 mg/kg DEN (Sigma, Santa Louis, MO) was i.p. injected into 2-weeks-old male mice. The mice were euthanized after 10 months. The DEN and TCPOBOP-induced HCC rodent models were generated according to a previous report.[43] Briefly, 100 mg/kg DEN was i.p. injected into 4-weeks-old male mice, and after 2 weeks, 3 mg/kg TCPOBOP (Sigma) was i.p. injected into the mice every two weeks for 8 times. Six months after the DEN treatment, mice were euthanized. All procedures followed the NIH guidelines for the care and use of laboratory animals.

Liver histology, TUNEL, and PCNA staining

Liver specimens were prepared and analyzed by pathologists at City of Hope Pathology Core Lab. Livers were fixed in 4% PBS-buffered formalin, dehydrated and embedded in paraffin, sectioned and processed for H&E and immunostaining. Necrosis and leukocyte infiltration (inflammation) were graded as described previously and below.[78] TUNEL and PCNA staining were used to quantify liver cell apoptosis and proliferation using kits from Roche (San Diego, CA) and Invitrogen (San Diego, CA), respectively.

The necrosis grading standard:

Score 0: No focus of necrosis;

Score 0.5: One focus of necrosis

Score 1: Greater than 1 focus of necrosis in at least two regions, but not diffusely scattered throughout the liver lobe

Score 2: Diffuse small foci of CN across an entire liver lobe

Score 3: Diffuse and large area of geographic necrosis

The inflammation grading standard:

Score 0: No inflammation

Score 1: At least one focus or neutrophil-rich inflammation in at least two low-power [×4] fields

Score 2: Greater than two foci of neutrophil-rich inflammation in at least two low-power fields

Score 3: Diffuse and large area of neutrophil-rich inflammation

Cell line and in vitro cell assay

Human HCC cell line Huh7 and HepG2 were purchased from ATCC. Huh7 and HepG2 cell lines with CAMK2γ overexpression was as described elsewhere. For in vitro insulin stimulation assay, HCC cell lines were starved overnight to decrease basal Akt/mTOR activity. The cells were pre-incubated with differently types of kinase inhibitors, including KN-93 (Calbiochem), LY294002 (LC labs), PD98058 (LC labs), and Rapamycin (LC labs) for 30 minutes. The cells then were treated with human recombinant insulin (10 nM) for indicated time. Treated cells were subject to lysis on ice and saved for further analysis.

Antibodies and Western blot

Anti-pCAMK2 and anti-CAMK2γ antibodies were ordered from Santa Cruz Biotechnology (Santa Cruz, CA). All the other antibodies were purchased from Cell Signaling Technology (Danvers, MA).

ALT Analysis

Serum was obtained by centrifuging mouse blood at 3,500 rpm at 4°C for 10 min. Serum ALT were measured at the City of Hope Helford Research Hospital.

Statistical analysis

Data were presented as mean±SEM. Two-tailed Student’s t test was used to determine the significance of differences between data groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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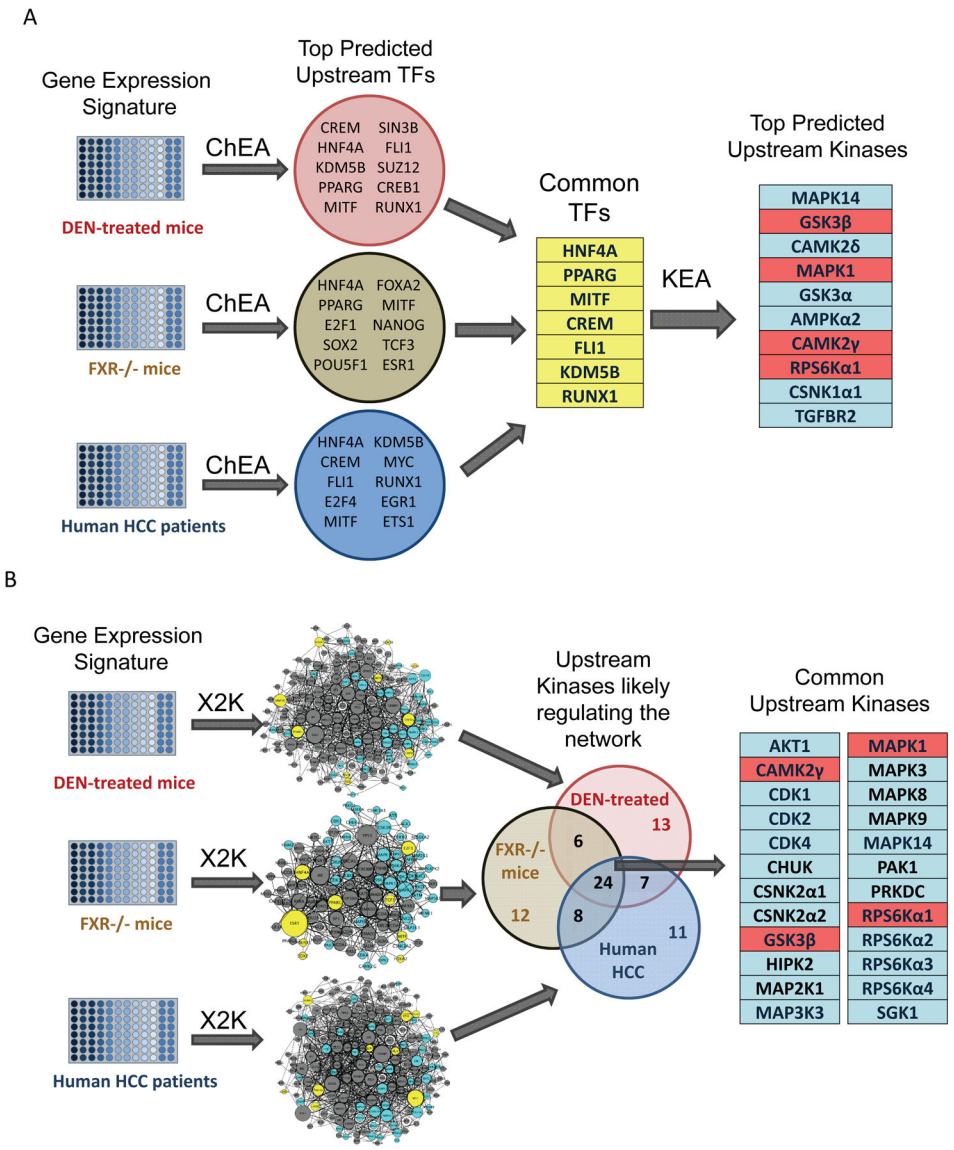

Figure 1. Gene profiling and pathway analyses of kinases involved in liver carcinogenesis

(A) Microarray data from mouse models and clinical HCC specimens were analyzed by ChIP-X Enrichment Analysis (ChEA) and Kinase Enrichment Analysis (KEA). The top hits of ChEA and KEA were listed for each dataset. (B) X2K analyses on the same datasets. Top 50 kinases in each dataset were selected and compared among the 3 data sets. Four kinases, CAMK2γ, GSK3β, MAPK1 (ERK2), and RPS6Kα1 (RSK1), were enriched in both KEA and X2K analyses, and are highlighted in the figure.

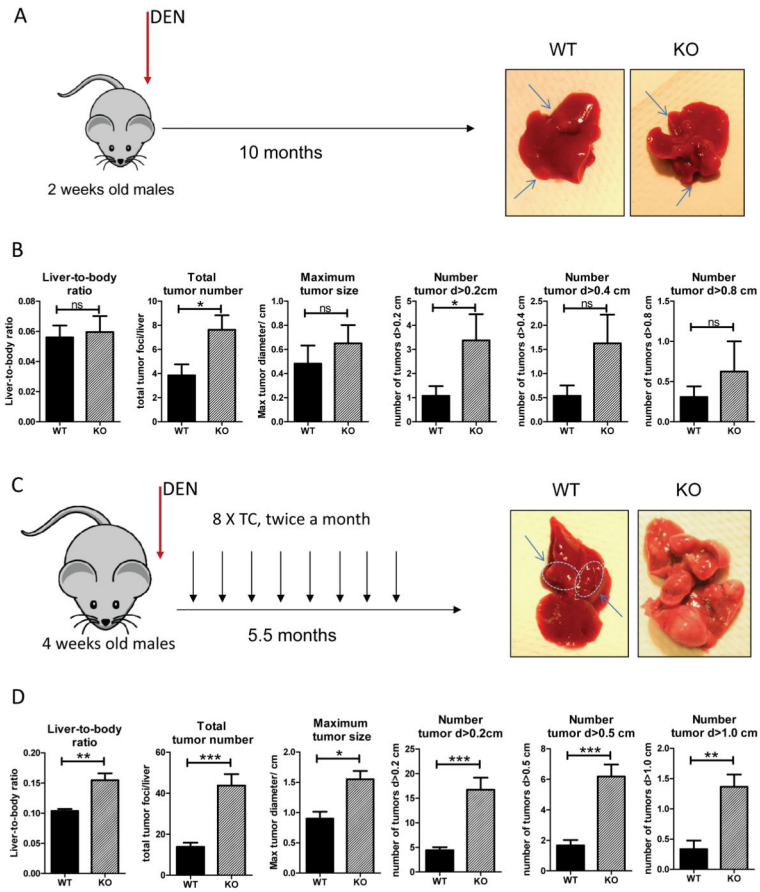

Figure 2. Enhanced DEN-induced hepatocarcinogenesis in CAMK2$\gamma^{-/-}$ mice

(A) A DEN-induced liver cancer model was compared in CAMK2$\gamma^{-/-}$ and wild-type (WT) mice. Representative images of liver tumors in the WT and CAMK2$\gamma^{-/-}$ mice after DEN injection. (B) Statistical analyses of liver size, tumor number, and tumor size in the mice. HCC was induced by a DEN plus TCPOBOP protocol. *, $p < 0.05$; **, $p < 0.01$; n=14 or 9. (C) Representative images of liver tumors in the WT and CAMK2$\gamma^{-/-}$ mice after DEN plus TCPOBOP injection. (D). Statistical analyses of liver size, tumor number, and tumor size in the mice. *, $p < 0.05$; **, $p < 0.01$; n=11 or 12.

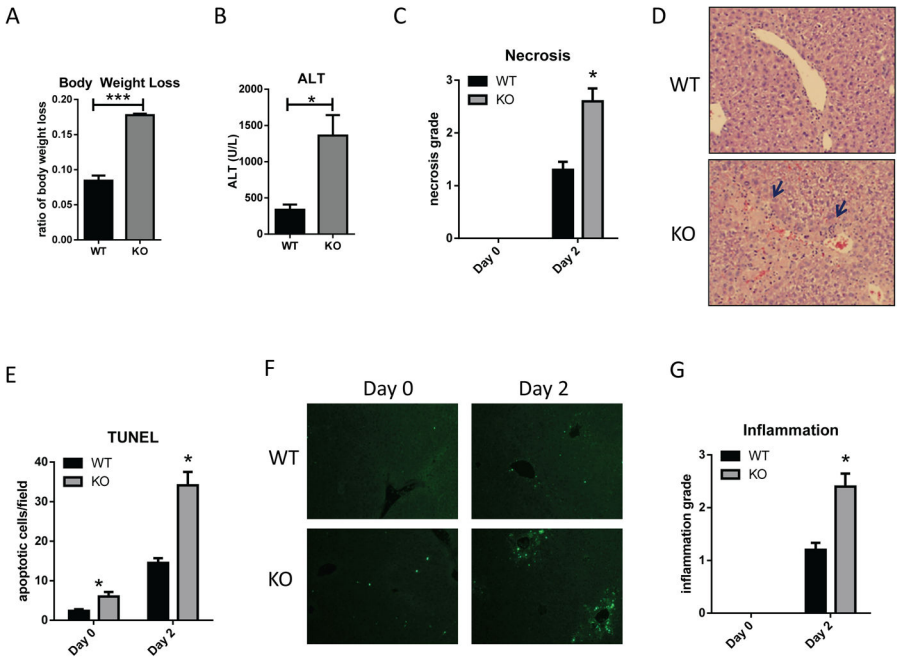

Figure 3. Enhanced acute responses and compensatory proliferation in CAMK2γ$^{-/-}$ livers

(A) Body weight loss and serum ALTs (B) 48 h after DEN injection. *, $p < 0.05$; ***, $p < 0.001$; n=5 or 6. (C) Grading of necrosis. (D) A representative H&E staining of the liver in the mice. (E) Quantification of apoptotic cells shown in the TUNEL staining. (F) Representative images of TUNEL staining. (G) Grading of inflammatory cell infiltration.

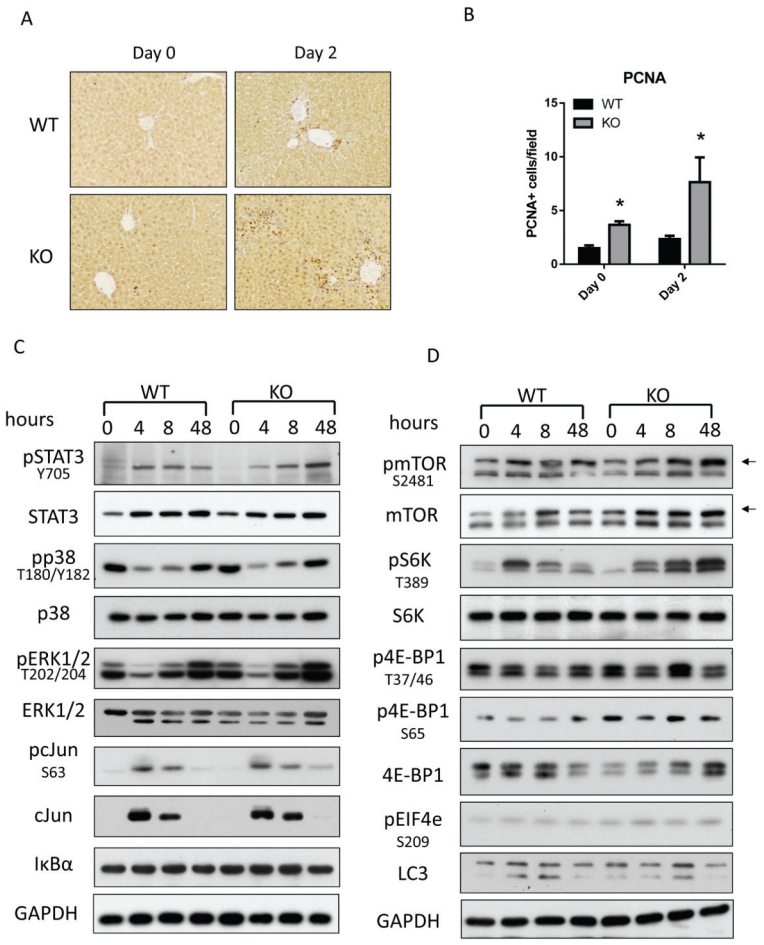

Figure 4. Upregulation of mTORC1 signaling in CAMK2γ$^{-/-}$ livers

(A) Representative images of PCNA staining. (B) Quantification of hepatocyte compensatory proliferation by PCNA staining. *, $p < 0.05$; ***, $p < 0.001$. (C) Key signaling pathways in the liver of DEN-treated mice were analyzed by western blotting. (D) Western blots of mTOR pathway components in the livers of DEN treated mice. Small arrow indicates the mTOR band.

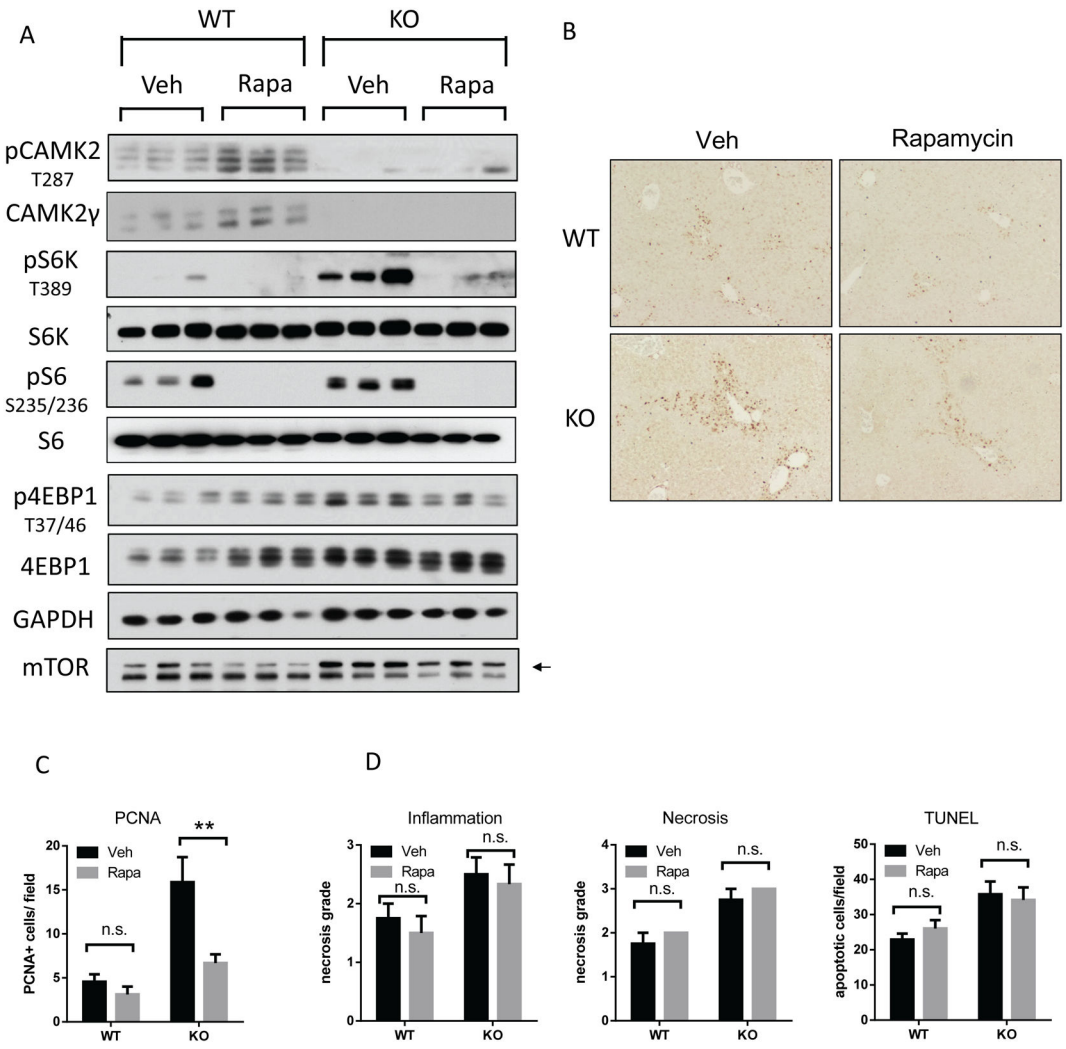

Figure 5. Inhibition of mTORC1 signaling by rapamycin in CAMK2γ$^{-/-}$ mice

CAMK2γ$^{-/-}$ mice and WT mice received either 5mg/kg rapamycin or 2% vehicle i.p. injection 2 h before the DEN challenge. The mice then received a second and a third rapamycin or vehicle injection 24 h and 46 h after the DEN challenge. At 48 h after the DEN injection, the mice were sacrificed and the liver were harvested for PCNA immunostaining and Western blotting analysis. (A) Immunoblotting analyses of mTORC1 signaling in the liver 48 h after DEN treatment. Small arrow indicates the mTOR band. (B) Representative images of PCNA staining in the liver 48 h after DEN treatment. (C) Quantification of proliferating hepatocytes by PCNA staining. (D) Quantification of hepatic inflammatory cell infiltration, centrilobular necrosis, and hepatocyte apoptosis. **, p <0.01; n=4–7.

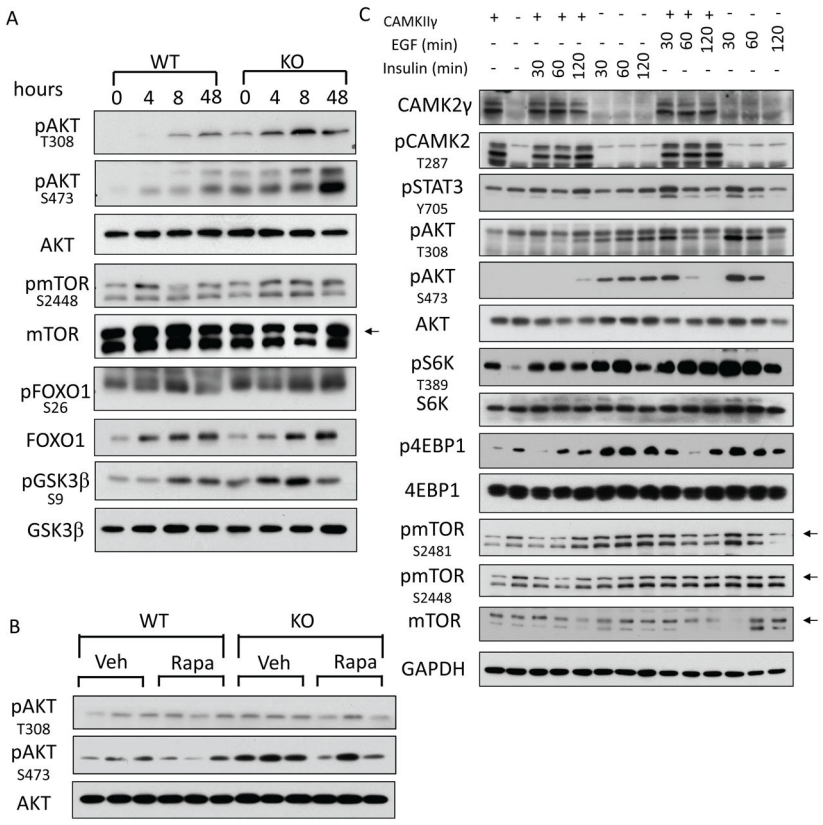

Figure 6. Suppression of AKT activities by CAMK2γ in liver cells

(A) AKT was hyperphosphorylated in the DEN-treated CAMK2$\gamma^{-/-}$ livers. Small arrow indicates the mTOR band. (B) AKT phosphorylation was unchanged in the rapamycin-treated liver 48 h after DEN treatment. (C) EGF (20 ng/ml) or insulin (10 nM) induced exaggerated activation of AKT in CAMK2$\gamma^{-/-}$ primary hepatocytes at the time points as indicated. Small arrow indicates the mTOR band.

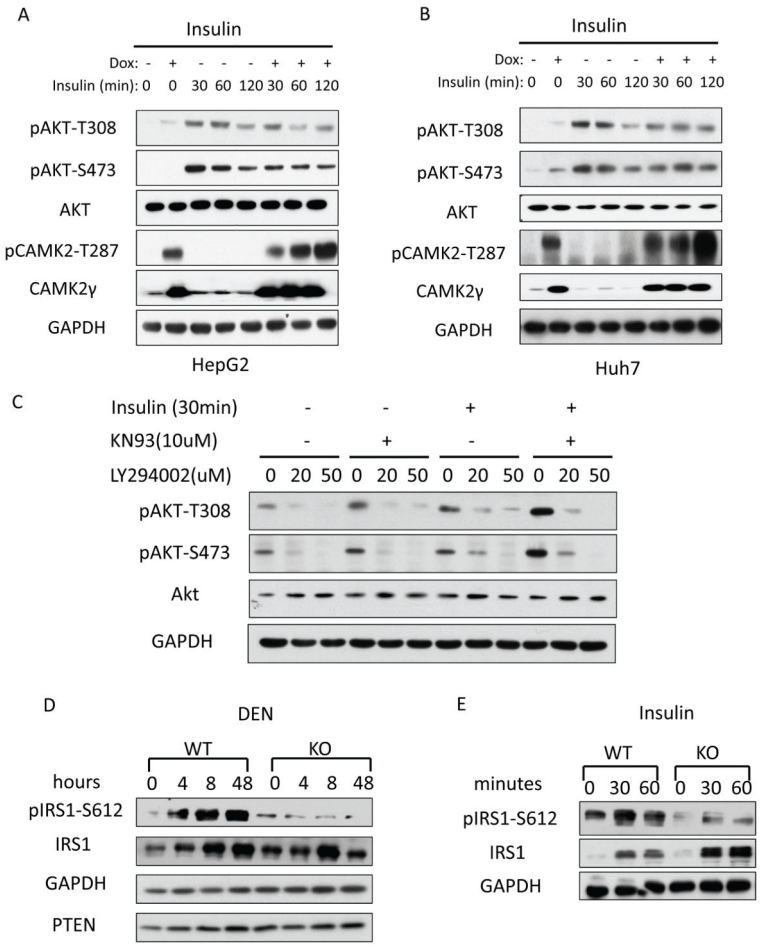

Figure 7. Regulation of AKT/mTOR pathway by CAMK2γ

(A–B) HepG2 and Huh7 cells with tetracycline-inducible CAMK2γ expression were cultured with Dox for 48 h to induce the expression of CAMK2γ. The cells were then cultured in serum free medium overnight to minimize basal AKT activities. Subsequently, the cells were treated with 10 nM insulin for the indicated time points. (C) Huh7 cells were starved in serum-free medium overnight and pretreated with a combination of DMSO, KN93, and LY294002 for 30 min. Cells were then stimulated with 10 nM insulin and harvested at the indicated time points. The PI3K inhibitor LY294002 suppressed the hyperactivation of AKT induced by CAMK2γ inhibitor KN93. (D) Phosphorylation of IRS1-S612 in the DEN-treated mouse livers. (E) Deletion of CAMK2γ abolished insulin-induced IRS1-S612 phosphorylation in mouse hepatocytes.